Platelet derived growth factor receptor alpha (PDGFRα) signaling in liver biology: Promises and Perils

by

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Hepatocellular carcinoma (HCC) is amongst the top common cancers and the third cause of cancer related death worldwide. It is a disease of dismal prognosis. Much effort has been devoted to identifying the major players involved in HCC to facilitate the development of efficacious treatments. Due to the commonalities between development and cancer, our lab used developing livers to identify genes that might play a crucial role in HCC. We identified increased expression of Platelet Derived Growth Factor Receptor Alpha (PDGFRα), its ligands and activity in early developing mouse livers, which coincided with ongoing cell proliferation. Blockade of PDGFRα signaling using a mouse specific PDGFRα blocker in embryonic liver cultures led to significantly decreased cell proliferation and survival. PDGFRα overexpression was also evident in HCC with around 63% of the patients showing around 7-fold up-regulation. PDGFRα upregulation was also identified as the chief molecular basis of enhanced tumorigenesis in hepatocyte-specific β-catenin knockout mice exposed to chemical carcinogen. In fact, blockade of PDGFRα in this model led to a significant abrogation of tumorigenesis.

Since most HCC develop in the background of cirrhosis where liver regeneration is ongoing and critical for maintenance of hepatic function, it is important to identify pathways that are dispensable for normal liver regeneration, but indispensable for tumor cell proliferation and viability. We sought to determine if PDGFRα, which is indispensable to HCC was important in
liver regeneration using partial hepatectomy (PHx) model. We identified a dramatic increase in total PDGFRα at 24hrs after PH, which was accompanied by its tyrosine phosphorylation. However, hepatocyte-specific Pdgfra knockout mice (KO) that lacked any spontaneous phenotype, showed no difference in hepatocyte proliferation at 40hrs. Interestingly, we identified an increase in total and phosphorylated EGFR and MET expression in the KO at 24hrs, which eventually led to a modest increase in hepatocyte proliferation at 72hrs. Interestingly, PDGFRA knockdown in human hepatoma cells did not lead to EGFR or MET upregulation indicating that PDGFRα is redundant in liver regeneration but not in HCC.

Thus we have uncovered important roles of PDGFRα in liver development, regeneration, and cancer.
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PREFACE

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1.0 INTRODUCTION

1.1 LIVER DEVELOPMENT

Embryonic liver development is a complex process that requires regulated signals from different cell types to assure the proper and efficient development of the liver [1, 2] as seen in Figure 1. The use of animal models have facilitated the discovery of many of the signals necessary for liver development in addition to revealing high evolutionary conservation for hepatic development between the various animal models [3]. Mouse liver initiates from the definitive foregut endoderm at embryonic day 8 of mouse gestation [4].

1.1.1 Patterning of the endoderm and hepatic competence

Endoderm patterning is a crucial step for normal development of organs that are derived from the endoderm such as the liver. During gastrulation, the endoderm is patterned into 3 different divisions culminating in the foregut, midgut, and hindgut. This process is aided by secreted factors, most notably bone morphogenic proteins (BMP) [2, 5, 6], fibroblast growth factor (FGF), Wnt, and retinoic acid (RA) [7] emanating from the surrounding mesoderm germ layer [2]. Precise and controlled expressions of these various signals are key to successful
endoderm patterning. Posterior endoderm formation is majorly dependent on Wnt/β-catenin [8, 9], FGF [10, 11], and BMP signaling pathways, while RA [7] signaling is crucial for proper foregut-hindgut boundary formation [2]. Wnt/β-catenin and FGF signaling act to suppress foregut fate and subsequently promote hindgut fate in the posterior endoderm. During foregut fate in the anterior endoderm, both FGF and Wnt/β-catenin signaling must be suppressed. Overstimulation or blockades of these critical signaling pathways have been shown to cause improper or failure of liver formation [2, 9, 12]. Patterning of the endoderm to make the foregut is a critical step for liver development since the foregut contains hepatoblasts and is therefore competent to develop into the liver [13, 14].

During endoderm formation, specific signals induce expression of endoderm transcription factors such as FOXA [15, 16] and GATA [17]. FOXA and other transcription factors are critical for inducing the endoderm to express liver specific genes [15, 18]. The importance of FOXA for hepatic competence was revealed by foxa1 and foxa2 double knockout studies. In this study, they showed an inability of liver specific genes to be expressed in the absence of foxa1 and foxa2. During liver development, signals from the developing heart are crucial for initiating the expression of these liver-specific genes in the endoderm [19-21].

1.1.2 Hepatoblasts

Hepatoblasts are the hepatic bipotential stem cells endowed with the ability to give rise to both hepatocytes and biliary epithelial cell lineages [22, 23]. These cells have a high nuclear to cytoplasmic ratio and are distributed between different cell types, including hematopoietic cells in the developing liver. Hepatoblasts express various markers including hepatocyte specific genes, such as hepatocyte nuclear factor 4-alpha (HNF4α) and albumin. They also express fetal
markers like α-feto-protein in addition to biliary epithelial cell marker like cytokeratin 19 (CK19). At Embryonic day 9 during mouse embryonic liver development, the liver diverticulum becomes conspicuous juxtaposed to the septum transversum [2, 3]. Hepatoblasts, which are contained in the liver diverticulum discharges and permeates the septum transversum. Interaction between hepatoblasts and endothelial precursor cells are critical for hepatoblast proliferation into the septum transversum and promoting morphogenesis and liver bud formation, which occurs at embryonic day 9.5 [24]. At embryonic day 10, the newly formed bud is now ready to expand to generate the liver [25-27].

Figure 1. Model of Liver Development.
Multiple growth factors and transcription factors are required for liver development. Adapted from Nejak-Bowen et al (Ref. 1)
The next phase of embryonic liver bud growth is characterized by expansion and proliferation of hepatoblasts. The liver undergoes robust growth between embryonic days 10 to 16 aided by various signals in a paracrine fashion [3]. During the expansion of the liver bud, hepatoblasts, expressing many receptors, receive several paracrine growth factor and cytokine signals (HGF, BMP, FGF) from the mesenchyme consisting of endothelial cells, stellate cells, and hematopoietic cells within the expanding bud [23, 28]. This results in activation of PI3K, MAPK, and β-catenin signaling to regulate hepatic morphogenesis include. Also NFκB signaling at this stage is known to play important role in hepatoblast survival [29]. Eventually hepatoblast differentiation into either hepatocyte or biliary epithelial cell lineage commences at around embryonic day 13.

1.1.3 Hepatoblast differentiation into hepatocyte and biliary epithelial cell lineage

Differentiation of hepatoblasts into the two cell lineages is a key step during liver development. Interaction between hepatoblasts and the developing portal vein is crucial in facilitating this transition. For differentiation into hepatocytes, hepatoblasts that away from the portal mesenchyme suppress markers for biliary development and subsequently up regulate hepatic factors such as C/EBPα and HNF4α. These factors are important for controlling hepatic gene expression [30, 31]. Factors including HGF, Oncostatin M and β-catenin signaling have been shown to be important for the proper execution of the hepatocyte lineage from hepatoblasts [32-34]. Epithelial morphology that defines mature hepatocytes become evident at embryonic day 17, where hepatocytes are seen arranged in hepatic cords with bile canaliculi on the apical surface [3].
In contrast, hepatoblasts that are situated near the portal vein form biliary epithelial cells. For differentiation towards a biliary lineage, periportal hepatoblasts receive signals from the mesenchyme in the form of TGFβ and down-regulate pro-hepatocyte transcription factors such as HNF4α and C/EBPα [3] and increase expression of biliary transcription factors such as Sox9, HNF-6, HNF-1β. Notch signaling is a critical regulator of this process as well [35, 36].

1.2 LIVER REGENERATION

Liver is distinct from all other organs in the body for its capacity to regenerate. The liver is equipped with the unique ability to regenerate not only after loss of liver mass but also after hepatic injury. Liver regeneration (LR) is experimentally studied as growth after partial hepatectomy (PHx) and has been instrumental in our understanding of the cellular and molecular basis of this process. What has been revealed is that unlike other tissues such as bone marrow and skin, which require progenitor cells for homeostasis, LR is executed via proliferation of all mature liver cells [37]. Impressively, throughout the regenerative process, the liver continues to perform all the essential functions for survival. LR can be induced either by administering hepatotoxic chemicals like carbon tetrachloride (CCl4), or by surgically removing parts of the liver (2/3 PHx), which is the more utilized and preferred method.

1.2.1 2/3 Partial Hepatectomy and the Regenerative Process

The most common method of inducing LR is surgical removal of three of five lobes from the
rodent liver, commonly referred to as a 2/3rd PHx [38] (Figure 2A). The remaining two lobes grow to recapitulate the original liver mass within approximately 5-7 days in the rat [39] and 14 days in the mouse. The entire regenerative process can be precisely timed due to the fact that the lobes can be removed within a brief period of time. This method of triggering LR is reliable and effective because the extent of excised liver lobes are determined by the experimenter. In addition, this method induces LR without causing inflammation or damage to surrounding tissues as seen with CCl4 administration.

At baseline, hepatocytes remain in a quiescent state and rarely undergo cell division, however after PHx, 95% expeditiously reenter cell division [40]. Proliferation of adult hepatic cell types is a distinguishing feature of LR. Hepatocytes, which are the principle cell type and accounting for about 70% of the liver mass are first to enter into DNA synthesis. After 2/3 PHx, the remaining 1/3 of hepatocytes undergo the first round of DNA synthesis, which peaks at 24 h in the rat and approximately 36 h in the mouse [39] (Figure 2B). A second round of DNA synthesis occurs constituting a smaller percent of hepatocytes, which reestablish the original number of hepatocytes [39]. During LR, proliferating hepatocytes produce growth factors for other cell types, including stellate cells, endothelial cells, and kupffer cells in a paracrine fashion [37, 39, 41-43]. At approximately 48hr post PHx, biliary epithelial cells and kupffer cells undergo DNA synthesis, followed by sinusoidal endothelial cells occurring at 96h [37] (Figure 2B). All these cell types work in a concerted effort to facilitate regeneration of the liver.
Figure 2. Schematic model of partial hepatectomy. (A) Removal of 2/3 of the mouse/rat liver lobes induces strong regenerative response causing the remaining two lobes to grow until restitution of the original liver mass is complete. (B) Time kinetics of DNA synthesis in different liver cell types during LR after PHx. The four major types of liver cells undergo DNA synthesis at different times. Adapted from Michalopoulos, GK and DeFrances, M (Ref. 37)

1.2.2 Signaling Pathways Regulating Liver Regeneration

PHx triggers signaling pathways and cascades that are tightly regulated. During this regenerative process, several genes are differentially expressed as exemplified by the rapid induction of over 100 genes not generally expressed at baseline by quiescent hepatocytes [40, 44] and may represent the entry of hepatocytes into cell cycle. One of the early events during LR is the matrix remodeling as indicated by a significant turnover of many matrix proteins such as matrix
metalloproteinases (MMPs) [45]. Matrix remodeling after PHx may be relevant since it sequesters important growth factors like hepatocyte growth factor (HGF) [46], which is crucial for initiation of LR. Several cytokine and growth factor pathways have now been identified to be relevant in initiating LR after PHx. Cytokines such as IL-6 and TNFα are upregulated and their loss affects normal LR kinetics since loss of either one leads to deficient LR [47, 48]. The two major growth factors important for the progression of the cell cycle after PHx are HGF and EGF and are known to induce the transcription of genes important for regulation of cell cycle entry [49, 50]. Within the first 3h during LR after PHx, matrix bound HGF are released in the plasma and becomes utilized followed by new HGF synthesis, while EGF is constantly available to the liver via the portal vein [39]. EGFR and MET, receptors for EGF, and HGF, respectively are activated within 30-60min post PHx [51]. Once activated, hepatocytes themselves secrete additional mitogenic growth factors including VEGF [52, 53], TGFα, FGF [54], and PDGF [55], which act either in a paracrine fashion to induce proliferation of other cell types in the liver or in an autocrine manner. Proliferations of other liver cells are important as these cells also secrete factors that act to further bolster the LR process. LR is a redundant process and as such no elimination of any single gene has been shown to completely abrogate LR after PHx with the possible exception of the HGF/c-Met signaling pathway [39].

Just as the initiation of the LR process is critical, it also important for the liver to terminate this process once the lost liver mass has been fully restored. Less is known about the termination process of LR. Based on both in vivo and in vitro data, TGFβ1 has been implicated in the termination of LR [43, 56], which produced by mesenchymal cells and is a known mitoinhibitor for most epithelial cells. It appears that the degradation of the matrix during PHx releases both mitogenic factors such as HGF and mitoinhibitory factors such as TGFβ1 into
circulation, however TGFβ1 is bound to the hepatic matrix and made inactive until it is required [57]. TGFβ1 has been shown to inhibit hepatocyte proliferation in culture [58] and when given at high doses, TGFβ1 has been shown to delay hepatocyte DNA synthesis peak after PHx [59].

Figure 3. Schematic representation of signaling between different hepatic cell types during LR

During LR, liver cells secrete cytokines and growth factors that are important for executing the regenerative response. Adapted from Michalopoulos, GK and DeFrances, M. (Ref. 43)
1.3 LIVER CANCER: HEPATOCELLULAR CARCINOMA

Hepatocellular cancer (HCC) is the most prevalent liver cancer and it continues to be a major health concern. It is the 5th most common cancer and the 3rd most fatal cancer worldwide [60-62]. There are over 500,000 people worldwide that develop HCC yearly and roughly about the same number die from the disease. Epidemiological data indicate that the frequency of HCC has been steadily increasing over the past two to three decades in the United States. In fact HCC is one of few types of cancers increasing in both frequency and mortality in USA [63, 64] and Europe [65]. Both the incidence and the mortality rates are higher in men than women [66]. The cellular and molecular mechanisms underpinning this devastating disease need further elucidation.

1.3.1 Cellular aberrations and signaling pathways in HCC

Over 85% of HCCs develop as a result of chronic liver diseases, with the majority occurring in patients with advanced stage cirrhosis [66-68]. There are certain risk factors that are known to contribute to the development of HCC. Some of these factors include but are not limited to, viral hepatitis (B and C), alcoholic liver disease, metabolic liver disease including non-alcoholic steatohepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and hemochromatosis [69]. Of the various risk factors in HCC, viral hepatitis appears to be the most prevalent cause of HCC worldwide [70]. Even though other risk factors are known to also play a role, what appears
to be at the center of initiation and progression of HCC is hepatocyte injury and death, followed by compensatory regeneration [66]. As a consequence of hepatocyte injury, and in response to inflammatory cytokines and oxidative stress, stellate cells and portal fibroblasts become activated and begin producing and depositing collagen. This abnormal collagen deposition leads to development of fibrosis and eventually cirrhosis [71]. Deposition of collagen replaces liver tissue over time, which prompts residual and surviving hepatocytes to proliferate and attempt to repair the damaged liver. Proliferation of hepatocytes is required for the maintenance of normal liver functions. However, proliferation of hepatocytes in this fibrotic and suboptimal environment renders these hepatocytes prone to genetic and epigenetic alterations as a result of continued inflammation and oxidative stress [66]. Proliferation of hepatocytes in this environment is thought to be driving tumorigenesis in most patients with chronic liver diseases [66].

In order to achieve efficacious therapies for HCC, it is important to uncover signaling pathways that are often utilized by tumor cells to proliferate and survive. Excessive activation of several pathways including HGF/MET, EGFR/Ras/MAPK, Wnt/β-catenin, PI3K/AKT and IGF signaling cascades are known to play roles in the initiation and exacerbation of HCC [66, 72]. Blockade of these signaling pathways has been shown to significantly decrease cancer cell growth and survival [73].

During tumorigenesis, certain angiogenic factors like PDGF, EGF, VEGF, and HGF, secreted by cancer cells in the tumor microenvironment can act in either a paracrine or autocrine fashion to sustain and exacerbate the tumorigenesis state in HCC [74]. As previously mentioned, the Wnt/β-catenin pathway is one of the more studied pathways in HCC and is of interest to our lab.
1.4 SIGNALING IN LIVER BIOLOGY

1.4.1 Wnt/β-catenin Signaling

The Wnt/β-catenin pathway remains suppressed in normal cells at baseline. When the pathway is not active, β-catenin, the key player in this signaling pathway is bound in a complex with Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3 (GSK3) and casein kinase (CK). In this bound complex, β-catenin is phosphorylated by CK1 and GSK3α/β at serine/threonine residues located at the N-terminal region [75]. Phosphorylation of β-catenin marks it for ubiquitination, and ensuing degradation by the proteasome. However, in the presence of Wnt, this degradation process is precluded. When Wnt proteins bind to the Frizzled receptor on the surface of cells, the canonical Wnt pathway becomes activated. The interaction between Wnt/Frizzled induces an association with the low-density lipoprotein receptor related protein (LRP) 5/6, and this complex then recruits Dishevelled, which is believed to inactivate GSKβ [76]. Upon GSKβ inactivation, β-catenin phosphorylation is prevented, which subsequently releases β-catenin from the Axin/APC/GSK3 complex. β-catenin then translocate to the nucleus, where it binds to lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) and displaces the transcriptional inhibitor Groucho. This β-catenin/(LEF/TCF) complex then induces activation of target genes important in various cellular processes such as proliferation and differentiation [77].

The Wnt/β-catenin pathway is known to play many critical roles in various aspects in liver biology. Utilization of antisense oligonucleotides against β-catenin gene (Ctnnb1) in embryonic liver cultures revealed important roles for β-catenin during liver development. The
authors reported decreased cell proliferation and survival [34]. Interestingly, over expression of constitutively active β-catenin in the developing liver was reported to lead to a three-fold increase in liver size and expansion of hepatocyte precursor cells [78]. Later, our lab reported a Foxa3-Cre driven conditional deletion of ctnnb1. This resulted in embryonic lethality at midgestation due to improper liver development [79]. Wnt/β-catenin signaling has been shown to be a key component for LR. In rats, following PHx, β-catenin is increased within minutes post PHx [80] and knockdown of β-catenin was shown to cause a decrease in liver weight to body weight ratio as a result of decreased cell proliferation [81]. This pathway has also been reported to be one of the major players underpinning liver disease [82-87].
1.4.2 Signaling in β-Catenin (ctnnb1) Conditional KO mice

The Wnt/β-catenin pathway as previously mentioned plays critical roles in many cellular processes. Global deletion of the β-catenin gene, ctnnb1 is embryonic lethal. Previous and ongoing studies from our lab have revealed critical roles for β-catenin in liver biology. Since global deletion of ctnnb1 is embryonic lethal, much of the work in the field has been significantly advanced due to the ability to conditionally delete ctnnb1. Recently, our lab proposed to conclusively address the role of β-catenin in liver growth and regeneration by using a conditional knockout approach to delete ctnnb1 in the liver [88]. To accomplish this task, floxed ctnnb1 (exons 2-6) mice were intercrossed with Albumin-Cre recombinase transgenic mice. Results from western blot and immunohistochemistry analysis showed considerable β-catenin deletion at 15 days post birth. We also showed that these conditional ctnnb1 knockout mice (Ctnnb1 (loxp/loxp); Alb-Cre (+/-)) were viable, although there was an appreciable decrease in their liver weight/body weight ratio at 1 month and was sustained throughout their normal life span. Ki-67 staining displayed basal hepatocyte proliferation. When two-thirds PHx was performed on KO
mice, these mice showed modest morbidity during the first 2-3 days. These mice displayed increased apoptosis at all stages of regeneration and also decrease in hepatocyte proliferation at the time of peak hepatocyte proliferation (40hrs) [88]. However, a rebound increase in hepatocyte proliferation was evident in the knockout mice at 3 days. There were several genes that were upregulated in Ctnnb1 KO mice during LR after PHx. One of the genes that was highly upregulated was the Platelet Derived Growth Factor Receptor Alpha (Pdgfra) [88].

Interestingly, our lab has also shown Ctnnb1 KO mice injected with Diethylnitrosamine (DEN), a chemical carcinogen developed enhanced tumorigenesis compared to control mice [73]. In this study, our lab showed significantly enhanced PDGFRα protein expression and downstream activation in KO mice indicating a significant role of this receptor in the enhanced tumorigenesis. To corroborate involvement of PDGFRα in enhanced tumorigenesis, another set of KO mice treated with DEN, were injected with Gleevec, a well-established tyrosine kinase inhibitor that also inhibits PDGFRα. After treatment with Gleevec, we observed a significant abrogation in tumorigenesis when compared with KO mice injected with DEN alone without Gleevec [73]. These results strongly suggest that PDGFRα may be playing an important role in liver biology and therefore merits further investigation.

1.5 PLATELET DERIVED GROWTH FACTOR RECEPTOR ALPHA SIGNALING

1.5.1 Introduction

Platelet-derived growth factor (PDGF) is a stimulating growth factor that elicits effects on growth and motility of connective tissue cells, such as fibroblasts and smooth muscle cells, but
also acts on other cell types, including capillary endothelial cells and neurons [89, 90]. In both human and mouse, there are four PDGF chains in the PDGF family: PDGF-A, PDGF-B, PDGF-C and PDGF-D. PDGFs signal via two receptors, PDGF receptor-alpha and PDGF receptor-beta (PDGFRα and PDGFRβ). PDGFs and PDGFRs are expressed in overlapping and distinct cell types. PDGFRα is mainly expressed in cells of mesodermal and neural origin and is involved in the regulation of cell migration, proliferation and differentiation. PDGFRα plays an important role in various biological processes such as embryonic development, wound healing and atherosclerosis, but also in tumorigenesis (31).

1.5.2 PDGFRα gene and protein structure

Human PDGFRα gene is located on chromosome 4q11-12, spanning approximately 65 kb of DNA and contains 23 exons. The gene structure is similar to that of the related receptor tyrosine kinase family members PDGFRβ, and c-KIT [91-93]. Exon 1 encodes the 5’ untranslated region of the mRNA, followed by a large first intron of 23 kb. Exon 2 is responsible for encoding the translation initiator codon AUG and signal sequence [94]. The PDGFRα gene encodes a full-length protein of 140 kDa, which consists of five extracellular immunoglobulin-like domains (ligand binding domains), a transmembrane domain and an intracellular tyrosine kinase domain with a characteristic inserted sequence. When fully glycosylated, the molecular mass of the protein increases to approximately 170 kDa and the extent of glycosylation varies among cell types [95].
1.5.3 PDGFR ligands and downstream signaling

PDGF ligands function mainly as homodimers, but can also form heterodimers as in the case of PDGF-AB. They bind with different affinities to the two receptors. PDGFA, PDGFB, and PDGFC can all bind to PDGFRα but PDGFαA and PDGFCC bind with the highest affinities. PDGFA and PDGFB are synthesized and secreted as functional dimers, however, PDGF-C is secreted as latent PDGF and must be cleaved to function [96, 97]. PDGF-B and PDGF-D bind to PDGFRβ with the highest affinity. PDGFRα functions as a homodimer (PDGFRαα) or as a heterodimer with PDGFRβ (PDGFRαβ), but mostly as a homodimer. Upon ligand binding, these receptors dimerize and autophosphorylate each other on tyrosine residues. Autophosphorylation activates the receptor kinase and provides docking sites for downstream signaling molecules [90, 98, 99]. As depicted in Figure 5, PDGFRα can engage several well-characterized signaling pathways including Ras-MAPK, Phosphatidylinositol 3-kinase (PI3K), and Phospholipase C-gamma (PLC-γ), which are all known to be involved in many cellular and developmental responses.

Of these signaling molecules, the PI3K pathway has been documented to be one of the more critical signaling molecules that bind to the phosphorylated PDGFRα. Briefly, PI3K binds the autophosphorylated receptor at Tyr-731 and Tyr-742, and phosphorylates PI(4,5)P2 to give phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3). There are several downstream targets of this pathway, most notably AKT. These downstream targets act to mediate cellular responses including actin reorganization, chemotaxis, cell growth, and anti-apoptosis [89, 90].
Figure 5. Simplified description of PDGFRα signaling.

Simplified description of PDGFRα signaling. Binding of PDGFs to the receptor induces homodimerization followed by autophosphorylation and activation of the receptor. Adapted and modified from Heldin, CH (Ref. 90).
1.5.4 Platelet Derived Growth Factor Receptor alpha signaling in Development

1.5.4.1 Expression patterns of PDGF receptor and ligands

PDGFRα and its ligands have been shown to play important roles during development and organogenesis [100, 101]. In the mouse, Pdgfra is expressed from the two cell stage onwards by all cells of the blastocyst, but later in development its expression becomes restricted to cells of mesodermal and neural origin and also in the epithelium in few cases [102]. In early post-implantation embryos, at embryonic days 6-7.5, Pdgfra mRNA can be found in extra-embryonic and embryonic endoderm, as well as in the embryonic mesoderm [103, 104]. At embryonic day 8.0-8.5, Pdgfra expression in the embryo becomes more regionalized to the somites and mesenchyme. At embryonic day 9.5, Pdgfra expression becomes restricted to the sclerotome and dermatome [103, 105]. Several cell types have been shown to express PDGF ligands during development. Expression of Pdgfa has been shown to be co-expressed with Pdgfra in the inner cell mass of the blastocyst. However as development progresses, Pdgfa expression becomes mostly restricted to the epithelial, muscle and nervous tissue [103, 105]. Interestingly, the expression pattern of Pdgfc has a large overlap with that of pdgfa, which were observed at embryonic days 9.5-12.5 of embryonic development [102, 106, 107]. Pdgfc is strongly expressed in but not limited to somatic myotome, skeletal muscle, gut, lung and kidney [102, 106, 108]. This indicates the general expression of receptor and ligand as non-overlapping.

1.5.4.2 Phenotypes in Pdgfra knockout and signaling mutants in mouse

Mouse studies involving deletion of Pdgfra have revealed the importance of this receptor during
development. Global deletion of Pdgfra is embryonic lethal and this occurs between embryonic days 11.5 to 15 [97]. Interestingly global deletion of Pdgfra reveals more severe phenotypes when compared to global deletion of its ligand Pdgfa, which indicates that PDGFC signaling via PDGFRα during development is also critical. Global deletion of Pdgfra displays significant defects in the development of many organs including but not limited to brain, [109], lungs, testes, kidney, and skin [97].

As previously mentioned, upon PDGF-receptor activation, there is a homo- or heterodimerization event that occurs. Receptor dimerization leads to transphosphorylation of tyrosine residues in the cytoplasmic region, after which various intracellular signaling pathways are activated [89, 97, 110]. In order to decipher the roles of specific PDGFRα-activated signaling pathways during development, knock-in mice were generated. In these mice, the endogenous Pdgfra was substituted by a transgene encoding mutant receptors that lacked the ability to activate PDGFRα specific downstream signaling molecules [111]. This study revealed the significance of certain signaling molecules, most notably PI3K, which was shown to be the major effector of PDGFRα signaling during embryogenesis. The authors show similar defects such as spina bifida and cervical vertebrae malformations when they compared Pdgfra-PI3K (αPI3K/αPI3K) mutants to Pdgfra null (-/-) mutants. However, defects seen in Pdgfra-PI3K (αPI3K/αPI3K) mutants were less severe than the defects observed in the Pdgfra null (-/-). Pdgfra-PI3K (αPI3K/αPI3K) mutants survived longer than Pdgfra null mice, but the mutation was still embryonic lethal and pups that survived passed birth died shortly afterwards. The authors next examined mice hemizygous for the F7 allele (F7/-), a mutation that disrupts binding sites for PI3K, SRC family kinases, PLCγ and SHP2. Interestingly, F7/- embryos did not show abnormalities that were worse than those observed in αPI3K/αPI3K homozygous mutants. The
inability of the *Pdgfra*-PI3K (α^{PI3K}/α^{PI3K}) mutants to fully phenocopy *Pdgfra* null mutants was suggested to be the result of PI3K signaling from the β-receptor via heterodimerization with *Pdgfra*. To address this, the authors generated double homozygous PI3K (α^{PI3K}/β^{PI3K}) mutants and indeed these mice phenocopied *Pdgfra* null mice [111].

PDGFRα signaling in liver development is not known, but our lab has recently reported robust expression of PDGFRα during early liver development and immunohistochemistry showed liver progenitor cell (hepatoblast) expression of this receptor. Our data suggests that PDGFRα maybe be important for liver development.

1.5.5 Platelet Derived Growth Factor Receptor alpha signaling in Cancer

PDGFRα signaling has been shown to be critical for the initiation of many cancers. Interestingly, this receptor has been shown to be involved in normal development of many organs, but also in the pathogenesis of the same organs. For example, PDGFRα plays a critical role in brain development, and cancers affecting the brain, such as gliomas are characterized by aberrant PDGFRα signaling [112]. In addition, PDGFRα is important for normal lung development and ironically PDGFRα signaling has been implicated as a mechanism of myofibroblast hyperplasia during pulmonary fibrosis [113]. This receptor has also been strongly implicated in advanced gastrointestinal cancer [114]. Furthermore, a recent report showed enhanced expression of PDGFA in addition to various mutations in the *PDGFRA* sequence in [115, 116] cholangiocarcinoma. Recently, our lab has reported significantly upregulated protein expression of PDGFRα and it’s ligands PDGFAA, and PDGFCC in tumors of human liver cancer tissue samples [117]. The authors show that blockade of PDGFRα signaling in various human cancer
cell lines using a human PDGFRα specific blocking antibody, 3G3, significantly reduced cancer cell proliferation and survival [117]. Implications of PDGFRα in the various aforementioned pathologies strongly make this receptor a viable potential therapeutic target in various cancers, especially HCC, which is a focal point of study in our lab.
2.0 ROLE AND REGULATION OF PLATELET DERIVED GROWTH FACTOR RECEPTOR ALPHA IN LIVER DEVELOPMENT

2.1 ABSTRACT

PDGFRα signaling is crucial and indispensable during development. Global inactivation of this receptor has been shown to be embryonic lethal. The critical role of this receptor during development has been shown in certain organs including, lung and brain. Our lab recently identified robust PDGFRα expression as early as embryonic day 11 during early liver development *albeit* with a gradual decrease until birth. We investigated the role and regulation of PDGFRα signaling during early liver development. We identified differential expression of PDGFRα ligands during development. PDGF-CC, a ligand for PDGFRα was expressed during early and PDGF-AA at late stages of hepatic development, while PDGF-BB was expressed at all times. We also identified notable PDGFRα activation in hepatic morphogenesis, which when interrupted by PDGFRα-blocking antibody led to decreased hepatoblast proliferation and survival in embryonic liver cultures. Based on ongoing proliferation, survival and differentiation of hepatoblasts and hepatocytes at early stages of hepatic development and concomitant PDGFRα signaling at the same stages, we conclude that PDGFRα may be an important mediator of these critical biological events in hepatic development.
2.2 INTRODUCTION

Liver development is a complex and temporal process, which entails a balanced regulation of proliferation and differentiation of the bipotential progenitor cells or hepatoblasts [118]. It involves several key steps such as competence, induction and morphogenesis. Many proteins have been shown to be essential in each of these phases and many more of these protein molecules are continuing to be discovered. With the idea of commonalities between development and cancer in mind, we employed developing livers to explore and identify genes that may possibly play a role in diseases affecting the liver such as hepatocellular carcinoma. Using developing livers, we previously identified high PDGFRα expression at embryonic days 12 and 14 with a gradual to significant decrease up to the adult stage by micro-array and western blot analysis [117]. Immunohistochemical analysis showed PDGFRα localization in liver progenitor cells (hepatoblasts) and also hematopoietic cells at early stages during liver development suggesting that PDGFR-α maybe playing a role in hepatoblast and hepatocyte proliferation and survival and is the major hypothesis. Other organs where PDGFRα signaling mediated cell proliferation has been implicated in their development includes but is not limited to: lungs, testes, kidney, and skin [97]. Mouse studies involving deletion of PDGFRα have delineated the indispensable role of this protein during development as embryonic lethality occurs between embryonic day 11.5 to 15 [97]. Intriguingly, much pathology affecting the aforementioned organs involves PDGFRα signaling. For example, PDGFRα plays a critical role in brain development, and most cancers affecting the brain, such as gliomas [119] are characterized by aberrant PDGFRα signaling [112]. In addition, PDGFRα is important for lung development and similarly PDGFRα signaling has been implicated as a mechanism of myofibroblast hyperplasia
during pulmonary fibrosis [113]. The fact that PDGFRα plays such a critical role in development and also is involved in many cancers further suggests that developmental studies can facilitate our understanding of the mechanisms that may play roles in cancer initiation and progression. The robust increase in PDGFRα gene and protein expression during early liver development merits some close attention as it could be mediating a critical process during liver development.

2.3 METHODS

2.3.1 Isolating Developing livers

Time pregnant mice were utilized to obtained embryos from different stages. Whole embryos (n≥3) were isolated from E10-E14 stages and fixed in 10% buffered formalin for paraffin embedding and OCT compound for cryosections. Embryonic livers (n=3) were harvested from E15-E19 stages, and additional livers (n=3) were isolated from postnatal day 1 (P1), P5, P10, P15, P20, P30 and 3-month adult mice.
Figure 6. Description of experimental design for the embryonic liver isolation process as described in the methods

2.3.2 Western Blot (WB) Analysis

Livers were isolated from embryos from each stage (E11-E19, P1, P30 and adults) and pooled (n>20 for E11-14; n>3 E15-adult) for whole cell lysate extraction in radio immunoprecipitation assay (RIPA) buffer and assessed by western blot. After autoradiography, the films were scanned to obtain integrated optic densitometry (IOD) using NIH Imager software. The average IOD for a protein was compared between the KO and WT groups and assessed for statistical significance by student t test and p<0.05 was considered significant.
### Table 1: Primary antibodies used for Western Blot

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFRα</td>
<td>1:900</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>1:300</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>phospho-PDGFRα Tyr572/574</td>
<td>1:800</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>phospho-PDGFRα Tyr849</td>
<td>1:800</td>
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<tr>
<td>phospho-PDGFRα Tyr742</td>
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</tr>
<tr>
<td>phospho-PDGFRα Tyr720</td>
<td>1:800</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PDGFA</td>
<td>1:200</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>PDGFB</td>
<td>1:200</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>PDGFC</td>
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<td>Santa Cruz</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1:2000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Actin</td>
<td>1:5000</td>
<td>Millipore</td>
</tr>
<tr>
<td>EGFR</td>
<td>1:200</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

### Table 2: Phospho specific PDGFRα Primary antibodies used for WB

<table>
<thead>
<tr>
<th>Tyr-PDGFRα (Antibody Source)</th>
<th>Downstream Signaling (ref.)</th>
<th>Biological endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr -742/731 (Invitrogen)</td>
<td>PI3K pathway [120-123]</td>
<td>• Proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Survival</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Motility</td>
</tr>
<tr>
<td>Tyr-572/574 (Invitrogen)</td>
<td>Src Kinase [124]</td>
<td>• Cell growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Motility</td>
</tr>
</tbody>
</table>
Tyr-720  (Cell Signaling)  
SHP2\textsuperscript{[125]}{(dephosphorylates PDGFR\textgreek{a})}  
• Negatively modulates PDGFR\textgreek{a} activation

Tyr-849 (Invitrogen)  
Ras\textsuperscript{[126]}{(?)}  
• Kinase insert domain

### 2.3.3 Histology, Immunohistochemistry (IHC)

Four-micron sections from paraffin-embedded (E11-13) liver tissues were subjected to IHC. PCNA-positive hepatocytes were counted under an Axioskop 40 (Zeiss) upright research microscope in four randomly selected fields per section at 400X magnification. PCNA counts between control and 1E10 treated livers were compared for statistical significant by student \(t\) test with \(p<0.05\) considered significant.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Concentration</th>
<th>Antigen Retrieval</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>1:50</td>
<td>Citrate Buffer</td>
<td>Neomarkers</td>
</tr>
<tr>
<td>HNF4\textgreek{a}</td>
<td>1:100</td>
<td>Citrate Buffer</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>PCNA</td>
<td>1:4000</td>
<td>Zinc Sulfate</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>PDGFR\textgreek{a}</td>
<td>1:50</td>
<td>Citrate Buffer</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>
2.3.4 Immunofluorescence

For immunofluorescence studies, double stains of PDGFRα and hepatoblast cell specific marker (HNF4α+) were performed to demonstrate subset of epithelial cells that are expressing PDGFRα during hepatic development. Briefly, cryosections were fixed for 10 minutes in 4% paraformaldehyde, washed in PBS, and blocked in 2% BSA for 45 minutes. Rabbit polyclonal PDGFRα antibody (Santa Cruz) and mouse HNF4α (Perseus Proteomics Inc.) was used at 1:40 and 1:300 dilution respectively in 0.5% BSA and incubated for 1h, washed in 0.5% BSA, and Cy3-conjugated anti-Rabbit antibody and Alexa 488-conjugated anti-mouse antibody (Molecular Probes) was applied at 1:700 and 1:600 dilution respectively in 0.5% BSA for 30 minutes. Washes were repeated in 0.5% BSA, PBS, followed by incubation with DAPI for 45 seconds. Sections were cover slipped in Gelvatol and sections visualized under Zeiss Axioscope microscope.

Table 4: Primary antibodies used for Immunofluorescence

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFRα</td>
<td>1:50</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>HNF4α</td>
<td>1:300</td>
<td>Perseus Proteomics Inc.</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>1:50</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>
2.3.5 Embryonic liver culture

As shown in Fig 7, E11 embryos from time pregnant mice were isolated and livers were micro-dissected with atraumatic instruments under stereomicroscope as described in [127]. Livers were cultured on 3μm pore sized tissue culture treated polycarbonate membrane in transwells in 6 well plates. 12 embryonic livers were cultured in DMEM in the presence of 5% FBS, 12 were cultured in 5% DMEM and treated with 50nM of 1E10-IMC-MAb (murine PDGFRα blocking antibody, obtained under Material Transfer Agreement from ImClone)[128, 129]. Livers were cultured for 72h in fresh media with or without 1E10 blocking antibody and spent media was replaced every 24h. 4 livers per six well were fixed in 10% formalin for paraffin embedding and histological characterization.
Figure 7. Experimental design for ex-vivo liver cultures

(A) Livers from E11 embryos are isolated & cultured on (B) Tissue culture treated polycarbonate membrane in transwells at 37°C in DMEM with 5% FBS containing 1E10 blocking antibody or without 1E10 for 72h.

2.3.6 Generation of Pdgfra Conditional Knockout Mice

Homozygous Pdgfra floxed (exons 1-4) and Foxa3 Cre mice (both in C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, ME). Homozygous floxed Pdgfra mice were bred to Foxa3-Cre mice and the offspring carrying a floxed Pdgfra allele and Foxa3-Cre were then bred to the homozygous floxed Pdgfra mice. The mice with floxed and floxed-deleted allele of Pdgfra are henceforth referred to as $Pdgfra^{loxp/loxp}; Foxa3-Cre^{+/-}$ or knockout (KO) mice and all other genotypes including $Pdgfra^{loxp/loxp}; Foxa3-Cre^{-/-}$ and $Pdgfra^{loxp/Wt}; Foxa3-Cre^{-/-}$ or $Pdgfra^{loxp/loxp}; Foxa3-Cre^{-/-}$ are referred to as wildtype controls (WT).

2.4 RESULTS
2.4.1 Increased expression and activation of PDGFRα during early hepatic development.

To validate previous findings [117], we utilized whole cell lysates from pooled livers from various prenatal developmental stages in mice to perform WB analysis for PDGFRα and PDGFRβ. PDGFRα is expressed at high levels in E11 and E12 livers with a gradual decrease in protein expression (Figure 8A). PDGFRβ was also similarly expressed (Figure 8A). To verify that PDGFRα was expressed in epithelial cell compartment of developing liver, we performed double IF for PDGFRα and HNF4α, a known marker for both hepatoblasts and hepatocytes. Membranous and cytoplasmic localization of PDGFRα was evident in several HNF4α-positive cells at E12.5 stage (Figure 8B). To address PDGFRβ localization, we examined its co-localization with HNF4α, but could not detect any at E12.5 (Figure 8B). To further substantiate the expression of PDGFRα in hepatoblasts, we tested HBC-3, a hepatoblast cell line, which showed comparable PDGFRα expression to E11 murine livers (Figure 8C). Next, we tested for the presence of ligands that may be responsible for engaging PDGFRα during early stages. Interestingly, while PDGF-CC was the predominant ligand at early stages, PDGF-AA became more prominent at E17 and beyond, while PDGF-CC levels deteriorated (Figure 8D). PDGF-BB appears to be minimally expressed throughout liver development.

To address if PDGFRα signaling is ongoing during hepatic development, we tested whole cell lysates for phosphorylation of PDGFRα at specific residues. We identified several sites to be phosphorylated on PDGFRα, prominently at Tyr-572/574, Tyr-720 and Tyr-742 at E11-E12 stages, whereas Tyr-849 showed only limited phosphorylation at E11 stage (Figure 8E). To test if some of the associated downstream signaling may be active at the corresponding times, we examined the state of PI3-kinase and AKT signaling, since these have been shown to be
regulated by PDGFRα especially when it is phosphorylated at Tyr-742 and other RTKs as well [120]. While total PI3K-p85 and AKT levels remained relatively unaltered during hepatic development until after birth, phospho-PI3K-p85 and phospho-AKT-Thr308 levels were observed mostly in the liver lysates at E11-E13 stages followed by decrease at all later stages coinciding with loss of PDGFRα and its phosphorylated forms (Figure 8F).

Our results therefore suggest that concomitant PDGFRα and PDGF-C expression induces activation of this RTK to contribute to hepatic morphogenesis.
Figure 8. Western blot and immunofluorescence analysis using developing livers

A) Representative WB shows highest expression of PDGFRα at E11 in liver lysates with gradual decrease over next days of gestational development in mice. (B) Left panel shows a representative double IF with PDGFRα (red) expressed in HNF4α-positive hepatoblasts (green) cells in E12.5 murine liver (arrowhead). Right panel shows PDGFRβ (red) expressed in non-HNF4α-positive cells (arrows) whereas HNF4α-positive (green) hepatoblasts (arrowheads) are negative for PDGFRβ (400X). (C) WB verifies PDGFRα expression in HBC3 hepatoblast cell line
and embryonic day 11 liver whole cell lysate. (D) WB shows high PDGF-CC but not PDGF-AA during early liver development, whereas PDGF-AA levels increase at later stages. (E) WB shows PDGFRα phosphorylation at multiple sites indicating its activation in embryonic livers at early stages of hepatic development. (F) WB shows tyrosine phosphorylation of PI3K-p85 and AKT depicting activation at corresponding early stages of hepatic development.

2.4.2 Blockade of PDGFRα signaling in embryonic liver cultures reveals its role in hepatoblast proliferation and survival.

To further investigate the role of PDGFRα signaling during early liver development, we utilized embryonic liver cultures and PDGFRα-blocking antibody (1E10-IMC), kindly provided by ImClone, whose efficacy and specificity has been recently shown [128]. Livers isolated from E10 embryos and cultured in the presence of 1E10-IMC and 4% serum as compared to 4% serum alone for 72 hours showed fewer hepatoblasts when examined by IHC for HNF4α (Fig. 9). To determine the mechanism of reduced hepatoblasts, we tested the organoid cultures for proliferation and any change in cell viability. 1E10-IMC treated embryonic livers showed dramatically fewer cells in S-phase as indicated by IHC for PCNA suggesting decreased cell proliferation after 72 hours of PDGFRα-blockade (Fig. 9). Simultaneously, these livers showed an increase in the numbers of TUNEL-positive cells indicative of ongoing apoptosis (Fig. 9). Thus, based on the expression of PDGFRα mostly in hepatoblasts, it is apparent that PDGFRα signaling is one of the contributing molecular pathways in hepatoblast proliferation and viability and hence in liver growth during ex vivo hepatic morphogenesis.
Figure 9. PDGFRα blockade in embryonic liver cultures reveals its role in hepatoblast proliferation.
Embryonic livers cultured in PDGFRα blocking 1E10-IMC MAb show by IHC decreased numbers of HNF4α-positive cells. In addition, there was a notable decrease in PCNA-positive cells and increase in numbers of TUNEL-positive cells after 1E10-IMC treatment compared to cells grown in 4% serum and shown in representative photomicrographs.

2.4.3 Loss of hepatoblast PDGFRα is not embryonic lethal as evidenced by viable pups.

Originally Pdgfra was conditionally deleted from hepatoblasts by interbreeding floxed Pdgfra mouse with Foxa3 cre mouse (mixed background). Out of 42 live pups, only one Pdgfra KO mouse was found by PCR indicating loss of Pdgfra was embryonic lethal (data not known). So to make sure that the mixed background was not the cause of embryonic lethality, Foxa3 mouse was bred into a pure C57BL6 background for 6 generations. Foxa3 mouse (C57BL6 background) was then bred to floxed Pdgfra mouse. Out of 36 live births, 4 Pdgfra KO pups and these pups were sacrificed after 1 month and livers were used for WB. As seen in figure 10, WB results show complete loss of Pdgfra in KO mice. As will be discussed in a later chapter, we recently showed significant EGFR upregulation and activation during liver regeneration after PHx in hepatocyte specific Pdgfra KO mice, which indicated a possible compensatory mechanism. Since we found surviving hepatoblast Pdgfra KO mice, we performed WB to test whether EGFR could be again compensating for loss of Pdgfra during liver development. Interestingly, we didn’t see any changes in EGFR expression between WT and KO mice as shown in figure 10. We also probed for PDGFRβ, which identical in structure, has similar functions, and has been shown to compensate for PDGFRα in cell lines. Interestingly, levels of PDGFRβ remained unchanged between WT and KO. These results suggest that perhaps another receptor tyrosine
kinase could be compensating for loss of *Pdgfra* or that PDGFRα signaling maybe dispensable in hepatoblasts.

![Western blot analysis](image)

**Figure 10. Western blot analysis confirms depletion of hepatoblast PDGFRα by Foxa3**

*Foxa3 driven deletion of hepatoblast *Pdgfra* shows no increase in EGFR or PDGFRβ in KO mice*

## 2.5 DISCUSSION

PDGFRα, a traditional serum growth factor receptor for mesenchymal cells, is expressed abundantly in human HCC cells and known to induce cell proliferation [89, 90, 97, 100, 117, 130]. To address the role and regulation of PDGFRα in normal liver growth, especially in epithelial cells of the liver, we investigated its status in liver development.

Previously we have reported highest expression of PDGFRα gene in the earliest stages of hepatic development, which was identified by microarray [117]. In the current study we verify that
hepatoblasts co-express HNF4α and PDGFRα. Indeed HNF4α is a known marker of hepatoblasts and differentiating hepatocytes during hepatic development [131]. However there are other cell types in developing livers that have been reported to express PDGFRα as well. One study has reported PDGFRα to be expressed in submesothelial cell fraction, a precursor of hepatic stellate cells, and yet another group has reported PDGFRα expression in a stromal cell that supports erythropoiesis [132, 133]. In the last study, the authors observed all PDGFRα-positive cells to express β1-integrin, which is also known to be expressed in hepatocytes during early hepatic development [134]. Utilizing previously characterized embryonic liver cultures and a mouse PDGFRα blocking antibody, we demonstrate rampant apoptosis and decrease in cell proliferation that eventually led to lower numbers of HNF4α-positive cells in the culture suggesting an important role of PDGFRα in hepatoblast biology. The effect is likely direct due to RTK blockade of PDGFRα on the hepatoblasts. Indeed, PDGFRα activation is associated with cell survival and proliferation in several cell types [89, 120]. To address the importance of PDGFRα signaling in-vivo, we conditionally deleted Pdgfra from hepatoblasts by interbreeding floxed Pdgfra mouse (C57BL6) with Foxa3 cre mouse (mixed background). Loss of Pdgfra appeared to be embryonic lethal since we found 1 Pdgfra KO mouse out of 42 live pups by PCR. To determine whether this observation was not a result of the mixed background of the Foxa3 cre mouse, we repeated the same experiment but this time we bred Foxa3 cre for 6 generations into C57BL6. This led to survival of additional Pdgfra KO mice (4 out of 36), which was still below the normal mendelian ratio. Our results suggest that some strains of mice might be more susceptible to Pdgfra loss in developing livers. Furthermore, based on our personal communications with Dr. Klaus Kaestner, Foxa3 cre transgenic line might exhibit some mosaicism. Nonetheless, our in vivo and in vitro results, taken together, indicate an important
role of PDGFRα signaling in liver development...Further studies using a more specific and robust cre to induce deletion of PDGFRα in hepatoblasts would be essential to conclusively demonstrate it’s role in liver development.

### 2.6 FUTURE DIRECTIONS

- We have obtained HBC-3 cells, which are clonal fetal murine hepatoblast cell line derived from an embryonic day 9.5 embryo [135]. When these cells are grown on fibroblast feeder layer, they maintain their stemness. When grown in DMSO on tissue culture plastic, they undergo hepatocyte differentiation and when grown on matrigel, they form bile ducts. We have data showing robust expression of PDGFRα in lysates from these cells. We will employ these cells to test the effects of PDGF-CC and PDGFRα specific blocking antibody (1E10). This will also allow us to assess the importance of PDGFRα in hepatoblasts. Based on our data, we anticipate that treatment of HBC-3 with PDGFCC will induce their proliferation and survival. We also anticipate that treatment of these cells with PDGFRα blocking antibody, 1E10, will lead to increased cell death and decreased proliferation. Additional roles in hepatic morphogenesis maybe revealed as well.

- Foxa3 Cre has been shown to be mosaic (Personal communication with Dr. Klaus Kaestner), where a subset of cells escapes deletion. In addition, Foxa3 is also expressed in extrahepatic tissues such as pancreas [136]. For these reasons, we have now obtained AFP-Cre mice that will be bred to homozygous Pdgfra floxed mice.. This strategy will
enable deletion of Pdgfra in hepatoblasts at embryonic days 9.5-11 when PDGFRα expression is highest. Tails of pups will be used for PCR analysis to determine whether KO pups are being produced at the normal mendelian ratio. This will conclusively allow us to determine whether PDGFRα is important or dispensable during liver development. Based on our ex-vivo data, we anticipate that loss of hepatoblast Pdgfra will be embryonic lethal.
3.0 β-CATENIN LOSS IN HEPATOCYTES PROMOTES HEPATOCELLULAR CANCER AFTER DIETHYLNITROSAMINE AND PHENOBARBITAL ADMINISTRATION TO MICE

3.1 ABSTRACT

HCC is the 5th commonest cancer worldwide. β-Catenin, the central orchestrator of the canonical Wnt pathway and a known oncogene is paramount in HCC pathogenesis. Administration of phenobarbital (PB) containing water (0.05% w/v) as tumor promoter following initial injected intraperitoneal (IP) diethylnitrosamine (DEN) injection (5µg/gm body weight) as a tumor inducer is commonly used model to study HCC in mice. Herein, nine 14-day male β-catenin knockout mice (KO) and fifteen wild-type littermate controls (WT) underwent DEN/PB treatment and examined for hepatic tumorigenesis at 8 months. Paradoxically, a significantly higher tumor burden was observed in KO (p<0.05). Tumors in KO were β-catenin and glutamine synthetase negative and HGF/Met, EGFR & IGFR signaling was unremarkable. A significant increase in PDGFRα and its ligand PDGF-CC leading to increased phosphotyrosine-720-PDGFRα was observed in tumor-bearing KO mice (p<0.05). Simultaneously KO livers displayed increased cell death, stellate cell activation, and hepatic fibrosis and cell proliferation. Further, PDGF-CC significantly induced hepatoma cell proliferation especially following β-
catenin suppression. Our studies also demonstrate that the utilized DEN/PB protocol in the WT C57BL/6 mice did not select for β-catenin gene mutations during hepatocarcinogenesis. Thus, DEN/PB enhanced HCC in mice lacking β-catenin in the liver may be a result of an inability to regulate cell survival leading to enhanced fibrosis and regeneration, through PDGFRα activation. β-catenin down regulation also made hepatoma cells more sensitive to receptor tyrosine kinases and thus may be exploited for therapeutics.

3.2 INTRODUCTION

HCC is the fifth most common cancer and the third cause of cancer death worldwide [62]. There is a strong need to delineate the molecular alterations responsible for the initiation and exacerbation of this disease. In order to study the cellular and molecular perturbations in HCC, many preclinical strategies employ the use of genetic and chemical models of carcinogenesis. Administration of diethylnitrosamine (DEN) alone or in conjunction with phenobarbital (PB) in mice is frequently used to induce HCC in mice.

One pathway of critical importance in HCC is the Wnt/β-catenin signaling. β-Catenin is the central effector of the canonical Wnt signaling, which is a highly conserved pathway regulating critical cellular processes such as proliferation, differentiation, survival and self-renewal [75, 137-139]. In the absence of Wnt, β-catenin is phosphorylated at amino-terminal serine and threonine residues and targeted for ubiquitination [140]. Upon binding of Wnt protein to its cell surface receptor Frizzled and co-receptor low-density lipoprotein– related protein 5/6 (LRP5/6), a signal is transduced through disheveled that allows for inactivation of degradation
complex comprised of glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli gene product (APC) and casein kinase Iα, which allows β-catenin to dissociate and translocate to the nucleus to bind to lymphoid enhancer-binding factor/T cell factor (LEF/TCF) family of proteins to transactivate target genes. The Wnt/β-catenin pathway has been implicated in a subset of HCCs where activating mutations in the β-catenin gene (CTNNB1) have been reported in 20%-40% of patients [85, 87]. Knockdown of β-catenin in hepatoma cells leads to decreased growth and survival. For the aforementioned reasons, β-catenin is a well-recognized oncogene and considered a valuable therapeutic target.

With this background, we hypothesized that lack of β-catenin in hepatocytes might protect against chemical-induced carcinogenesis especially in a model where HCC is conceived through tumor induction by DEN and tumor promotion through the continuous use of PB [141, 142]. We used male conditional hepatocyte-specific β-catenin knockout (KO) mice and wild-type littermate controls (WT) to study tumorigenesis in response to DEN/PB. We report a paradoxical increase in hepatic tumorigenesis in the absence of β-catenin that was attributable to enhanced injury, fibrosis and ensuing regeneration, which appear to be driven by a rather non-classical epithelial receptor tyrosine kinase receptor PDGFRα. We also demonstrate that the commonly employed DEN/PB protocol in C57BL/6 mice does not induce tumorigenesis through β-catenin mutations as is observed in C3H mice. Lastly, β-catenin inhibition led to PDGFRα activation and thus may make the hepatoma cells more amenable to receptor tyrosine kinase inhibition for therapies.
3.3 MATERIALS AND METHODS

3.3.1 Animal studies

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. Mice with conditional deletion of β-catenin in hepatocytes with genotype- Ctnnb1$^{loxp/loxp}$;Alb-Cre$^{+/-}$ are referred to as knockout mice (KO) and have been described previously [88]. Littermates with any of the following genotypes- Ctnnb1$^{loxp/loxp}$;Alb-Cre$^{-/-}$, Ctnnb1$^{loxp/WT}$;Alb-Cre$^{+/-}$, Ctnnb1$^{loxp/WT}$;Alb-Cre$^{-/-}$ are referred to as wildtype or WT. These mice are in C57BL/6 background. Male KO (n = 9) and WT (n= 15) mice were injected intraperitoneally with DEN (Sigma-Aldrich, Inc.) at a dose of 5μg/gram body weight at postnatal day 14 and from day 28 onwards the drinking water available ad libitum contained phenobarbital (PB) (0.05% w/v). Water containing fresh PB was prepared weekly for the duration of the studies. Mice were sacrificed at 8 months and liver collected for histology and protein analysis.

3.3.2 Western blot Analysis

Total tissue lysates prepared in radio immuno-precipitation assay (RIPA) buffer containing 1% IgePAL CA-630, 0.5% Sodium Deoxycholate, 0.1% SDS in 1x PBS along with protease and
phosphate inhibitor (1:100) (Thermo Scientific). Proteins were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis in 4-15% gels and then transferred to Immobilon-P membranes (Millipore, Bedford, MA) in transfer buffer containing 10% methanol. Membranes were probed with primary antibodies (see below) in Tris-buffered saline with Tween-20 containing 5% nonfat milk or BSA. Horseradish peroxidase–conjugated secondary antibodies were used at 1:50,000 dilution and signal assessed with Super Signal West Pico chemiluminescence substrate (Pierce, Rockford, IL) and autoradiography. The films (Molecular Technology Sales, St. Louise, MI) were scanned to obtain integrated optic densitometry (IOD) using NIH Imager software. The average IOD for a protein was compared between the KO and WT groups and assessed for statistical significance by student \( t \) test and \( p<0.05 \) was considered significant.

**Table 5: Other primary antibodies used for western blot**

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<th>Source</th>
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</tr>
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<td>MET</td>
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</tr>
<tr>
<td>Glutamine Synthetase</td>
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<td>Santa Cruz</td>
</tr>
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</table>
3.3.3 Histology

Four-micron sections of paraffin-embedded liver tissues were used for histology and immunohistochemical staining. Hematoxylin and Eosin (H&E) staining was employed to identify tumor foci. Typically four representative lobes from each mouse were included on each slide. Tumor foci were identified based on characteristic attributes such as basophilic cytoplasm and mitotic figures. For comparison, total number of microscopic nodules were counted and average numbers compared for statistical significant by student $t$ test with $p<0.05$ considered significant.

3.3.4 Immunohistochemistry

For immunohistochemistry, antigen retrieval was achieved both by steam cooking or boiling the slides in microwave in citrate buffer for 20 minutes or 10 minutes, respectively. The sections were inactivated for endogenous peroxide, blocked and incubated with primary antibody overnight at room temperature or for one hour at room temperature, washed and incubated with appropriate biotin-conjugated secondary antibody for 30 minutes. Sections were washed, incubated with ABC reagent, washed and incubated with DAB. Sections were next counterstained with Shandon hematoxylin solution (Sigma) and cover slipped using Cytoseal XYL (Richard Allen Scientific, Kalamazoo, MI). For negative control, the primary antibody was omitted in the protocol. Slides were viewed under an Axioskop 40 (Zeiss) upright research microscope and digital images obtained. Collages were prepared using Adobe Photoshop CS4 software. For PCNA and TUNEL, numbers of positive cells were counted in four random 400X fields in five representative livers from each group and averages compared for significance.
between groups by student t test. P value of less than 0.05 was considered significant. For quantitative analysis of β-catenin- and PDGFRα-positivity in tumors, all microscopic foci in KO and WT livers were assessed. Any tumor foci showing cytoplasmic and/or nuclear β-catenin staining were labeled as being β-catenin-positive and any foci exhibiting cytoplasmic staining for PDGFRα as compared to surrounding non-tumor areas were labeled as being PDGFRα-positive. This enabled us to calculate percentage of β-catenin and/or PDGFRα-positive tumors in each group after DEN/PB treatment.

For measurement of fibrosis, trichrome staining was performed by the Department of Pathology Histology Services. Photomicrographs were taken at 50x magnification and % area of fibrosis measured using Adobe Photoshop as previously described.

<table>
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<th>Antibody Target</th>
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<td>Citrate Buffer</td>
<td>Abcam</td>
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<tr>
<td>Glutamine Synthethase</td>
<td>1:50</td>
<td>Citrate Buffer</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

### 3.3.5 Cell culture and treatment

Hep3B cells (ATCC) were grown to 100% confluence in 10% FBS and EMEM media. Cells were trypsinized and plated in 6 well plates containing 2 ml of 10% FBS EMEM media and serum-starved overnight. Transfection was performed using pre-validated β-catenin and control siRNA (Ambion) using lipofectamine as described previously [143]. After 24 hours, the media
was discarded and 1 ml of 5% trichloroacetic acid (TCA) was added to each well and plates placed in 4°C for 15 minutes. Cells were washed, dried and suspended in 1 ml of 0.33M NaOH for 20 minutes, and 300 μl of total mixture from each well was added to 3 ml of scintillation fluid and then placed in scintillation counter. Experiment was repeated at least twice and each condition was done in triplicates. Average counts per minute (CPM) were compared between different conditions for statistical significance by student t test with value of less than 0.05 considered significant.

3.4 RESULTS

3.4.1 β-Catenin loss in hepatocytes leads to enhanced hepatocarcinogenesis in mice in response to DEN/PB.

WT and KO male mice (C57BL/6) were given a single dose (5μg/gram body weight) of DEN injection at postnatal day 14 days and two weeks later allowed ad libitum access to PB containing drinking water (0.05% w/v) for 8 months at which time mice were examined for liver tumors as seen in Figure 11A. Intriguingly, the mice lacking β-catenin in hepatocytes displayed significantly enhanced tumorigenesis compared to WT mice that was grossly appreciable as larger and greater numbers of tumors (Fig. 11B). H&E staining was employed to also determine the microscopic tumor foci in both groups of animals (Fig. 11C). The total numbers of foci were counted in representative sections from four lobes from the KO and WT, which show significantly more tumors in KO as compared to the WT (p<0.05) (Fig. 11D).
Figure 11. Enhanced tumorigenesis in β-catenin KO mice exposed to DEN/PB regimen.

(A) Experimental strategy summarizing DEN/PB treatment in KO and WT mice. (B) Representative photographs of tumor-bearing livers in DEN/PB treated KO and WT mice at the time of harvest at 8 months of age. (C) DEN/PB induced microscopic tumor foci (outlined by arrowheads) visualized by H&E in WT and KO livers at 8 months of age.
age. (D) A significant increase in microscopic tumor foci in KO as compared to WT (p<0.05). Tumors were counted from H&E stained sections representing 4 liver lobes from each KO and WT animals on DEN/PB protocol.

3.4.2 β-Catenin KO livers after DEN/PB treatment shows increased cell death, stellate cell activation and fibrosis and tumor proliferation.

Next, we addressed the cellular mechanisms that may be the basis of enhanced tumorigenesis in KO. We identify higher numbers of TUNEL-positive hepatocytes in KO at 8 months after DEN/PB as compared to similarly treated WT, suggesting greater cell death (Fig. 12A). There was an accompanying increase in hepatic parenchymal cell proliferation in KO that exceeded that of WT (Fig. 12A). In addition, KO mice exhibited a dramatic increase in the numbers of α-smooth muscle actin, which identifies activated stellate cells that are responsible for collagen deposition and fibrosis (Fig. 12A). Concomitant to stellate cell activation, we observed enhanced fibrosis by Masson Trichrome staining in the KO as compared to the WT (Fig. 12B). In fact only one of the 15 WT animals after DEN/PB challenge showed fibrosis, which was comparable to fibrosis in the KO. All together, our results suggest that greater tumorigenesis in KO livers occurred concomitantly with significantly greater cell death, proliferation (Fig. 12C), and hepatic fibrosis.
Figure 12. Tumorigenesis in KO mice following DEN/PB treatment is associated with greater injury, fibrosis and regeneration.

(A) Representative tumor-bearing KO livers show increased parenchymal cell death (TUNEL), greater stellate cell activation (α-SMA) and increased parenchymal cell proliferation (PCNA) as compared to WT. (B) Increased hepatic fibrosis (blue) in tumor-bearing KO livers is evident by Masson Trichrome staining as compared to control. (C) PCNA- and TUNEL-positive cells were counted in 4 random sections of 5 representative KO and WT livers. A significant increase in both PCNA and TUNEL positive parenchymal cells was evident in KO livers as compared to WT after DEN/PB treatment.

3.4.3 Tumors in KO livers after DEN/PB are not composed of β-catenin-positive hepatocytes.

Since there have been reports from our lab and others that in response to specific injuries, there is pressure on hepatocytes that may have escaped cre-mediated deletion, our first goal was to ascertain if livers exposed to DEN/PB from KO group showed any reappearance of β-catenin especially when compared to the WT. A representative WB showed that all KO livers from DEN/PB exposed mice expressed dramatically lower levels of β-catenin as compared to WT by western blots (Fig. 13A). This was also verified by a detailed immunohistochemical analysis included in a forthcoming section (Table 7 and Fig. 14). The low level of β-catenin in KO livers represents its presence in the non-parenchymal cells of the liver that show no albumin cre-mediated recombination. Similarly analysis presented from representative KO and WT livers also showed absence of glutamine synthetase, a known target gene of β-catenin signaling in the KO (Fig. 13A). Cyclin-D1, another prominent target of β-catenin in liver and elsewhere was
increased in most WT, whereas 8/9 KO showed very low or absent cyclin-D1 in response to DEN/PB (Fig. 13A and not shown). c-Myc, another target of β-catenin, was ironically higher in KO as compared to WT mice after DEN/PB exposure as shown in a representative western blot (Fig. 13A). Increased c-Myc in KO livers is most likely due to PDGFRα and PDGFCC increase since PDGF has been shown to stimulate c-Myc mediated proliferation via the PI3K/Akt pathway [144]. KO livers after DEN/PB continue to be negative for β-catenin after 8 months.

### 3.4.4 Tumors in KO livers after DEN/PB are not associated with activation of traditional HCC associated receptor tyrosine kinases.

Due to a paradoxical increase in DEN/PB-induced tumorigenesis in KO, we next explored possible molecular mechanisms. Both epidermal growth factor (EGF) signaling and hepatocyte growth factor (HGF) signaling are critical players in the development and exacerbation of HCC [145]. We explored both total and phosphorylated status of these receptor tyrosine kinases (RTKs) in livers from DEN/PB-treated KO and WT mice. Interestingly we noted a decrease in total and phosphorylated levels of MET, the HGF receptor in KO animals as compared to WT (Fig. 13B). Both total and phosphorylated levels of EGFR between WT and KO animals remain unaltered (Fig. 13B). We also examined insulin like growth factor receptor, another signaling pathway implicated in HCC [145]. We noted higher levels of its phosphorylation in the WT as compared to KO (Fig. 13B). Thus, classical RTKs do not appear to be responsible for enhanced HCC in KO but p-MET and P-IGFR are prominently induced in tumor bearing WT mice suggesting their important role in HCC.
Figure 13. Molecular signaling in tumor-bearing B-catenin KO and WT mice.

(A) Representative western blot analysis from 5 WT and 4 KO tumor-bearing livers shows low β-catenin, absent GS and dramatically lower cyclin-D1 in KO whereas c-Myc levels were increased. Actin verifies equal loading. (B) Examination of RTK in the same sets of animals shows notably lower phospho-MET and phospho-IGFR and only marginally lower phospho-EGFR, in KO than WT livers by western blots. GAPDH verifies comparable loading.
3.4.5 Immunohistochemical characterization of KO and WT liver tumors for β-catenin and PDGFRα.

We next characterized the tumors observed in the WT and KO by immunohistochemistry for β-catenin localization. Around 31% of total tumor foci in WT mice showed nuclear β-catenin (Table 7, Fig. 14). Out of 63 observed foci in 9 KO mice, only two were composed of β-catenin-positive tumor cells that exhibited its nuclear/cytoplasmic localization (data not shown) while others were negative (Fig. 14). This substantiated the observations in Figure 13A and also demonstrates that almost all tumors in this group were comprised by β-catenin-negative hepatocytes and cannot be due to expansion of cells, which may have retained β-catenin due to incomplete albumin-cre recombination.

We next investigated PDGFRα signaling, which has been implicated in HCC and is involved in tumor growth, angiogenesis, and maintenance of tumor microenvironment [117, 130, 146]. 54.5% of all tumor foci in the WT mice showed cytoplasmic PDGFRα expression (Table 7). We found around 94% of tumor foci in KO to be strongly positive for PDGFRα in the cytoplasm of tumor cells (Table 7, Fig. 14). Only four foci were negative for PDGFRα. The two tumor foci that were β-catenin-positive in the KO livers were simultaneously positive for PDGFRα.

In an additional analysis, 9 of the 55 tumors observed in the WT were concomitantly positive for both PDGFRα and nuclear β-catenin, while others were positive for either one of the two (Table 7, Fig. 14). A small fraction of tumors were negative for both these proteins. The two tumors that were composed of β-catenin-positive hepatocytes in the KO were also positive for PDGFRα signaling (Table 7). Overall, PDGFRα expression was higher and also in greater
numbers of tumor foci in the KO as compared to the WT 8 months after DEN/PB exposure.
Figure 14. β-catenin and PDGFRα immunohistochemistry in tumors in WT and KO mice exposed to DEN/PB.
Tumors in WT were heterogeneous and were either positive for both β-catenin and PDGFRα, or for either one of them. In KO, almost all tumors lacked any β-catenin and showed intense PDGFRα-positivity.

Table 7: Summary of Immunohistochemical findings of microscopic tumor foci

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<th>SAMPLE</th>
<th>NUMBER OF TUMOR FOCI</th>
<th>β-CATENIN-POSITIVE FOCI</th>
<th>PDGFRα-POSITIVE FOCI</th>
<th>NUMBER OF FOCI POSITIVE FOR BOTH N/C β-CATENIN and PDGFRα</th>
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59
3.4.6 DEN/PB induced increased tumorigenesis is associated with activation of PDGFRα signaling.

To further verify PDGFRα increase in the KO over WT after DEN/PB, we utilized western blots analysis. PDGFRα protein levels were higher in the KO than the WT livers (Fig. 15A), and this difference was statistically significant (Fig. 15B). There was a modest increase in PDGFRβ levels in the KO livers (Fig. 15A). We also identified an increase in total protein levels of selective PDGFRα ligand PDGF-CC while PDGF-AA and PDGF-BB remained unaltered between the two groups. To determine consequences of enhanced PDGF-CC/PDGFRα levels in
KO we assessed levels of PDGFRα phosphorylation at several specific tyrosine residues using antibodies listed in methods. As shown in densitometric analyses, a significant increase in Tyr720-PDGFRα (p<0.05) (Fig. 15D) but not in Tyr849-PDGFRα (Fig. 15E) or Tyr572/574- and Tyr754-PDGFRα (not shown) was observed in the KO. These observations demonstrate PDGFRα activation in KO, which may be playing an important role in hepatocarcinogenesis.
### DEN+PB

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### B

![Graph showing average integrated optical density](image17)

**p = 0.002**

### C

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### D

![Graph showing average integrated optical density](image26)

**p = 0.036**

### E

![Graph showing average integrated optical density](image27)

**p = 0.323**
Figure 15. Tumor-bearing β-catenin KO mice display active PDGFRα signaling when compared to WT. (A) Representative western blots from 8 KO and 9 WT show a dramatic increase in total levels of PDGFRα and modest increase in PDGFRβ in the KO. Actin loading verifies equal loading. (B) Average integrated optical density (IOD) obtained from scanned autoradiographs shown in Fig. 5A revealed significantly higher PDGFRα levels in KO (p=0.002). (C) Western blot from representative samples shows a dramatic increase in PDGF-CC, a ligand for PDGFRα in KO whereas PDGF-AA and BB remained unremarkable between the two groups. (D) Bar graph depicts a significant increase in Tyr720-PDGFRα in KO as compared to WT (p<0.05). (E) Insignificant differences were evident in Tyr-849-PDGFRα between the WT and KO.

3.4.7 PDGFRα ligand stimulates hepatoma cell proliferation only upon β-catenin suppression.

To further ascertain the relevance of PDGFRα signaling in absence of β-catenin, we utilized human hepatoma cells. PDGF-CC induced an insignificant increase in Hep3B cell proliferation in almost confluent cell cultures (Fig. 16). β-Catenin knockdown when compared to control siRNA transfection, significantly lowered thymidine incorporation in Hep3B cells (p<0.0005) (Fig. 16). Only upon β-catenin silencing, was PDGF-CC treatment able to induce significant DNA synthesis in Hep3B cells as compared to HCl (p<0.005). Thus β-catenin suppression enabled PDGF-CC to be mitogenic to Hep3B cells.
Figure 16. β-catenin suppression increases mitogenicity to PDGF-CC in human hepatoma cell culture.

PDGF-CC (10ng/ml) treatment does not increase DNA synthesis as compared to HCl treatment of Hep3B cells. β-Catenin knockdown led to significant decrease in thymidine incorporation as compared to control siRNA (p<0.0005). However PDGF-CC treatment led to a significant increase in thymidine incorporation in β-catenin-suppressed as compared to control siRNA-transfected cells (p<0.005).

3.5 DISCUSSION

To understand the molecular and cellular basis of HCC in patients, several preclinical models are in use including DEN or DEN/PB regimens in rodents. DEN is a commonly used carcinogen to induce HCC in rodent models, however it has high strain specificity. In C57BL/129Sv x C3H/He mice, a strain more susceptible to hepatocarcinogenesis, DEN injection at 6 weeks of age at a
dose of 90 μg/gm body weight, induces HCC through Ha-Ras mutations, while inclusion of PB in drinking water after 3 weeks of DEN, promotes tumorigenesis due to β-catenin mutations [147]. However, another study in male B6C3F1 mice, obtained by interbreeding female C57BL/6J and male C3H/HeJ mice, injected DEN at 10 μg/gm body weight at 3 weeks of age without PB, showed HCC via β-catenin mutations [148]. Another model utilizes DEN at a dose of 5μg/gm body weight in C57BL/6 mice, a strain relatively resistant to HCC. Here, DEN induces DNA adducts in hepatocytes undergoing cell division, and eventually leads to development of HCC [149, 150]. Inclusion of PB enhances tumorigenesis via its tumor promoting ability [141, 142]. In our study, we show that DEN/PB induced liver tumors in the C57BL/6 mice did not exhibit nuclear/cytoplasmic localization of β-catenin and hence not selectively cause HCC via β-catenin mutations. This could be because of either the timing of DEN/PB administration, since we injected at 2 weeks after birth as opposed to Dr. Schwarz’s group, where DEN was administered at 6weeks after birth. The other possibility is strain difference of mice, since we used pure C57BL/6 as compared to C57BL/129 X C3H/He mice utilized by Dr. Schwarz’s group.

Many pathways broadly categorized into Ras/MAPK, PIK3CA/AKT, and Wnt/β-catenin signaling, have been shown to be of significance in HCC [69, 145]. β-Catenin, the central orchestrator of Wnt signaling, is a known oncogene due to its implications in a variety of cancers, including 20%-40% of all HCCs [151]. Intriguingly though, overexpression of either wild type or mutant form of β-catenin in murine livers is unable to induce spontaneous HCC [152-155]. However, in the presence of another ‘hit’ in the form of a transgene or a chemical carcinogen, these various mice show enhanced tumorigenesis [154, 156]. Paradoxically, conditional loss of β-catenin in hepatocytes in C57BL/6 mice led to an unexpectedly higher
susceptibility to DEN-induced HCC [73]. Another group also recently demonstrated increased injury, fibrosis, and HCC in KO mice in response to DEN/PB, albeit in C3H/N mice [157]. In the current study, we provide evidence that β-catenin KO mice in C57BL/6 background subjected to DEN-mediated tumor induction at P14 followed by tumor promotion 2 weeks later by PB also led to a dramatically higher tumor burden.

DEN or DEN/PB induced HCC in any strain of mice is not typically associated with any hepatic fibrosis. However, in the β-catenin conditional null mice DEN/PB exposure led to development of HCC, which was associated with hepatic fibrosis and is in line with recent studies by others and us [73, 157]. We also saw an increase in cell death and resulting increase in cell proliferation. In fact we identified increased stellate cell activation, a modest increase in PDGFRβ and ensuing hepatic fibrosis. These data indicate that β-catenin loss makes livers more prone to genotoxic injury and eventually tumorigenesis mimicking the predominant scenario of human HCC where tumors often occurs in cirrhotic background [158]. Role of β-catenin in regulating redox state has been implied in many recent studies where its interactions with HIF1α, FOXO3 and others may be critical [159, 160]. It will thus be important to understand the basis of such ‘tumor suppressive’ roles of β-catenin in HCC that may eventually require careful selection of patients to be treated with anti-β-catenin therapies [66].

To ascertain the molecular basis of HCC in the absence of β-catenin, we were interested in signaling pathways that are well known in the development and exacerbation of HCC. MET and IGFR phosphorylation was increased in WT but not in KO mice while EGFR activation was only mildly elevated in WT. Thus while these RTK’s may be playing an important role in tumorigenesis in the WT exposed to DEN/PB, their participation in KO is unlikely. Based on our previous findings in DEN-induced tumorigenesis in KO [73] and independent studies showing an
important role of PDGFR signaling in HCC [117, 130, 146], we investigated its expression and activation in DEN/PB studies. PDGFRα levels were significantly upregulated in KO after DEN/PB-exposure. In addition, PDGF-CC a selective ligand of PDGFRα, whose overexpression in liver-specific transgenic mice has been shown to induce cirrhosis and HCC [161], was also increased in KO. In fact PDGF-CC was localized to hepatocytes in KO exposed to DEN/PB (data not shown) suggesting an autocrine loop of signaling. The results obtained from thymidine incorporation assay in the Hep3B cells, corroborates in vivo findings. While decreased cell proliferation was observed in hepatoma cells after β-catenin knockdown [143], PDGF-CC promoted their mitogenesis more robustly only after β-catenin suppression that leads to PDGFRα upregulation [73]. This also verifies PDGFRα signaling as a means of escape from β-catenin therapeutic inhibition.

PDGFRα overexpression in the presence of increased PDGF-CC led to increased Tyr720-PDGFRα in tumor bearing KO but not WT livers after DEN/PB. Phosphorylation at tyrosine 720 is known to activate phosphatase SHP2, which in turn dephosphorylates Src, leading to its activation [89]. Src activation has been shown to induce c-Myc, an important proto-oncogene [124], which was concomitantly elevated in KO. Other tyrosine sites in PDGFRα showed inconspicuous changes in phosphorylation between the KO and WT and thus may be of lesser relevance in the current tumorigenesis model.

Only around 3% of all tumors in the 9 KO’s at 8 months after the DEN/PB regimen were β-catenin-positive tumors that may be due to ‘leaky’ cre-recombinase. β-catenin-negative tumors were also negative for GS and mostly negative for cyclin-D1. We have not followed any animals in this study beyond 8 months. Others have recently reported extensive spontaneous repopulation in KO livers albeit at 18-20 months of age [162]. One group has reported a more robust
spontaneous repopulation and hepatic adenomatosis in KO, which has not been reported by any other group working with the β-catenin conditional knockout mice [163]. The only instance of extensive repopulation in the KO mice in our experience was observed after continuous administration of diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 5 months [164]. In fact KO mice have been studied after PHx, methionine-choline deficient diet, alcohol diet or DEN and have exhibited lack of hepatic repopulation with β-catenin-positive hepatocytes [73, 88, 160, 165].

3.6 FUTURE DIRECTIONS

- Generate Pdgfra/β-catenin conditional double KO mice and perform the same DEN/PB induced tumorigenesis studies and assess whether there will be abrogation of increased tumorigenesis. This will conclusively corroborate our results that PDGFRα activation is the escape mechanism for increased tumorigenesis in β-catenin conditional KO mice. Based on both in vivo and cell culture results, we anticipate that Pdgfra/β-catenin mice will have significantly reduced tumorigenesis when compared to β-catenin KO alone.

- Our lab has previously shown increased PDGFRα protein expression 24hr after β-catenin knockdown [73]. We show here that treatment of Hep3B cells with PDGFCC induced insignificant changes in Hep3B cell proliferation. However, we observed a significant increase in Hep3B cell proliferation upon PDGFCC treatment 24hr after β-catenin knockdown. Based on these observations, we will perform siRNA knock down of β-catenin in Hep3B cells and obtain lysates. Lysates will be utilized for WB analysis using
site-specific PDGFRα phospho antibodies to identify which sites are activated after β-catenin knockdown. Based on our in vivo data, we anticipate that several PDGFRα sites, especially the SHP2 site will be highly activated after β-catenin knockdown. If the phosphorylation of the PDGFRα-SHP2 site is increased after β-catenin suppression as anticipated, we will then utilize SHP2 site mutant to address the importance of this phosphorylation site of PDGFRα upon β-catenin silencing. We anticipate that transfection of Hep3B cells with the PDGFRα-SHP2 mutant 24hr after β-catenin suppression will abrogate PDGFCC induced Hep3B cell proliferation.
4.0 ROLE AND REGULATION OF PDGFRα SIGNALING IN LIVER REGENERATION

4.1 ABSTRACT

Aberrant PDGFRα signaling is evident in a subset of hepatocellular cancers (HCCs). However, its role and regulation in hepatic physiology remains elusive. In the current study we examine PDGFRα signaling liver regeneration (LR). We identified temporal PDGFRα overexpression, which is regulated by EGF and TNFα, and its activation at 3 hours (3h)-24h after partial hepatectomy (PHx). Through generation of hepatocyte-specific Pdgfra knockout (KO) that lack an overt phenotype, we show absent PDGFRα compromises Erk and AKT activation at 3h after PHx, which however is alleviated by temporal compensatory increases in EGF receptor (EGFR) and HGF receptor (MET) expression and activation along with rebound activation of Erk and ATK at 24h. These untimely increase in EGFR and MET allow for normal hepatocyte proliferation at 48h in KO, which however is aberrantly prolonged up to 72h. Intriguingly, such compensation was also visible in primary KO hepatocyte cultures but not in HCC cells after siRNA-mediated PDGFRα knockdown. Thus, temporal activation of PDGFRα in liver development is important in hepatic morphogenesis. In LR, despite increased signaling, PDGFRα is dispensable due to EGFR and MET compensation, which is unique to normal
hepatocytes and not HCC cells.

4.2 INTRODUCTION

Platelet-derived growth factor receptor-alpha (PDGFRα) is a receptor tyrosine kinase (RTK) expressed chiefly on mesenchymal cells including fibroblasts and smooth muscle cells [95, 97, 100]. In addition, it is also expressed on other cell types including neurons and endothelial cells. Its activation is elicited by platelet derived growth factors, especially AA and CC, which induce potent effects on the growth, motility, and survival, thus regulating function of these cells [89]. Following engagement, PDGFRα tyrosine phosphorylation can occur at diverse residues to elicit activation of distinct downstream effectors. Specifically relevant are downstream activation of phosphatidylinositide 3-kinases (PI3K) and AKT as well as Erk signaling [90, 120, 166].

Our lab has recently identified PDGFRα expression in hepatoblasts during early liver development coinciding with the time of peak proliferation that gradually decreases to low levels. In an adult liver only low PDGFRα expression is evident, however, its expression is dramatically increased in a significant subset of hepatocellular carcinomas (HCC) and its inhibition in human HCC cells leads to reduced tumor cell proliferation and viability [117, 130].

It is thus pertinent to further investigate the role and regulation of PDGFRα in liver growth. In the current study, we investigate the role of this RTK in a major model of hepatic growth. Liver regeneration (LR) after PHx is commonly used to study importance of signaling molecules in hepatic growth. The process of LR requires an orderly interplay between many cell types and several signaling pathways [39, 41]. The cellular and molecular mechanisms
responsible for LR exhibit significant redundancy to allow completion of the process as shown by studies in genetic models or after chemical intervention.

In the current study we demonstrate that adult murine hepatocytes indeed express PDGFRα albeit at low levels. However, after PHx, we observe temporal early upregulation and activation of PDGFRα. Through generation of hepatocyte-specific Pdgfra knockout mice (KO) by interbreeding floxed Pdgfra [167] and albumin-cre animals [168], we demonstrate that its conditional loss from hepatocytes is well tolerated. When subjected to PHx, LR proceeds uneventfully owing to compensatory increases in the expression of epidermal growth factor receptor (EGFR) and MET, the hepatocyte growth factor (HGF) receptor. Such redundancy is unique to LR and not HCC growth making PDGFRα an attractive therapeutic target. Thus we show an important role of PDGFRα in various aspects of liver growth

4.3 MATERIALS AND METHODS

4.3.1 Generation of Pdgfra Conditional Knockout Mice

Homozygous Pdgfra floxed (exons 1-4) and albumin-Cre mice (both in C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, ME). Homozygous floxed Pdgfra mice were bred to albumin-Cre mice and the offspring carrying a floxed Pdgfra allele and albumin-Cre were then bred to the homozygous floxed Pdgfra mice. The mice with floxed and floxed-deleted allele of Pdgfra are henceforth referred to as Pdgfra<sup>loxP/loxP</sup>; Alb-Cre<sup>+/−</sup> or knockout (KO)
mice and all other genotypes including $Pdgfra^{loxp/loxp}$; Alb-Cre$^{-/-}$ and $Pdgfra^{loxp/Wt}$; Alb-Cre$^{-/-}$ or $Pdgfra^{loxp/loxp}$; Alb-Cre$^{-/-}$ are referred to as wildtype controls (WT).

### 4.3.2 Animal studies and PHx.

All experiments on mice were performed under the strict guidelines of the National Institutes of Health and the Institutional Animal Use and Care Committee at the University of Pittsburgh. Eight to 12 week-old female KO and littermate female controls were subjected to PHx as described previously [88, 154]. Mice (n$>3$ per genotype) were sacrificed at specified time-points post surgery as indicated in results and figures. Baseline and regenerating livers were harvested and stored at -80°C until use. Livers were also fixed in 10% formalin to be used for paraffin embedding or placed in OCT compound for cryosectioning.

### 4.3.3 Serum Biochemistry

Serum biochemical measurements were performed by the University of Pittsburgh Department of Pathology Lab Support Services. Blood from male or female KO along with sex and age-matched WT was collected at 3-10 months (Table 9) for serum biochemistry Total bilirubin, alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured on samples taken prior to sacrifice at different time points.

### 4.3.4 Western Blot (WB) Analysis

Total tissue lysates were prepared from independent or pooled (n$\geq3$) livers in the radio immuno-
precipitation assay (RIPA) buffer and assessed by WB as discussed elsewhere. After autoradiography, the films were scanned to obtain integrated optic densitometry (IOD) using NIH Imager software. The average IOD for a protein was compared between the KO and WT groups and assessed for statistical significance by student \( t \) test and \( p<0.05 \) was considered significant.

Table 8: EGFR and Met phospho specific antibodies used for western blot

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Concentration</th>
<th>Source</th>
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<tbody>
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<td>phospho-EGFR1173</td>
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<td>Santa Cruz</td>
</tr>
<tr>
<td>phospho-EGFR1068</td>
<td>1:300</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>phospho-MET1234</td>
<td>1:200</td>
<td>Cell Signaling</td>
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4.3.5 Real time-PCR

RNA was extracted from livers using Trizol (Invitrogen) as per the manufacturer’s instructions. RNA from each sample was reverse transcribed with SuperScript III First –Strand Synthesis System for RT-PCR with an RNase H treatment (Invitrogen). Also, equal amounts of RNA from three age- and sex-matched KO mouse livers were pooled to make cDNA. A total of 0.1 \( \mu \)g cDNA along with 1x Power SYBR-Green PCR Master Mix (Applied Biosystems) and the appropriate primers (see below) were used for each real-time PCR reaction. The samples were analyzed for each condition in triplicates to enable statistical analysis. The Applied Biosystems StepOnePlus Real-Time PCR System was used for the analysis of the transcripts with the StepOne v2.1 software. Comparative \( \Delta\Delta CT \) was used for analysis of the data, and calculations
were made without the StepOne software.

The real-time PCR analysis was performed with a liver-specific reference gene (cyclophilin-A). Primer efficiencies were performed for each primer, and only similar efficiencies were used for analysis. The following real-time PCR primers were designed using Primer-BLAST (NIH): mouse Pdgfra forward: 5’- TCC TTC TAC CAC CTC AGC GAG -3’; mouse Pdgfra reverse: 5’- CCG GAT GGT CAC TCT TTA GGA AG -3’; mouse cyclophilin-A forward: 5’- CCC CAC CGT GTT CTT CGA CA -3’; mouse cyclophilin-A reverse: 5’- TCC AGT GCT CAG AGC TCG AAA -3’.

4.3.6 Histology, Immunohistochemistry (IHC) and Immunofluorescence (IF)

Four-micron sections from paraffin-embedded liver tissues were subjected to IHC [154]. PCNA-positive hepatocytes were counted under an Axioscope 40 (Zeiss) upright research microscope in four randomly selected fields per section at 400X magnification. PCNA counts between WT and KO livers after PH were compared for statistical significant by student t test with p<0.05 considered significant.

For IF, cryosections were fixed for 10 minutes in 4% paraformaldehyde, washed in PBS, and blocked in 2% BSA for 45 minutes. Rabbit polyclonal PDGFRα antibody (Santa Cruz) was used at 1:40 dilution in 0.5% BSA and incubated for 1h, washed in 0.5% BSA, and Cy3-conjugated anti-Rabbit antibody (Molecular Probes) was applied at 1:700 dilution in 0.5% BSA for 30 minutes. Washes were repeated in 0.5% BSA, PBS, followed by incubation with DAPI for 45 seconds. Sections were cover slipped in Gelvatol and sections visualized under Zeiss Axioscope microscope.
4.3.7 Cell culture and treatment

Human hepatoma cells (Hep3B; ATCC) cultured in serum free EMEM were treated with 20ng/ml EGF (BD, Durham NC), 40ng/ml HGF (Kind gift from Snow Brand milk company), 25ng/ml TNFα (R&D systems, Minneapolis MN), 3uM/ml EGFR inhibitor (SelleckBio, Houston TX) for 24hr. Cells were washed and lysed in RIPA buffer and assessed by WB. Briefly, Hep3B cells were plated in 10cm dish plates containing EMEM with 10% FBS and serum starved for 4hr for synchronization. Cells were treated with the various cytokine or growth factors and harvested 24hr later.

4.3.8 Cell culture and Transfection

Human hepatoma cells (Hep3B; ATCC) were plated and cultured in six well plates in EMEM with 10% FBS. The next day cells were serum starved for 14hr to allow for synchronization. Cells were then transfected with 30uM PDGFRA siRNA (Ambion®, Austin TX) or control for 6h. After 6h transfection, 1ml of EMEM media with 4% FBS was added to each well for overnight and cells were harvested at 24h and 48h for western blot analyses.

4.3.9 Collagenase Perfusion

A modified 2-step collagenase perfusion and mouse hepatocyte culture was done as described previously. Primary hepatocytes from WT and Pdgfra KO mice were isolated and plated on collagen coated six well plates in MEM media with 10% FBS for 2h. Afterwards the media was discarded and replaced with basic mouse growth media (MGM) or MEM media with 10% FBS.
Cells in basic MGM were untreated or treated with 20ng/ml EGF (BD, Durham NC) 40ng/ml HGF (Kind gift from Snow Brand milk company), 25ng/ml TNFα (R&D systems, Minneapolis MN), combined EGF/HGF and harvested 24h later for WB analyses.

4.4 RESULTS

4.4.1 Temporal increase in PDGFRα protein and activity after PHx.

To determine if PDGFRα may have a role in another model of regulated hepatic growth, we assessed its expression during the process of LR after PHx especially since hepatocytes are known to secrete PDGFs during this process [43]. We examined whole cell lysates from pooled livers (n≥3) from different times after PH. A dramatic increase in PDGFRα protein level was evident at 24h during LR (Fig. 17A and C). No change in PDGFRα expression in sham surgery was observed (not shown). To further verify this observation, PDGFRα expression was examined in individual livers from four mice harvested at 24h after PHx. All four samples showed a clear increase in PDGFRα expression at this time during LR (Fig. 17B). While no other phospho-specific forms of PDGFRα were detectable (data not shown), increased phosphorylation at Tyr-572/574 was observed in regenerating livers from 3h-28h while 24h sham sample lacks such an increase (Fig. 17C). Concomitant to an early increase in p-PDGFRα, there was a notable increase in both p-Erk and p-AKT that indicates the role of PDGFRα in regulating the activity of these important mediators of LR (Fig. 17C). To determine the mechanism of enhanced protein expression of PDGFRα, we examined mRNA expression and
identified a 2.0 fold increase in \textit{Pdgfra} in pooled 24h livers as compared to pre-hepatectomy samples (Fig. 17D). To address the molecular basis of increased PDGFR\(\alpha\) expression after PHx, we examined the effects of known growth factors and cytokines that are known to critical initiators of LR [39]. A human hepatoma cell line Hep3B was treated for 24h with HGF, EGF and TNF\(\alpha\) as indicated in methods and cells tested for changes in PDGFR\(\alpha\) protein expression by WB. EGF treatment brought about an increase in PDGFR\(\alpha\) levels, which was abrogated by concomitant treatment of cells with an EGFR inhibitor (Fig. 17E). TNF\(\alpha\) treatment also led to an increase in total PDGFR\(\alpha\). Thus, EGF and TNF\(\alpha\) induce the expression of PDGFR\(\alpha\) during early LR.
Figure 17. Temporal increase in PDGFRα expression and activation during LR after PHx.

(A) Representative WB from pooled livers shows increased PDGFRα protein expression after PHx with peak expression evident at 24h post-PHx. GAPDH verifies comparable loading. (B) WB using whole cell lysates from 4 individual animals show a notable increase in total PDGFRα at 24h after PHx as compared to normal livers at baseline, while GAPDH shows comparable loading. (C) Representative WB shows activation of PDGFRα.
(phosphorylated at Tyr 572, 574), Erk (phosphorylated at Thr202 and Tyr204 of Erk1 or Thr185 and Tyr187 of Erk2) and AKT (phosphorylated at Thr308) at various time-points after PHx using a site-specific PDGFRα. Abbreviation: SH-sham surgery sample. (D) Real-Time PCR showing 2-fold increase in Pdgfra 24hr post-PHx (p=0.0001). (E) Lysates from Hep3B cells treated with various growth factors for 24h shows increased PDGFRα expression in response to EGF and TNFα treatment. The increase by EGF was abrogated by concomitant use of EGFR inhibitor AG-1478. GAPDH depicts equal loading.

4.4.2 Pdgfra conditional KO mice lack an overt phenotype.

To address the function of PDGFRα during LR, we generated hepatocyte-specific conditional Pdgfra KO mice. Mice were born in normal Mendelian ratio and did not exhibit any apparent phenotype. Genotype was verified by the presence of homozygous floxed Pdgfrα allele and cre-recombinase by PCR (Fig. 18A). The livers from KO and WT livers showed unremarkable gross differences (not shown). No differences were observed in liver weight to body weight ratios between KO and WT mice (Fig. 18B) and serum biochemistry showed insignificant differences between WT and KO animals (Table 1). KO livers (≥2 months old) were utilized for WB, IHC and real-time PCR. A decrease in total PDGFRα was evident in KO by WB as compared to the control livers (Fig. 18C). No changes in PDGFRβ, or PDGFRα ligands such as PDGF-AA or PDGF-CC were observed in the KO versus WT in the livers from age-and sex-matched littermates (Fig. 18C). Since there was significant remnant expression of PDGFRα in KO livers, we next tested PDGFRα by IHC in KO and WT livers. Indeed PDGFRα continued to be expressed in non-parenchymal cells in the KO livers as compared to WT since albumin-cre will specifically delete floxed gene from hepatocytes (Fig. 18D). Real-time PCR also showed around
50% decrease in Pdgfra expression in KO livers, which was significantly lower than littermate controls (Fig. 18E).

Figure 18. Generation of Pdgfra KO mice reveal lack of overt phenotype.
(A) Genotyping PCR identifies KOs (lanes 2 and 4) by the presence of cre recombinase (lower panel) and floxed PDGFRα allele (242 bp) (upper panel). WT controls were identified by the absence of cre (lane 3) or presence of cre
in animals that harbor WT PDGFRα allele (451 bp) and floxed allele (lane 1). (B) Graph depicting insignificant change in liver weight to body weight ratios between WT (n=6) and KO (n=5) at baseline. (C) WB in lower panel shows significant decrease in total PDGFRα protein in the KO livers. No changes in levels of PDGFRβ and PDGFRα ligands including PDGF-AA and CC were observed. (D) IHC of WT (left) livers show expression of PDGFRα at hepatocyte membrane (arrows). PDGFRα KO (right) show reduced PDGFRα staining which was localized to non-parenchymal cells (arrowheads). (400X) (E) Real-Time PCR showing a significant decrease of Pdgfra expression in KO livers (p=0.0001).

Table 9: Serum biochemistry results comparing WT and Pdgfra KO mice

<table>
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<tr>
<th>Genotype</th>
<th>Sex</th>
<th>AGE (Months)</th>
<th>Total Bilirubin (mg/dl)</th>
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<th>Alanine transaminase (ALT) (IU/L) Normal (7-56)</th>
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</table>
4.4.3 *Pdgfra* KO mice show normal hepatocyte proliferation after PHx.

Eight weeks or older female littermate KO and WT were subjected to PHx. Since peak PDGFRα expression was observed at 24h, we first determined its levels at this time in KO. KO livers lack any increase in PDGFRα at 24h after PHx as compared to WT, indicating the predominant increase in PDGFRα protein to be in the parenchymal cell compartment (Fig. 19A). IF at 24h during LR demonstrates PDGFRα expression to be dramatically lower in KO and evident in non-parenchymal cells whereas WT show strong membranous localization in the hepatocytes (Fig. 19B).

The KO mice were followed after PHx for any morbidity. There appeared to be a temporal restricted activity in the KO around 24h after PHx, however all KO mice recovered from surgery and were indistinguishable from WT at all later time points. Based on the role of PDGFRα in liver development and in HCC [117], we compared hepatocyte proliferation between the two groups of animals during LR. We hypothesized that PDGFRα induction and activation at 24h may be one of the upstream signaling cascades regulating hepatocyte proliferation during LR, which usually peaks at around 48h after PHx in C57BL6 mice, although cell cycle initiation occurs much earlier. However, we found comparable and high number of hepatocytes in S-phase as detected by IHC for PCNA at 48h in KO and WT (Figure 19C-D). Intriguingly, at 72h, while PCNA staining was expectedly lower in WT, KO mice continued to display several hepatocytes in S-phase (Fig. 19C-D). However, no further growth advantage was evident at later stages and KO and WT mice had comparable restoration in hepatic mass at 14 days after PHx.
4.4.4 *Pdgfra* KO mice show no compensatory changes in PDGF signaling but show enhanced EGFR and MET expression and activation during LR.

Due to lack of any defect in cell proliferation in KO at 48h and in fact a continued proliferation at higher than normal levels at 72h we wondered if any compensatory mechanisms could account for such observation. We focused on 24-72h, which represent the times of activation of cell cycle and of ongoing cell proliferation during the LR process. First, liver lysates were assessed for PDGF signaling which was unremarkable for any differences in PDGF-AA, PDGF-CC and also for PDGFRβ, which has been shown to compensate for PDGFRα loss *in vitro* [166] (Fig. 20A). We next investigated expression for RTKs, such as EGFR and MET, which are major drivers of LR and also associated with hepatocyte proliferation [39]. Interestingly, WB showed increased EGFR and MET protein prominently at 24h in the regenerating KO livers (Fig. 20B). Quantification after normalization to loading control revealed around 3.5-fold increase in EGFR and 2.5-fold increase in MET above the WT levels at 24h (Fig. 20C-D). To address the state of RTK signaling, we assessed lysates for phosphorylation of EGFR and MET. Elevated levels of Tyr1068-EGFR, Tyr1173-EGFR and Tyr1234-MET were observed *in lieu* of PDGFRα-activation at 24h after PHx in KO livers (Fig. 20E). Thus, the anomalous increases in EGFR and MET expression and activity at 24h ensure hepatocyte proliferation at 48h in PDGFRα KO that is comparable to WT, but the aberration eventually became apparent at 72h when proliferation in WT receded while KO hepatocytes continued to proliferate *albeit* temporally and without chronic consequences.
Figure 19. Continued proliferation in Pdgfra KO livers during LR after PHx

(A) Representative WB verifies abrogation of surge in PDGFRα expression in KO livers at 24h after PHx as compared to WT. (B) IF shows PDGFRα localizing to hepatocyte membrane (red) at 24h after PHx in WT liver (upper panel) whereas it is localized to non-parenchymal cells and is conspicuously absent from hepatocyte membranes in KO (lower panel). (400X). (C) PCNA IHC in WT liver and KO liver at 48h and 72h post PHx shows enhanced nuclear staining (arrowhead) and mitosis (arrows) in KO especially at 72h. PCNA-negative hepatocytes are indicated with red arrowheads. (100X). (D) Bar graph of PCNA index of WT and KO livers shows comparable cells in S-phase at 48h whereas a significant increase is observed in KO at 72h (*p<0.05).
Figure 20. Loss of hepatocyte *Pdgfra* induces temporal increase in EGFR and MET expression and activation at 24hr after PHx.
(A) Representative WB from pooled livers shows no changes in protein expression of PDGFRβ, PDGF-AA or PDGF-BB at various time points after PHx in KO when compared to WT. (B) Representative WB shows decreased p-AKT and p-Erk in KO as compared to WT at 3h during LR. Enhanced EGFR and MET protein expression in KO livers at 24h after PHx coincides with increased p-AKT and p-Erk at this time in KO as compared to WT. (C) Quantification of changes in EGFR protein after PHx shows a 2.5-fold increase in KO livers at 24h post PHx. Normalized to baseline WT. (D) Quantification of changes in MET protein levels after PHx shows 2-fold increase over baseline. Normalized to baseline WT. (E) Representative WB shows enhanced protein expression of phosphorylated forms of EGFR and MET as indicated, especially at 24h after PHx.

4.4.5 **Inhibition of PDGFRα signaling induces EGFR or MET expression in vitro only in hepatocytes and not hepatoma cells.**

To determine if EGFR and MET are globally induced upon PDGFRα inhibition, we first tested the impact of PDGFRα loss in primary hepatocytes, which were isolated by collagenase perfusion from WT and KO mice and cultured for 24h as described in methods. An increase in total EGFR and MET expression was evident in KO hepatocytes as compared to WT after 24h in culture (Figure 21A).

Next we tested the impact of PDGFRα knockdown in human hepatoma cells on EGFR and MET expression. After transfection of PDGFRα siRNA but not control siRNA for either 24h or 48h led to a robust PDGFRα decrease in Hep3B cells (Figure 21A). However, no change in either EGFR or MET protein levels were observed at the corresponding times indicating that only primary hepatocytes but not HCC cells had the capability to enhance the expression of the two RTKs upon PDGFRα inhibition.
Figure 21. EGFR and MET expression are induced after Pdgfra loss in normal mouse hepatocytes but not in HCC cells.

(A) Primary hepatocytes isolated from age- and sex-matched WT and KO livers upon culture for 24h show increased expression of EGFR and MET in KO. Densitometric analysis on the representative WB shows at least 2.0 fold increase in MET and 2.5-fold increase in EGFR levels in the KO hepatocytes. (B) A representative WB shows PDGFRα-siRNA and not control-siRNA transfection of Hep3B cells leads to a notable decrease in PDGFRα expression, at both 24h and 48h. However no changes in EGFR or MET were detectable at either time point.

4.5 DISCUSSION

PDGFRα, a traditional serum growth factor receptor for mesenchymal cells, is expressed
abundantly in human HCC cells and known to induce cell proliferation [117]. To address the role and regulation of PDGFRα in normal liver growth, especially in epithelial cells of the liver, we investigated its status in a model of surgically induced LR. We observed an increase in PDGFRα activation and expression at 3-24h after PHx. There was an associated increase in AKT and Erk signaling that are known to be important in LR and also shown to be downstream effectors of PDGFRα signaling [166, 169, 170]. At the same time PDGFRα is activated as evident by its phosphorylation status, which may be due to an autocrine mechanism since PDGFs are known to be secreted by hepatocytes during LR, although no changes in their total protein is observed during LR [43]. We next wanted to address possible mechanisms of PDGFRα upregulation after PH. Many important cytokines and growth factors including TNFα, EGF and HGF are secreted very shortly during liver regeneration after PHx and are important for this process [41, 56, 171-174]. Our laboratory recently showed NFκB as a possible mechanism for PDGFRα protein upregulation after knockdown of CTNNB1 (β-catenin) in human hepatoma cells [73]. NFκB plays an important role during liver regeneration [175] and TNFα is known to activate NFκB [173, 176, 177]. Based on these reports, we speculated that EGF, HGF, or TNFα could be inducing PDGFRα increase during liver regeneration after PH. To address this, we cultured and treated human hepatoma cells (Hep3B) with EGF, HGF, and TNFα for 24h and harvested cells for WB analyses. Treatment of Hep3B cells with EGF and TNFα respectively induced a salient increase in PDGFRα protein levels while HGF had no effect. To address the role of PDGFRα, we generated Pdgfra-conditional KO mice lacking Pdgfra in hepatocytes. These mice showed no overt phenotype and this is not surprising because of normally low expression of PDGFRα in the liver and perhaps due to existing redundancy in RTK signaling pathways. Also, remnant expression of PDGFRα in KO is due to its presence in non-parenchymal cells such as endothelial
cells and hepatic stellate cells [132, 178]. When the KO mice were subjected to PHx, we observed a notable decrease in PDGFRα expression at 3h and 24h supporting the increased expression and activity of PDGFRα in hepatocytes during the early LR period. Simultaneously, a decrease in p-Erk and AKT activity was evident at 3h in the KO. Intriguingly though, increased p-AKT and p-ERK levels were evident in the KO at 24h, which corresponded to increased EGFR and MET expression over the WT levels. MET and EGFR are paramount in normal LR ad in regulating hepatocyte proliferation [179, 180]. This excessive EGFR and MET expression and activity in absence of PDGFRα enabled comparable hepatocyte proliferation at 48h in KO and WT. These findings again highlight the existing molecular redundancy that exists in normal LR process where blockade of a single signaling pathway is readily compensated by an alternate signaling cascade [39, 56]. However, this untimely and non-scheduled aberration appears to be responsible for transiently prolonging hepatocyte proliferation in PDGFRα in KO at 72h as reflected by increased hepatocytes in S-phase when compared to WT where proliferation has receded. However all later time points in KO are unremarkable for any proliferative changes when compared to the WT. These observations highlight a fine balance that exists between various signaling mechanisms during the process of normal LR [39, 41].

Interestingly, the inverse relationship between EGFR and PDGFRα was also described in EGFR-suppression studies. shRNA mediated silencing of EGFR during LR led to an increase in both gene and protein expression of PDGFRα [180]. Similarly, deletion of Pdgfra lead to enhanced stimulation of the EGFR pathway in mouse embryonic fibroblasts [166]. The exact mechanism of the relationship between PDGFRα and EGFR will need further studies although EGFR and PDGFRα may be physically interacting as has shown to occur between EGFR and PDGFRβ [102, 181, 182].
Hepatic fibrosis and cirrhosis as a result of chronic liver diseases are precursors of HCC. Around 80% of all HCC have underlying cirrhosis [69]. In fact chronic liver injury leads to hepatocyte death, inflammation, fibrosis, oxidative stress and hepatocyte proliferation [158]. The regenerating nodules that occupy cirrhotic liver are considered critical for maintenance of hepatic function, but proliferation in these nodules in a suboptimal environment also makes them prone to genotoxic insult, eventually evolving into dysplastic nodules and HCC. Since several signaling pathways are commonly relevant in LR and HCC, molecular targeting can be a concern in HCC in chronic liver diseases. Thus it is critical to identify targets that may be dispensable for LR but are critical for HCC. In our current study, we show that PDGFRα signaling, which has been previously shown to be a valid, biologically relevant, therapeutic target in HCC [117, 130, 183], may be dispensable for LR because of redundancy with other RTKs like EGFR and MET. Intriguingly, such redundancy is unique to normal hepatocytes and not HCC cells as siRNA mediated PDGFRα knockdown in Hep3B cells did not induce the expression of these RTKs. Although additional studies will be necessary, we propose that selective targeting of PDGFRα in eligible HCC patients may be efficacious even in chronic liver diseases without a concern of impairing hepatic functions. Humanized PDGFRα-blocking antibody is in fact on the horizon for treatment of various cancers [128, 184, 185].

4.6 FUTURE DIRECTIONS

- EGFR floxed and c-met floxed mice are available [186, 187]. Using similar breeding strategy outlined in the methods, we plan to breed these mice to Pdgfra floxed mice in order to generate double Pdgfra/EGFR or Pdgfra/c-Met conditional double KO mice. We
will then perform PHx on these mice to conclusively address the importance of these possible compensations. We show that lack of hepatocyte *Pdgfra* do not delay LR and this appears to be due to MET and EGFR signaling. Lacks of hepatocyte c-Met or EGFR alone have been shown to delay LR [179, 180, 186, 187]. It will be interesting to determine whether there are any additive or synergistic defects in either of the double KO mice compared to *EGFR*, c-Met, or *Pdgfra* single KO after PHx.

- As displayed in figure 22, we have also identified enhanced PDGFRα protein expression in wild type mice 12 weeks after bile duct ligation. We next plan to do immunohistochemistry to determine if hepatocytes are responsible for increased PDGFRα expression after BDL. If hepatocytes show high expression, we plan to perform BDL on our conditional *Pdgfra* KO mice and determine whether loss of *Pdgfra* accelerates or negatively impacts the regenerative process. It is unclear whether hepatocyte proliferation after BDL is protective or part of the pathological process. Based on this, it is difficult to predict an outcome but nonetheless it will be interesting to decipher the effects of hepatocyte *Pdgfra* loss during this process.

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*Figure 22. WB analysis of PDGFRα levels in bile duct ligated mice.*
WB showing significantly upregulated PDGFRα levels in livers of mice 12 weeks after Bile duct ligation (BDL) compared to baseline line levels. Ponceau stain verifies comparable loading
5.0 GENERAL DISCUSSION

5.1 IDENTIFICATION OF OTHER DEVELOPMENTAL SIGNALS AS A WAY TO GENERATE HEPATIC LIKE CELLS.

The liver performs functions that are critical for survival. Some of these functions include processing nutrients from food, making bile, removing toxins from the body and synthesizing proteins. However when the liver becomes disease stricken, these basic functions of the liver are precluded and this can ultimately lead to death of the patient. Fortunately, the liver is equipped with extraordinary innate regenerative capacity to re-grow when a portion of the liver mass is removed or when the liver is damaged [39, 41]. Currently, the most efficacious treatment of choice for patients with end stage liver disease is orthotopic liver transplantation (OLT) [2, 188, 189]. However, this form of treatment has been met with many challenges in that it demands major surgery in addition to the threat of morbidity in case of graft failure and lifelong immunosuppressant, but the biggest challenge is the paucity of liver donors and this mandates careful research for alternate options. As a way to circumvent the need of whole liver transplant, researchers have been employing hepatocyte transplantation as an alternative method especially as a bridge to transplantation. However this method has also been met with many challenges
including engraftment efficiency, cell viability, and obtaining optimum hepatocyte function [188]. The use of bio-artificial liver devices to support limited functions is also becoming a potential option [190, 191].

Figure 23. Diagram showing hepatocyte differentiation from embryonic stem or induced stem cells and the direct reprogramming of fibroblasts.

Exogenous factors that induce differentiation are written in black and transcription factors that are required for fibroblast reprogramming are written in red of adult hepatocyte from various cell types.

Adopted and modified from Shin, D and Monga, SP (Ref. 2).

In order to circumvent the challenges of limited cell source and shortage of organ donors, cell based hepatocyte transplantation and generation of hepatic tissue from various stem cell
sources have become very attractive. Researchers have made numerous attempts to generate adult hepatocytes from various cell sources including but not limited to ES cells, iPS cell and mesenchymal stem cells [2, 189]. For a long period of time, efforts to generate hepatic tissue from ES cells have been challenging and often times results vary with inconsistencies in generating a homogenous cell population in addition to fully mature cells [192, 193]. Recent attempts to generate hepatocytes from these cell sources have been tremendously aided by the growing understanding and further elucidation of embryonic liver development process [2, 189].

![Figure 24. Schematic representation of the differentiation process of ES and iPS cells to mature hepatocyte.](image)

Key factors are required to differentiate ES cells and iPS cells into mature hepatocytes. After differentiation, some cells appear to be fully differentiated and some cells remain immature hepatocytes so perhaps other factors are required to obtain fully differentiated mature hepatocyte. Based on our development data, I propose that perhaps addition of PDGFCC and PDGFAA are required to achieve mature hepatocytes. Adopted and modified from Shin, D and Monga, SP (Ref. 2).
Different researchers employ different but yet similar differentiation protocols in efforts to generate mature functional hepatocytes. Amid the slight differences in protocols, the general consensus has been to model the stepwise differentiation seen during liver development. For generation of hepatocytes from ES cells, it is critical to show that mouse and human ES cell can efficiently generate definite endoderm tissue, which is critical for liver development, by treating these cells with varying concentrations of known factors that are required for endoderm formation. As shown in Figure 23, treatment of ES cells with critical developmental factors at specific times facilitates generation of mature hepatocytes. Using different developmental protocols on mouse and human ES cells, cultures have been generated where 70% of the cells resemble mature hepatocytes and perform many hepatocyte functions including albumin secretion, expression of hepatic enzymes, and drug metabolism [189]. Furthermore, continued understanding of the developmental process has allowed others to generate mature hepatocyte like cells from fibroblasts by overexpression of hepatocyte specific transcription factors [194, 195].

These results show great promise, however the expression of fetal markers by a population of these generated hepatic-like cells suggests these cells are not fully differentiated and that there are still certain unidentified factors required to fully differentiate these cells. As shown in Figure 8, we have identified robust PDGFRα expression and activation during early liver development. We show that hepatoblasts in addition to other cell types express this receptor during liver development. In addition, we show that there is differential expression of PDGFRα ligands, where PDGFC is expressed early, while PDGFA is expressed during later stages of liver development. Finally, we show that blocking of PDGFRα signaling in ex vivo liver cultures leads to decreased cell proliferation, increased cell death, and decreased HNF4α positive cells.
These novel results strongly suggest that PDGFRα and its ligands play essential roles during liver development. As shown in figure 24, we speculate that perhaps PDGFs could be part of certain key missing factors that could also be included in the developmental protocols to efficaciously achieve much higher populations of mature hepatocytes from ES cells or other cell types.

We believe that a more in depth understanding of pathway cross talks and regulations will be key to obtaining a functional and mature hepatocyte from cells. Over the past decade, one of the major findings is that many of these signaling pathways and transcription factors seem to have completely different functions at precisely different times-points during the course of liver development. Detailed understanding of these pathways and discovery of other important genes will facilitate the production of large quantities of human liver tissue that can be used to not only treat diseased patients but also to test the toxicity of new drugs.

5.2 TARGETING PLATELET DERIVED DROWTH FACTOR RECEPTOR-ALPHA FOR HCC THERAPY

Liver cancer is ranked amongst some of the most deadly cancers [196]. Hepatocellular Carcinoma (HCC) is the fifth most common cancer and the third cause of cancer death worldwide [62]. Five hundred million individuals are infected with Hepatitis B or C and a proportion will progress to liver failure and cancer [197]. With these statistics in mind, it is without doubt that there is an urgent need to understand the mechanism of liver carcinogenesis in order to facilitate the development of new cancer therapy that will ameliorate this problem. Hepatic fibrosis and cirrhosis as a result of chronic liver diseases are precursors of HCC.
Approximately 80% of all HCC have underlying cirrhosis [69]. In fact chronic liver injury leads to hepatocyte death, inflammation, fibrosis, oxidative stress and hepatocyte proliferation [158]. The regenerating nodules that occupy cirrhotic liver are considered critical for maintenance of hepatic function, however proliferation in these nodules in this abnormal environment also makes them susceptible to genotoxic insult, eventually leading dysplastic nodules and HCC. Since several signaling pathways are commonly relevant in LR and HCC, molecular targeting can be a concern in HCC in chronic liver diseases. Thus it becomes important to identify targets that may be dispensable for LR but are critical for HCC.

There is preponderance of literature showing many parallel pathways critically involved in both liver regeneration and liver cancer. Examples of such pathways include EGFR and MET signaling pathways. Both of these pathways are crucial for the liver regeneration process as signified by their early activation after PHx [37, 42, 56, 180]. Interestingly both of these pathways are also well document therapeutic targets for HCC. Blockade of EGFR has been shown to inhibit HCC cell proliferation [198, 199]. A recent report showed that HCC patients with an active HGF/c-Met signaling pathway show significantly worse prognosis and inhibition of this pathway significantly inhibited tumor growth and tumor cell proliferation [200]. Another pathway that is a major player in liver regeneration is Wnt/β-catenin signaling pathway. Conditional deletion of β-catenin gene, ctnnb1 in the liver led to a 24hr delay during liver regeneration. Interestingly, the Wnt/β-catenin pathway has been implicated in a subset of HCC where activating mutations in the β-catenin gene (CTNNB1) have been reported in around 30% of patients [85, 87]. This makes β-catenin a critical therapeutic target. Interestingly, our lab has reported increased tumorigenesis in liver specific conditional Ctnnb1 KO as a result of robust hepatocyte PDGFRα expression and PDGFRα-PI3K-Akt signaling. This strongly corroborates
PDGFRα as a relevant therapeutic target in HCC as has been shown [117, 130, 183]. It has been shown that PDGFRα signaling plays a critical role in HCC, but whether this receptor is critical for normal liver regeneration is not yet known. We show that loss of hepatocyte Pdgfra does not compromise normal liver regeneration after PHx due to EGFR and MET upregulation possibly driving increased hepatocyte proliferation. Interestingly, we failed to observe EGFR or MET increase when we knocked down PDGFRA in human hepatoma cell line. Our results clearly show that signaling is PDGFRα dispensable for normal liver regeneration but it’s blockade is detrimental for liver cancer. Also these results are indications that normal hepatocytes maybe more intelligent than abnormal hepatocytes. As mentioned earlier, proliferation of hepatocytes inside regenerating nodules that occupy cirrhotic liver are considered critical for maintenance of normal hepatic function. Our current findings also show that PDGFRα signaling, which has been previously shown to be a valid, biologically relevant, therapeutic target in HCC [117, 130, 183], may be dispensable for LR because of redundancy with other RTKs like EGFR and MET. Our results suggest that not only would inhibition of PDGFRα kill cancer cells in HCC, it will allow for normal hepatocytes to grow in the regenerative nodules by upregulating EGFR and MET levels to maintain hepatic function.

While our results show promise for targeting PDGFRα signaling in HCC, it will be relevant to conduct more experiments to show whether these results are specific to the liver or whether such paradigms exist in other organs or cancers. Perhaps in other cancers such as glioblastomas or colon cancer, where PDGFRα, EGFR, and MET signaling/cross-talks have been shown to be critical [201-204], PDGFRα blockade may lead to EGFR and MET upregulation to exacerbate tumorigenesis. In fact the significance of activation of alternate pathways is immense in oncology as tumors often become resistant to chemotherapy through
various mechanisms including activation of alternate pathways. Since PDGFRα inhibition is imminent for many forms of tumors, our study divulges EGFR and MET as possible ‘escape pathways’ in cells that may have to be concomitantly or sequentially targeted for a more effective response.


