The Role of RECK in the Pathogenesis of Nonalcoholic Steatohepatitis

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The Role of RECK in the Pathogenesis of Nonalcoholic Steatohepatitis

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DEDICATION

I would like to dedicate this dissertation to my wife, Maria, for her limitless kindness, patience, and love. Thank you, Maria, for everything, this would not have been possible without your support.

And to Chloe, the best dog ever, who passed away on July 1st, 2022.

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ABBREVIATIONS

α-SMA – α-smooth muscle actin

AAV – Adeno-associated virus

ADAM10 – A disintegrin and metalloproteinase domain-containing protein 10

ADAM17 – A disintegrin and metalloproteinase domain-containing protein 17 (also known as TACE)

ADAMTS10 – ADAM Metallopeptidase with Thrombospondin Type 1 Motif 10

Adv – Adenovirus

AFLD – Alcoholic fatty liver disease

AREG - Amphiregulin

AUC – Area under the curve

CD – Control diet

DAMPS – Damage-associated molecular patterns

DNL – *De novo* lipogenesis

ECM – Extracellular matrix

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

ERK – Extracellular signal-regulated kinase

FFA – Free fatty acid

FXR – Farsenoid Receptor X

GFP – Green fluorescent protein

GTT – Glucose tolerance test

H&E – Hematoxylin and eosin

HCC – Hepatocellular carcinoma

HPC – Hepatocyte progenitor cell

HSC – Hepatic stellate cell

IL – Interleukin

IR – Insulin resistance

KC – Kupffer cell

KO – Knock-out

LSEC – Liver sinusoidal endothelial cell

MDM – Myeloid-derived macrophage

MMP – Matrix metalloproteinases

NAFLD – Nonalcoholic fatty liver disease

NAS – NAFLD activity score

NASH – Nonalcoholic steatohepatitis

OCA – Obeticholic acid

PAMPs – Pathogen-associated molecular patterns

PRRs – Pattern recognition receptors

RECK – Reversion inducing cysteine rich protein with kazal motifs

ROS – Reactive oxygen species

TACE – TNFα converting enzyme

TGF-β – Transforming growth factor β

TLR – Toll-like receptor

TNFα – Tumor necrosis factor α

WD – Western diet

WT – Wild-type

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ABSTRACT

Reversion-inducing cysteine-rich protein with Kazal motifs, or RECK, is a cell surface-anchored glycoprotein and a critical regulator of the extracellular matrix (ECM). It has been examined extensively as an anti-metastatic peptide in the field of oncology, though its role in regulating inflammation and fibrogenesis has recently been postulated. As both inflammation and fibrosis are inherent in the pathology of nonalcoholic steatohepatitis (NASH), this dissertation explores the potential of RECK to serve as a hepatoprotective agent. NASH is a growing medical concern across the globe, for which there are currently no therapeutics available. Studies here show RECK is downregulated in the liver tissue of individuals with progressive NASH. Furthermore, utilizing transgenic rodent models, RECK is demonstrated to play a regulatory role in the development of NASH. Mechanistically, RECK may be exerting these effects through the ADAM10/ADAM17-AREG-EGFR signaling axis, as alterations in this pathway correlated with experimental manipulations of RECK. Furthermore, experiments here revealed hepatic stellate cells (HSCs) to be downstream targets in this process, as exposure to exogenous AREG induced a pro-fibrotic activation phenotype in these cells. This dissertation thus demonstrates the importance of hepatocellular RECK and attempts to provide a mechanistic explanation for this novel finding. Thus, RECK is identified as a potential therapeutic target which may be utilized in the treatment of NASH.

CHAPTER 1: Background and Aims

Nonalcoholic fatty liver disease (NAFLD) is a spectrum disorder of the liver spanning from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH), to the end-stage of liver fibrosis and cirrhosis (1). NAFLD is a highly prevalent condition with the incidence in the general population estimated to be approximately 10-30%, and as high as 80-100% in obese populations, in the United States (2, 3). Progression of the disease, from simple steatosis, to NASH, and, ultimately, cirrhosis depends on several factors, including changes in lipid metabolism, insulin resistance, inflammatory cytokines, and oxidative stress (4). The progression towards NASH correlates with an impaired liver function and places affected individuals at greater risk of cardiovascular, liver-related, and all-cause mortality (5). Furthermore, NAFLD progression to NASH is the most rapidly increasing indication for liver transplantation in the United States (6). Currently, there are no pharmaceutical therapies FDA-approved for the treatment of NASH. Therefore, understanding the mechanisms of NASH progression and identifying potential therapeutic targets is of vital importance. This work will explore molecular mechanisms of NASH progression as well as identify a novel target to potentially treat this debilitating disease.

Mechanisms of NAFLD Development

The pathophysiology of NAFLD progression is poorly understood, as the mechanisms of disease development are complex and multifactorial. Currently, the most widely accepted model for pathogenesis of NAFLD is the ‘multiple-hit’

hypothesis, stating that several insults work simultaneously to induce and progress disease (1, 7). These 'hits' may consist of dietary, environmental, and/or genetic factors. At the core, insulin resistance (IR) of various tissue types is the major contributor towards NAFLD pathogenesis; IR of the adipose tissue disrupts insulin-mediated suppression of lipolysis, causing free fatty acids (FFA) to be released into the systemic circulation and ultimately taken up by the liver for conversion into triglycerides. IR of the skeletal muscle reduces glucose uptake, and IR of the hepatocytes themselves disrupts insulin-mediated suppression of hepatic glucose output. This results in elevated serum insulin and glucose; potent triggers of *de novo* lipogenesis (DNL), converting excess circulating carbohydrates – from both IR-mediated processes as well as excess dietary intake – into lipids. As much as 25% of liver triglycerides can be attributed to DNL in NAFLD patients (8), with dietary intake of lipids contributing as well. As the synthesis of triglycerides within hepatocytes requires the production of lipotoxic intermediaries, these intermediaries can build up over time, leading to production of reactive oxygen species (ROS), hepatic injury and further IR, and ultimately triggering hepatocellular death. These apoptotic and necrotic processes release cytokines activating immune pathways, recruiting and activating bone-marrow derived leukocytes as well as innate repair pathways. Finally, gut dysbiosis has also been linked with NAFLD development and progression, with gut-derived endotoxins contributing towards hepatic inflammation (9-12).

In addition, hepatocyte mitochondrial dysfunction has been shown to play a critical role in development of NAFLD (13-15). Lipotoxic intermediaries have demonstrated the ability to trigger organelle damage, including of the mitochondria. Damaged or injured mitochondria have reduced β-oxidation functionality, reducing the ability to oxidize FFAs entering the liver, further contributing to liver triglyceride accumulation. Coupled with hepatic inflammation and inflammatory cytokine production, as well as generation of ROS, mitochondrial function rapidly deteriorates in NAFLD. This creates a cycle whereby additional fats accumulate in hepatocytes, more of which undergo cell death, recruiting and triggering more immune mechanisms. Taken together, these processes contribute towards the pathogenesis and progression of NAFLD and NASH.

Mechanisms of Inflammation in NASH Development

NASH, the progressive form of NAFLD, is characterized by the presence of lobular inflammation, as well as hepatocellular death and profibrogenic processes. Within the context of NASH, inflammation occurs in the absence of pathogens (termed 'sterile inflammation') and once begun, the inflammatory response remains a chronic condition (16). Damage- and Pathogen-Associated Molecular Patterns (DAMPs and PAMPs, respectively), can originate from a variety of regions; and indeed more than 20 DAMPs have been implicated in triggering sterile hepatic inflammation in the liver alone (17). PAMPs from the GI can spread to the liver via the portal tract, for example, through dysfunction of the

epithelial layer of the gut (16). In fact, alterations in the gut microbiome alone are sufficient to induce a NASH phenotype through dysregulation of the gut epithelium (18). Hepatocytes are known to express Pattern Recognition Receptors (PRRs) in addition to immune cells found within the liver (19). In response, hepatocytes can produce acute phase proteins, cytokines, chemokines, and – are the main source of – complement proteins, aiding in the triggering of the inflammatory response. Furthermore, metabolic processes of hepatocytes associated with early stages of NAFLD can prompt pro-inflammatory signaling and leukocyte recruitment, including lipotoxicity, generation of ROS, and the unfolded protein response (20). ROS, for example, is a well-known activator of macrophages towards an M1 (or pro-inflammatory) polarization, triggering and exacerbating inflammation (21).

Other cell types are also involved in triggering and promoting inflammation within the liver as well in the context of NAFLD/NASH. Hepatic macrophages are made up of predominantly two major populations; the resident Kupffer cell (KC) population (which are self-renewing) and the myeloid-derived macrophages (MDMs) (22). An imbalance of these populations (with MDMs dramatically outnumbering KCs in late NASH) are thought to be associated with disease progression (16). Furthermore, saturated fats, such as palmitic acid, are known to trigger MDMs towards an M1 phenotype, whereas KCs are better able to resist the responding transition (23). This in turn may play a role in the progression of the disease as KCs become outnumbered by MDMs, thus further driving a proinflammatory phenotype. Other lipids, such as FFAs, also push macrophages

towards an M1 polarization (24), which in turn triggers the production and release of several pro-inflammatory cytokines, such as IL1β, IL-6, CCL2, CCL6, and TNF-α (22). These peptides further the recruitment of myeloid-derived leukocytes, such as neutrophils, and the activation of KCs (20).

Dendritic cells also constitute a major part of the resident immune cell population within the liver, and serve as antigen presenting cells, recruiting other phagocytic cells to injury sites, such as can occur in regions of hepatocellular apoptosis and necrosis (19, 20). Dendritic cells mature in response to inflammatory triggers, and in turn activate natural killer T cells and promote T cell proliferation (19), exacerbating inflammation.

Mechanisms of Fibrogenesis in NASH Development

A key process in NASH development is fibrogenesis of the liver. Fibrogenesis is accomplished through activation of the hepatic stellate cells (HSCs); cells which, under normal conditions, are located within the Space of Disse between the sinusoidal endothelial cells and hepatic parenchyma. These cells in a healthy liver are considered quiescent, and are notable for their large lipid droplets containing Vitamin A. However, upon activation –triggered primarily through production and release of TGF-β by KCs and other macrophages - these cells will expel these Vitamin A lipid droplets and become the mobile and self-replicating myofibroblasts. Myofibroblasts then produce and deposit fibrin and collagen throughout the liver (25). In chronic liver diseases such as NASH, continuous activation and replication of these cells leads to an overt

accumulation of these ECM proteins, the definition of fibrosis. Ultimately, continuous activation of the deposition of ECM components and inflammatory pathways will lead to fibrosis and cirrhosis of the liver and may later lead to development of hepatocellular carcinoma. Indeed, inhibiting activation of HSCs has been shown to inhibit fibrosis and inflammation in models of NASH (26-28). Therefore, therapies aimed at preventing HSC activation and the overt accumulation of fibrosis and inflammation may provide novel avenues for NAFLD treatment.

The Extracellular Matrix: At the Crossroads of Inflammation and Fibrosis

The extracellular matrix (ECM) is a complex and dynamic component of multicellular organisms, regulating crucial cellular processes such as proliferation, differentiation, migration, adhesion, and tissue remodeling (29). As such, dysregulation of the ECM has been linked to several pathological conditions, including cancer development, fibrosis, and inflammation (29-31). Indeed, there is much evidence to support the idea that ECM dysfunction plays an active role in the pathogenesis and progression of NASH (32-34), and changes within the ECM are known to occur well before the process of fibrogenesis begins (35). Therefore, regulators of the ECM serve in critical roles in these conditions and have been explored as potential therapeutic targets in a variety of diseases.

RECK: A Well-Established ECM-Regulator

One such regulator of the ECM is Reversion Inducing Cysteine Rich Protein with Kazal Motifs (RECK), a cellular membrane-anchored glycoprotein (36). At the NH3-terminal, there are five cysteine repeats followed by two epidermal growth factor (EGF)-like repeats hypothesized to be required for proper interaction between RECK and its targets (37, 38). At the COOH terminus, there are three serine protease inhibitor (SPI)-like domains, that play a role in inhibiting target peptides through 'physical trapping' or 'reversible tight binding' (37). RECK itself is anchored to the cell membrane via a GPI-anchor located at the COOH-terminal (37).

RECK influences ECM remodeling primarily through regulation of the activity of several matrix metalloproteinases (MMPs) (39). This regulation of ECM components, combined with the observation that RECK is downregulated in cancers that metastasize, prompted many studies aimed at better understanding RECK's potential as a metastasis-suppressor (38, 40-42). However, it is currently unclear how RECK may influence inflammatory and fibrogenic pathways in the context of NAFLD and NASH. Clues to RECK's potential action may be gathered however through examination of its well-documented role in cancer.

RECK and Cancer

The ECM plays a critical role in the development and progression of cancer. Dysregulated ECM remodeling by tumor cells alters cell signaling, angiogenesis, and tissue biomechanics (43). These changes in the tumor microenvironment can allow for local tissue invasion as well as distal metastasis of cancer cells (43). Therefore, regulators of the ECM have drawn much interest in the oncological realm, and RECK, a key player in ECM remodeling, is no exception.

Reduced RECK is a characteristic feature of many cancers (44). In fact, interest in regulators of the gelatinases, MMP2 and MMP9, stems from their identification as prognostic indicators in a number of tumors, including liposarcoma (45), breast cancer (46), oral squamous cell carcinoma (47), and ovarian cancer (48). Tumors expressing higher concentrations of these gelatinases are in general linked to poorer prognosis and overall survival (29, 49, 50). Furthermore, RECK is known to complex with MT1-MMP, and inhibit its proteolytic activity at the cell membrane and internalization (51). Increased expression of MT1-MMP in tumors has also been linked to poorer prognosis and reduced overall survival, independent of other gelatinases (52). The underlying theory is that sustained activation of these gelatinases promotes excess ECM degradation allowing for local and distant tumor invasion, as well as furtherance of angiogenesis. For example, MMP9 is known to promote the release of vascular endothelial growth factor (VEGF), a pro-angiogenic mediator (37, 44, 53). Interestingly, RECK was first identified as a gelatinase inhibitor, reducing

ECM breakdown and preventing angiogenesis in several tumor types (54-57), including colorectal, gastric, and liver. Across all tumor categories, preserving RECK expression was shown to inhibit MMP2 and MMP9 activity, and improve prognosis by decreasing invasion and metastasis (38).

As alluded to above, RECK expression is either downregulated or undetected in various invasive cancers (37). By contrast, tumors that expressed normal or elevated RECK levels show reduced tissue invasion and metastasis (37). The mechanisms underlying RECK downregulation in cancer are hypothesized to be multifactorial and tumor specific; however, the general mechanism appears to involve increased Sp1 binding to the RECK promoter, resulting in its reduced transcription (38). It remains unclear, however, whether RECK downregulation occurs prior to, concurrently, or following tumor metastasis. Regardless, reduced RECK expression appears to worsen prognosis (40, 42, 58). RECK has also been shown to interact and inhibit other cellular pathways involved in cancer progression and metastasis, such as the Notch and EGFR/RAS signaling cascades. Both Notch (59) and EGFR/RAS (47, 48) are implicated in inflammation and fibrosis, which are explored further below.

RECK and Liver-Related Tumorigenesis

Within the context of the liver, RECK's role has been assessed in the pathogenesis of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). In line with other oncological studies, several groups have found that

overall prognosis and survival were significantly improved when tumors – either HCC or CCA – expressed relatively greater amounts of RECK (54, 58, 60, 61). What remains unanswered is how and when RECK expression is altered in these individuals – i.e., does RECK suppression precede tumorigenesis, or occurs during progression and metastasis?

Several mechanisms are implicated in RECK downregulation. For example, many single nucleotide polymorphisms (SNPs) are identified in the RECK gene within given populations (62-64). These SNPs appeared more frequently in patients diagnosed with HCC versus healthy controls (62, 65), with Chung *et al.* outlining specific SNPs in RECK that are relevant to liver cancer in humans (62). Their group identified two SNPs of interest in the development of HCC; individuals with the RECK promoter rs10814325 polymorphism saw increased risk of developing HCC compared to wild-type carriers, while HCC patients carrying the rs11788747 had higher risk of developing distant metastasis than wild-type carriers (62). This leads to the hypothesis that singular changes within the RECK protein structure itself or promoter polymorphisms could have a significant impact on its activity and ultimately on tumor progression. Hypermethylation of its promoter has also been shown to downregulate RECK expression (58), which also led to poorer prognosis in individuals with HCC (58).

Several micro-RNAs (miRs) are also shown to target RECK in HCC. For example, miR-135b, upregulated in HCC tissues, not only targets RECK post-transcriptionally (66), but also promotes HCC cell motility and invasiveness in vitro (67). Huang, et al. reported increased miR-21 and reduced RECK

expression in CCA patients with lymph node metastasis or perineural invasion (68). In that study, silencing miR-21 dramatically decreased CCA cell invasion and metastasis, which was rescued by the forced expression of RECK.

These experiments suggest a direct link between reduced RECK expression and invasion and metastasis of liver cancers. However, several questions remain unanswered. For example, what other mechanisms play a role in RECK regulation, and when RECK expression is altered, is this a dynamic process that can change over time or is activity static and only serves as a predisposing factor in these cases? It is also unclear whether RECK expression is altered prior to the formation of HCC in situations of NAFLD or cirrhosis. Of note, Furumoto, *et al.* found that approximately half of individuals with HCC recruited into the study already had reduced RECK expression. However, they did not delineate cases based on predisposing factors leading up to HCC, such as which patient had NASH prior to recruitment into the study (60).

There is currently no published literature determining whether RECK is already downregulated or silenced in NAFLD, and if this causes an exacerbation of symptoms, including development and progression towards NASH, cirrhosis, and HCC. Individual heterogeneity in RECK expression due to various genetic and environmental factors may govern the development of each, or even all these processes. Therefore, we further examined the literature to determine whether RECK was found to be involved in pathways and physiological processes leading up to and including progression of NASH and liver fibrosis.

RECK and Inflammation

RECK regulation of the ECM also modulates inflammation. For example, RECK/MMP-mediated ECM remodeling plays a role not only in tumor cell spread, but leukocyte infiltration into tissues as well. RECK-mediated inhibition of MMP2 and MMP9 expression and activity (44, 69, 70) has been shown to regulate inflammation in a variety of tissues and models. In models of experimental autoimmune encephalomyelitis (EAE), CD4+T cell invasion requires local MMP2 and MMP9-mediated parenchymal basement membrane breakdown (71, 72). MMP2 and MMP9 knock-out (KO) mice have reduced inflammatory cell influx into bronchoalveolar lavage fluid in experimental asthma models (73, 74). In models of acute pyelonephritis, it is known that there is a direct correlation between levels of MMP2 and MMP9 in the kidney and the severity of inflammation (75). Nascimento et al. found that MMP9 was involved in the early phases of temporomandibular joint inflammation in a rodent model, while MMP2 was involved in later phases of inflammation of the joint capsule. Additionally, they found that using doxycycline, a non-specific MMP inhibitor, diminished the inflammatory response (76). Furthermore, MMP9 is established as a mediator of inflammation within the intestinal muscularis in rodent models of post-operative ileus; inhibition of MMP9 activity reduced immune cell infiltration into intestinal muscularis, and MMP9-KO mice were protected from the inflammation and dysmotility associated with post-operative ileus (77). Finally, Ries, *et al.* found that inflammatory cytokines upregulate MMP2 and MMP9 in cultured human mesenchymal stem cells, which in turn allowed for chemotactic

migration through reconstituted basement membranes (78), suggesting a complex interplay between inflammatory cytokines, MMP activity, and immune cell infiltration through a basement membrane. Given such, RECK may be a central regulator in controlling leukocyte extravasation into other tissues as well, such as liver in NASH.

The chronic inflammatory state of obesity has been shown to closely associate with metabolic syndromes, such as NASH. In the context of obesity and inflammation, elevated MMP2 expression and MMP9 activity were found in a mouse model of obesity and positively correlated with inflammatory cytokine expression (79). Even more compelling is that MMP9 has already been shown to be involved in the active recruitment of CD11b+ leukocytes (80) and migration of neutrophils (81) in the post-ischemic liver. Lingwal, *et al.* examined swine islet cell transplantation into the liver of C57BL/6 mice via the portal vein and found that the transplantation drove an increase in MMP9 activity, which corresponded with massive inflammation in the liver (82). Using MMP9-KO mice, they found hepatic inflammatory infiltrates were significantly lower. More specifically, a positive correlation was observed between hepatic MMP9 expression and activity and CD11b+ leukocyte infiltration. Further, using pharmacological gelatinase inhibitors in vitro and in vivo, they reported a significant decrease in KC migration towards TNFα or IL1β expressing loci (82). These results suggest that the gelatinases, MMP2 and MMP9, are critical in the inflammatory processes of the liver, and, through inhibiting the activity of these matrixins, reductions in inflammatory infiltrates could be achieved. As the downregulation of RECK

clearly disrupts ECM integrity in the liver through dysregulation of MMP activity – as evidenced by the spread and invasiveness of HCC and CCA when RECK concentrations are lowered, as well as in the Lingwal, *et al.* study (82) – we could ask two critical questions that require further investigation: (i) would RECK downregulation lends itself to increased invasion of inflammatory cells into the liver in cases of NAFLD and NASH? and (ii) could restoring RECK reduces the amount of inflammation in these patients?

Beyond MMPs, RECK is also a known inhibitor of ADAM17 (A Disintegrin and Metalloproteinase Domain-containing protein 17) (83). Known also as TNFα-Converting Enzyme (TACE), ADAM17 plays a pivotal role in inflammation (84, 85). Of note, TNF-α expression has been shown to be upregulated in NASH (86), and plays a role in the development and progression of NAFLD (87). Therefore, regulating TNF-α release by targeting ADAM17 may be an effective strategy to blunt hepatic inflammation. However, identifying a pharmaceutical inhibitor of this enzyme has remained elusive. It is therefore plausible that sustaining or inducing RECK has the therapeutic potential to inhibit ADAM17 and overt inflammation in the liver as a result of metabolic dysregulation.

In addition to ADAM17, RECK has also been shown to inhibit ADAM10, both of which play a role in the activation of the pro-inflammatory Notch signaling cascade (59, 83). In fact, RECK has been shown to inhibit Notch signaling in neural tissues (59) and during angiogenesis (88). An increase in the Notch signaling pathway has been implicated in several proinflammatory conditions, such as rheumatoid arthritis (89) and uveitis (90). Increased Notch activity,

specifically Notch2, is known to regulate monocyte cell fate and inflammation in response to Toll Like Receptor (TLR) signaling (91). Both canonical and non-canonical Notch activity have been found to be increased in response to inflammatory mediators (92), thereby creating a positive feedback loop of Notch➔inflammation➔Notch signaling. In the realm of NAFLD, the number of hepatocytes expressing a major Notch outcome product – Hes Family BHLH Transcription Factor 1 (Hes1) – is significantly elevated in patients with severe NASH (93), suggesting overt activation of this pathway. Since RECK modulates the Notch pathway via direct regulation of ADAM17 and ADAM10, strategies that sustain or induce RECK expression have the therapeutic potential in NASH.

In addition to Notch signaling, both ADAM10 and ADAM17 are shown to be crucial in regulating the epidermal growth factor receptor (EGFR) signaling cascade. For example, RECK's inhibition of the ADAMs could prevent the release of membrane-anchored EGFR ligands, such as amphiregulin, and suppress EGFR activation. Indeed, RECK's ability to downregulate EGFR activity has already been reported (94, 95). This is of particular interest in the context of NASH, as EGFR has been implicated in hepatocyte and liver regeneration, and HCC development. EGFR signaling is also implicated in mitochondrial dysfunction, apoptosis of hepatocytes and hepatic stellate cells (HSCs), and liver necrosis (96-98). Pharmacological inhibition of EGFR has shown to reduce high-fat diet-induced liver injury in mouse models of NAFLD (99, 100), suggesting that targeting EGFR signaling may prove to have therapeutic

potential in human NASH. Sustaining or inducing RECK may be a viable strategy to modulate EGFR activity and thus inhibit NASH.

RECK and Fibrogenesis

Fibrosis results from an excess accumulation of ECM components. The downregulation of RECK has been linked to fibrosis in several tissues. In a mouse model of Western diet-induced obesity, RECK protein levels were found to be decreased in the kidney and correlated positively with renal fibrosis (101). We previously reported reduced RECK expression in the fibrotic heart. We also reported reduced RECK expression and increased angiotensin-II-induced fibroblast migration and proliferation, and their reversal by ectopic RECK overexpression (102-104).

As previously reported by our group, RECK regulates fibrosis in part by inhibiting activation of MMP2 and MMP9 (102-104). These gelatinases perform a much wider range of functions than the cleavage of ECM components and can be considered to have more of a ‘processing’ than ‘degradation’ role in maintaining the ECM (105). MMPs have been studied extensively in the context of hepatic fibrosis (106-109). During hepatic fibrogenesis, collagen deposition from HSCs is markedly increased, and paradoxically both MMP2 and MMP9 are highly upregulated in these cells (110). For example, MMP2 is an autocrine proliferator and activator of HSCs (111), promoting further ECM deposition. In an animal model of CCA where RECK was found to be decreased, increased MMP2

was associated with periductal fibrosis (54). Importantly, it was suggested that serum MMP2 levels could serve as a diagnostic marker to assess the level of liver fibrosis in patients with NASH (112). Furthermore, a positive correlation was reported between serum MMP2 concentrations and liver function as assessed via bilirubin and albumin production, and prothrombin time (113). While both gelatinases are upregulated in the context of fibrogenesis, paradoxically hepatic fibrosis was exacerbated in MMP2-KO mice (114). This suggests not only a complex relationship between gelatinase function and activity in the context of hepatic fibrosis, but also activation of compensatory mechanisms. Therefore, rather than ablating their expression, inhibiting MMP activity sequentially could blunt progression of fibrosis. As such, sustained RECK expression may have the therapeutic potential in NASH by targeting time-dependent or sequential activation of MMPs.

Notch signaling, and RECK's modulation of this pathway, may further serve to alter fibrogenesis. Activation of HSCs, classically, is mediated through TGF-β signaling (115), promoting Notch activity and fibrosis. Importantly, pharmaceutical Notch inhibitors prevent TGF-β-mediated HSC activation *in vitro* (116) and limit HSC activation and hepatic fibrosis in an animal model of fibrosis (117). In fibroblasts, Hes1 was shown to promote *Col1A1* and *Col1A2* transcription, and type I collagen deposition (118); however, it is unclear whether this holds true in HSCs as well. As has already been outlined above, RECK inhibits the Notch pathway by targeting ADAM10 and ADAM17 activity; whether this is sufficient to alter fibrosis in NASH patients is unknown.

EGFR signaling is also involved in tissue fibrosis. Its increased activity positively correlated with several pulmonary pathologies; individuals affected by the SARS outbreak of 2003 saw extensive lung fibrosis, which was suggested to be induced by a hyperactive host response to EGFR-mediated lung injury (119). More specifically, in a review by Stolarczyke and Scholte examining chronic obstructive pulmonary disease and cystic fibrosis, extensive evidence was found linking hyperactivity of the EGFR/ADAM17 signaling axis to ADAM17-cleavage of amphiregulin, an EGFR ligand (120). In a rodent model of lung injury resulting from chronic allergies, Morimoto, *et al.* found that amphiregulin/EGFR signaling activated eosinophils to an inflammatory state with enhanced production of osteopontin, an important profibrotic protein. Furthermore, they found that amphiregulin was produced by memory Th cells, further contributing to pulmonary fibrosis (121). Chronic kidney disease (CKD) is associated with fibrosis (122); EGFR is activated following renal injury, and studies have suggested its potential inhibition as a treatment for CKD (123).

In the context of NASH, it has been found that treatment of isolated Kupffer cells with CXCL6 increases EGFR phosphorylation and TGF-β induction (124). These results were confirmed by the same authors *in vivo* using a carbon tetrachloride (CCl_4) model of NASH (124). Increases in EGFR phosphorylation was observed in hepatocytes, activated HSCs, and macrophages in fibrotic livers in response to CCl_4. Furthermore, *EGFR* gene ablation (EGFR-KO) markedly reduced hepatic fibrosis and α-SMA expression in livers in response to CCl_4 (125). EGF and EGFR are also upregulated in humans with chronic liver injury.

However, in rodent models, it was shown that EGF was downregulated in liver fibrosis, but amphiregulin and EGFR were significantly increased (126). Overall, these reports indicate that overactivation of the EGFR signaling pathway may be linked to overt ADAM17 activity and NASH progression. Due to RECK's inhibition of ADAM17 and consequent downregulation in EGFR signaling, it is plausible that sustaining or inducing RECK has the potential to prevent or even reverse hepatic fibrosis seen in NASH. A more comprehensive analysis of potential signaling pathway is necessary to better understand the protective role of RECK in NAFLD, NASH and HCC.

RECK and the Development of NASH

While the previously mentioned studies do point to the potential for RECK to be involved in the processes of inflammation and fibrogenesis, there remains a substantial lack of evidence for the involvement of RECK in the pathogenesis and development of NASH. Therefore, this dissertation will focus on the role RECK plays in the development of NASH. We focused on RECK in the hepatocyte specifically, as it is the most abundant cell in the liver, comprising roughly 50-70% of the cellular population. We also examined downstream targets of RECK in the context of NASH to ascertain mechanisms related to its activity in hepatocytes and the liver at large.

Project Hypotheses

Using these strategies, we tested the hypothesis that RECK is protective against the development and progression of NASH. We hypothesized that with higher levels of hepatocyte RECK expression, there will be diminished hepatic inflammation and fibrosis; conversely, we expected hepatocellular RECK-depletion to exacerbate inflammation and fibrosis. Furthermore, we explored molecular mechanisms through which RECK may operate to ameliorate Western-diet-induced NASH. We hypothesized that tissue effects of RECK will rely specifically on alteration of the ADAM10/17-AREG-EGFR pathway, and involve crosstalk between various cell populations within the liver.

Innovation

The role of RECK in the pathogenesis of NAFLD, and specifically NASH, has not been fully examined. Preliminary studies from our lab (included in **Chapter 2**) suggested that hepatocyte-specific overexpression of RECK is protective against the development of inflammation and fibrosis associated with an animal model of diet-induced fatty liver disease, whereas hepatocyte-specific knock-down of RECK in the same animal model of diet-induced fatty liver disease tends to worsen inflammation and fibrosis in the liver. Thus, the innovative aspects of the proposed study are as follows:

- Given preliminary data demonstrating an exacerbated NAFLD phenotype in RECK-knock-down mice, this study investigated the role of hepatocellular RECK in NAFLD development using mice that had *RECK* completely abated in all hepatocytes.
- Given preliminary data demonstrating a mitigated NAFLD phenotype in RECK-overexpression mice, this study investigated the role of hepatocellular RECK in NAFLD development using mice that overexpress *RECK* in hepatocytes.
- Additionally, we performed experiments that uncover novel mechanisms involving RECK's involvement in EGFR signaling, and how this may influence NAFLD progression.

In short, this project used the novel $RECK^{Hep-/-}$ and RECK-Hep^{Tg} mouse models our group has developed and characterized to evaluate the role of RECK in the liver in NASH development. In doing so, we have attempted to determine through which mechanism RECK may be working to influence disease progression. These data will identify a potential therapeutic target that could further be capitalized upon to treat individuals with NASH.

Impact

The outcomes of the studies outlined here will lead to a better understanding of the role of RECK in the pathogenesis and development of NASH. Furthermore, these studies may lead to the use of RECK as a novel therapeutic target in the treatment of NASH, as there are currently no FDA-

approved pharmaceutical agents available. In addition, exploring the underlying mechanisms of RECK's involvement in fibrosis and inflammation in the liver will lead to a deeper understanding of the molecular drivers of NASH development, and may assist in guiding future experiments identifying potential NAFLD therapeutics.

CHAPTER 2: RECK as a Critical Regulator of NASH Development

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ABSTRACT

RECK, a membrane-anchored glycoprotein, is a critical regulator of the ECM. It has been studied for decades as an anti-metastatic gene, though its role in regulating inflammation and fibrogenesis has recently been postulated. As both inflammation and fibrosis are major components in the context of NAFLD and NASH, we hypothesized that RECK may serve to regulate these processes as a hepatoprotective agent. Thus, experiments here demonstrate the downregulation of RECK in the liver tissue of individuals with worsening NASH. Furthermore, utilizing models of hepatocellular RECK depletion and overexpression, manipulation of RECK negatively correlated with disease phenotypes in a diet-induced rodent model of NASH. *In vitro* studies examining potential RECK mechanisms have identified the ADAM10/ADAM17-AREG-EGFR signaling cascade to be an important process, as alterations in this pathway correlated with *in vivo* manipulations of RECK. Furthermore, hepatic stellate cells are shown to be a crucial downstream target of these pathways as *in vitro* AREG exposure induces transcription of pro-fibrotic activation markers. This study therefore finds that hepatocellular RECK plays a protective role against the development of fatty liver disease in mice by reducing histological evidence of inflammation and fibrosis, which worsens when RECK is reduced. This highlights RECK as a potential therapeutic target which may be utilized for the treatment of NAFLD.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum disorder, spanning from simple steatosis to nonalcoholic steatohepatitis (NASH) to liver cirrhosis and can ultimately develop into hepatocellular carcinoma. NAFLD is a growing problem affecting primarily obese individuals (though lean individuals too can become afflicted), and is quickly becoming the leading cause for liver transplantations in the Western world (127). Currently, there are no FDA-approved pharmaceutical agents available for treatment. While simple steatosis is considered relatively benign, clinical signs begin to present once individuals progress into NASH. NASH is characterized by the presence of inflammation in addition to excessive lipid accumulation, with fibrosis potentially present. Inflammation and fibrosis are inextricably linked as key components of the healing and regenerative process. However, in instances of chronic liver disease, they become overactive and dysregulated, leading to cirrhosis, cancer, and ultimately organ failure (128).

The membrane-anchored glycoprotein Reversion Inducing Cysteine Rich Protein with Kazal Motifs (RECK) has been shown to be involved in ECM remodeling. It has been extensively characterized in the realm of oncology, where RECK is a known metastasis-inhibitor, promoting patient survival rates in a variety of cancers by decreasing rates of tumor spread (60, 129, 130). More recently, the role of RECK has been explored in the context of fibrosis formation in the heart, having proven capable of a regulatory role in the ECM whereby it ultimately reduces the rate of cardiac fibrosis (102-104). In addition, other roles

of RECK within the ECM have been proposed by our group as being a potential regulator of inflammation and fibrosis within the context of NASH (131).

In this study, RECK was hypothesized to be decreased in the livers of human patients diagnosed with NAFLD as well as rodent models of obesity. Then, utilizing transgenic RECK manipulated mouse models, ablation of RECK in hepatocytes of mice fed a Western-style diet (WD) was hypothesized to cause exacerbated liver inflammation and fibrosis. Conversely, hepatocellular RECK overexpression in mice fed a WD would be protective against overt inflammation and fibrosis. Multiple *in vitro* experiments using primary mouse hepatocytes explored the potential of RECK to modulate cellular responses to inflammatory challenges.

We found RECK significantly decreased in the livers of human patients with worsening NAFLD and fibrosis. In addition, ablation of RECK within hepatocytes of mice worsened histological evidence of hepatic pathology. Furthermore, WD feeding in hepatocellular-RECK-overexpressing mice induced significantly decreased indicators of inflammation and fibrosis. Through *in vitro* experimentation, hepatocellular RECK is demonstrated to modulate activity of the sheddases, A Disintegrin And Metalloproteinase Domain-containing protein 10 and 17 (ADAM10 and ADAM17, respectively), subsequent cellular release of amphiregulin (AREG) and activation of Epidermal Growth Factor Receptor (EGFR) on local hepatic stellate cells (HSCs).

Taken together, these experiments demonstrate the hepatoprotective potential of RECK, and serve to highlight this peptide as a possible mediator of

NASH progression. Thus, RECK may be considered a novel therapeutic target in the treatment of NASH.

RESEARCH DESIGN AND METHODS

Human Liver Tissue

Liver samples were obtained from adults with clinical obesity undergoing elective bariatric surgery at the University of Missouri Hospital, Columbia, MO. Prior to inclusion, participants gave written informed consent to the protocol, which was approved by the institutional review board at the University of Missouri (protocol #2008258) and conducted in accordance with the World Medical Association's Declaration of Helsinki. Briefly, liver wedge biopsies were processed for H&E and trichrome staining, as well as for Western blot analysis. Degree of NAFLD severity was determined histologically by a blinded hepatopathologist; patients were clustered into three groups based on NAFLD Activity Score (NAS) – No Disease (NAS=0; *n*=7), Moderate (NAS=3-4; *n*=8), and Severe (NAS≥5; *n*=8). NAS inclusion criteria for patients with ANFLD were based on an alcohol intake of ≤20g/day and histologically confirmed steatosis with or without necroinflammation and/or fibrosis. Other causes of liver disease were excluded based on history, laboratory data, and histological features.

Animal Protocol

All animal protocols were approved by the University of Missouri and the Harry S Truman Memorial Veteran's Affairs Hospital Animal Care and Use Committees and conformed to the Guide for the Care and Use of Laboratory Animals. To test the effects of RECK activity in response to a WD, transgenic *RECK* mice were generated. Owing to *RECK* gene deletion being embryonically lethal, for the RECK-knock-out model, RECK-loxP targeted ($RECK^{fl/fl}$) mice were generated on a C57BL/6 background (original C57BL/6 strain acquired from Taconic Biosciences; Albany, NY, USA) in which exons 2 and 3 of the *RECK* gene are flanked by loxP sites. Deletion of this region leads to a frame shift and immediate premature translation stop codon. In addition, an overexpression mouse model was generated in which a CAG-CAT promotor sequence was inserted immediately upstream of the promotor region of the *RECK* gene (CAG-$RECK^{+}$), also on a C57BL/6 background (originally acquired from Taconic Biosciences). Conditional expression of this promoter was achieved through additional insertion of loxP sites flanking an inhibitory sequence, preventing use of the CAT-CAG promoter in the absence of Cre-recombinase. Excision of loxP sites in both cases of $RECK^{fl/fl}$ and CAG-$RECK^{+}$ mice was accomplished through one of two ways: 1) Mice were injected with hepatocyte-targeting AAV8-TBG-Cre ($1x10^{12}$ gc) via tail vein to alter *Reck* expression in hepatocytes and induce changes in *Reck* gene expression later in life; AAV8-TBG-GFP injected mice served as controls; or 2) mice were crossed with homozygous Albumin-Cre [B6.Cg-Tg(Alb-Cre)21Mgn/J] mice (no. 003574, Jackson Labs; Bar Harbor, ME,

USA) to generate RECK$^{Hep-/-}$ and RECK-Hep^{Tg} mice, with litter mates that did not inherit (and thus did not express) the Albumin-linked cre-recombinase allele being used as controls - designated RECK$^{fl/fl}$ and CAG-RECK^{+}, respectively.

For mice used in the AAV-arm of the study, male mice were injected with either the AAV8-TBG-Cre or AAV8-TBG-GFP at approximately 10 weeks of age. Following this, they received a high-sucrose WD (45% kcal fat, 17% kcal sucrose) with 1% cholesterol (D12110704; Research Diets; New Brunswick, NJ, USA) for 8 weeks. This resulted in a total of four groups (*n* = 7-10): AAV8-TBG-GFP-RECK$^{fl/fl}$, AAV8-TBG-Cre-RECK$^{fl/fl}$, AAV8-TBG-GFP-CAG-RECK^{+}, and AAV8-TBG-Cre-CAG-RECK^{+}. Following 8 weeks of WD-feeding, tissues were collected.

For animals used in the germline manipulated arm of the study, male mice that were crossed with Albumin-cre-expressing animals, starting at 10 weeks of age, received either a semi-purified control diet (CD; 10% kcal fat) (D12110704; Research Diets, New Brunswick, NJ, USA) or a WD identical to that described above for 24 weeks. In addition, animals receiving WD were also supplied with water containing 0.125 mol/L fructose and 0.105 mol/L glucose *ad libitum*. This resulted in a total of eight groups (*n* = 8-12): CD-RECK$^{fl/fl}$, WD-RECK$^{fl/fl}$, CD-RECK$^{Hep-/-}$, WD-RECK$^{Hep-/-}$, CD-CAG-RECK^{+}, WD-CAG-RECK^{+}, CD-RECK-Hep^{Tg}, and WD-RECK-Hep^{Tg}. Animal room temperatures were maintained between 20 and 21 degrees Celsius with a twelve-hour light/dark cycles. Food intake by cage and body weights were recorded weekly, and body composition was assessed every 4 weeks via NMR-MRI. Two weeks prior to the 24-week

endpoint, food was removed 12 hours preceding to a 2 µg/kg intraperitoneal injection of glucose as previously (132). Briefly, following the glucose injection, blood samples were collected at the 15-, 30-, 60-, 90-, and 120-minutes post-injection time-points from the tail vein, and blood glucose concentrations were measured to assess glucose-tolerance. Finally, following 24-weeks of diet-feeding, mice were anesthetized with pentobarbital sodium (80-100 mg/kg) and then exsanguinated following a 12-hour overnight fast. Livers were excised immediately, and portions were either flash frozen in liquid nitrogen for gene and protein analysis or placed in 10% formalin for liver histology. A diagram outlining *in vivo* studies is depicted in **Fig 2.1**.

Histological Analysis of Liver Tissue

Fresh liver was placed in 10% formalin for at least 24 hours before being imbedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E), trichrome, or picrosirius red (PSR) by IDEXX BioAnalytics (Columbia, MO, USA) for histological evaluation. NAS and fibrosis staging of liver sections were conducted by a trained and blinded observer. The NAS assessment consists of histologically examining the liver for steatosis, lobular inflammation, and hepatocyte ballooning utilizing H&E staining. Steatosis refers to the amount of hepatic surface area affected by excess lipid accumulation within cells on a low to medium power examination scored as a 0 (<5%), 1 (5-33%), 2 (33-66%), or 3 (>66%). Lobular inflammation refers to the number of white blood cell infiltrate foci present per 200x powered field, scored as a 0 (no foci), 1 (<2 foci), 2 (2-4

foci), or 3 (>4 foci). Hepatocyte ballooning refers to the presence of hepatocytes that have pathologically swollen with or without the presence of lipid vacuoles, indicating a trend towards apoptosis and necrosis of the cell, and is scored as 0 (no ballooned hepatocytes), 1 (rare but definite ballooned cells), or 2 (many/most cells are prominent with ballooning). The NAS is then indicated as a sum of these scores. Evaluated separately is the fibrosis score using PSR or trichrome staining; this score consisted of a grade of 0 (no fibrosis present), 1 (perisinusoidal or periportal fibrosis), 2 (perisinusoidal and periportal fibrosis), 3 (bridging fibrosis), or 4 (cirrhosis).

Primary Hepatocyte Isolation and Culture

A separate cohort of standard chow (Formulab 5008, Purina Mills, St Louis, MO, USA)-fed C57BL/6J mice (no. 000664, Jackson Laboratory) was used for primary hepatocyte studies. Primary hepatocytes were isolated from 10- to 15-week-old mice using a two-step collagenase method described previously (133, 134). The collagenase-cell suspension was filtered through a 100-micron filter and centrifuged first at 50 *g* for 6 minutes at 4°C. The resultant crude isolated hepatocyte pellets were resuspended in a 43.5% Percoll solution (11 ml Percoll, 11 ml growth medium [Williams E, no. 12551-032; Thermo Fisher Scientific; Waltham, MA, USA], 10% FBS, 4 mM L-glutamine, 100 U penicillin-100 mg streptomycin (no.15140122; Thermo Fisher Scientific), 2 ng/ml rat EGF, 100 nM insulin, 100 nM dexamethasone, 0.1% BSA, and 10 mM sodium pyruvate, and 3.3 mL PBS) and centrifuged at 40 *g* for 15 minutes at 4° C without braking.

Cellular preparations were assessed for viability and number using trypan blue and manual hemocytometry, and preparations with <90% viability were discarded. Isolated hepatocytes were then plated on type 1 collagen-coated plates for 2 days. Treatment with metabolic challenge conditions, pharmaceutical agents, and exogenous peptides occurred during the final 24 hours immediately prior to collection. Growth medium (Williams Medium E, 10% FBS, 4mM L-glutamine, 1% penicillin-streptomycin, insulin, transferrin, and selenium (ITS), 100nM dexamethasone, 10 mM sodium pyruvate, 0.1% BSA) was changed daily throughout culture.

Small Interfering RNA Transfection and Viral Infection of Primary Hepatocytes

To reduce expression of RECK *in vitro*, Silencer Select small interfering (si)RNAs were introduced via reverse transfection according to the manufacturer's instructions. Briefly, duplex siRNAs for RECK (siRECK) (no. s79243/s123244; Thermo Fisher Scientific) or a scrambled negative control (siSCR) (no. 4390843; Thermo Fischer Scientific) were complexed to lipofectamine RNAi Max (no. 13778, Thermo Fisher Scientific) in the appropriate volume of OptiMEM (no. 31985070, Thermo Fisher Scientific) for 15 minutes. Following pre-plating, cells were pelleted (50 *g* for 3 min), resuspended in growth medium without antibiotics, and added to wells containing the appropriate siRNA-lipofectamine complex in Opti-MEM. Alternatively, for *in vitro* RECK overexpression studies, primary hepatocytes were transfected with an adenovirus encoding an plasmid-

expressing RECK (Adv-RECK) (Vector Biolabs; Malvern, PA, USA) or control Adv-β-Gal (Adv-β-Gal) ($1x10^4$ to $1x10^8$ particles/cm^2) for 6 hours as previously reported (104).

Metabolic Challenge, Exogenous Peptide Exposure, and Pharmaceutical Agent Delivery

Following isolation and for 24 hours prior to cell collection, primary hepatocytes were metabolically challenged with exposure to TNFα (50 ng/ml; Millipore Sigma, St Louis, MO, USA). Concomitantly, cells were exposed to either dimethyl sulfoxide (DMSO) as a control, an ADAM10/17 inhibitor (GW280264X; 10 μM; no. AOB3632, Aobious Inc., Gloucester, MA, USA), a selective EGFR inhibitor (Erlotinib; 5nM; Millipore Sigma). Additional studies were completed in the presence or absence of exogenous AREG (100 ng/ml; Millipore Sigma).

Whole Liver and Primary Hepatocyte Processing for Western blot and qRT-PCR

Cells prepared for Western blot analysis were washed with ice-cold PBS and lysed with cell lysis buffer (1% Triton X-100, 100 mM NaCl, 20 mM Tris, 2 mM EDTA, 10 mM $MgCl_2$, 10 mM NaF, 40 mM β-glycerol phosphate, protease inhibitor, and phosphate inhibitors [Millipore Sigma]). Samples were sonicated and centrifuged at 15,000 *g* for 25 minutes, and the supernatant was evaluated for total protein content using Pierce BCA protein assay (no. 23225, Thermo

Fisher Scientific). Cells prepared for RNA extraction were washed with ice-cold PBS and lysed in buffer RLT (QIAGEN, Hilden, Germany) with 1% (vol/vol) β-mercapatoethanol and sonicated. RNA was isolated using the RNeasy mini kit (Qiagen) per the manufacturer's instructions. Whole liver samples were stored long-term at -80° C until all samples were collected to allow for batch processing. Samples were fractured using a mortar and pestle before being weighed, while kept frozen on liquid nitrogen or dry ice. For Western blot, 50 mg of liver tissue were weighed, and sonicated in cell lysis buffer (described above) before centrifugation at 15,000 *g* for 25 minutes, and the supernatant was evaluated for total protein content using BCA. For RNA extraction, 20 mg of whole liver tissue were sonicated in 350 µL buffer RLT. RNA was isolated using the RNeasy mini kit per the manufacturer's instructions.

Western Blots

Western blot analyses were completed using whole-liver homogenate and isolated hepatocyte lysate. Primary antibodies used are as follows: RECK (RECK[D8C7]; no. 3433, Cell Signaling Technology, Danvers, MA, USA), monocyte chemoattractant protein-1 (MCP-1; no. 2027, Cell Signaling Technology), platelet derived growth factor β (PDGF-β; no. 178409, Abcam, Cambridge, MA, USA), transforming growth factor β1 (TGF-β1[56E4]; no. 3709, Cell Signaling Technology), tumor necrosis factor α (TNFα; no. 3707, Cell Signaling Technology), ADAM10 (ADAM10[A-3]; no. 48400, Santa Cruz Biotechnology, Dallas, TX, USA), ADAM17 (TACE; no. 3500367, Millipore

Sigma), amphiregulin (AREG; no. 180722, Abcam), EGFR (EGFR[D38B1]; no. 4267, Cell Signaling Technology), phospho-EGFR (Tyr1068) (pEGFR(Y1068) [D7A5] XP; no. 3777, Cell Signaling Technology), and phospho-EGFR (Tyr1173) (pEGFR(Y1173)[53A5]; no. 4407, Cell Signaling Technology). Primary antibodies were used at 1:1,000 dilution, and secondary antibodies at 1:5,000 dilution. Blots were analyzed via densometric analysis (Image Lab v5.1, Bio-Rad Laboratories Inc., Hercules, CA). Total protein was assessed with Amdio black (0.1%; Millipore Sigma) to control for differences in protein loading and transfer, as previously described (135). Western blots were normalized to total protein content.

Quantitative Real-Time PCR

Once RNA was extracted from frozen liver tissue and primary hepatocytes using a commercially available kit (no. 74104; QIAGEN), a cDNA library was synthesized (Promega, Madison, WI, USA). Purity and quality of RNA and cDNA were assessed via Nanodrop spectrometer (model ND-1000, Nanodrop, Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using SYBER Green reagents (172-5121; Bio-Rad Laboratories, Inc.) and primer pairs listed in **Table 2.1** (Millipore Sigma), or TaqMan (Thermo Fisher Scientific) for RECK (Mm00443824_m1) and cyclophilin B (Mm00478295_m1). PCR product melt curves were assessed to determine primer specificity. Data are represented relative to either glyceraldehye-3-

phosphate dehydrogenase (GAPDH) or cycoliphillin B (PPIB) using the $2^{-\Delta\Delta CT}$ method.

Sheddase Activity Measurement

Cell suspensions from cultured primary hepatocyte *in vitro* experiments were used to assess ADAM10/ADAM17 sheddase activity. Briefly, separate kits assessing ADAM10 and ADAM17 were obtained (no. AS-72226 and AS-72225, Anaspec, Fremont, CA, USA). Cold assay buffer was added to freshly harvested cell suspensions, incubated for 10 min, and centrifuged for 5 min at 10,000 *g* at 4°C; supernatant was collected and stored at -80° until use. Samples were processed according to manufacturer's instructions; final fluorescence intensity was measured at Ex/EM=490nm/520nm and plotted against a reference standard to determine relative activity.

Amphiregulin Secretion Assessment

Culture media from primary hepatocyte *in vitro* experiments was used to assess release of AREG from cells. A sandwich ELISA kit was utilized to obtain measurements (no. ab224882, Abcam). Briefly, cell culture supernatant following experiments was harvested and stored at -80° until use. Samples were thawed, spun for 10 minutes at 2,000 *g* at room temperature to remove debris. Samples were processed according to manufacturer's instructions; optical

density was measured at 450 nm and plotted against a reference standard to determine released AREG content.

Statistical Analysis

Statistical analyses were completed in Prism GraphPad 9.0.2 with an alpha level of $p < 0.05$ used to determine statistical significance of all comparisons. Studies were analyzed either using a one-way or two-way ANOVA where appropriate, with a Fisher least significant difference *post hoc* test used, when a significant interaction term ($p < 0.05$) was detected. For *in vitro* studies, data were analyzed with either two-way or one-way ANOVA with or without repeated measures or paired *t* test as appropriate. A biological replicate for *in vitro* was defined as the cells isolated from a single mouse, and each condition was tested in technical triplicate and averaged. Data from such experiments, and *n* size listed in the figure legends are representative of independent biological replicates. Data were graphed in the same program and are presented as mean ± standard error (SEM).

RESULTS

RECK is downregulated in livers of human patients diagnosed with NASH and rodents fed an obesogenic diet

To determine whether the condition of NASH in humans is associated with changes in RECK expression, liver samples obtained through biopsy of human patients undergoing bariatric surgery were analyzed. When adjusted on the histological basis of NAS, a significant decrease in RECK content was observed. Similar findings were observed when livers were assessed instead based on histological fibrosis score (**Fig 2.2 A and B**). RECK expression, as well as MMP2, MMP13, and TIMP1 (downstream targets of RECK activity), was also measured in mice fed a WD for 16 weeks. WD feeding significantly reduced RECK content and targets of RECK inhibition were significantly increased (**Fig 2.2 C**) indicating a reduction in RECK expression and its corresponding activity.

Confirmation of RECK Manipulation in Animal Models

Further exploration of RECK's effects on NASH development utilized newly generated transgenic RECK mouse models. To confirm transgenic alteration of hepatocellular-specific *Reck*-manipulation, presence of the floxed *Reck* gene was observed in both transgenic $RECK^{fl/fl}$ and $RECK^{hep-/-}$ mice, along with the presence of an albumin-cre driver in $RECK^{hep-/-}$ mice (**Fig 2.3 A**). $RECK^{Hetero+/-}$ mice have only one floxed *Reck* allele and were not used in

experiments. Whole liver analysis from these animals demonstrated a significant reduction of RECK mRNA in RECK$^{hep-/-}$ mice, but not in protein (p=0.35), when compared to control RECK$^{fl/fl}$ mice (**Fig 2.3 B, D and F**). This was possibly due to differential expression of *Reck* in various cell populations of the liver. When comparing hepatic cell populations isolated from WT C57BL/6J male mice, relative *Reck* expression is greatest in HSCs, followed by non-parenchymal cells (including KCs), and finally hepatocytes, which express the least relative amount of RECK (**Fig 2.4**). Therefore, changes in *Reck* expression in hepatocytes may be masked when assessing whole liver RECK protein due to the abundance of RECK from other cellular sources. Nevertheless, gene and protein expression from isolated hepatocytes showed a significant reduction of RECK protein and mRNA in RECK$^{hep-/-}$ versus RECK$^{fl/fl}$ mice on a CD (**Fig 2.3 C, E and F**).

Confirmation of genetic manipulation and altered *Reck* expression was also accomplished in CAG-RECK^{+} mice. Verification of insertion of the CAG-CAT sequence upstream of the *Reck* gene promoter is demonstrated in both CAG-RECK^{+} and RECK-Hep^{Tg} animals, along with the presence of the albumin-cre driver in the RECK-Hep^{Tg} mice (**Fig 2.3 G**). All CAG-RECK^{+} animals have a single CAG-CAT construct inserted as overexpression of RECK can be achieved by targeting a single *Reck* allele. Though whole liver protein and mRNA assessment did not reveal significantly different expression between CAG-RECK^{+} and RECK-Hep^{+} animals (p=0.59 and 0.75 respectively), isolated hepatocytes from these animals demonstrated a significant increase in RECK protein and mRNA expression (**Fig 2.3 H through L**). Again, a lack of response

in the whole liver is likely due to masking effects by non-hepatocyte *Reck* expression and is therefore not likely a consequence of failed transgenic hepatocyte expression, as isolated hepatocytes demonstrated significant changes in relative *Reck* expression.

Animal Characteristics

No differences were observed for body weight, body fat percentage (%), liver or epididymal fat mass, or heart:body mass ratio (mg/g), though there were clear effects of WD feeding on all these characteristics in $RECK^{fl/fl}$ and $RECK^{Hep-/-}$ mice (**Table 2.2**). Interestingly, $RECK^{Hep-/-}$ displayed a significantly lower serum glucose AUC in response to a GTT compared to $RECK^{fl/fl}$, with main effect of genotype lost when WD-fed animals are added (**Fig 2.5 A and B**). WD feeding significantly increased serum glucose during the GTT (specifically at the 30-, 60-, 90-, and 120-minute time points) and the AUC compared to CD-fed animals.

In CAG-$RECK^{+}$ and RECK-Hep^{+} animals, following 24-weeks of WD-feeding, RECK overexpression significantly increased body mass and body fat percentage as compared to WD-fed control animals, which cannot be accounted for with feed intake alone (**Table 2.3**). WD-fed animals, regardless of genotype, displayed significantly elevated liver and epididymal fat pad mass. WD-feeding elevated serum glucose and the AUC during the GTT (specifically at the initial time point, and 60- and 90-minute time points) but no effect was observed arising

from genotype (**Fig 2.5 C and D**). Additional animal morphological data, including female animal data, are included the **Supplemental Data** chapter.

Decreased hepatocellular RECK exacerbated liver disease in WD-fed mice

Following 24 weeks of diet feeding, hepatocellular RECK depletion significantly exacerbated histological evidence of hepatocyte ballooning and inflammation (**Fig 2.6 A and B**). This was reflected in overall NAS score, as well as fibrosis score (**Fig 2.6 C through E**), though these effects were driven primarily by changes in CD-fed animals. This may be due to a maximal effect in WD-fed animals (i.e. histological evidence of pathology is not capable of extending beyond what is already observed here) and therefore, hepatocellular RECK depletion would be unable to further exacerbate histological evidence of dysfunction. Thus, significant changes may be observed primarily in CD-fed mice. Effects of hepatocellular RECK depletion in female mice fed 24 weeks of diet feeding are including the **Supplemental Data** chapter.

As a secondary approach to establish the importance of RECK expression in hepatocytes in adult mice, a separate group of $RECK^{fl/fl}$ adult male mice were injected with either an AAV8-TBG-Cre (AAV-Cre) or an AAV8-TBG-GFP (AAV-GFP) at 10 weeks of age via tail vein to knockdown hepatocyte RECK (**Fig 2.7 A**). Following 8 weeks of WD feeding, RECK knockdown significantly increased histological evidence of inflammation and fibrosis compared to AAV-GFP control mice (**Fig 2.7 B through D**). In addition, RECK knockdown significantly

increased expression of inflammatory markers TNFα and PDGFβ, markers of HSC activation αSMA and Col1A1, and RECK targets MMP2, MMP9, and TIMP1. In addition, whole liver vimentin mRNA trended up (p=0.06), while RECK knockdown had no effect on TGF-β1 expression (p=0.41) (**Fig 2.7 E**).

Increased hepatocellular RECK reduced liver damage in WD-fed mice

WD induced increases in inflammation and fibrosis were significantly attenuated with hepatocellular RECK overexpression in RECK-Hep^{Tg} mice compared to CAG-RECK^{+} controls (**Fig 2.8 A and B**). Histological scoring confirmed increased hepatic steatosis, hepatocellular ballooning, inflammation, and overall NAS score due to WD-feeding, as well as fibrosis score. Histological evidence of inflammation and fibrosis were significantly attenuated with RECK overexpression (**Fig 2.8 C through E**), while overall NAS trended down (p=0.07). Effects of hepatocellular RECK overexpression in female mice fed 24 weeks of diet feeding are including the **Supplemental Data** chapter.

To further validate the protective role of hepatocyte-specific RECK overexpression, male CAG-RECK^{+} mice were injected with AAV-Cre or AAV-GFP at 10 weeks of age via tail vein and fed a WD for 8 weeks. AAV-Cre driven RECK overexpression (**Fig 2.9 A**) significantly reduced hepatocellular ballooning, hepatic inflammation, and a correspondingly decreased NAS (**Fig 2.9 B through D**). Fibrosis was slightly down but failed to achieve significance (p=0.24), though this may be due to an 8-week WD trial being insufficient to induce an insult

potent enough to trigger extensive fibrosis. Nevertheless, RECK overexpression significantly decreased TNFα, TGFβ1, the HSC activation marker αSMA, and the RECK targets MMP2 and MMP9, while PDGFβ (p=0.47), vimentin (p=0.44), col1a1 (p=0.46), and TIMP1 (p=0.67) failed to demonstrate any changes (**Fig 2.9 E**).

Manipulated hepatocellular RECK altered whole liver expression of inflammatory, ECM, HSC activation components

Whole tissue analysis was completed in livers collected from transgenic-RECK mice fed a WD or CD for 24 weeks. In $RECK^{fl/fl}$ and $RECK^{hep-/-}$ mice, WD significantly upregulated expression of several inflammatory markers and ECM components and regulatory cytokines – specifically IL33, iNOS, TNFα, Arg1, IL1Ra, Col1a1, MMP9, PDGFβ, and TGFβ1. Hepatocellular-RECK-knockout did not significantly exacerbate WD induced elevation of inflammatory markers, but did significantly upregulate the markers of HSC activity, MCAM and αSMA (**Fig 2.10 A through D**). However, in the context of MCAM mRNA expression, this effect of genotype on HSC activity was primarily driven by an increase in CD-fed $RECK^{hep-/-}$ mice, while both CD- and WD-fed $RECK^{hep-/-}$ animals experienced significant increases in αSMA.

Conversely, in CAG-$RECK^{+}$ and RECK-Hep^{Tg} animals, RECK overexpression significantly attenuated WD-induced increases of several inflammatory, ECM components, and HSC activity markers compared to control

(**Fig 2.11 A through D**) – specifically IL33, TNFα, IL1Ra, Col1a1, PDGFβ, TGFβ1, and αSMA. The main effect of genotype was primarily driven by changes in WD-fed animals.

Manipulated hepatocellular RECK altered ADAM sheddase activity, amphiregulin release, and EGFR activity

To examine effects of RECK depletion in hepatocellular physiology, an *in vitro* siRNA system targeting RECK mRNA (siRECK) in primary murine hepatocytes was compared with cells transfected with a scrambled mRNA sequence (siSCR) control (**Fig 2.12 A and B**). RECK depletion induced a modest increase in ADAM10 protein (p=0.15), but no change in ADAM17 protein. ADAM10 and ADAM17 mRNA and activity also failed to respond to RECK knockdown (**Fig 2.12 C through E**). AREG expression and secretion into culture media was measured as well. RECK-knockdown significantly upregulated secreted AREG and mRNA content, with AREG protein content trending up (p=0.07) as well. This effect was reversed when cells were exposed to a dual-ADAM10/ADAM17 inhibitor (GW280264X or referred here as 'ADAM10/17inhib') in conjunction with RECK-knockdown (**Fig 2.12 F through H**), suggesting effects on AREG were mediated, at least in part, through these sheddases.

To further explore RECK's function in primary hepatocytes, RECK overexpression in primary hepatocytes was accomplished via transfection using an adenovirus encoding a plasmid that either overexpressed RECK (Adv-RECK)

or a control protein (Adv-β-Gal) (**Fig 2.13 A and B**). RECK overexpression significantly reduced ADAM10 and ADAM17 activity, though neither mRNA nor protein was altered (**Fig 2.13 C through E**). Overexpression of RECK also significantly decreased AREG mRNA expression with a corresponding downward trend in cellular protein (p=0.13); however, RECK overexpression did not alter AREG secretion into media, while addition of the ADAM10/17 inhibitor significantly lowered it (**Fig 2.13 F through H**).

As AREG is a ligand for the EGFR receptor, EGFR expression and phosphorylation in RECK-manipulated hepatocytes was then examined. RECK knock-down significantly increased EGFR mRNA and protein expression, as well as phosphorylation at the Tyr1068 site (but not at the Tyr1173 site; p-value of 0.89) (**Fig 2.14 A and B**). RECK overexpression significantly decreased EGFR mRNA and protein content, while phosphorylation at the Tyr1068 site trended down (p=0.10) and phosphorylation at the Tyr1173 site trended up (p= 0.12) (**Fig 2.14 D and E**). To explore what effects EGFR expression and activity had on immunological signaling in hepatocytes, inflammatory cytokine expression of IL1β, IL1RA, and IL33 was also measured. RECK overexpression significantly increased IL1β mRNA; others analyzed showed no significantly different expression (**Fig 2.14 C and F**).

To determine if the effects of RECK-overexpression were mediated by AREG signaling, exogenous AREG was added back into the media of primary hepatocytes. As shown in **Fig 2.14**, RECK overexpression was sufficient to significantly decrease EGFR expression and induce a modest decrease in

phosphorylation at the Tyr1068 site (p=0.10). The addition of exogenous AREG failed to alter EGFR expression or phosphorylation, nor change expression of inflammatory cytokines measured, beyond the baseline response to RECK overexpression (**Fig 2.15 A and B**). This suggests exogenous AREG is insufficient to alter hepatocyte EGFR signaling, but in the presence of RECK overexpression, exogenous AREG prevents decreases in EGFR expression and phosphorylation.

Finally, siRNA-treated cells were exposed to exogenous AREG and EGFR inhibition simultaneously. EGFR inhibition significantly downregulated phosphorylation of the EGFR site Tyr1068 (**Fig 2.15 C**), and significantly reduced IL1Ra (**Fig 2.15 D**) despite the presence of exogenous AREG and regardless of RECK expression. No other changes in the cell expression of other inflammatory cytokines were observed, however. This may be due to a maximal effect of the EGFR inhibitor, preventing EGFR signaling to be rescued even in the presence of exogenous amphiregulin. Furthermore, as exposing hepatocytes to AREG appeared to have minimal effects, it is unknown if AREG itself can change the expression of various EGFR and its downstream targets within a pure hepatocyte population, or if whole tissue effects are mediated through other cell populations.

Altered AREG-EGFR signaling in human bariatric surgery patients, WD-fed mice

To determine whether the AREG-EGFR pathway was altered in previously analyzed human bariatric surgery samples and diet-fed rodent liver samples, and support the theory that changes in this pathway correlate with changes in RECK, components of the signaling cascade were measured. In the liver of individuals undergoing bariatric surgery, worsening histological evidence of NASH correlated with significant increases in AREG and pEGFR(Tyr1173) protein content, with expression of both greatest in the most severely affected livers (**Fig 2.16 A**). This was mirrored in the livers of mice in which WD induced a significant increase in hepatic AREG content (**Fig 2.16 B**).

In examination of liver samples taken from the viral-mediated-RECK-manipulation experiment, hepatocellular RECK-knockdown significantly increased hepatic AREG protein content and trended an increase in the phosphorylation of EGFR at the Tyr1173 site (p=0.07) (**Fig 2.16 C**). Forced overexpression of hepatocellular RECK significantly decreased these same targets (**Fig 2.16 D**).

In germline manipulated mice, RECK-knockout significantly elevated EGFR protein but did not alter expression or content of other components (including ADAM10, ADAM17, and AREG, nor phosphorylation rates of EGFR at the Tyr1068 and Tyr 1173 sites) (**Fig 2.17 A and B**). Likewise, in germline hepatocyte specific RECK-overexpression animals, AREG mRNA expression was significantly decreased; however, ADAM10, ADAM17, and EGFR, and

phosphorylation rates of EGFR at the Tyr1068 and Tyr1173 sites, were not changed in response to RECK manipulation (**Fig 2.17 C and D**). Interestingly, WD-feeding significantly increased ADAM17 protein content compared to CD-fed animals (p=0.03), which only trended up when RECK was overexpressed (p=0.14). As these animals have had hepatocellular RECK altered since conception, it may be that compensatory mechanisms are differentially influencing components, and/or masking overt effects of RECK on these pathways.

Exposure of exogenous AREG to HSCs induced activation

Because of the lack of robust changes observed in the ADAM10/17-AREG-EGFR pathway in RECK manipulated cells, further exploration of alternative explanations was necessary to determine why RECK-manipulation had an impact on liver histology in WD-fed mice. Other studies have found that RECK inhibits the sheddases ADAM10 and ADAM17 (83), which in turn are necessary for the release of AREG (136) allowing for juxtracrine signaling. Therefore, we hypothesized that HSCs, being proximal to hepatocytes, may be targets of released hepatocellular AREG, and thus mediated whole tissue effects from hepatocyte RECK manipulation. Furthermore, previous literature has already demonstrated that AREG serves to induce HSC activation (137), an important step in hepatic fibrogenic processes. Thus, isolated primary HSCs obtained from adult WT-C57BL/6J mice were exposed to TGF-β, mimicking the primary cytokine signal these cells would receive in a typical NASH setting to

establish a baseline. Exposure to exogenous AREG induced HSC activation markers Col1a1 and αSMA, which was attenuated with an EGFR inhibitor (Erlotinib), demonstrating extracellular AREG is sufficient to induce HSC activation through EGFR (**Fig 2.18**).

DISCUSSION

RECK is well-established as a regulator of the ECM (38, 42, 44, 50), primarily through inhibition of MMPs and sheddases demonstrated in oncological studies (55, 61, 64, 88, 138, 139). Based on these data, we proposed RECK modulates the processes of hepatic inflammation and fibrosis - critical steps in the development of late-stage NAFLD. As demonstrated here, RECK is downregulated in human patients with advanced NAFLD as well as in obesogenic animal models. Altered hepatocellular expression of RECK changed the progression of NAFLD in rodent models, in that RECK-reduction worsened signs of hepatic pathology while RECK-overexpression mitigated it. Furthermore, manipulation of RECK correlated with changes in the expression and activity of the ADAM10/ADAM17-AREG-EGFR signaling cascade involving hepatocellular and HSC crosstalk and has led to the proposed mechanism outlined in **Fig 2.19**. These novel data demonstrate the potential for RECK to serve as a therapeutic target in the treatment of NAFLD.

We have previously proposed the potential for RECK to mitigate the process of inflammation (131). Through manipulation of various ECM components, RECK may limit the infiltrative ability of inflammatory cells. For example, RECK-mediated inhibition of the gelatinases, MMP2 and MMP9, has already been shown to modulate inflammation in experimental models of autoimmune encephalomyelitis (71, 72) and asthma (73, 74) via invasion restriction of immune cells. Furthermore, RECK can alter release of

proinflammatory TNFα from the cell surface and signaling capabilities via inhibitory effects on ADAM17 (83, 84). Additionally, through modulation of the related ADAM10, RECK may limit activity of the proinflammatory Notch signaling cascade (59). In the present study, effects of RECK on ADAM10/17 and downstream AREG-EGFR signaling were examined. This is an area of particular interest, as RECK has already demonstrated an ability to inhibit EGFR activity (94, 95), and EGFR inhibitors have proven efficacious in decreasing disease severity of NAFLD models (99, 100, 140). Furthermore, AREG itself induces activation of human HSCs (137), a finding supported by rodent models in the present study. Thus, RECK may be able to ameliorate inflammation through inhibition of sheddase of activity of the ADAMs, decreased release of membrane-bound AREG, and diminished EGFR activity. RECK depletion through transgenic germline manipulated and virally induced means exacerbated hepatic inflammation in rodent models of NAFLD. Furthermore, RECK overexpression, again in both transgenic germline manipulated, and virally induced animal models, diminished histologic evidence of inflammation as well as cellular markers of inflammation. However, *in vitro* RECK manipulation of primary hepatocytes here failed to significantly alter inflammatory cytokine production (a schematic outlining proposed mechanisms and relevant hypotheses demonstrated in **Fig 2.20**). This may be due to the relatively low levels of inflammatory cytokine production by hepatocytes, and thus concentrations of these peptides are so negligible in a primary hepatocyte population that altering RECK is insufficient to significantly impact production. Furthermore, as

demonstrated in experiments examining effects of AREG on isolated HSCs, it may be released AREG from hepatocytes is influencing inflammation through its action on nearby Kupffer cells and other leukocytes. Therefore, further studies are needed examining what effects juxtracrine AREG signaling has on Kupffer cell and leukocyte activation in the liver. Nevertheless, the data presented here support the hypothesis of RECK ameliorating inflammation associated with NAFLD, potentially through modulation of cellular crosstalk pathways.

Downregulation of RECK has been linked to fibrosis in previous studies by other groups. For example, decreased RECK levels correlated with fibrosis within the kidney (101); we have previously reported reduced RECK expression and increased fibroblast migration and proliferation within the heart (102-104). Here, we demonstrate the connection between RECK expression and fibrosis within the liver. In germline RECK-depleted animals, hepatocyte RECK-knockout significantly increased histological evidence of fibrosis, though this effect was, interestingly, driven primarily by animals fed a CD. This may be due to a maximal capacity effect within the liver, whereby animals fed a WD for 24 weeks have such extensive fibrosis that reducing RECK is unable to worsen the phenotype beyond this. Viral-induced RECK depletion however, significantly increased fibrosis in animals fed a WD for 8 weeks – a much milder fibrotic-inducing insult, which may have allowed for room in worsening of clinical signs. Conversely, RECK overexpression significantly reduced histological evidence of fibrosis in mice fed a WD. In addition, these changes correlated with activation markers in HSCs (specifically MCAM and αSMA). This is of particular interest

concerning our proposed RECK-ADAM10/17-AREG-EGFR pathway, as AREG has demonstrated the ability to induce HSC activation markers *in vitro* here, as well as in previously mentioned studies (137) elsewhere. Therefore, hepatocellular RECK manipulation appears to have consequences on HSC activation, a critical step in the profibrotic processes associated with NAFLD (4, 141). This also aligns with previous literature examining the role of EGFR activation in promoting fibrosis in other tissues as well; in a literature review examining chronic obstructive pulmonary disease and cystic fibrosis, extensive evidence was found linking the hyperactivity of the ADAM17 cleavage of AREG to EGFR activation and subsequent fibrosis (120). In the context of NASH, it has already been shown that *Egfr* gene ablation in rodents significantly reduced hepatic fibrosis and αSMA expression in livers in response to a CCl_4 challenge. Therefore, data presented here fit in the context of previous literature examining the proposed ADAM10/17-AREG-EGFR signaling cascade as being pro-fibrotic.

A limitation of this study is the restriction of RECK manipulation in single cell-type in the liver. As has been alluded to, it is the crosstalk between different hepatic cell populations that coordinates inflammation and fibrosis in the context of NASH. By targeting RECK in the hepatocytes, our goal was to influence the phenotype of the most abundant cell type within the liver, however, as results suggest, it is the signaling peptides from hepatocytes that have significantly altered (ie secreted AREG, as demonstrated here). This in turn appears to have consequences on the physiology of neighboring cell populations, for example, activation of HSCs. However, what remains unexplored here is effects of RECK

manipulation within other cell populations – such as HSCs and KCs. As previously mentioned, relative RECK expression in HSCs is significantly greater than in hepatocytes, and trends upward (p=0.12) in non-parenchymal cells which includes KCs and liver sinusoidal endothelial cells (**Fig 2.4**). Thus, consequences of RECK manipulation there may have an even greater effect on liver physiology in NASH. Nevertheless, it is noteworthy that RECK manipulation in hepatocytes alone had significant effects on inflammation and fibrosis in WD-induced mouse models of NASH as shown here.

Collectively, this study demonstrates the importance of RECK in regulating inflammation and fibrosis within the NAFLD-afflicted liver. RECK is decreased in the livers of patients with moderate to severe NASH, as well as in animal models of the disease. Transgene overexpression of hepatocellular RECK minimized the disease phenotype in rodent models, while hepatocellular RECK depletion increased inflammation and fibrosis. Utilizing primary hepatocytes *in vitro*, hepatocyte RECK demonstrated the ability to alter ADAM10 and ADAM17 activity and thus, cellular AREG liberation. When examining inflammatory cytokine production from these cell populations, there were minimal effects observed on the phenotype of hepatocytes themselves. However, exogenous AREG induced the transcription of activation markers in primary HSCs, in agreement with previous literature. Furthermore, inhibiting downstream signaling of AREG and EGFR decreased the expression of activation markers in HSCs. These data highlight the potential of RECK as a novel target in the treatment of NAFLD.

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Disclosures

No conflict of interest, financial or otherwise, are declared by the authors.

Author Contributions

RJD, BC, RSR were involved in study concept and design; CB provided the transgenic RECK mouse models; RJD, RPC, GMM, CT, IA, and RSR helped with data acquisition; RJD, GMM, CT, IA, and RSR analyzed and interpreted results; RJD and RSR provided statistical analysis of the data; RJD drafted the manuscript, while RPC, GMM, CB, and RSR reviewed the manuscript and provided important intellectual content; RJD, RPC, GMM, CT, IA, CB, and RSR approved the final version of the manuscript; RJD, CB, EJP, JAI, and RSR obtained funding.

TABLES AND FIGURES

Primers	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
αsma	AAACAGGAATACGACGAAG	CAGGAATGATTTGGAAAGGA
adam10	CTTTGGATCTCCACATGATC	AACAGTTGTTCCTCTTCTTC
adam17	AGCTTATTACAACCCAACTG	CAGCTTCCTTTGTGAGAATAG
arg1	GATCACATCCCAAAATTCCC	CTTCATCTTTCTTCCCACAC
areg	GACATGCAATTGTCATCAAG	GACAAAGATAGTGACAGCTAC
col1α1	ACGCCATCAAGGTCTACTGC	ACTCGAACGGGAATCCATCG
desmin	CAGGATCAACCTTCCTATCC	CTGTCTTTTTGGTATGGACTTC
egfr	GGCATCATGGGGGAGAACAA	TATGAAGAGGAGGCCACCCA
gapdh	CTTCAACAGCAACTCCCACTC	GCCGTATTCATTGTCATACCAGG
il1β	GCTACCTGTGTCTTTCCCGT	CATCTCGGAGCCTGTAGTGC
il1ra	CAGAAGACCTTTTACCTGAG	GGCACCATGTCTATCTTTTC
il33	GAACATGAGTCCCATCAAAG	CAGCTGGTTATCTTTTACTCC
inos	TCAACTGCAAGAGAACGGAGAA	ACATTCTGTGCTGTCCCAGTG
mcam	CTTCACTATCAACAAGAAGGAC	CATCAGACACATAGTTCACC
mmp2	GAGATCTTCTTCTTCAAGGAC	AATAGACCCAGTACTCATTCC
mmp9	CTTCCAGTACCAAGACAAAG	ACCTTGTTCACCTCATTTTG
mmp13	CTTTAGAGGGAGAAAATTCTGG	CATCATCATAACTCCACACG
pdgfβ	GATTGACATCCTGCCTGACC	CATGGAACTCCACCAAATCC
ppib	TGGAGATGAATCTGTAGGAC	CAAATCCTTTCTCTCCTGTAG
tgfβ1	AAGTTGGCATGGTAGCCCTT	GCCCTCCATACCAACTATTGC
timp1	CATCCTCTTGTTGCTATCAC	CATGAATTTAGCCCTTATGACC
tnfα	GTGACAAGCCTGTAGCCCAC	GCAGCCTTGTCCCTTGAAGA
vimentin	GAACCTGAGAGAAACTAACC	GATGCTGAGAAGTCTCATTG

Table 2.1: List of Syber primers used throughout the studies.

	CAG-RECK^{+}:CD	RECK-Hep^{Tg}:CD	CAG-RECK^{+}:WD	RECK-Hep^{Tg}:WD
Final Body Weight (g)	31.4 ± 1.6	30.3 ± 0.6	34.7 ± 1.3^{D}	39.8 ± 1.5^{DG}
Body fat (%)	20.0 ± 2.2	16.4 ± 1.2	25.9 ± 2.3^{D}	33.7 ± 2.3^{DG}
Delta change body fat (%)	12.4 ± 2.3	9.5 ± 1.2	17.2 ± 2.1	25.6 ± 2.5^{DG}
Liver (mg)	1130.4 ± 101.4	1009.8 ± 37.8	1714.4 ± 151.2^{D}	1845.3 ± 211.0^{D}
Epi fat (mg)	923.9 ± 149.4	700.3 ± 57.4	1604.9 ± 206.0^{D}	1909.4 ± 187.6^{D}
Heart:Body weight (mg/g)	4.5 ± 0.2	4.6 ± 0.1	4.1 ± 0.3	3.6 ± 0.1^{D}
Average weekly food intake (g)	18.6 ± 0.9	19.1 ± 0.9	17.1 ± 1.1	17.9 ± 1.1

Table 2.2: Animal characteristics of RECK$^{fl/fl}$ and RECK$^{hep-/-}$ mice fed either a CD or a WD for 24 weeks. Data are presented as mean ± SEM (n=8-12/group). D indicates main effect of diet ($p \leq 0.05$).

	CAG-RECK^{+}:CD	RECK-Hep^{Tg}:CD	CAG-RECK^{+}:WD	RECK-Hep^{Tg}:WD
Final Body Weight (g)	31.4 ± 1.6	30.3 ± 0.6	34.7 ± 1.3^{D}	39.8 ± 1.5^{DG}
Body fat (%)	20.0 ± 2.2	16.4 ± 1.2	25.9 ± 2.3^{D}	33.7 ± 2.3^{DG}
Delta change body fat (%)	12.4 ± 2.3	9.5 ± 1.2	17.2 ± 2.1	25.6 ± 2.5^{DG}
Liver (mg)	1130.4 ± 101.4	1009.8 ± 37.8	1714.4 ± 151.2^{D}	1845.3 ± 211.0^{D}
Epi fat (mg)	923.9 ± 149.4	700.3 ± 57.4	1604.9 ± 206.0^{D}	1909.4 ± 187.6^{D}
Heart:Body weight (mg/g)	4.5 ± 0.2	4.6 ± 0.1	4.1 ± 0.3	3.6 ± 0.1^{D}
Average weekly food intake (g)	18.6 ± 0.9	19.1 ± 0.9	17.1 ± 1.1	17.9 ± 1.1

Table 2.3: Animal characteristics of CAG-RECK^{+} and RECK-Hep^{Tg} mice fed either a CD or a WD for 24 weeks. Data are presented as mean ± SEM (n=8-12/group). D indicates main effect of diet ($p \leq 0.05$); G indicates main effect of genotype ($p \leq 0.05$).

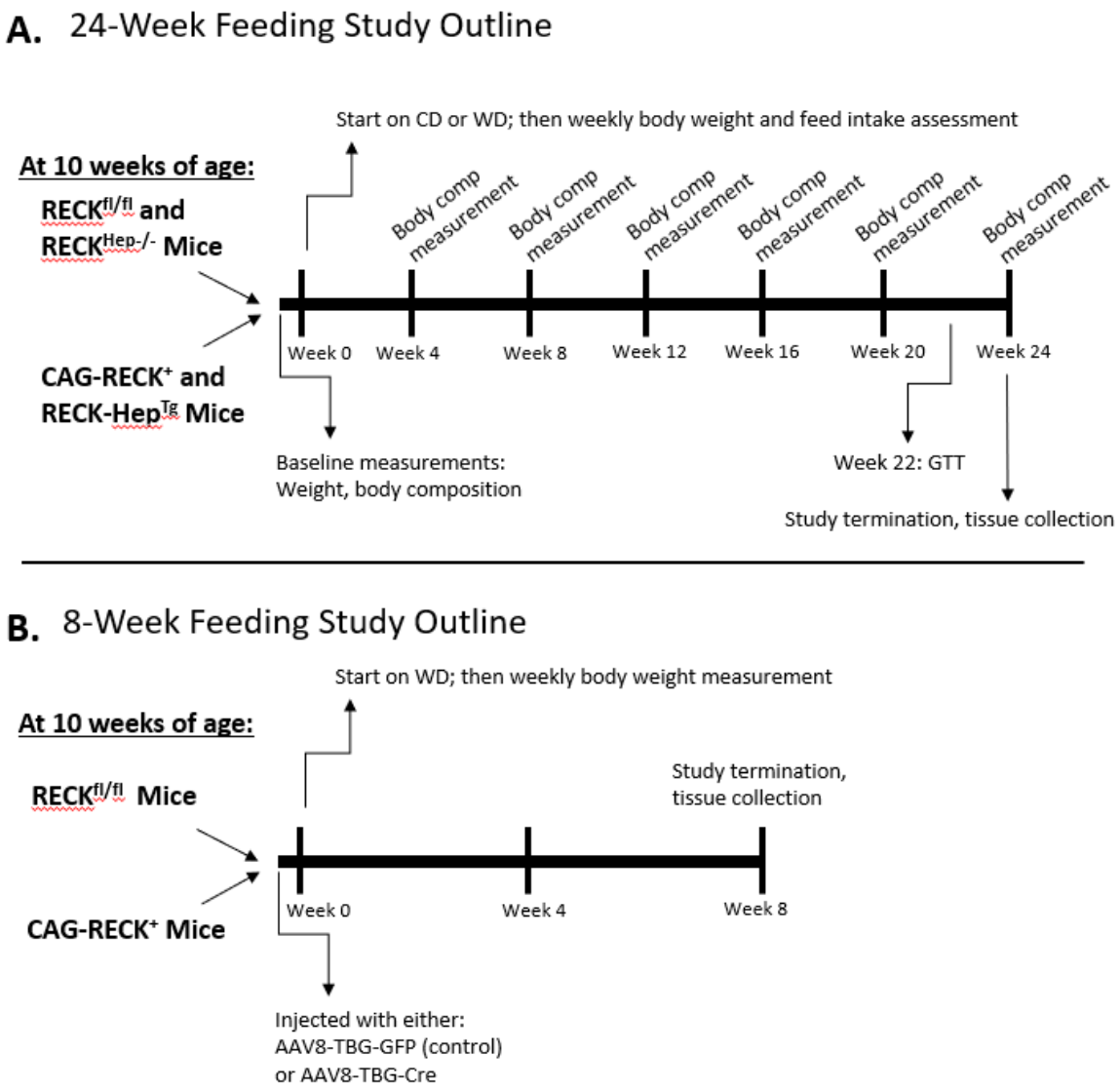

Figure 2.1: Schematics showing study timeline. **A.** 24-week feeding study outline (n=8-12/group) and **B.** 8-week feeding study outline (n=7-9/group).

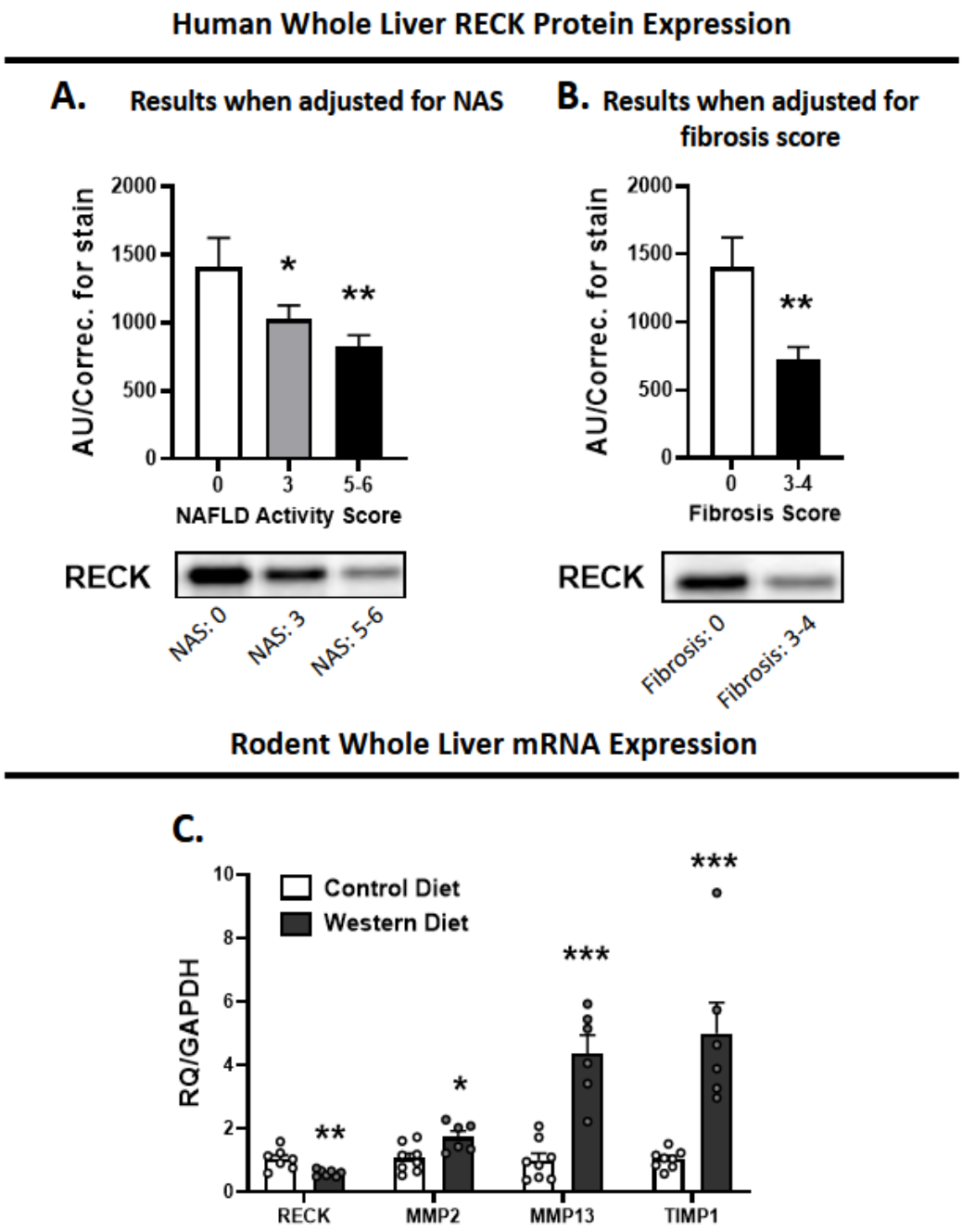

Figure 2.2: Downregulation of RECK in human patients with NAFLD, mice fed a WD. **A.** Adjusted for NAFLD Activity Score, human patients presenting for bariatric surgery had diminished hepatic RECK protein content, correlating with worsening disease; **B.** this was true also when adjusted for fibrosis score (n=8-9/group). **C.** Feeding WT C57BL/6 mice a WD for 16-weeks significantly lowered

hepatic RECK, and increased RECK-inhibitory target, mRNA content compared to CD-fed cohorts (n=6-8/group)

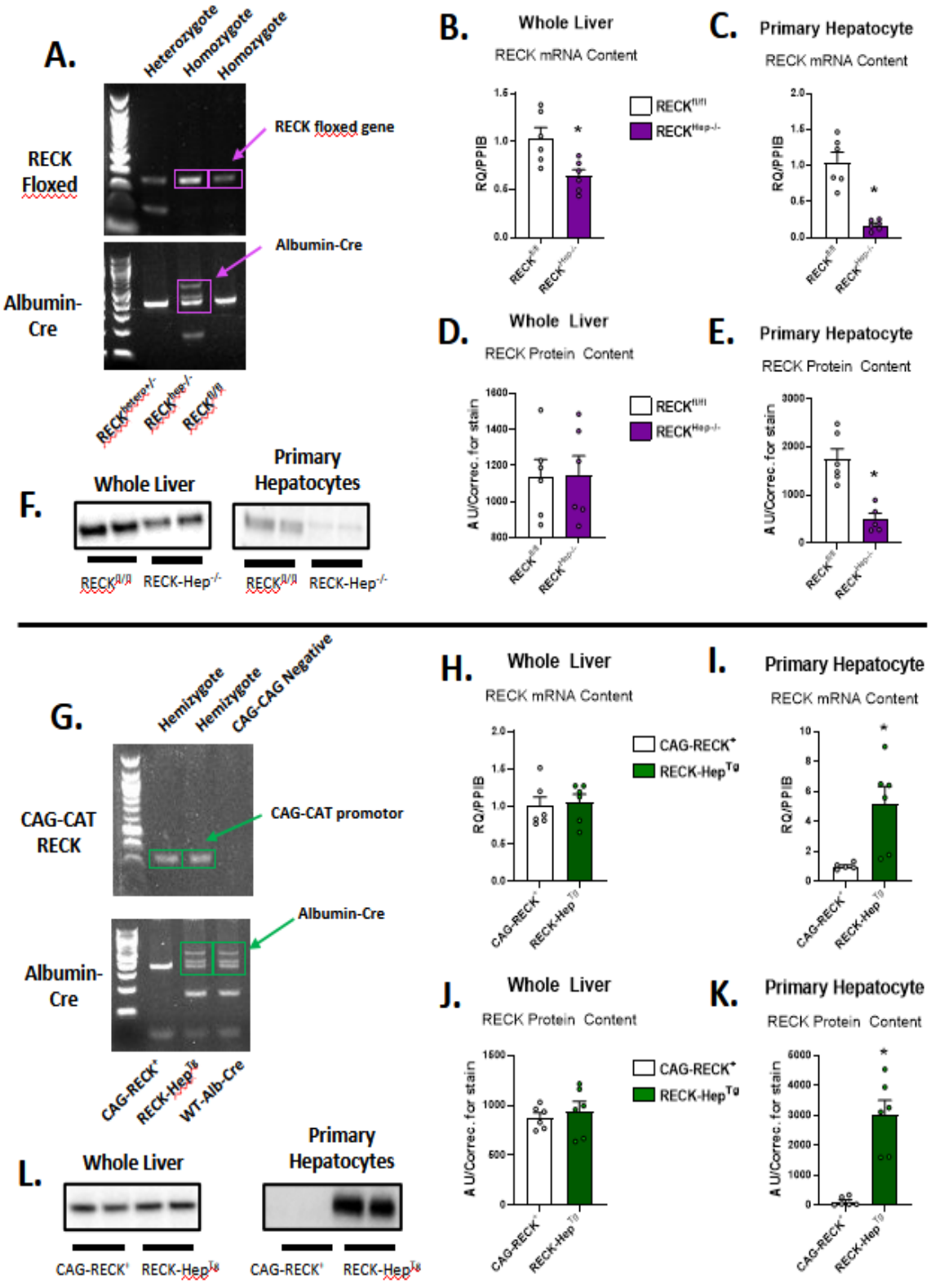

Figure 2.3: Confirmation of transgenic *Reck* manipulation in mouse models using primary hepatocytes and whole liver tissue collected from animals on a CD.

A. Genotyping images displaying floxed *Reck* gene and the presence of Albumin-linked Cre recombinase (Albumin-Cre) in the $RECK^{fl/fl}$ and $RECK^{hep-/-}$ murine lines. **B.** mRNA expression and **C.** total protein content of whole livers and isolated primary hepatocytes (**D.** and **E.**, respectively). **F.** Representative Western images from whole livers (left) and isolated primary hepatocytes (right) from $RECK^{fl/fl}$ and $RECK^{hep-/-}$ mice fed CD (n=6/group). **G.** Genotyping images displaying insertion of the CAG-CAT promotor upstream of the *RECK* gene and the presence of Albumin-Cre in the CAG-$RECK^{+}$ and RECK-Hep^{Tg} mouse lines. mRNA expression and total protein content of whole livers (**H.** and **I.**, respectively) and isolated primary hepatocytes (**J.** and **K.**). **L.** Representative Western images from whole livers (left) and isolated primary hepatocytes (right) from CAG-$RECK^{+}$ and RECK-Hep^{Tg} animals fed CD (n=6/group).

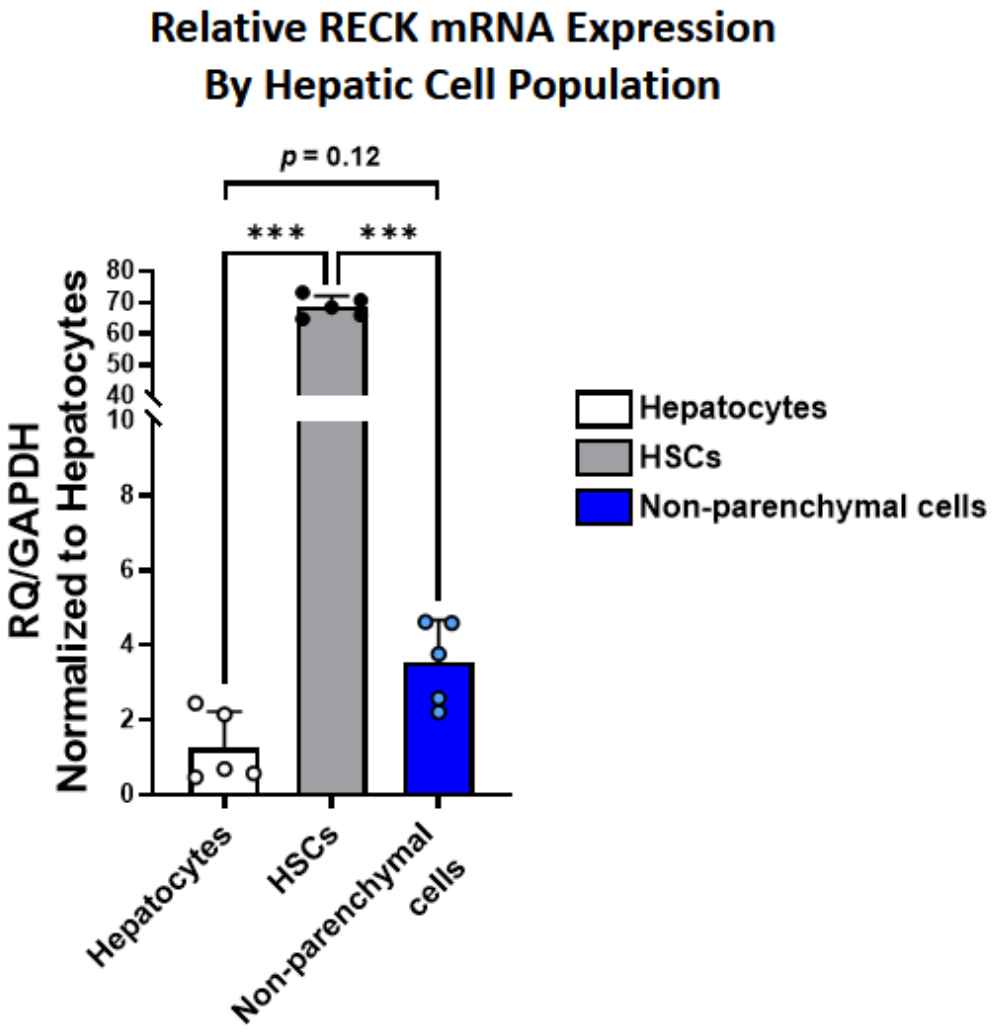

Figure 2.4: Relative comparison of RECK mRNA expression in isolated primary hepatic cell populations isolated from adult WT C57BL/6J male mice, n=5. *** indicates $p \leq 0.001$

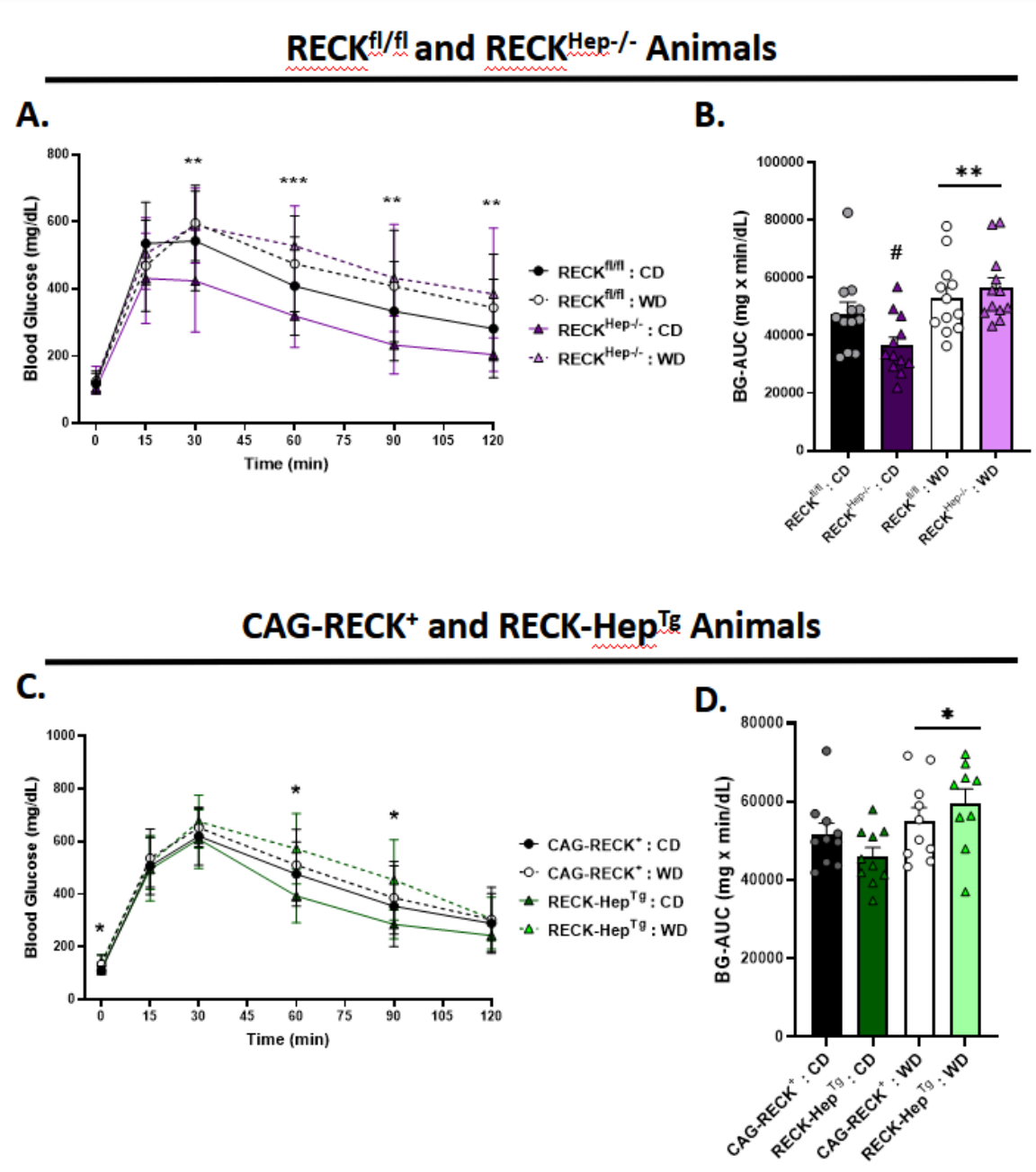

Figure 2.5: Effects of hepatocellular RECK manipulation on glucose tolerance. Following 22 weeks of either CD or WD feeding, RECK$^{fl/fl}$ animals underwent glucose tolerance testing (**A.**); data is also presented as area under the curve (**B.**). The same was accomplished with CAG-RECK^{+} and RECK-Hep^{Tg} mice (**C.**

and **D.**). n=8-12/group. * indicates main effect of diet, # indicates main effect of genotype, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

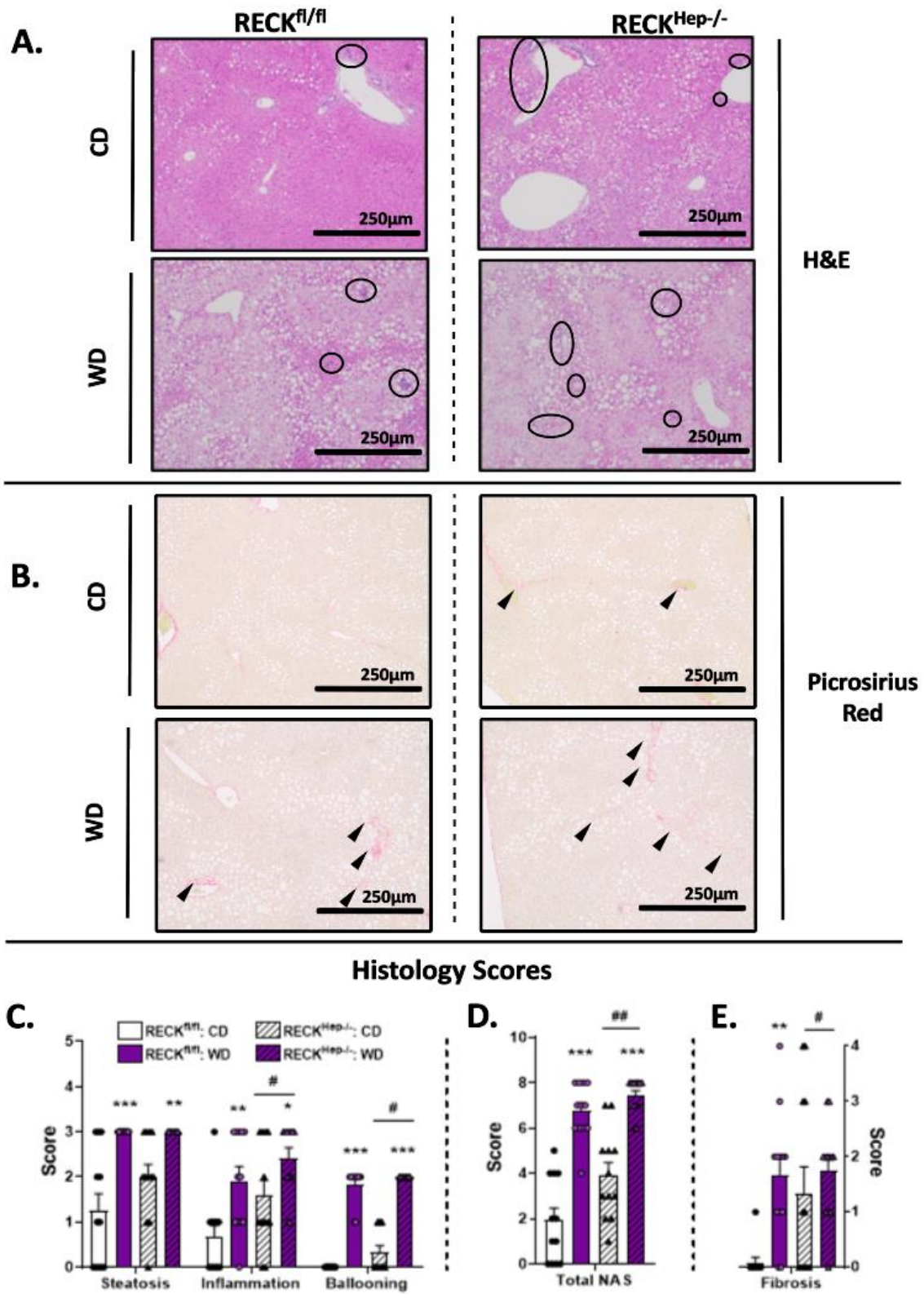

Figure 2.6: Effects of germline hepatocellular RECK deletion on liver histology.

A. Representative liver H&E and **B.** picosirius red staining from the RECK$^{fl/fl}$

(left) and RECK$^{Hep-/-}$ (right) mice fed CD or WD for 24 weeks. **C.** Histological scoring and **D.** total NAFLD activity scores based on H&E images, and **E.** fibrosis scores based on PSR images (n=8-12/group). Data presented as mean ± SEM. * indicates main effect of diet, # indicates main effect for genotype. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

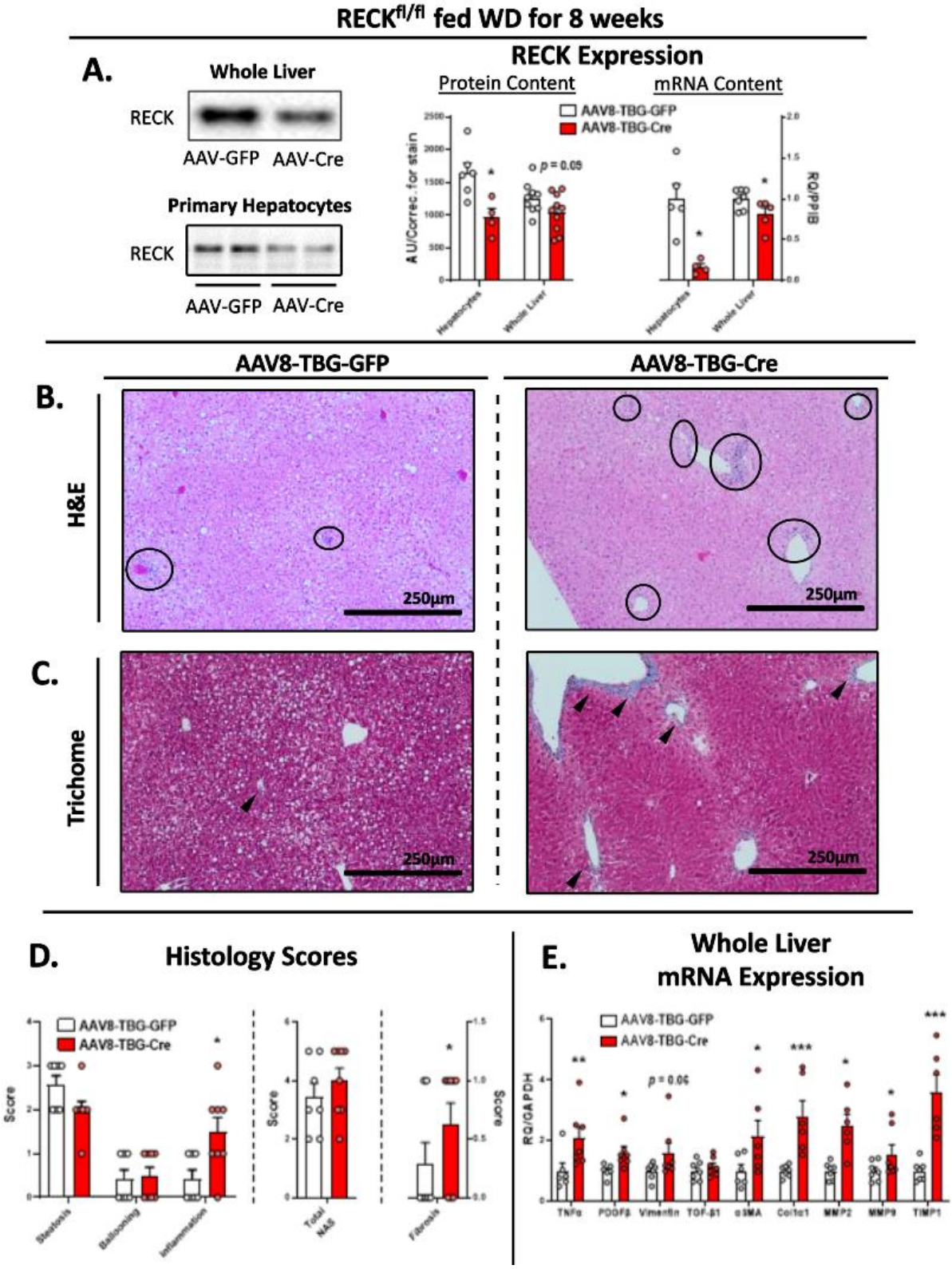

Figure 2.7: Effects of induced hepatocellular RECK depletion on liver histology.

A. Representative Western blots, protein and mRNA content of RECK from whole

liver tissue and isolated primary hepatocytes from RECK$^{fl/fl}$ animals injected with either AAV8-TBG-Cre or AAV8-TBG-GFP and fed a WD for 8 weeks. **B.** Representative liver H&E and **C.** trichrome staining from these animals. **D.** Histological scoring with total NAFLD activity scores based on H&E images, and fibrosis scores based on trichrome images (n=7-9/group). **E.** Whole liver mRNA content of inflammatory, fibrosis, and HSC activation markers. Data presented as mean ± SEM. * indicates main effect of virus. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

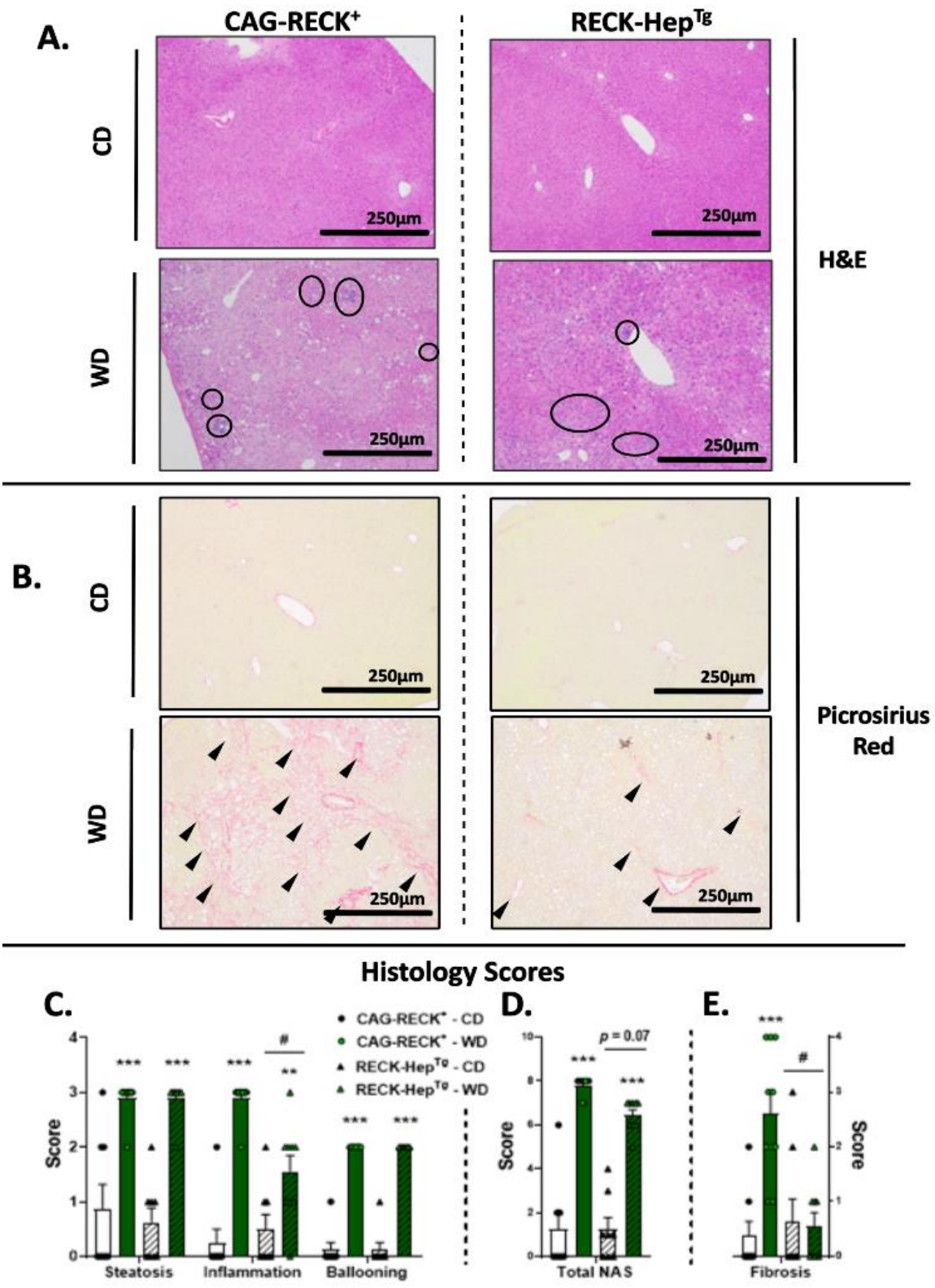

Figure 2.8: Effects of germline hepatocellular RECK overexpression on liver histology. **A.** Representative liver H&E and **B.** picrosirius red staining from the

CAG-RECK^{+} (left) and RECK-Hep^{Tg} (right) mice fed CD or WD for 24 weeks. **C.** Histological scoring and **D.** total NAFLD activity scores based on H&E images, and **E.** fibrosis scores based on PSR images (n=8-12/group). Data presented as mean ± SEM. * indicates main effect of diet, # indicates main effect for genotype. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

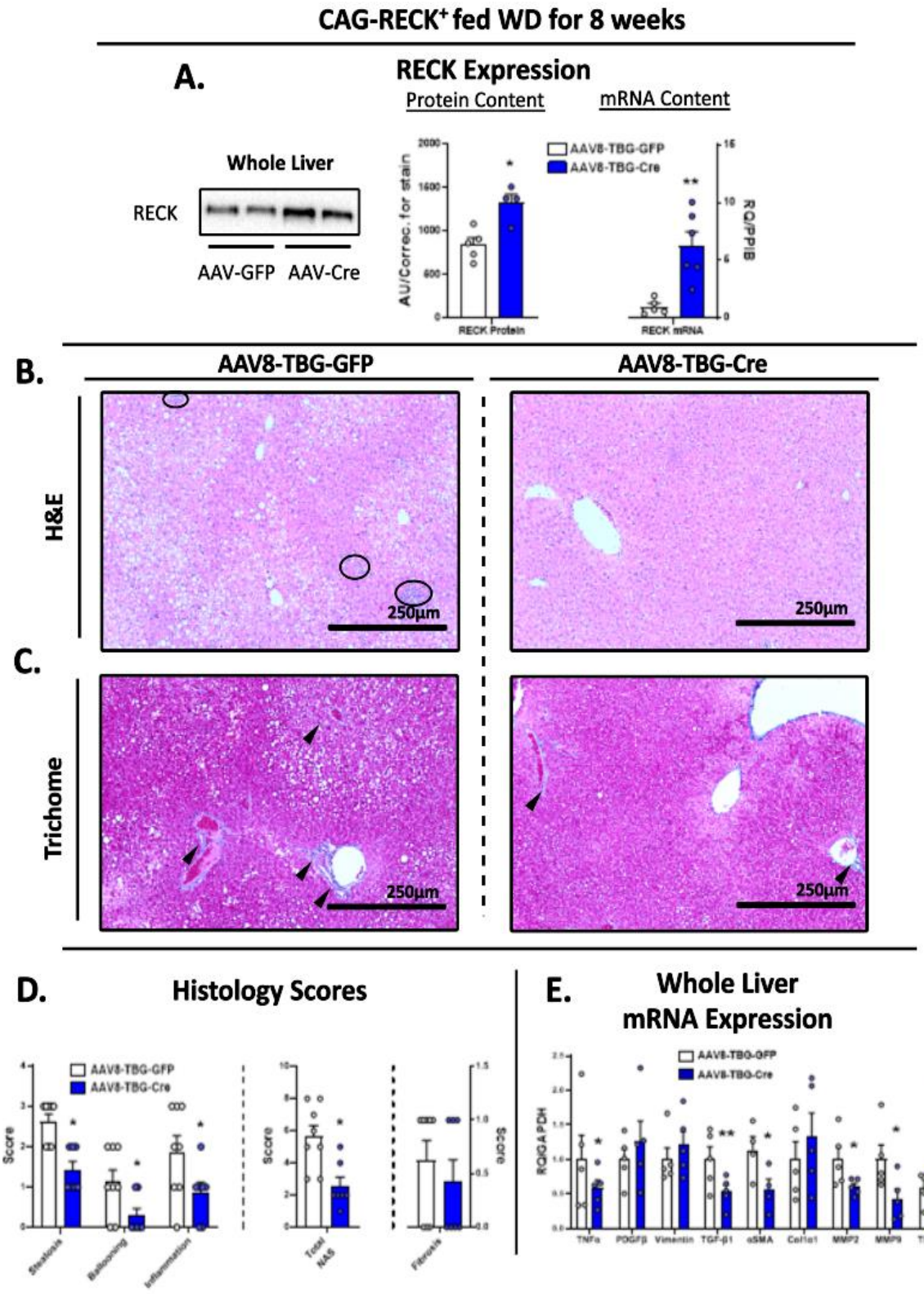

Figure 2.9: Effects of induced hepatocellular RECK overexpression on liver histology. **A.** RECK representative Westerns, protein and mRNA content of

whole liver tissue and isolated primary hepatocytes from CAG-RECK^{+} mice injected with either an AAV-GFP or AAV-Cre and fed a WD for 8 weeks. **B.** Representative liver H&E and **C.** trichrome staining from these animals. **D.** Histological scoring with total NAFLD activity scores based on H&E images, and fibrosis scores based on trichrome images (n=7-9/group). **E.** Whole liver mRNA content of inflammatory, fibrosis, and HSC activation markers was performed (n=5/group). Data presented as mean ± SEM. * indicates main effect of virus. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

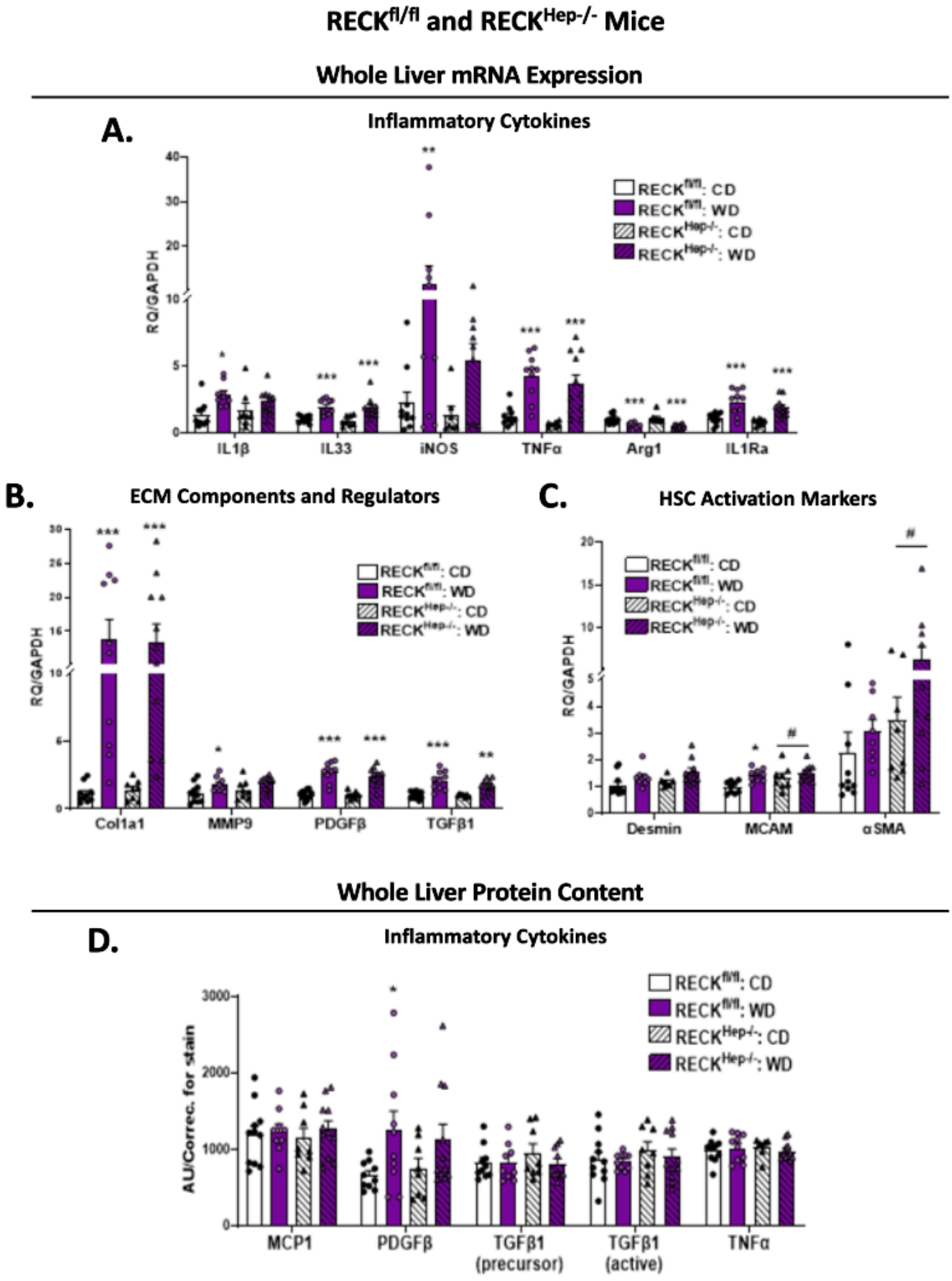

Figure 2.10: Effects of germline hepatocellular RECK depletion on inflammatory markers, extracellular matrix components and regulators, and HSC activation

markers. Whole liver mRNA expression from RECK$^{fl/fl}$ and RECK$^{hep-/-}$ mice fed either a CD or WD for 24 weeks, probing for **A.** inflammatory markers, **B.** components and regulators of the ECM, and **C.** markers of HSC activation, as well as **D.** whole liver protein content assessing for inflammatory cytokines. For all experiments, n=8-12. Data presented as mean ± SEM. * indicates main effect of diet, # indicates main effect of genotype. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

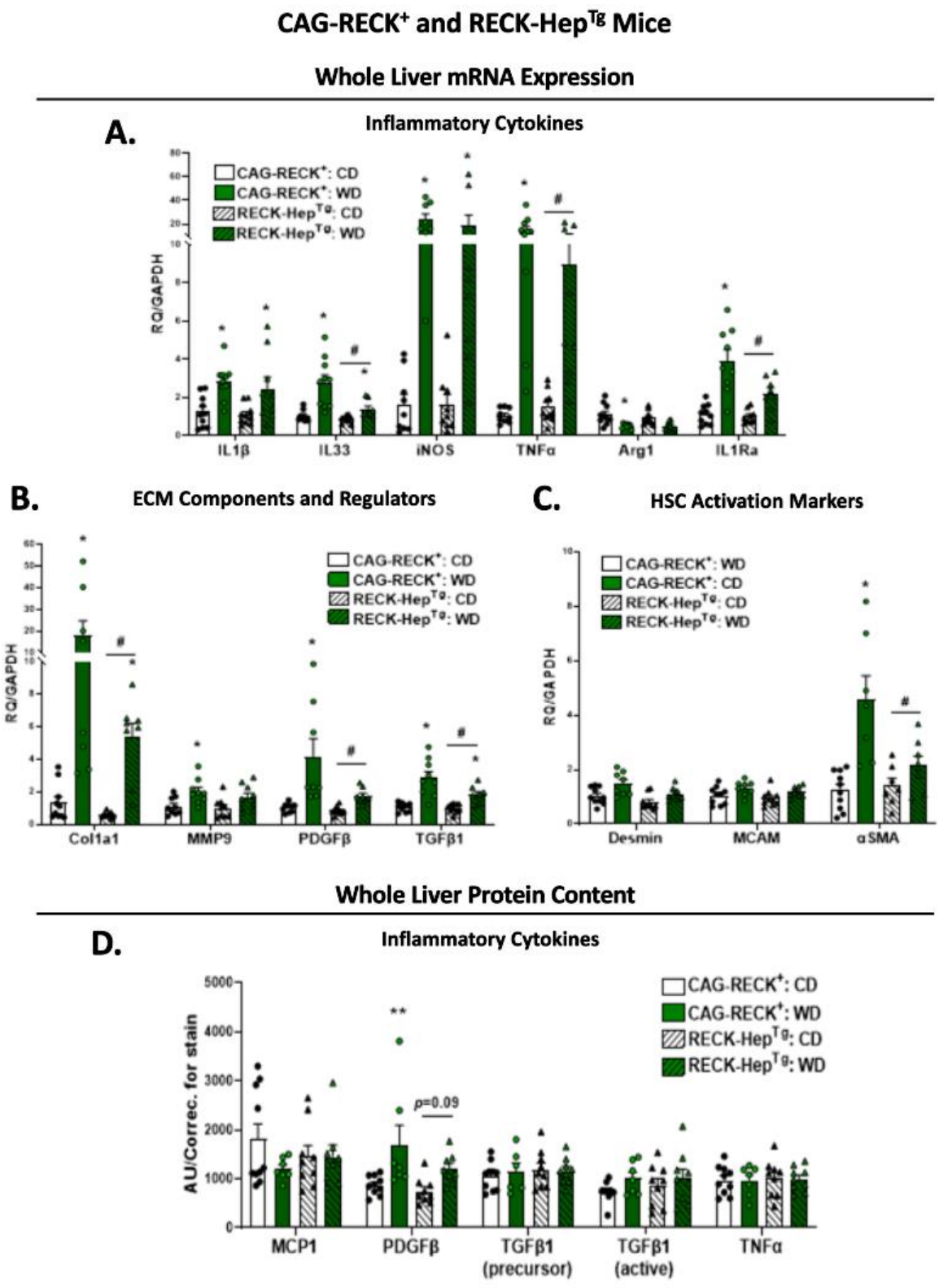

Figure 2.11: Effects of germline hepatocellular RECK overexpression on inflammatory markers, extracellular matrix regulators and components, and HSC

activation cytokines. Whole liver mRNA expression from CAG-RECK^{+} and RECK-Hep^{Tg} mice fed either a CD or WD for 24 weeks, probing for **A.** inflammatory markers, **B.** regulators and components of the ECM, and **C.** HSC activation markers, as well as **D.** whole liver protein content assessing for inflammatory cytokines. For all experiments, n=8-12. Data presented as mean ± SEM. * indicates main effect of diet, # indicates main effect of genotype. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

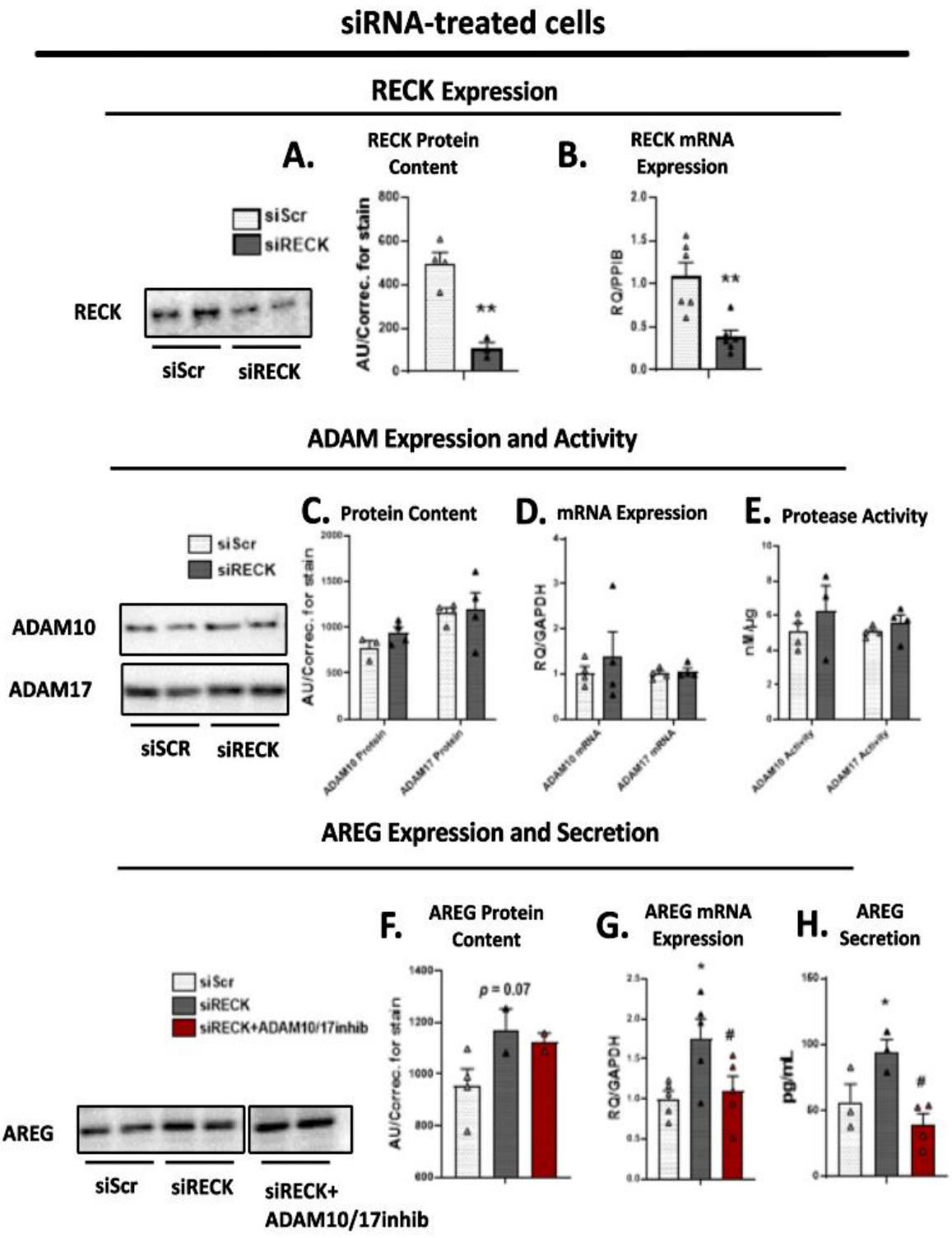

Figure 2.12: Effects of *in vitro* RECK knockdown on ADAM10 and ADAM17 expression and activity, and AREG expression and secretion. Primary

hepatocytes harvested from WT adult male C57BL/6 mice were treated with either an siRNA targeting a scrambled sequence of RNA (siScr) or RECK mRNA (siRECK) while in media containing 50 μg/ml TNF-α. Probing for RECK, siRECK treated cells expressed lower RECK protein (**A.**) and mRNA (**B.**). Cells were assessed for ADAM10 and ADAM17 protein content (**C.**), mRNA expression (**D.**), and protease activity as assessed via a commercially available fluorescent kit (**E**.). Cells were also treated with 10 μM GW280264X (ADAM10/17inhib) or control substrate (DMSO) and assessed for AREG protein content (**F.**), mRNA expression (**G.**), and secreted AREG in cell media (**H.**), n=4-6/group for all experiments. Non-sequential representative Western images are from the same blot with interceding unquantified samples removed. * indicates significance compared to control, # indicates significance compared to RECK-manipulated control (vs. siRECK). * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

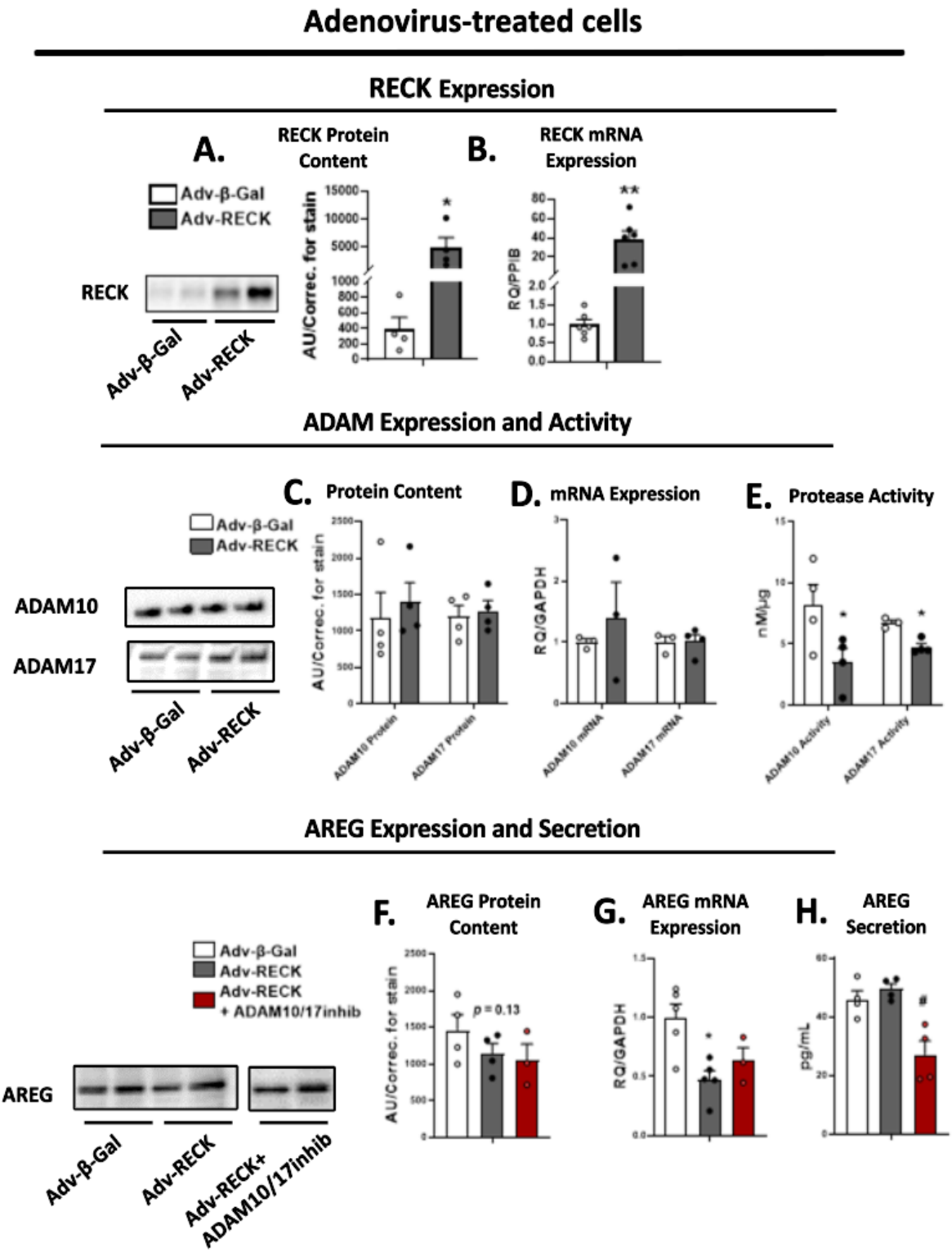

Figure 2.13 Effects of *in vitro* RECK overexpression on ADAM10 and ADAM17 expression and activity, and AREG expression and secretion. Primary

hepatocytes harvested from WT adult male C57BL/6 mice were treated either with an adenovirus encoding for a RECK plasmid (Adv-RECK) or a control protein (Adv-β-Gal) while in media containing 50 μg/ml TNFα. Probing for RECK, Adv-RECK treated cells expressed significantly higher RECK protein (**A.**) and mRNA (**B.**). Cells were assessed for ADAM10 and ADAM17 protein content (**C.**), mRNA expression (**D.**), and protease activity as assessed via a commercially available fluorescent kit (**E**.). Cells were also treated with 10 μM GW280264X (ADAM10/17inhib) or control substrate (DMSO) and probed for AREG protein content (**F.**), mRNA expression (**G.**), and secreted AREG in cell media (**H.**), n=4-6/group for all experiments. Non-sequential representative Western images are from the same blot with interceding unquantified samples removed * indicates significance compared to control, # indicates significance compared to RECK-manipulated control (Adv-RECK). * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

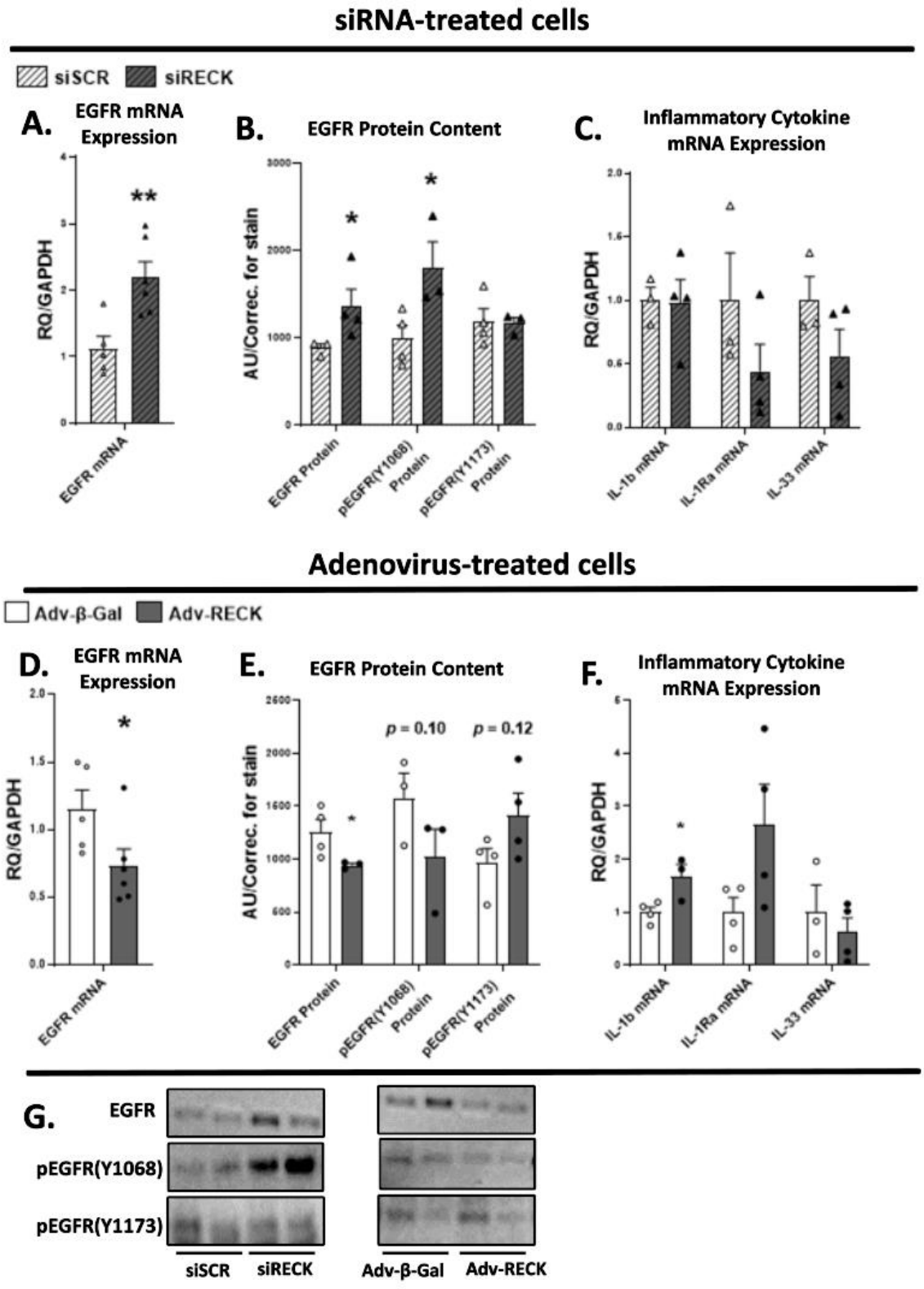

Figure 2.14: Effects of *in vitro* RECK manipulation on EGFR expression and activity, and inflammatory cytokine production. On the left, primary hepatocytes

harvested from WT adult male C57BL/6 mice were treated with either an siRNA targeting a scrambled sequence of RNA (siSCR) or RECK mRNA (siRECK) while in media containing 50 μg/ml TNF-α. On the right, primary hepatocytes in media containing 50 μg/ml TNF-α were treated either with an adenovirus encoding for a RECK plasmid (Adv-RECK) or a control protein (Adv-β-Gal). Cells were assessed for EGFR mRNA (**A.** and **D.**), as well as EGFR protein and phosphorylated EGFR (pEGFR(Y1068) and pEGFR(Y1173) (**B.** and **E.**). Inflammatory cytokine production was also probed for (**C.** and **F.**). Representative Western blot images (**G.**). For all experiments, n=3-4. * indicates significance compared to control; * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

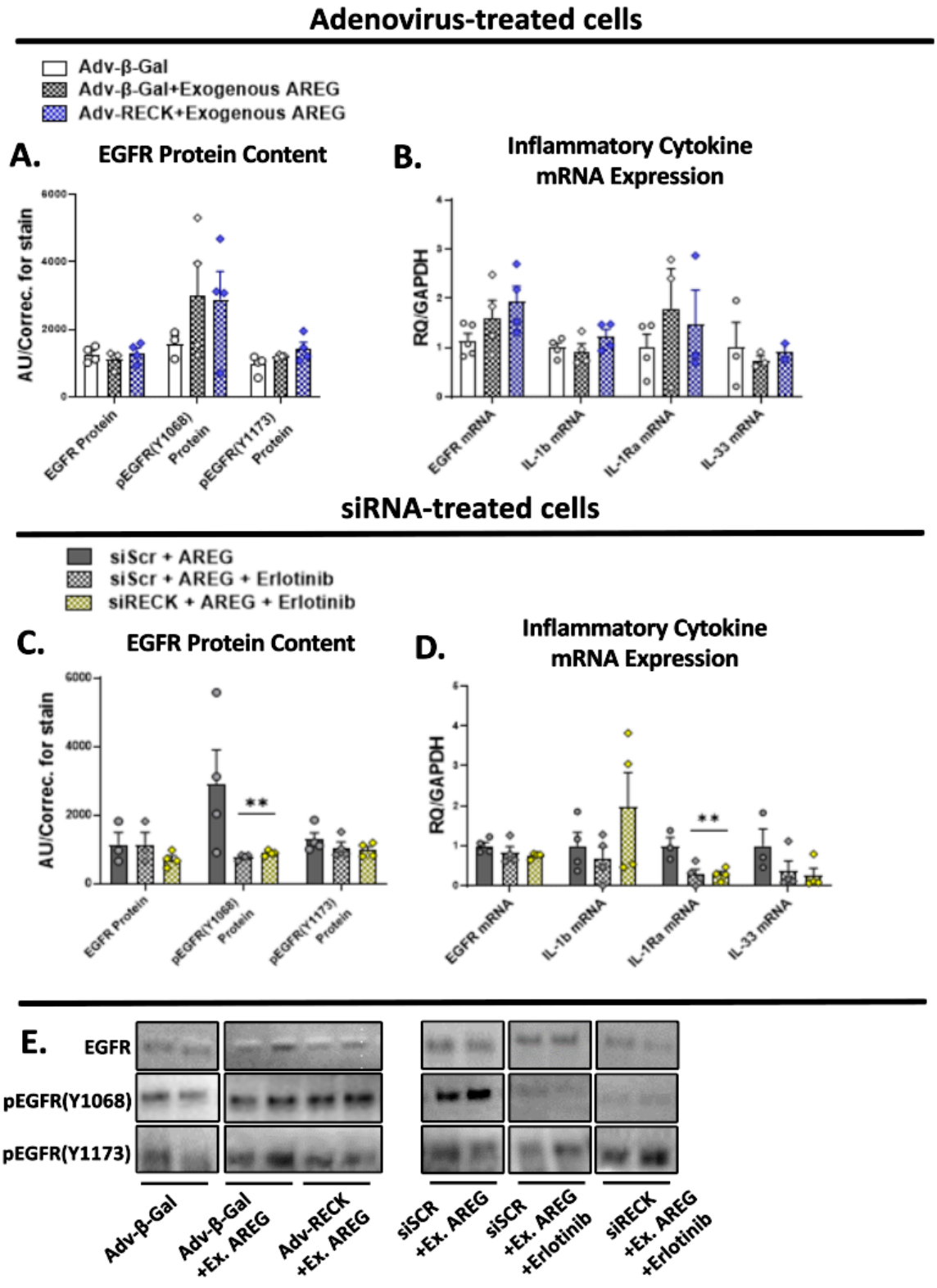

Figure 2.15: Effects of *in vitro* RECK manipulation on EGFR expression and activity, and inflammatory cytokine production in conjuncture with exogenous

AREG exposure. Using primary hepatocytes harvested from WT adult male C57BL/6 mice treated with either siSCR or siRECK, and another set treated either with Adv-RECK or Adv-β-Gal. Cells were assessed for EGFR, pEGFR(Y1068), and pEGFR(Y1173) content (**A.** and **C.**). Inflammatory cytokine production was also probed for (**B.** and **D.**). Representative Western images for the above experiments (**E.**). For all experiments, n=3-4. Non-sequential representative Western images are from the same blot but interceding unquantified samples are removed. * indicates significance compared to control condition, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

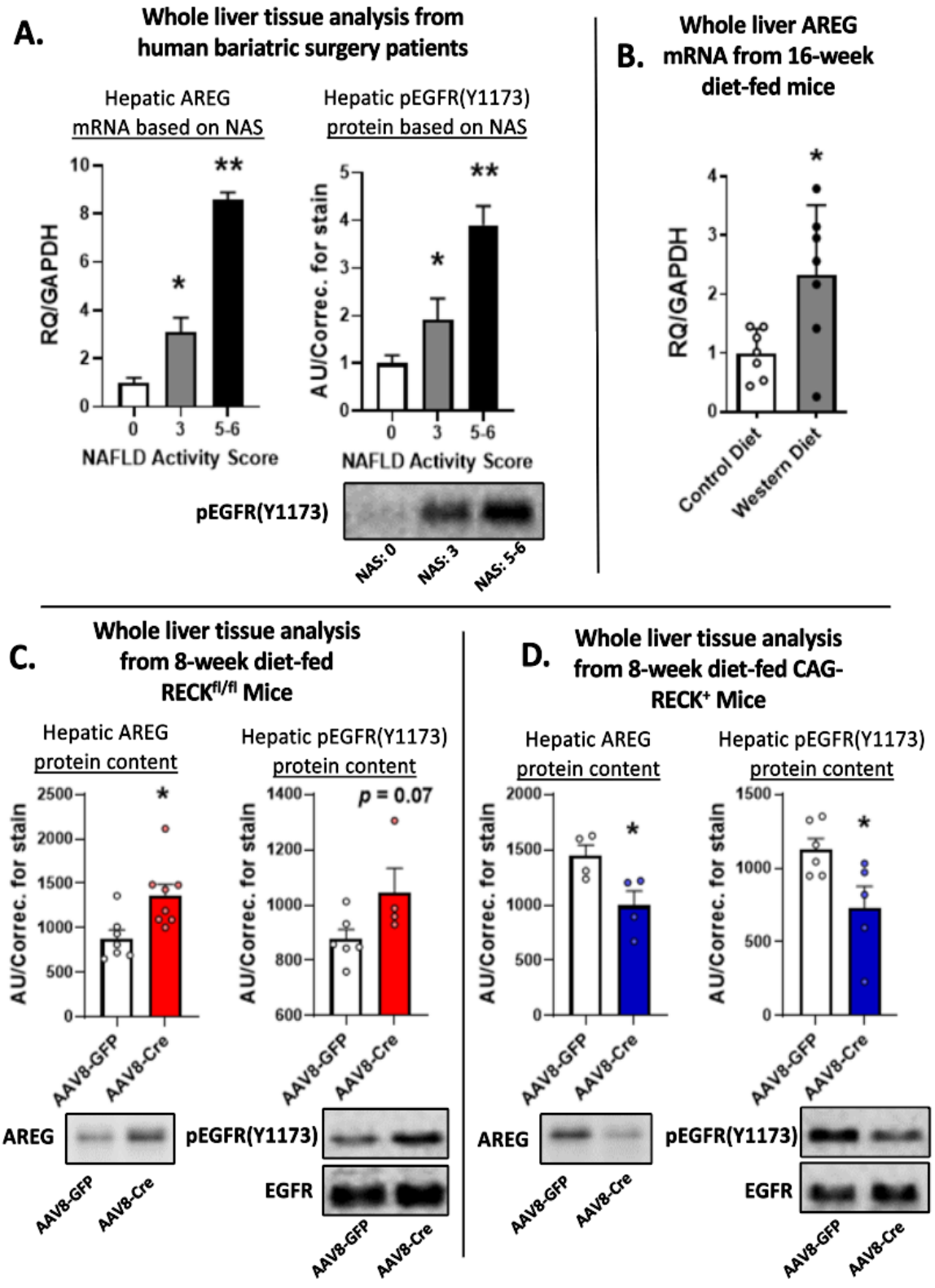

Figure 2.16: Altered AREG content and EGFR phosphorylation in liver tissue of human patients undergoing bariatric surgery and WD-fed mice. **A.** Hepatic AREG

mRNA and pEGFR(Y1173) was increased in human patients with worsening NAFLD as evidenced by histological correlation (n=8). **B.** Mice fed a WD for 16-weeks had increased hepatic AREG mRNA (n=7). We also assessed hepatic AREG and pEGFR(Y1173) protein from $RECK^{fl/fl}$ mice (**C.**) and CAG-REG^{+} mice (**D.**) injected with an AAV-Cre or control virus (AAV-GFP) and fed a WD for 8 weeks (n=6-8). * indicates significance compared to controls, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

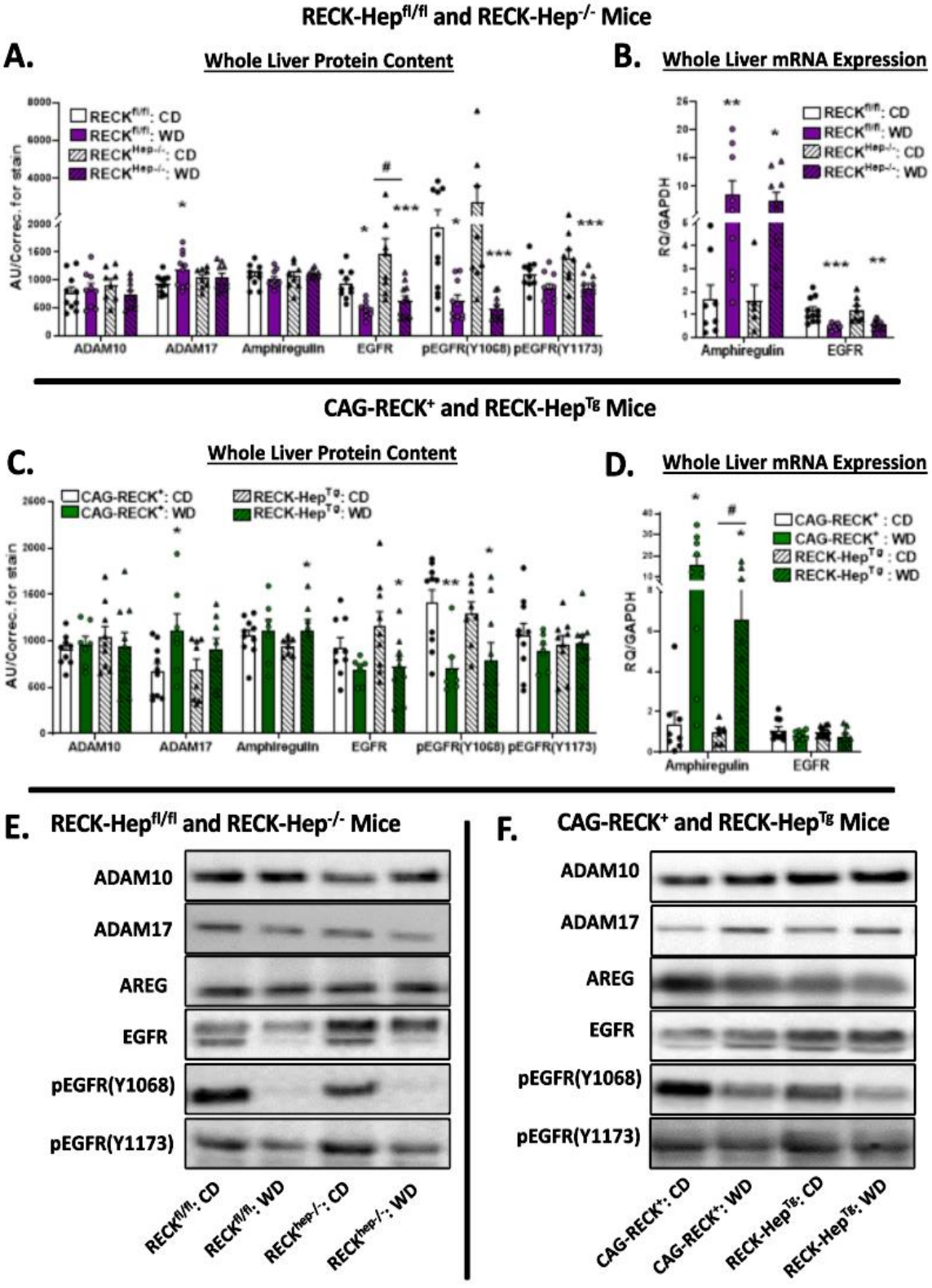

Figure 2.17: Assessment of downstream RECK targets in whole liver tissue of transgenic mice fed a CD or WD for 24 weeks. Whole liver protein content of

RECK targets was assessed in RECK$^{fl/fl}$ and RECK-Hep$^{-/-}$ (**A.**), and CAG-RECK^{+} and RECK-Hep^{Tg} mice (**C.**), as well as mRNA content of AREG and EGFR (**B.** and **D.**). Representative Western images from RECK$^{fl/fl}$ and RECK-Hep$^{-/-}$ (**E.**) and CAG-RECK^{+} and RECK-Hep^{Tg} (**F.**) animals. For all experiments, n=7-12. * indicates a main effect of diet, # indicates a main effect of genotype, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

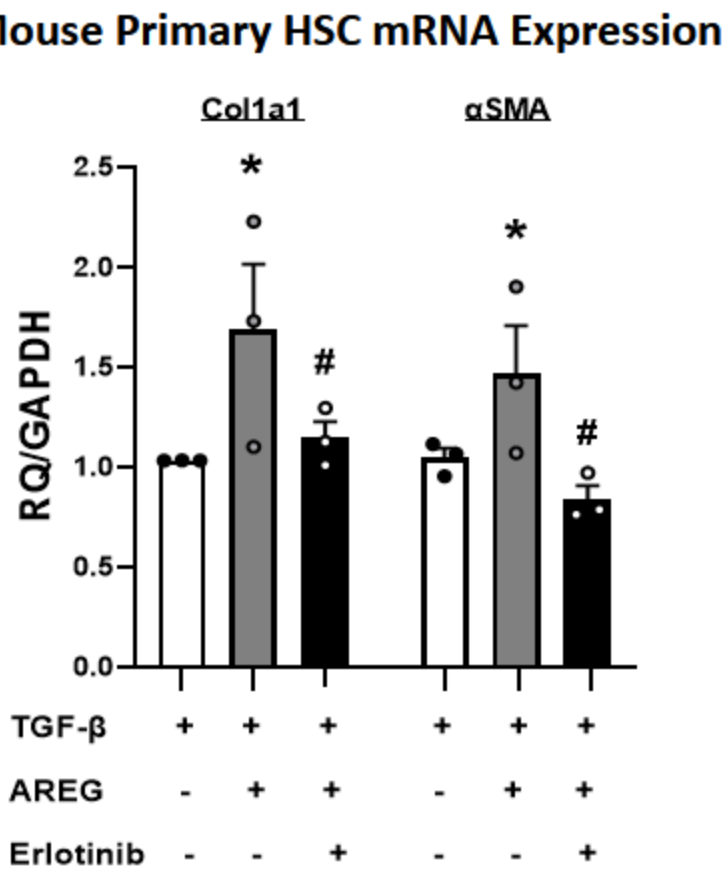

Figure 2.18: Effects of exogenous AREG and EGFR inhibition on primary mouse HSC activity, n=3-4. * indicates significance from control condition (vs. TGF-β only treatment), # indicates significant from control and AREG treatment (vs. TGF-β and AREG treatment), * indicates $p \leq 0.05$

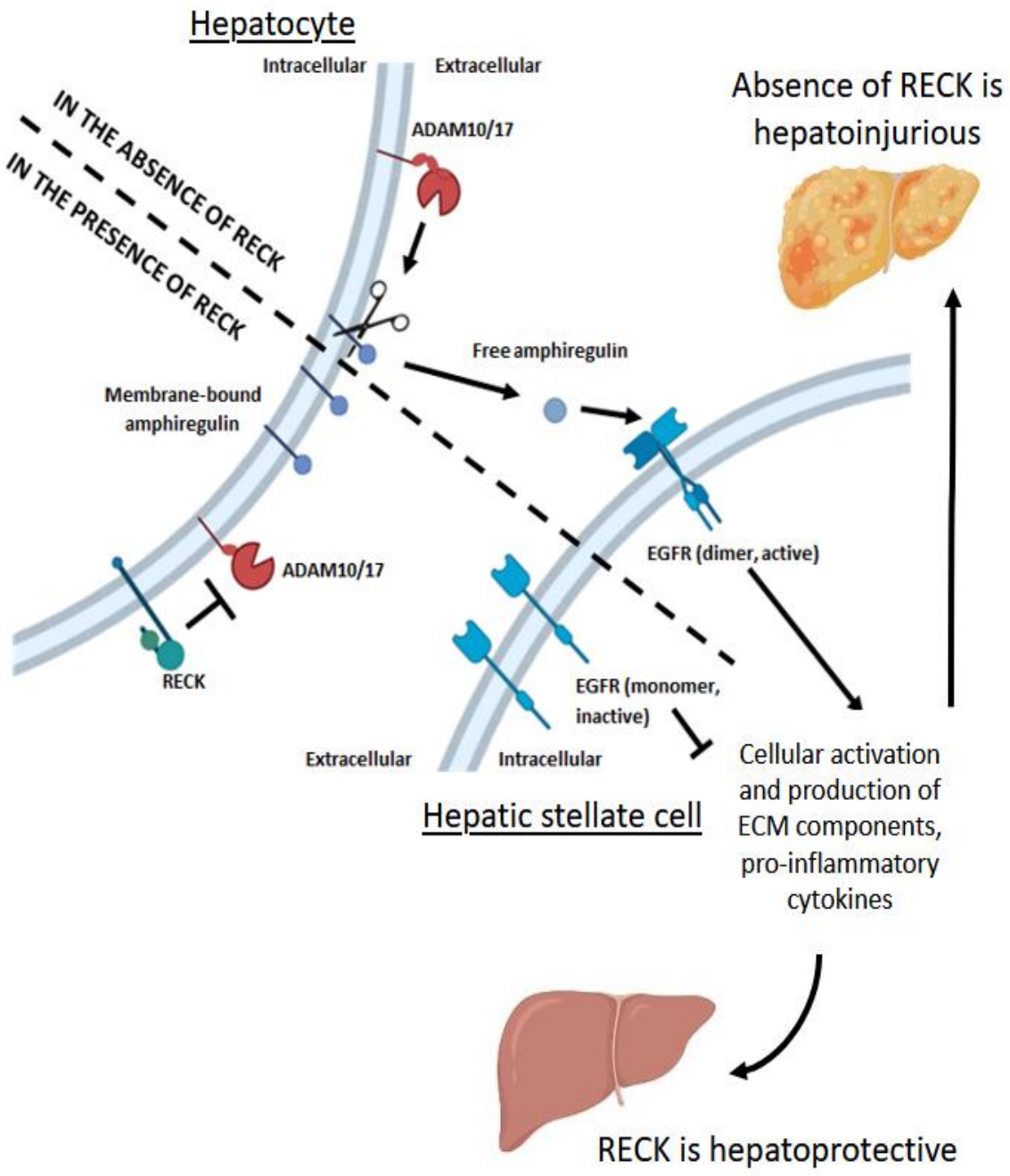

Figure 2.19: Proposed mechanism by which RECK activity alters EGFR signaling through direct inhibition of ADAM10 and ADAM17 and subsequent inhibition of AREG release from its membrane-bound location. The top-right of

the diagram depicts the events that occur in the absence of RECK, while the bottom-left demonstrates events whereby RECK is present.

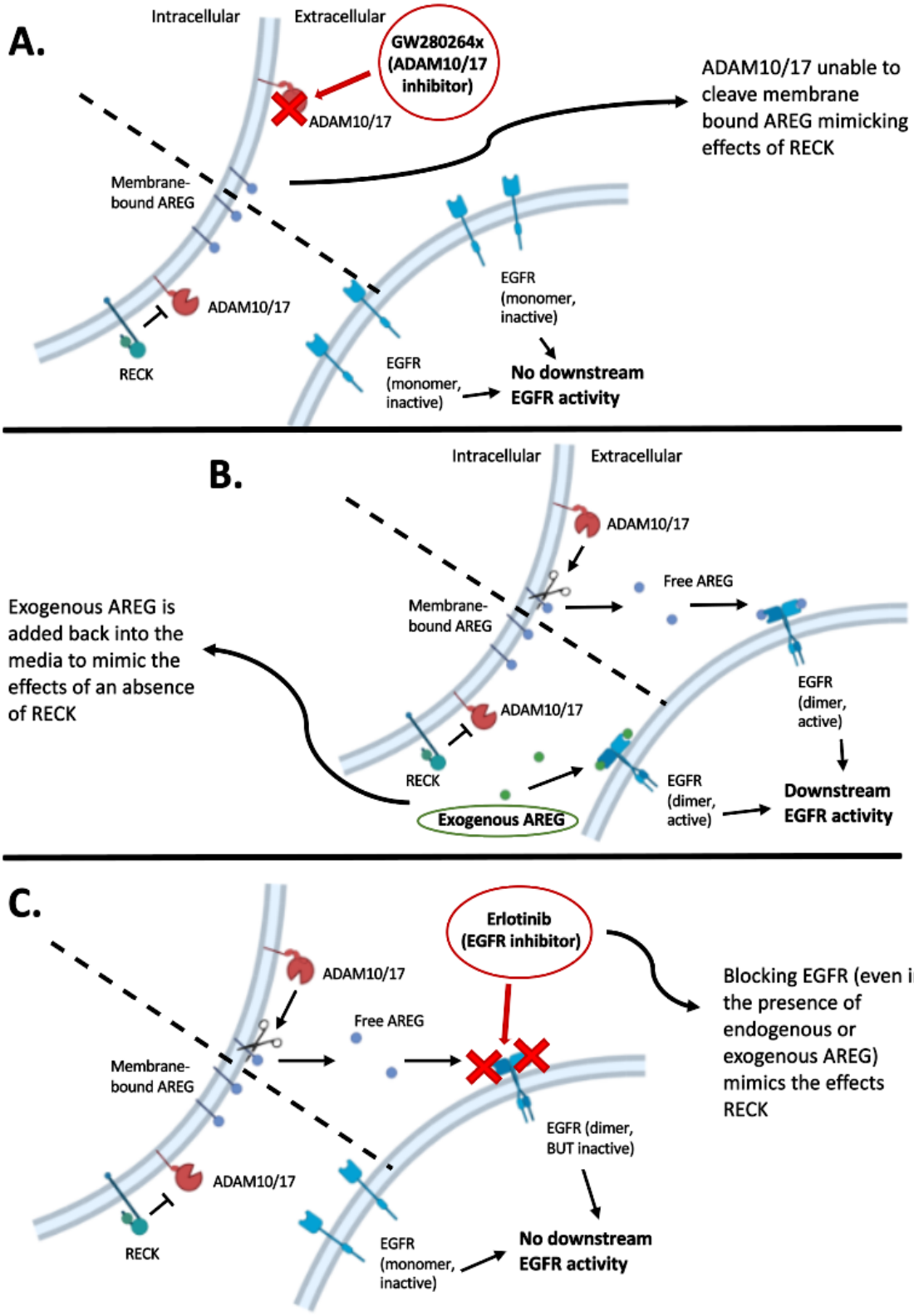

Figure 2.20: Schematics detailing the hypothesized consequences of cell culture experiments performed in this study as they pertain to the proposed mechanism

of RECK altering EGFR signaling through sheddases. **A.** Effects when a pharmacological ADAM10/ADAM17 inhibitor is added to the media. **B.** Effects when exogenous AREG is added to cell media. **C.** Effects when a pharmacological EGFR inhibitor is added to media.

CHAPTER 3: Summary, Limitations, Future Directions

SUMMARY

Nonalcoholic fatty liver disease is a spectrum disorder spanning from simple steatosis to steatohepatitis and fibrosis, to cirrhosis and, finally, hepatocellular carcinoma. This condition disproportionately affects individuals who are obese and overweight, and, correlating with increasing rates of obesity, has seen a dramatic increase in prevalence over the last several decades (127, 142, 143). Because of its association with life-threatening disease and lack of approved pharmacological interventions, studies investigating cellular mechanisms of NAFLD are needed, and potential pharmaceutical targets are required (144). The presence of hepatic inflammation and fibrosis is a hallmark of late-stage NAFLD, thus indicating needs for liver transplants in patients with severe disease. These processes both rely heavily on proper functioning and homeostasis of the extracellular matrix, and so identifying key regulators in that space is a potential avenue for further exploration.

As such, this dissertation focused on the role of manipulating hepatocellular RECK via multiple approaches to examine the potential this ECM regulator has on the progression of inflammation and fibrosis in the liver. RECK was selected as a potential target because of previous literature examining its anti-fibrotic capacity in other organs (102-104), as well as the correlation observed between decreasing RECK content in the liver and worsening histological evidence of NAFLD in human patients (**Fig. 2.2**). Furthermore, in

rodent models of the disease, RECK was also found decreased, and its inhibitory targets increased. Given this, we determined to examine the role of RECK in the development of NAFLD, and specifically, within the inflammatory condition of NASH.

Germline-RECK-manipulated mice were first fed either a CD or a WD for 24 weeks to induce a moderate-to-severe phenotype of NAFLD. Mice fed a WD did develop hepatic inflammation, fibrosis, hepatocyte ballooning, and extensive steatosis, which was significantly attenuated in RECK-overexpression mice, though RECK depletion did not incur a significant worsening of phenotype as expected. This may be because the WD-phenotype was already so extensively severe that further worsening may not have been possible. Hepatocellular RECK-depletion did however induce a significant increase in inflammation and fibrosis in CD-fed animals. These data support the hypothesis that hepatocellular RECK is a critical regulator in hepatic inflammation and fibrosis.

Germline manipulation of RECK could lead to compensatory mechanisms that would diminish the response in adult mice (for example, increased methylation of the *Reck* gene in cells that would otherwise express it; or alternative regulatory mechanisms contributing towards the limitation of proposed downstream pathways that would prevent their overactivation in the face of RECK depletion). Therefore, experiments utilizing an induced model of RECK manipulation, specifically through viral induction, were necessary. In addition, potential therapeutic utilization of RECK activity in NAFLD treatment in human patients would necessarily require manipulation of an established baseline of

RECK activity and could thus be considered more analogous to treatment in human patients. In these mice, hepatocyte RECK reduction significantly worsened of fibrosis and inflammation histologically and induced an upregulation of several inflammatory cytokines. Likewise, RECK-overexpression significantly reduced histological evidence of inflammation and inflammatory cytokines. Taken together, this would suggest that inducing changes in RECK is sufficient to alter the disease course of NAFLD, and thus underlines the potential for RECK to be used as a target for treatment in this disease.

The next goal of this study was to determine which mechanisms RECK may be involved in to effect such changes NAFLD progression. Using proteomics data from RECK-knockdown mouse livers (data not shown), significant alterations were identified in AREG/EGFR signaling pathway proteins. Examining the literature also led us to identify RECK as a regulator of the sheddases, ADAM10 and ADAM17, which were necessary for the cleavage and release of amphiregulin from the cell surface, whereby it is then able to bind to the g-coupled protein receptor, EGFR in nearby cells, triggering downstream signaling. Previous work has suggested EGFR signaling is altered in NAFLD progression and that inhibiting this signaling may be hepatoprotective (99, 100), thus the aim of the *in vitro* experiments was to manipulate RECK in this ADAM10/17-AREG-EGFR pathway. Manipulating RECK did change the expression and secretion of AREG from primary cultured hepatocytes, and this correlated with ADAM10/17 activity. However, this did not appear to have much of an effect on the hepatocytes themselves, as when they were treated with

exogenous AREG (or EGFR inhibitors), the expression of proinflammatory cytokines was not significantly altered.

Though significant changes to primary hepatocytes were not observed in these studies, this may have been due to the lack of other cell populations present, and that altered AREG secretion from hepatocytes would be sufficient to influence phenotypes of other hepatic cells. Therefore, in analyzing the whole liver tissue taken from human bariatric surgery patients, evidence emerged to support that the proposed RECK-ADAM10/17-AREG-EGFR pathway was altered in these samples as well. Data from diet-fed animal studies supported these findings, as RECK manipulation did indeed alter various proposed pathway components as well. Therefore, to determine what effects AREG would have in a primary HSC culture, cells isolated from WT-C57BL/6 mice were also exposed to exogenous AREG. AREG was sufficient to induce expression of markers of HSC activation, and, when exposed to an EGFR inhibitor, this returned expression to baseline.

Taken together, this supports the hypothesis that hepatocellular RECK is critical in the development of NASH, and RECK is hepatoprotective against damage associated with metabolic disease. Evidence provided suggests hepatocellular RECK is influencing the AREG-EGFR signaling cascade, and that this may lead to activation of HSCs. These novel data help to further understand RECK and its regulatory mechanisms in the liver, and thus identify a possible target that could be used to help prevent progression of NASH in the future.

LIMITATIONS

Animal models

In general, several inherent limitations and variables must be considered when using diet-induced fatty liver disease transgenic rodent models, as is the case here. First, Cre-recombinase can be 'leaky', meaning there could be changes in cells other than hepatocytes (i.e. expression or uptake of the Cre-recombinase in other cell types). In the case of albumin-linked Cre-drivers, it is already known that cholangiocytes also express albumin for example, and so in the present study, we must assume that cholangiocyte RECK has also been manipulated in the animals containing *Albumin*-linked Cre-recombinase. Similarly, viruses that were used to induce RECK changes may have been able to infect non-hepatocyte cells despite hepatocyte targeting sequences; and furthermore, the viruses are unlikely to infect every single hepatocyte, meaning that there would be a great deal of heterogeneity amongst hepatocytes regarding RECK expression. However, as we demonstrated in our experiments, both in whole liver tissue and, perhaps more importantly, in primary hepatocytes, RECK is successfully altered in these models. Furthermore, this may lend additional credence to the concept that RECK is hepatoprotective itself, as its manipulation in even just a subset of hepatocytes is sufficient to significantly alter the observed phenotypes.

A notable exclusion in the data here is anything relating to female mice. While the human samples were from both males and females, all rodent data included is from males. These experiments were replicated in a smaller set of

female rodents; results are consistent with what was observed throughout, but less robust. This may be due to female mice generally being resistant to developing diet-induced fatty liver disease (145). This could be linked to the presence of estrogen, as it is generally observed that there is a resilience in females populations compared to males (127, 143) which vanishes when considering the above-50 population. In any case, the data was not included here, as the understanding between estrogen signaling and pathophysiology of NAFLD is multifactorial, complex, and poorly understood. Therefore, to simplify the interpretation of results, we have focused on males in the present study.

A great number of factors inherent to rodent studies may enhance variability when considering these data. As the total feed consumption of these mice would have important consequences on their ability to develop obesity and fatty liver disease, we do measure feed consumption in these animals. However, while we assess overall feed consumption in cages, there could be individual differences of feed intake within cages. Furthermore, animals are group-housed, when possible, but separated if fighting between cohorts takes place. As single-housing does alter how much diet rodents will consume (146), this is another inherent variable that may influence the outcomes of the studies.

Lastly, while all studies took place in a single location with a designated strain of mice, we must consider these conditions as well. As gut microbiome is known to play a role in the development of NAFLD in human and rodent models (9-11, 18), where these animals are housed, the sort of caging environment, the water they receive – everything may play a role in affecting the gut microbiome

and thus, progression of liver disease and the phenotype observed. Strain of mouse, and where mice are acquired is already known to impact gut-microbiome composition (147). Diet-induced rodent obesity models display a significant difference in disease phenotype when considering strains, with C57BL/6 mice showing a more moderate phenotype in response to a WD (148). It is unclear what response might be obtained if an alternative rodent strain was used, such as the 129Sv strain, which has shown to be particularly susceptible to developing obesity, or the CD-1 and BALB/c strains, shown to be more protected against developing obesity in response to a WD.

Taken together, there are many factors that one must consider when completing any animal study and these studies are no different. However, many variables have been accounted for or considered, for example, through assessing RECK expression in all animals following experiments, by using age-matched cohorts, and completing experiments and processing all samples at a single location.

Assessments of EGFR pathway activity

The main assessment of the ADAM10/17-AREG-EGFR pathway was through measurement of expression and content of individual components as well as phosphorylation of EGFR. While this may give an overall representative image of pathway activity, other means and methods of assessment must be considered. Given that hepatocellular ADAM10 and ADAM17 activity itself is

what drives release of AREG, the quantity of mRNA or protein present in samples may not be able to accurately represent the molecular crosstalk that is occurring outside of transcription or translation. Rather, activity assays, such as the one used in **Fig 2.10 E and H**, would potentially be more meaningful when interpreting these data. However, much like measuring Westerns or qRT-PCR, these assays would still provide only a singular snapshot of sheddase activity if used *in vivo*, and it is unclear at this time how RECK activity may change over time in response to a WD. Furthermore, as demonstrated previously in terms of differential RECK expression in various hepatic cell populations, it is unknown what the relative expression of the ADAMs are within these same groups, and how the activity of these peptides may differ between them. Thus, results may again be skewed by differential ADAM activity between hepatic cell types.

Second, assessment of phosphorylation of EGFR occurred at two sites: Tyr1068 and Tyr1173; however, EGFR has several other phosphorylation sites, and the presence or absence of phosphate groups at one site may alter signaling based in conjunction with another site (149). Thus, there are several combinations and expression patterns of downstream signaling cascades which may turn differentially alter the expression of various genetic markers. Assessing downstream genetic targets of EGFR activity (such as PI3K or RAS/RAF/MEK/ERK phosphorylation) may provide further insight, though, again, this may only serve to indicate that a specific component of downstream EGFR signaling is occurring in any given cell-type, and that other mechanisms are inactive or inhibited that are masked or unmasked in whole tissue. Thus, it is

difficult to obtain a complete picture of EGFR activity and its consequences in NAFLD, and a further understanding of EGFR signaling in NAFLD progression is needed. Though this is beyond the scope of this study, we are interested to see work completed by other groups in this area.

FUTURE DIRECTIONS

Are the effects of RECK mediated by mechanisms outside of the ADAM10/17-AREG-EGFR pathway?

Though the proposed mechanistic hypothesis here, pertaining to how RECK influences NAFLD progression, is based upon cell culture data and proteomics analysis, many other pathways could be involved outside of the ADAM10/17-AREG-EGFR axis. RECK manipulation influences hepatocyte AREG secretion, but ADAM10 and ADAM17, as well as the classic RECK targets, MMP2 and MMP9, have many roles beyond EGFR-ligand release.

For example, ADAM10 and ADAM17 are also involved in the Notch pathway. The Notch signaling pathway is a complex, highly conserved juxtracrine signaling system allowing neighboring cells to influence the expression of various peptides in cells expressing the appropriate Notch receptors (150). When Notch-ligands bind to their appropriate receptors, the ligand applies a ‘pulling force’ resulting in a conformational change in the receptor itself and exposing cleavage sites for ADAM10 and ADAM17. Once this cleavage occurs, the remaining downstream processes of Notch signaling can

proceed. In hepatocytes, Notch signaling is important in glucose and lipid metabolism (151). Overt activation of the pathway, however, can lead to steatosis, metabolic dysregulation, and hepatic fibrosis. Indeed, Zhu, et al. have shown that inhibiting the Notch signaling cascade in hepatocytes can modulate fibrosis in the liver in an animal model of late-stage NASH (93). Furthermore, Notch signaling in hepatic stellate cells is a key component of their activation (117). Thus, RECK may be inhibiting Notch through ADAM10 and ADAM17 and indeed, it has already been demonstrated that this process occurs in neuronal tissues (59). Therefore, it is possible that RECK's inhibition of Notch may be driving its hepatoprotective effects.

Furthermore, ADAM10 and, particularly, ADAM17 are major sheddases in the release of several proinflammatory cytokines TNFα, IL1, IL6, and IL15 (152, 153). Additionally, these proteases also are involved in cell adhesion of leukocytes during inflammation. Both are responsible for shedding of CX3CL1 and CXCL16 proteins, potent chemoattractant peptides involved in the recruitment of various leukocytes (154, 155). Therefore, ADAM10 and ADAM17 play important roles in the progression of inflammation outside of the AREG-EGFR pathway, and further examination of these signaling mechanisms in the context of RECK is warranted.

Other ECM modulating-peptides, such as MMP2 and MMP9 could also be involved in RECK's control over NAFLD pathophysiology. MMP2 and MMP9 are specialized regulators of the ECM and together are considered gelatinases – so named for the ECM substrate they were initially found to cleave (156). However,

they have also been shown to liberate inflammatory cytokines and chemokines (such as TNFα) from cell-surface anchors (72), thus capable of contributing towards the progression of NASH. RECK direct inhibitory regulation of these gelatinases is well-documented (54, 69) and therefore is another potential source of its hepatoprotective effects.

These areas require further research, such as assessment of downstream Notch activity in RECK-manipulated animals, or broad examination of gelatinase activity. Manipulation of either Notch signaling or gelatinase expression in the presence or absence of RECK may shed additional light on their involvement in NAFLD pathophysiology.

How does RECK manipulation in other hepatic cell types alter NAFLD outcomes?

Studies here focused exclusively on RECK manipulation in hepatocytes, primarily due to the abundance and functional role they serve in the liver. However, RECK is ubiquitously expressed, and it is possible that manipulating RECK in other cell lines may also prove meaningful in the progression of NAFLD. Indeed, when comparing RECK expression in key hepatic cells, hepatocytes themselves express lower RECK levels than hepatic nonparenchymal cells or HSCs (**Fig 2.4**).

HSCs, the main source of ECM in the liver, under homeostatic conditions, are considered inactive and relatively inert, residing primarily in the Space of

Disse in liver sinusoids. However, during times of hepatic insult and stress, HSCs activate, and produce an abundance of ECM material. As RECK could be considered a key ECM regulator, it is unknown what RECK's manipulation on the HSC could mean in the context of its activation and, thus, in NAFLD. Indeed, future experiments targeting RECK in HSCs are already underway. We have acquired a transgenic mouse strain that expresses Cre-recombinase specifically in hepatic stellate cells and intend to cross these animals with our $RECK^{fl/fl}$ and CAG-$RECK^{+}$ mice to produce animals that have germline deletion and overexpression of RECK specifically in HSCs (respectively). Current hypotheses state that RECK manipulation in HSCs would have similar effects to RECK manipulation in hepatocytes, though potentially more so (or only) on the fibrogenic portion of the disease. However, as recent literature suggests that HSCs may also influence inflammatory processes as well within the liver (157, 158), and therefore RECK in HSCs may influence not only fibrogenesis but inflammation as well.

Similar experiments are planned targeting RECK in Kupffer cells – a nonparenchymal resident hepatic macrophage of the liver. Utilizing cell-specific Cre-recombinase to assess the effects of RECK manipulation in a Kupffer cell (Clec4c-Cre) population as well as AAVs specifically designed to transfect KCs will aid in understanding how RECK in KCs impacts NASH development. As KCs are a primary initiator of the immune response in the liver, as well as activator of HSCs, our hypothesis states that altering RECK in these cells may have a robust effect on limiting inflammation and fibrosis in the livers of WD-fed

mice. However, it should be noted that recent literature examining the role of KCs in the liver and NAFLD, have found that while these cells are actually better suited to the injurious environment presented in the face of excessive hepatic steatosis (159, 160), the limited population of KCs are replaced over time by myeloid-derived macrophages (which lack the same antioxidant capacity of the KCs) throughout the disease progression. Therefore, it may be that manipulating RECK in these cells may prove useless in later disease stages as KCs have already been replaced by other macrophages. Furthermore, RECK may serve to play a negative role in KCs, as KC-MMP9 has been demonstrated to play a role in fibrosis resolution in NAFLD (106). Therefore, RECK-mediated MMP9 inhibition may reduce fibrosis resolution in the liver. Thus it is unclear whether targeting RECK in KCs would have positive or negative impact on NAFLD progression.

Lastly, another cell of interest specific to the liver is the liver sinusoidal epithelial cell (LSEC). These cells play a critical role in the development of NAFLD, not only for the liver, but for the entire body as well. Under normal conditions, LSECs are fenestrated, which allows for blood flowing from the GI tract through the liver, to be exposed to hepatocytes for filtering. However, upon hepatic insult, these cells defenestrate – meaning gaps between the cells close – as a means of protecting hepatocytes from potentially toxic and damaging agents that are absorbed from the GI. These components may then enter the systemic blood stream however, damaging distant organs, while also, hepatocytes receive a diminished blood supply, limiting the absorption of necessary nutrients and

supplies, and decreasing the excretion of waste and toxic intermediaries. Thus, defenestration of the LSECs may also cause damage to hepatocytes alone. This fenestration/defenestration process necessitates extensive ECM remodeling, and what role RECK may play in this process is unknown and could warrant further exploration a well.

Given the position of RECK as ECM regulator, it can be postulated that it plays a major role in cellular crosstalk between populations and types of cells. Therefore, examining the effects on how RECK manipulation influences these signals and the matrisome itself, is an important next step in furthering understanding of its function.

Does RECK manipulation alter the progression of hepatic fibrosis/inflammation in other diseases? In other tissues involving inflammation/fibrosis?

Fibrogenesis is an important component of disease across several organs and can be triggered by a multitude of processes (161). In conditions of obesity, similar to the liver, adipose tissue becomes inflamed and fibrotic, leading to a worsening of insulin resistance and glucose homeostasis (162). Both cardiac and renal fibrosis have also been strongly associated with obesity and metabolic dysfunction (101, 122, 163). Therefore, this raises the question of whether RECK manipulation in these extra-hepatic tissues might also have a similar effect in dampening inflammation and fibrosis? As is already demonstrated, RECK

expression in cardiac fibroblasts is closely correlated with fibrosis (102-104), and so future studies may examine this relationship in the context of NAFLD. Thus, further work is necessary examining RECK's in diseases outside of the oncology realm and focus instead on the basic processes of inflammation and fibrosis.

Within the liver, another significant disease resulting in inflammation and fibrosis is alcoholic fatty liver disease (AFLD), which has remained a consistent syndrome in approximately 5% of Americans (164). Though the origin of the initial insult is different (mediated instead through ethanol's metabolism by alcohol dehydrogenase and cytochrome P450 2E1 resulting in toxic acetaldehyde), AFLD still results in hepatic steatosis, inflammation, and fibrosis (141). Interestingly, a recent study has emerged linking a reduction in hepatoxicity associated with AFLD via inhibition of EGFR (165), similar to studies previously cited here that have found EGFR inhibition effective against the progression of NAFLD (99, 100), and similar to what we have proposed here in the context of NAFLD. Therefore, RECK may be a suitable modulator of AFLD pathophysiology and treatment as well, and in doing so may mediate its effects through the ADAM10/17-AREG-EGFR pathway proposed here. Further studies on its ability to alter disease course is therefore warranted.

RECK has been previously examined extensively in oncological research and has been shown to prevent metastasis of tumors. A consequence of all tumor formation is alterations within the ECM (43), and thus RECK's involvement (or lack thereof) may play a critical role. RECK has already been explored in the context of hepatocellular carcinoma aggressiveness and metastasizing ability

(58, 60-62, 65-67, 166). However, there are currently no studies examining the mechanistic role of RECK in primary HCC growth. As HCC is not an uncommon sequela of advanced NASH, and as RECK is downregulated in NAFLD (demonstrated by studies here), is its downregulation is linked towards HCC development? That is, could RECK prevent the transition from advanced NASH to HCC primarily, through prevention of deterioration of the ECM into a pro-tumor environment? Indeed, EGFR inhibition has already been linked to reduced HCC development (167). Therefore, further studies are necessary to examine the potential for RECK to alter this later stage NAFLD consequence, and correlative studies examining RECK expression in NAFLD prior to, and following, HCC development may provide a deeper understanding. Potential future studies using, for example, the CCl_4-induced model of HCC in RECK-transgenic mice may also lead to mechanistic insights as well.

Is RECK involved in cellular processes outside of sheddase/MP inhibition?

It has been well-established in the literature that RECK directly inhibits protease activity on the cell-surface, including MMP2 and MMP9, and ADAM10 and ADAM17 (as discussed previously throughout this dissertation). What has thus far been neglected is what other cell-surface proteins RECK may be binding to and interacting with. Survey studies, such as co-immunoprecipitation examining RECK-binding partners would help identify other novel targets and pathways. Regardless, it must be considered that RECK may be involved in cellular processes outside of direct inhibition of cell-surface proteins. For

example, a 2007 study by Miki, *et al.* proposed a role for RECK in endocytosis of its target peptides (51), though no follow-up on RECK's endocytic capabilities followed. It may be possible, for example, that RECK's regulation of cell-surface proteins is not, as we may suppose, a consequence of direct binding-interactions, but rather a regulatory system whereby RECK is able to target membrane proteins for endocytosis. This would have the obvious consequence of preventing membrane proteins from enacting their typical surface functions, thus altering the ECM and cell-cell signaling pathways. This could fit with data presented here, i.e., why changes in amphiregulin release and ADAM10/17 activity, without any changes (or minimal changes) in mRNA or protein expression are observed. Thus, exploring the effects of RECK manipulation on endocytosis, through studies, for example, utilizing pulse-chase assesses with cell-impertinent probes, would allow for assessment of how RECK manipulation may influence endocytosis of cell surface peptides.

RECK is also positioned to exert influence on the ECM at large, i.e. the overall composition and activity of the matrisome; we have already demonstrated here that RECK alters pathways that are present and plays a role in structural ECM regulation. However, the matrisome is more than just a scaffold for cells; it can serve as a repository as well for several various growth factors, changes can provide methods of communication between neighboring cells, and it can allow for ingress or egress of various cell types or secondary messengers (35). Therefore, further work using isolated ECMs could provide additional insight into how the matrisome is impacted in the absence or presence of RECK. This in

turn may have important consequences in the progression of disease. For example, tissue fibrosis is heavily dependent upon orientation of fibrin strands (168, 169); therefore, assessing quantity or expression of fibrin and collagen may not be as useful as determining the orientation of protein strands. Studies observing how RECK may influence such a characteristic would prove useful in understanding how RECK controls ECM homeostasis and development throughout disease.

What other components might be involved in the ADAM10/17-AREG-EGFR pathway?

RECK is sufficient to alter NAFLD disease course and RECK may do so through its influence over the ADAM10/17-AREG-EGFR signaling cascade. Given literature already indicating that inhibiting EGFR signaling does prove clinically useful in prevent a NAFLD phenotype in WD-fed rodents, alternative sources of EGFR control should be considered. Other regulators may prove to be even more efficacious than RECK in modulating EGFR signaling and would therefore be even more fruitful in exploring as therapeutic targets in the treatment of NAFLD. There are several upstream components involved in the EGFR signaling cascade; EGFR itself has five well-established ligands. Thus, future experiments could begin with manipulating each of these five ligands alone and in concert with others (as different ligands are able to induce a variety of changes when bound to the EGFR-receptor-domain and thus impact the pattern of EGFR phosphorylation and downstream activity) (149). This could lead to potentially

new therapeutic targets beyond RECK that may be able to change the disease course of NAFLD.

Furthermore, downstream components of EGFR signaling may also be targeted for a more refined approach towards managing NAFLD. EGFR signaling is important in many hepatic cell types in the context of NAFLD, and thus manipulating this cascade in individual cells may serve to effectively minimize inflammation and fibrosis in the management of the disease. Future studies need to address the downstream EGFR signaling cascade and how alterations in this pathway can affect disease outcomes.

CHAPTER 4: Expanded Literature Review

Mechanisms of RECK's Involvement in NASH

Experiments here have demonstrated that manipulating the RECK protein is sufficient to alter progression of NASH. Decreased RECK levels correlated with increases in fibrosis and inflammation in the livers of human patients with NASH, as well as in rodent models of obesity. RECK overexpression significantly attenuated WD-induced inflammatory and fibrotic pathways. Further understanding however, is needed in determining the mechanisms through which RECK may confer this protection on the liver against NASH progression. In the previously described experiments, we have attempted to establish a connection between RECK, the sheddases ADAM10 and ADAM17, AREG, and EGFR – the activity of which has previously been linked to NASH progression. Further examination of this relationship, as well as others, in this section, is intended to thoroughly investigate mechanisms through which RECK is likely able to ameliorate NAFLD progression. In addition, upstream regulation of RECK itself, alternative RECK isoforms, and the implications of RECK as a potential therapeutic agent are examined as well.

Amphiregulin and EGFR in NAFLD, and Interactions with RECK

One area of interest in the study of NAFLD is the activity of EGFR, as its activation is already known to be increased in patients with NAFLD (98). Furthermore, inhibition of EGFR activity has already been shown to reduce liver fibrosis and steatosis (99, 100, 167). EGFR has five known ligands; one of which

is AREG (170). AREG is upregulated in human patients with NAFLD (137), as well as rodent models of NASH, without an increase in other EGFR ligands, such as EGF or TGFα (99).

RECK activity is negatively correlated with EGFR transactivation (94). This is primarily accomplished through RECK's inhibition of ADAM10 and ADAM17 (59, 88, 171), which are required for to release membrane-bound EGFR ligands, including AREG (120, 170, 172-176). Binding of EGFR to one of its ligands leads to activation of three major signaling pathways: the RAS/RAF/MAPK pathway, the PI3K/Akt pathway, and the JAK/STAT pathway. PI3K/Akt signaling is known to upregulate SREBP-1, a master transcriptional regulator of fatty acid biosynthesis, and eventually promotes *de novo* lipogenesis and decreases fatty acid beta-oxidation (177). Furthermore, inhibiting EGFR has been shown to improve glucose tolerance (140), and as glucotoxicity has been shown to be a means through which NASH develops (178), this may be another avenue through which EGFR inhibition may be beneficial in the context of NASH. As all EGFR downstream pathways promotes cell survival (179), in the context of activated HSCs and inflammatory cell types, this would reduce survival and proliferation of these cells, allowing for a cessation of the profibrotic and proinflammatory processes inherent in NASH.

Beyond liberating EGFR ligands from cell surfaces and allowing for juxtracrine EGFR signaling, RECK may also inhibit EGFR transactivation more directly through regulation of MT1-MMP/MMP14. It was initially established that MT1-MMP is able to, in the presence of sphingosine 1-phospate, stimulate EGFR

transactivation (180, 181). RECK has demonstrated the ability to inhibit MT1-MMP on the cell surface (39), thus, through a potential secondary method, inhibit EGFR activity.

The Role of the Notch Pathway in NAFLD, and Interactions with RECK

Another area of interest is the role of the Notch pathway in NAFLD, and the potential for the influence of RECK. The Notch signaling pathway is a complex, highly conserved pathway first identified in *Drosophila* (150, 182). A juxtracrine signaling system, the Notch pathway allows neighboring cells to influence the expression of various proteins in cells exposing the appropriate Notch receptors. Specific Notch ligands on the signal-sending cell bind to receptors on the signal-receiving cell. The ligand applies a 'pulling force' to the receptor resulting in a conformational change in the receptor itself, exposing the cleavage site for ADAM10 and ADAM17. Following this cleavage (known as the S2 cleavage), an additional cleaving event occurs within the cell by γ-secretase (the S3 cleavage). This releases the Notch intracellular domain (NICD) to translocate into the nucleus and form a complex with the RBPJ (recombination signal binding protein for immunoglobulin κ-J region, also known as CSL), displacing the co-repressor proteins bound to RBP-κJ, and thus allowing for transcription of downstream Notch signaling products (150).

Canonical Notch signaling is mediated by four receptors (NOTCH1-4) and five ligands (Delta-like 1 (DLL1), DLL3, DLL4, Jagged 1 (JAG1) and JAG2) (150,

182). While some redundancy exists between the binding of various ligands with receptors, there is also some level of specificity to each pairing. For example, in the liver, JAG1-NOTCH2 signaling is key for biliary specification and development (151, 183).

In hepatocytes, Notch signaling is important in glucose and lipid metabolism (151, 184). Notch signaling coordinates with FoxO1 to regulate hepatic glucose metabolism (185-187). In patients with insulin resistance, there is a positive correlation with Notch activity and gluconeogenesis (188). Notch also activates mTOR, which in turn induces *SREBP-1c* and promotes lipogenesis, thus contributing to the development of steatosis in the liver (189). Over activation of these pathways lead not only to steatosis, but also dysregulated metabolism, suggesting a role of Notch in the development of metabolic diseases in the liver – all of which contributes further to inflammation and fibrosis. And indeed, the number of hepatocytes expressing a major Notch outcome product – Hes1 – is significantly higher in human patients suffering from severe NASH (93). Therefore, inhibiting Notch in hepatocytes should prove efficacious in treating metabolic dysregulation in the liver. In line with this idea, levels of steatosis are reduced in high-fat-diet (HFD) fed RBPJ-KO mice (190). Furthermore, Zhu, et al. showed that inhibiting Notch signaling in the hepatocyte can modulate fibrosis in the liver in an animal model of hepatic fibrosis. Their group showed that Notch-specific deletion in hepatocytes attenuated NASH-associated liver fibrosis, whereas forced hepatocyte activation of Notch, in both control- and NASH-diet

animals, saw increased activation of hepatic stellate cells – ultimately contributing to liver fibrosis (93).

Though there are advantages to decreasing Notch activity in the face of metabolic disease and chronic inflammation and fibrosis in the liver, Notch also plays a protective role in ischemia/reperfusion injury through the repression of ROS (191). Whether this protective mechanism exists throughout other forms of liver injury is currently unexplored. Notch signaling is also key in liver repair following insult, specifically in hepatic progenitor cells (HPCs), where signaling is necessary for the differentiation into cholangiocytes and reconstruction – as well as maintenance - of the biliary tree (192). Therefore, though some evidence suggests limiting Notch activity may prove beneficial in a mature liver, an overt reduction of the pathway in hepatocytes may prove detrimental, and other potential targets should be considered.

Additional targets, and the main source of ECM components such as fibrin and collagen in the liver, are the HSCs. These cells constitute about 5-8% of the total liver cells and under quiescent conditions store Vitamin A in lipid droplets. In situations involving liver injury however, these cells activate, becoming proliferative, fibrogenic myofibroblasts. As noted previously, activation of HSCs, classically, is mediated through TGF-β signaling (115). TGF-β has recently been shown to help drive fibrosis by promoting Notch activity, and ultimately activation, in isolated mouse HSCs, while pharmaceutical Notch inhibitors prevented TGF-β -mediated HSC activation(116). Furthermore, pharmaceutical Notch inhibition in an animal model of fibrosis significantly limited activation of HSCs and

subsequent hepatic fibrosis (117). Lastly, in fibroblasts, Hes1 protein has been shown to bind to and promote *Col1A1* and *Col1A2* transcription – the genes that generate type I collagen (118); whether this holds true in HSCs is not established, however. As reducing HSC activation and subsequent ECM production is a significant step towards reducing hepatic fibrosis and amplification of the inflammatory process, finding regulators of such processes as Notch activity, is critical.

It is believed that RECK inhibits ADAM10 and ADAM17 activity through direct binding of these proteases (59, 83). ADAM10 and ADAM17 are necessary for progression of the Notch signaling cascade. Once the signal-sending cell binds to the signal-receiving cell, endocytosis of the Notch receptor reveals a cleavage site for these ADAMs just outside of the cell membrane. Without this critical step, noted as the S2 cleavage, subsequent steps are blocked, and the NICD – the component that induces transcription of Notch products – cannot be released from its membrane-bound anchor to translocate into the nucleus. Therefore, RECK would theoretically inhibit the Notch signaling pathway preventing downstream transcription of target genes, and indeed this has proven to be the case both in developing neural tissues (59) and in angiogenesis (88). However, what remains to be elucidated is whether RECK can inhibit Notch activity in HSCs and whether this is significant in liver disease and the progression of hepatic inflammation and fibrosis. Though exploration of this process is beyond the scope of this study, it worthwhile to note this alternative mechanism of RECK's influence in NAFLD and NASH.

RECK and the Regulation of MMP Activity in NAFLD

MMP2 and MMP9 are specialized regulators of the ECM and together considered gelatinases – so named for the ECM substrate they were initially found to cleave (156). Functionally however, they perform a much wider range of functions than the cleavage and breakdown of collagens. They also cleave and/or process an array of cytokines, chemokines, ECM receptors, and can have more of a 'processing' than 'degradation' role in the ECM (105). As such, they have been shown to cleave inflammatory cytokines and chemokines from cell-surface anchors (such as TNFα (193)), which then allows for these peptides to perform paracrine functions (72). In addition, MMP2 is known to promote the processing of pro-MMP9 to the active form of MMP9 outside of the cell, indicating that the gelatinases do serve self-regulatory functions as well (194). Due to their influence in the ECM and processing of various cytokines and chemokines, these gelatinases have been investigated in the realm of inflammation and fibrosis.

The gelatinases are linked through several mechanisms to the development and progression of inflammation. First, through their cleavage of membrane-bound proinflammatory cytokines allowing for release and ultimately promoting inflammation in the microenvironment surrounding the cells (72, 195). They have been found to increase the activity of proinflammatory cytokines, including TNFα (193, 196) and IL1β (195). Perhaps of more importance, the gelatinases are critical in the remodeling of the basement membrane of the endothelium, allowing for both the ingress and egress of immune cells (197).

While the liver sinusoidal endothelial cells do not produce and maintain a basement membrane under normal homeostatic conditions, upon injury to the liver, they begin to secrete collagen type IV, creating a basement membrane and separating the sinusoidal lumen from the liver parenchyma (198). The gelatinases then are critical in regulating the basement membrane to allow for translocation of leukocytes into the liver, thus promoting an inflammatory environment (105). Indeed, in the brain, MMP2 and MMP9 are required to allow for white blood cell penetrance into the tissue parenchyma (72) – animals in which both are knocked-out are found to be immune to certain experimental forms of auto-immune encephalitis (199). And while the implications for their importance in regulating the blood-brain-barrier is clear, gelatinase activity has been linked in leukocyte migration in other body tissues as well (200, 201). For example, in an animal model of peritonitis, it was found that mice lacking plasminogen, a potent activator of MMP9, lacked any significant macrophage penetrance in a model of peritonitis; adding activated MMP9 directly to these mice rescued this phenotype (202). Conversely, in a rodent model of inflammatory lung disease, MMP2-deficiency was noted to have an impeding effect on leukocyte egression from the tissue (73), suggesting that MMP2 may play a role in resolution of inflammation. However, given MMP2's role in activating MMP9, and MMP9's clear role in promoting inflammation, it could be considered that MMP2 does indirectly promote inflammation. Given this complicated relationship, it is less clear what role these MMPs have in the liver and how they may promote inflammation.

Elevated and chronic levels of inflammation lead to pathological accumulations of fibrin and collagens, ultimately resulting in fibrosis and, in the case of the liver as scar tissue replaces normal parenchyma, cirrhosis. As gelatinases are intimately involved in the remodeling of the ECM, they have been extensively studied in the context of hepatic fibrosis (105-109). During hepatic fibrogenesis, collagen deposition from HSCs is markedly increased, and MMP2 and 9 are both highly upregulated (110). MMP2 is expressed by HSCs, and is an autocrine proliferator and activator of HSCs (111), promoting further ECM deposition contributing to fibrosis. Indeed, serum MMP2 levels can be used diagnostically to assess the level of liver fibrosis in human patients (112). Furthermore, there existed a significant correlation between serum MMP2 concentrations and liver function (as assessed via bilirubin and albumin production, as well as prothrombin time) (113). Serum MMP9 was not as strongly correlated with liver disease in the context of fibrosis in these studies, but this may be due to its local roles in promoting inflammation and ECM remodeling within the liver itself. And while both gelatinases are upregulated in the context of fibrogenesis, it has also been shown in an animal model of hepatic fibrosis that whole body knock-out of MMP2 exacerbates fibrosis (114). This suggests a complicated relationship exists between gelatinase function and activity in the context of hepatic inflammation and fibrosis. While completely ablating these MMPs may exacerbate clinical signs, regulating their activity in the ECM may prove to be an efficacious means of modulating hepatic inflammation and fibrosis. Though it seems counterintuitive to reduce the enzymes

responsible for breaking down ECM when an abundant and dysregulated ECM is the problem, several key points from aforementioned studies must be considered. First, production of these ECM components would not occur if the liver was not under a largely chronic and dysregulated inflammatory condition – of which these gelatinases contribute to through the processing and release of proinflammatory cytokines. Second, these gelatinases serve several functions, one of which is to activate HSCs as has already been described. As HSC activation leads to ECM production, this is another mechanism by which they contribute to the progression of fibrosis. Third, the gelatinases are integral for leukocyte migration into tissues, which further promotes and exacerbates a proinflammatory environment; limiting access of leukocytes into tissues may help limit hepatic inflammation. Lastly, while whole body knockouts of MMP2 have been shown to exacerbate hepatic fibrosis in animal models, a more targeted approach at limiting gelatinase activity could have a different effect in models of liver disease.

RECK is a well-established post-transcriptional inhibitor of MMP activity (specifically MMP2, MMP9, and MT1-MMP) via direct inhibition of protease activity, as well as regulation of cellular release and possible sequestration of the MMPs at the cell surface (37, 54, 69). As MMPs are required to exit the cell to undergo extracellular processing to perform their proteolytic actions, RECK may also be considered to inhibit their pro-enzyme forms as well. Additional evidence supports the idea that RECK can inhibit MMP9 at the gene expression level as well (69). It should be noted however that there exists some debate within the

scientific community regarding RECK's regulation over the gelatinases (70), though the vast majority agree RECK plays an inhibitory role over the activity and expression of these peptides. RECK's regulation over MMP2 and MMP9 has been examined very little in the liver; however, to date only one study has found that promoting RECK activity (through induction of FXR) correlated with a decrease in MMP9 activity within the liver (203). And while we have supplied preliminary data showing upregulation of RECK expression correlated with a significant reduction in MMP9 expression, and that a downregulation of RECK expression correlated with a significant increase in MMP2 expression, further studies are needed. The impact that this might have on clinical disease within the liver has not yet been established.

Alternative RECK Targets and NAFLD

Evidence examining the involvement of RECK in alternative pathways, which may have a bearing in the pathogenesis of NASH, must be considered. One such pathway is the Wnt/b-Catenin signaling pathway. Several studies have been conducted examining the role of RECK in modulating the Wnt pathway, generally finding that RECK is a positive effector of the Wnt pathway (204-206). Interestingly, in conjunction with their studies examining Wnt signaling, Mahl, et al. found that human mesenchymal stem cells would migrate significantly faster in RECK-depleted cells, which is mediated through RECK's effects on the Wnt-signaling pathway (207). This has implications as well in the context of NAFLD, as activated HSCs migrate through liver tissue depositing collagen and fibrin,

leading to liver fibrosis and cirrhosis (4). Mechanistically, evidence supports RECK's effect on the Wnt signaling pathway by specifically binding directly to, and stabilizing, the ligand Wnt7, thus allowing for binding to Gpr124 before presenting the Wnt7 ligand to the Frizzled Receptor and allowing for canonical Wnt signaling (208, 209). However, it is unclear what, if any, impact this has on the development of NASH. Canonical Wnt signaling has been correlated with the development and progression of primary liver cancers, as well as implicated in the progression of NASH and liver fibrosis (144). As the Wnt pathway consists of several ligands and receptors, and is capable of canonical and non-canonical activity, further examination of this pathway and RECK's involvement would be necessary to determine what effects this has on the development of NASH.

RECK has also been shown to interact with ADAMTS10. Similar to ADAM10 and ADAM17, ADAMTS10 is a metallopeptidase, however it is a member of the ADAMTS family – unique from the ADAM family in that ADAMTS peptides are exclusively secreted from cells and do not contain membrane-anchorage sites. Recent studies have found that RECK binds to and colocalizes with ADAMTS10 whereby reciprocal activity occurs – RECK appears to stabilize ADAMTS10, while ADAMTS10 has demonstrated a weak ability to cleave RECK in a dose-dependent manner *in vitro* (210). Furthermore, RECK appears to only be capable to alter the gelatinase/collagenase activity of MT1-MMP/MMP14 only in the presence of ADAMST10 (211), and this may also be the case with other MMPs as well. Interestingly, a recent study has emerged that found that ADAMTS10 is upregulated in human liver samples of patients with NASH and

liver fibrosis, but not in cases of simple steatosis (212). Taken together, this may demonstrate ADAMTS10 as being upstream of RECK, and, when it is upregulated, may inhibit RECK's ability to modify its own downstream targets and thus allow for progression of NASH and fibrosis in cases of fatty liver disease. Further research is necessary examining this potential signaling crosstalk.

RECK has also demonstrated an ability to interact with CD13/Aminopeptidase N/Alanine aminopeptidase, inhibiting the proteolytic activity of CD13 in a cholesterol-dependent manner (51). RECK and CD13 have been found to be colocalized on the cell-surface *in vitro* (213), and in a proteomics study examining the livers of high-fat diet-fed mice, CD13 has been found to be upregulated (214). CD13 has a myriad number of functions relating to cholesterol uptake and regulation, peptide cleavage, endocytosis and cell membrane homeostasis, as well as cell-cell signaling (215, 216). It has been designated a protein of interest in oncological studies, and research is currently ongoing examining the inhibition of this peptide in a variety of cancers (215). It is unknown how much of an impact CD13 has on the progression of NASH and thus, what RECK's influence on this peptide could mean in the context of NAFLD is unclear. Further studies examining the role of CD13 in NASH would be necessary to elucidate the implications of RECK's inhibition of it.

Targeting RECK in the Treatment of Disease

RECK plays a regulatory role in the management of the ECM and shown to be protective in the context of various cancers, and, here, in the pathogenesis of NASH. This highlights multiple disease states in which an upregulation of RECK may be considered therapeutic, and indeed, upregulation of RECK has been proposed and achieved through various means. For example, HDAC inhibitors and nonsteroidal anti-inflammatory drugs upregulate RECK both *in vitro* and *in vivo* (217), however these agents are non-specific and can have several off-target effects. More recent studies examining means of inducing RECK have attempted more specified targeting of RECK using pharmaceutical agents. Empagliflozin, which has been used to improve glucose regulation in people with type 2 diabetes, is correlated with inducing RECK (218, 219). Several agents have been examined in downregulating or inhibiting peptides, miRNAs, or other mechanisms that in turn lead to an inhibition of RECK. Dihydroatemisinin (DHA), a semi-synthetic derivative of artemisinin, inhibits miR-21, which in turn inhibits RECK activity, thus, treatment with DHA increases RECK activity (220). Furthermore, DHA mitigates migration and invasion of ovarian cancer cells *in vitro*, demonstrating that elevating RECK levels may have therapeutic relevance (221). Casticin demonstrated the ability to inhibit the methylation of RECK *in vitro* in a line of gastric cancer cells, significantly increasing both protein and mRNA content (222). In HCC cell lines, salvianolic acid B, a caffeic acid phenethyl ester analog, was shown to inhibit mortalin which allowed for upregulation of RECK (223). Oral obeticholic acid (OCA) was associated with a

reduction in MMP2 and MMP9 activity, as well as a recovery of RECK, which was downregulated in response to hepatic ischemia/reperfusion injury (224) in rats. This is particularly interesting, given the proposed use of OCA in the treatment of NAFLD (225, 226); which then raises the question – is OCA working through the upregulation of RECK activity?

Beyond pharmaceutical means of upregulating RECK, a select number of groups are currently exploring alternative methods. Viral vectors used in gene therapy are being assessed and may be a means by which RECK upregulation can be delivered to specific cell types. One study demonstrated a means of upregulating RECK protein content via transfection of double-stranded RNA complementary to the promotor region of the *RECK* gene in a tumor cell line (227). This also had the effect of suppressing MMP expression and activity. Demonstrated here, experiments have manipulated RECK expression *in vitro* using cultured primary hepatocytes, though *in vivo* models relied upon the Cre-lox system to alter *Reck* expression in animal models. A means of viral transduction that overexpresses RECK in the absence of the Cre-lox system would be of interest as the therapeutic implications are still unknown.

Finally, diet and exercise remain the mainstays of the treatment of NAFLD, though what effects, if any, this has on RECK are currently unexplored. Additional studies are needed examining the effects of traditional NAFLD treatments – including calorie restriction, various dietary interventions, different forms of exercise such as high-intensity-interval training – and what these methods do to RECK expression. These methods may have some ability to

induce RECK, though no studies have examined this idea yet. Therefore, additional research exploring the manipulation of RECK through traditional NAFLD treatment would be of great interest.

Alternative Splicing of RECK and Ramifications

It is estimated that as many as 86% of eukaryotic mRNA can be alternatively spliced – a mechanism by which multiple transcripts can be produced from a single mRNA precursor (228, 229). Coordinated splicing networks regulate tissue and organ development, and is critical for cellular homeostasis and function (230). RECK mRNA can also be alternatively spliced, and, to date, several alternatively spliced RECK variants have been identified. Trombetta-Lima, et al. described the first variants in 2015, characterizing RECK-B (consisting of 9 exons with 1,548 base pairs, leading to a 248 amino acid isoform that is 40 kDa) and RECK-I (consisting of 9 exons with 1,101 base pairs, leading to a 220 amino acid isoform that is 37 kDa) (139). This contrasts with canonical RECK, the mRNA of which consists of 21 exons leading to a protein that consists of 971 amino acids and is ~125 kDa. Trombetta-Lima, et al. found that the epidermal growth factor-like and serine protease-like domains, hallmarks of canonical RECK, are not present in the isoforms (139). This is particularly of interest in the functionality of these isoforms, as, specifically, the serine protease-like domain confers the ability to inhibit MMP9 activity, and thus may be required for other canonical RECK functions (36). In addition, Trombetta-Lima, et al. found that RECK-B, *in vitro*, promotes anchorage-independent clonal growth in

multiple cell lines, however, these same cells do not display any modulation of MMP2 or MMP9, indicating that RECK-B may serve to be pro-oncogenic though this activity may not require alteration of MMP activity (139). Lastly, they found that these splice variants do have some level of tissue specificity as well as differences in expression depending on developmental stages (139), suggesting that their expression is tightly regulated.

Other groups have found similar RECK isoforms, all of which are shorter than the canonical version (70, 231-235), though common trends have emerged from these studies. First, these shorter isoforms seem to have the potential to influence fibroblast migration, generally promoting it – as opposed to canonical RECK, which limits fibroblastic migration (231, 232). Second, these alternatively spliced variants tend to be unable to alter MMP activity (70, 231, 232) – another hallmark of canonical RECK. Lastly, the expression of these isoforms may serve as another prognostic indicator in cancer patients as canonical RECK have; specifically, that a higher ratio of isoform expression to canonical expression in tumors is an indicator of poorer patient survival (139, 233, 235). Of note, Vancheri, et al. found a similar observation to the cancer studies, however they examined RECK isoform expression in coronary artery disease patients and concluded that canonical RECK may be protective against atherosclerosis (234). As coronary artery disease and atherosclerosis are associated with inflammatory processes, this adds to the idea that RECK may be protective against such mechanisms, and, potentially, alternatively spliced isoforms of RECK, that may

serve to work against the mechanisms of canonical RECK, could be considered pro-inflammatory.

Taken together, this raises the question of alternatively spliced RECK in liver disease, and how it may affect outcomes in studies such as ours, examining the effects of inflammation and fibrosis in response to a tissue insult. This will require further exploration into specific RECK isoforms, and their levels of expression in comparison to canonical RECK, when considering utilizing RECK as a therapeutic target in NASH.

Regulators of RECK

As the role of RECK has been extensively examined in various diseases and conditions, upstream regulators of RECK are therefore of great importance, as they may be used to indirectly manipulate this peptide. RECK itself is highly regulated by several factors, both at the transcriptional and post-transcriptional levels (44). Several studies examining the role of non-coding miRNAs have demonstrated their ability to post-transcriptionally suppress (236-241), or upregulate (242) RECK. Additionally, a long non-coding RNA sequence known as Growth Arrest Specific 5 (GAS5) has also been shown to upregulate RECK protein expression through via inhibitory binding of miRs-21 and 135b – both promotors of RECK degradation (67, 243).

RECK is also regulated is via promotor methylation. In certain cancers, hypermethylation of the *RECK* promotor region as associated with a reduction in

RECK mRNA and protein content (58, 244). Histone deacetylase (HDAC) activity also regulates expression through histone acetylation of the *RECK* gene (244). As noted previously here, HDAC inhibitors have demonstrated the ability promote *RECK* expression through alteration of Specificity Protein 1 (SP1), and its ability to bind to its promotor (245, 246).

The *RECK* gene also contains two SP1-binding sites in its promoter region, through which activation of SP1 via the extracellular signal-regulated kinase (ERK) pathway can downregulate RECK (138, 247), which allows for regulation via several other signaling cytokines. Angiotensin-II and IL18, both of which activates ERK, suppresses RECK expression via SP1 (103, 104). Additionally, estrogen also suppresses RECK expression in mouse uterine epithelial cells; however, the mechanism is unknown (248). Lastly, farnesoid X receptor (FXR) has been demonstrated to upregulate RECK expression within the murine liver (203). This is of particular interest, as FXR has been established to regulate glucose metabolism, as well as play an anti-inflammatory and suppress fibrosis in the liver (249). As a was previously described, in a recent study examining the efficacy of OCA (a bile acid derivative and potent FXR agonist) in the management of hepatic ischemia/reperfusion injury, Ferrigno, et al. found that hepatic RECK levels were rescued in the group treated with OCA, which correlated with a reduction in MMP2 and MMP9, as well as pro-inflammatory mediators (224). Given the RECK's involvement in regulating pro-inflammatory and fibrotic processes, it may be possible that FXR's well-documented hepatic protection in disease is mediated through its upregulation of

RECK itself. Thus, further studies are needed examining the interaction between RECK and its established regulators, including FXR.

CHAPTER 5: Supplemental Data

Additional Animal Characteristics for 24-Week Fed Mice

No differences were observed regarding body weight over time, final body fat percentage, and final liver wet weight, though there were clear effects of WD feeding on all these characteristics in $RECK^{fl/fl}$ and $RECK^{Hep-/-}$ mice (**Fig. S.1 A through C**). Interestingly, RECK-Hep^{Tg} gained significantly more weight over time as compared to CAG-$RECK^{+}$ animals on a WD, though no difference in weight gain was observed between groups on a CD (**Fig. S.1 D**). Furthermore, RECK-Hep^{Tg} mice had significantly higher final body fat percentage as compared to CAG-$RECK^{+}$ animals, which was driven primarily by the WD-fed group (**Fig S.1 E**). However, hepatocellular RECK-overexpression did not alter final liver mass (**Fig S.1 F**).

Animal Characteristics and Histology for RECK-Manipulated 24-Week Fed Female Mice

In a separate cohort of all female mice fed the 24-week diet, no differences were observed for body weight, body fat percentage (%), liver or visceral fat mass, or heart:body mass ratio (mg/g), though there were clear effects of WD feeding on all these characteristics in $RECK^{fl/fl}$ and $RECK^{Hep-/-}$ mice (**Table S.1**). In female CAG-$RECK^{+}$ and RECK-Hep^{+} animals, RECK overexpression significantly reduced the final body fat percentage as compared to CD-fed control animals despite no significant differences in feed intake (**Table**

S.2). This significant decrease in total body fat was not, however, observed when comparing WD-fed CAG-RECK^{+} and RECK-Hep^{Tg} animals. WD-fed animals, regardless of genotype, displayed significantly elevated liver and visceral fat pad mass, and a significant decrease in heart:body weight ratio.

Following 24 weeks of diet feeding, in female mice, hepatocellular RECK depletion did not significantly exacerbated histological evidence NAFLD, though inflammation was moderately elevated (p=0.11) in the RECK$^{Hep-/-}$ animals; this was reflected in overall NAS score as well (**Fig S.2 A through E**). Interestingly, WD-feeding significantly increased inflammation and fibrosis in female RECK$^{Hep-/-}$ animals only, whereas no significant difference was observed in these parameters between RECK$^{fl/fl}$ animals fed a CD compared to those fed a WD.

In female RECK-Hep^{Tg} and CAG-RECK^{+} mice, WD induced increases in hepatocellular ballooning was significantly attenuated with hepatocellular RECK overexpression in RECK-Hep^{Tg} mice compared to CAG-RECK^{+} controls (**Fig S.3 A and B**). Histological scoring confirmed increased hepatic steatosis, hepatocellular ballooning, and overall NAS score due to WD-feeding (**Fig S.3 C**). Interestingly, inflammation was significantly increased in WD-fed CAG-RECK^{+} as compared to CD-fed controls, while WD-feeding failed to significantly upregulated inflammation in RECK-Hep^{Tg} animals. Furthermore, RECK-overexpression induced a moderate reduction in total NAS (p=0.06), driven primarily by a reduction in WD-fed RECK-Hep^{Tg} animals (**Fig S.3 D**). There were no significant changes in fibrosis in female mice in response to WD-feeding or RECK overexpression (**Fig S.3 E**).

SUPPLEMENTAL FIGURES AND TABLES

	RECK$^{fl/fl}$: CD	RECK$^{Hep-/-}$: CD	RECK$^{fl/fl}$: WD	RECK$^{Hep-/-}$: WD
Final Body Weight (g)	26.6 ± 0.6	26.2± 1.2	38.4 ± 1.4^{D}	40.6 ± 1.1^{D}
Body fat (%)	27.7 ± 1.7	26.7 ± 2.1	45.0 ± 2.1^{D}	47.2 ± 1.6^{D}
Delta change body fat (%)	13.7 ± 1.7	12.5 ± 2.5	32.2 ± 1.9^{D}	33.8 ± 1.8^{D}
Liver (mg)	1104.3 ± 43.1	1148.6 ± 50.5	1751.4 ± 143.6^{D}	2089.8 ± 149.0^{D}
Visceral fat (mg)	1046.5 ± 84.4	970.0 ± 185.7	2628.6 ± 172.5^{D}	2921.3 ± 134.7^{D}
Heart:Body weight (mg/g)	3.8 ± 0.1	4.3 ± 0.1	2.9 ± 0.1^{D}	2.7 ± 0.1^{D}
Average weekly food intake (g)	15.7 ± 0.6	15.7 ± 0.6	16.0 ± 0.5	16.0 ± 0.5

Table S.1: Animal characteristics of female RECK$^{fl/fl}$ and RECK$^{hep-/-}$ mice fed either a CD or a WD for 24 weeks. Data are presented as mean ± SEM (n=5-9/group). D indicates main effect of diet ($p \leq 0.05$).

	CAG-RECK^{+} : CD	RECK-Hep^{Tg} : CD	CAG-RECK^{+} : WD	RECK-Hep^{Tg} : WD
Final Body Weight (g)	23.4 ± 0.9	22.0 ± 0.8	34.1 ± 4.2^{D}	34.2 ± 0.7^{D}
Body fat (%)	21.6 ± 1.6	17.3 ± 1.1^{G}	35.5 ± 1.9^{D}	39.0 ± 1.2^{D}
Delta change body fat (%)	9.5 ± 2.5	6.6 ± 2.1	23.7 ± 1.6^{D}	27.5 ± 1.5^{D}
Liver (mg)	904.5 ± 51.6	869.1 ± 37.2	1729.5 ± 410.6^{D}	1493.4 ± 57.3^{D}
Visceral fat (mg)	668.6 ± 76.4	454.9 ± 43.1	1766.5 ± 343.4^{D}	1952.5 ± 167.6^{D}
Heart:Body weight (mg/g)	4.6 ± 0.1	4.7 ± 0.1	3.6 ± 0.3^{D}	3.5 ± 0.2^{D}
Average weekly food intake (g)	15.2 ± 0.5	15.2 ± 0.5	15.2 ± 0.4	15.2 ± 0.4

Table S.2: Animal characteristics of female CAG-RECK^{+} and RECK-Hep^{Tg} mice fed either a CD or a WD for 24 weeks. Data are presented as mean ± SEM (n=3-5/group). D indicates main effect of diet ($p \leq 0.05$); G indicates main effect of genotype ($p \leq 0.05$).

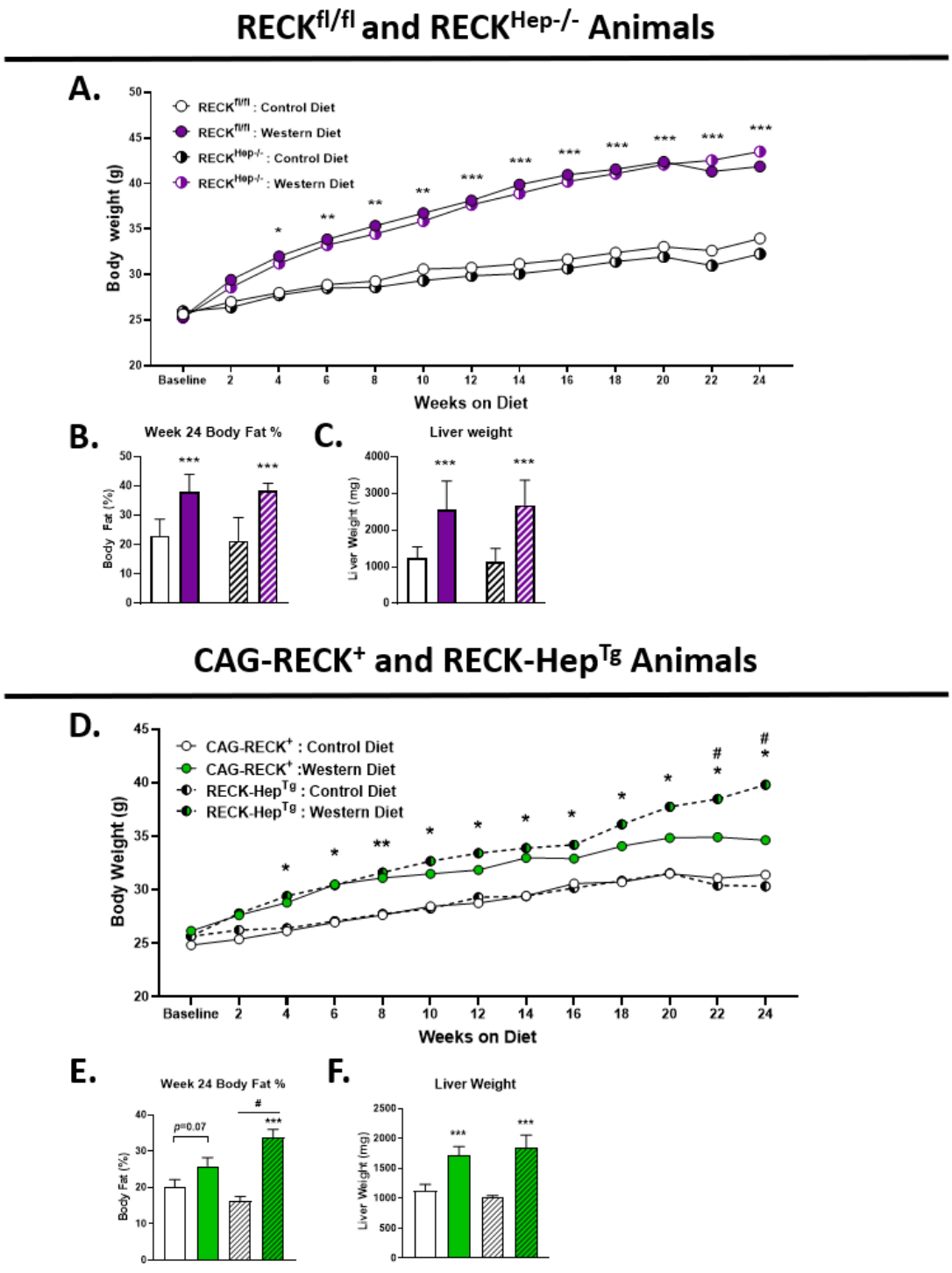

Figure S.1: Animal characteristics of male mice over time. A. Weight gain over time of RECK$^{fl/fl}$ and RECK$^{hep-/-}$ mice fed either a CD or a WD for 24 weeks. B.

Body fat percentage and C. liver weights at completion of the study. For male CAG-RECK^{+} and RECK-Hep^{Tg} mice, D. body weight over time, E. final body fat, and F. final liver weight, are also presented. Data are presented as mean ± SEM (n=7-12/group). * indicates main effect of diet, # indicates main effect of genotype, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

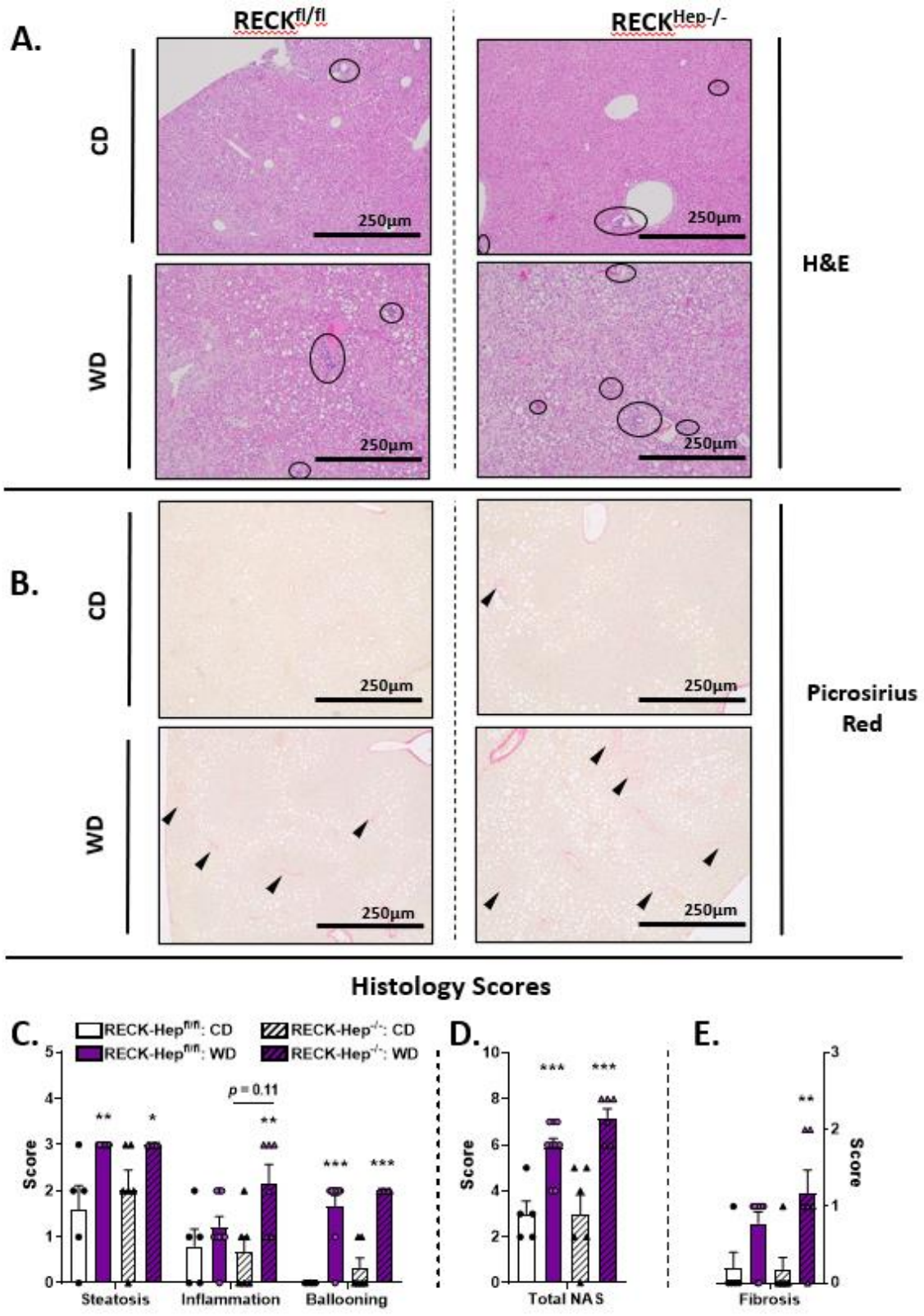

Figure S.2: Effects of germline hepatocellular RECK deletion on female liver histology. A. Representative liver H&E and B. picosirius red staining from the

RECK$^{fl/fl}$ (left) and RECK$^{Hep-/-}$ (right) female mice fed CD or WD for 24 weeks. C. Histological scoring and D. total NAFLD activity scores based on H&E images, and E. fibrosis scores based on PSR images (n=5-9/group). Data presented as mean ± SEM. * indicates main effect of diet, # indicates main effect for genotype. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

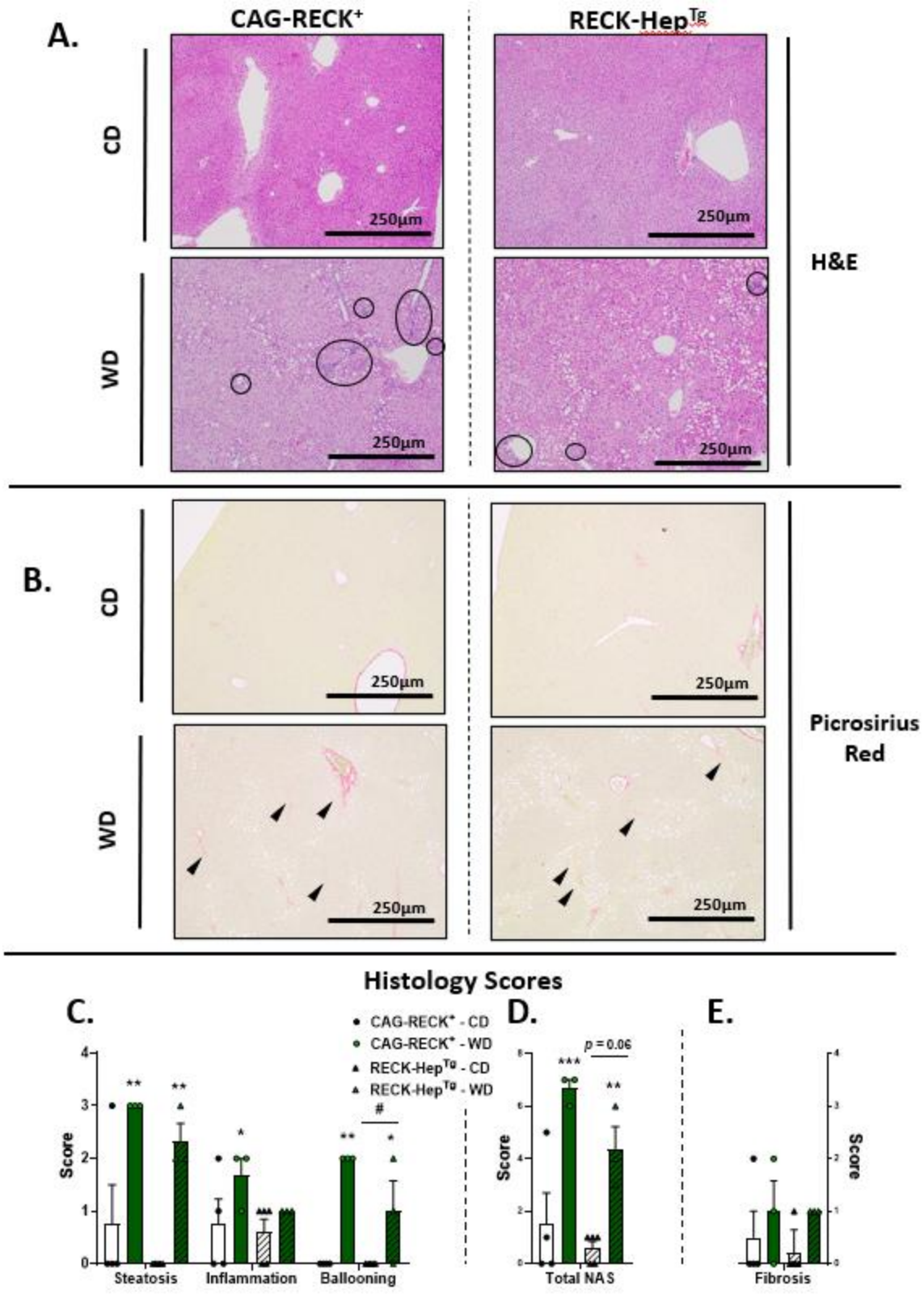

Figure S.3: Effects of germline hepatocellular RECK overexpression on female liver histology. A. Representative liver H&E and B. picrosirius red staining from

the CAG-RECK^{+} (left) and RECK-Hep^{Tg} (right) female mice fed CD or WD for 24 weeks. C. Histological scoring and D. total NAFLD activity scores based on H&E images, and E. fibrosis scores based on PSR images (n=8-12/group). Data presented as mean ± SEM. * indicates main effect of diet, # indicates main effect for genotype. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

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APPENDIX A: Curriculum Vitae

Education

Doctor of Philosophy, Veterinary Pathobiology, University of Missouri – Columbia **Oct 2018 – Sep 2022**
Residency in Laboratory Animal Medicine, University of Missouri – Columbia **Jul 2017 – Jun 2021**
Doctor of Veterinary Medicine, University of Wisconsin – Madison **Sep 2012 – May 2017**
Masters, Comparative Biomedical Sciences, University of Wisconsin – Madison **Jun 2014 – Aug 2015**
Bachelor of Science, Biology, University of Wisconsin – Madison **Sep 2006 – May 2010**

Veterinary/Research Experience

Candidate for Doctor of Philosophy – Rector Lab, University of Missouri **Oct 2018 – Present**

- Assisted in experiment design and implementation, data collection and analysis, manuscript review and writing; presented several seminars, abstracts at national and local meetings, and co-written manuscripts relating to NAFLD and the role of cellular processes inherent in the disease pathogenesis
- Primary skills learned/utilized: Rodent colony management, rodent models of obesity and liver disease, rodent terminal surgery, RNA isolation and cDNA synthesis, qRT-PCR analysis, Western blotting, primary cell isolation and culture, mitochondrial isolation, fatty acid oxidation using C14-Palmitate, mitochondrial respiration using the Oroboros system, and histological preparations, stains, and analysis

Veterinary Resident in Comparative Medicine, University of Missouri **Jul 2017 – Jun 2021**

- Participated in and administered the direct veterinary care of laboratory animal cases involved in the research animal program at the University of Missouri; participated in surgeries, oversaw the diagnosis and treatment implementation of veterinary cases, worked directly with PIs and staff, and participated in many other research-support and animal welfare duties. Attended weekly rounds, monthly IACUC meetings, and regular laboratory animal medicine seminars. Guest taught courses for veterinary studies, trained research staff and students on proper handling and surgical techniques, provided continuing training opportunities for husbandry staff
- Completed a first-year research project in the lab of Dr. Chris Baines working on the characterization of mitochondrial proteins and their roles in disease

Candidate for Master's Degree – Svaren Lab, University of Wisconsin **Jun 2014 – Aug 2015**

- Assisted in experimental design and implementation, data collection and analysis; presented several talks and abstracts at local conferences relating to the study based on the manipulation of a gene associated with the pathogenesis of a peripheral neuropathy (Charcot-Marie-Tooth Syndrome) via pharmaceutical compounds
- Primary skills learned/utilized: immortalized cell line experiments, experimental reagent analysis using rodent models, RNA isolation and cDNA synthesis, qRT-PCR, Western blotting

Student Researcher – McLellan Lab, University of Wisconsin **May 2013-Oct 2013**

- Completed as a Merial Summer Scholars Project with the School of Veterinary Medicine
- Processed ocular tissue samples using various stains, immunohistochemistry, etc directed at characterizing the pathogenesis and progression of glaucoma in a novel feline model of disease
- Created and established an immunofluorescence protocol to be run on tissue samples
- Presented a poster on the work at a national meeting of veterinary students and researchers

Research Specialist – Nork Lab, University of Wisconsin **June 2010 – Aug 2012**

- Processed tissue samples for numerous projects, including immunohistochemistry, various light microscopy stains, sectioning, etc directed at understanding the basic pathogenesis of glaucoma
- Received training and worked with various laboratory animal species, including cynomologous and rhesus macaques, dogs, cats, pigs, and rabbits
    - Assisted in surgical procedures, ophthalmologic testing and diagnosing of disease states, administration of treatments (IM, IV, and topical medications), placing IV catheters, assist in data collection, collection of tissues post-mortem
- Conducted poster presentation, talks, and created a manuscript on a project examining cellular mechanisms of pathogenesis in glaucoma

Student Researcher - Sutula Lab, University of Wisconsin **Sept 2008 – June 2010**

- Completed as senior thesis requirement for the Bachelor of Science in Biology.
- Primary skills learned/utilized: Rodent models of post-traumatic epilepsy, rodent models of behavior assessment, histology grading, manuscript writing and review

Student Lab Assistant - Shaver Lab, University of Wisconsin **May 2008 – June 2010**

- Collection of samples from dairy cattle, including blood, milk, and liver tissue, as well as assisting in surgical procedures in a project determining the effects of vitamin D administration to lactating dairy cattle and subsequent milk production
    - Received training to draw blood samples, assisted in surgical procedures, collected and processed various tissue samples
- Weighing, processing, and analysis of samples (including examining for glucose levels, folates, and trace minerals present), interpreting protocols
- General lab maintenance, involving washing, cleaning and autoclaving materials, etc.

Publications

- **Ryan Dashek**, Yusuke Higashi, Nitin A Das, Andrea J Carpenter, Jacob J Russell, Luis A Mertinez-Lemus, R Scott Rector, Bysani Chandrasekar. (2022). Empagliflozin inhibits intermittent hypoxia-induced TRAF3IP2/NF-κB/HIF-1α/IL-6-dependent human aortic smooth muscle cell proliferation. Submitted to Cellular Signaling, May 2022; under revision.
- Rory P Cunningham, Mary P Moore, **Ryan J Dashek**, Grace M Meers, Takamune Takahashi, Ryan D Sheldon, Andrew A Wheeler, Alberto Diaz-Arias, Jamal A Ibdah, Elizabeth J Parks, John P Thyfault, R Scott Rector. (2021). Critical Role for Hepatocyte-Specific eNOS in NAFLD and NASH. *Diabetes*. 2021 Nov;**70**(11):2476-2491. PMID: 34380696
- **Ryan J Dashek**, Connor Diaz, Bysani Chandrasekar, R Scott Rector. (2021). The Role of RECK in Hepatobiliary Neoplasia Reveals its Therapeutic Potential in NASH. *Frontiers in Endocrinology.* 2021 Oct;**12**:770740. PMID: 34745017
- Samantha A Gerb, **Ryan J Dashek**, Aaron C Ericsson, Rachel Griffin, Craig L Franklin. (2021). The Effects of Ketamine on the Gut Microbiome on CD1 Mice. *Comparative Medicine*. 2021 Aug;**71**(4):295-301. PMID: 34301347
- Mary P Moore, Rory P Cunningham, **Ryan J Dashek**, Justine M Mucinski, R Scott Rector. (2020). A fad too far? Dietary strategies for the prevention and treatment of NAFLD. *Obesity (Silver Spring).* 2020 Oct;**28**(10):1843-1852. PMID: 32893456
- Paula J Klutho, **Ryan J Dashek**, Lihui Song, Christopher P Baines. (2020).Genetic manipulation of SPG7 or NipSnap2 does not affect mitochondrial permeability transition. *Cell Death Discovery*. **6**(1): 1-3.

- Langberg Tomer, **Ryan Dashek**, Bernard Mulvey, Kimberly Miller, Susan Osting, Carl E Stafstrom, Thomas P Sutula. (2016).Distinct behavioral phenotypes in novel "fast" kindling-susceptible and "slow" kindling-resistant rat strains selected by stimulation of the hippocampal perforant path. *Neurobiology of Disease.* **85**:122-9.
- T Michael Nork, Charlene BY Kim, Kaitlyn M Munsey, **Ryan J Dashek**, James N Ver Hoeve. (2014). Regional choroidal blood flow and multifocal electroretinography in experimental glaucoma in rhesus macaques. *Investigative Ophthalmology and Visual Sciences*. **55**:7786-7798.
- **Ryan J Dashek**, Charlene BY Kim, Carol A Rasmussen, Elizabeth A Hennes-Beean, James N VerHoeve, T Michael Nork. (2013). Structural and functional effects of hemiretinal endodiathermy axotomy in cynomolgus macaques. *Investigative Ophthalmology and Visual Sciences.* **54**:3479-3492.

Selected Abstracts/Presentations

- Connor Diaz, **Ryan Dashek**, Bysani Chandrasekar, R Scott Rector. A Mechanistic Role for RECK in the Regulation of Hepatocellular Inflammation. Presented as a poster at Health Sciences Research Day at the University of Missouri School of Medicine in Columbia MO, November 2021.
- **Ryan Dashek**, Rory Cunningham, Mary Moore, Vivien Jepkemoi, Grace Meers, Bysani Chandrasekar, Scott Rector. Hepatocellular RECK Overexpression Attenuates NASH Susceptibility. Presented as a poster at the virtual National AALAS Meeting (October 2020), and the Research and Creative Arts Forum held virtually at the University of Missouri (November 2020)
- Rory Cunningham, Mary Moore, **Ryan Dashek**, Luigi Boccardi, Vivien Jepkemoi, Grace Meers, Bysani Chandrasekar, Scott Rector. Hepatocellular RECK Knock-Down Exacerbates NASH Susceptibility. Presented as a poster at the Veterans Affairs Research Poster Symposium meeting in Columbia, MO, December 2019.
- **Ryan Dashek,** Samantha Gerb, Aaron Ericsson, Rachel Griffin, Craig Franklin. Daily Ketamine Administration Does Not Alter the Gut Microbiome of CD-1 Mice. Presented as a poster at the National AALAS Meeting in Denver, CO, October 2019.
- **Ryan Dashek,** Paula Klutho, Lihui Song, Christopher Baines. Characterization of NipSnap2 as a Potential Mediator of Mitochondrial Permeability Transition. Presented as a poster at the National AALAS Meeting in Baltimore, MD (October 2018), University of Missouri Life Sciences Week in Columbia, MO, and the College of Veterinary Medicine Research Day (May 2019) in Columbia, MO.
- **Ryan Dashek**. An Overview of Rabbit Parasitic Diseases. Presented as a seminar at the regional Show-Me Exotics Symposium in Columbia, MO, January 2019.
- **Ryan Dashek**, John Moran, Jeff Johnson, John Svaren. Transcriptional regulation of *Pmp22* through pharmaceutical compounds. Presented as Master's thesis at University of Wisconsin-Madison, August 2015.
- **Ryan J Dashek,** Richard R Dubielzig, Leandro BC Teixiera, Gillian J McLellan. Tissue localization of TGF-β2 and its receptors in a feline model of congenital glaucoma. Presented at Merial Summer Scholars Meeting at MSU, August 2013.
- **Ryan J Dashek**, Charlene BY Kim, Carol A Rasmussen, Elizabeth A Hennes-Beean, James N VerHoeve, T Michael Nork. Structural and functional effects of hemiretinal endodiathermy axotomy in cynomolgus macaques. Project was presented in April 2011 at the Association for Research in Vision and Ophthalmology annual meeting, and in October 2011 at the University of Wisconsin Ophthalmology Department Grand Rounds.

Grants/Research Funding

- Supported by NIH T32 Training Grant (Project Number 2T32OD011126-43) from Jul 2018 to Jun 2021
    - Supported work in the laboratory of Dr. R. Scott Rector towards completion of PhD; grant managed by Drs. Craig Franklin and Elizabeth Bryda
- ACLAM Foundation Grant – Proposal submitted Dec 2018, full grant submitted Feb 2019
    - Grant Application Title: Effects of Oral Antibiotics on the Physiology and Health of the Gut Microbiome in Laboratory Rabbits
    - Selected as finalist for that year (2018); ultimately was not selected for funding
- Phi Zeta Research Award - College of Veterinary Medicine, University of Missouri – Submitted Oct 2018
    - Co-authored with Samantha Gerb and Craig Franklin
    - Grant Application Title: The Effects of Ketamine on the Microbiome
    - Funded; amount: $1000
- 12-Month Mentored Research Project Award – School of Veterinary Medicine, University of Wisconsin – Submitted Jan 2013, awarded Apr 2013
    - Supported work in the laboratory of Dr. John Svaren towards completion of Master's degree
- Merial Summer Scholars Award – School of Veterinary Medicine, University of Wisconsin – Submitted Jan 2012, awarded Apr 2012
    - Supported work in the laboratory of Dr. Gillian McLellan from May 2013 to Sept 2013

Teaching Experience

- In the role of University of Missouri – Resident in Comparative Medicine:
    - V_PBIO 6010 Laboratory Animal Medicine – Spring Semester, 2018 - Present
        - Taught three lectures: 'Amphibian Biology, Husbandry, and Diseases', 'Rabbit Biology and Husbandry' and 'Rabbit Diseases'; aided in handling labs
    - LAB_AN 9437 Pathology of Laboratory Animals – Fall Semester, 2021
        - Taught two lectures on the 'Pathology of the Laboratory Rabbit'; assisted in one histopathology lab
    - LAB_AN 9468 Biology of Laboratory Animals – Fall Semester 2020
        - Taught one lecture on the 'Biology of the Laboratory Rabbit'
    - ANSCI 3085/8085 Elements of Experimental Surgery – Fall Semester, 2018-2020
        - Taught two lectures: 'Anesthetics and Analgesics of Rodent Surgery' and 'Instrumentation When Performing Experimental Animal Surgery'; aided in surgery labs
    - Created two extern learning activities for visiting and rotating veterinary students: 'Research Study Design PBL' and 'An Overview of Rabbit Health Topics' both presented throughout the year on a rotating basis
    - Mentored a veterinary student (Rachel Griffin) in completing a veterinary research project examining microbiome responses to ketamine administration in mice from April 2019 to May 2019
    - Office of Animal Resources Technician Class – Taught 'Medical Record Keeping and Terminology' in June 2018

    - Taught mouse handling and rat handling workshops for individuals working with these species in a laboratory animal setting from July 2017 to July 2018
- In the role of University of Missouri – PhD Student in Veterinary Pathobiology:
    - NEP 8030 Etiology of Obesity – Spring Semester, 2021
        - Taught 'Cellular Mechanisms in the Pathogenesis of NAFLD'
    - Have personally mentored, or assisted in mentoring, several undergraduate students (Vivien Jepkemoi, Corey Diemer, Nicole Wieschhaus), veterinary students (Rachel Griffin), and medical students (Connor Diaz, Faith Vietor) in completing research projects

Select Scientific/Veterinary Meetings Attended

- **Experimental Biology**
    - April 2022, Philadelphia, PA
        - Poster Presentation
- **National American Association for Laboratory Animal Science (AALAS) Meeting:**
    - October 2021, Kansas City, MO
        - Selected as a sponsored participant by IDEXX Analytics
    - October 2020, virtual
        - Poster Presentation
    - October 2019, Denver, CO
        - Poster Presentation
    - October 2018, Baltimore, MD
        - Poster Presentation
- **University of Missouri's Research and Creative Arts Forum (local):**
    - November 2020, virtual
        - Platform session
    - April 2019
        - Poster Presentation
- **University of Missouri's CVM Research Day (local):**
    - May 2022
        - Poster Presentation
    - May 2019
        - Poster Presentation
    - May 2018
        - Poster Presentation

Extramural Activities

- Served as a member of the Post-Doctoral Committee of the Comparative Medicine Program helping enact changes and improvements to the structure of the program: November 2018 – present
    - Served as chairman of the committee: January 2021 – January 2022
- Participated in the 3-Minute Thesis Challenge: October 2021
- Coordinated Veterinary Researchers Summer Program (VRSP) Student laboratory animal medicine activity schedule: May 2021 – July 2021

- Participated in the NIH Series on Program Funding and Grants Administration: November 2021, November 2020
- Attended the ACLAM Boards Prep Short-Course hosted virtually by the University of Wisconsin and the Wisconsin National Primate Research Center: May 2020
- Participated in Science on Wheels: October 2019 – March 2020
- Attended the Precision Mouse Modeling Symposium hosted by the Mutant Mouse Resource and Research Center in Columbia, MO: September 2019
- Attended the National Veterinary Scholars Symposium in Worcester, MA to meet with potential trainees and represent the University of Missouri's Comparative Medicine Program: July 2019

Honors and Awards

- Selected as 2022 recipient of 'Mizzou 18'
- 1st for Poster Presentation in the category of 2nd- and 3rd-year resident posters won at CVM Research Day May 2019
- 2nd for Poster Presentation in the category of Veterinary/Biomedical Sciences at the Research and Creative Arts Form hosted by the Graduate Professional Council April 2019
- Awarded the Douglas D Randall Young Scientists Travel Grant in November 2018

APPENDIX B: Abstracts of Published Manuscripts

A Fad too Far? Dietary Strategies for the Prevention and Treatment of NAFLD

Citation:

Moore MP, Cunningham RP, **Dashek RJ**, Mucinski JM, Rector RS. A fad too far? Dietary strategies for the prevention and treatment of NAFLD. Obesity (Silver Spring). 2020 Oct;28(10):1843-1852. doi: 10.1002/oby.22964. Epub 2020 Sep 6. PMID: 32893456; PMCID: PMC7511422.

Abstract:

Nonalcoholic fatty liver disease (NAFLD) is a major health problem, and its prevalence has increased in recent years, concurrent with rising rates of obesity and other metabolic diseases. Currently, there are no FDA-approved pharmacological therapies for NAFLD, and lifestyle interventions, including weight loss and exercise, remain the cornerstones for treatment. Manipulating diet composition and eating patterns may be a sustainable approach to NAFLD treatment. Dietary strategies including Paleolithic, ketogenic, Mediterranean, high-protein, plant-based, low-carbohydrate, and intermittent fasting diets have become increasingly popular because of their purported benefits on metabolic disease. This review highlights what is currently known about these popular dietary approaches in the management of NAFLD in clinical populations with mechanistic insight from animal studies. It also identifies key knowledge gaps to better inform future preclinical and clinical studies aimed at the treatment of NAFLD.

The Effects of Ketamine on the Gut Microbiome of CD1 Mice

Citation:

Gerb SA, **Dashek RJ**, Ericsson AC, Griffin R, Franklin CL. The Effects of Ketamine on the Gut Microbiome on CD1 Mice. Comp Med. 2021 Aug 1;71(4):295-301. doi: 10.30802/AALAS-CM-20-000117. Epub 2021 Jul 23. PMID: 34301347; PMCID: PMC8383998.

Abstract:

The intestinal microbiota of an organism can significantly alter outcome data in otherwise identical experiments. Occasionally, animals may require sedation or anesthesia for scientific or health-related purposes, and certain anesthetics, such as ketamine, can profoundly affect the gastrointestinal system. While many factors can alter the gut microbiome (GM), the effects of anesthetics on the composition or diversity of the GM have not been established. The goal of the current study was to determine whether daily administration of ketamine would significantly alter the microbiome of CD1 mice. To achieve this goal, female CD1 mice received daily injections of ketamine HCl (100 mg/kg) or the equivalent volume of 0.9% saline for 10 consecutive days. Fecal samples were collected before the first administration and 24 h after the final dose of either ketamine or saline. Samples were analyzed by 16S rRNA sequencing to identify changes between groups in diversity or composition of GM. The study found no significant changes to the GM after serial ketamine administration when treated mice were housed with controls. Therefore, ketamine administration is unlikely to alter the GM of a CD1 mouse and should not serve be a confounding factor in reproducibility of research.

Critical Role for Hepatocyte-Specific eNOS in NAFLD and NASH

Citation:

Cunningham RP, Moore MP, **Dashek RJ**, Meers GM, Takahashi T, Sheldon RD, Wheeler AA, Diaz-Arias A, Ibdah JA, Parks EJ, Thyfault JP, Rector RS. Critical Role for Hepatocyte-Specific eNOS in NAFLD and NASH. Diabetes. 2021 Nov;70(11):2476-2491. doi: 10.2337/db20-1228. Epub 2021 Aug 11. PMID: 34380696; PMCID: PMC8564406.

Abstract:

Regulation of endothelial nitric oxide synthase (eNOS) in hepatocytes may be an important target in nonalcoholic fatty liver disease (NAFLD) development and progression to nonalcoholic steatohepatitis (NASH). In this study, we show genetic deletion and viral knockdown of hepatocyte-specific eNOS exacerbated hepatic steatosis and inflammation, decreased hepatic mitochondrial fatty acid oxidation and respiration, increased mitochondrial H_2O_2 emission, and impaired the hepatic mitophagic (BNIP3 and LC3II) response. Conversely, overexpressing eNOS in hepatocytes in vitro and in vivo increased hepatocyte mitochondrial respiration and attenuated Western diet-induced NASH. Moreover, patients with elevated NAFLD activity score (histology score of worsening steatosis, hepatocyte ballooning, and inflammation) exhibited reduced hepatic eNOS expression, which correlated with reduced hepatic mitochondrial fatty acid oxidation and lower hepatic protein expression of mitophagy protein BNIP3. The current study reveals an important molecular role for hepatocyte-specific eNOS as a key regulator of NAFLD/NASH susceptibility and mitochondrial quality control with direct clinical correlation to patients with NASH.

The Role of RECK in Hepatobiliary Neoplasia Reveals its Therapeutic Potential in NASH

Citation:

Dashek RJ, Diaz C, Chandrasekar B, Rector RS. The Role of RECK in Hepatobiliary Neoplasia Reveals Its Therapeutic Potential in NASH. Front Endocrinol (Lausanne). 2021 Oct 20;12:770740. doi: 10.3389/fendo.2021.770740. PMID: 34745017; PMCID: PMC8564138.

Abstract:

Non-alcoholic fatty liver disease (NAFLD) is a multimorbidity disorder ranging from excess accumulation of fat in the liver (steatosis) to steatohepatitis (NASH) and end-stage cirrhosis, and the development of hepatocellular carcinoma (HCC) in a subset of patients. The defining features of NASH are inflammation and progressive fibrosis. Currently, no pharmaceutical therapies are available for NAFLD, NASH and HCC; therefore, developing novel treatment strategies is desperately needed. Reversion Inducing Cysteine Rich Protein with Kazal motifs (RECK) is a well-known modifier of the extracellular matrix in hepatic remodeling and transition to HCC. More recently, its role in regulating inflammatory and fibrogenic processes has emerged. Here, we summarize the most relevant findings that extend our current understanding of RECK as a regulator of inflammation and fibrosis, and its induction as a potential strategy to blunt the development and progression of NASH and HCC.

Hepatocyte-Specific eNOS Deletion Impairs Exercise-Induced Adaptations in Hepatic Mitochondrial Function and Autophagy

Citation:

Cunningham RP, Moore MP, **Dashek RJ**, Meers GM, Jepkemoi V, Takahashi T, Vieira-Potter VJ, Kanaley JA, Booth FW, Rector RS. Hepatocyte-specific eNOS deletion impairs exercise-induced adaptations in hepatic mitochondrial function and autophagy. Obesity (Silver Spring). 2022 May;30(5):1066-1078. doi: 10.1002/oby.23414. Epub 2022 Mar 31. PMID: 35357089; PMCID: PMC9050943.

Abstract:

Objective: Endothelial nitric oxide synthase (eNOS) is a potential mediator of exercise-induced hepatic mitochondrial adaptations.

Methods: Here, male and female hepatocyte-specific eNOS knockout ($eNOS^{hep-/-}$) and intact hepatic eNOS ($eNOS^{fl/fl}$) mice performed voluntary wheel-running exercise (EX) or remained in sedentary cage conditions for 10 weeks.

Results: EX resolved the exacerbated hepatic steatosis in $eNOS^{hep-/-}$ male mice. Elevated hydrogen peroxide emission (~50% higher in $eNOS^{hep-/-}$ vs. $eNOS^{fl/fl}$ mice) was completely ablated with EX. Interestingly, EX increased [1-^{14} C] palmitate oxidation in $eNOS^{fl/fl}$ male mice, but this was blunted in the $eNOS^{hep-/-}$ male mice. $eNOS^{hep-/-}$ mice had lower markers of the energy sensors AMP-activated protein kinase (AMPK)/phospho- (p)AMPK and mammalian target of rapamycin (mTOR) and p-mTOR, as well as the autophagy initiators serine/threonine-protein kinase ULK1 and pULK1, compared with $eNOS^{fl/fl}$ mice. Females showed elevated electron transport chain protein content and markers of mitochondrial biogenesis (transcription factor A, mitochondrial, peroxisome proliferator-activated receptor-gamma coactivator 1α).

Conclusions: Collectively, this study demonstrates for the first time, to the authors' knowledge, the requirement of eNOS in hepatocytes in the EX-induced increases in hepatic fatty acid oxidation in male mice. Deletion of eNOS in hepatocytes also appears to impair the energy-sensing ability of the cell and inhibit the activation of the autophagy initiating factor ULK1. These data uncover the important and novel role of hepatocyte eNOS in EX-induced hepatic mitochondrial adaptations.

APPENDIX C: Abstracts of Scientific Presentations

A Mechanistic Role for RECK in the Regulation of Hepatocellular Inflammation

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RECK (Reversion Inducing Cysteine Rich Protein with Kazal Motifs), a membrane-anchored glycoprotein, modifies the extracellular matrix involved in various cancers, including hepatocellular carcinoma (HCC). Its role in regulating inflammatory and fibrogenic processes has also been postulated. Both inflammation and fibrosis contribute to progression of nonalcoholic steatohepatitis (NASH) to HCC. Here, we tested the hypothesis that inducing or sustaining RECK expression will inhibit proinflammatory amphiregulin and epidermal growth factor receptor (EGFR) signaling, progression of NASH and the development of HCC using an in vitro cell culture model employing isolated adult mouse primary hepatocytes. Ectopic RECK overexpression (gain-of-function) was achieved by the adenoviral (Adv) transduction of murine RECK cDNA. Adv.GFP served as a control. RECK expression was silenced (loss-of-function) by transducing RECK-specific siRNA using lipofectamine. Nonspecific siRNA served as a control. Hepatocytes were exposed to TNFα in the presence or absence of inhibitors against ADAM 10 (A Disintegrin and Metalloproteinase Domain-Containing Protein 10) and/or ADAM17 – two well-established pro-inflammatory sheddases. Results show that RECK overexpression inhibited TNFα-induced ADAM10/17 activity, and amphiregulin (an EGFR ligand) and EGFR expression. In the presence of ADAM10/17 inhibitors, no further reduction was observed in amphiregulin and EGFR activity, suggesting a maximal inhibitory effect of RECK on EGFR activity. In contrast, silencing RECK significantly increased amphiregulin secretion, and this effect was reversed by inhibition of ADAM10/17, indicating RECK signals via regulation of these sheddases. Thus, RECK not only regulates sheddase activity of ADAM10 and 17, but also downstream amphiregulin and EGFR signaling under pro-inflammatory stimuli. Because increased and sustained EGFR activity contributes to progression of NASH to HCC in preclinical models, our results indicate that inducing RECK has the potential to inhibit hepatocellular inflammation in the setting of NASH and HCC.

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Hepatocellular RECK Overexpression Attenuates NASH Susceptibility

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Nonalcoholic steatohepatitis (NASH) is characterized by hepatic steatosis, inflammation, and fibrosis. Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) has been shown to exert anti-fibrotic effects in the heart, though its role in liver inflammation and fibrosis is unknown. In this study, we assessed the effects of hepatic RECK overexpression specifically in cultured murine primary hepatocytes and immortalized AML-12 hepatocyte cell line *in vitro*, and transgenic mice that overexpress RECK in hepatocytes *in vivo*. Transgenic mice were generated by injecting hemizygous RECK-CAG mice with AAV8-TBG-Cre ($1x10^{12}$) to overexpress RECK in hepatocytes. Hemizygous RECK-CAG transgenic mice injected with AAV8-TBG-Scr-GFP served as controls. Mice were fed a western diet (WD; 45% fat, 17% sucrose, 1% cholesterol) for 8 weeks. *In vitro*, adenoviral-mediated RECK overexpression (Adv-RECK) in both cultured primary mouse hepatocytes and in AML-12 hepatocytes decreased pro-inflammatory TNFα and IL-1β mRNA expression ($p \leq 0.05$). Moreover, RECK overexpression in both cell types suppressed LPS-induced TLR4 mRNA expression ($p \leq 0.05$). *In vivo*, AAV8-TBG-Cre significantly increased hepatic RECK expression at both mRNA and protein levels ($p < 0.001$ vs AAV8-GFP control mice). Notably, hepatic RECK overexpression in mice significantly attenuated WD-induced NASH, including attenuated histological hepatic steatosis, inflammation, and ballooning, and tended to decrease hepatic fibrosis. These improvements corresponded with significant reductions in gene expression for markers of inflammation, fibrosis, and hepatic stellate cell activation (TNFα, αSMA, TGFβ1, ADAMs 10 and 17, MMPs 2 and 9, and TIMP1). These data represent the first observations that targeted RECK overexpression in hepatocytes *in vivo* and *in vitro* attenuates inflammation, NASH, and fibrosis. Future studies are needed to elucidate the precise mechanisms by which RECK elicits these hepato-protective effects. Funding sources: VA grants I01-BX004220 and IK6BX004016

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Daily Ketamine Administration Does Not Alter the Gut Microbiome of CD-1 Mice

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The gut microbiota (GM) is a critical player in the physiology of animals in both health and disease. Many factors have been shown to play a role in modulating the composition of the GM of animals used in research including, diet, husbandry practices, and certain experimental treatments. In turn, the GM has been shown to influence the outcome of various experimental measurements. Currently there is a lack of research examining the effects of various anesthetic agents on the GM of mice. The hypothesis of this study is that daily ketamine administration significantly alters the GM composition of CD-1 mice. Twenty adult female CD-1 mice were split into two groups: one group receiving daily intraperitoneal injections of 100 mg/kg ketamine daily, and one group receiving daily injections of an equivalent volume of saline, both for ten days in order to model an experimental protocol involving chronic ketamine exposure. Feces were collected immediately before the initiation of experimental protocol, and then again following the completion of the ten days. Animals were weighed every other day throughout the experiment to ensure that they did not lose a significant (>10% total body weight) amount of body mass and to adjust the ketamine dosage as necessary. DNA was extracted from fecal samples and amplified by PCR using bacterial 16S rRNA primers, and then processed for next generation sequencing. Sequence data were analyzed using PAST 3.25 software and groups were compared using permutational multivariate analysis of variance (PERMANOVA). PERMANOVA analysis revealed no significant effects on the GM by daily administration of 100 mg/kg ketamine for ten days. These results indicate that daily ketamine administration does not significantly alter the GM of mice used in research.

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Characterization of NipSnap2 as a Potential Mediator of Mitochondrial Permeability Transition

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The loss of cells underlies the basis of many diseases. A common cause of cell death is the mitochondrial permeability transition (MPT), characterized by a sudden increase in inner mitochondrial membrane permeability leading to ATP depletion, mitochondrial swelling, rupture, and cell death. This process is mediated by the MPT pore, a non-selective channel in the inner membrane. However, components of the pore itself are not well defined, with the matrix protein cyclophilin-D (CypD) being the only defined regulator of the MPT pore. To identify novel pore components we conducted proteomic analyses of CypD-binding proteins and identified a putative mitochondrial protein called NipSnap2. More recently, an shRNA-based screening study suggested that knockdown of NipSnap2 could attenuate oxidative stress-induced MPT and cell death. We hypothesized that NipSnap2 is a crucial component of the MPT pore. To assess NipSnap2's role in MPT, mouse embryonic fibroblasts were cultured and transfected with a control siRNA or an siRNA targeted against NipSnap2 to decrease protein levels. A parallel set of cells was infected with a control virus or a virus coded to produce NipSnap2, elevating levels of the protein. Verification that NipSnap2 protein levels were altered in the respective groups was determined using Western blot analysis. Sets of transfected and infected cells then underwent a calcium retention capacity (CRC) assay, an index of MPT. Depletion of NipSnap2 by siRNA did not affect CRC compared to control cells, nor did overexpression of NipSnap2. Together, these results suggest that NipSnap2 is not an essential component of the MPT.

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Vita

Ryan Joseph Dashek was born to Mark and Lynn Dashek in Franklin, Wisconsin. Following completion of high school at Franklin High School in 2006, Ryan attended the University of Wisconsin in Madison, Wisconsin where he received a Bachelor of Science degree in Biology with an emphasis in Neurobiology in 2010. He worked for two years in the laboratory of Dr. T Michael Nork in the Ophthalmology Department at the University of Wisconsin as a Research Specialist. Following this, he began his veterinary medical studies at the School of Veterinary Medicine at the University of Wisconsin in 2012. During this time, he participated in a mentored-research program where he earned a Master's of Science in Biomedical Sciences under the supervision of Dr. John Svaren in 2015. He graduated with his Doctor of Veterinary Medicine degree in 2017 before beginning his laboratory animal medicine residency in the Comparative Medicine Program at the University of Missouri. He completed his residency training in 2020 and his Doctor of Philosophy degree in the Pathobiology Area Program in 2022 in the laboratory of Dr. R Scott Rector. Ryan plans to continue working at the University of Missouri as a Research Assistant Professor with the intent to eventually establish his own research program expanding his research into other areas of liver disease and physiology.