EMERGING ROLES OF PLATELET-DERIVED GROWTH FACTOR RECEPTOR ALPHA IN CHRONIC LIVER INJURY: POTENTIAL THERAPEUTIC TARGET IN HEPATIC FIBROSIS

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Alexander Kikuchi, Ph.D.

Platelet-derived growth factor receptor α (PDGFRα) is a tyrosine kinase receptor that plays a role in cell survival, proliferation, and differentiation, and is involved in liver development, regeneration and chronic liver injury states such as hepatic fibrosis and cirrhosis. Hepatic stellate cells (HSCs) are the primary mediators of hepatic fibrosis through their activation from a quiescent state in response to the presence of pro-fibrotic growth factors such as PDGFs. Proliferation and migration are key outcomes of this transition, facilitating collagen deposition and migration of activated HSCs to sites of liver injury. We confirm the upregulation of PDGFRα in pericentral hepatocytes in CCl_4-induced liver injury as well as HSCs/myofibroblasts in carbon tetrachloride (CCl_4), bile duct ligation (BDL), and 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver injury. After ruling out a significant contribution of hepatocyte PDGFRα in hepatic fibrosis using Alb-Cre and FoxA3-Cre *Pdgfra-/-* animals, we examine PDGFRα signaling in primary human HSCs (HHSteCs) in combination with human PDGFRα–specific inhibitory monoclonal antibody Olaratumab to test the hypothesis that PDGFRα signaling in HSCs promotes hepatic fibrosis. Olaratumab-mediated PDGFRα inhibition resulted in decreased HHSteC proliferation and motility, while lacking an effect on transcriptional expression of fibrosis-associated genes. Furthermore, Olaratumab reduced activation of downstream signaling effectors involved in proliferation and motility including Akt, mTOR, Erk1/2, FAK, and p38 MAPK suggesting that PDGFRα contributes to mitogenesis and actin reorganization through diverse downstream mediators. This evidence was corroborated with findings that HSC-specific Lrat-Cre

Pdgfra-/- mice showed reduced CCl_4-induced fibrosis after 4 weeks (early fibrosis) followed by reduced ALT/AST levels at 8 weeks (advanced fibrosis). This was accompanied by increased macrophage infiltration and increased TUNEL-positive HSCs/myofibroblasts concomitant with a decrease in TUNEL-positive hepatocytes, suggesting that PDGFRα loss in HSCs may promote injury resolution in advanced fibrosis by limiting HSC/myofibroblast survival. These findings support a distinct pro-fibrotic role of PDGFRα in HSCs during chronic liver injury in both mice and human primary cells and provides an important pre-clinical foundation for the future testing of therapeutic PDGFRα inhibition in hepatic fibrosis.

PUBLICATIONS

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2) **Alexander Kikuchi** and Satdarshan Monga. PDGFRα in Liver Pathophysiology: Emerging Roles in Development, Regeneration, Fibrosis, and Cancer. *Gene Expression* **16,** 109127 (2015).

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TABLE OF CONTENTS

1.0 BACKGROUND: CHRONIC LIVER INJURY AND ROLE OF PDGFRα IN LIVER PATHOPHYSIOLOGY 1

1.1 OVERVIEW OF CHRONIC LIVER INJURY 1

1.1.1 Hepatic Fibrosis 2

1.1.2 Cirrhosis 2

1.1.3 Hepatocellular Carcinoma 3

1.2 ANIMAL MODELS OF CHRONIC LIVER INJURY 3

1.2.1 Carbon Tetrachloride 5

1.2.2 Bile Duct Ligation 5

1.2.3 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) 6

1.3 CELLULAR ROLES DURING HEPATIC FIBROSIS 7

1.3.1 Hepatic Stellate Cells 7

1.3.2 Kupffer Cells 9

1.3.3 Sinusoidal Endothelial Cells 9

1.3.4 Hepatocytes 10

1.3.5 Cholangiocytes 11

1.3.6 Portal Fibroblasts 12

1.3.7 Inflammatory Cells 12

1.4 PDGF SIGNAL TRANSDUCTION 13

1.5 PDGFRα IN LIVER DEVELOPMENT 15

1.6 PDGFRα IN LIVER REGENERATION 18

1.7 PDGFRα IN LIVER FIBROSIS 19

1.7.1 Relative Contributions of PDGFRα vs. PDGFRß in HSC Activation: Reconciling the Evidence 20

1.7.2 TGF-ß/PDGFRα Crosstalk in HSCs 24

1.7.3 PDGFRα in Portal Myofibroblast Activation During Cholestatic Liver Injury 27

1.7.4 PDGFRα Cellular Localization: Expression Patterns in Chronic Liver Injury 28

1.7.5 PDGF Sources in Injured Liver 30

1.8 THERAPEUTIC INHIBITION OF PDGF SIGNALING IN LIVER DISEASE 32

1.8.1 PDGF Ligand Neutralization 32

1.8.2 PDGFR Inhibitors 33

1.8.3 A Note on the Rationale for PDGFRα –Specific Targeting Inhibitors .. 34

2.0 PDGFRα LOCALIZATION AND SIGNALING IN MURINE LIVER DURING CHRONIC LIVER INJURY 36

2.1 PDGF SIGNALING STATUS IN CHRONIC LIVER INJURY 36

2.2 PDGFRα LOCALIZATION DURING CHRONIC LIVER INJURY 38

2.2.1 PDGFRα Expression in Parenchymal Cells of Fibrotic Liver 39

2.2.2 PDGFRα Expression in Non-Parenchymal Cells of Fibrotic Livers 42

2.3 PDGFRα LOSS IN HEPATOCYTES DOES NOT AFFECT HEPATIC FIBROSIS 46

2.3.1 PDGFRα Loss in Epithelial Cells does not Affect Hepatic Fibrosis..... 46

2.4 DISCUSSION: IMPLICATIONS OF PDGFRα EXPRESSION AND NULL FUNCTIONALITY IN HEPATOCYTES 49

3.0 PDGFRα CONTRIBUTES TO HUMAN HEPATIC STELLATE CELL PROLIFERATION AND MIGRATION..... 51

3.1 PDGFRα EXPRESSION IN HUMAN HEPATIC STELLATE CELLS..... 52

3.2 CONTRIBUTION OF PDGFRα SIGNALING TO HUMAN HSC PROLIFERATION 54

3.2.1 PDGF Treatment Induces PDGFRα-dependent HHSteC Proliferation, but not Pro-Fibrotic Gene Expression 54

3.2.2 Olaratumab inhibits PDGF-BB mediated HSC proliferation through blockade of PDGFRα and downstream signaling 57

3.3 CONTRIBUTION OF PDGFRα SIGNALING TO HUMAN HSC MIGRATION..... 61

3.3.1 Olaratumab Inhibits HHSteC Migration Attributable to Autocrine Signaling..... 61

3.3.2 Olaratumab Inhibits Baseline PDGFRα Activation in HHSteCs and Downstream Cell Motility Signaling 63

3.4 DISCUSSION: DISSECTING THE ROLE OF PDGFRα IN HUMAN HSCS 66

4.0 LOSS OF PDGFRα IN MICE AMELIORATES HEPATIC FIBROSIS DURING CHRONIC LIVER INJURY 74

4.1 BACKGROUND: PRINCIPLES AND DEVELOPMENT OF HSC-SPECIFIC TRANSGENE EXPRESSION IN MICE 74

4.2 VALIDATION STUDIES OF LRAT-CRE PDGFRα KO STRAIN 76

4.2.1 Loss of PDGFRα in Liver Lysates of Lrat-Cre *Pdgfra-/-* Mice 76

4.2.2 Loss of PDGFRα in HSCs and Myofibroblasts of Lrat-Cre *Pdgfra-/-* Mice 78

4.3 CHARACTERIZATION OF LRAT-CRE PDGFRα KO MICE 78

4.3.1 Biliary Fibrosis is Unaffected in Lrat-Cre *Pdgfra-/-* Mice Following Cholestatic Liver Injury 78

4.3.2 Reduced Hepatic Fibrosis in Lrat-Cre *Pdgfra-/-* Mice Following Short Term Hepatotoxic Liver Injury 79

4.3.3 Reduced Mid-Zonal Distribution of HSCs Following Biliary and Hepatotoxic Liver Injury 83

4.3.4 Reduced Hepatocellular Injury and Increased HSC/Myofibroblast Cell Death in Lrat-Cre *Pdgfra-/-* Mice Following CCl_4 85

4.3.5 Increased Inflammatory Response and Hepatic Macrophage Infiltration in Lrat-Cre *Pdgfra-/-* Mice Following Long Term CCl_4-induced Injury 88

4.4 DISCUSSION: DOES PDGFRα PLAY A SUBSTANTIAL ROLE IN HEPATIC FIBROSIS AND LIVER INJURY? 89

5.0 DISCUSSION AND FUTURE STUDIES 97

5.1 PDGFRα AND PDGFRß IN HEPATIC STELLATE CELLS: INTERCHANGEABLE OR DISCRETE FUNCTIONS? 97

5.2 PROSPECTS FOR PDGFRα-SPECIFIC INHIBITORS IN HEPATIC FIBROSIS 99

5.3 BEYOND FIBROSIS: STUDIES OF PDGFRα INHIBITION IN LIVER CANCER 102

5.3.1 Potential Roles of PDGFRα in Tumor Biology: Modulation of Angiogenesis and Hypoxic Response in Chronic Liver Injury and Cancer 104

5.4 FUTURE STUDIES 106

6.0 MATERIALS AND METHODS 110

6.1 CELL CULTURE AND ASSAYS 110

6.1.1 Preparation and Cell Culture Treatments 110

6.1.2 AlamarBlue Proliferation Assay 111

6.1.3 Transwell Migration Assay 111

6.2 PROTEIN EXTRACTION AND WESTERN BLOTTING 112

6.2.1 Whole Cell Lysate Preparation: Cell Lysates 112

6.2.2 Whole Cell Lysate Preparation: Liver Tissue 112

6.2.3 Western Blot Analysis 113

6.2.4 Immunoprecipitation 113

6.3 RNA EXTRACTION AND SEMI-QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) 114

6.4 ANIMALS AND MODELS OF LIVER INJURY 114

6.4.1 Mouse Model Development 114

6.4.2 Serum Liver Function Tests 115

6.4.3 Carbon Tetrachloride 115

6.4.4 Bile Duct Ligation Surgery 115

6.4.5 DDC Injury 116

6.5 IMAGE ANALYSIS 116

6.5.1 Immunohistochemistry 116

6.5.2 Immunofluorescence 117

6.5.3 Fibrosis Quantification 117

APPENDIX A 118

BIBLIOGRAPHY 123

LIST OF TABLES

Table 1. Summary of PDGF and PDGFR isoform expression in select liver cells in normal and pathologic states. 109

Table 2. Primary Antibodies used for WB, IHC, IF, and IP. 121

Table 3. Primers used for RT-PCR. 122

LIST OF FIGURES

Figure 1: ***PDGF Signaling Pathway.*** **14**

Figure 2: ***TGF-ßR/PDGFRα Signaling Crosstalk in HSC.*** **26**

Figure 3: ***PDGF Sources and Cell Interactions in Injured Liver.*** **31**

Figure 4: ***PDGFRα Signaling is Upregulated During Chronic Liver Injury.*** **37**

Figure 5: ***Absence of PDGFRα in Hepatocytes and Cholangiocytes following Cholestatic Liver Injury.*** **40**

Figure 6: ***PDGFRα Co-localization in Pericentral Hepatocytes during CCl_4 Injury.*** **41**

Figure 7: ***PDGFRα is not Expressed in Sinusoidal Endothelial Cells during Chronic Liver Injury.*** **43**

Figure 8: ***PDGFRα Co-localization in Hepatic Stellate Cells during Chronic Liver Injury.*** **44**

Figure 9: ***PDGFRα Co-localization in Myofibroblasts during Chronic Liver Injury.*** **45**

Figure 10: ***PDGFRα Expression is Retained in FoxA3-Cre Pdgfra-/- Livers.*** **47**

Figure 11: ***FoxA3 Pdgfra-/- Mice Show No Change in Hepatic Fibrosis or Hepatocellular Injury During CCl_4 Injury.*** **48**

Figure 12: ***PDGFRα Expression and Activity in Hepatic Stellate Cells.*** **53**

Figure 13: ***PDGFRα Signaling Contributes to HHSteC Proliferation, but not Activation, in the Presence of Exogenous PDGF Stimulation*** **56**

Figure 14: ***Olaratumab Inhibits PDGFRα Activation and Downstream Proliferative Signaling Mediators*** **59**

Figure 15: ***Densitometric Quantification of Olaratumab-Mediated Changes in Protein Phosphorylation of HHSteCs in the Presence of Exogenous PDGF Stimulation.*** **60**

Figure 16: ***Olaratumab Inhibits Migration of HHSteCs in the Absence of Exogenous PDGF Ligand, Due to Autocrine Baseline Signaling.*** **62**

Figure 17: ***Olaratumab Inhibits Baseline PDGFRα Signaling in HHSteCs Along with Downstream Effectors.*** **64**

Figure 18: ***Densitometric Quantification of Olaratumab-Mediated Changes in Protein Phosphorylation in HHSteCs at Baseline.*** **65**

Figure 19: ***Proposed Effect of PDGFRα Blockade on HSC Migration and Proliferation through Inhibition of Specific Downstream Signaling.*** **69**

Figure 20: ***LratCre Pdgfra-/- Mice show Reduced Total and HSC/Myofibroblast-specific PDGFRα following Chronic Liver Injury.*** **77**

Figure 21: ***Biliary Fibrosis is Unaffected in LratCre Pdgfra-/- Mice following Cholestatic Liver Injury.*** **81**

Figure 22: ***Lrat-Cre Pdgfra-/- Mice show Reduced Hepatic Fibrosis During Early, but not Advanced Hepatotoxic Liver Injury.*** **82**

Figure 23: ***Lrat-Cre Pdgfra-/- Mice show Reduced Mid-Zonal Distribution of HSCs Following Biliary and Hepatotoxic Liver Injury.*** **84**

Figure 24: ***Hepatocellular Injury Following BDL and DDC Unaffected in Lrat-Cre Pdgfra-/- Mice.*** **86**

Figure 25: ***Reduced Hepatocellular Injury and Increase HSC/Myofibroblast Cell Death in Lrat-Cre Pdgfra-/- Mice Following CCl_4.*** **87**

Figure 26: ***Increased Inflammatory Response and Hepatic Macrophage Infiltration in Lrat-Cre Pdgfra-/- Mice Following Long-Term CCl_4-induced Injury:*** **89**

Figure 27: ***Proposed Timeline of the Impact of PDGFRα Loss in HSCs During CCl4-induced Chronic Liver Injury:*** **..... 96**

1.0 BACKGROUND: CHRONIC LIVER INJURY AND ROLE OF PDGFRα IN LIVER PATHOPHYSIOLOGY

1.1 OVERVIEW OF CHRONIC LIVER INJURY

Chronic liver disease is a significant cause of morbidity worldwide. In the U.S. alone, around 5.5 million Americans suffer from hepatic fibrosis and cirrhosis [1]. Hepatic fibrosis, similar to fibrotic diseases in other solid tissues, is primarily a wound healing response in which myofibroblasts stemming from resident tissue fibroblasts propagate the accumulation and qualitative changes to the extracellular matrix (ECM). The etiologies of hepatic fibrosis are wide ranging and many different forms of repeated injury to the liver can result in the common outcome of hepatic fibrosis. Some of the most common types of chronic liver injury leading to cirrhosis in developed countries include alcoholic liver disease, cholestatic liver disease (including primary/secondary biliary cirrhosis, biliary atresia/neonatal hepatitis, congenital biliary cysts, and neonatal hepatitis), chronic viral hepatitis (Hepatitis B, C), hemochromatosis, and non-alcoholic fatty liver disease (NAFLD)[2]. Chronic liver injury and its sequelae, hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), are discussed below.

1.1.1 Hepatic Fibrosis

Hepatic fibrosis, a manifestation of chronic liver disease, is a wound healing response that results in excessive, dysregulated collagen deposition from activation of hepatic stellate cells (HSC). This could be a result of inflammation and the release of numerous paracrine and autocrine growth factors and inflammatory chemokines from injured hepatocytes, resident macrophages, infiltrating inflammatory cells, and HSC themselves. Hepatic fibrosis can result from a variety of injurious stimuli to the liver including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, chronic alcohol exposure, non-alcoholic steatohepatitis, primary biliary cirrhosis, or autoimmune hepatitis [3]. The convergence of each of these injurious stimuli on a similar fibrotic injury response has made the identification of therapeutic targets to prevent or reverse fibrosis a priority. Importantly, early fibrosis is potentially reversible if hepatic injury can be curbed or repair enhanced [4-6]. However failure to curb hepatic injury in the setting of fibrosis may eventually lead to the development of cirrhosis, setting the stage for liver failure or (in a subset of patients) liver cancer.

1.1.2 Cirrhosis

In the setting of chronic liver injury, the persistence of hepatocyte cell death and fibrotic response can lead to cirrhosis which is characterized by the presence of regenerative nodules disrupting the normal architecture as well as causing altered blood flow and portal hypertension[7]. In addition to the degeneration/necrosis of hepatocytes and replacement of parenchyma with fibrotic liver tissue that is associated with hepatic fibrosis, cirrhosis characteristics include regenerative nodules, defenestration of sinusoidal endothelial cells, venous occlusion, and ultimately loss of liver

function. Unlike hepatic fibrosis which is clinically silent, loss of liver function in cirrhosis is associated with specific clinical signs including ascites formation, portal hypertension, and varices[8, 9]. In a subset of cirrhosis patients, hyperplastic nodules undergo increasing genomic stability as a result of unrelenting hepatocyte necrosis and proliferation, eventually forming dysplastic nodules that can lead to HCC[10].

1.1.3 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most common type of liver cancer (83% of all cases) and is the 5th most common neoplasm worldwide [10]. It is a disease of grim prognosis with a 5 year survival rate of only 8.9% in the U.S.A. [10] Currently, tumor resection and liver transplantation are the only curative treatments available[11]. While orthotopic liver transplantation is effective in the treatment of HCC, it is associated with high morbidity, cost, life-long immunosuppressive therapy and a shortage of donor organs. In the last decade, molecular therapies have been explored as a potential option for HCC treatment. Sorafenib, a multikinase inhibitor targeting Raf, VEGFRs, and PDGFRα/ß, is currently the only chemotherapeutic which has been shown to be effective in the treatment of HCC [12, 13].

1.2 ANIMAL MODELS OF CHRONIC LIVER INJURY

Animal models are crucial tools for the investigation of the pathogenesis of hepatic fibrosis during chronic liver injury. Much of what is known about hepatic fibrosis and the signal transduction pathways and cell interactions arising in the injured liver come from these models, which provide

important advantages over clinical research including i) defined timepoints of injury and controlled injury conditions, ii) shorter time frame for disease development, iii) relatively larger liver sampling compared to human liver biopsy, iv) ability to use invasive terminal procedures (ex: liver perfusion and HSC isolation) in a non-post mortem setting, and vi) the use of genetically modified animals. This last point will be discussed later in this dissertation in Section 4.1.

Animal studies are also important complements to culture activated models of HSCs in vitro (discussed further below) due to the ability to study cells in intact organs with dynamic cell-cell interactions, cell-matrix crosstalk, and exposure to immune, vascular, metabolic, endocrine and other physiologic/pathophysiologic stimuli.

Despite the indispensable nature of animal models in the study of liver injury, the utility of these models is limited by the fact that rodents and humans often do not succumb to similar hepatotoxic agents. For example, mice are extremely averse to alcohol and rapid metabolism of alcohol in mice prevents high alcohol blood levels. Alternatively, Hepatitis C virus (HCV), which is a leading cause of infectious chronic liver injury in the developed world, does not infect rodent hepatocytes. Chronic liver injury in the setting of metabolic disease such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are complex diseases that cannot be recapitulated with a single injury agent.

The complexity of these diseases has led to the development of a variety of agents used to induce chronic liver injury including hepatotoxic chemicals (ex: CCl_4, TAA), physical surgery (ex: BDL), Special feed diets (ex: high fat diets), immune reaction, and genetic modification (ex: MDR2 knockout). The extreme diversity of injurious stimuli leading to hepatic fibrosis necessitates the use of complementary animal injury models to demonstrate the broad relevance of any findings at the molecular level. In light of this consideration, the animal studies shown in

the following chapters are performed on at least two models of animal injury, one hepatotoxic liver injury model (CCl_4) and one model of cholestasis (BDL, DDC).

1.2.1 Carbon Tetrachloride

Chronic administration of carbon tetrachloride (CCl_4) is a widely used and extensively characterized hepatotoxic model of liver injury in mice and rats due to its high degree of reproducibility, ease of administration, and predictable timecourse of injury and recovery encompassing early and late stages of fibrosis, cirrhosis, and eventually HCC. The mechanism of CCl_4-induced liver injury is well studied. CCl_4 is metabolized in pericentral hepatocytes by the cytochrome P450 family (CYP family) of enzymes to form the reactive radical compound trichloryl methyl (CCL_3*). Reaction with this compound ultimately results in centrilobular necrosis through oxidative stress, mitochondrial dysfunction, membrane dysfunction, and reduced protein synthesis[14]. Resulting hepatotoxic damage and inflammation over repeated exposures (ex: injections) followed by short recovery periods in between injections emulates periods of injury and recovery which characterize human pathology. With biweekly injections, mice develop robust fibrosis after 4 weeks, and a pre-cirrhotic/cirrhosis phenotype at 8-12 weeks of injection. Continued administration leads to the development of HCC, making CCl_4 a very versatile model.

1.2.2 Bile Duct Ligation

Bile duct ligation is the archetypal model of obstructive cholestatic liver injury. Ligation of the common bile duct leads to rapid ductular proliferation, portal inflammation, and portal fibrosis. Unlike hepatotoxic liver injury models targeting pericentral hepatocytes, cholangiocytes and

periportal hepatocytes bear the brunt of injury in BDL. This last point is particularly important because human hepatic fibrosis is more commonly distributed in the peri-portal rather than peri-central regions[15] – making cholestatic liver injury models a crucial tool for investigators to verify translational relevance.

In contrast to other models of cholestasis, BDL has been extensively characterized based on the relative contributions of hepatic stellate cells and portal fibroblasts to the overall transdifferentiated myofibroblast population. Using the absence of Vitamin A in Col-GFP expressing cells to distinguish between HSCs and portal fibroblasts, Iwaisako et al showed that the majority of myofibroblasts arising early timepoints post-BDL (5 days) are derived from Vitamin A-negative portal fibroblasts rather than HSCs[16]. At increasing timepoints post-surgery, the proportion of myofibroblasts derived from HSCs increases until it eventually becomes the majority source of myofibroblasts. In our studies described below (Chapters 2-4), we analyze livers 5 days and 2 weeks post-BDL. These two timepoints were chosen to reflect the differential contributions of portal fibroblasts and HSCs to the activated myofibroblast population at each of these times[17].

1.2.3 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)

DDC-induced liver injury is a well-studied xenobiotic model of sclerosing cholangitis which is used widely for the study of chronic cholestatic liver disease[18]. DDC is easily administered as a modified diet and does not involve invasive surgery in contrast to BDL. Furthermore, while BDL models secondary biliary fibrosis/cirrhosis as a result of large bile duct obstruction, DDC models primary biliary fibrosis/cirrhosis resulting from the formation of porphoryin plugs leading to cholestasis in the smaller bile ducts and contributing to a phenotype of sclerosing cholangitis,

ductular proliferation, peri-ductular fibrosis, and portal-portal fibrosis. Similar to other models of primary biliary cirrhosis, DDC is characterized by a reactive biliary epithelial cell (BEC) phenotype in which pro-inflammatory and pro-fibrogenic cytokines are secreted by BECs. The resulting injury and inflammation contribute to the activation and proliferation of myofibroblasts and peri-ductal fibrosis, which eventually form the characteristic 'onion skin-type' sclerosis histology which is a key hallmark of sclerosing cholangitis in humans[18].

1.3 CELLULAR ROLES DURING HEPATIC FIBROSIS

Hepatic fibrosis is a complex process that involves interactions between many cell types within the liver [4]. While myofibroblasts are the primary mediators of fibrosis [19], other cells influence and modulate the activity of myofibroblasts and their HSC precursors through the release of damage associated molecular patterns (DAMPS), reactive oxygen species (ROS), growth factors, cytokines, and chemokines that can stimulate quiescent HSCs to undergo activation and subsequent transdifferentiation to a myofibroblast form. In the following sections, we outline some of the different cellular roles of resident and infiltrating cells in the liver during chronic liver injury.

1.3.1 Hepatic Stellate Cells

Hepatic stellate cells, formerly known as Ito cells, lipocytes, pericytes, or fat-storing cells, are vitamin-A storing fibroblasts residing between hepatocyte sinusoids and sinusoidal endothelial cells known as the Space of Disse. In the normal liver, HSCs are thought to consist of 5-8% of total liver cells[20]. In their quiescent state, HSCs are thought to function as storage cells for

retinoids (vitamin-A and its metabolites) and also maintain balanced turnover of ECM and regulate sinusoidal blood flow through their close association with sinusoidal endothelial cells (described further below). However in the presence of inflammation and other injurious stimuli, HSCs undergo a process of activation representing a continuum of changes in gene expression, morphology, and functional characteristics. These changes span the start of activation from a quiescent state to fully transdifferentiated myofibroblast and subsequent reversion or apoptosis (in the case of injury resolution). This process has been generally categorized into three major stages: initiation, perpetuation, and resolution[21].

Initiation consists of early changes in gene expression that make HSCs more receptive to future stimuli, can be triggered by variety of factors including paracrine growth factors, changes in surrounding ECM, and ROS and DAMPs released by damaged cells. Both resident liver cells (ex: hepatocytes, cholangiocytes, endothelial cells, etc.) as well as infiltrating inflammatory cell populations (ex: platelets, macrophages, etc.) contribute to the paracrine signals that initiate the process of HSC activation. Specific cellular roles and factors are detailed in the sections below.

Once initiation of HSCs from a quiescent state has occurred, HSCs begin to undergo a series of discrete functional changes and begin to generate their own growth factors, cytokines, and chemokines which propagate the process of activation both in an autocrine as well as in a paracrine manner towards other HSCs. Due to the self-perpetuating nature of this process, this stage of HSC activation has been termed ‘perpetuation’ and represents the bulk of fibrogenic changes that occur during HSC activation. Among these changes are increased proliferation, migration, contractility, fibrogenesis, and retinoid loss.

Following regression of liver injury stimuli, myofibroblasts reduce in number through apoptosis or reversion to a quiescent phenotype. Lineage tracing experiments have demonstrated

that roughly half of myofibroblasts undergo apoptosis while the other half revert towards a semi-quiescent HSC state following spontaneous recovery in a CCl4-induced injury model[22]. Gene expression analysis of reverted HSCs show that they retain an intermediate profile of fibrosis-associated genes between completely quiescent and activated HSCs.

1.3.2 Kupffer Cells

Kupffer cells (KCs) are specialized self-renewing macrophages that reside in the lining of walls of liver sinusoids that form part of the reticuloendothelial system (RES). In contrast to monocyte-derived macrophages, KCs reside exclusively intravascularly and do not migrate[23]. In the absence of liver injury, KCs act primarily as antigen presenting cells that are positioned to sample the portal circulation and induce tolerogenic immune responses[24]. KCs are activated in response to several stimuli associated with chronic liver injury such as viral infection, alcohol, iron deposition, and high fat diet. Once activated, KCs are sources of important growth factors, cytokines, and chemokines that activate HSCs and rapidly recruit macrophages from circulation.

1.3.3 Sinusoidal Endothelial Cells

Sinusoidal endothelial cells (SECs) make up the endothelial lining of the sinusoid and are characterized by fenestrae on their surface which facilitate the filter and exchange of fluids and solutes and particulates between the sinusoidal blood and hepatocytes. SECs have intimately tied signaling feedback loops with HSCs due to their close proximity. For example, maintaining SECs in a quiescent, ‘differentiated’ phenotype relies in part on VEGF secreted by hepatocytes and HSCs. Alternatively, SECs share complex paracrine communications with HSCs and can

dramatically influence the ability of HSCs to remain quiescent or become activated. For example, Kruppel-like factor 2 (KLF2) expression in SECs triggers vasodilator, antithrombotic and anti-inflammatory genes that deactivates HSCs in co-culture[25]. During chronic liver injury, SECs lose their fenestrations in a process known as capillarization[25]. Structurally, HSCs are wrapped around SECs and increased contractility of HSCs that accompanies their activation along with decreased nitric oxide production from SECs during liver injury can lead to vasoconstriction and raise portal pressure.

SECs also play central roles in intrahepatic angiogenesis, the creation of new blood vessels from existing ones, This often occurs in tandem with fibrogenesis and serves several pathologic functions that propagate further hepatic fibrosis including the alleviation of hypoxia from expanding myofibroblast populations, the facilitation of movement of infiltrating inflammatory cells, and (in the case of cirrhosis/HCC) oxygenation of expanding cords of hepatocytes[26]. Angiogenesis is particularly relevant to the pathogenesis of HCC, discussed further below (Section 5.3.1). SECs are therefore a key consideration in any cell-based targeting approach to modulating liver injury.

1.3.4 Hepatocytes

As the primary parenchymal cells of the liver, hepatocytes play many roles in response to chronic liver injury and are the targets of many hepatotoxic agents and pathogenic processes. Chronic liver diseases promote compensatory hepatocyte regeneration and cell death (apoptosis/necrosis) that triggers the activation of quiescent HSCs through the release of ROS, DAMPs, cytokines & growth factors to influence myofibroblast activation [27, 28] and proliferation [29 30]. Among the growth factors secreted by hepatocytes that play an important role in HSC activation are vascular endothelial

growth factor (VEGF), insulin-like growth factor 1 (IGF-1)[31], and PDGF (see Figure 3 and Table I). In addition, hepatocytes are a major source of matrix metalloproteinases (MMP-2, MMP-3, and MMP-13) and tissue inhibitors of matrix metalloproteinase (TIMP-1 and TIMP-2) which are integral parts of the remodeling of fibrotic tissue during chronic liver injury[32].

Robust hepatocyte regeneration and clearance of necrotic hepatocyte cell debris and apoptotic bodies are thought to be important processes in recovery from liver injury – both for the restoration of liver metabolic function as well as mitigation of pro-inflammatory and pro-fibrotic signals that can exacerbate liver injury. However, the rapid turnover of hepatocytes as a result of chronic injury stimulus can eventually lead to genomic instability and dysplasia and (in some cases) can progress to cirrhosis or even HCC.

1.3.5 Cholangiocytes

Under physiologic conditions, cholangiocytes lining the bile ducts of the biliary tree of the liver actively contribute to the volume and composition of biliary secretions under various hormone-regulated events. Initially following injury, cholangiocytes become activated and begin to proliferate in order to compensate for loss of biliary epithelial cells during injury and retain secretory functions. In response to injury, cholangiocytes undergo a neuroendocrine-like transdifferentiation in which cholangiocytes synthesize and react to a number of neuroendocrine factors including secretin, VEGF, follicle-stimulating hormone (FSH), histamine, estrogens, and others (see Maroni et al for review[33]). These factors not only sustain cholangiocyte proliferation but modulate immune response, the angiogenesis and structural changes associated with ductular reaction, and the progression of hepatic fibrosis.

1.3.6 Portal Fibroblasts

Cholangiocyte injury during biliary fibrosis is also associated with the activation of portal fibroblasts. These cells are a heterogenous group of peri-portal fibroblasts that have been shown to contribute substantially to the myofibroblast population of biliary fibrosis – particularly in early stages of cholestatic liver injury. Lineage tracing studies in mice following BDL show that the contribution of portal fibroblasts to the overall myofibroblast (Col1a1-positive) population may be up to 73% at 5 days post-BDL and 49% at 17 days post-BDL[16]. The relative contribution of portal fibroblasts to the myofibroblast population decreases with progressive liver injury, with HSCs resuming the role of main precursor to myofibroblast differentiation at later BDL timepoints (20 days post-surgery)[16]. Due to this phenomenon, portal fibroblasts have been described as "first responders" for the initiation of fibrosis following biliary injury[34]. The varying contribution of portal fibroblasts to the myofibroblast population in BDL underscores the need to evaluate biliary fibrosis separately from other forms of liver injury (ex: hepatotoxicity) when trying to identify potential therapeutic targets in chronic liver injury.

1.3.7 Inflammatory Cells

Liver injury is accompanied by infiltrating immune cells which have a variety of pro- and anti-fibrogenic effects through their effects on HSCs, ECM, and other resident liver cells. For example, monocyte-derived macrophages can propagate fibrosis by promoting NF-κB mediated HSC survival through the release of IL-1 and TNF. In contrast, these macrophages can also assist in fibrosis resolution by the release of ECM degradation mediators MMP-12 and MMP-13, as well as the killing of HSCs through the release of TNF-related apoptosis-inducing ligand (TRAIL)[35].

Natural killer (NK) cells are also involved in HSC killing through FasL and TRAIL. B Lymphocytes also play an anti-fibrotic role through an Ig-independent mechanism that reduces ECM production but does not reduce the number of myofibroblasts[36].

In addition to exerting effects on HSCs and fibrogenesis, inflammatory cells are central to the removal of cell debris from necrosis. In particular infiltrating hepatic macrophages are important phagocytic cells that aid in the resolution of fibrosis by removing cell debris from dying hepatocytes, HSCs, and other inflammatory cells that can otherwise stimulate fibrotic activity[37].

1.4 PDGF SIGNAL TRANSDUCTION

PDGFs are cysteine-knot-type growth factors that have been identified as four different disulfide-bonded polypeptide chains (A, B, C, D) which form five known dimer configurations: AA, AB, BB, CC, DD (see Fig. 1) [38-41]. Each of these ligand dimers binds differentially to PDGFRs: type III receptor tyrosine kinases (RTK) that possess five extracellular IgG domains and an intracellular kinase domain separated by a transmembrane helix [42]. PDGFRs exist as α or ß monomers in the plasma membrane that are bound by dimeric PDGF ligands simultaneously to form αα, αß, and ßß receptor dimers, and upon binding trigger reciprocal tyrosine phosphorylation of specific residues of each receptor [43, 44]. Phosphorylation of tyrosine residues in the kinase domain increases catalytic efficiency and serves as binding sites for signaling molecules including other kinases as well as non-enzymatic adaptor molecules.

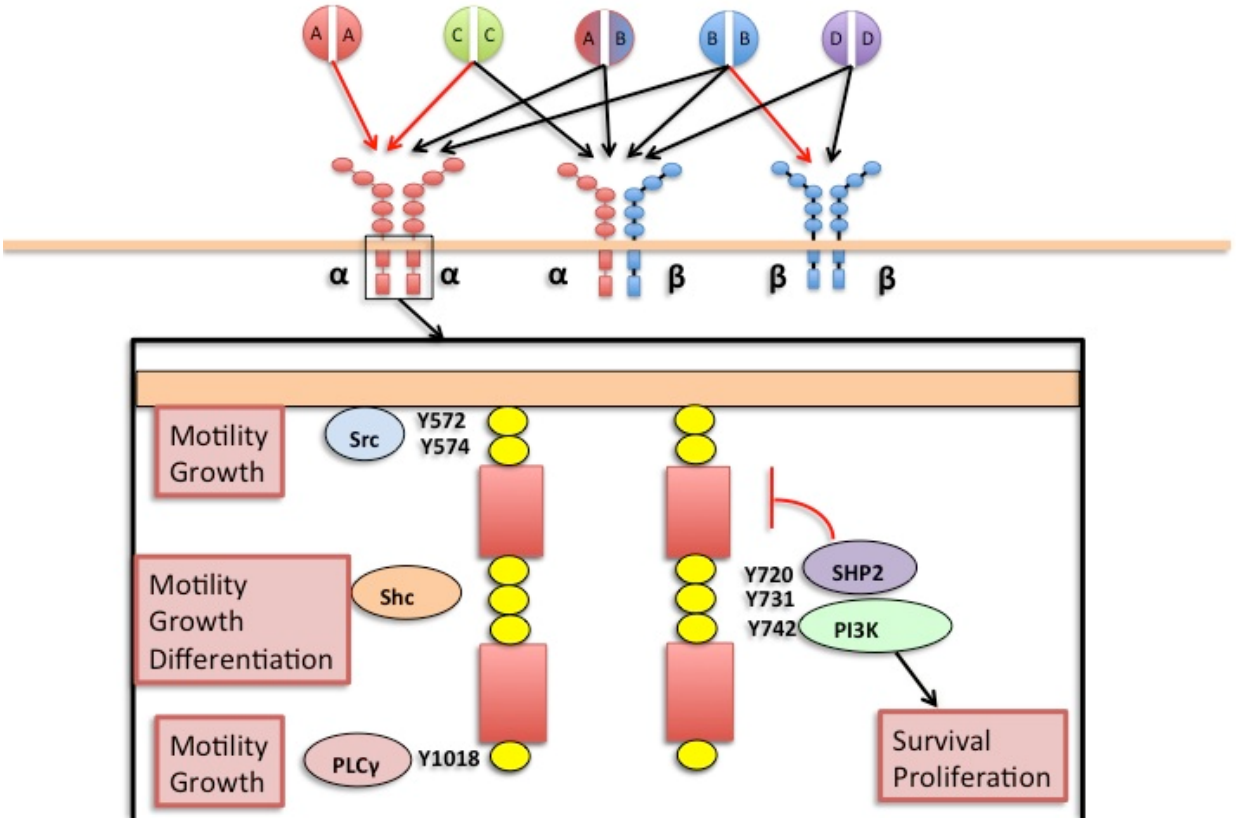


Figure 1: ***PDGF Signaling Pathway.*** Differential binding of PDGF ligands to PDGFRs highlighting key tyrosine phosphorylation residues of the intracellular kinase domain of PDGFRα homodimer and their downstream effectors (boxed insert). Red arrows: known *in vivo* ligand binding. Black arrows: documented *in vitro* ligand binding only. Red bracket: autoinhibitory activity (SHP2). Not included are Tyr 754, and Tyr849, which are also signaling tyrosine residues.

PDGFRα and PDGFRß have distinct but overlapping sets of ligands and downstream effectors. While the differences between PDGFRα and PDGFRß function in various cell types are likely primarily due to their spatiotemporal pattern of expression, there are some discrete differences between α and ß forms as Crk binding is specific to PDGFRα and PDGFRαß heterodimers confer increased mitogenicity compared to α and ß homodimers due to sustained activation of Ras and Erk2 [45]. However the physiologic roles of PDGFRαß dimers are not yet clear. Downstream effectors of PDGFRα signaling include enzymes such as PI3K, MAPK, PLCγ,

Src, and Shp-2, as well as non-enzymatic adaptor molecules such as Crk, Shc, and Grbs. These downstream mediators are important for a variety of cell processes including proliferation, cell survival, cell growth, and differentiation (see Fig. 1). Specific downstream mediators and tyrosine residue phosphorylation sites involved in PDGFR signaling have been previously reviewed [39, 44].

1.5 PDGFRα IN LIVER DEVELOPMENT

Studies of both PDGFRα and PDGFRß have demonstrated that they are essential in embryonic development. Mice lacking either PDGFRα or PDGFRß are embryonic lethal [46, 47], with PDGFRα homozygous null mutant embryos showing incomplete cephalic closure and apoptosis of migrating neural crest cells as well as skeletal and vascular abnormalities. In the context of embryonic livers, PDGFRα is present in important mesenchymal and mesothelial subpopulations that modify the microenvironment to support developmental processes. For example, PDGFRα may mark an important population of mesenchymal progenitor cells that promote hepatoblast differentiation through direct contact and growth factor secretion. These cells, isolated by expression of Dlk-1 and PDGFRα from embryonic day 13.5 (E13.5) murine livers, show direct and indirect effects on hepatoblast maturation through direct contact and transwell co-culture experiments, respectively [48]. This study is consistent with previous evidence of mesenchymal stem cell isolation using PDGFRα [49] as well as mesenchymal-supported hepatoblast maturation [50]. Thus, PDGFRα may mark a small, but active subpopulation of mesenchymal stem/progenitor cells that indirectly influence the development of hepatoblasts in fetal liver development.

Consistent with a supportive role of PDGFRα+ cells in hepatoblast development, PDGFRα+ stromal cells in murine fetal liver were also found to be necessary for erythropoiesis

[51]. In this study, it was shown that the PDGFRα+ fraction of murine fetal liver is necessary for the expansion of erythrocyte progenitor colonies *in vitro*, while maternal injection of anti-PDGFRα monoclonal antibody led to inhibition of erythropoiesis. In addition, exogenous PDGF-AA and PDGF-BB stimulated erythropoietin (EPO) production in fetal liver cells. These studies demonstrate an important role of PDGFRα signaling in EPO production and hematopoiesis in the liver, though a specific relationship between PDGFRα signaling and EPO production was not elucidated in this study.

While traditionally considered a receptor of mesenchymal cells, we observe both cytoplasmic and perinuclear expression of PDGFRα in a subset of epithelial cells during mouse embryonic liver development with peak expression from E10 to E12 [52, 53]. In contrast to the mesenchymal cell profiles from isolated PDGFRα+ cells reported by others [48], we show that a subset of HNF4α+ hepatoblasts from embryonic liver tissue express PDGFRα and that inhibition of PDGFRα signaling in embryonic liver cultures results in decreased survival and proliferation of these cells. This could be a cumulative effect of PDGFRα suppression in various aforementioned cell types. Following this mid-gestational period, PDGFRα expression dramatically decreases throughout murine fetal liver development and remains low in adult murine liver.

In combination with previous findings that PDGFRα marks a population expressing mesenchymal markers, the finding of PDGFRα in a subset of hepatoblasts brings to light the possibility that this receptor may be expressed in epithelia developing from a mesenchymal subpopulation - a process known as mesenchymal-to-epithelial transition (MET). Such an occurrence has been previously been reported in mouse hepatic stem cells *in vivo*, which co-express markers of both epithelial (CK8/18) and mesenchymal (vimentin) markers at similar

embryonic timepoints [54]. In fact the mesenchymal population characterized in this study was isolated based on intermediate expression of Dlk-1, a known marker of hepatoblasts and (at low expression) also a marker of mesothelial precursors [55]. The contribution of mesenchyme to a subset of hepatoblasts and eventually to hepatocytes was also supported more recently by the fact that vascular endothelial growth factor receptor 2 (VEGFR2) – a known mesodermal marker – was also expressed in hepatic progenitors capable of contributing to a substantial portion of adult parenchyma shown by lineage tracing studies [56].

PDGFRα expression was also identified in mesothelial and submesothelial cells of E12.5 murine livers, which were proposed to be precursors of hepatic stellate cells [57]. As with the above-mentioned studies, PDGFRα was used primarily as an identifying marker and a specific role of PDGFRα signaling was not elucidated. In the case of PDGFRα expression in mesothelial and submesothelial cells of the liver, it can be speculated that PDGFRα plays a pro-proliferative response which may be important for expansion of this HSC precursor population during development.

The presence of PDGFRα in mesenchymal, mesothelial, and epithelial cells of the developing liver may provide insight on its importance in adult liver pathophysiology. For example, the expression of PDGFRα in mesothelial precursors of HSC including 'sub-mesothelial cells' and their transitional cell counterparts [57], as well as it's potential expression in a subset of hepatoblasts, may signify that PDGFRα is serving as part of a modulatory proliferative transcription program which is upregulated in liver development and pathology while being suppressed in quiescent, non-proliferative states. Further investigation of the effects of PDGFRα inhibition in an *in vivo* or *ex vivo* developmental context will help to shed light on the function of this receptor in supporting hepatoblast maturation, erthyropoiesis, or mesothelial/submesothelial

migration and HSC formation. Eventually since tumorigenesis often represents reawakening of the developmental programs that may contextually encompass epithelial-to-mesenchymal transition, PDGFRα modulation may provide novel therapeutic opportunities in HCC.

1.6 PDGFRα IN LIVER REGENERATION

Our lab has previously investigated the role of PDGFRα in liver regeneration using the well-known 2/3 partial hepatectomy (PH) model in which 2/3 of the liver mass is surgically removed and compensatory regeneration is subsequently studied at discrete, well characterized timepoints [58]. In control mice, PDGFRα activation was evident at 3 hours although its total levels were unequivocally elevated at 24 hours. For further studies, we first generated mice lacking PDGFRα in hepatocytes (Albumin-cre excision of floxed *Pdgfra*). These mice were indistinguishable from their littermates. When subjected to PH, an initial delay in Akt signaling by 3 hours post-PH was soon offset by upregulation of EGFR and hepatocyte growth factor receptor Met. Both epidermal growth factor receptor (EGFR) and Met have been shown to be crucial mediators of normal liver regeneration [59]. In combination with previous findings of *Pdgfra* and *Pdgfa* upregulation in rats during shRNA-mediated inhibition of EGFR following 24 hour PH, our results suggests a potential reciprocal regulation between PDGFRα and EGFR [60]. These studies exemplify the well-known phenomenon of growth factor signaling compensation in liver regeneration [58]. Rather than diminish the importance of the PDGFRα signaling axis in hepatocyte regeneration in this model, these results attest to the signaling 'flexibility' that is a well-recognized theme in PH. Similar to most growth factors in liver regeneration following PH, ligands of PDGFRα appear to play a significant, but replaceable role.

PDGF ligands including ligands for PDGFRα, are generally known for their mitogenic effects in mesenchymal-derived stromal cells of the liver. There is however important evidence that hepatocytes themselves may respond to PDGFs. A recent study that examines the effect of growth factors on murine hepatocytes reveals a modest but significant and direct mitogenic effect of PDGF-AB on primary murine hepatocytes [61]. The importance of this finding is underscored by the fact that prior to this study, only HGF and ligands of EGFR were identified as direct mitogens on primary hepatocytes in chemically defined medium [58]. Evidence of PDGF-induced mitogenesis of hepatocytes *in vitro* or *in vivo* in the context of liver regeneration is sparse at this time. However, due to the increasing emergence of PDGFRα signaling as a therapeutic target in pathologic liver states (see below), the elucidation of regenerative hepatocyte PDGFRα signaling may be important to fully interpret the effects of therapeutic PDGFRα inhibition. Together, these studies suggest that PDGFRα signaling may occur in the hepatic parenchyma during liver regeneration – possibly contributing to mitogenesis. This is in contrast to models of chronic liver injury (discussed below) where PDGFRα seems to be located primarily in the NPCs.

1.7 PDGFRα IN LIVER FIBROSIS

Findings in the literature regarding PDGFRα signaling in chronic liver injury strongly support a pro-fibrotic role of this receptor. However, cell-specific studies of PDGFRα designed to delineate the function of this receptor in individual cell populations in the liver are currently lacking. Different resident cell populations play potentially antagonistic pro- or anti-fibrotic roles in the setting of chronic liver injury[62] and it is therefore conceivable the biologic endpoints of PDGFRα signaling (ie survival, proliferation) may contribute to injury progression in specific cells (ex:

activated HSC) while ameliorating injury in others (ex: hepatocytes). In addition our knowledge of downstream targets of PDGFRα signaling in liver pathogenesis is limited and elucidation of these will be important for the identification of potential therapeutic targets.

The role of PDGFRα signaling in the setting of fibrosis is currently unclear, as many studies present compelling data leading to differing conclusions on its contributions and relative importance compared to its related isoform PDGFRß in HSC activation and proliferation. In the following sections, we discuss some of the evidence for the localization and function of PDGFRα in the fibrotic liver, highlighting conflicting results and interpretations in the literature.

1.7.1 Relative Contributions of PDGFRα vs. PDGFRß in HSC Activation: Reconciling the Evidence

Though PDGFRß has long been established as a functional marker of activated HSCs [63], PDGFRα has only recently emerged as a potential mediator of HSC activation in hepatic fibrosis. Early studies of PDGFR isoforms in HSC emphasized the importance of PDGFRß due to the upregulation of this isoform at mRNA and protein level in contrast to the constant levels of PDGFRα reported following carbon tetrachloride (CCl_4) or bile duct ligation (BDL)-mediated injury in rats [64]. Over the next couple of decades, PDGFRα expression in HSCs of fibrotic livers became increasingly evident. PDGFRα mRNA is highly expressed in α-smooth muscle actin (αSMA) positive NPCs of cirrhotic human livers localized in the perisinusoidal region [65]. This study also showed that PDGFRα is upregulated in stromal and sinusoidal cells in human livers during cirrhosis and reported a strong correlation between expression of PDGFRα and PDGFRß in human livers to the histology activity index (HAI, Knodell's score) and type III collagen

deposition [65]. These findings were subsequently affirmed when PDGFRα upregulation was also observed in whole cell lysates of rat livers treated with CCl_4 [66], and has most recently been confirmed in the murine BDL [67] and CCl_4 models [68]. The exception of this trend is a study in BDL rats, indicating a potential difference in PDGFRα signaling role in toxic and cholestatic fibrosis models (discussed further below) [69].

Findings from studies of PDGF signaling in isolated rat HSC and culture activated myofibroblasts indicate that PDGFRαα homodimer is not likely to be the primary PDGFR isoform involved in HSC activation/proliferation as evidenced by studies showing that culture-activated HSCs showed selective proliferative response to PDGF-B and PDGF-D isoforms and lacked mitogenic response to PDGF-AA (specific for αα homodimer, see Fig. 1) [69]. There is however some discrepancy between findings in this model system as an earlier study showed a small but significant (2-3 fold) proliferative effect of PDGF-AA [70]. Of particular importance is a study that noted a comparable level of PDGF-AA-induced mitogenicity in HSC lines isolated from patients [71]. This study also showed that PDGF-AA may help activated HSCs overcome proliferative inhibition from ECM molecules such as collagen I. It is worth mentioning that part of the discrepancy between the mitogenic responses of HSCs to PDGF-AA between studies may be related to the specific concentration of ligand used. The studies showing mitogenicity of PDGF-AA in rat [70] and human derived HSCs [71] both showed maximal proliferative stimulation of HSCs at 10ng/mL PDGF-AA. In contrast, the study of rat culture-activated HSC, which showed no effect of PDGF-AA only utilized a single and higher concentration (50ng/ml).

Despite the relatively minor role of PDGFRα in proliferation of culture activated HSCs, Hayes et al recently showed that PDGFRα is upregulated in HSCs following CCl_4-mediated fibrosis in mice and that activation of PDGFRα may contribute to hepatic fibrosis since fibrosis

was reduced following CCl_4-mediated injury in mice heterozygous for PDGFRα [68]. While previous studies have reported PDGFRα expression in HSC from animals [64, 66] and patients [65], the study from Hayes et al is the first report indicating that genetic reduction of PDGFRα signaling *in vivo* reduces hepatic fibrosis in chronic liver injury, thus paving the way forward for possible therapeutic inhibition.

What are possible explanations for the seeming discrepancies of the profibrotic contribution of PDGFRα signaling between culture-activated HSC and murine HSCs heterozygous for PDGFRα? The answer is unclear at the moment, but may involve one or more factors, including (i) an effect on PDGFRαß heterodimer expression, (ii) a lesser role of PDGFRαα signaling in HSC activation/proliferation, and (iii) differences in receptor isoform signaling function. With regards to (i), PDGFRαß heterodimer is not known to interact with PDGF-AA (Fig. 1) but still requires PDGFRα expression. If PDGFRαß is playing an active role in HSC activation, PDGFRα might only contribute to HSC proliferation and myofibroblast activation through its ability to complex with the ß receptor. This explanation is consistent with the findings of close PDGFRα and PDGFRß co-localization in fibrotic livers [65, 68], as well as the presence of PDGFRα phosphorylation in chronic liver injury ([67], unpublished observations).

While PDGFRαß heterodimer function is a plausible explanation for these studies, PDGFRα is still likely to contribute to HSC activation through (ii) its homodimer form as PDGF-AA (a ligand specific to PDGFRαα homodimer) did show a significant, albeit lesser, effect on proliferation/intracellular calcium in culture-activated HSCs. Furthermore, transgenic mice overexpressing PDGF-A in hepatocytes spontaneously develop fibrosis [72]. This study lends strong support to the notion that PDGF-AA/PDGFRαα signaling alone is at least sufficient to initiate hepatic fibrosis in mice – though whether hepatic fibrosis is propagated by active PDGFRαα

signaling, or is reliant on the subsequent autocrine or paracrine release of other factors (such as other PDGF isoforms) is not assessed in this study.

Finally, future investigations should consider that (iii) downstream signaling functions of PDGFRs in HSCs may be isoform specific. Studies of chemically-defined mouse embryonic fibroblasts have shown that PDGFRαα, αß, and ßß activate distinct downstream signaling pathways [73]. Primary cell culture studies to determine differences of PDGFRα and PDGFRß signaling in genetically PDGFR-defined HSCs or myofibroblasts may ultimately be necessary to fully understanding the specific roles of PDGF/PDGFR in HSC activation, survival, or proliferation.

The development of several transgenic murine models overexpressing specific forms of PDGF in hepatocytes under albumin promoter-controlled transgene expression support a potential role of PDGFRα in the development of fibrotic changes in the liver. In addition to transgenic overexpression of PDGF-A (described above), PDGF-B overexpression in hepatocytes leads to development of spontaneous fibrosis in mice [72, 74]. Similarly, the overexpression of PDGF-C, a known ligand of both PDGFRα and PDGFRß, in transgenic mice leads to the spontaneous development of liver fibrosis, steatosis, and HCC [75]. Both PDGFRß and PDGFRα were upregulated in whole liver lysates in this model. In addition, PDGF-C overexpression in hepatocytes causes expansion of NPC populations including sinusoidal endothelial cells and activated HSCs [76], supporting potential PDGFRα expression in both of these populations. It should be noted however that neutralization of PDGF-C in other murine strains by genetic knockout or neutralizing antiserum does not confer protection to BDL-induced liver injury [67]. Data from this study indicates that PDGF-C may primarily mediate its fibrotic effects through PDGFRß rather than PDGFRα, as PDGFRß mRNA and total/phosphorylated protein level - not PDGFRα - is

exclusively upregulated in response to PDGF-C neutralization. These authors confirm that this is not due to differential expression of other PDGF isoforms. Thus, at least in the context of murine experimental biliary fibrosis it appears that PDGFRß, not PDGFRα, is the primary activated receptor in response to PDGF-C in a pathophysiological (non-overexpressed) setting. Nevertheless PDGFRα is still substantially upregulated and phosphorylated in these settings, indicating activation of this receptor in biliary fibrosis.

Evidence suggesting the presence of PDGFRα in HSC and activated myofibroblasts sheds new light on much of the current literature regarding PDGFR signaling in HSCs and activated myofibroblasts in hepatic fibrosis/cirrhosis – the majority of which focus exclusively on assessment of PDGF-BB/PDGFRß signaling. In light of the fact that PDGF-BB activates both PDGFRα and PDGFRß, much of this data can be reinterpreted to consider a potential contribution of PDGFRα isoform.

1.7.2 TGF-ß/PDGFRα Crosstalk in HSCs

Thus far, we have primarily considered only ligand-dependent mechanisms of PDGFR activation in hepatic fibrosis. However, a recent study sheds new light on a potential ligand-independent role of PDGFRα in HSCs. Liu et al show compelling evidence that PDGFRα appears to be necessary for SMAD2 signaling downstream of TGF-ß receptor in human HSCs *in vitro* [77]. This was demonstrated through the shRNA knockdown of PDGFRα in human HSC and HSC cell line LX-2, which led to a decreased RNA expression of TGF-ß receptor I (TßRI) and SMAD2 phosphorylation activity of TGF-ß receptor II (TßRII). SMAD-2 is a key mediator of fibrosis in myofibroblasts in the setting of acute and chronic liver injury [78], indicating a potential role of PDGFRα in this important arm of TGF-ß signaling. This study brings to light a novel mechanism

of indirect PDGFRα activation triggered by interaction of PDGFRα with TßRII (summarized in Fig. 2). The modulation of PDGFRα expression in response to TGF-ß is consistent with previous findings in other fibroblast populations including scleroderma skin fibroblasts [79]. Though PDGFRα activation in the absence of direct ligand binding has been previously reported [80], this is the first report indicating that PDGFRα is necessary for a major fibrotic signaling pathway in the liver. Combined with previous studies showing TGF-ß-induced PDGFRα in a Ras-mutant murine hepatocyte model of EMT[81], there may be a reciprocal regulation between TGF-ß and PDGF signaling.

Experimental RTK inhibitors often function by preventing the activating interaction of ligands and their receptors, either by binding ligands or receptors themselves to prevent phosphorylation. The findings by Liu et al suggest that PDGFRα may function in chronic liver injury not only through RTK autophosphorylation following ligand binding, but also through a ligand independent mechanism involving monomeric PDGFRα. Further investigation of the extent of the latter form of PDGFRα signaling *in vivo* will be particularly relevant to predict the effectiveness of targeted PDGFRα inhibitors, which may only prevent ligand binding. Ligand-independent PDGFRα activation has been shown previously in the setting of proliferative vitreal retinopathy in which mitochondrial ROS triggers the activation of Src family kinases (SFK) leading to phosphorylation of monomeric PDGFRα [82]. In another example, the PDGFRα-specific inhibitor Olaratumab (discussed further below) failed to inhibit bone marrow-induced Akt activation in metastatic prostate cells *in vitro* and *in vivo* as a result of ligand-independent transactivation of PDGFRα [83, 84]. Despite these examples, exclusive monomeric activation of PDGFRα during liver injury is unlikely due to the overwhelming evidence that PDGF ligands play a central role in the initiation and progression of fibrosis [53, 72, 74, 75, 85]. Thus it is likely that ligand-

independent PDGFRα activation through TGF-ß signaling arm is only one mode of activation of downstream PDGFRα signaling.

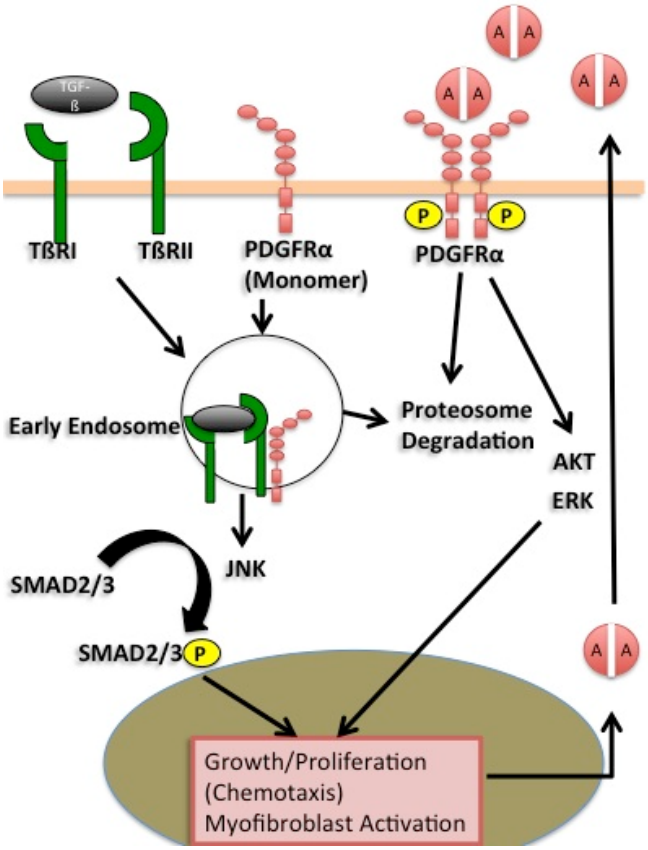


Figure 2: ***TGF-ßR/PDGFRα Signaling Crosstalk in HSC.*** Ligand dependent and independent signaling pathways are shown. During ligand-independent signaling, PDGFRα is recruited to TßRI/TßRII complexes by TGF-ß stimulation. Through interaction with TßRII, PDGFRα promotes internalization and trafficking of TGF-ß receptors into the early endosomes, where phosphorylation of SMADs occurs and TGF-ß signaling is activated. Knockdown of PDGFR- blocks endocytosis of TGF-ß receptors, thereby inhibiting phosphorylation of SMADs. Activation of SMAD2/3 has been shown to lead to the upregulation of PDGF-A mRNA, which may indicate an autocrine mechanism of PDGFRα activation in HSC[71, 86]. Abbreviations: TGF-ß receptor II (TßRII). Adapted from [77].

While the full nature of PDGFRα signaling in this model is not elucidated in this study, previous studies suggesting PDGF-induced activation of SMADs may offer insight. Treatment of rat primary HSC in culture with TGF-ß results in a selective increase of PDGF-A mRNA expression [87], which may implicate an autocrine activation of PDGFRα in HSC (see Fig. 2). It has previously been shown that co-treatment of cultured HSC with TGF-ß and PDGF (unspecified

isoform) leads to c-Jun N-terminal kinase (JNK) mediated activation of SMAD2/3 [88]. *In vitro*, high TGF-ß1 concentrations in a study of aortic smooth muscle cells and fibroblasts were shown to cause differential modulation of PDGF-AA (increased) and PDGFRα (decreased) [86] responsible for an inhibition of PDGF-AA mediated growth. In light of the study by Liu et al, it is interesting to speculate that in addition to transcriptional regulation of PDGF-A and PDGFRα by TGF-ß, posttranscriptional regulation may also be occurring via direct binding and internalization of PDGFRα by ligand-activated TßRs in aortic smooth muscle cells and fibroblasts of the aforementioned study.

1.7.3 PDGFRα in Portal Myofibroblast Activation During Cholestatic Liver Injury

PDGFRα expression in cholestatic liver injury was initially reported in myofibroblasts isolated from mice subjected to BDL at various timepoints [64]. However, in contrast to PDGFRß, PDGFRα mRNA was not upregulated following BDL. Similarly, a more recent study of PDGFR and PDGF expression following BDL in rats shows that PDGFRα protein expression remains relatively unchanged, or only slightly elevated [69], in contrast to increased PDGFRß expression.

Portal fibroblasts (PFs) are thought to play an important role in the initiation of fibrosis following cholestasis, particularly in early response to biliary injury (see section 1.3.6) [17]. The question of whether PFs possess PDGFRs and are responsive to PDGF signaling is unclear at this time due to contradictory reports. PDGF-BB-mediated expansion of isolated peribiliary fibroblasts from rats that have undergone BDL express functional PDGFRß that contributes to myofibroblastic differentiation as measured by α-SMA expression [89]. In addition, peribiliary myofibroblast conversion as measured by α-SMA was reduced upon treatment with PDGFR inhibitor STI571 (Gleevec). On the other hand, primary rat PFs isolated by Wells et al show no

mitogenic activity in response to PDGF-BB stimulation *in vitro* [90]. Li et al demonstrate an interesting dichotomous effect of PDGF-BB on isolated rat PFs: exposure to PDGF-BB inhibited differentiation of PFs as measured by α-SMA but promoted proliferation [91] on collagen I-coated polyacrylamide gel supports. Finally, a study of murine BDL-derived activated PFs demonstrated that these fibroblasts were unresponsive to PDGF in contrast to HSCs [17].

Thus, the range of PDGFs and their receptors expressed in PFs during biliary fibrosis requires further investigation in order to elucidate the potential autocrine or paracrine mechanisms of PDGF signaling in this population. While PDGFRα and PDGFRß have been previously reported in isolated HSCs during cholestatic liver injury [64], the absence or presence of PDGFRα in PFs prior to myofibroblastic changes remains unknown, as (to our knowledge) only PDGFRß has been confirmed in isolated PFs [89]. This will be an interesting question to address in future studies, since PFs actively contribute to the myofibroblast population in early cholestatic injury [17] and biliary fibrosis and can be attenuated by targeted inhibition of PDGF signaling [92].

1.7.4 PDGFRα Cellular Localization: Expression Patterns in Chronic Liver Injury

As an autocrine and paracrine signaling factor, insight on the actions of PDGFRα signaling may be elucidated by their cellular localization in normal and pathogenic liver states. Localization of PDGFRα is most clearly demonstrated in NPCs of the liver including HSC and EC. Early reports of PDGFRα localization in normal and cirrhotic human livers identify PDGFRα expression in stromal cells of portal tracts as well as some sinusoidal EC and EC of the centrilobular veins [64, 65]. Another group reported that mice with thioacetamide (TAA)-induced liver injury showed upregulated PDGFRα localizing in a sinusoidal pattern and in NPC [85]. Consistent with a sinusoidal pattern of expression in cirrhosis, PDGFRα is overexpressed in EC of HCC associated with high

metastatic potential [93] and increased recurrence in patients. This is in line with evidence that tumor fibroblasts may become resistant to anti-VEGF therapy through the expression of PDGF-C [94]. While one recent study denied the expression of PDGFRα in EC during CCl_4-mediated liver injury in mice [68], it should be noted that this conclusion was based on lack of co-localization with CD31 (PECAM), whose expression is low in liver sinusoidal EC (LSEC) following CCl_4 treatment and thus may not be a sensitive marker in this model[95].

Currently the cellular localization of PDGFRα in hepatocytes during chronic liver injury is unclear. In situ immunostaining of human normal and cirrhotic liver shows no PDGFRα expression in hepatocytes [65]. A recent study also reported an absence of hepatocyte PDGFRα in a murine CCl_4 model [68]. In contrast, PDGFRα is reportedly upregulated in regenerating rat hepatocytes following CCl_4-mediated fibrosis [66] and contrary to the reported findings of other labs, we have found low level expression of PDGFRα in hepatocytes of human and murine liver [53]. Further support for the presence of PDGFRα in hepatocytes stems from the finding that isolated murine hepatocytes proliferate in response to PDGF-AB in a chemically defined serum free growth media, and PDGF-AA or PDGF-BB exposure increases bromodeoxyuridine staining in these hepatocyte cultures [61]. In contrast, another group has reported that primary hepatocytes in culture do not respond to PDGF-CC[75]. It should be noted that hepatocytes are heterogenous and different subpopulations (for example periportal versus pericentral) may express different receptors due to their differing metabolic roles or depending on the zonality of liver injury. Therefore, some subpopulations of hepatocytes may specifically upregulate PDGFRα/PDGFA signaling over others, as was evidenced in rat livers subjected to CCl_4 in which pericentral hepatocytes selectively expressed PDGF-A [66].

It remains undetermined whether a potential upregulation of PDGFRα in hepatocytes would be a reparative or pathologic response in chronic liver injury. Considering that hepatocyte survival and proliferation play crucial roles in liver regeneration and fibrosis, and detrimental roles in injury sequelae such as liver cancer, these findings warrant further investigation of PDGFRα signaling in hepatocytes. Elucidating the contribution of hepatocyte PDGFRα activation in disease pathogenesis, the signaling arms activated and their downstream cellular events will not only improve understanding of the pathobiology of this disease process but will also be relevant in validating PDGFRα as a therapeutic target. For these studies, hepatocyte-specific conditional knockouts of PDGFRα may lend themselves well [52].

1.7.5 PDGF Sources in Injured Liver

During liver injury, PDGFs are secreted by both resident and infiltrating cells of the liver including hepatocytes, Kupffer cells, cholangiocytes, infiltrating macrophages, and HSC themselves (summarized in Fig. 3, Table I). PDGFs from all of these sources likely converge on HSCs to trigger their activation and myofibroblast conversion, as well as proliferation and migration. PDGFs are also likely to exert autocrine effects on cell populations that express PDGFRs in addition to PDGF ligands, such as hepatocytes and activated HSCs. Kupffer cells and infiltrating macrophages are considered one of the primary sources of PDGFs involved in activating HSC. PDGF-B is expressed by infiltrating macrophages and Kupffer cells in patients with chronic hepatitis/cirrhosis, the expression of which correlates with inflammation and severity of fibrosis [96]. Hepatocytes influence HSC activation via activation of Kupffer cells as well as directly through the secretion of PDGFs and other signals during liver injury. Freshly isolated rat hepatocytes have

been reported to express mRNA for PDGF-A and PDGF-C, while PDGF-B mRNA was present in low amounts and PDGF-D mRNA was absent [87].

Perhaps one of the most important sources of PDGFs in fibrosis is from HSCs themselves. Freshly isolated rat HSCs express PDGF-A, B, and low levels of D, while transdifferentiated myofibroblasts (HSC plated for 8 days) also express PDGF-C [87]. Platelets are also known to be important secretory sources of many molecules and growth factors involved in liver regeneration including PDGFs [97]. Supporting this, freeze-dried platelets storing growth factors including PDGF are able to promote hepatocyte proliferation in mice [98].

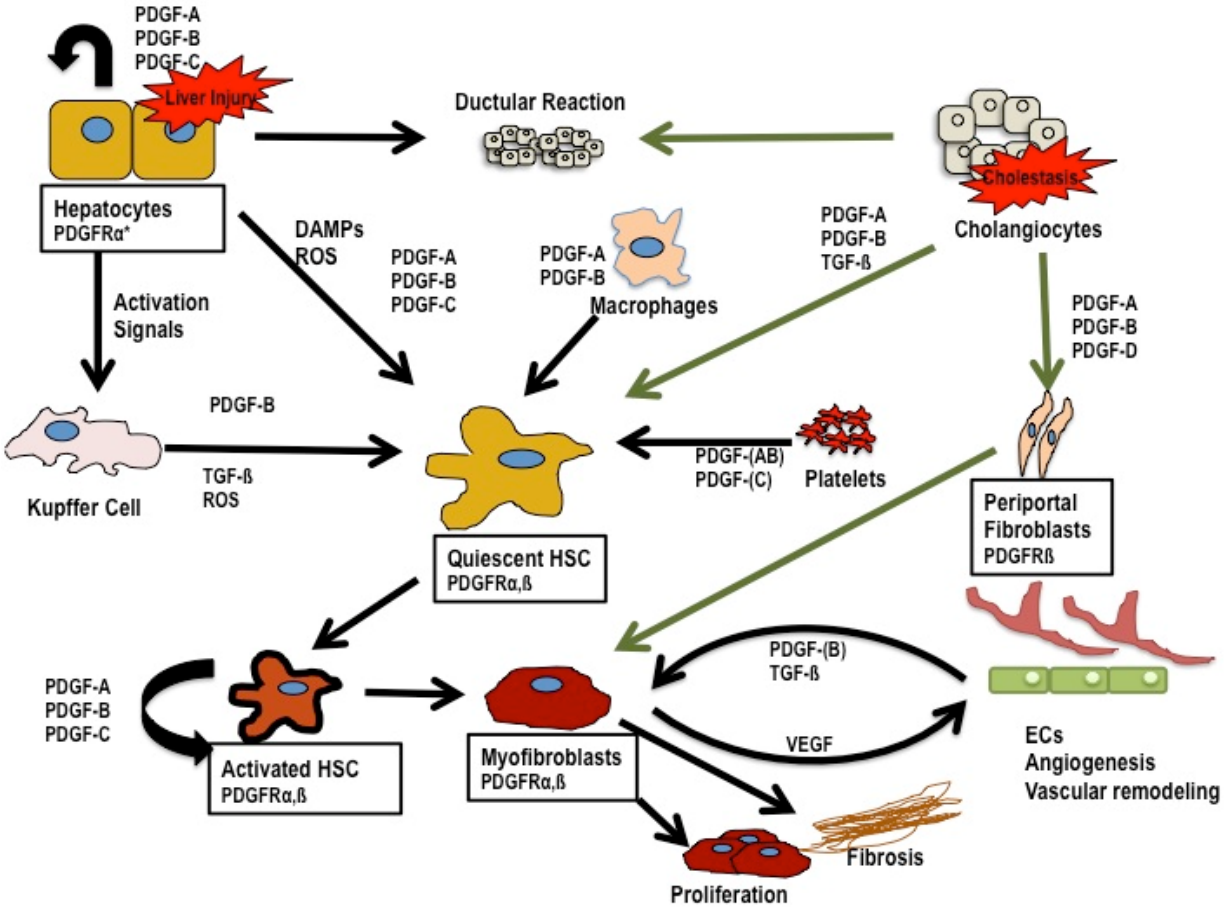


Figure 3: ***PDGF Sources and Cell Interactions in Injured Liver.*** Shown are known or predicted sources of PDGF secretion during liver injury, and potential interactions between resident and infiltrating liver cell populations, during toxic or cholestatic liver injuries. Curved arrows represent potential autocrine stimulation. Green arrows represents events specific for cholestatic liver injury. Abbreviations: damage associated molecular patterns (DAMPs), hepatic stellate cell (HSC), platelet-derived growth factor (PDGF), reactive oxygen species (ROS), transforming growth factor beta (TGF-ß). Asterisk (*) represents discrepancy between studies.

The sources of PDGFs are most likely determined by the origin of the liver injury. In contrast to the lack of cholangiocyte PDGF positivity in tissue specimens from cirrhotic patients [65], cholangiocytes from patients with biliary atresia do demonstrate strong expression of PDGF-AA and PDGF-BB [99].

1.8 THERAPEUTIC INHIBITION OF PDGF SIGNALING IN LIVER DISEASE

Therapeutic inhibition of PDGF signaling focuses on three major approaches: the regulation of PDGF ligands, the inhibition of functional PDGF-PDGFR interactions using tyrosine kinase inhibitors (TKIs), and the inhibition of downstream intracellular signaling kinases. In this section, we focus on the first two approaches which specifically affect PDGF signaling.

1.8.1 PDGF Ligand Neutralization

Attempts to regulate the activity of PDGF ligands have focused on PDGF-B monomer and PDGF-BB dimers due to the prominent role of this isoform as the most mitogenic PDGF ligand towards HSCs[21, 70]. One example of direct targeting of PDGF ligand is the development of MOR8457, a selective PDGF-BB monoclonal neutralizing antibody. Preclinical studies of MOR8457 in mice with biliary fibrosis showed a reduction in hepatic fibrosis and fibrosis-associated gene expression that was comparable or greater than that of similar doses of the non-selective multi-TKI Imatinib[100]. However, it is worth pointing out that these compounds were administered using different methods (weekly i.p. injections for the former, daily oral gavage for the latter). Similar

reductions in fibrosis was achieved using the neutralizing anti-PDGF-B monoclonal antibody AbyD3263 in mice injured via BDL or concanavalin A (ConA)[101].

1.8.2 PDGFR Inhibitors

The development of tyrosine kinase inhibitors has been an important avenue for the research of new treatments for fibrosis and advanced liver disease. Many of the most successful inhibitors to date have co-targeted the components of the platelet-derived growth factor (PDGF) family of ligands and their receptors. While the ß isoform of this receptor: platelet-derived growth factor receptor beta (PDGFRß) has been at the forefront of PDGF signaling in the liver due to its important roles in myofibroblast activation during fibrosis [63, 64], several new studies have shown an emerging role of platelet-derived growth factor receptor alpha (PDGFRα) in liver pathophysiology that may identify this receptor as an important therapeutic target.

Receptor tyrosine kinases are critical pharmacologic targets. Evidence from the development of both small molecule TKIs as well as monoclonal antibody inhibitors support a role of PDGFRα and PDGFRß in cancer and liver injury states such as fibrosis and cirrhosis [13, 102]. PDGFRs are co-targeted by several small molecule pharmacologic agents such as imatinib, sunitinib, and sorafenib, which are multi-tyrosine kinase inhibitors each targeting a discrete set of tyrosine kinases [103].

Many multi-TKIs that target PDGFRs appear to have activity against both α and ß isoforms [93, 104, 105]. As such, it is often difficult to delineate whether specific effects of these inhibitors stem from inhibition of PDGFRα, PDGFRß, or both receptors. Nevertheless, preclinical and clinical studies of multi-TKIs provide important evidence that PDGFRα is a potential therapeutic target in cancer. Imatinib has shown activity in gastrointestinal stromal tumors (GISTs), which do not

express mutations in c-KIT. This activity is likely due to demonstrated inhibition of PDGFRα which is mutated in many GISTs with normal c-KIT [106] and shares adjacent chromosomal location on human chromosome 4 as well as close amino acid homology with c-kit. Sorafenib, a multikinase inhibitor targeting Raf, VEGFRs, and PDGFRα/ß, is currently the only chemotherapeutic which has been shown to be effective in the treatment of HCC [12, 13]. Sorafenib has also been shown to have beneficial effects in animal models of hepatic fibrosis and portal hypertension. Partial portal vein ligation (PPVL) in rats, a model of portal hypertension, showed a decrease in portal pressure and splanchnic inflammation as well as a decrease in TGF-ß, TGF-ßR1, and TIMP2 potentially leading to reduced fibrogenesis [107]. Sorafenib also reduced intrahepatic fibrosis, inflammation, and neovascularization in rats undergoing BDL.

A major impetus for the development of PDGFR inhibitors stems from their role in angiogenesis, as described in the preceding sections. Rats subjected to PPVL experienced decreased splanchnic neovascularization, pericyte coverage of new vessels, portal pressure, superior mesenteric artery blood flow and resistance when treated with a combination of VEGFR inhibitor Rapamycin and PDGFR inhibitor Gleevec compared to treatment with either agent alone [108]. Beneficial effects of combined VEGF/PDGF signaling inhibition in portal hypertension are supported by subsequent studies showing improved hemodynamics in PPVL rats treated with Sorafenib [109]

1.8.3 A Note on the Rationale for PDGFRα –Specific Targeting Inhibitors

The development of specific and potent inhibitors such as Olaratumab that are already in clinical use, may present therapeutic and safety advantages [110, 111] due to reduction in off-target effects that allow for higher dosing. For instance, Olaratumab shows around 100-fold increased effect on

PDGF-mediated cell proliferation compared to the multi-tyrosine kinase inhibitor Imatinib [112]. This potential for reduced side effects is particularly relevant when considering that the current multi-TKIs that co-target PDGF receptors and have shown anti-hepatic fibrosis activity are metabolized by cytochrome P450 (CYP450) enzymes in the liver and are poorly tolerated by patients. Hepatotoxicity is a major limiting factor in the use of these agents – especially those who already have liver dysfunction[113]. The prospects for the therapeutic application of Olaratumab and other PDGFRα –specific inhibitors will be further discussed in Chapter 5, incorporating a discussion of the relevant data included in the intervening chapters.

2.0 PDGFRα LOCALIZATION AND SIGNALING IN MURINE LIVER DURING CHRONIC LIVER INJURY

Due to unresolved questions regarding the cellular origin of PDGFRα in hepatic fibrosis (see Section 1.7.4), we sought to examine the cellular localization of PDGFRα in chronically injured murine livers using co-immunofluorescence confocal microscopy. In the following sections, we present data supporting the expression of active PDGFRα signaling in murine livers following chronic liver injury, and specifically examine the presence (or absence) of this receptor in hepatocytes, cholangiocytes, sinusoidal endothelial cells, HSCs, and myofibroblasts.

2.1 PDGF SIGNALING STATUS IN CHRONIC LIVER INJURY

To examine the relevance of PDGF signaling in chronic liver injury, we utilized two well-characterized models of chronic liver injury: repeated CCl_4 injections and BDL. These models are described in detail in Section1.2 above. In assessing whether PDGFRα may play a functional role in the progression of chronic liver injury, we tested its expression as well as the expression of associated ligands in the presence of chronic CCL_4-induced liver injury.

Following 4 week CCL_4 liver injury, we saw distinct increases in PDGFRα as well as PDGF-A, and PDGF-C ligands in whole liver lysates (Fig. 4A). This increase in expression was further pronounced at the 8 week CCl_4 liver injury timepoint which is consistent with increased numbers of activated HSCs and myofibroblasts associated with advanced fibrotic liver injury

represented by the 8 week time course. Similarly, we also see a corresponding upregulation of PDGFRß – a well-known marker of HSCs.

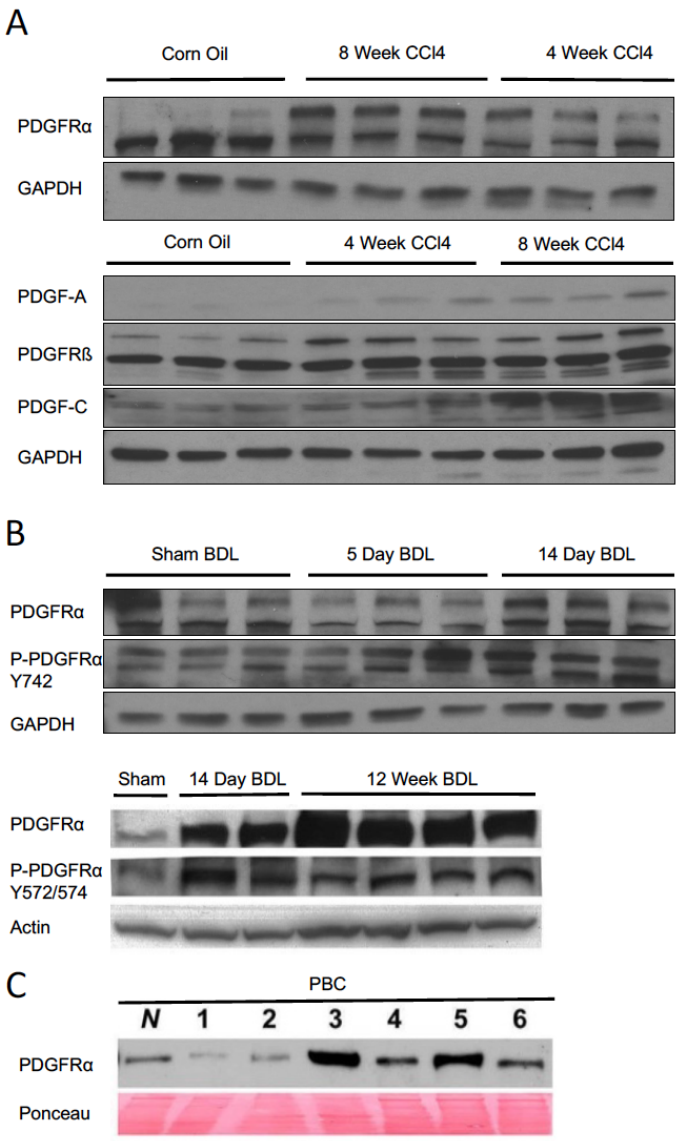


Figure 4: ***PDGFRα Signaling is Upregulated During Chronic Liver Injury.*** **(A)** PDGFRα expression as well as ligands of PDGFRα (PDGF-A and PDGF-C) are progressively upregulated at 4 weeks and 8 weeks of CCl_4-induced liver injury in parallel with PDGFRß compared with corn oil injected controls. **(B)** Similar upregulation of PDGFRα is observed in mice after BDL compared to sham-operated control animals. In addition, increases in activating PDGFRα phosphorylation is detected at both Y742 and Y572/574. **(C)** PDGFRα upregulation is observed in human liver biopsies from patients with primary biliary cirrhosis (PBC) compared to normal liver (N).

To determine whether PDGFRα activity was also upregulated in cholestatic liver injury, we examined its expression in murine livers 5 days and 14 days post-BDL. Compared to sham-operated control animals, post-BDL murine livers had increased levels of PDGFRα as well as phosphorylated PDGFRα at signaling tyrosine residue Y742 (Fig. 4B). Furthermore, analysis of PDGFRα expression in liver tissue from patients with primary biliary cirrhosis (PBC) revealed elevations in several individuals compared to normal liver control (Fig. 4C), providing evidence of PDGFRα signaling involvement in human chronic liver disease. Based on this evidence, we concluded that PDGFRα signaling was involved in both early and advanced hepatic fibrosis, and involved in both chronic hepatotoxic (CCL_4) and cholestatic (BDL) liver injury.

2.2 PDGFRα LOCALIZATION DURING CHRONIC LIVER INJURY

In light of evidence that PDGFRα signaling was upregulated in whole liver lysates of chronically injured murine and human livers, we sought to determine the specific cell types expressing PDGFRα during chronic liver injury to inform our efforts to generate genetic knockout murine models and to infer potential efficacy and consequences of therapeutic PDGFRα inhibition. Based on the ambiguity of PDGFRα expression in various PC and NPC cell populations (reviewed in Section 1.7.4), we examined the potential co-localization of cell markers for multiple liver cell types involved in the progression of hepatic fibrosis using both CCl_4 and BDL models of chronic liver injury.

2.2.1 PDGFRα Expression in Parenchymal Cells of Fibrotic Liver

Epithelial (parenchymal) cells of the liver include hepatocytes and cholangiocytes, which are both derived from endoderm-derived fetal hepatoblasts during liver development. In addition to playing important roles in hepatic growth and regeneration, parenchymal liver cells play an important role in the wound healing fibrotic response of the injured liver – including the release of pro-fibrotic growth factors, reactive oxygen species, and DAMPs which can propagate overall fibrotic response.

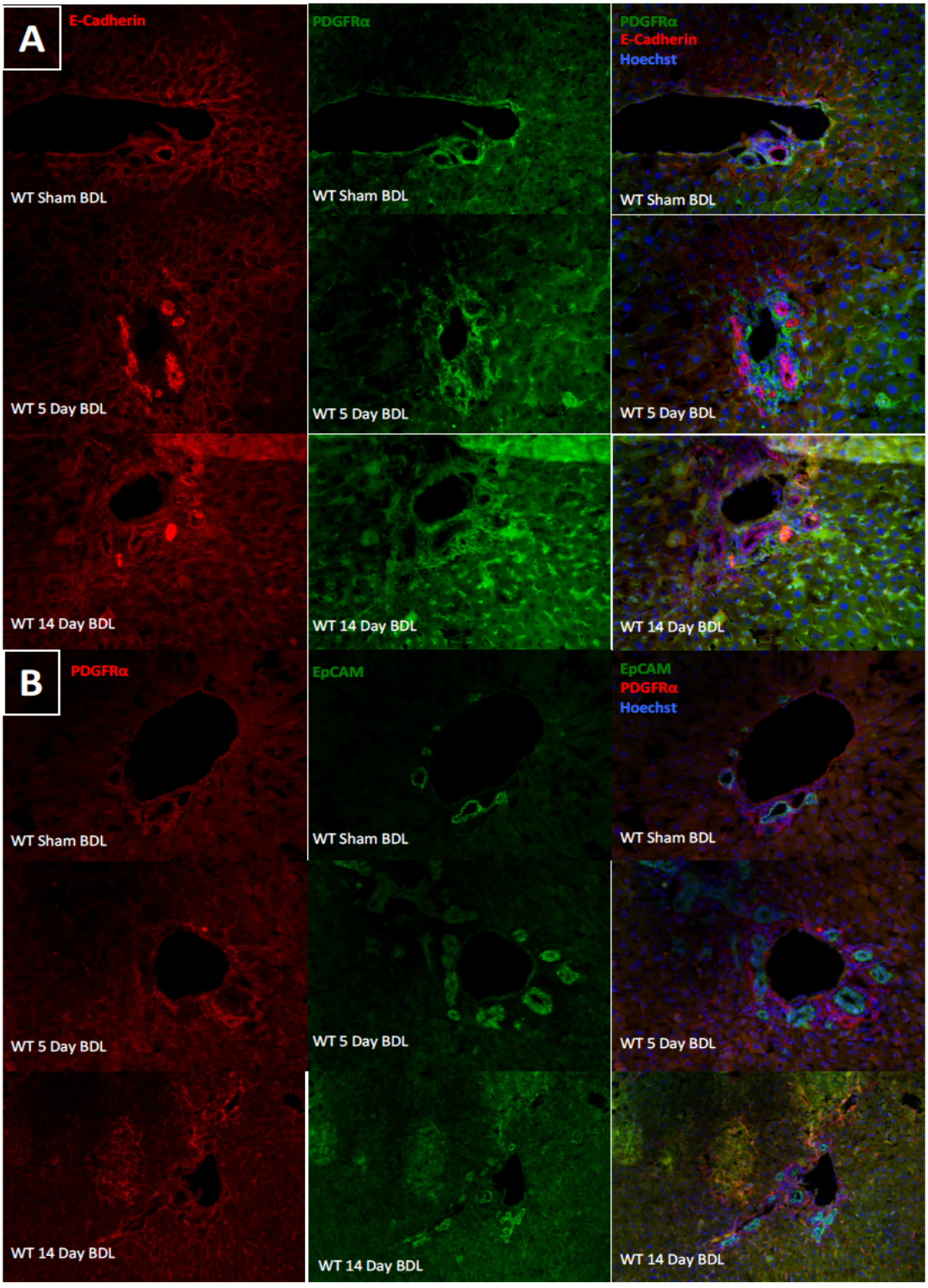


Figure 5: ***Absence of PDGFRα in Hepatocytes and Cholangiocytes following Cholestatic Liver Injury.*** PDGFRα co-immunofluorescence with either **(A)** epithelial marker E-Cadherin (hepatocytes, cholangiocytes) or **(B)** cholangiocyte marker EpCAM showed no co-localization at 5 days or 14 days post-BDL injury.

In order to determine whether PDGFRα is expressed in parenchymal cells following chronic liver injury, we performed co-immunofluorescence analysis of PDGFRα with the epithelial marker E-cadherin and cholangiocyte marker EpCAM in post-BDL or sham-operated murine livers at 5 days and 14 days post-surgery (Fig. 5). No co-localization of PDGFRα with either marker was observed, suggesting that PDGFRα is not expressed in either hepatocytes or cholangiocytes following cholestatic liver injury. In contrast, co-immunofluorescence of PDGFRα and E-cadherin in CCL4-injured murine livers revealed a subset of pericentral hepatocytes which were positive for both markers (Fig. 6).

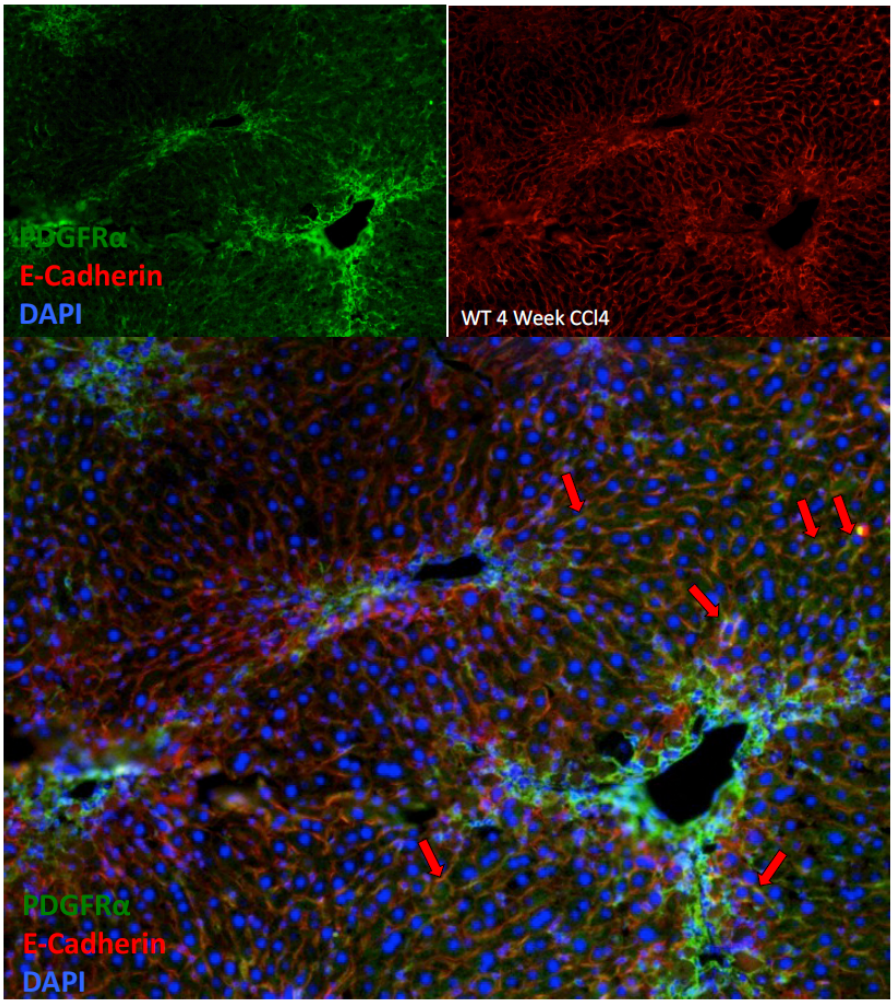


Figure 6: ***PDGFRα Co-localization in Pericentral Hepatocytes during CCl_4 Injury.*** PDGFRα expression was detected in a subpopulation of E-Cadherin-positive pericentral hepatocytes (arrows) in murine livers following 4 weeks of CCl_4-induced chronic liver injury.

2.2.2 PDGFRα Expression in Non-Parenchymal Cells of Fibrotic Livers

Non-parenchymal cells of the liver include HSCs, Kupffer cells, sinusoidal endothelial cells (SECs), and other cell types that affect the progression and outcome of hepatic fibrosis (refer to section 1.3 for details). Several studies have shown evidence suggesting the presence of PDGFRα in HSCs[68] and SECs (refer to Section 1.7.4). To assess whether PDGFRα is expressed in SECs, we performed co-immunofluorescence of PDGFRα and LYVE-1 in murine livers following either CCl_4 (Fig. 7A) or BDL-induced (Fig. 7B) liver injury. In CCl_4-treated murine livers, distinct separation between PDGFRα and LYVE-1 was observed suggesting relative absence of PDGFRα in SECs in the setting of toxic liver injury. Interestingly, we observed closer proximity of PDGFRα and LYVE-1 in co-immunofluorescence of post-BDL murine livers (Fig. 7B). To interrogate potential overlap between PDGFRα and LYVE-1 more closely, we examined high resolution serial confocal images (1μm step size) of post-BDL murine livers which showed PDGFRα cells closely adherent to – but distinct from – LYVE-1 positive cells (Fig. 7C).

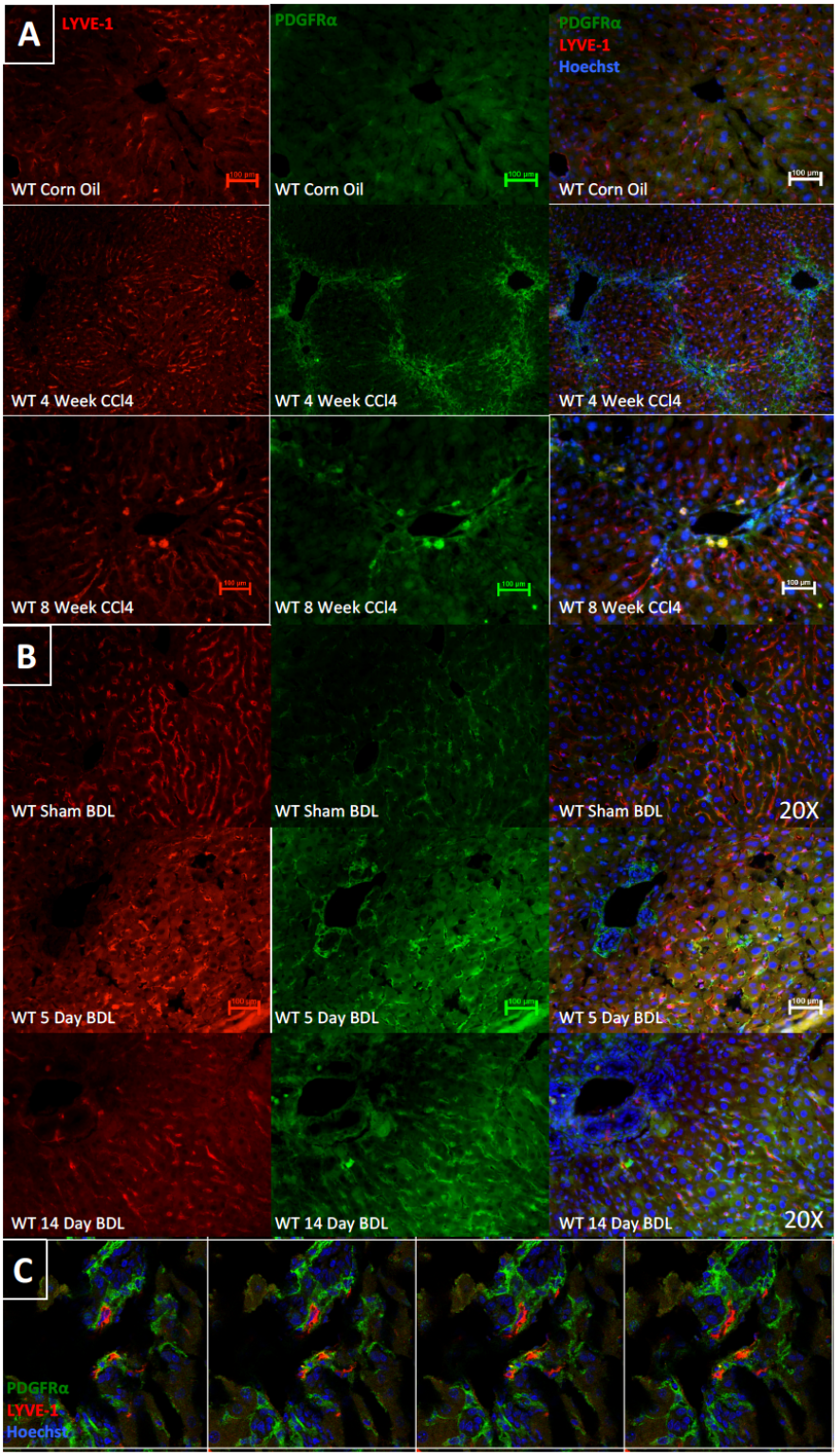


Figure 7: ***PDGFRα is not Expressed in Sinusoidal Endothelial Cells during Chronic Liver Injury.*** **(A)** Representative co-immunofluorescence images in wildtype mice show no co-localization of PDGFRα in LYVE-1 positive sinusoidal endothelial cells (SECs). **(B)** Co-immunofluorescence images in wildtype mice following BDL showed ambiguous overlap of PDGFRα in LYVE-1 positive SECs. **(C)** Absence of co-localization was determined by confocal immunofluorescence imaging confirmed close proximity but separate expression of PDGFRα and LYVE-1.

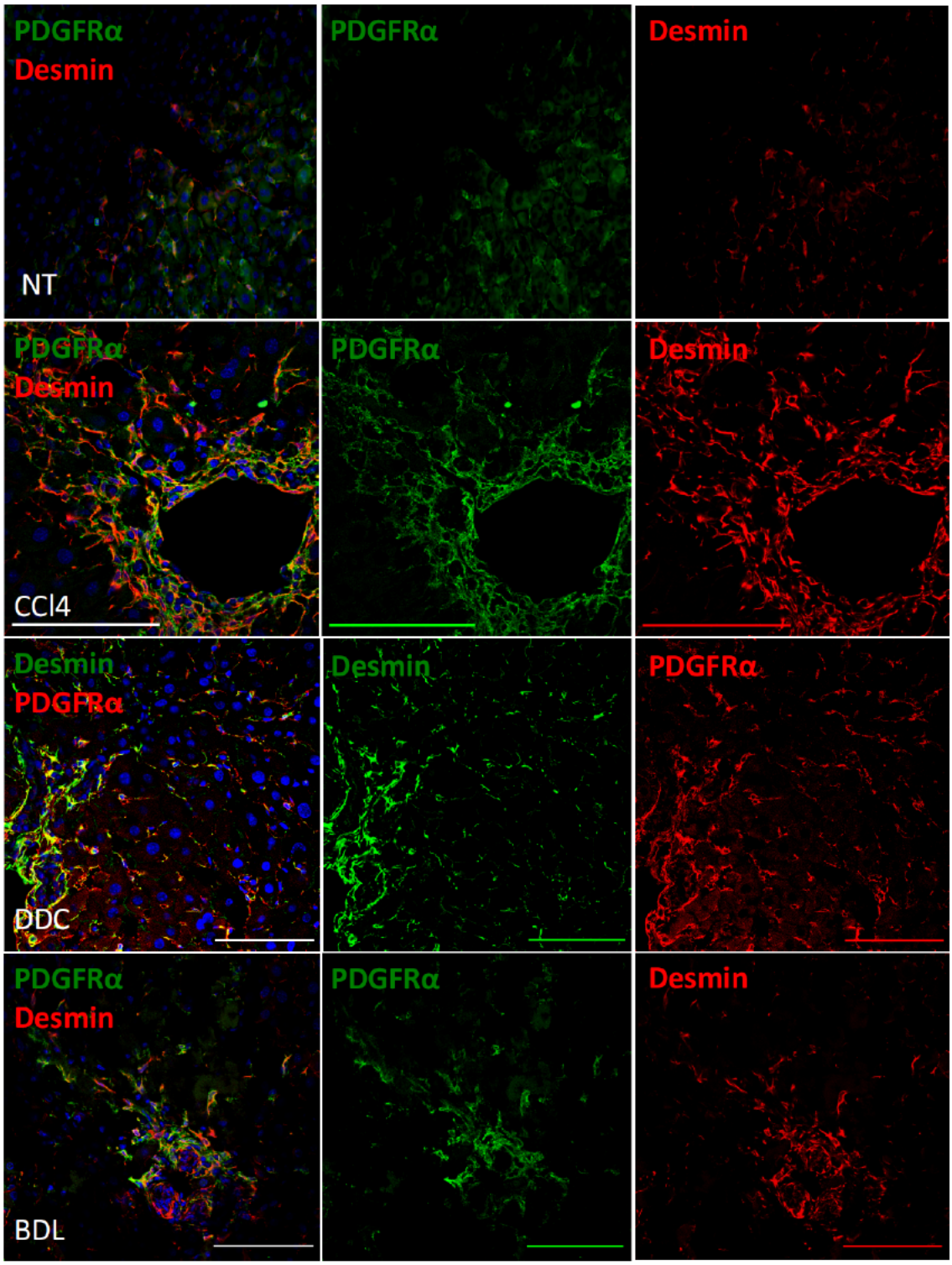


Figure 8: ***PDGFRα Co-localization in Hepatic Stellate Cells during Chronic Liver Injury.*** Confocal immunofluorescence images of murine livers following 8 week CCl4, 16 day DDC, or 14 day BDL show PDGFRα expression co-localized to desmin, an established marker of HSCs. Non-treated (NT) livers are shown for comparison. Scale bars represent 100μm length.

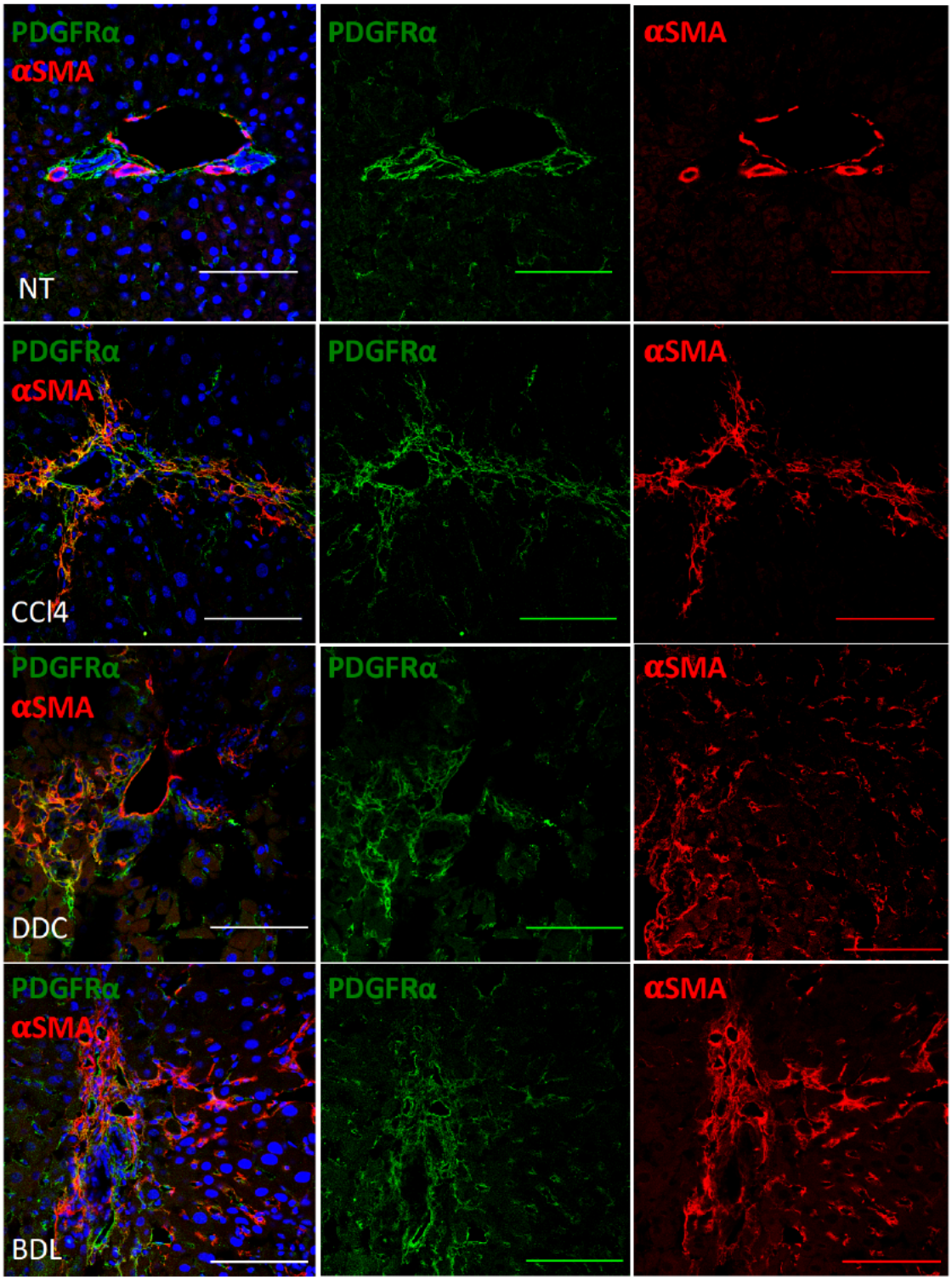


Figure 9: ***PDGFRα Co-localization in Myofibroblasts during Chronic Liver Injury.*** Confocal immunofluorescence images of murine livers following 8 week CCl4, 16 day DDC, or 14 day BDL show PDGFRα expression co-localized to αSMA, an established marker of myofibroblasts. Non-treated (NT) livers are shown for comparison. Scale bars represent 100μm length.

The spindular morphology and close proximity of PDGFRα-positive cells to LYVE-1 positive SECs suggested that PDGFRα positive cells may be HSCs. In order to confirm this, we performed co-immunofluorescence of PDGFRα with the HSC marker desmin (Fig. 8A) as well as the myofibroblast marker αSMA (Fig. 8B). Confocal immunofluorescence imaging showed close co-localization of PDGFRα with both markers in CCL_4, BDL, and DDC diet-induced chronic liver injury. Of particular note, PDGFRα was expressed in desmin-positive HSCs in non-injured (NT) livers at baseline while maintaining distinct separation from αSMA. Together, these findings suggest the presence of PDGFRα in quiescent and activated HSCs as well as myofibroblasts.

2.3 PDGFRα LOSS IN HEPATOCYTES DOES NOT AFFECT HEPATIC FIBROSIS

2.3.1 PDGFRα Loss in Epithelial Cells does not Affect Hepatic Fibrosis

Based on our findings that a subset of pericentral hepatocytes may express PDGFRα following CCl_4-induced liver injury (Fig. 6), we sought to determine whether PDGFRα loss in epithelial cells would affect the outcome of chronic liver injury *in vivo*. To this end, we have generated hepatocyte-specific *Pdgfra-/-* mice by crossing floxed *Pdgfra* animals with mice expressing Albumin promoter-driven (Alb-Cre) or Foxa3 promoter-driven (Foxa3-Cre) Cre recombinase. Foxa3 is a member of the Foxa gene family of forkhead box containing transcription factors which plays an important role in early liver specification in conjunction with Foxa1 and Foxa2 to open compacted chromatin regions containing liver-specific regulatory regions in the developing foregut endoderm[114]. Since hepatoblasts originate from foregut endoderm, Foxa3-cre recombination is expected to cause floxed gene excision in both hepatocytes and cholangiocytes[115].

Albumin - a more advanced marker of hepatocyte differentiation - is expressed in bipotential hepatoblasts along with other hepatocyte and biliary epithelial cell associated genes[115]. Our lab has published numerous studies using both Foxa3-Cre and Albumin-cre mediated recombination of floxed genes[116, 117] .

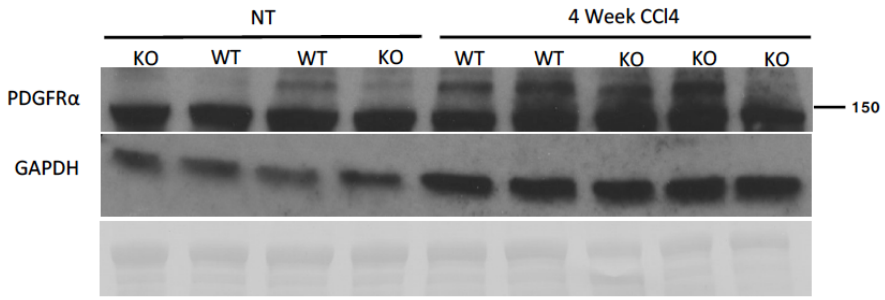


Figure 10: ***PDGFRα Expression is Retained in FoxA3-Cre Pdgfra-/- Livers.*** PDGFRα expression is upregulated following 4 weeks of CCl_4-induced liver injury, but is not consistently downregulated in Foxa3-Cre *Pdgfra-/-* animals.

The resultant Foxa3-Cre *Pdgfra-/-* mice showed no substantial reduction of PDGFRα in whole liver lysates (Fig. 10) following CCl_4-induced liver injury. In addition, these mice did not show changes in overall fibrosis as assessed by Picrosirius Red staining of liver sections as well as αSMA immunohistochemistry (Fig. 11A,B). Hepatocellular injury was assessed using serum markers for liver injury including ALT and AST, however both Foxa3-Cre *Pdgfra-/-* and littermate controls showed similar levels of injury induction following CCl_4 (Fig. 11C). Furthermore, no difference in hepatocyte proliferation/regeneration was observed as indicated by similar levels of PCNA immunohistochemistry (Fig.11A,B). Based on these findings, we conclude that PDGFRα expression in pericentral hepatocytes following toxic liver injury is not a significant contributor to the propagation or recovery from hepatic fibrosis – either through an effect on hepatocyte-death driven fibrogenesis or hepatocellular regeneration. Similar results were seen in Alb-Cre *Pdgfra-/-* animal cohorts following CCl_4-mediated chronic liver injury (data not shown).

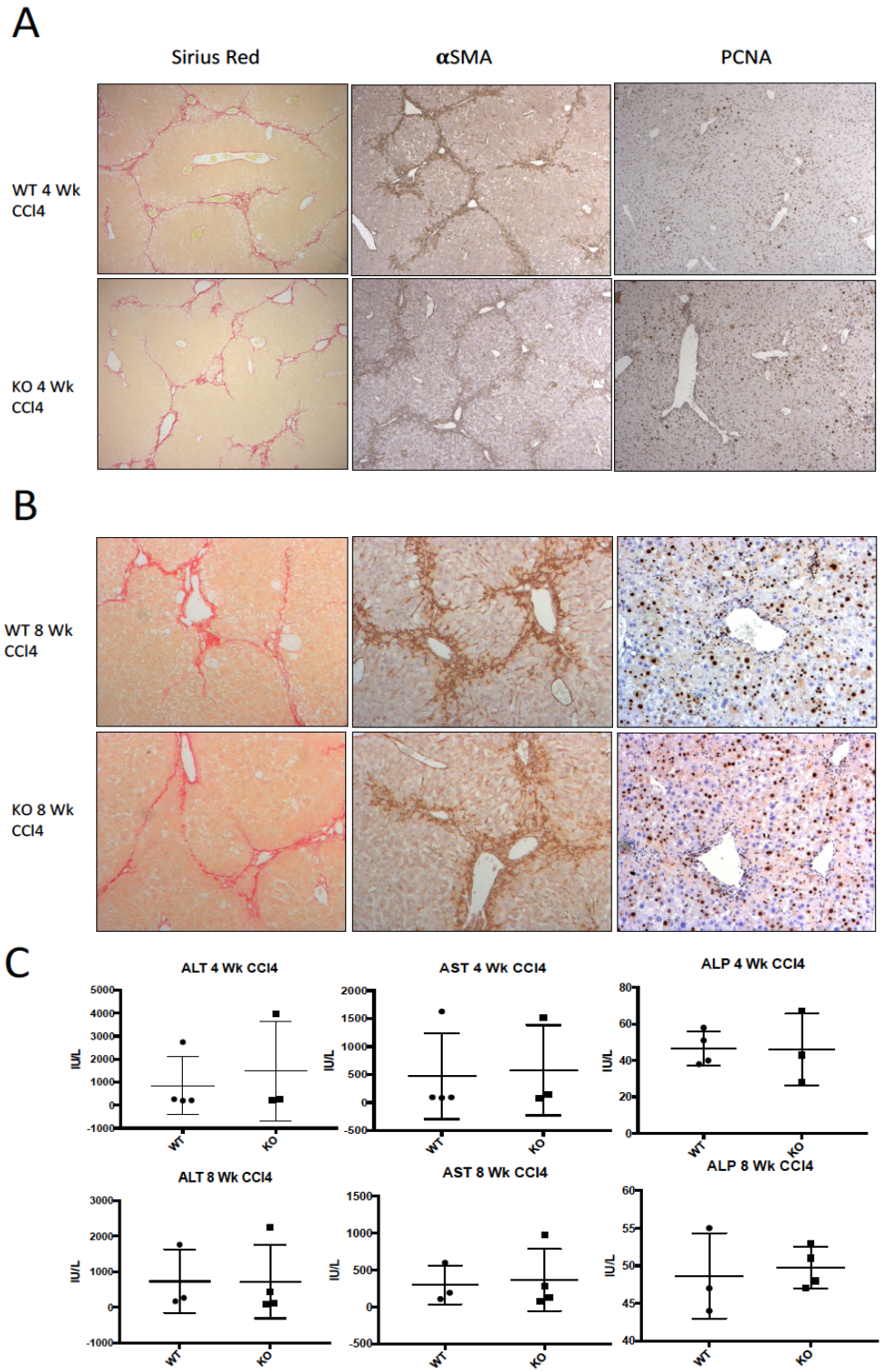


Figure 11: ***FoxA3 Pdgfra-/- Mice Show No Change in Hepatic Fibrosis or Hepatocellular Injury During CCl_4 Injury.*** Sirius red staining, αSMA, and PCNA immunohistochemistry of liver sections from 4 week **(A)** and 8 week **(B)** CCl_4-treated mice show no changes in Foxa3-Cre *Pdgfra-/-* (KO) mice compared to littermate controls (WT). **(C)** Serum liver function tests including ALT, AST, and ALP are similar in KO and WT controls following 4 weeks and 8 weeks of CCl4-induced liver injury.

2.4 DISCUSSION: IMPLICATIONS OF PDGFRα EXPRESSION AND NULL FUNCTIONALITY IN HEPATOCYTES

Specific RTK expression in multiple cell types is an important factor to consider in the development of any prospective targeted RTK inhibition during chronic liver injury. Different resident cell populations play potentially antagonistic pro- or anti-fibrotic roles in the setting of chronic liver injury[62] and it is therefore conceivable the biologic endpoints of PDGFRα signaling (ie survival, proliferation) may contribute to injury progression in specific cells (ex: activated HSCs/myofibroblasts) while ameliorating injury in others (ex: hepatocytes). Precluding the availability of clinically viable HSC-targeted drug delivery, non-specific effects of RTK inhibition must be carefully considered. This is especially relevant in cases of chronic liver injury, in which hepatotoxicity of CYP450-metabolized drugs is exacerbated by decreased liver function. For many clinically approved RTK inhibitors, hepatotoxicity is the major limiting factor in their utilization[113] – providing a major impetus for the development of targeted RTK inhibition (see Section 5.2).

While studies have shown that PDGFRα is expressed in HSCs[68], studies of PDGFRα localization in other cell types in the liver has not previously been undertaken. In this chapter, we explored the expression of PDGFRα and its localization in two commonly utilized models of chronic liver injury to show its specific expression in a subset of pericentral hepatocytes and HSCs/myofibroblasts. Importantly, we rule out the possibility of PDGFRα expression in two cell populations relevant to chronic liver injury: cholangiocytes (in post-BDL livers) and SECs (both CCl_4- and BDL-induced liver injury).

Through the characterization of two models of hepatocyte-targeting PDGFRα loss (Foxa3-Cre and Alb-Cre), we show that PDGFRα expression in a subset of peri-central hepatocytes does

not contribute to the progression of hepatic fibrosis during CCl_4-mediated liver injury. This data confirms the absence of potential exacerbation of liver injury occurring from off-target PDGFRα loss/inhibition in hepatocytes, which could conceivably affect hepatocyte survival and proliferation – thereby worsening the outcome of chronic liver injury.

Lastly, we are the first to confirm through high resolution confocal immunofluorescence co-localization imaging that PDGFRα is expressed in desmin-positive HSCs and αSMA-positive myofibroblasts in fibrotic liver sections. Based on these findings, we proceed to study the functional and mechanistic effects of PDGFRα inhibition in human HSCs *in vitro* (Chapter 3), as well as the pathophysiological consequences of PDGFRα loss in HSCs in multiple mouse models of chronic liver injury (Chapter 4).

3.0 PDGFRα CONTRIBUTES TO HUMAN HEPATIC STELLATE CELL PROLIFERATION AND MIGRATION

In the previous chapter, we showed evidence that PDGFRα is expressed in both HSCs and myofibroblasts. In this chapter, we examine the role of PDGFRα in human HSCs and show that selective inhibition of PDGFRα with Olaratumab inhibits HSC proliferation and migration *in vitro*, but not pro-fibrotic gene expression. We show that these cell functions are differentially mediated by either exogenous or autocrine PDGF stimulation, and demonstrate the involvement of multiple downstream signaling pathways through analysis of phosphorylation of key signaling mediators involved in cell proliferation and migration including Erk1/2, p38, Akt, mTOR, FAK, and CrkII/L.

During the process of liver injury, activated HSCs undergo a process of transdifferentiation towards a myofibroblast phenotype characterized by increased proliferation as well as altered gene transcription[118]. During this process, HSCs become motile and migrate to the sites of injury and inflammation guided by chemotactic stimuli such as growth factors like PDGFs, TGFß1, and type I collagen[119-121]. In the setting of chronic liver injury, HSCs continue to migrate through hepatic lobule to the areas of injury and facilitate the progression of hepatic fibrosis[122].

The downstream signaling targets of PDGF-induced cellular activity are diverse and affect multiple cell functions. Among these targets are mitogen activated protein kinases such as Erk1/2 and p38 which are central in the activation, proliferation, and migration of HSCs[123-127]. Factors such as Akt are common targets of PDGF signaling and have been demonstrated to function downstream of FAK - which plays a dual role in cell adhesion as well as PDGF-induced HSC proliferation[128]. Another functionally versatile signaling kinase is mTOR which is active in cellular processes associated with proliferation[129] and migration[130, 131]. Our findings are also

consistent with studies showing that mTOR inhibition may attenuate experimental hepatic fibrosis[25], potentially through reduction in proliferation and migration of activated HSCs. Adaptor proteins also play an important function in PDGF signaling exemplified by CrkII and Crk-like (CrkL), whose formation of a stabile complex with PDGFRα is one of the few identified distinctions between downstream effectors of PDGFRα and PDGFRß[44, 132, 133].

While the cellular function and downstream signaling mediators of PDGFRß signaling in HSCs have been extensively studied[134-136], the precise role and contribution of PDGFRα in these cells has not been adequately elucidated[137]. Olaratumab (LY3012207, IMC-3G3, Eli-Lilly) is a humanized monoclonal antibody that targets PDGFRα with high potency and specificity and has been utilized in both preclinical and clinical studies[112, 138-141].

3.1 PDGFRα EXPRESSION IN HUMAN HEPATIC STELLATE CELLS

In order to identify suitable HSCs for studies of PDGFRα, we examined its expression and activation in primary human hepatic stellate cells (HHSteCs) and LX-2, an immortalized HSC line. While PDGFRß was present in both cell types, expression of PDGFRα was more profound in HHSteCs (Fig. 12A). Furthermore, increased PDGFRα phosphorylation in response to PDGF-BB stimulation was also evident (Fig. 12A). LX-2 cells also showed PDGFRα phosphorylation in response to PDGF-BB, *albeit* to a lesser extent (Fig. 12A). Therefore, due to greater basal PDGFRα expression in HHSteCs and activation in response to the highly mitogenic and well-characterized pro-fibrotic ligand PDGF-BB, we opt to use these cells as a representative model of PDGFRα-expressing HSCs for further study.

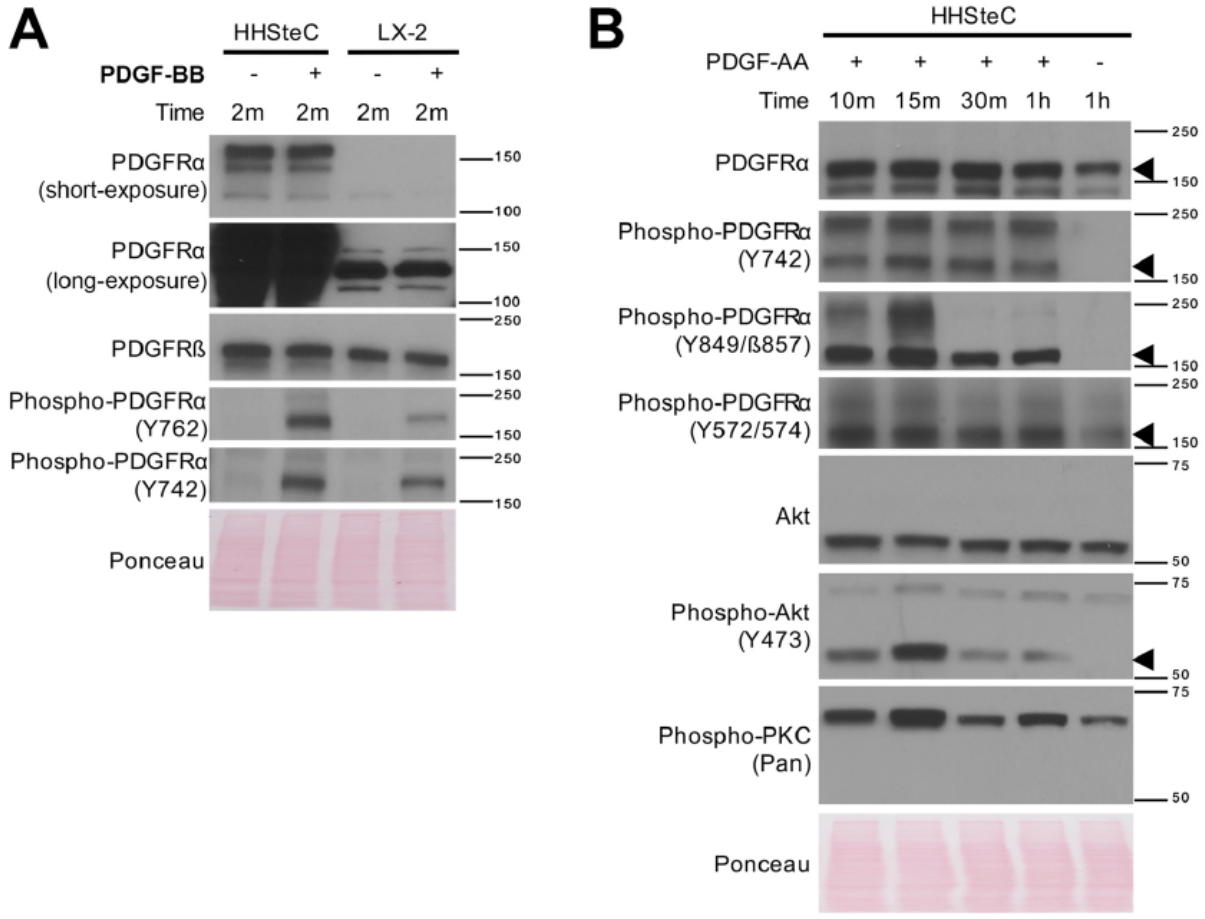


Figure 12: ***PDGFRα Expression and Activity in Hepatic Stellate Cells.*** **(A)** HHSteC and LX-2 cells showed differential expression of PDGFRα at baseline and corresponding increase in phosphorylated receptor following PDGF-BB (10ng/mL) treatment for 2 minutes. **(B)** HHSteCs stimulated with PDGF-AA (10ng/mL) for the indicated time periods show increased PDGFRα phosphorylation at Y742, Y849, and Y572/574. Associated downstream signaling mediators Akt and PKC also showed increased phosphorylation in response to PDGF-AA treatment.

To assess if PDGFRα is functional in HHSteCs, these cells were treated with PDGFRα-specific ligand PDGF-AA. This led to phosphorylation of PDGFRα at key tyrosine signaling residues (Fig. 12B). Furthermore, PDGF-AA treatment induced phosphorylation of downstream Akt and PKC (Fig. 12B).

To block PDGFRα, we employed the fully human anti-PDGFRα antibody Olaratumab [110, 111]. To validate efficacy and specificity of Olaratumab, ELISA-based cell free binding assay was performed. Olaratumab showed a dose-dependent binding to immobilized PDGFRα extracellular domain (ECD) (data not shown). Furthermore, the antibody concentration required for 50% maximum binding to PDGFRα ECD was calculated to be around 0.06 nM as also reported previously [139]. Olaratumab did not cross-react with a human PDGFRß ECD (data not shown).

3.2 CONTRIBUTION OF PDGFRα SIGNALING TO HUMAN HSC PROLIFERATION

3.2.1 PDGF Treatment Induces PDGFRα-dependent HHSteC Proliferation, but not Pro-Fibrotic Gene Expression

PDGF-BB signaling through PDGFRß plays a well-known central role in processes associated with HSC activation including proliferation and transdifferentiation to an activated myofibroblast state accompanied by the expression of pro-fibrogenic genes. However, the specific contribution of PDGFRα in these processes is unknown. To test the contribution of PDGFRα to HSC proliferation, we used alamarBlue cell viability assay following 24-hour exposure to PDGF-AA and PDGF-BB in the presence of PDGFRα-specific blocker Olaratumab or control IgG (Fig. 13A). We observed an increase in HHSteC proliferation in response to PDGF-AA and PDGF-BB when compared to non-treated (NT) controls. While Olaratumab alone was insufficient to affect HHSteC proliferation (Fig. 13A, B), incubation of these cells with Olaratumab in the presence of either

exogenous PDGF-AA or PDGF-BB resulted in a decrease in proliferation comparable or greater than that of Mitomycin C - a known inhibitor of DNA synthesis (Fig. 13A).

Since HSC activation from a quiescent state is associated with increased expression of transcriptional targets including genes for structural extracellular matrix proteins (ex: COL1A1, FN1), cytokines (ex: TGFB1), neural markers (ex: SYP), and the contractile filament alpha smooth muscle actin (ACTA2), we next examined any effect of Olaratumab on pro-fibrotic gene expression. Olaratumab treatment for 24 hours led to no significant change in expression of any of these genes (Fig. 13C). To determine if the absence of PDGF was responsible for the lack of effect of Olaratumab on HHSteC activation, we also examined gene expression of the above-mentioned targets following PDGF-AA or PDGF-BB exposure. Neither exogenous PDGF-AA or PDGF-BB significantly induced the expression of these genes (data not shown).

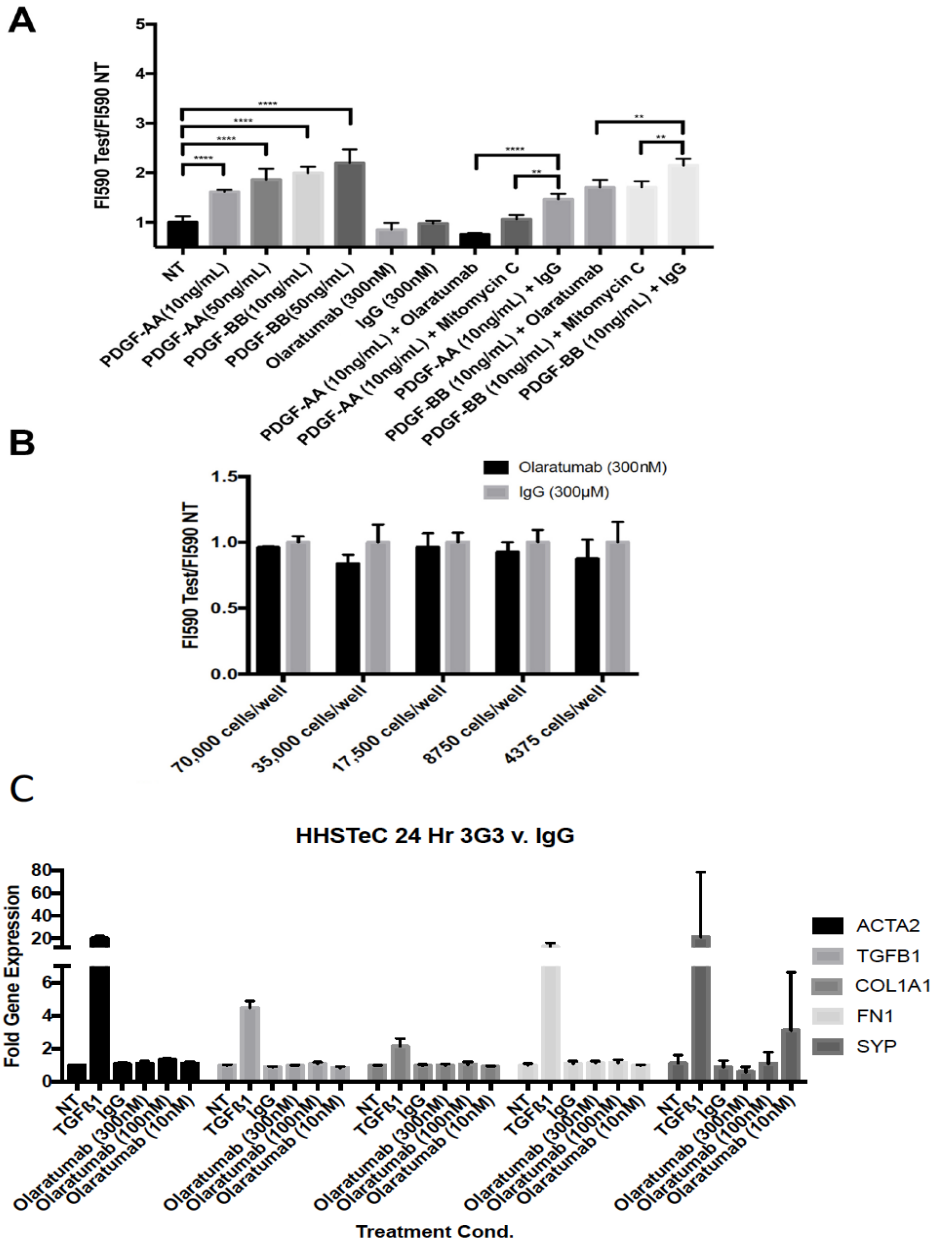


Figure 13: ***PDGFRα Signaling Contributes to HHSteC Proliferation, but not Activation, in the Presence of Exogenous PDGF Stimulation.*** **(A)** Proliferation of HHSteCs was measured using alamarBlue dye following a 24-hour incubation with PDGF-AA, PDGF-BB, Olaratumab, or IgG at the indicated concentrations. PDGF-AA and PDGF-BB induced significant proliferation of HHSteCs. HHSteCs were also exposed simultaneously to PDGF and Olaratumab, PDGF and IgG, or PDGF and mitomycin C (DNA synthesis inhibitor). Both Olaratumab and mitomycin C caused a reduction in PDGF-induced proliferation compared to IgG control. **(B)** PDGFRα inhibition using Olaratumab tested over 24 hours at various plating densities showed no significant change in proliferation compared to IgG-treated controls. **(C)** RT-PCR shows no significant changes in expression of ACTA2, TGFB1, COL1A1, FN1, or SYP in HHSteCs following Olaratumab treatment at multiple concentrations. TGFβ1 (2ng/mL) treatment was included as a positive control. All assays were performed in triplicates and results are normalized to their respective non-treatment (NT) conditions. Adjusted P value of <0.01 represented as **; adjusted P value of <0.0001 represented as ****.

3.2.2 Olaratumab inhibits PDGF-BB mediated HSC proliferation through blockade of PDGFRα and downstream signaling

In light of our findings that PDGFRα signaling contributes to HHSteC proliferation in the presence of exogenous PDGF ligands, we sought to determine the downstream signaling that is enhanced by PDGF ligand and blocked by Olaratumab treatment. In order to assess the most physiologically relevant signaling pathways involved, we tested the effect of Olaratumab in the presence of PDGF-BB: a non-selective isoform of PDGF with a central role in HSC mitogenesis, chemotaxis, and activation during hepatic fibrosis[21, 120, 142]. HHSteCs were pre-treated with Olaratumab (300nM) or human IgG (300nM) for 30 minutes prior to exposure to PDGF-BB for various times (range 5 minutes to 6 hours).

PDGF-BB treatment led to increased phosphorylation of PDGFRα at all phosphorylation sites for which phospho-specific antibodies were available (Fig. 14A,B). Phosphorylation of PDGFRα in HHSteC cells in response to PDGF-BB was readily evident at all sites within 5 minutes of treatment and peaked at 30 minutes with gradual decrease at 2 and 6 hours. Olaratumab pre-treatment of HHSteCs led to notable decreases in phosphorylation of PDGFRα at multiple tyrosine residues including Y754, Y849, Y762, and Y1018 (Fig. 14A,B). In contrast, Y742 and Y572/574 were unaffected.

We next examined downstream effectors of PDGFRα associated with PDGF-induced proliferation such as Erk1/2 [126], p38 [124], FAK [128], Akt and mTOR [143], using phospho-specific antibodies including T202/Y204 for Erk1/2, T180/Y182 for p38 MAPK, Y397/Y576/Y577/Y925 for FAK, S473 for Akt, and S2448/S2481 for mTOR. PDGF-BB treatment increased phosphorylation of Erk1/2, p38 MAPK, Akt, mTOR and FAK as compared

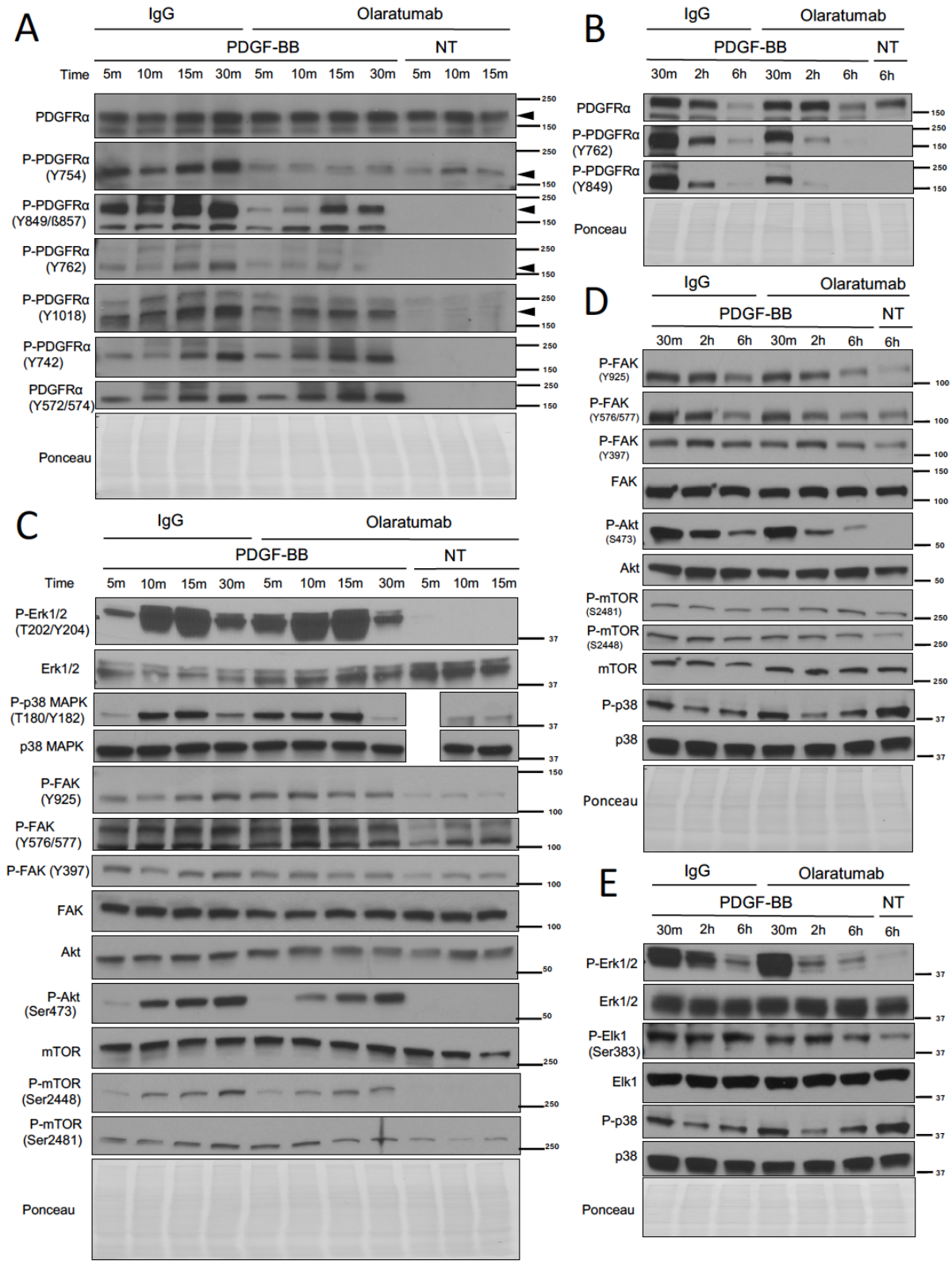

A
IgG
Olaratumab
PDGF-BB
NT
Time
5m 10m 15m 30m 5m 10m 15m 30m 5m 10m 15m
PDGFRα
P-PDGFRα (Y754)
P-PDGFRα (Y849/ß857)
P-PDGFRα (Y762)
P-PDGFRα (Y1018)
P-PDGFRα (Y742)
PDGFRα (Y572/574)
Ponceau
B
IgG
Olaratumab
PDGF-BB
NT
30m 2h 6h 30m 2h 6h 6h
PDGFRα
P-PDGFRα (Y762)
P-PDGFRα (Y849)
Ponceau
C
IgG
Olaratumab
PDGF-BB
NT
Time
5m 10m 15m 30m 5m 10m 15m 30m 5m 10m 15m
P-Erk1/2 (T202/Y204)
Erk1/2
P-p38 MAPK (T180/Y182)
p38 MAPK
P-FAK (Y925)
P-FAK (Y576/577)
P-FAK (Y397)
FAK
Akt
P-Akt (Ser473)
mTOR
P-mTOR (Ser2448)
P-mTOR (Ser2481)
Ponceau
D
IgG
Olaratumab
PDGF-BB
NT
30m 2h 6h 30m 2h 6h 6h
P-FAK (Y925)
P-FAK (Y576/577)
P-FAK (Y397)
FAK
P-Akt (S473)
Akt
P-mTOR (S2481)
P-mTOR (S2448)
mTOR
P-p38
p38
Ponceau
E
IgG
Olaratumab
PDGF-BB
NT
30m 2h 6h 30m 2h 6h 6h
P-Erk1/2
Erk1/2
P-Elk1 (Ser383)
Elk1
P-p38
p38
Ponceau

Figure 14: ***Olaratumab Inhibits PDGFRα Activation and Downstream Proliferative Signaling Mediators in HHSteCs in the Presence of Exogenous PDGF Stimulation.*** **(A, B)** HHSteCs were pretreated with either Olaratumab (300nM) or human IgG (300nM) for 30 minutes prior to PDGF-BB (10ng/mL) exposure for the indicated time periods. Representative western blots show decrease in phosphorylation of tyrosine signaling residues in PDGFRα in cells pretreated with Olaratumab including Y754, Y762, Y849, and Y1018, but not at Y742 and Y572/574. **(C, D)** Representative western blots show decreased phosphorylation of p38 MAPK (T180/Y182), FAK (Y397, Y576/Y577, Y925), Akt (Ser473), and mTOR (Ser2448, Ser2481) after PDGFRα blockade with Olaratumab (300nM) prior to PDGF-BB (10ng/ml) treatment. **(E)** Decreased phosphorylation of Erk1/2 (T202/Y204) and downstream transcription factor Elk1 (Ser383) was also observed. Ponceau staining is included as a loading control. All Western blots were repeated twice on lysates from experimental conditions that were performed in triplicate and pooled for analysis.

to non-treated cells. The peak phosphorylation of Erk and p38 MAPK occurred at 15 minutes followed by a gradual decrease up to 6 hours after PDGF-BB (Fig. 14C-E). While earlier time points were unchanged, a notable decrease in Erk1/2 phosphorylation was evident at 2-6 hours in the Olaratumab group (Fig. 14E, 15). Decreased phosphorylation of p38 MAPK was evident at only 30 minutes in the Olaratumab group (Fig. 14C-D, 15). Consistent with decreased phosphorylation of Erk1/2 and p38, we observed decreased phosphorylation of Elk-1, a known downstream target of Erk1/2 and p38 [144], in Olaratumab pre-treated cells from 30 minutes to 6 hours. PDGF-BB treatment also led to increased FAK phosphorylation that peaked at 30 minutes to 2 hours. Olaratumab pre-treatment modestly affected FAK phosphorylation at Y397 at 15-30 minutes and Y925 and Y576/577 at 2 hours (Fig. 14C-D, 15). PDGF-BB treatment led to a sustained mTOR phosphorylation at both S2481 and S2448, which was reduced by Olaratumab for up to 30 minutes (Fig. 14C-D, 15). Akt phosphorylation after PDGF-BB treatment was also sustained but peaked at 30 minutes to 2 hours (Figure 14C-D). Olaratumab decreased Akt phosphorylation at all time points with maximal affect at 2-6 hours (Fig. 14C-D, 15).

Given Olaratumab's selective inhibition of PDGFRα, as well as the universal binding of PDGF-BB to both PDGFRα and PDGFRβ receptors, our results provide evidence of an important and independent contribution of the PDGFRα in regulating multiple proliferative signaling pathways in human HSCs.

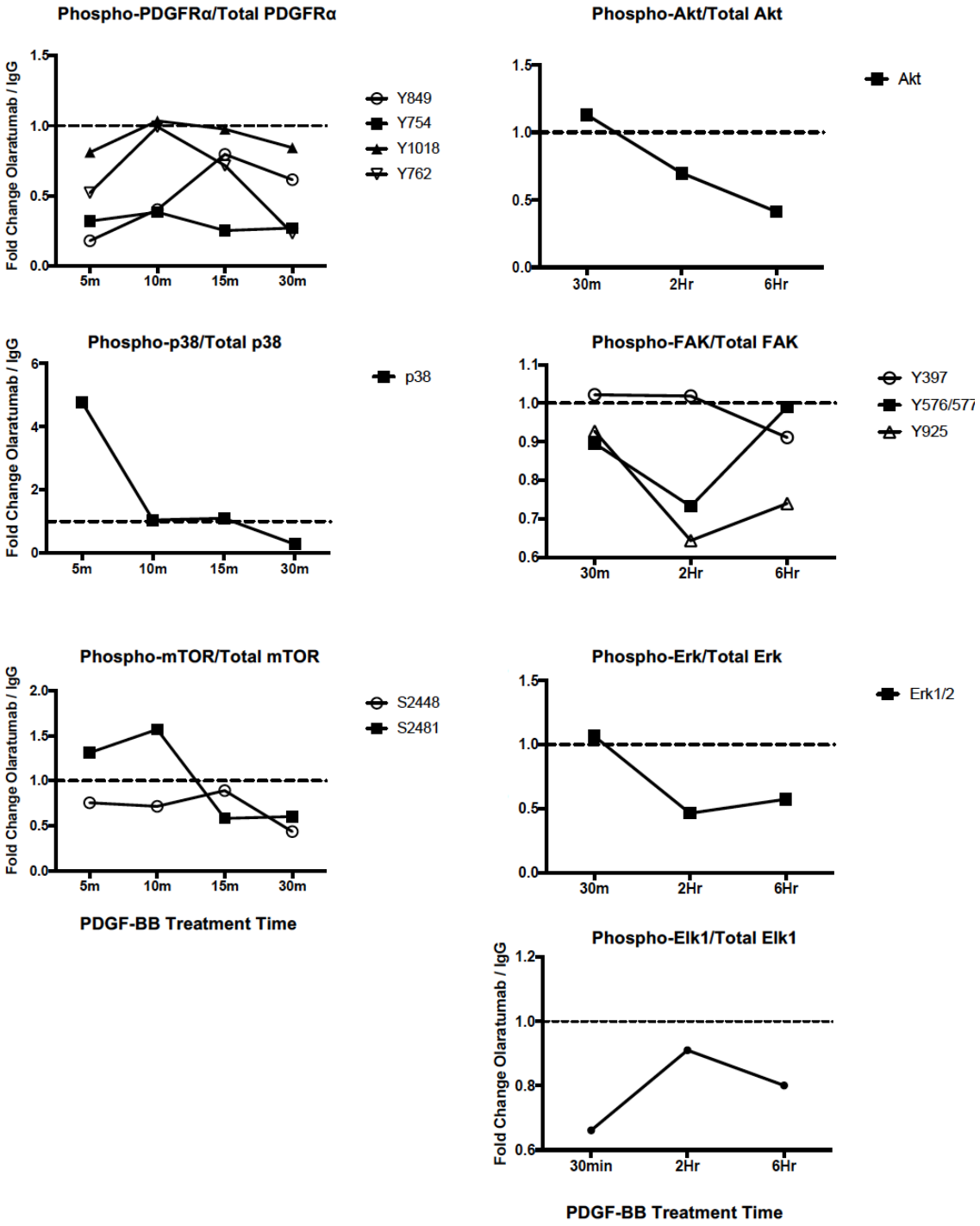


Figure 15: ***Densitometric Quantification of Olaratumab-Mediated Changes in Protein Phosphorylation of HHSteCs in the Presence of Exogenous PDGF Stimulation.*** Densitometry of key phosphorylated protein changes from representative Western blots shown in Figure 3. Values are normalized to total protein levels and shown as the ratio of the signal of Olaratumab-treated HHSteCs to IgG-treated HHSteCs. The dashed line represents the value at which the relative phosphorylated protein signals of Olaratumab-treated HHSteCs and IgG-treated HHSteCs are equal.

3.3 CONTRIBUTION OF PDGFRα SIGNALING TO HUMAN HSC MIGRATION

3.3.1 Olaratumab Inhibits HHSteC Migration Attributable to Autocrine Signaling

PDGF has previously been reported to be a potent chemoattractant for HSCs - stimulating cell migration[126, 145, 146]. To examine whether PDGFRα signaling affects HSC migration, we performed transwell migration assays on HHSteCs exposed to Olaratumab, PDGF-BB, human IgG, or serum-free medium (NT). No significant change in cell migration following PDGF-BB treatment was observed - indicating that exogenous PDGF ligand stimulation did not substantially contribute to the migratory response of HHSteCs (Fig. 16A). In contrast, Olaratumab treatment alone significantly reduced HHSteC migration relative to IgG-treated controls (Fig. 16B).

The lack of migratory response to exogenous PDGF-BB and the simultaneous migratory inhibition to PDGFRα inhibition with Olaratumab suggests ongoing autocrine PDGFRα activation that may be contributing to migration of HHSteCs. HSC cell lines and primary HSCs typically undergo a process of activation towards a differentiated myofibroblast state as they are cultured *in vitro* [41] which is likely to induce autocrine secretion of activating ligands characteristic of myofibroblast transdifferentiation. To confirm this hypothesis, we analyzed PDGF gene expression of PDGF-A, -B, -C, and -D from RNA isolated from non-treated, low passage HHSteCs. RT-PCR analysis showed mRNA expression of all PDGFs in non-treated HHSteCs (Fig. 16C). We also observed presence of PDGF-BB protein in cell lysates of non-treated HHSteCs (Fig. 16D). Secretion of PDGF-BB, PDGF-CC, and PDGF-DD was also confirmed by WB analysis of the concentrated cell culture media collected from the non-treated

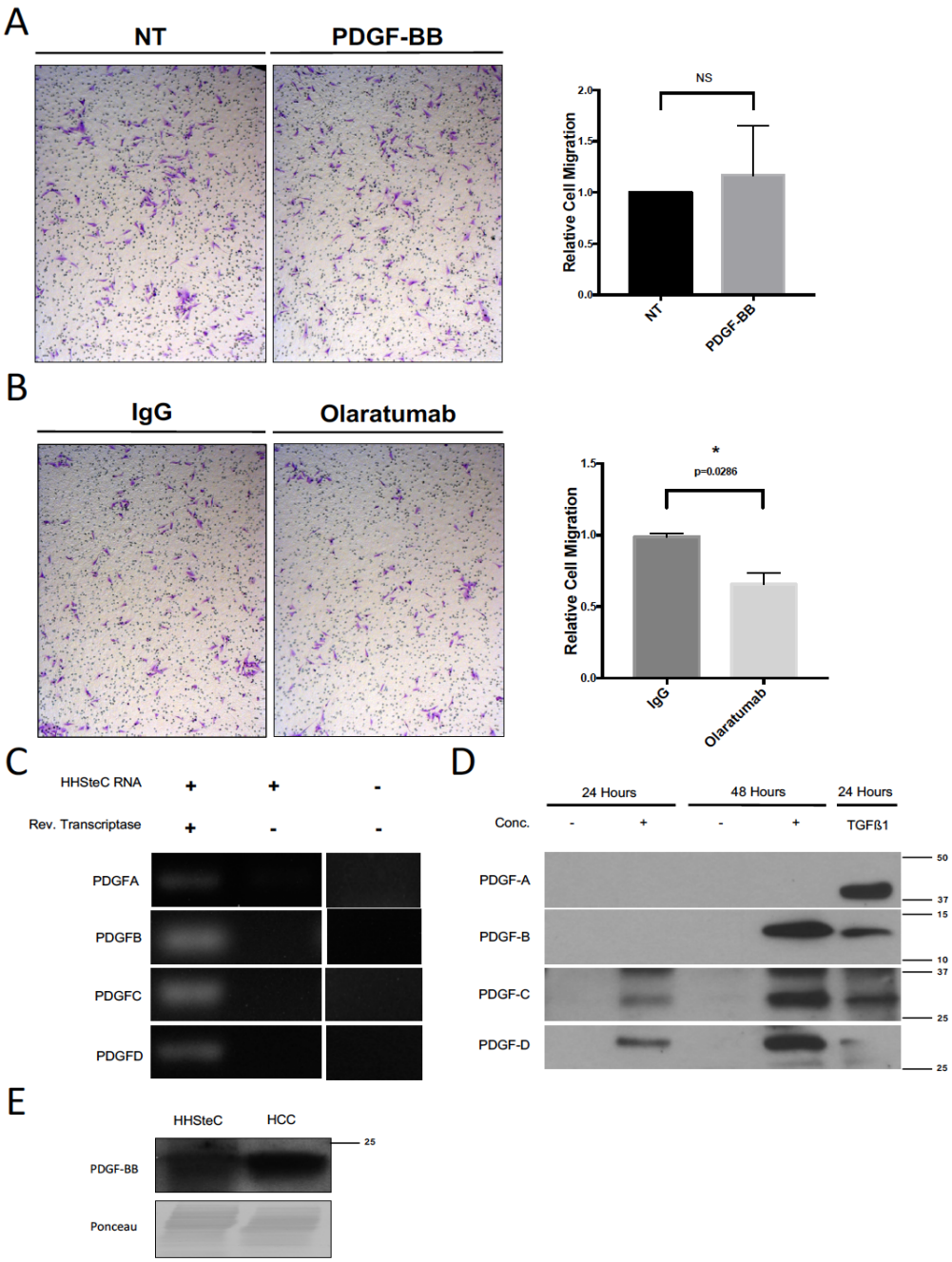


Figure 16: ***Olaratumab Inhibits Migration of HHSteCs in the Absence of Exogenous PDGF Ligand, Due to Autocrine Baseline Signaling.*** Representative images from HHSteC transwell migration assays and quantification (right panel) shows insignificant difference (NS) between no treatment (NT) versus PDGF-BB treatment. **(B)** Decreased HHSteC migration following Olaratumab versus IgG-treated controls after 3 hours relative to IgG-treatment and quantified in right panel (p=0.0286). **(C)** RT-PCR of cDNA derived from non-treated HHSteCs (left lane) shows baseline expression of PDGFA, PDGFB, PDGFC, and PDGFD compared to HHSteC RNA in the absence of reverse transcriptase (middle lane) or primer only control (right lane) (separated due to non-inclusion of technical replicates). **(D)** Representative western blots of PDGF ligands detected in concentrated baseline HHSteC media after 24 or 48 hours of serum starvation indicates autocrine secretion of PDGF-B, -C, and –D, but not PDGF-A. Concentrated supernatant from TGF-ß1-activated HHSteCs are shown for comparison. **(E)** Representative western blot shows PDGF-BB expression in non-treated HHSteC whole cell lysates and hepatocellular carcinoma (HCC) sample as a positive control. Ponceau staining is included as a loading control.

HHSteCs cultured for 24 or 48 hours (Fig. 16E). Taken together these data indicate a role of autocrine PDGFRα signaling in HHSteC migration.

3.3.2 Olaratumab Inhibits Baseline PDGFRα Activation in HHSteCs and Downstream Cell Motility Signaling

Since Olaratumab affected HHSteC migration specifically in the absence of exogenous PDGF stimulation, we next assessed downstream signaling affected by Olaratumab in the absence of PDGF ligand to address the potential mechanism by which PDGFRα mediates cell migration. Whole cell lysates from HHSteCs treated with Olaratumab or IgG control for 30 minutes, 1 hour and 3 hours were examined for levels of phosphorylated PDGFRα and its downstream effectors. Lysates from HHSteCs treated with PDGF-BB for 30 minutes serve as a positive control (Fig. 17).

We observed a notable decrease in PDGFRα phosphorylation at only Y762 and Y849, while other residues like Y754 remained unaffected following Olaratumab treatment (Fig. 17A, 18). Relative decreases in phosphorylation of Erk1/2 at 1- and 3-hours, as well as p38 MAPK at all timepoints, were observed after Olaratumab treatment (Fig. 17B, 18). Reduced phosphorylation of Elk-1 is consistent with these findings as Elk-1 is a known downstream target of both Erk1/2 and p38 MAPK (Fig. 17B) [144]. Olaratumab treatment led to a decrease in mTOR phosphorylation at Serine 2448 especially at 30 minutes and ,1-hour time point but an increase in phosphorylation at Serine 2481 at all times (Fig. 5C). This suggests a shift away from mTOR complex 1 (mTORC1) [147] - the rapamycin-sensitive form of mTORC whose inhibition has been previously shown to affect migration of activated HSCs[148].

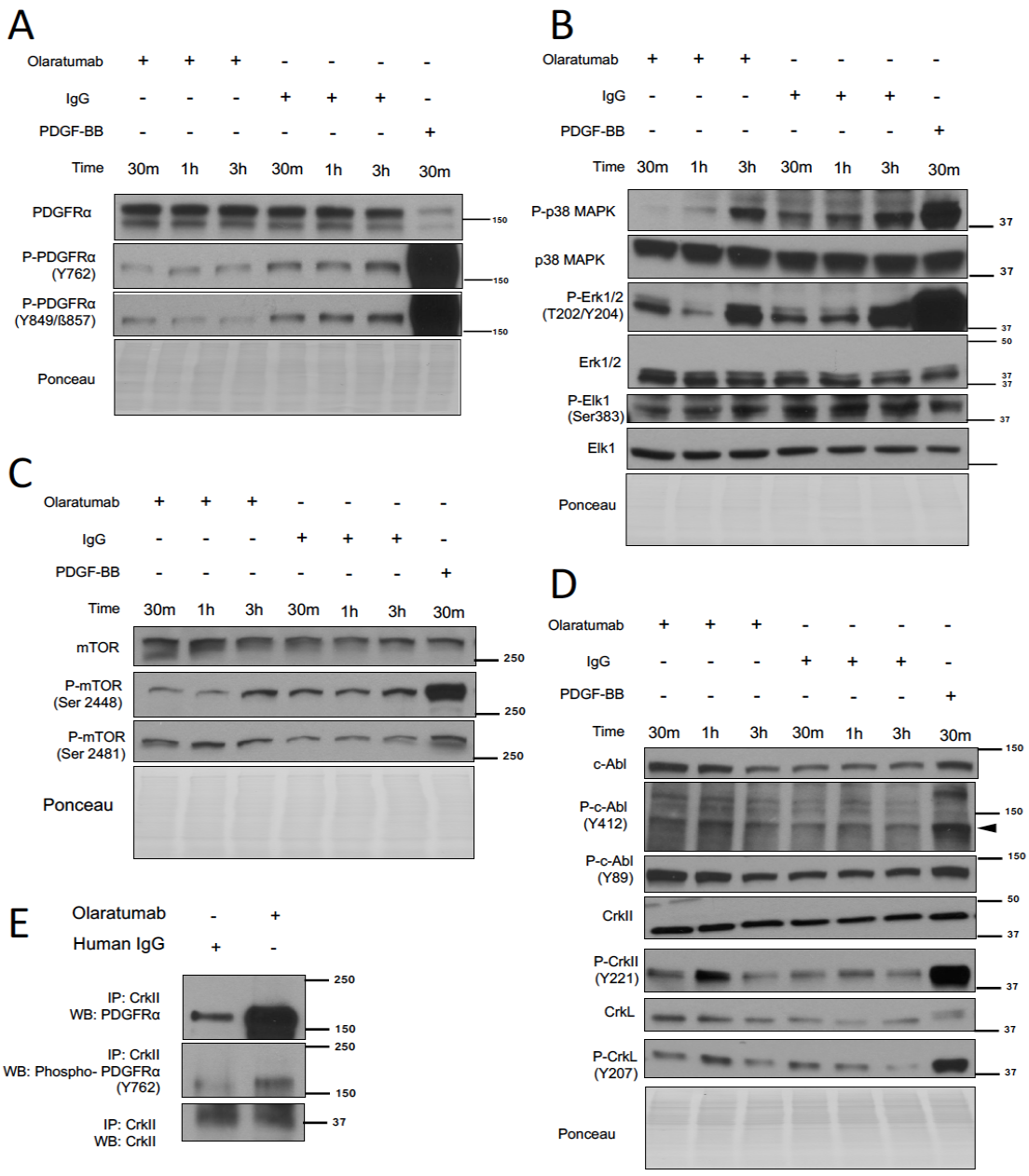


Figure 17: ***Olaratumab Inhibits Baseline PDGFRα Signaling in HHSteCs Along with Downstream Effectors.*** **(A)** Representative western blots from HHSteC treatment with Olaratumab (300nM) for the indicated time period showing decreased PDGFRα phosphorylation at Y762 and Y849 compared to IgG-treated controls. PDGF-BB treatment included as a positive control. **(B)** Representative western blots from HHSteC treatment with Olaratumab (300nM) for the indicated times showing decreased Erk and Elk-1 phosphorylation compared to IgG-treated controls. PDGF-BB treatment serves as a positive control. **(C)** Representative western blots show Olaratumab treatment decreased phosphorylation of mTOR at Ser2448 and increased phosphorylation at Ser2481. Olaratumab also decreased p38 phosphorylation as well. **(D)** Representative western blots show Olaratumab treatment increased Abl expression and phosphorylation at Y412 and Y89 and increased phosphorylation at inhibitory tyrosine residues of CrkII (Y221) and CrkL (Y207). **(E)** Immunoprecipitation of HHSteC lysates using anti-CrkII shows increased binding of CrkII to both total PDGFRα and Phospho-PDGFRα Y762 following Olaratumab treatment. Ponceau staining is included as a loading control. All Western blots were repeated twice on lysates from experimental conditions that were performed in triplicate and pooled for analysis.

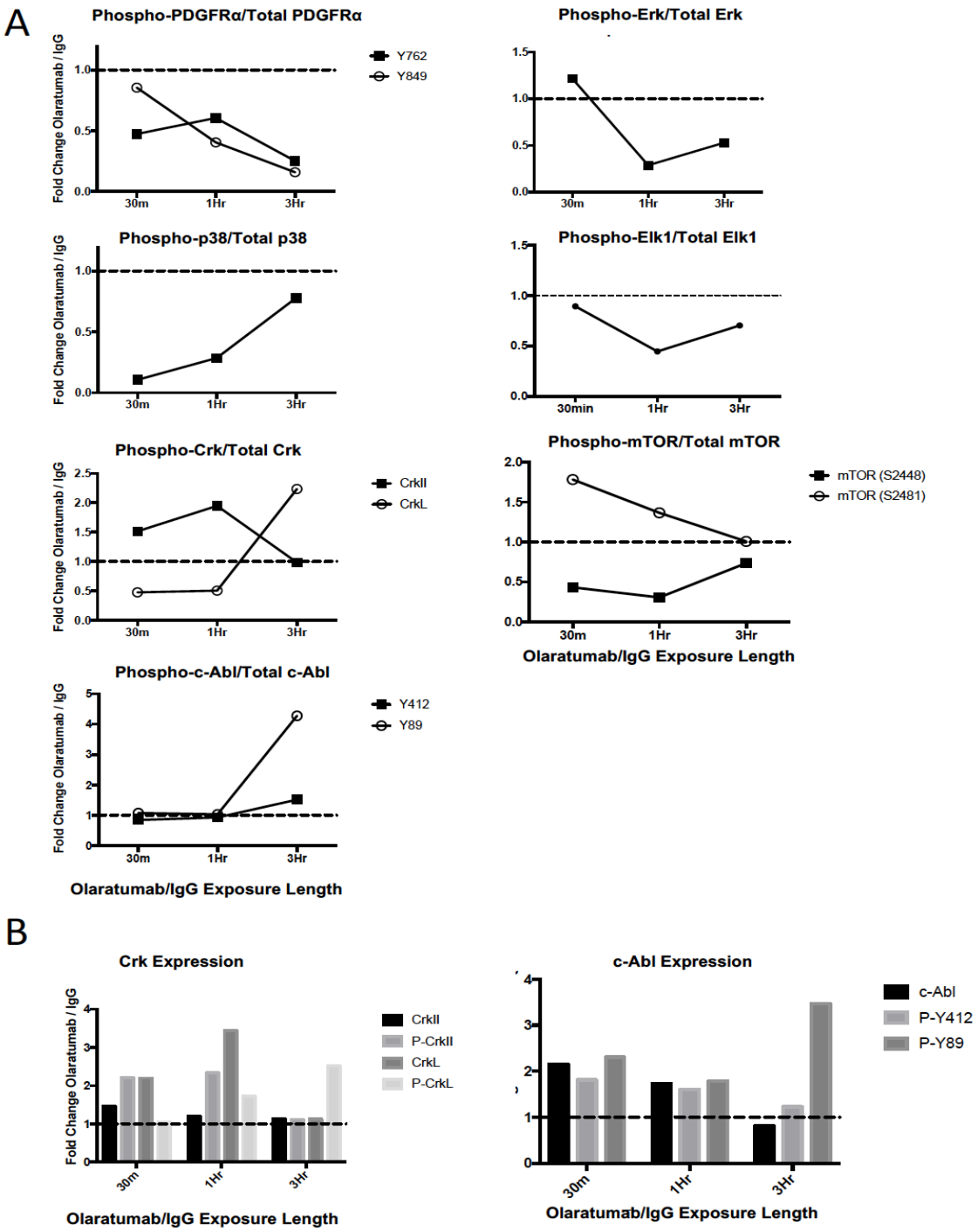


Figure 18: ***Densitometric Quantification of Olaratumab-Mediated Changes in Protein Phosphorylation in HHSteCs at Baseline.*** **(A)** Densitometry of key phosphorylated protein changes from representative Western blots shown in Figure 6. Values for each phosphorylated protein signal are normalized to total protein levels and shown as the ratio of the signal of Olaratumab-treated HHSteCs to IgG-treated HHSteCs. **(B)** c-Abl phosphorylated and total protein are represented separately to highlight the increase in both total and phosphorylated protein. The dashed line represents the value at which the relative phosphorylated protein signals of Olaratumab-treated HHSteCs and IgG-treated HHSteCs are equal.

We next examined the effect of Olaratumab on signaling mediators more specifically associated with cell migration. CrkII and its isoform CrkL are signaling adaptor proteins that play an active role in actin reorganization associated with cell motility[149]. Phosphorylation of CrkII (Y221) and CrkL (Y207) by Abl kinase results in sequestration of SH2 and SH3 binding domains of Crk proteins leading to inhibition of downstream signal activation[40]. Olaratumab treatment of HHSteCs led to increased phosphorylation of both CrkII and CrkL, suggesting decreased binding activity of these adaptor proteins (Fig. 17D). Consistent with inhibition of Crk signaling we observe activation of Abl kinase, indicated by phosphorylation of Abl at Y412[150] and Y89[151, 152] (Fig. 17D). The inhibition of Crk signaling downstream of PDGFRα inhibition is also consistent with the specific inhibition of PDGFRα phosphorylation at Y762 (Fig. 17A), which has been shown to be necessary for PDGF-dependent Crk signaling[133]. Although PDGFRα has been shown to bind directly to Crk proteins potentially leading to stabilization of a non-phosphorylated CrkII-PDGFRα complex and prolonging the signaling activity of CrkII, we noted Olaratumab to strongly induce CrkII protein interaction to both total PDGFRα as well as Phospho-PDGFRα Y762 (Fig. 17E). Thus, the overall basis of PDGFRα blockade on human HSC migration appears to be through a cumulative effect on modulation of Erk, p38 MAPK, mTORC1 and CrkII/CrkL.

3.4 DISCUSSION: DISSECTING THE ROLE OF PDGFRα IN HUMAN HSCS

The development of specific and potent inhibitors such as Olaratumab offers an opportunity to study exclusive PDGFRα function in human HSCs using a clinically viable therapeutic agent. Recently a phase II clinical trial for the use of Olaratumab in soft tissue sarcoma patients led to the FDA granting accelerated approval for this drug[110, 111]. In addition to its potential therapeutic

utility, Olaratumab also offers a means to study the specific signaling pathways activated by PDGFRα independent of PDGFRß. To our knowledge, our study is the first to directly examine the role of PDGFRα in human HSCs through pharmacologic inhibition of PDGFRα. Further, we examine the effect of PDGFRα-specific inhibition on downstream signaling mediators *in vitro*. Using the PDGFRα-specific inhibitor Olaratumab, we show that human hepatic stellate cells respond to exogenous or autocrine PDGFs in part through PDGFRα and specific downstream signaling to contribute to proliferation and migration, but not transdifferentiation to activated myofibroblasts (Fig. 13).

After confirming the expression of PDGFRα co-localized to HSCs and activated myofibroblasts in multiple models of murine chronic liver injury (Chapter 2), we show that human HSCs respond to exogenous or autocrine PDGFs in part through PDGFRα-specific downstream signaling to contribute to proliferation and migration, but not pro-fibrotic gene expression (Fig. 12). Baseline blockade of PDGFRα signaling in HHSteCs affected migration but not proliferation or pro-fibrotic gene expression. Exogenous PDGF-BB did not induce migration or pro-fibrotic gene expression but induced HHSteC proliferation, which was suppressed by PDGFRα blockade. These findings point to distinct biological outcomes of autocrine versus paracrine PDGF-BB/PDGFRα signaling axis. A possible explanation is a distinct cellular response to dose of ligand available at baseline versus during exogenous treatment. Suffice to say that both distinct and common signaling events were associated with the two biological outcomes of PDGFRα blockade. The major translational significance of the current study is the use of a clinically relevant biological in human HSCs and its effectiveness in suppressing two key processes of HSCs for treatment of hepatic fibrosis, which remains a major unmet clinical need.

Due to the central role of PDGF signaling in HSC proliferation, we tested the effect of PDGF as well as Olaratumab on HHSteC proliferation *in vitro*. We found that both PDGF-AA and PDGF-BB promote HHSteC proliferation, which was inhibited by Olaratumab. PDGFRß plays an important role in HSC proliferation. Studies in rat HSCs have shown PDGFRß to have higher mitogenic activity than PDGFRα [69, 70]. Since PDGF-BB is the predominant mitogen for HSCs during hepatic fibrosis, we wanted to address the relative contribution of PDGFRα towards HSC proliferation. In our study, Olaratumab blockade prior to PDGF-BB treatment of HSCs led to decreased HSC proliferation that was associated with inhibition of several phosphorylation sites in PDGFRα and of multiple downstream effectors including Erk1/2, p38, Elk-1, FAK, mTOR and Akt. In particular, Erk1/2, Elk-1, FAK, and Akt showed sustained reduction in activating phosphorylation after Olaratumab-mediated PDGFRα inhibition. Erk1/2 is known for its role in HSC proliferation [126], while its downstream target Elk-1 promotes gene expression associated with proliferation[153] and migration[144]. FAK has been shown to serve as a sensor for the detection of integrin-mediated binding to ECM and simultaneously acts as a signaling node for PDGF-induced proliferation in HSCs via FAK/PI3K/Akt pathway [128]. FAK also promotes Erk-mediated cell proliferation through phosphorylation at Y925 and Y397, two of the tyrosine residues affected by Olaratumab [154]. Akt affects cell proliferation through diverse sets of mechanisms [155, 156]. In contrast, the role of p38 in HSC proliferation is more ambiguous as inhibition of p38 in primary rat HSCs reduced proliferation[157] while p38 was necessary for PPARδ - induced LX-2 proliferation[158]. Our findings corroborate the results of the latter study, which may reflect a species-specific difference in the role of p38 in activated HSCs.

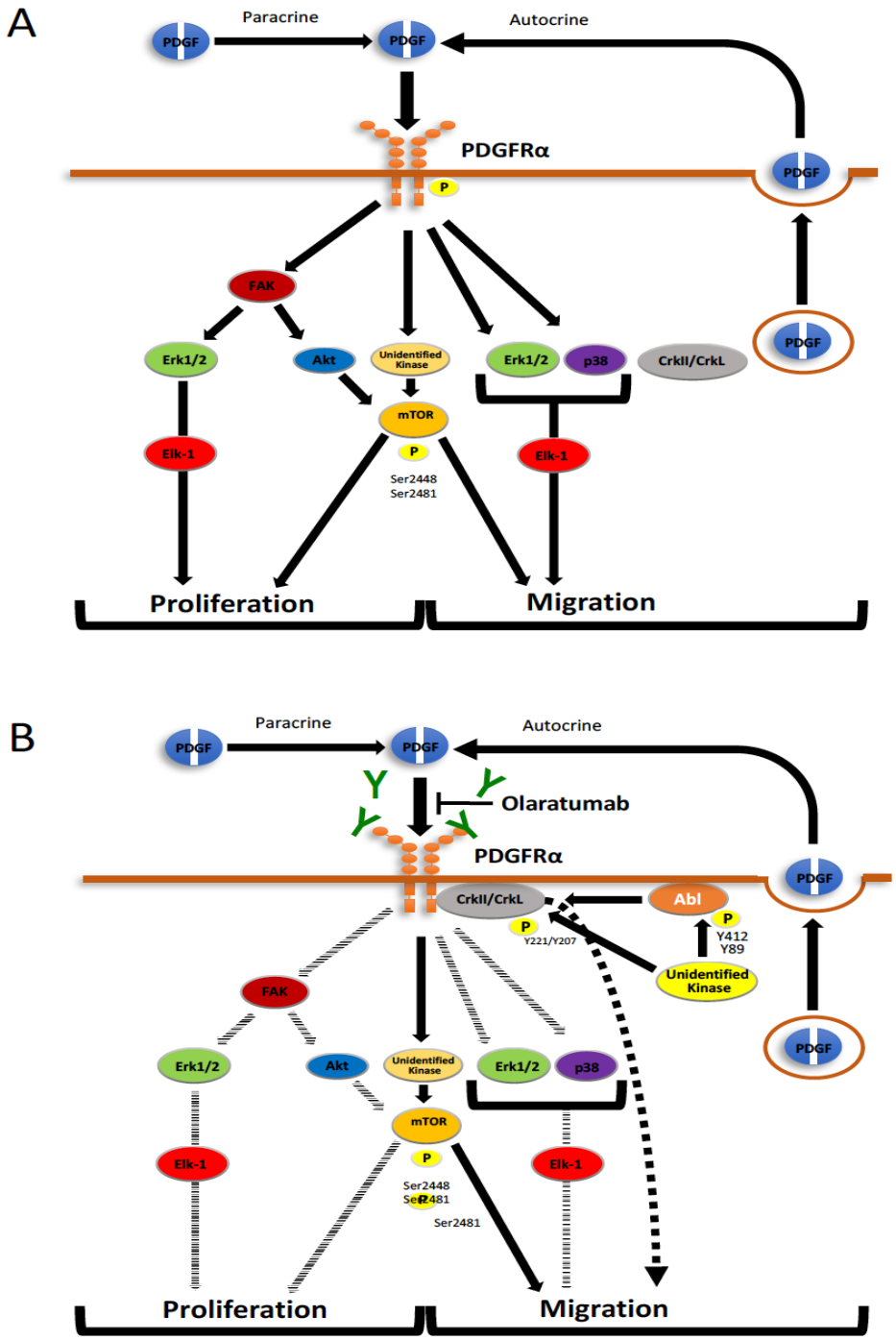


Figure 19: ***Proposed Effect of PDGFRα Blockade on HSC Migration and Proliferation through Inhibition of Specific Downstream Signaling.*** **(A)** In response to PDGFRα activation by autocrine and/or exogenous PDGF, HSC proliferation and migration are induced through activation of FAK, Erk1/2, p38, Akt, mTOR, and CrkII/CrkL signaling. **(B).** Blockade of PDGFRα by Olaratumab decreases the above mentioned signaling mediators, with FAK, Erk1/2, mTOR and Akt impacting proliferation and Erk1/2, p38, CrkII/L, decreased mTORC1 and increased mTORC2 regulating migration. Decreases in signaling activity of downstream targets is represented by dashed arrows.

It is important to note that PDGF-BB treatment led to an increase in phosphorylation of PDGFRα at Y754, which was decreased by Olaratumab. Y754 is an indicator of formation of PDGFRαß heterodimer[159], suggesting that Olaratumab mediates part of its effect on HSC proliferation through the inhibition of heterodimer formation. However, since Olaratumab inhibits phosphorylation of other signaling residues of PDGFR (which are more specific for PDGFRα homodimer) in the presence of exogenous PDGF-BB, we conclude that there is a definite and unique contribution of PDGFRα to HSC proliferation through Erk1/2, Elk-1, FAK and Akt (Fig. 14). A small increase in Erk1/2, Akt, mTOR, and FAK after initial Olaratumab treatment may be due to its potential transient and partial agonistic activity.

It was intriguing to note that expression of fibrosis-associated genes did not show a response to Olaratumab or PDGF-AA treatment in HHSteCs, suggesting that PDGFRα may be dispensable in HSC pro-fibrotic gene induction. Together, these data suggest that PDGFRα in HSCs may have a more narrowly defined function than its counterpart PDGFRß, a finding consistent with earlier investigations showing less potent activation of HSCs as a result of PDGFRα signaling[136, 160] as well as studies of rat HSCs showing that PDGFRß has higher mitogenic activity than PDGFRα[161, 162]. Notably, our studies do not preclude a ligand-independent contribution of PDGFRα signaling to HSC activation. This is particularly relevant in light of studies showing that PDGFRα is necessary for TGFß1-mediated SMAD activation following interaction and internalization of a PDGFRα monomer/TGFß Receptor II complex[77].

In our study, the Y762 residue of PDGFRα was one of the tyrosine residues specifically affected by Olaratumab treatment. This tyrosine residue has special significance because its phosphorylation has been shown to be necessary for the ability of PDGFRα directly bind the SH2 domain of Crk[133]. Indeed, one of the earliest substrate differences identified between PDGFRα

and PDGFRß was among the amino acids surrounding Y771 of PDGFRß and the homologous Y762 residue of PDGFRα[163]. Initially it was thought that out of the two PDGF receptor isoforms, only PDGFRα bound to Crk[133]. Later studies showed that PDGFRß bound to Crk as well, albeit in a much more transient nature - likely due to the relative inefficiency of Crk phosphorylation by PDGFRα compared to PDGFRß[132, 149]. The prolonged direct binding of PDGFRα to Crk proteins is considered to be one of the only major differences in signaling function between PDGFRα and PDGFRß[44], underscoring the significance of our findings in the setting of PDGFRα-specific inhibition by Olaratumab.

The prolonged binding of PDGFRα to Crk proteins is considered a major difference in signaling function between PDGFRα and PDGFRß [44]. Upon phosphorylation of Y221 on CrkII, and Y207 on CrkL, their SH2 domain is sequestered, leading to inhibition of Crk activity [164]. During its effect on cell migration, Olaratumab reduced phosphorylation of Y762-PDGFRα, which was associated with increased phosphorylation of CrkII and CrkL. Olaratumab treatment increased c-Abl activation as indicated by increased phosphorylation, which in turn is known to phosphorylate and inactivate CrkII/CrkL [40]. The relatively stable interaction of CrkII/CrkL and PDGFRα, in contrast to rapid phosphorylation by PDGFRß, has been speculated to lead to prolonged activity of CrkII/CrkL, leading us to hypothesize that inhibition of Y762 phosphorylation by Olaratumab may dissociate the Crk/PDGFRα complex and lead to early phosphorylation of CrkII/CrkL by another kinase like c-Abl [132]. Surprisingly, Olaratumab resulted in a dramatic increase in binding of CrkII to both total and Y762-phosphorylated PDGFRα. It is likely that Olaratumab-mediated enhanced phosphorylation and hence inhibition of Crk activity occurs despite enhanced PDGFRα-CrkII association. Further studies will be critical to uncover the precise mechanism by which Olaratumab alters CrkII/L phosphorylation.

Migration of HSCs is an important mechanism of perpetuation of hepatic fibrosis. PDGF ligands, specifically PDGF-BB are known chemotactic stimuli for HSCs [142]. We did not observe a significant increase in migration of HHSteCs following PDGF-BB treatment. However, HHSteCs expressed and secreted PDGF ligands at baseline, indicating potential autocrine PDGFRα signaling. Indeed, activation of HSCs as a result of cell culture is well-known in both primary [165, 166] and immortalized HSCs [167]. Olaratumab blocked HHSteC migration, which was associated with decreased PDGFRα phosphorylation at Y762 and Y849. Among residues not affected was Y754, ruling out the role of PDGFRαß heterodimer in HSC migration. Previous studies have shown that PDGF-AA-specific for the PDGFRαα homodimer is not chemo-attractive for HSCs in contrast to other PDGF ligand dimers [119, 168]. However, our study clearly shows a reproducible effect of PDGFRα inhibition on HHSteC migration, which was associated with notable decreases in phosphorylation of well-known mediators of HSC migration like Erk1/2 and p38 MAPK [169] and others like mTOR (Y2448) and Crk.

Olaratumab treatment of HHSteCs in the setting of both exogenous PDGF and autocrine signaling shared a reduction in Erk1/2 as well as mTOR. However in contrast to mTOR phosphorylation changes in the presence of exogenous PDGF, we observed differential changes in mTOR phosphorylation suggestive of a shift away from mTOR complex 1 (mTORC1) signaling to mTOR complex 2 (mTORC2) signaling (Fig.17C)[147]. Due to the apparent association of autocrine PDGFRα activation with migratory signaling in HHSteCs, this may mean that mTORC1 plays a more predominant downstream effector of PDGFRα. This finding is consistent with studies showing that mTOR inhibitors that primarily affect the rapamycin-sensitive mTORC1 have shown promise in reducing experimental fibrosis[170-172] - including through reduced proliferation and migration of HSCs in hepatic fibrosis[148]. Of particular note, rapamycin has been demonstrated to

reduce PDGF-induced migration of HSCs *in vitro*[143]. Further studies will be needed in order to definitively assess the role of mTOR downstream of PDGFRα signaling in HSCs.

Overall our study concludes that PDGFRα contributes to human HSC proliferation and migration independent of pro-fibrotic gene expression. These findings suggest that Olaratumab, alone or in combination, may have therapeutic activity in the pathogenesis of hepatic fibrosis. However, future research of potential therapeutic approaches aimed at inhibiting the PDGFRα pathway in *in vivo* models, including potential combinations, is unpredictable and would need to be performed prior to clinical investigation.

4.0 LOSS OF PDGFRα IN MICE AMELIORATES HEPATIC FIBROSIS DURING CHRONIC LIVER INJURY

In the previous chapter, we provided evidence that PDGFRα is functionally important to mitogenesis and cell migration in human primary HSCs. Our next aim was to determine whether loss of PDGFRα in HSCs *in vivo* affected the progression of chronic liver injury. In order to address this question, we have acquired *Lrat-cre* mice from our collaborator Dr. Robert Schwabe at Columbia University[173]. Using this strain, we have generated a novel murine model of Cre-lox recombination using the promoter for lecithin retinyl acyl-transferase (*Lrat*) to drive Cre expression in floxed *Pdgfra* mice in a HSC-specific manner. By subjecting resulting *Lrat*Cre *Pdgfra* knockout (*Pdgfra*-/-) animals and their wildtype littermate (WT) controls to multiple models of chronic liver injury, we sought to test the hypothesis that PDGFRα expression in HSCs contributes to the progression of hepatic fibrosis.

4.1 BACKGROUND: PRINCIPLES AND DEVELOPMENT OF HSC-SPECIFIC TRANSGENE EXPRESSION IN MICE

Animal models of chronic liver injury resulting in hepatic fibrosis and cirrhosis-like phenotypes (see Section 1.3) have developed in conjunction with advances in the identification of promoters driving transgene expression in quiescent and/or activated HSCs. Some of the promoters used to identify activated HSCs include components of promoters for collagen α-1(I), collagen α-2(I), and αSMA[21]. While effective, such promoters lead to targeted transgene expression only in

myofibroblasts and cells that have undergone a significant degree of activation. Due to the need to study factors contributing to HSC activation from a quiescent state in the non-injured liver, promoters targeting quiescent HSCs have also been widely used.

With increasing evidence that the large majority of myofibroblasts arise from transdifferentiation of HSCs[173], and due to the need to analyze the process of transdifferentiation of quiescent HSCs to activated HSCs and myofibroblasts, promoters targeting specific transgene expression in quiescent HSCs have become widely used in studies of hepatic fibrosis. The most prominent promoter used for targeting transgene expression in quiescent HSCs is glial fibrillary acidic protein (GFAP), an intermediate filament protein first discovered in astrocytes[174]. Despite the widespread use of GFAP promoter for HSC-targeted transgene expression, these models must be rigorously tested and validated for HSC-specific transgene expression, due to lineage tracing studies showing that the human or murine promoter for GFAP not reliably target HSCs and have robust cholangiocyte expression[173].

The search for more reliable promoters expressed specifically in non-activated HSCs led to the consideration of lecithin: retinol acyltransferase (Lrat). Lrat plays an important role in the formation of retinyl ester lipid droplets in HSCs which is necessary for the storage of retinoids (vitamin A and its metabolites) and is one of the primary characteristics of quiescent HSCs. In a seminal study demonstrating efficiency and specificity of Lrat promoter expression, Lrat-cre mice have been shown to express Cre in 99% of HSCs, and through fate tracing in toxic and cholestatic liver injury models, these HSCs were shown to give rise to 82-96% of myofibroblasts[173]. These studies, as well as findings from our own lab (unpublished) showing non-specifically transgene expression in GfapCre mice, have led us to use Lrat-Cre animals for the targeted expression of Cre transgene in HSCs.

4.2 VALIDATION STUDIES OF LRAT-CRE PDGFRα KO STRAIN

4.2.1 Loss of PDGFRα in Liver Lysates of Lrat-Cre *Pdgfra-/-* Mice

We have previously shown that PDGFRα is upregulated at the level of whole liver cell lysates following chronic liver injury in multiple models of chronic liver injury (Fig. 4). In order to confirm loss of PDGFRα expression in Lrat-Cre *Pdgfra-/-* mice, we examined whole liver lysates through Western blot and found that PDGFRα expression was lost or reduced in Lrat-Cre *Pdgfra-/-* livers in representative samples from CCl_4, BDL, and DDC models of liver injury (Fig.20A). The loss of nearly all PDGFRα expression in many *Pdgfra-/-* livers support the finding that PDGFRα is primarily expressed in HSCs and myofibroblasts, while the presence of residual PDGFRα expression in some *Pdgfra-/-* livers suggest possible expression of PDGFRα in infiltrating inflammatory cells or incomplete Cre recombination.

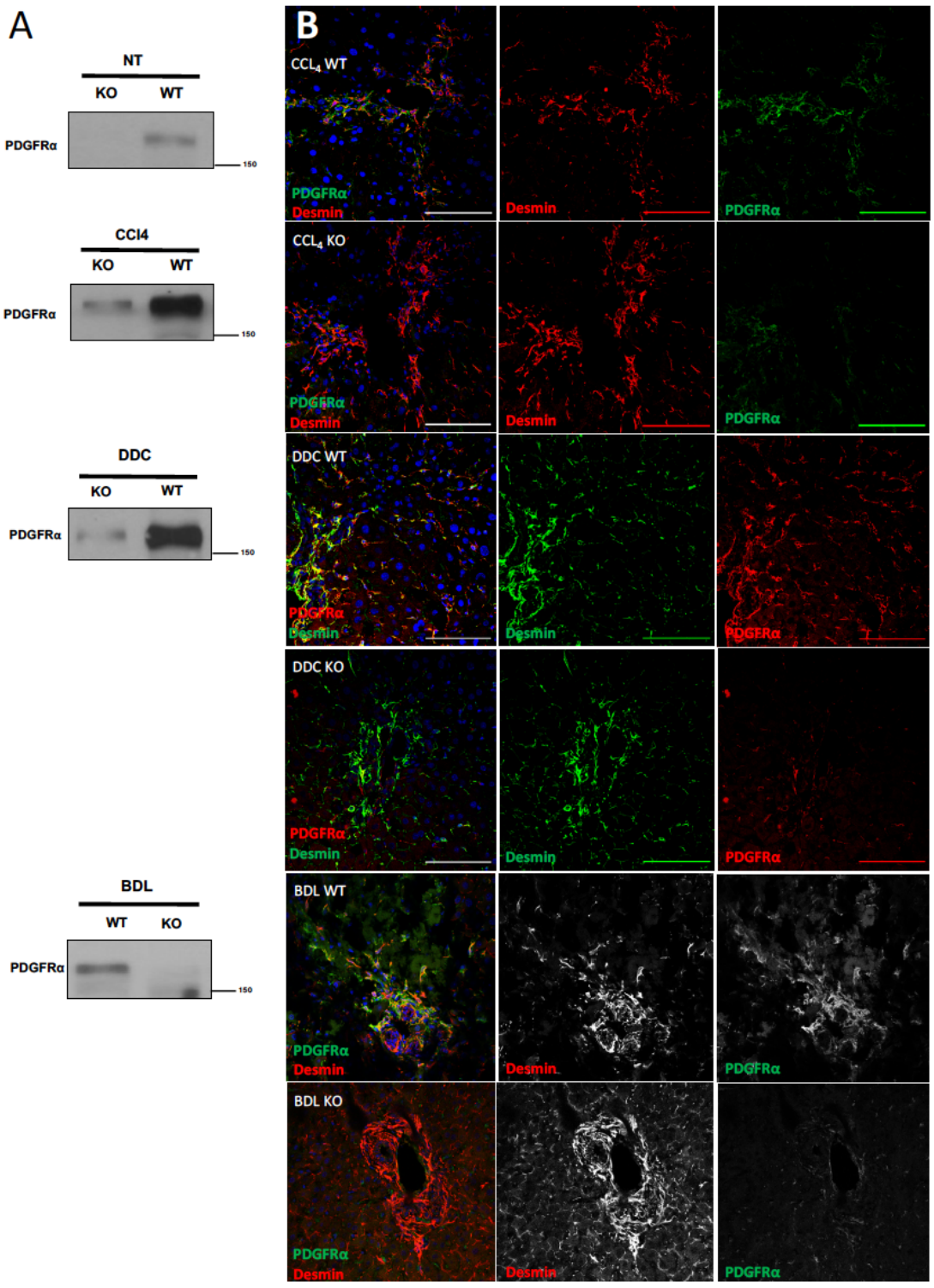


Figure 20: ***LratCre Pdgfra-/- Mice show Reduced Total and HSC/Myofibroblast-specific PDGFRα following Chronic Liver Injury.*** **(A)** Representative Western blots showing reduction of total PDGFRα expression in whole liver lysates of Lrat-Cre *Pdgfra-/-* (KO) and littermate controls (WT). **(B)** Representative confocal immunofluorescence microscopy showing reduction in PDGFRα expression in desmin-positive HSCs of Lrat-Cre *Pdgfra-/-* mice following 4 week CCl4, 16 day DDC, or 14 day BDL chronic liver injury. Scale bar represents 100µm.

4.2.2 Loss of PDGFRα in HSCs and Myofibroblasts of Lrat-Cre *Pdgfra-/-* Mice

In order to more specifically confirm loss of PDGFRα expression in HSCs and myofibroblasts in Lrat-Cre *Pdgfra-/-* mice, we performed co-localization immunofluorescence confocal microscopy of PDGFRα with either desmin (HSCs) or αSMA (myofibroblasts) in CCl_4, DDC, and BDL-injured livers of *Pdgfra-/-* and WT mice (Fig.20B). In each condition, we saw clear loss of PDGFRα in desmin-positive and αSMA-positive cells in *Pdgfra-/-* animals compared to WT littermate controls.

4.3 CHARACTERIZATION OF LRAT-CRE PDGFRα KO MICE

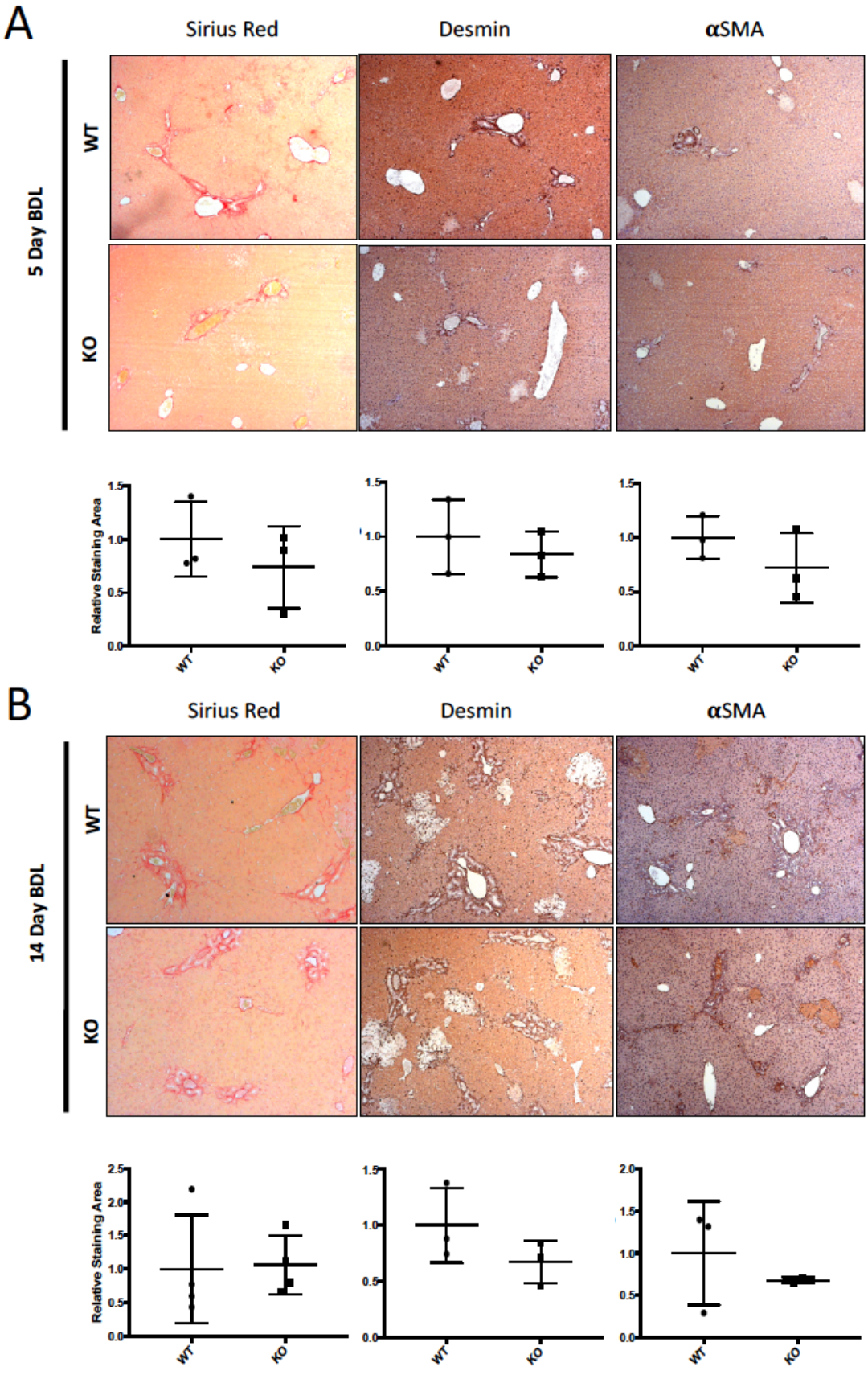
4.3.1 Biliary Fibrosis is Unaffected in Lrat-Cre *Pdgfra-/-* Mice Following Cholestatic Liver Injury

We first examined the phenotype of Lrat-Cre *Pdgfra-/-* mice in two models of cholestatic liver injury to examine the effect of PDGFRα loss in biliary fibrosis. Specifically, we examined fibrosis at 5 days and 14 days post-BDL as well as following 16 days of DDC-supplemented diet. Though we observed a qualitative reduction in collagen deposition in Lrat-Cre *Pdgfra-/-* mice at both 5 days post-BDL and 16 day DDC timepoints (Fig. 21A, C), this change was not significant when quantified under either condition. In addition, no changes in desmin or αSMA IHC was observed in any of the timepoints tested, including 14 day post-BDL (Fig. 21B). Collagen was quantified using polarized light microscopy for BDL samples. Collagen immunofluorescence was performed on DDC sections for quantification due to the detection of porphoryin plugs in brightfield and

polarized light microscopy which interfere with quantification. Based on these findings, we conclude that HSC-specific PDGFRα loss does not affect hepatic fibrosis in the setting of biliary injury.

4.3.2 Reduced Hepatic Fibrosis in Lrat-Cre *Pdgfra-/-* Mice Following Short Term Hepatotoxic Liver Injury

In order to determine whether loss of PDGFRα in Lrat-Cre *Pdgfra-/-* mice affected the progression of hepatic fibrosis in hepatotoxic liver injury, we next assessed LratCre *Pdgfra-/-* mice following 4 weeks or 8 weeks of CCl_4-induced liver injury. We found that after 4 weeks of CCl_4-induced liver injury, fibrosis was significantly reduced in Lrat-Cre *Pdgfra-/-* mice as assessed by quantification of Picrosirius red images taken with polarized light (brightfield images shown) (Fig. 22A). These changes were also reflected in desmin immunohistochemistry showing a reduction of the total number of HSCs in LratCre *Pdgfra-/-* mice (Fig. 22A). In contrast, no changes in fibrosis was seen following 8 week CCl_4 treatment (Fig. 22B). These findings suggest that PDGFRα does have an effect on hepatic fibrosis in hepatotoxic liver injury that is limited to early stages of fibrosis (4 week CCl_4) which is subsequently compensated in more advanced stages of fibrosis (8 week CCl_4).


A
Sirius Red
Desmin
αSMA
5 Day BDL
WT
KO
Relative Staining Area
1.5
1.0
0.5
0.0
WT
KO
B
Sirius Red
Desmin
αSMA
14 Day BDL
WT
KO
Relative Staining Area
2.5
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0.0
WT
KO

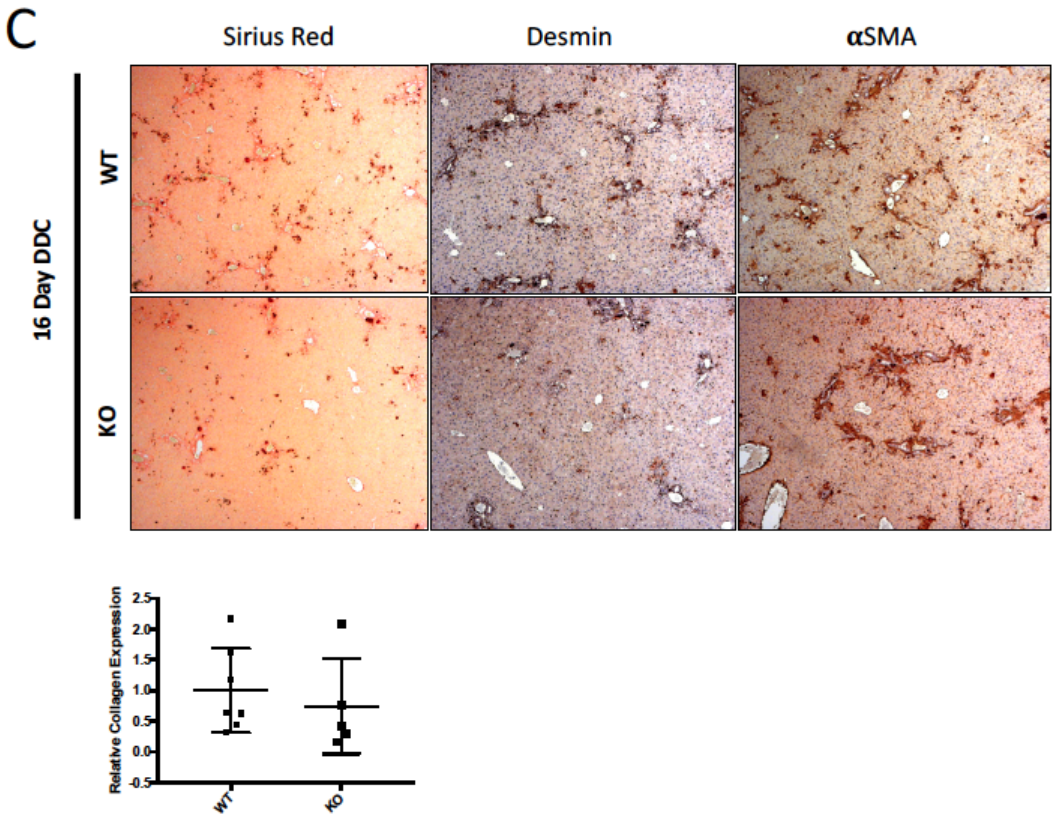

Figure 21: ***Biliary Fibrosis is Unaffected in LratCre Pdgfra-/- Mice following Cholestatic Liver Injury.*** Lrat-Cre *Pdgfra-/-* mice showed no significant change in fibrosis or HSC/myofibroblast population expansion as assessed by Sirius Red staining, desmin, and αSMA IHC. Biliary fibrosis was assessed at **(A)** 5 days and **(B)** 14 days post-BDL injury, as well as **(C)** 16 days DDC-induced liver injury. Quantification is shown. For DDC sections, collagen was quantified by immunofluorescence imaging.

Notably, no change in αSMA immunohistochemistry was observed at either timepoint, indicating that HSC transdifferentiation to myofibroblast phenotype may not be affected by PDGFRα loss.

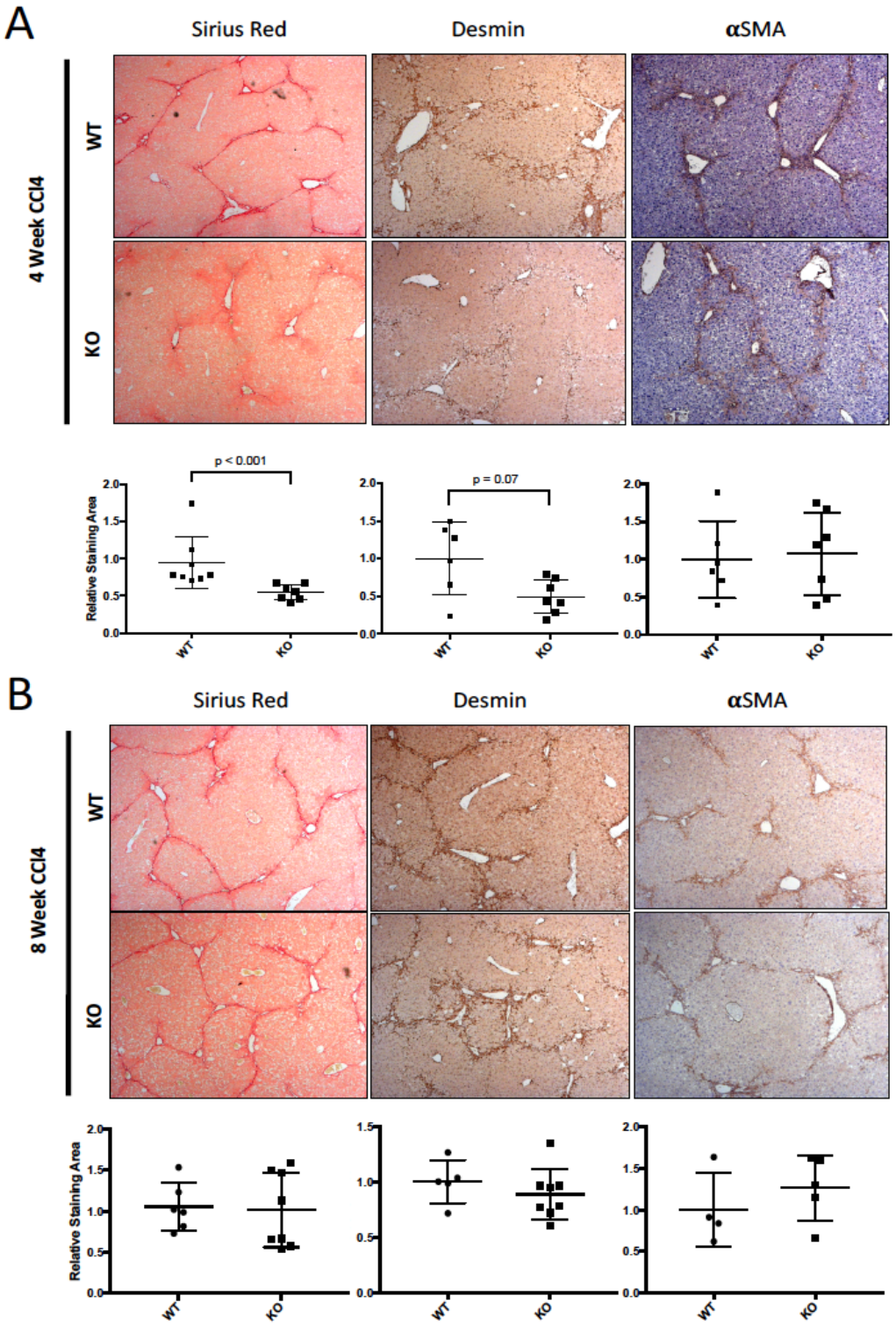

Figure 22: ***Lrat-Cre Pdgfra-/- Mice show Reduced Hepatic Fibrosis During Early, but not Advanced Hepatotoxic Liver Injury.*** **(A)** Lrat-Cre *Pdgfra-/-* mice showed reduced fibrosis as assessed by Sirius red staining and desmin IHC at 4 weeks CCl4-induced liver injury. No change in αSMA IHC was observed at this timepoint. **(B)** No change in hepatotoxic fibrosis was seen in Lrat-Cre *Pdgfra-/-* mice at the 8 week CCl_4 timepoint. Quantification of Sirius red collagen staining is shown.

4.3.3 Reduced Mid-Zonal Distribution of HSCs Following Biliary and Hepatotoxic Liver Injury

Migration is an important function of activated HSCs which facilitates their ability to reach areas of liver injury and initiate the deposition of ECM associated with hepatic fibrosis. Despite the lack of significant changes in overall levels of fibrosis in BDL- and DDC-induced liver injury models, closer analysis of HSC distribution in these models revealed a distinct decrease in mid-zonal HSC distribution in *Pdgfra-/-* livers as observed by desmin IHC (Fig. 23). In the absence of changes in overall desmin IHC, these findings may reflect a reduction in the ability of HSCs to migrate in the setting of biliary fibrosis. These changes were also observed in *Pdgfra-/-* animals following CCl_4-induced liver injury.

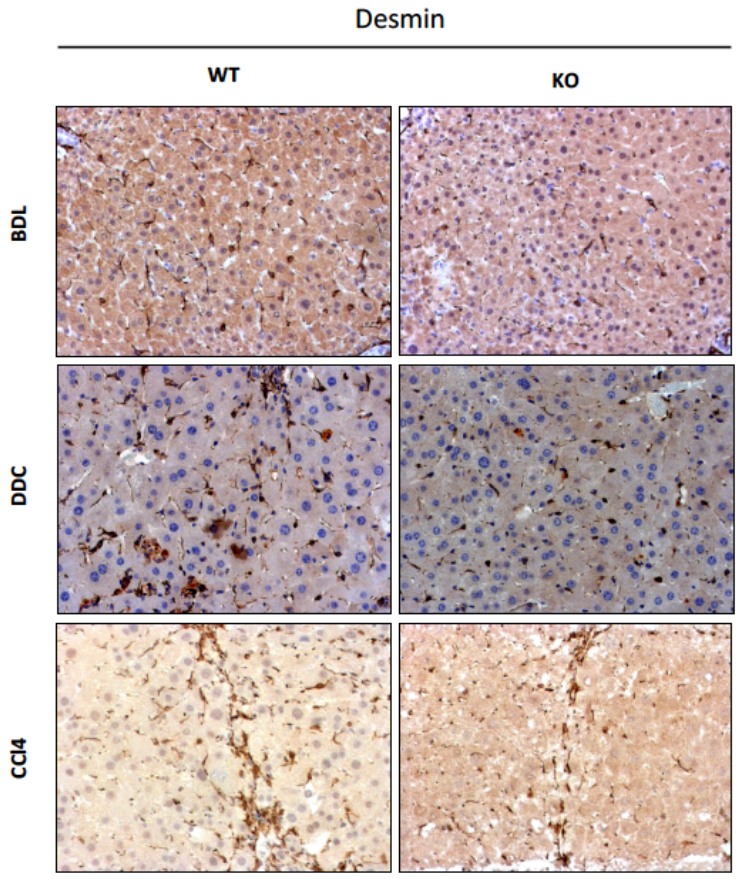

Figure 23: ***Lrat-Cre Pdgfra-/- Mice show Reduced Mid-Zonal Distribution of HSCs Following Biliary and Hepatotoxic Liver Injury.*** Representative higher magnification images of desmin IHC-stained liver sections highlight reduced distribution of HSCs in mid-zonal areas in Lrat-Cre Pdgfra-/- livers compared to WT controls. Images represent 5 day BDL, 16 day DDC, and 4 week CCl_4 timepoints.

4.3.4 Reduced Hepatocellular Injury and Increased HSC/Myofibroblast Cell Death in Lrat-Cre *Pdgfra-/-* Mice Following CCl_4

Analysis of liver function tests (LFTs) in cholestatic models of liver injury (BDL and DDC) showed no significant difference in the level of hepatocellular injury as indicated by similar ALT/AST in Lrat-Cre *Pdgfra-/-* animals (Fig. 24). In addition, early fibrosis timepoints in CCl_4 injury model revealed similar levels of hepatocellular injury (indicated by ALT, AST) as well as cholangiocyte injury (ALP). Interestingly however, while serum ALT/AST values continued to progress in WT animals, ALT/AST values in Lrat-Cre *Pdgfra-/-* animals plateaued and stayed at a similar level to the 4 week-treated cohort (Fig. 25A). In order to further investigate the potential reason for this discrepancy, we examined hepatocyte regeneration by PCNA immunohistochemistry as well as relative levels of necrosis and apoptosis assessed by TUNEL staining of liver sections in *Pdgfra-/-* and WT animals. While we did not see an overall difference in the level of hepatocyte proliferation assessed by PCNA (data not shown), we did see an intriguing inverse relationship between TUNEL staining of pericentral hepatocytes and TUNEL staining of spindular-shaped HSCs between *Pdgfra-/-* and WT animals (Fig. 24B). Specifically, TUNEL staining of Lrat-Cre *Pdgfra-/-* liver sections showed generally fewer TUNEL positive hepatocytes but larger numbers of TUNEL positive HSCs. The latter finding suggests that PDGFRα may promote survival of activated HSCs or myofibroblasts in advanced hepatic fibrosis, and that loss of PDGFRα in these cells may adversely affect cell survival, leading to increased apoptosis/necrosis of HSCs. The finding that Lrat-Cre *Pdgfra-/-* animals possessed decreased TUNEL positive hepatocytes is consistent with a decrease in overall hepatocellular injury as indicated by lower ALT/AST values. In order to determine a possible cause for this discrepancy, we next examined inflammatory cell response in these animals.

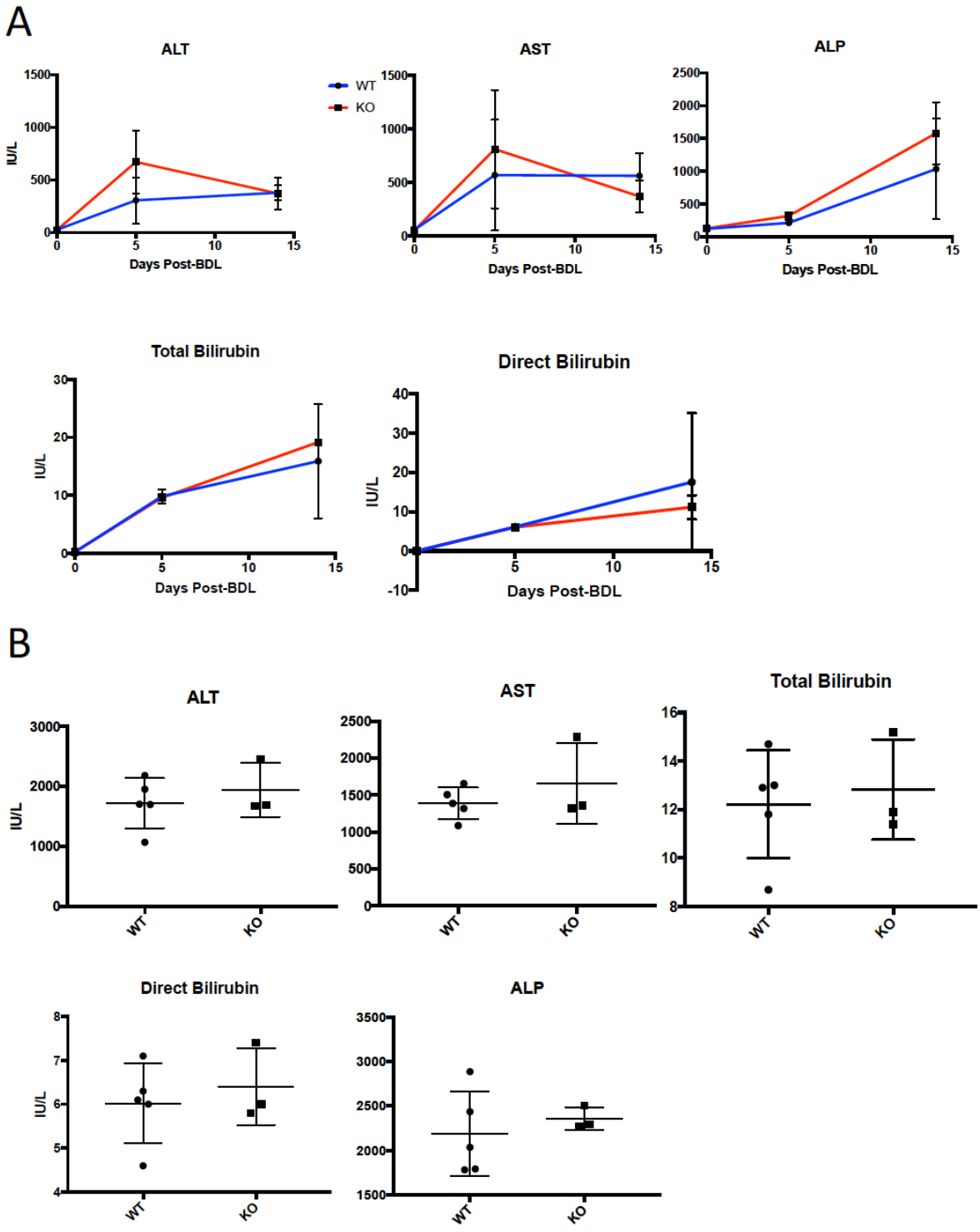

Figure 24: ***Hepatocellular Injury Following BDL and DDC Unaffected in Lrat-Cre Pdgfra-/- Mice.*** Liver function tests from harvested animal serum were performed and showed insignificant differences in ALT, AST, ALP, total and direct bilirubin values between Lrat-Cre *Pdgfra-/-* (KO) and littermate controls (WT) following **(A)** BDL or **(B)** DDC.

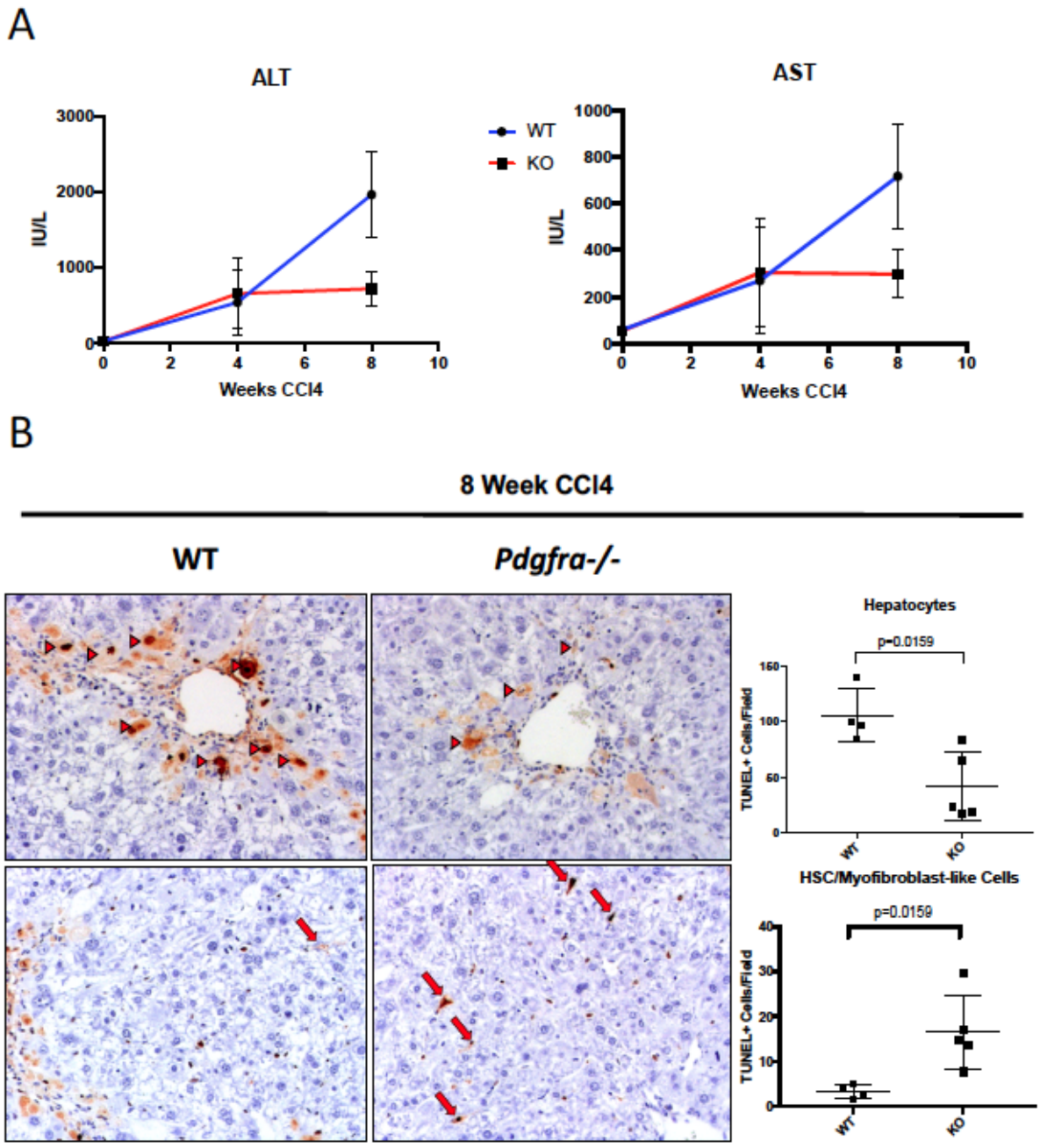

Figure 25: ***Reduced Hepatocellular Injury and Increase HSC/Myofibroblast Cell Death in Lrat-Cre Pdgfra-/- Mice Following CCl4.*** **(A)** ALT and AST values in Lrat-Cre Pdgfra-/- remained stable between 4 weeks and 8 weeks of CCl4-induced liver injury and were significantly reduced at the 8 week timepoint compared to littermate controls. **(B)** TUNEL staining of Lrat-Cre Pdgfra-/- liver sections following 8 week CCl4-induced liver injury shows increased numbers of TUNEL positive HSCs/myofibroblasts (arrows) while lacking the robust levels of TUNEL positive pericentral hepatocytes (arrowheads) in comparison to littermate controls.

4.3.5 Increased Inflammatory Response and Hepatic Macrophage Infiltration in Lrat-Cre *Pdgfra-/-* Mice Following Long Term CCl_4-induced Injury

To further elucidate the cause of reduced hepatocyte necrosis in *Pdgfra-/-* animals after 8 week CCl_4-induced liver injury, we examined general measures of inflammation including CD45 (leukocyte common antigen) and F4/80 (macrophage marker) immunohistochemistry. Despite reduced levels of fibrosis between WT and *Pdgfra*-/- animals at 4 week CCl_4 liver injury, we saw no differences in either CD45 or F4/80 IHC at this timepoint (Fig. 26). In contrast, we saw a notable increase in both CD45 and F4/80 IHC in *Pdgfra*-/- animals at the 8 week CCl_4 timepoint. Notably, the increase in both CD45 positive cells as well as F4/80 positive macrophages was primarily observed in fibrotic foci centered around pericentral regions. Since the major function of hepatic macrophages as part of the innate immune response is the clearance of cellular debris and apoptotic bodies, it is likely that the increased presence of macrophages in these regions leads to the relative reduction in necrotic hepatocytes in *Pdgfra*-/- animals at the 8 week CCl_4 timepoint compared to littermate controls (previously shown in Fig. 25B). This finding is also consistent with the improved transaminase profiles shown in Figure 25A.

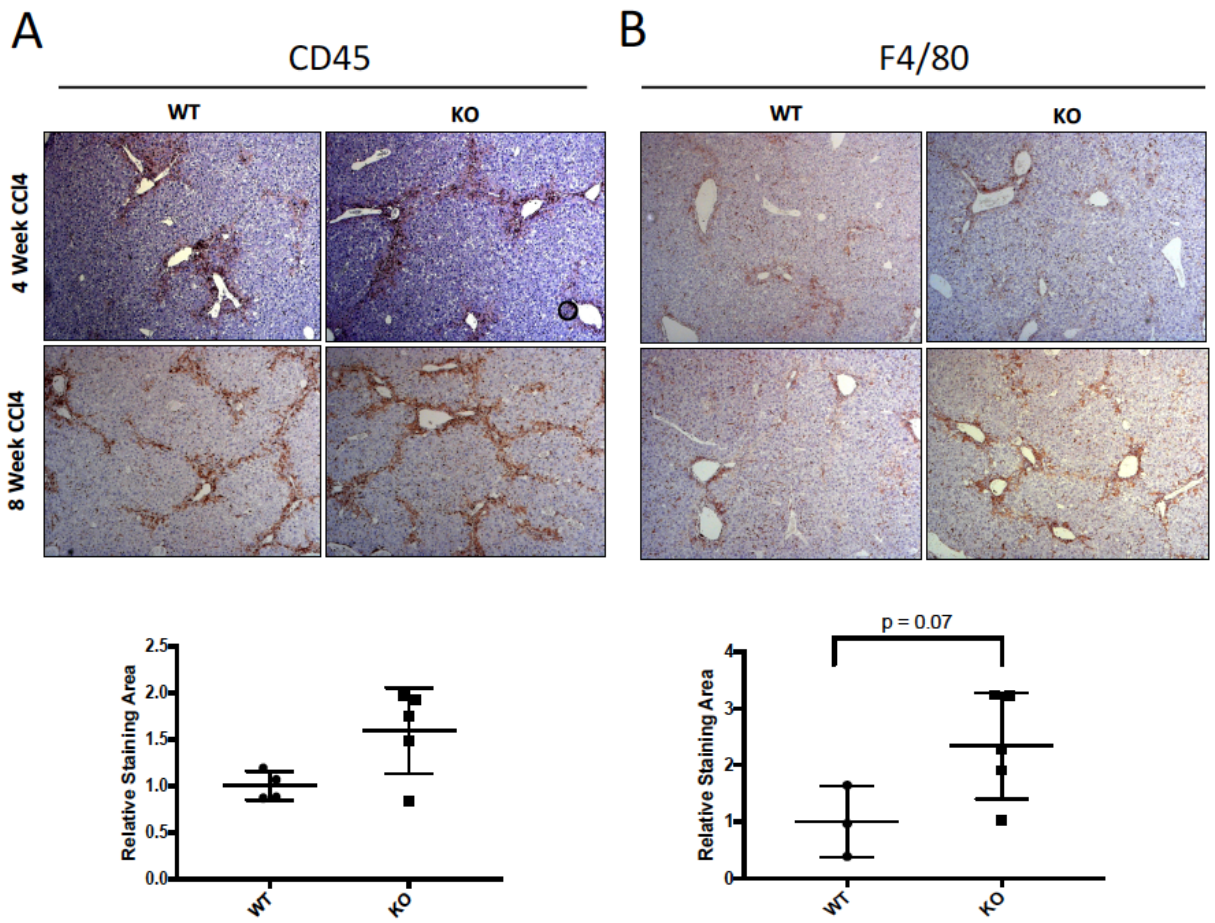

Figure 26: ***Increased Inflammatory Response and Hepatic Macrophage Infiltration in Lrat-Cre Pdgfra-/- Mice Following Long-Term CCl_4-induced Injury:*** CD45 **(A)** and F4/80 **(B)** immunohistochemistry of liver sections from 4 week and 8 week CCl_4-treated Lrat-Cre *Pdgfra-/-* (KO) mice and littermate controls (WT) are shown. No change in CD45 positive or F4/80 positive cells is observed at 4 week timepoint between KO and WT mice but an increase in both cell types is observed in KO mice at 8 week timepoint.

4.4 DISCUSSION: DOES PDGFRα PLAY A SUBSTANTIAL ROLE IN HEPATIC FIBROSIS AND LIVER INJURY?

Our study is the first to examine the loss of PDGFRα in a HSC-specific manner using the HSC-specific promoter for Lrat, in order to direct Cre expression for the excision of floxed *Pdgfra* in a highly specific and efficient manner. This model is rigorously validated by confocal co-localization immunofluorescence to show loss of PDGFRα in HSCs/myofibroblasts of KO

animals. Previous studies have identified PDGFRα expression in HSCs and shown their upregulation in HSCs following CCl_4-induced chronic liver injury as well as reduced fibrosis in mice with heterozygous expression of PDGFRα[68]. In this study, mice heterozygous for PDGFRα were used due to the fact that global *Pdgfra* null animals are embryonically lethal. While heterozygous loss of PDGFRα prevents the developmental defects associated with global PDGFRα loss, these animals do not lose PDGFRα in a cell-specific manner and by definition would likely retain some PDGFRα expression HSCs. Our study provides proof of principle that PDGFRα loss specifically in HSCs ameliorates early CCl_4-induced fibrosis *in vivo*. In addition, we utilize multiple models of chronic liver injury including CCl_4, BDL, and DDC, in order to comprehensively test the validity of our findings across different modes of chronic liver injury.

Studies of chronic liver injury on the mechanisms of hepatic fibrosis progression have focused on the potential for therapeutic intervention to prevent or reverse fibrosis. The ultimate therapeutic goal of such intervention is to prevent the progression of hepatic fibrosis to end stage liver diseases such as cirrhosis or HCC, which accompany devastating clinical consequences. There is however established clinical and pre-clinical evidence that advanced fibrosis and cirrhosis are less reversible (or irreversible) compared to earlier stages of fibrosis[4-6]. In our study, we observed decreased hepatic fibrosis in Lrat-Cre *Pdgfra-/-* animals following 4 week CCl_4-induced liver injury but not after 8 weeks of CCl_4 (Fig. 22). This finding is consistent with a contributory role of PDGFRα with the initiation and early perpetuation of hepatic fibrosis and suggests that while PDGFRα inhibition may play an important role in ameliorating early fibrosis, it is not likely to be sufficient as a monotherapy for the complete prevention or reversion of advanced fibrosis.

Furthermore, Lrat-Cre *Pdgfra-/-* animals did not show a significant reduction in fibrosis in either models of biliary fibrosis (BDL, DDC), even when biliary fibrosis was assessed at relatively

early liver injury timepoints (5 days post-BDL, 16 days DDC). A potential explanation for the discrepancy in findings between hepatotoxic liver injury (CCl_4) and cholestatic liver injury (BDL, DDC) is the presence of a small population of portal fibroblasts which does not express Lrat. These cells have been previously identified as a precursor contributing to the myofibroblast population in biliary fibrosis and consequently may retain PDGFRα expression in our model[173]. Mederacke et al found that while the majority of myofibroblasts originated from Lrat-tdTomato-expressing cells in both toxic (CCl_4, TAA) and cholestatic (BDL, DDC) forms of liver injury, the overlap between tdTomato expression and myofibroblast marker αSMA was considerably less in cholestatic liver injury (82-85%) compared to toxic liver injury (93-96%) models.

Earlier in this document we outlined the conflicted evidence for a major role of PDGF signaling in portal fibroblast fibrogenicity (see section 1.7.3). Further studies will be needed to determine whether PDGFRα is expressed specifically in the portal fibroblast subpopulation, and whether potential retention of PDGFRα in this population can help to supplement the myofibroblast supply in a compensatory manner in Lrat-Cre *Pdgfra-/-* animals during biliary fibrosis. If so, the discrepancy in our findings in hepatotoxic and biliary fibrosis may reflect a limitation of the Lrat-Cre model of transgene expression, rather than a redundant or inconsequential role of PDGFRα in biliary fibrosis. In this regard, pharmacologic inhibition of PDGFRα in cholestatic liver injury models may be useful to circumvent these issues.

Another limitation of our current model is the inability to distinguish *in vivo* contributions of PDGFRα signaling in HSCs compared to transdifferentiated myofibroblasts. Studies have shown a differential response and sensitivity to pro- or anti-fibrogenic growth factors between HSCs and myofibroblasts. For example, cultured rat HSCs showed reduced TGFß1-induced proliferation inhibition in their transdifferentiated (myofibroblast-like) form – despite similar

levels of TGFß receptor expression in both forms- due to changes in relative ligand-binding affinity of TGFß receptors[175]. Similarly, there is evidence that PDGF-induced proliferation in HSCs may be an earlier event in the progression of HSC transdifferentiation. Evidence that HSC proliferation in response to PDGF is an early response of HSCs in cholestatic liver injury[176]. Based on this evidence, it is possible that higher numbers of myofibroblasts present in more advanced stages of liver fibrosis (represented in our study by the 8 week CCl_4 cohorts) may be less dependent on PDGFRα signaling for the perpetuation of fibrosis.

In Chapter 3 we showed that PDGFRα contributes to proliferation and migration of human HSCs, while lacking a direct effect on the expression of fibrosis-associated genes including ACTA2 (αSMA). These findings provide a functional explanation for how loss of PDGFRα *in vivo* may alter the progression of hepatic fibrosis. For example, our finding that desmin IHC is reduced in *Pdgfra-/-* livers following 4 week CCl_4 injury supports the notion of impaired HSC proliferation (Fig. 22A). Increased HSC death at this timepoint is unlikely to explain the reduction in desmin positivity, as *Pdgfra-/-* and WT livers showed similar low levels of TUNEL staining at this timepoint (data not shown). Also consistent with our findings in human HSCs, no changes in αSMA IHC were seen under any conditions, suggesting a lack of an effect of PDGFRα on myofibroblast transdifferentiation.

Examination of the mid-zonal regions of *Pdgfra-/-* livers following desmin IHC also suggests a possible effect of PDGFRα loss on HSC migration. Migration of HSCs presumably facilitates the movement of activated HSCs towards areas of injury and inflammation and is likely to contribute the formation of characteristic “bridging fibrosis” seen in advanced hepatic fibrosis. Based on these assumptions, we posit that a reduction in the mid-zonal distribution of HSCs in *Pdgfra-/-* livers (Fig. 23) may be partially due to a defect in HSC migration – particularly in biliary

fibrosis where overall HSC proliferation appears to be unaffected due to similar levels of desmin positivity (Fig. 21). These findings are consistent with our findings that Olaratumab inhibits migration in human HSCs (Fig. 16). At this time however we cannot rule out the possibility that the observed changes in mid-zonal HSC distribution are primarily driven by anti-proliferative or anti-survival effects of PDGFRα loss – especially in CCl_4-induced liver injury. Future studies incorporating HSC isolation and subsequent migration assays will be required to show whether migration of HSCs is significantly affected in Lrat-Cre *Pdgfra-/-* mice.

ALT and AST values are the most commonly used clinical surrogate measures of hepatocyte death upon which liver injury and disease is screened and monitored in patients. During CCl_4-mediated liver injury, we saw a remarkable plateau of ALT and AST between the 4 week and 8 week timepoints in which ALT/AST levels did not increase in Lrat-Cre *Pdgfra*-/- animals compared to littermate controls. Consistent with these changes in ALT/AST, we observed decreased TUNEL-positive hepatocytes in *Pdgfra*-/- animals representing an overall reduced level of hepatotoxicity compared to littermate controls. Of particular interest was a concomitant increase in TUNEL-positive HSCs in *Pdgfra*-/- mice at this timepoint.

Apoptosis is a known mechanism of myofibroblast reduction during regression of fibrosis[22]. While PDGFRα is known to play a role in cell survival in mesenchymal cell types[177] against apoptotic stimuli, studies thus far have not reported a contribution of PDGF receptors in promoting HSC survival independent from their effect on HSC transdifferentiation to myofibroblasts during liver injury. For example, PDGFRß siRNA does not induce apoptosis of HSCs isolated from rats post-BDL[178]. In another study of activated HSCs isolated from rat livers post-BDL, PDGF-AB did not reduce rat HSC apoptosis in response to serum deprivation[179]. Therefore, our findings may indicate a novel and distinct pro-survival function of PDGFRα

signaling in HSCs and suggest that loss of PDGFRα in myofibroblasts of Lrat-Cre *Pdgfra-/-* mice may make these cells more vulnerable to premature apoptosis. One way in which the effect of PDGFRα signaling on HSC/myofibroblast survival may be through TGFß-conferred anti-apoptosis which has previously been reported in rat HSCs *in vitro*[180, 181]. This could conceivably result from the PDGF ligand-independent interactions of PDGFRα and internalized TGFß receptor I/TGFß Receptor II heterodimer complexes following binding to TGFß which has been demonstrated in human HSCs[77].

While PDGF is known to promote myofibroblast transdifferentiation from HSCs, we posit that increased resistance to apoptosis as a result of myofibroblast transdifferentiation[182] is not the driving force behind the increase in TUNEL positive HSCs/myofibroblasts in *Pdgfra -/-* animals at 8 weeks CCl_4. This is because we did not observe a change total number of transdifferentiated myofibroblasts as assessed by αSMA IHC. This finding is consistent with our previous findings in primary human HSCs *in vitro,* in which we have shown that inhibition of PDGFRα using Olaratumab did not have an effect on the expression of fibrosis-associated gene expression (Fig. 13).

The substantial increase in F4/80 positive cells in *Pdgfra-/-* animals centered at the pericentral hepatocyte foci of injury at 8 weeks of CCl_4-induced injury suggest that hepatic macrophages may be driving an accelerated immune-mediated clearance of apoptotic/necrotic hepatocytes and cell debris in these animals. We posit that this effect may be responsible for the overall reduction in liver injury seen at this timepoint as reflected by ALT/AST serum values. Immune-mediated clearance of cell debris resulting from hepatocyte apoptosis/necrosis is considered an important step for effective hepatocyte regeneration. Furthermore, it has previously been shown that hepatic macrophage depletion in rats following CCl_4-mediated injury has

differential effects during different periods of injury and recovery – with macrophage depletion early in liver injury alleviating fibrosis and macrophage depletion during CCl_4 recovery resulting in slower recovery and decreased fibrosis regression[183]. This dual nature of hepatic macrophages on chronic liver injury may explain the discrepancy between our findings in *Pdgfra*-/- mice fed DDC-supplemented diet for 16 days – a short term injury course in which fibrosis and inflammation were positively correlated – and those treated with CCl_4 for 8 weeks where inflammation was negatively correlated with injury outcome. Therefore, loss of PDGFRα in HSCs/myofibroblasts and its effects on survival may indirectly promote a beneficial inflammatory response in later stages of chronic liver injury that improves clearance of hepatocyte debris and ameliorates further progression of liver injury. This proposed phenomenon is illustrated in Figure 27.

Our model is naturally limited by the lack of inducibility of Cre transgene expression in HSCs. This limitation precludes us from drawing clear distinctions between the effect of PDGFRα loss in quiescent HSCs compared to activated HSCs and fully transdifferentiated myofibroblasts. Nevertheless, our study provides clear evidence of a substantial contribution of PDGFRα to the progression of hepatic fibrosis. Future studies will be needed to confirm whether PDGFRα inhibition in a therapeutic manner will be effective in reversing the course of fibrosis (see section 5.2 below).

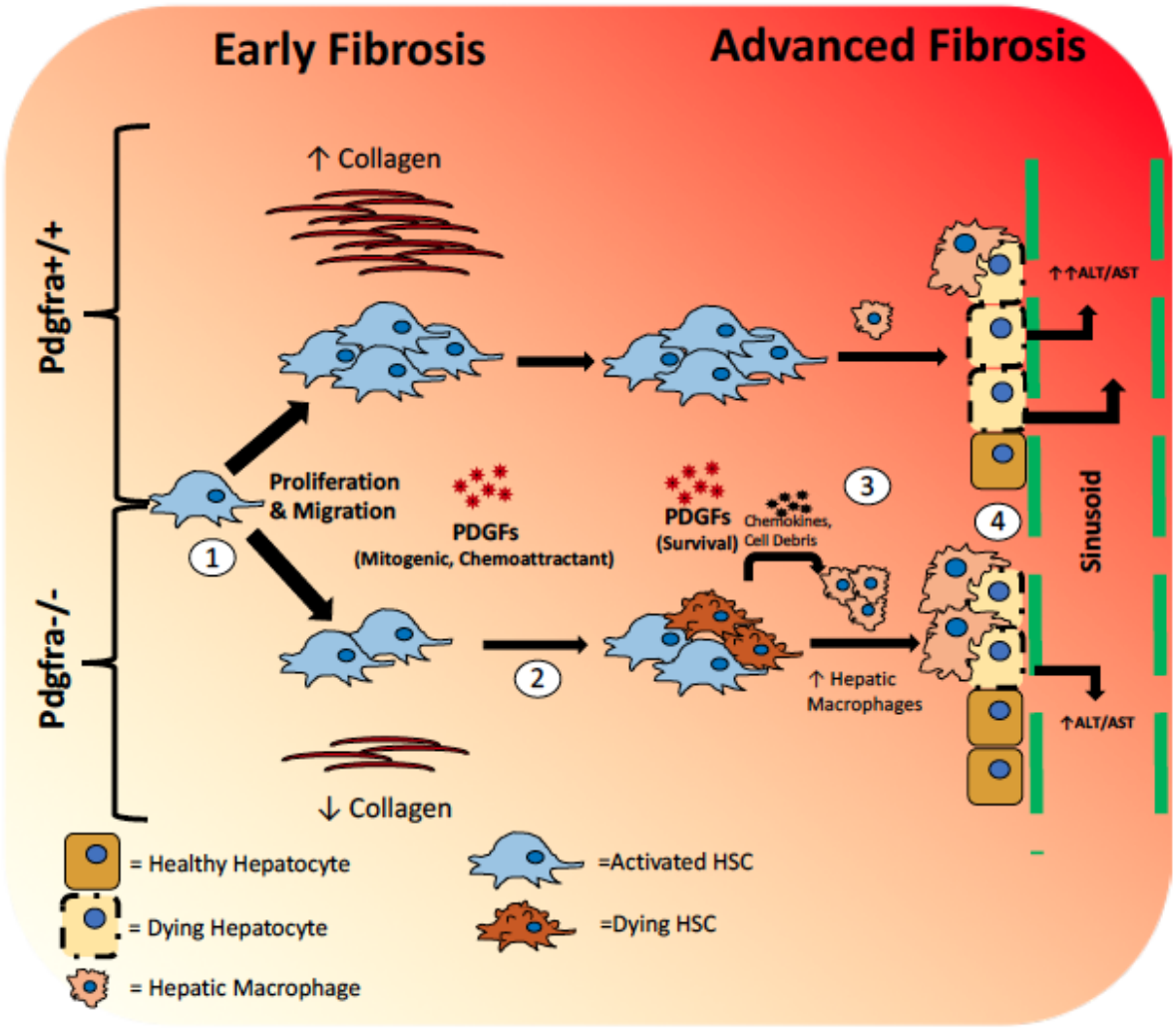

Figure 27: ***Proposed Timeline of the Impact of PDGFRα Loss in HSCs During CCl4-induced Chronic Liver Injury:*** **(1)** During early stages of liver fibrosis (represented at 4 weeks of CCl_4 administration), reduction in HSCs is observed as well as decreased hepatic fibrosis – likely as a result of impaired HSC proliferation and migration. **(2)** As chronic liver injury progresses (represented at 8 weeks of CCl_4 administration), HSCs/myofibroblasts in *Pdgfra*-/- animals undergo increased levels of cell death which is hypothesized to be the result of decreased response to PDGF survival signals. **(3)** The presence of increased inflammatory cells and hepatic macrophages in *Pdgfra*-/- animals may be the result of pro-inflammatory cell debris and chemokines released from dying HSCs/myofibroblasts. **(4)** The resulting increased influx of hepatic macrophages in *Pdgfra*-/- animals results in improved clearing of necrotic hepatocytes near injury foci, ameliorating overall liver injury. This improvement in hepatocellular injury is detected as reduced levels of ALT/AST in Lrat-Cre *Pdgfra-/-* serum following 8 weeks of CCl_4 compared to littermate controls (*Pdgfra*+/+).

5.0 DISCUSSION AND FUTURE STUDIES

5.1 PDGFRα AND PDGFRß IN HEPATIC STELLATE CELLS: INTERCHANGEABLE OR DISCRETE FUNCTIONS?

PDGFRα and PDGFRß have mostly overlapping downstream signaling pathways and functions and respond to similar ligands as described in Section 1.4. Due to these structural and functional similarities, it is reasonable to question whether their functions in HSCs are distinct and whether one isoform is able to sufficiently compensate for the other in the event of receptor loss or inhibition. This question has therapeutic implications and will need to be answered if isoform-specific inhibitors such as Olaratumab are to be considered for therapeutic use in chronic liver injury. While our current studies do not explicitly compare PDGFRß-specific loss or inhibition in HSCs, we do show that PDGFRα inhibition *in vitro* and downregulation *in vivo* has a distinct functional contribution in HSCs separate from expression of PDGFRß.

Recent studies examining the effects of PDGFRß loss in HSCs in murine CCl_4 and BDL injury models support the notion that PDGFRß is a major contributor to the fibrogenic potential of HSCs[135]. In comparing the findings of this study with our study in vivo, it is apparent that both PDGFRß and PDGFRα promote HSC proliferation in chronic liver injury – as early fibrosis timepoints in each of these studies (1 week and 4 weeks, respectively) show decreased fibrosis and HSC numbers as assessed by Sirius red staining and desmin IHC. In contrast, PDGFRα loss in our model did not result in changes in αSMA IHC while PDGFRß deficient mice showed a significant reduction of this measure of HSC activation.

Furthermore, the level of fibrosis reduction achieved in these studies suggest that PDGFRß is a more predominant mediator of fibrosis than PDGFRα. Nevertheless, our data indicate that PDGFRα plays a role in the progression of early fibrosis as well as recovery from injury in later fibrosis. Combined with our studies of Olaratumab-mediated PDGFRα inhibition in human primary HSCs showing that PDGFRα inhibition did not result in changes of fibrosis-associated gene expression, our studies point towards a divergence in function between PDGFRα and PDGFRß where PDGFRß contributes to both HSC proliferation and activation, while PDGFRα contributes to proliferation only.

One interesting and unanswered question is the relative contributions of PDGFRα and PDGFRß to the survival of HSCs/myofibroblasts in the presence of fibrosis resolution. As discussed above in Section 4.4, our data points to a potential pro-survival effect of PDGFRα which is lost in HSCs of *Pdgfra*-/- mice following 8 weeks of CCl_4-induced liver injury. Since loss of PDGFRα at this timepoint had no effect on total or activated HSC numbers measured by desmin or αSMA IHC, respectively, this change cannot be accounted for by increased HSC proliferation or myofibroblast transdifferentiation. In contrast, the effect of PDGFRß loss *in vivo* on HSC apoptosis/necrosis has not yet been investigated, though studies of isolated rat HSCs following BDL suggest that siRNA-mediated loss of PDGFRß does not affect HSC survival[178].

Interestingly, while Kocabayoglu et al[135] investigated different timepoints of CCl_4-induced injury (1 week and 6 weeks) compared to our study (4 weeks and 8 weeks), a similar pattern of ALT/AST reduction was seen in both of these models. Specifically, while PDGFRß loss and PDGFRα loss at early timepoints (1 week and 4 weeks, respectively) did not result in a difference between ALT/AST values between knockout and littermate controls, a similar reduction in ALT/AST was seen at the later timepoints assessed in these studies (6 weeks and 8 weeks,

respectively). This similar trend is remarkable because it suggests that inhibition of PDGFRα or PDGFRß individually may improve the outcome of advanced liver injury, even while expression of its sister isoform receptor in these HSCs is retained. In other words, the beneficial effects of one PDGF receptor isoform loss *in vivo* does not appear to be averted by compensatory signaling of its sister isoform – at least at the timepoints tested in these studies.

These findings are also noteworthy because they suggest that an improvement in liver injury outcome may not be contingent upon reduction of overall hepatic fibrosis. While it is generally agreed that hepatic fibrosis is a pathogenic process that broadly correlates with liver dysfunction and hepatotoxicity, it remains unclear to what extent fibrotic changes in the liver drive chronic liver injury and loss of function, rather than vice versa. On this point – while the resolution of hepatic fibrosis has been a major goal of our study and the many preceding it, it has not conclusively been shown that resolution of hepatic fibrosis alone will resolve chronic liver injury in patients without abatement of the source of injury and hepatocellular repair/regeneration. For these reasons, we consider PDGFRα alone or in combination with PDGFRß to be viable therapeutic targets that warrant further study and consideration in the treatment of chronic liver injury.

5.2 PROSPECTS FOR PDGFRα-SPECIFIC INHIBITORS IN HEPATIC FIBROSIS

The development of specific inhibitors of PDGFRα has shown promising results in preclinical and clinical studies. The use of Olaratumab in our studies in human HSCs (Chapter 3) as well as the therapeutic effect of PDGFRα loss in mice during chronic liver injury (Chapter 4) provides important proof-of-concept evidence of the potential effectiveness of specifically targeting

PDGFRα signaling in chronic liver conditions such as fibrosis, cirrhosis and HCC (discussed further in next section). While multi-TKI small molecules targeting multiple receptors and pathways have therapeutic effects, they may also be prone to a lower therapeutic index due to toxicity from bystander receptor activation. Inhibitory monoclonal antibodies targeting single receptors have several advantages including higher specificity, lower off-target tissue toxicity, and the use of higher drug doses with lower risk of developing drug resistance due to sub-therapeutic dosing[110, 111]. For instance, Olaratumab shows approximately 100-fold increased effect on PDGF-mediated cell proliferation compared to the multi-TKI Imatinib (Gleevec) [112]. In addition, antibodies generally have a longer half-life (necessitating less frequent dosing) and less variation in clearance among individuals than most small molecule therapies[184].

Current multi-TKIs that co-target PDGF receptors and have shown anti-fibrotic activity in the liver are metabolized by CYP450 enzymes of hepatocytes and are poorly tolerated by patients. For these agents, hepatotoxicity remains a major limiting factor for effective dosing – a problem which is exacerbated in patients with chronic liver injury who are likely to have decreased liver dysfunction at baseline[113]. Currently Sorafenib – the only moleculary therapeutic approved for treatment of HCC - has been shown to have major concerns for liver toxicity at relatively low doses[185]

We have previously shown that Olaratumab decreases proliferation of various hepatoma and HCC cell lines [53]. In addition, our previous studies in ß-catenin KO mice suggest that PDGFRα may be an important co-therapeutic target in ß-catenin inhibition of HCC [186]. Based on the high frequency of PDGFRα overexpression [53, 187] and the relative success of Sorafenib (a PDGFRα co-inhibitor) in human HCCs, blockade of PDGFRα signaling may indeed have therapeutic value in liver cancer. In addition to Olaratumab, other PDGFRα-specific inhibitors are available for use in

preclinical models such as the murine PDGFRα inhibitor APA5 [188-191]. Combined with the potential for genetic modulation of PDGFRα demonstrated in the data presented here and in previous studies [52, 68], an abundance of potential preclinical models for PDGFRα modulation are available and await future investigation in liver regeneration, injury, and cancer.

The development of specific inhibitors of PDGFRα has shown promising results in preclinical and clinical studies. Olaratumab is a unique, directed antibody therapy that exclusively targets PDGFRα and is currently being tested in clinical trials[112]. Olaratumab has been shown to have anti-tumor properties in several preclinical studies including lung cancer xenografts[138], glioblastoma and leiomyosarcoma xenografts[139], and ovarian carcinoma[140] . The use of highly-specific small molecule inhibitors also allows the close study of functions and signaling pathways attributable to specific receptors – an endeavor which is obscured by the use of multi-tyrosine kinase inhibitors. As an example, the multi-kinase inhibitor sorafenib which has shown some promise in a preclinical context for the treatment of hepatic fibrosis remains not fully understood in its mechanism of action.

Though our studies suggest that PDGFRα plays a limited role in the perpetuation of hepatic fibrosis – with effects on collagen deposition limited to early stages of hepatic fibrosis (Fig. 21-22) – the observed effects on overall hepatocellular injury and inflammation (Fig. 25-26) suggest that PDGFRα inhibition may have therapeutic activity that is not directly tied to levels of fibrosis. Studies closely examining *in vivo* loss or inhibition of PDGFRα following the establishment of fibrosis, and during the resolution of chronic liver injury, will be critical to determine its future utility as an anti-fibrotic therapy. This is discussed in further detail below (Section 5.4).

5.3 BEYOND FIBROSIS: STUDIES OF PDGFRα INHIBITION IN LIVER CANCER

In patients with cirrhosis, hyperplastic hepatocytes succumb to increasing genomic instability as a result of unrelenting necrosis and regeneration in the face of chronic liver injury. In a subset of these patients, these regenerative hepatocytes eventually form dysplastic nodules and give rise to HCC [10]. HCC is the most common type of liver cancer with a 5 year survival rate of only 8.9% in the U.S.A. [10] Currently non-palliative treatments for cirrhosis and HCC are limited to radiofrequency ablation, transarterial chemoembolization, resection, and liver transplantation [11]. The latter is the most effective but is associated with high morbidity, cost, life-long immunosuppressive therapy and a shortage of donor organs. Thus, there is a strong need for the identification of new therapeutic targets associated with the pathogenesis of these conditions as well as effective methods of inhibition.

Studies from our lab and others suggest a role of PDGFRα dysregulation in hepatocarcinogenesis (full review in [192]). We have previously shown that the majority of human HCCs overexpress PDGFRα and that a subset of these tumors also show an upregulation of PDGF-AA and PDGF-CC[53]. This study also demonstrates that several human and rat cell lines of hepatoma and HCC also exhibit increased expression and activation of PDGFRα, and *in vitro* inhibition of PDGFRα in these human cell lines using Olaratumab led to significant decreases in DNA synthesis and cell survival. PDGFRα overexpression was also detected in 46/63 (73.0%) patients who did not undergo neoadjuvant chemo/radiotherapy [187]. In this study, a significant clinical correlation was found between vascular invasion in resected HCCs that overexpress PDGFRα as well as those that overexpress PDGFRß compared to those that did not. In addition, the co-expression of PDGFRα, PDGFRß, and VEGF was identified by multivariate analysis to be

an independent prognostic factor of disease-free and overall survival in this cohort. Furthermore, PDGFRA is upregulated in K19 positive HCCs from patients, which are associated with increased tumor size, microvascular invasion, metastasis, and poor differentiation [193]. Lastly, a study found high intratumoral expression of PDGFRα and PDGFRß in a small subset of HCCs, which were independently associated with poor overall survival [194]. The seeming discrepancy between the number of patients expressing 'high' levels of PDGFRα in this study compared to other studies in which the majority of patients overexpress PDGFRα [53, 187] may be explained by the categorization of patients into 'high' or 'low' expression groups, in which only tissues staining with the highest intensity on a five tier scale were categorized as 'high' – rather than direct comparison of PDGFRα upregulation compared to adjacent normal liver tissue.

Findings in patients are corroborated in preclinical animal models of HCC. Mice lacking the secreted proteoglycan decorin - a tumor suppressor inhibiting both EGFR and the hepatocyte growth factor receptor Met - have dysregulated PDGFRα signaling in TAA-induced hepatocarcinogenesis leading to more severe cirrhosis and HCC [85]. This was possibly due to impaired sequestration of secreted PDGF ligands by decorin in the ECM and increased production of PDGF.

These findings suggest that unregulated PDGFRα signaling is pathogenic and may promote hepatocarcinogenesis. The number of studies suggesting a role of PDGFRα in promoting hepatocarcinogenesis has been a driving impetus for the further study of specific roles of PDGFRα in liver cancer. Some of the potential modes of action and regulation of PDGFRα in HCC are discussed in the following sections.

5.3.1 Potential Roles of PDGFRα in Tumor Biology: Modulation of Angiogenesis and Hypoxic Response in Chronic Liver Injury and Cancer

HCC is a highly-vascularized tumor for which PDGFRs represent potential alternative targets to supplement traditional VEGFR inhibitors. While PDGFRß has been the most well documented PDGF receptor for angiogenic effects including vessel stability [195] and maturation [196], there is evidence for a role of PDGFRα in angiogenesis as well.

Studies have shown that specific PDGFRα blockade results in the downregulation of angiogenic factors which may be an important mode of growth inhibition in tumors [141]. Furthermore PDGFRα is a co-target of several anti-angiogenic drugs [197], some with anti-fibrogenic effects [198]. PDGFRα expression in endothelial cells (ECs) [199, 200] and vascular smooth muscle cells [201], as well as liver sinusoidal ECs [64] has been reported. In addition, the presence of PDGFRα in liver EC in liver cancer is strongly supported by findings in HCC (described below) indicating its upregulation in pathologic angiogenesis.

There is substantial evidence that PDGFRα signaling in HCC is associated with metastasis and tumor progression, mediated at least in part by pathologic angiogenesis. PDGFRα is overexpressed in EC of HCC associated w/ high metastatic potential in a murine xenograft model and increased recurrence of HCC in patients [93]. In fact, PDGFRα is one of the only known tumor EC markers in HCC that correlates with metastasis. Higher tumor recurrence rate and lower survival in human HCCs expressing high PDGFRA was reaffirmed in a study by Zhu et al [202]. This study also employed a murine xenograft model using an HCC cell line and transfected human umbilical vein endothelial cells (HUVECs) to show evidence that tumor progression may be the result of dysregulation of PDGFRA by BRCA1, which is in turn regulated by microRNA 146a

(MiR-146a). This study shows a potential regulatory mechanism of PDGFRα expression in ECs of HCC, and introduces a new potential therapeutic target upstream of PDGFRα (MiR-146a).

While the precise role of PDGFRα/PDGFA signaling in HCC progression is unknown, studies indicate that this signaling arm is likely to be an important escape pathway for pathologic angiogenesis in the setting of HCC. One murine HCC model showed increased PDGFA expression in the liver following drug resistance development to IFN-alpha [203], an antiviral with known anti-angiogenic effects [204]. This is consistent with evidence that tumor fibroblasts may become resistant to anti-VEGF therapy through the expression of PDGF-C [94]. Furthermore, it has been shown that VEGFA can activate PDGFRα and PDGFRß, likely due to the close homology between PDGFR and VEGFR [205]. Thus, PDGFRα signaling may be an important alternative therapeutic target in addition to VEGFRs, and may explain why sorafenib (a multi-tyrosine kinase receptor inhibitor targeting VEGFRs and PDGFRs) is currently the only clinically approved targeted therapy for HCC [12, 13].

PDGF signaling is also important for communication between HSCs and ECs of the liver during angiogenesis to coordinate the formation and stabilization of neovessels. Co-transplantation of Ras-transformed hepatocytes with myofibroblasts in a murine model of HCC enhances tumor growth in a PDGF-dependent manner [206]. Studies in rats undergoing BDL demonstrate that PDGF-BB promotes HSC-driven vascular tube formation through ephrinB2 signaling[207]. The authors in this study hypothesize that this phenomenon is responsible for a decrease in portal pressure in BDL rats following Imatinib treatment. Given the known expression of PDGFRα on HSCs [65, 68], and HCC-associated EC [93], it is possible that PDGFRα activation by PDGF ligands may play an active role in these processes.

Hypoxia is a well-known driver of pathologic angiogenesis. Though the specific response of PDGFRα in hypoxic liver tissue has not been reported, potential activity of this receptor can be gleaned from hypoxia-induced modulation of PDGF ligands, especially PDGF-A. PDGFA as well as PDGFB are downregulated in HIF-1α deficient mice, signifying a link between hypoxia and the release of these profibrogenic mediators [208]. HIF-1α/ß in hepatocytes *in vitro* do not appear to significantly affect the production of PDGF-A or PDGF-B mRNA but, rather, promote other angiogenic factors including VEGF. Combined with this group's previous findings in HIF-1α deficient mice, these data indicate that HIF-1 is regulating PDGF-A and –B expression in NPCs. HIF-2α is also shown to be a likely mediator of this effect [209]. Investigation of PDGFR localization and activation in response to hypoxia will be an important complement to studies of HIF-induced ligand production in order to discern the precise effects of PDGFRs in hypoxic response and angiogenesis.

5.4 FUTURE STUDIES

Earlier in this document we outlined the conflicted evidence for a major role of PDGF signaling in portal fibroblast fibrogenicity (see section 1.7.3). One limitation of the murine model utilized in Chapter 4 is the presence of a small population of portal fibroblasts which do not express Lrat which have been previously identified as contributing to myofibroblasts and would retain PDGFRα expression in our model[173]. Mederacke et al found that while the majority of myofibroblasts originated from Lrat-Cre expressing tdTomato-expressing cells in both toxic (CCl_4, TAA) and cholestatic (BDL, DDC) forms of liver injury, the overlap between tdTomato expression and myofibroblast marker αSMA was considerably less in cholestatic liver injury (82-85%) compared

to toxic liver injury (93-96%) models. This data mirrors our own findings in BDL and DDC in which some PDGFRα expression is retained in myofibroblasts of knockout animals (Fig. 20 and data not shown). Thus additional studies will be needed to determine if portal fibroblasts specifically express PDGFRα, and whether PDGF signaling affects their contribution to biliary fibrosis.

Based on our findings described in Chapter 4 that PDGFRα may contribute to HSC survival, it remains to be answered whether PDGFRα inhibition will have beneficial effects on the resolution of fibrosis. In order to answer this question, we will assess levels of hepatic fibrosis, liver injury, and hepatocellular regeneration in a model of fibrosis resolution (8 weeks CCl_4 administration followed by 7 days recovery). Mechanisms of cell death will also need to be more closely examined to determine whether increased TUNEL positivity of HSCs/myofibroblasts is the result of apoptosis, necrosis, or necroptosis – a recently elucidated pathway of regulated necrosis[210].

Our current findings using Olaratumab to inhibit PDGFRα signaling in human HSCs *in vitro*, as well as our findings that early hepatic fibrosis is ameliorated in Lrat-Cre *Pdgfra-/-* mice, provides an important foundation for future studies of therapeutic PDGFRα inhibition in hepatic fibrosis. One approach synthesizing the strengths of the two approaches mentioned above is the therapeutic administration of PDGFRα inhibitor to *in vivo* models of chronic liver injury. The human-specific inhibitory nature of Olaratumab, as well as our inability to obtain sufficient quantities of murine-targeting PDGFRα-specific inhibitor precluded us from undertaking such a study at this time. However, such studies will be important in order to strengthen the findings outlined in this dissertation and to avoid influence of HSC-specific PDGFRα loss during normal liver development in LratCre *Pdgfra-/-* animals (see Section 1.5).

Future studies utilizing robust HSC isolation will also be needed in our current animal models as well as the prospective study mentioned above. HSC isolation will allow direct assessment of proliferative, migratory, and survival responses from HSCs isolated from Lrat-Cre *Pdgfra-/-* animals compared to littermate controls. Such studies would begin to clarify the distinct role of HSC PDGFRα signaling in the propagation of hepatic fibrosis.

Cell Type	Normal Liver	Biliary Liver Injury	Toxic Liver Injury	HCC	CCA	Ref.
HSCs **(Myofibroblast-specific)**	α, ß A, B, D**	α, ß A, B, D	α, ß (D)	(α, ß)	NR	64, 66, 68, 69, 77, 85, 87, 88
Portal Fibroblasts **(Peribiliary myofibroblasts only)**	NR	ß B, D	(ß)	NR	NR	69, 89, 99
Kupffer Cells	NR	NR	B	NR	NR	66, 96, 211
Endothelial Cells	α, ß	α, ß	α*	α, ß	A	64, 66, 69, 212, 213
Hepatocytes	- A, B**, C	NR	α* A, B**, C	α	α	53, 66, 85, 87
Bile Duct Epithelia	-	A, B, D	D	-	A	66, 99, 212, 214
Platelets	NR	NR	AB, C	NR	NR	98
Infiltrating Macrophages	NR	NR	A,B	NR	NR	96, 211

Table 1. Summary of PDGF and PDGFR isoform expression in select liver cells in normal and pathologic states. Key: A, B, C and D represent various PDGF ligands; α/β = PDGF receptors. Parentheses = expression specific to activated forms (myofibroblasts); NR = PDGF/PDGFR expression not reported in the evaluated literature; * = Discrepancy between studies; ** = Predicted expression only; '-' = Lack of PDGF/PDGFR expression specifically reported.

6.0 MATERIALS AND METHODS

6.1 CELL CULTURE AND ASSAYS

HHSteCs (Cat. # 5300, Lot # 10326) were purchased on two occasions from ScienCell Research Laboratories (Carlsbad, CA) and were cultured according to the company's provided protocol. Cells were used prior to 6th passage to reduce culture activation of HSCs *in vitro*.

6.1.1 Preparation and Cell Culture Treatments

Cells were used prior to 6th passage and serum starved 12 hours prior to treatment by washing cells twice with sterile, cold PBS followed by serum-free Stellate Cell Medium (ScienCell). LX-2 cells were provided by Dr. Scott Friedman (Mount Sinai, NY) and cultured according to protocol from commercial provider (Millipore, Billerica, MA). Cells were cultured/expanded in TPP-treated T75 tissue culture flasks (Sigma-Aldrich, St. Louis, MO). Recombinant human PDGF-AA (R&D Systems, Minneapolis, MN) or PDGF-BB (Sigma), and TGFß1 (R&D Systems) was diluted with serum-free media and exposed to cells for the indicated time periods following two washes with sterile, cold PBS. For PDGFRα inhibition, PDGFRα-specific human monoclonal antibody inhibitor Olaratumab (LY3012207, IMC-3G3) or human IgG (Equitech, Kerrville, TX) was diluted in serum free media and used at the indicated concentrations following two washes with sterile, cold PBS.

6.1.2 AlamarBlue Proliferation Assay

For proliferation assays, HHSteCs were plated on 48-well plates (BD Falcon) at an initial plating density of 50,000 cells/well. Cells were serum starved the following day by gentle washing with chilled PBS followed by culturing in serum-free media overnight. Cells were treated the next day with serum-free media (NT), PDGF-AA, PDGF-BB, Olaratumab, or IgG at the indicated concentrations for 24 hours. Next, 20μl of AlamarBlue Cell Viability Reagent (Thermo Scientific) was added to each well and incubated at 37ºC for 1 hour before measuring fluorescence readings at 590nm wavelength. Statistical analysis and graphs were performed in Prism Version 7.0a (GraphPad Software) using one-way and two-way ANOVA.

6.1.3 Transwell Migration Assay

All cells were serum starved for 12 hours prior to use in migration assays. Eight μm-pore polycarbonate filters (Costar) were pre-coated with 100μL collagen I (Sigma-Aldrich) and washed 2 times with sterile PBS prior to adding cell suspension. Immediately prior to addition of cell suspension, Olaratumab, control IgG, or PDGF-BB were added to the lower chamber in serum free culture medium. HHSteCs were suspended in serum-free media and plated at 2000 cells in 100μl/well. After incubation at 37°C for 3 hours, well inserts were fixed with 4% paraformaldehyde and stained by crystal violet. The number of migrated cells that was determined by counting the number of cells per representative 10X field by light microscopy. Statistical analysis and graphs were performed in Prism Version 7.0a (GraphPad Software) using non-parametric Student's t-tests.

6.2 PROTEIN EXTRACTION AND WESTERN BLOTTING

6.2.1 Whole Cell Lysate Preparation: Cell Lysates

Whole cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer containing 1% IgePAL CA-630, 0.5% Sodium Deoxycholate, 0.1% Sodium dodecyl sulfate in Phosphate Buffered Saline (PBS). After adding protease/phosphatase inhibitor cocktail (Sigma Aldrich), 200 µl of RIPA was used per T-75 flaks (75 cm^2). Lysates were centrifuged at 14,000 RPM for 5 minutes at 4oC in order to remove clear supernatant to a new 1.5 ml tube while disposing of the pellet (non-soluble fraction). Samples were then stored at -80oC until utilization or protein concentration was measured via BCA protein assay (Pierce) to ensure equal protein concentrations for subsequent assays.

6.2.2 Whole Cell Lysate Preparation: Liver Tissue

At time of harvest, mice were anesthetized by isoflurane inhalation and subsequently killed by cervical dislocation. After sacrifice, the livers were extracted, washed in PBS, and then the tissue was flash frozen in liquid nitrogen and stored at -80oC until use. At time of use, tissue was homogenized in RIPA with protease/phosphatase inhibitor manually via glass mortar and pestle on ice. Lysates were removed to a fresh 1.5 ml tube and centrifuged at 14,000 RPM for 5

minutes at 4oC in order to remove clear supernatant to a new 1.5 ml tube while disposing of the pellet. Samples were then used for protein applications or stored at -80oC until utilization.

6.2.3 Western Blot Analysis

Western blots were performed on cell culture lysates homogenized in RIPA buffer with protease/phosphatase inhibitors (Thermo Scientific, Waltham, MA). Protein was quantified using bicinchoninic acid (BCA) protein assay and Western blotting was performed as previously described using commercially available primary antibodies from Santa Cruz Biotechnology (Dallas, TX) and Cell Signaling Technology (Danver, MA) outlined in Table 2 [53]. For HHSteC studies, Western blots from pooled samples from three technical replicates from each batch of stellate cells were performed at least twice. Representative Western blots from three pooled technical replicates for each timepoint are shown. Densitometry was performed on these representative Western blots. For detection of PDGFs in HHSteC media, media was concentrated after 24 or 48 hours of serum starvation using Amicon Ultra-15 Centrifugal Filters (Millipore) and resuspended in sample buffer prior to gel electrophoresis as previously described.

6.2.4 Immunoprecipitation

Co-immunoprecipitation studies were performed with 500µg protein lysate in RIPA buffer with protease/phosphatase inhibitors. 2µg of mouse monoclonal anti–CrkII (Santa Cruz Biotechnology) primary antibody was incubated with sample for 3 hours followed by overnight conjugation to Protein G PLUS-Agarose beads (Santa Cruz Biotechnology, sc-2002). Total and phospho-PDGFRα was detected using the same antibodies indicated in Table 1.

6.3 RNA EXTRACTION AND SEMI-QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR was performed on RNA isolated using TRIzol (Thermo Scientific) RNA extraction from adherent cell cultures. Samples for each target were assessed as part of the same qPCR reaction and gel analysis. RNA extraction was performed using Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA) and following the manufacturer's protocol. Real time PCR was performed using Power SYBR Green (Thermo Scientific), 100 ng of cDNA, and 0.2 μmol/L of forward and reverse primers. Primers used in this study are listed in Table 3. Data was analyzed using the comparative ΔΔCt method or PCR product was run on 0.8% agarose gel with ethidium bromide and detected by UV lamp. Statistical analysis and graphs were performed in Prism Version 7.0a (GraphPad Software, San Diego, CA) using one-way ANOVA.

6.4 ANIMALS AND MODELS OF LIVER INJURY

6.4.1 Mouse Model Development

All animal experiments were performed under the guidelines of the National Institutes of Health and the Institutional Animal Use and Care Committee at the University of Pittsburgh. The studies performed in the current report were approved by the Institutional Animal Use and Care Committee at the University of Pittsburgh.

For the generation of *Pdgfra* knockout strains, homozygous *Pdgfra* floxed (exons 1-4) were crossed with Foxa3-Cre, Alb-Cre or Lrat-Cre mice for the creation of F1 generation with

mice heterozygous for floxed *Pdgfra* as well as Cre allele. These mice were subsequently backcrossed with homozygous *Pdgfra* floxed mice to create Cre-positive homozygous floxed *Pdgfra* animals at a mendelian ratio of ¼. Lrat-Cre mice of a mixed background strain were provided by Dr. Robert Schwabe at Columbia University[173]. Foxa3- and Albumin-Cre mice and homozygous *Pdgfra* floxed (exons 1-4) were obtained from Jackson Laboratories (Bar Harbor, ME).

6.4.2 Serum Liver Function Tests

All liver function tests were performed by the UPMC Dept. of Pathology Division of Clinical Chemistry Automated Testing Laboratory.

6.4.3 Carbon Tetrachloride

Mice were injected intraperitoneally twice weekly with CCl_4 (1:3 dilution in corn oil) at 0.5µl/g body weight for 4 weeks or 8 weeks. Animals were sacrificed 48 hours following last injection for liver and serum harvesting.

6.4.4 Bile Duct Ligation Surgery

In BDL, the peritoneal cavity is opened under anesthesia to expose the common bile duct and is cut between two 5-0 silk ligatures. Directly following surgery and at 24 and 48 hours post-surgery, mice were administered subcutaneous injection of 5mg/kg ketoprofen for analgesia. Livers and serum were collected at 5 days or 2 weeks post-BDL. These two timepoints were chosen to reflect

the differential contributions of portal fibroblasts and HSCs to the activated myofibroblast population at each of these times[17].

6.4.5 DDC Injury

Animals were fed 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet *ad libitum* for 16 days. Livers and serum were subsequently collected for analysis.

6.5 IMAGE ANALYSIS

6.5.1 Immunohistochemistry

Liver tissue fixed for 48 hours in 10% formalin solution were embedded in paraffin and cut into 4-micrometer sections for immunohistochemistry. Slides were deparaffinized in xylene and rehydrated in graded alcohol washes (100%, 95%, 70%) prior to dH20 washing. Heat-induced antigen retrieval was performed on sections by microwaving them for 12 minutes in citrate buffer (10mM sodium citrate + 0.05% Tween 20, pH6.0) (PCNA) or pressure cooking sections for 20 minutes in EDTA buffer (1mM EDTA + 0.05% Tween 20, pH9.0) (desmin, αSMA, CD45 IHC). For F4/80 IHC, Proteinase K buffer (Millipore, Cat.#21627) was used for antigen retrieval. TUNEL was performed using ApopTag peroxidase kit (Millipore).

6.5.2 Immunofluorescence

Liver tissue was fixed in 2% formaldehyde for 2 hours prior to 24 incubation in 30% sucrose solution. Following flash freezing in optimal cutting temperature (OCT) compound, tissue was cut in 6 micrometer thick sections and stored at -20ºC. For immunofluorescence staining, tissue was rehydrated with PBS followed by 10 minute detergent permeabilization using 0.1% Triton X-100 in PBS. Next slides were incubated in dH20 for 30 minutes at 37ºC followed by blocking with 2% bovine serum albumin (BSA) in PBS for 45 minutes. Slides were incubated in primary antibody solution overnight at designated concentrations (Table 2) in 0.5% BSA in PBS (PBB). The following day, slides were washed with PBB prior to secondary antibody detection using species-specific antibodies conjugated to Alexafluor 488 or Alexafluor 555 diluted 1:500 in PBB for 1 hour. Slides were then washed sequentially with PBB and PBS followed by 30 second Hoeschst counterstain and slide covering. Epifluorescence images were obtained using a Nikon Eclipse Ti microscope and NIS Elements ver4.40. For confocal imaging, serially stacked, 1µm thick images were obtained using an Olympus FluoView 1000 microscope and FV1000 ASW ver. 4.2 software.

6.5.3 Fibrosis Quantification

Polarized light images of Picrosirius Red-stained liver sections were taken using an Olympus Provis microscope and MagnaFire software (ver. 2.1B). These images were quantified using NIS Elements ver. 4.51.

APPENDIX A

[SUPPLEMENTAL TABLES]

Target	Host Species	Size (kDa)	WB Dil.	IP Dil.	IF/IHC Dil.	Diluent	Company	Catalogue Number
αSMA	Rb	42	--	--	1:200 (IHC)	1%BSA + 1% Triton X-100	Abcam	ab5694
αSMA	Ms	42	--	--	1:1000 (IF)	0.5% BSA	Sigma	C6198
Akt	Rb	60	1:1000	--	--	5% BSA	CST	4685
P-S473-Akt	Rb	60	1:1000	--	--	5% BSA	CST	4060
c-Abl	Rb	135	1:1000	--	--	5% BSA	CST	2862
P-Y89-c-Abl	Rb	135	1:1000	--	--	5% BSA	CST	3098
P-c-Abl Y412	Rb	135	1:1000	--	--	5% BSA	CST	2865
CD45	Rt	220	--	--	1:100	PBS	SCBT	sc-53665
CrkII	Rb	42	1:1000	--	--	5% BSA	CST	3492
P-Y221-CrkII	Ms	42	1:1000	--	--	5% Milk	CST	3491
CrkL	Rb	39	1:1000	--	--	5% BSA	CST	3182
P-Y207-CrkL	Rb	39	1:1000	--	--	5% BSA	CST	3181
Desmin	Rb	52	--	--	1:200 (IHC)	1%BSA + 1% Triton X-100	Novus Bio	NB120-15200
Desmin	Rb	52	--	--	1:200 (IF)	0.5% BSA	Thermo	RB-9014
E-Cadherin	Rt	35	--	--	1:500 (IF)	0.5% BSA	BD Biosci	BD610182
Elk-1	Rb	47	1:1000	--	--	5% BSA	CST	9182
P-S383 Elk-1	Rb	47	1:1000	--	--	5% BSA	CST	9181
Erk1/2	Rb	42,44	1:1000	--	--	5% BSA	CST	4695
P-T202/Y204-Erk1/2	Rb	42,44	1:1000	--	--	5% BSA	CST	4370
F4/80	Rt	160	--	--	1:200 (IHC)	PBS	Bio-Rad	MCA497GA
FAK	Rb	125	1:1000	--	--	5% BSA	CST	13009
P-Y397-FAK	Rb	125	1:1000	--	--	5% BSA	CST	8556
P-Y576/577-FAK	Rb	125	1:1000	--	--	5% BSA	CST	3281

P-Y925-FAK	Rb	125	1:1000	--	--	5% BSA	CST	3284
P-S2448-mTOR	Rb	289	1:500	--	--	5% BSA	CST	2971
P-S2481-mTOR	Rb	289	1:1000	--	--	5% BSA	CST	2974
p38	Rb	40	1:1000	--	--	5% BSA	CST	8690
P-T180/Y182-p38	Rb	43	1:200	--	--	5% Milk	CST	4511
PCNA	Ms	29	--	--	1:4000 (IHC)	PBS	SCBT	sc-56
PDGF-A	Ms	31	1:200	--	--	5% Milk	SCBT	sc-9974
PDGF-B	Rb	14	1:1000	--	--	5% Milk	Abcam	ab23914
PDGF-C	Gt	30	1:200	--	--	3% BSA	R&D Sys	AF1560
PDGF-D	Gt	30	1:1000	--	--	5% BSA	R&D Sys	AF1159
PDGFRα	Rb	190	1:1000		--	5% BSA	CST	3174
PDGFRα	Gt	190	--	--	1:40 (IF)	0.5% BSA	R&D Sys	AF1062
PDGFRß	Rb		1:1000	--	--	5% BSA	CST	3169
P-Y572/574-PDGFRα/ß	Rb	190	1:1000	--	--	5% BSA	Invitrogen	44-1000G
P-Y742-PDGFRα	Rb	190	1:1000	--	--	5% BSA	Invitrogen	44-1006
P-Y762-PDGFRα	Rb	190	1:1000	--	--	5% BSA	CST	24188
P-Y849/ßY857-PDGFRα	Rb	190	1:1000	--	--	5% BSA	CST	3170
P-Y1018-PDGFRα	Rb	190	1:1000	--	--	5% BSA	CST	4547
Pan-phospho-PKC	Rb	78,80, 82,85	1:1000	--	--	5% BSA	CST	9371
LYVE-1		35	--	--	1:200	0.5% BSA	Abcam	Ab14917

Table 2. Primary Antibodies used for WB, IHC, IF, and IP. Abbreviations: CST – Cell Signaling Technology; Gt – goat; Ms – mouse; Rb – rabbit; Rt – rat; SCBT – Santa Cruz Biotechnology.

Primer	Sequence (5’ to 3’)	Amplicon (bp)
ACTA2 Fwd	TTC ATC GGG ATG GAG TCT GCT GG	141
ACTA2 Rev	TCG GTC GGC AAT GCC AGG GT	141
COL1A1 Fwd	TCG TCA CAG ATC ACG TCA TCG	120
COL1A1 Rev	AAT CAC CTG CGT ACA GAA CGG	120
PDGFA Fwd	CAC ACC TCC TCG CTG TAG TAT TTA	220
PDGFA Rev	GTT ATC GGT GTA AAT GTC ATC CAA	220
PDGFB Fwd	ACT CGA TCC GCT CCT TTG ATG A	111
PDGFB Rev	GCT CGC CTC CAG AGT GGG	111
PDGFC Fwd	TCA CAG CCC AAG GTT TCC TC	100
PDGFC Rev	CCA CAC CAG CGC CCT AAT AT	100
PDGFD Fwd	GAA CAG CTA CCC CAG GAA CC	100
PDGFD Rev	CTT GTG TCC ACA CCA TCG TC	100
FN1 Fwd	GGC TGA CAG AGA AGA TTC CCG AGA G	87
FN1 Rev	CCA GTT TAG ATG GAT CTT GGC AGA GAG AC	87
SYP Fwd	GCA ATG GGT CTT CGC CAT CT	134
SYP Rev	GCC TGA AGG GGT ACT CGA AC	134
TGFB1 Fwd	CCC TGG ACA CCA ACT ATT GC	75
TGFB1 Rev	TGC GGA AGT CAA TGT ACA GC	75
GAPDH Fwd	GAA GGT GAA GGT CGG AGT C	226
GAPDH Rev	GAA GAT GGT GAT GGG ATT TC	226

Table 3. Primers used for RT-PCR.

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