A novel role for CEACAM1 in hepatic stellate cell activation in the progression of non-alcoholic steatohepatitis

Sumona Ghosh

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by

Sumona Ghosh

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Dr. Sonia M. Najjar, Committee Chair

Dr. Edwin R. Sanchez, Committee Member

Dr. Stanislaw Stepkowski, Committee Member

Dr. Jennifer Hill, Committee Member

Dr. Edith Mensah-Osman, Committee Member

Dr. Yatrik M. Shah, Committee Member

Dr. Marcia McInerney, Committee Member

Dr. Patricia Komuniecki,
Dean of the College of Graduate Studies

The University of Toledo

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An Abstract of

A Novel Role for CEACAM1 in Hepatic Stellate Cell Activation in the Progression of Non-Alcoholic Steatohepatitis

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Hyperinsulinemia, a characteristic of insulin resistance, is a key factor in the pathogenesis of a number of metabolic disorders such as obesity, fatty liver disease and type 2 diabetes mellitus. Regulation of both insulin secretion, from the beta cell, and insulin clearance, mainly by the liver, is vital to maintain insulin sensitivity. Insulin clearance is mainly mediated by Carcinoembryonic antigen-related cell adhesion molecule (CEACAM1). Inactivation of CEACAM1 in the liver, or global null mutation of CEACAM1, decreases hepatic insulin clearance resulting in hyperinsulinemia and subsequent insulin resistance.
High fat feeding for 30 days results in a >50% decrease in hepatic CEACAM1 expression and induces insulin resistance in C57BL/6 mice. Specific induction of CEACAM1 in the liver, driven by Apolipoprotein A1 promoter, protects against diet-induced insulin resistance by maintaining normal insulin clearance, even after 4 months of high fat feeding. This data promotes CEACAM1-dependent insulin clearance pathways as a molecular underpinning of metabolic diseases such as diet-induced obesity and non-alcoholic steatohepatitis (NASH). Long term high fat feeding induces features of NASH in Ceacam1 null mice: steatosis, inflammatory infiltration, oxidative stress and progressive fibrosis, thus implicating a role for CEACAM1 in the pathogenesis of NASH. Basal fibrosis observed in Ceacam1 null mice suggests a role for CEACAM1 in the initiation of fibrogenesis. Stable knockdown of CEACAM1 in cultured LX2 human stellate cells causes a state of activation including loss of lipid content, increased proliferation and expression of extracellular matrix components, generally observed in diseased liver. Together, the data provide an \textit{in vivo} and \textit{in vitro} demonstration of the critical role of CEACAM1 in the pathogenesis of metabolic diseases, in particular, obesity and NASH.
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Introduction

I. INSULIN RESISTANCE IN OBESITY AND TYPE 2 DIABETES MELLITUS

Type 2 diabetes mellitus (T2DM) continues to be a growing worldwide epidemic, with an estimated 285 million people afflicted in 2010 (Campbell et al., 2010). T2DM is increasing in parallel to obesity, in particular, visceral obesity (Utzschneider and Kahn, 2006). Obesity/T2DM occurs when carbohydrate and lipid metabolism are not properly regulated by insulin (Saltiel, 2001). A major risk factor for the development of obesity/T2DM is insulin resistance. As the majority of patients with T2DM in the United States are overweight (Bajaj and Defronzo, 2003), this insulin resistance is frequently associated with obesity. In a healthy insulin-sensitive individual, the rise in glucose concentrations following a meal stimulates release of insulin from pancreatic β cells. Insulin acts to suppress hepatic glucose production while also stimulating glucose uptake by the liver and peripheral tissues, thereby decreasing circulating glucose to sustain normal glucose levels (Bajaj and Defronzo, 2003). Additionally, insulin is antilipolytic, causing a decrease of circulating plasma free fatty acid (FFA) levels (Groop et al., 1991). This reduction in plasma FFA, in turn, allows for more efficient glucose uptake in peripheral tissues.
The onset of insulin resistance occurs with rising levels of plasma glucose as insulin is no longer able to suppress hepatic glucose production and induce sufficient glucose uptake in the periphery. This rise in circulating glucose is counteracted by an increase in insulin secretion by the pancreatic β cell. This compensatory increase in insulin secretion, coupled with a decrease in insulin clearance results in both fasting and random hyperinsulinemia, preceding the development on T2DM (Saad et al., 1989). With the onset of insulin resistance, suppression of lipolysis is lost, thereby resulting in increased plasma FFAs further contributing to insulin resistance states in both liver and muscle (DeFronzo, 1992). Insulin resistance is frequently associated with a number of other pathological complications including cardiovascular disease, stroke, hypertension, kidney disease and non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH).

II. INSULIN SIGNALING

Insulin signaling begins with the binding of insulin to the insulin receptor (IR). The insulin receptor is comprised of 2 α subunits and 2 β subunits linked by disulfide bonds. Upon insulin binding, the β subunit of the IR undergoes autophosphorylation with subsequent activation of IR tyrosine kinase. IR tyrosine kinase then can go on to phosphorylate a number of specific intracellular proteins, including IRS-1, IRS-2, IRS-3, IRS-4, Grb2, Shc (Saltiel and Kahn, 2001) and CEACAM1 (Rees-Jones and Taylor, 1985). In the muscle, IRS-1 is the major docking protein to interact with IR tyrosine kinase to regulate the PI-3 kinase pathway for glucose transport (Ruderman et al., 1990) and glycogen synthase activity (Saltiel and Kahn, 2001). In the liver, IRS-2 is a major docking protein to undergo tyrosine phosphorylation to mediate hepatic glucose
production and gluconeogenesis (Kerouz et al., 1997). However, other proteins with SH2 domains, such as Shc, interact with IRS-1 to become phosphorylated to activate the Ras/mitogen-activated protein (MAP) signaling pathway to mediate cell growth and proliferation (Saltiel and Kahn, 2001).

III. **CEACAM1**

The Ceacam1 gene is comprised of 9 exons, the 7th of which is alternatively spliced during mRNA processing to yield two isoforms: CEACAM1-4L and CEACAM1-4S. These two isoforms differ by their intracellular domain. The intracellular domain of CEACAM1-4L contains phosphorylation sites (Ser503 and Tyr488). CEACAM1 is found
in most cell types including immune cells.

III.1 ROLE OF CEACAM1 IN INSULIN CLEARANCE

As a substrate of the insulin receptor tyrosine kinase, hepatic CEACAM1 (-4L) promotes insulin endocytosis and degradation to mediate insulin sensitivity (Rees-Jones and Taylor, 1985). Basal phosphorylation of Ser503 is required for insulin-stimulated Tyr488 phosphorylation (Najjar et al., 1995). Following insulin-stimulated phosphorylation, the insulin-receptor complex is internalized to the endosomes through clathrin-coated vesicles for subsequent dissociation. Insulin is degraded while the insulin receptor is recycled and returns back to the cell surface (Carpentier, 1993; Rabkin et al., 1984). Supporting this mechanism, L-SACC1 mice, expressing a dominant-negative, phosphorylation-defective S503A mutation in the CEACAM1 protein, exclusively in the
liver, displayed hyperinsulinemia as a result of faulty insulin clearance (Poy et al., 2002). This abnormality also held true in Ceacam1 knockout (Cc1+/−) mice (DeAngelis et al., 2008). Additionally, both lines of mice (L-SACC1 and Cc1−/−) developed visceral adiposity accompanied by abnormally high levels of plasma FFA, plasma triglycerides and hepatic triglycerides (DeAngelis et al., 2008; Poy et al., 2002). This suggests improving insulin sensitivity via the regulation of hepatic CEACAM1 may alleviate the deleterious effects of hyperinsulinemia to the liver. This hypothesis is tested in Chapter 1.

III.2 ROLE OF CEACAM1 IN LIPOGENESIS

CEACAM1 also plays a role in regulating hepatic de novo lipogenesis (Najjar et al., 2005). It does so via the enzyme, fatty acid synthase (FAS). FAS enzyme is responsible for converting malonyl-CoA to palmitate to undergo subsequent elongation into long chain fatty acids (LCFA). Under fasting conditions, a decrease in malonyl CoA is favored as it acts as an allosteric inhibitor of carnitine palmitoyl transferase-1 (CPT-1) (McGarry and Foster, 1980). CPT-1 supports the transfer of LCFA to the mitochondria for oxidation to sustain gluconeogenesis during fasting. Under fasting conditions, FFA’s are mobilized from the adipose tissue to be transported to the liver for subsequent conversion into LCFA-CoA to activate PPARα resulting in an increase in fatty acid β-oxidation (Pegorier et al., 2004; Schoonjans et al., 1996). However, under chronic states of hyperinsulinemia (obesity), SREBP-1c is activated resulting in activation of lipogenic enzymes such as FAS (Horton et al., 2002; Shimomura et al., 1999). This unnecessary increase in lipogenic activity further contributes to the adverse effects and pathogenesis caused by obesity.
Conversely, we found that insulin acutely decreases hepatic FAS activity (Najjar et al., 2005). This acute effect, however, depends on preceding insulinemic conditions. The decrease in FAS activity occurs as the binding of insulin to insulin receptor induces CEACAM1 phosphorylation and subsequent binding of FAS (Najjar et al., 2005). Binding of FAS prohibits its lipogenic activity thereby reducing hepatic triglyceride synthesis (Najjar et al., 2005). This acute effect of insulin on interaction of CEACAM1 and FAS indicates a potential mechanism by which hepatic insulin sensitivity strives to be maintained. It is worth noting that this provides a second role for hepatic CEACAM1 in maintaining healthy insulin and fat metabolism.

III.3 ROLE OF CEACAM1 IN INFLAMMATION

CEACAM1 plays an anti-inflammatory role in T-cells (Gray-Owen and Blumberg, 2006). T-cell activation causes redistribution of CEACAM1 to the cell surface, leading to its homophilic binding with CEACAM1 expressed on a different cell type. This anti-inflammatory task of CEACAM1 is dependent on the presence and phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIM) within the cytoplasmic domain (Nagaishi et al., 2006). Homophilic binding of the two CEACAM molecules results in phosphorylation of the ITIM and subsequent recruitment and binding of SHP-1 to CEACAM1 in the T-cell. This halts further inflammation and redistribution of CEACAM1 to the cell surface, as CEACAM1 returns back into the cell (Gray-Owen and Blumberg, 2006). Similar to its function in T cells, CEACAM1 also acts as an inhibitory B cell co-receptor (Lobo et al., 2009). Because inflammation plays a key role in the progression of non-alcoholic fatty liver disease (NAFLD) to non-alcoholic
steatohepatitis (NASH), it is important to decipher whether the anti-inflammatory effect of CEACAM1 at all contributes to the pathogenesis of NASH.

IV. NON-ALCOHOLIC STEATOHEPATITIS

NAFLD is the hepatic manifestation of the metabolic syndrome and is a clinical consequence of obesity. NAFLD is often associated with insulin resistant states, as seen in obesity, type 2 diabetes mellitus and the metabolic syndrome (Utzschneider and Kahn, 2006). Studies have shown significant reductions in glucose disposal, impaired ability of insulin to suppress hepatic glucose production and blunted inhibition of fatty acid oxidation in, all indications of insulin resistance associated with NAFLD (Bugianesi et al., 2005; Marchesini et al., 2001; Utzschneider and Kahn, 2006). Body fat distribution also seems to play a vital role in the pathogenesis of fatty liver disease. Higher than normal stores of intra-abdominal fat has both a high correlation with insulin resistance and may also be a source of excess FFAs that contribute to the NAFLD phenotype (Utzschneider and Kahn, 2006). However, the contribution of insulin resistance to the development of NASH remains questionable as the majority of animal models used to study NASH, such as feeding the mice with methionine-choline deficient (MCD) diet, does not induce insulin resistance. MCD diet feeding results in steatosis by impairment in β-oxidation, but fails to cause insulin resistance and obesity, both characteristics observed in human NASH (Anstee and Goldin, 2006). A model in which the full spectrum of NAFLD/NASH is present, as observed in human NASH, is lacking. Thus an animal model exhibiting the gamut of NASH in its entirety, is vital to advancing our knowledge and potential therapies for the disease.
NAFLD is mainly characterized by the presence of hepatic macrosteatosis. Progression from NAFLD to NASH occurs with the onset of inflammation, oxidative stress and fibrosis (Boppidi and Daram, 2008; Fujii and Kawada, 2012). NASH has been described to follow a “two-hit” hypothesis (James and Day, 1998). The first hit consists of steatosis, hepatic fat accumulation due increased de novo hepatic lipogenesis coupled with decreased export (Boppidi and Daram, 2008; Utzschneider and Kahn, 2006). Studies using stable isotope labeling techniques have demonstrated approximately 60% of liver triacylglycerol in NAFLD subjects is derived from circulating FFAs, 26% from de novo lipogenesis and about 15% from dietary intake suggesting that overproduction of FFAs from excess adipose tissue is the primary source of hepatic triglyceride accumulation (Donnelly et al., 2005). As previously mentioned, both L-SACC1 and Cc1−/− mouse lines develop hyperinsulinemia as a result of aberrant insulin clearance (DeAngelis et al., 2008; Poy et al., 2002). Mounting hyperinsulinemia causes insulin resistance and eventual hepatic steatosis as a result of activation of SREBP-1c and subsequent accumulation of triglycerides (Shimano et al., 1997). Because hyperinsulinemia lowers ApoB synthesis and stability, decreased ApoB from the liver may further contribute to hepatic fat accumulation (Charlton et al., 2002; Taghibiglou et al., 2000). As well as aiding in fat metabolism regulation via FAS, CEACAM1 also plays a role in decreasing de novo cholesterol synthesis (Lee et al., 2008). Because mitochondrial accumulation of free cholesterol, due to a decrease in cholesterol trafficking protein, Neimann Pick Type C-1 (NPC-1), has also been indicated to play a role in NASH development (Mari et al., 2006).
The second hit in the development of NASH is characterized by oxidative stress brought on by excessive lipid peroxidation, inflammation and fibrosis (Boppidi and Daram, 2008; Fujii and Kawada, 2012). Inflammatory infiltration can often arise simply from an increase in visceral obesity, as a number of adipokines are fat-derived including TNF-α and IL-6 (Fontana et al., 2007; Hotamisligil et al., 1993). Visceral obesity has also been correlated with an increase in NF-κB activation subsequent activation of its downstream inflammatory pathways (Cai et al., 2005). Additionally, increased visceral adiposity is correlated with an increase in plasma FFA levels, contributing to insulin resistance via activating the JNK pathway and further increasing TNF-α release (Begriche et al., 2006; Nguyen et al., 2005).

Hepatocellular apoptosis is a major component contributing to the pathogenesis of NASH. Not only has hepatocyte apoptosis been shown to be increased in patients with NASH, but it also correlates with the severity of the disease (Feldstein et al., 2003). A potential mechanism through which apoptosis occurs may be by increased death receptor, Fas. This death receptor can be upregulated via Nuclear Factor-κB (NF-κB) activation. Activation of the Fas receptor induces mitochondrial dysfunction by caspase 8 cleavage of Bid which is then translocated to the mitochondria. Along with the production of mitochondrial reactive oxygen species (ROS), cytochrome c is then released from the mitochondria for subsequent cleavage of caspase 3, resulting in initiation of apoptosis (Feldstein et al., 2003). Apoptosis, along with release of pro-fibrogenic cytokines, activate hepatic stellate cells (Hinds et al.) to differentiate into collagen type-1 producing cells to induce
fibrosis (Bataller and Brenner, 2005; Kisseleva and Brenner, 2007).

IV.1 FIBROSIS IN NON-ALCOHOLIC STEATOHEPATITIS

Activation of hepatic stellate cells (HSCs) is the major event in fibrogenesis (Friedman, 2003). These cells originate from either the endoderm or the septum transversum. Hepatic stellate cells are located in the subendothelial space, between hepatocytes and sinusoidal endothelial cells (Friedman, 2008). In a healthy liver, HSCs have spindle shaped bodies with elongated nuclei. Under normal conditions, quiescent HSCs store about 80% of the body’s ingested Vitamin A, primarily in the form of retinyl palmitate, contained in lipid droplets (Sauvant et al., 2011). The composition of these lipid droplets are largely affected by dietary intake and also contain significant amounts of triglycerides, free fatty acids and cholesterol (Friedman, 2008). Quiescent HSCs also
proliferate slowly and are thought to mediate cell-cell communication between neighboring hepatocytes and endothelial cells (Sauvant et al., 2011).

However, in a diseased liver, as observed in non-alcoholic steatohepatitis, hepatic stellate cells become activated. Upon activation, stellate cells lose their Vitamin A-rich lipid droplets, begin to proliferate excessively and start to secrete a higher amount of extracellular matrix (ECM) proteins including alpha-smooth muscle actin (alpha-SMA) and collagen type I (Friedman, 2008; Sauvant et al., 2011). HSC activation occurs on a spectrum, such that phenotypic changes occurring early in activation may be significant different from changes observed with chronic injury and activation (Friedman, 2008).

Stellate cell activation is comprised of two phases: initiation and perpetuation (Friedman, 2008). Initiation, or preinflammatory stage, is reflected by changes in gene expression causing the cells to become more sensitive to external stimuli for further activation. This phase of activation is predominantly due to paracrine stimulation from neighboring cell types such as endothelial cells, Kupffer cells, hepatocytes and platelets (Friedman, 2008). Additionally, initiation induces secretion of various chemoattractants for the recruitment of inflammatory cells leading into the perpetuation phase of activation
The perpetuation phase of activation includes phenotypic changes in proliferation, fibrogenesis, contractility and retinoid/lipid loss (Friedman, 2008). Increases in proliferation and mitogenic activity have been found to occur primarily through PDGF signaling, particularly via the PI 3-kinase pathway (Lechuga et al., 2006). However, other mitogenic compounds such as vascular endothelial cell growth factor and EGF have also been implicated to play a potential role in increased proliferation observed in activated HSCs. Activated stellate cells drive fibrosis not only through increased proliferation, but also by increased matrix production per cell. Expression of major matrix components, collagen type I and α-smooth muscle actin are increased in the activated HSCs. A number of signaling pathways may contribute to this fibrogenic phenotype, however TGF-β and
downstream Smad signaling have been widely implicated to play a predominant role (Inagaki and Okazaki, 2007). Finally, activation of stellate cells results in retinoid/lipid loss. The mechanism by which this occurs remains elusive, however previous studies indicate intracellular hydrolysis of esters preceding export may contribute to this phenomenon (Friedman et al., 1993).

IV.2 CURRENT TREATMENTS FOR FIBROSIS IN NON-ALCOHOLIC STEATOHEPATITIS

As NAFLD/NASH patients are generally obese and possess increased intra-abdominal fat stores that may contribute to their insulin resistant phenotype, lifestyle changes, particularly weight loss, is a major target for therapy (Utzschneider and Kahn, 2006). Studies have shown weight loss intervention to achieve reduced liver transaminases, and decreased hepatic fat accumulation (Huang et al., 2005). Metformin treatment is often used to treat patients with type 2 diabetes mellitus. Metformin reduces plasma glucose levels by reducing hepatic glucose production via the activation of AMP kinase (AMPK) (Zhou et al., 2001). Activation of AMPK also results in decreased lipogenesis and increased fat oxidation (Zhou et al., 2001).

Current therapies for hepatic fibrosis, particularly, aim to: (1) reduce inflammation and immune response to avoid chronic stellate cell activation; (2) directly inhibit hepatic stellate cells activation; (Dietrich et al.) counteract proliferative, fibrogenic and pro-inflammatory response of activated stellate cells; (4) induce stellate cell apoptosis (Friedman, 2003). Corticosteroids are currently used as anti-inflammatory agents in liver disease patients, but have been found to have no significant effects on
hepatic fibrosis (Friedman, 2003). Reducing stellate cell activation, directly, has been most successfully demonstrated by reduction of oxidative stress. Anti-oxidants, including alpha-tocopherol (Vitamin E), have been demonstrated to decrease fibrogenesis (Houglum et al., 1997).
References


Aims

Insulin resistance, characterized by hyperinsulinemia, is a vital component to the pathogenesis of several metabolic disorders, including obesity, type 2 diabetes and non-alcoholic steatohepatitis (NASH). Proper mediation of both insulin secretion and insulin clearance are important in maintaining insulin sensitivity. Carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) regulates hepatic insulin clearance. The cause-effect relationship between insulin resistance and hyperinsulinemia has not been well identified. Additionally, the role of insulin resistance in the pathogenesis of NASH has been questionable. This thesis aims to address these questions.

- In Chapter 1, we investigated whether hyperinsulinemia causes insulin resistance in the context of diet-induced obesity.

- In Chapter 2, we investigated whether insulin resistance predisposes to non-alcoholic steatohepatitis using Ceacam1 null mice.

- In Chapter 3, we investigated a potential role for Ceacam1 in fibrosis, an integral feature of NASH.
Delineating the Cause-effect Relationship between Hyperinsulinemia and Insulin Resistance

Qusai Y. Al-Share\textsuperscript{1,2*}, Anthony M. DeAngelis\textsuperscript{1,2*}, Sumona Ghosh\textsuperscript{1,2*}, Thomas A. Bowman\textsuperscript{1,2}, Sadeesh K. Ramakrishnan\textsuperscript{1,2}, Payal R. Patel\textsuperscript{1,2}, Lucia Russo \textsuperscript{1,2}; Christian K. Raphael\textsuperscript{1,2}; William Philbrick\textsuperscript{4}, Rohit Kulkarni, and Sonia M. Najjar\textsuperscript{1,2,†}

\textsuperscript{1}Center for Diabetes and Endocrine Research and Departments of \textsuperscript{2}Physiology and Pharmacology and \textsuperscript{3}Medical Microbiology and Immunology at the University of Toledo College of Medicine, Health Science Campus, Toledo, OH 43614; \textsuperscript{4}Naomi Berrie Diabetes Center, Columbia University, New York, NY 10032; and \textsuperscript{5}Department of Internal Medicine, Section of Endocrinology and Metabolism, Yale University School of Medicine, New Haven, CT 06520

*Authors contributed equally to these studies

†Address correspondence to:

Sonia M. Najjar, Ph.D.
College of Medicine
University of Toledo, Health Science Campus
3000 Arlington Avenue, Mail stop 1008
Toledo, Ohio, 43614
Tel: (419) 383-4183
FAX: (419) 383-2871
e-mail: sonia.najjar@utoledo.edu
Footnotes:

**Nonstandard abbreviations used.** CEACAM1, CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 1; Ceacam1, gene encoding CEACAM1 protein; CcI\(^{-/-}\), global Ceacam1 null mouse; L-SACC1, liver-specific S503A CEACAM1 mutant mouse; BL6, C57BL/6 genetic background; RD, regular diet; HF, high fat diet; FFA, free fatty acids; FATP-1, fatty acid transport protein-1; FAS, fatty acid synthase.

**Conflict of interest:** The authors have declared that no conflict of interest exists.
The development of insulin resistance is vital to the manifestation of a number of metabolic diseases. A state of insulin resistance is commonly characterized by the presence of hyperinsulinemia. The cause-effect relationship between insulin resistance and hyperinsulinemia remains somewhat elusive, as peripheral insulin resistance induces compensatory insulin secretion from pancreatic β-cells (Polonsky et al., 1988; Valera Mora et al., 2003) resulting in hyperinsulinemia while impaired insulin clearance also contributes to mounting hyperinsulinemia to exacerbate insulin resistance by both down-regulating insulin receptors and increasing de novo lipogenesis.

Considerable evidence in humans supports the view that impaired hepatic insulin extraction causes chronic hyperinsulinemia in obesity (Escobar et al., 1999; Polonsky et al., 1988). Moreover, modest weight loss (10%) is associated with a greater improvement of insulin clearance than insulin secretion, which requires a more marked weight loss (Jones et al., 2000). Studies on the role of the carcinoembryonic-related cell adhesion molecule 1 (CEACAM1) in insulin clearance provide more convincing evidence that hyperinsulinemia causes insulin resistance (Poy et al., 2002b). Mice with liver-specific Ceacam1 inactivation (L-SACC1) or with global null mutation (Cc1−/−), exhibit impairment of insulin clearance and hyperinsulinemia, which causes insulin resistance (DeAngelis et al., 2008b; Park et al., 2006). These mice are also viscerally obese, in part due to redistribution of triglyceride from liver to white adipose tissue (WAT).
Ceacam1 mutant mice demonstrate that impaired insulin clearance causes insulin resistance and visceral obesity. Reciprocally, increased visceral obesity causes insulin resistance by activating FFA- and adipokines-mediated pathways (Lazar, 2005; Sjöholm and Nystrom, 2005; Tataranni and Ortega, 2005). Increased FFA supply causes insulin resistance in liver, at least in part, by impairing insulin clearance through mechanisms involving activation of PKC-delta (Chen et al., 2006) and reducing insulin receptor number (Svedberg et al., 1990). FFA supply also contributes to lipid accumulation in hepatocytes, which in turn induces local inflammatory response (Bigorgne et al., 2008) and elevates TNFα production (Boden, 2006) to inactivate insulin signaling (Cai et al., 2005; Hotamisligil et al., 1993). Similarly, a rise in WAT-derived adipokines modulates the intra-hepatic pro-inflammatory milieu (de Wit et al., 2008; Li et al., 2005) to contribute to pathways leading to insulin resistance.

The most common form of visceral obesity is diet-induced. Prolonged high fat intake causes visceral obesity and insulin resistance, which has been explained in part with the development of a chronic inflammatory state (Chiang et al., 2009; Shi et al., 2006). However, as opposed to other cardinal features of obesity (hyperinsulinemia, insulin resistance, dysplidemia), inflammation (characterized by elevation in WAT-associated and circulating adipokines) constitutes a delayed component of the progression of the disease, and in response to high-fat diet, it emanates from a sustained period of nutrient overload (>3 months) (Kim et al., 2008). Based on previous work underscoring the central role for CEACAM1 in pathways regulating insulin metabolism and de novo lipogenesis (Najjar et al., 2005), we investigated
whether CEACAM1 is implicated in the early pathogenesis of diet-induced insulin resistance. We herein, show that hyperinsulinemia causes insulin resistance, and is not simply a consequence thereof.
RESEARCH DESIGN AND METHODS

Generation of mice and animal husbandry. Like the L-SACC1 mouse (Poy et al., 2002b), L-CC1 transgenic mice with liver-specific over-expression of rat CEACAM1 were generated using the proximal 490 bp-fragment 5' of the translation start site in the human apolipoprotein (Apo) A-I promoter/enhancer element. The ApoAI/WT Ceacam1 minigene, containing intron 1, the BGH polyadenylation signal and a Flag-tag at the 3' end, was excised and injected in the pronuclei of single-cell fertilized mouse embryos from SJLXC57Bl/6J matings (Yale Transgenic Facility). 11 F0 founders were identified by PCR analysis of tail gDNA and liver-specific transgene expression was confirmed by Western analysis using the rat-specific CEACAM1 polyclonal antibody (Poy et al., 2002b) and a monoclonal anti-FLAG M2 antibody (SIGMA cat# F1804). Two lines were identified and backcrossed six times either on the FVB or on the C57BL/6 (BL6) background by mating with wild-type mice from Jackson Laboratories.

All mice were kept in a 12h-dark/light cycle. Male mice (2-3 months of age) were fed ad libitum either a standard chow (Teklad 2016) with low 12% fat content (RD) or a 45% high fat (HF) diet (Research Diets, Catalog# D12451), deriving fat from lard and soy oil and consisting of 36.3% Saturated fat (SFA), 45.3% monounsaturated fat (MUFA) and 17.4% Omega 6 polyunsaturated fatty acids (PUFA). All procedures were approved by the Institutional Animal Care and Utilization Committee.

Analysis of metabolic parameters. Following an overnight fast, mice were anesthetized at 1100h. Whole venous blood was drawn from the retro-orbital sinuses to carry out radioimmunoassay analysis of serum levels of insulin and leptin (Linco Research,
Billerica, MA). Serum free fatty acids (FFA) were determined using NEFA C kit (Wako, Richmond, VA) and serum triglycerides using the Pointe Scientific Triglyceride kit (Canton, MI), as described previously (DeAngelis et al., 2008b). Hepatic triglyceride was measured as described previously (Park et al., 2006).

**Body composition and hyperinsulinemic-euglycemic clamp.** White visceral adipose tissue (WAT) was excised, weighed, and visceral adiposity expressed as percentage of total body weight (BWt). Whole body fat and lean mass were measured by \(^1\)H-magnetic resonance spectroscopy (MRS; Echo Medical Systems, Houston, TX), and a 2-hour hyperinsulinemic-euglycemic clamp was performed in awake overnight-fasted mice (n=10-12) with primed and continuous infusion of human regular insulin (Humulin) at a rate of 2.5 mU·kg\(^{-1}\)·min\(^{-1}\), as previously described (DeAngelis et al., 2008a; Park et al., 2006). Glucose metabolism was estimated with a continuous infusion of [3-\(^3\)H] glucose (PerkinElmer Life and Analytical Sciences) for 2 hours prior to (0.05 \(\mu\)Ci/min) and throughout the clamps (0.1 \(\mu\)Ci/min).

**Daily food intake.** Mice (n\(\geq5\) per age group) were housed individually in a 12-hour dark-light cycle and provided standard chow *ad libitum*. Food was weighed daily at 1200 hours for 7 consecutive days. Daily food Intake was calculated as food mass from previous day minus remaining food in the cage.
**Indirect calorimetry.** Indirect calorimetry was performed in awake mice (6-month-old; n=6 per group), as previously described (Hong et al., 2009). Mice were placed in metabolic cages (TSE Systems, Bad Homburg, Germany) for 3 days and provided food and water *ad libitum*. Spontaneous locomotor activity was measured with an optical beam device used for the quantitative measurement of horizontal and vertical movement (XYZ-axis). Oxygen consumption (VO$_2$) and CO$_2$ production (VCO$_2$) were sampled every 30 minutes for 5 seconds and data normalized per fat free lean mass. Respiratory exchange ratio was calculated from the VCO$_2$/VO$_2$ ratio.

**Intraperitoneal glucose tolerance test.** Following an overnight fast, awake mice were injected intraperitoneally with glucose (1.5 g/kg body weight) before drawing blood from clipped tail for glucose measurement (DeAngelis et al., 2008b).

**Insulin tolerance test.** Following a 6 hour-fast starting at 0800h, awake mice were injected intraperitoneally with Regular Human insulin (Novo Nordisk, 0.75U/kg body weight) before drawing blood from clipped tail for glucose measurement (DeAngelis et al., 2008b).

**Primary hepatocytes isolation and fatty acid treatment.** 0.1 mM of a fatty acid mixture that simulates dietary fat composition [45:35:20 of Oleic acid (OA, monounsaturated fat): palmitic acid (PA, saturated fat): linoleic acid (LA, omega 6 polyunsaturated)] was used
to treat cells.

**Primary hepatocyte isolation.** Animals were anesthetized using pentobarbital at 55mg/kg body weight. The ventral side of the animal was sterilized using ethanol after fixing the mice on its back. Incisions are made with sterile scissors to expose the abdominal cavity. Intestinal contents are carefully moved in order to expose the portal vein. Once the portal vein was located, a loose knot was made using a suture thread, above the liver including all the blood vessels supplying the cranial portion. Perfusion system was pre-cleaned with 70% ethanol and then with Hanks – EGTA solution. Portal vein was cannulated carefully using a 23 G needle, avoiding puncturing the portal vein. Once inside the portal vein, the needle was held in position using a clamp. Surgical knot was tightened above the liver and a cut was made in the inferior vena cava close to the bladder in order to drain the infused solution. The liver was flushed with 15 ml of Hanks EGTA solution at 37 °C. Once liver turned pale, liver tissue was digested by perfusing with 15 ml of L-15 media containing 5.5 mM glucose and 0.08% collagenase type II at 1ml/min. Successful perfusion results in visible cracks in the liver. Perfused liver was dissected out in a petri dish with 10 ml of collagenase type 2 solution. Under the hood, hepatocytes were removed by disrupting glissons capsule (liver capsule) using sterile curved forceps. Primary hepatocytes were filtered using sterile 70µM cell strainers into a 50ml polypropylene tubes. Hepatocytes were pelleted by centrifugation at 400 rpm for 5 min at 4 °C. Cells were washed with sterile 1X PBS twice followed by spinning and pelleting the cells. Once washed, cells were dispensed in 5 to 10 ml of Williams E complete media containing 10mM lactate, 10 nM dexamethasone, 100 nM insulin, 10% fetal bovine
serum and 1% antibiotics. Viable cell counting was performed using tryphan blue stain.

**Insulin internalization assay.** For insulin internalization assay using primary hepatocytes, cells were plated onto 12 well cell culture dish at a cell density of 2.5 x 10^5/well. Cells were plated in Williams E media containing 10% FBS along with other supplements as mentioned above. 24 hours after plating, media changed to Williams E media containing 10% dialyzed FBS with other growth supplements as above. Following treatment of primary hepatocytes with 30µM WY for 18 hours, insulin internalization assay was performed. Briefly, cells were washed twice with 1X PBS and then incubated with 1 ml of KRP buffer containing 30,000 cpm [I^{125}] insulin (Human I^{125} insulin, Perklin-Elmer, Waltham, MA) for 5 hours on ice to allow insulin binding to the hepatocytes. Hepatocytes were washed once with 1X PBS to remove unbound insulin and then the plates were incubated with 1 ml KRP buffer for 5-90 min at 37C. At the end of incubation, cells were washed once with 1X PBS then incubated with 900µl acidic PBS (pH 3.5) for 10 min on ice to remove the surface bound insulin. Hepatocytes were washed once with 1X PBS and lysed with 0.4N NaOH to remove cell-associated insulin.

**In vivo insulin clearance:** In vivo insulin clearance was performed as described previously (Xu et al., 2009) with slight modifications. Briefly, overnight fasted mice were anesthetized using pentobarbital at 55 mg/kg body weight. Human [I^{125}] insulin (Perklin Elmer) was injected via tail vein at 1640 Bq per mouse. Immediately after injection
timing was started and blood sampling was done via retro-orbital bleeding for every 10 sec for up to 2 minutes. Radioactivity in blood samples were counted using γ-counter and the clearance rate was calculated as the percentage of the radioactivity at 10 seconds post intra venous injection.

**Immunohistochemistry** Tissues were formalin-fixed and paraffin-embedded. Slides were placed in Antigen Retrieval solution after deparaffinizing and hydration, quenched in hydrogen peroxide solution for 30 minutes, and blocked with normal goat serum (VECTASTAIN Elite ABC peroxidase enzyme systems kit PK-6101; Vector Laboratories, Burlingame, California). Slides were then incubated with rabbit polyclonal UCP1 antibody (1:100) (ab23841, Abcam, Cambridge, UK) at 4°C overnight. Following three washes with TBS for 5 minutes each, slides were incubated in biotinylated goat anti-rabbit IgG secondary antibody (ABC Vectastain kit) at room temperature for 30 minutes, treated with ABC solution for 30 minutes at room temperature, and stained with DAB Vectastain kit SK-4100.

**Analysis of insulin signaling.** Following an overnight fast, some mice were re-fed for 6-7 hours to elicit a rise in insulin levels (Najjar et al., 2005), before the liver was excised and lysed, and proteins analyzed by 7% SDS-PAGE (Invitrogen) and immunoprobing with polyclonal antibodies against phospho-Akt (Ser\(^{473}\), Cell Signaling Technology Inc., Danvers, MA) followed by α-Akt antibody (Cell Signaling Technology) for normalization. Blots were incubated with horseradish peroxidase-conjugated anti-goat
IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-mouse IgG (Amersham Biosciences, Sunnyvale, CA), or anti-rabbit IgG (Amersham) antibodies prior to detection by enhanced chemiluminescence (ECL; Amersham) and quantifying by densitometry.

**Western analysis.** Following analysis by 4-12% SDS-PAGE, proteins were immunoblotted with polyclonal antibodies against endogenous mouse CEACAM1 (α-CC1) (Najjar et al., 2005), rat CEACAM1 (α-rCC1) (DeAngelis et al., 2008b), fatty acid synthase (α-FAS) (Najjar et al., 2005), PPARα (Santa Cruz), CD36 (Santa Cruz) and FATP-1 (Santa Cruz). For normalization, membranes were reprobed with monoclonal antibodies against Actin, GAPDH and Tubulin (Sigma-Aldrich Corp., St. Louis, MO or Santa Cruz). Proteins were detected by Odyssey® Infrared Imaging System, scanned, and their density measured using Image J software and calculated as percentage of the amount of protein loaded.

**Northern analysis.** Liver and cellular mRNA was purified using Trizol (Invitrogen) followed by the MicroPoly (A) Pure kit (Ambion) and analysis by probing with cDNAs for *Ceacam1*, using the Random Primed DNA Labeling Kit (Roche), prior to reprobing with β-actin cDNA to normalize against the amount of mRNA applied.
**Semi-quantitative Real-Time RT-PCR.** Total hepatic RNA was isolated with PerfectPure RNA Tissue Kit (5 Prime) and total adipose tissue RNA was isolated with RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized by ImProm-II™ Reverse Transcriptase (Promega), using 1µg of total RNA and primers (Table S1). cDNA was evaluated with real-time quantitative PCR (Step One Plus, Applied Biosystems). The relative amounts of mRNA were calculated by comparison to the corresponding standards and normalized relative to β-actin. Results are expressed as mean ± SEM in fold change relative to controls.

**Statistical analysis.** Data were analyzed with SPSS software using one-factor ANOVA analysis or two-tailed Student’s t-test and graphed with GraphPad Prism 4 software. 

*P*<0.05 were statistically significant.
RESULTS

**High-fat intake causes insulin resistance.** High-fat (HF) feeding for 9-30 days progressively increased visceral obesity and subsequently, lipolysis and serum Non-esterified free fatty acids (NEFA) in male C57BL/6 (BL6) mice (Table 1). In parallel, it caused peripheral insulin resistance, as shown by the development of glucose and insulin intolerance (Fig. 1A, B respectively), after 21-30 days. Western analysis showed that when mice were fed a regular diet (RD), insulin release upon 7 hours of refeeding (358.2 ±47.76 vs 39.13±3.162 pM at fasting; \( P<0.05 \)) induced Akt phosphorylation in all insulin target tissues [liver, soleus muscle and white adipose tissue (WAT)] (Fig. 2). In mice fed a HF diet for 30 days; however, the rise in insulin (1128.±70.53 vs 38.72±1.408 pM at fasting; \( P<0.05 \)) induced Akt phosphorylation in muscle and WAT, but not liver (Fig. 2). Together, this demonstrated that hepatic insulin resistance is an early event in diet-induced insulin resistance.

**High-fat intake impairs insulin clearance and causes hyperinsulinemia.** HF feeding for 30, but not 9 days, caused hyperinsulinemia (Table 1). This could be caused at least in part, by impaired hepatic insulin clearance, as demonstrated by: 1) the significant decrease in steady-state C-peptide/insulin molar ratio by HF intake for 30, but not 9 days (Table 1); 2) the higher amount of plasma residual \(^{125}\)I-insulin within 90 sec following intravenous tail injection in HF-fed than RD-fed mice (measured by the Area Under the Curve representing the decrease in plasma insulin with post-injection time) (Fig. 3B with...
graph); and 3) maintenance of low glucose levels 3 hours after insulin injection in mice fed a HF diet for 30 days (Fig. 1), consistent with the possibility that injected insulin was cleared less efficiently in these mice. Additionally, higher glucose induction of acute-phase insulin release (Fig. 3A) suggests that hyperinsulinemia could be caused in part, by an increase in insulin secretion, likely to compensate for peripheral insulin resistance.

High-fat intake decreases hepatic CEACAM1 levels. Because CEACAM1 plays a key role in promoting insulin clearance (DeAngelis et al., 2008a; Poy et al., 2002a; Xu et al., 2009), which mainly occurs in liver and to some extent in kidney, we examined the effect of HF diet on CEACAM1 expression. Northern and western analyses (Fig. 4A,B) showed that HF feeding decreased CEACAM1 mRNA and protein levels by up to 40% after 9-15 days and by >50% after 21-30 days in liver. HF feeding for 30 days caused a similar lowering effect on CEACAM1 protein levels in kidney (Fig. 5). The suppressing effect of HF on CEACAM1 was cell-autonomous, as supported by the decrease in CEACAM1 protein content in primary hepatocytes treated with a fatty acid mixture (0.1 mM) that simulates the dietary fat composition (Fig. 6).

Cause-effect relationship between reduced CEACAM1 levels and insulin resistance. Reminiscent of the normal metabolic phenotype of $Cc^{+/-}$ heterozygous mice (DeAngelis et al., 2008b), loss of <50% of CEACAM1 after 9-15 days of HF feeding was associated with normal insulin and glucose tolerance (Fig. 1A-B). This gene-dosage effect between CEACAM1 levels and insulin resistance in response to HF prompted us to investigate the
cause-effect relationship between hyperinsulinemia (caused by impaired CEACAM1-dependent insulin clearance pathways) and insulin resistance in response to diet. To this end, we generated mice (termed L-CC1) overexpressing FLAG-tagged rat CEACAM1 in liver, using the Apolipoprotein A1 (ApoA1) promoter (Poy et al., 2002b).

Immunoblotting (Ib) liver lysates with α-FLAG (not shown) and α-rat CEACAM1 (α-rCC1) antibodies (Fig. 7), indicated that ApoA1 drove liver-specific expression, as assessed by the absence of rat CEACAM1 in intestine, the other main site of ApoA1 production (Rader, 2006). Unlike endogenous mouse CEACAM1, which underwent a >55% reduction (Fig. 7, Ib: α-mCC1, transgenic rat CEACAM1 protein level remained intact, even after 4 months of HF intake (Fig. 7, Ib: α-rCC1), owing to ApoA1 induction by HF.

Although HF intake may slightly alter insulin clearance in L-CC1 mice, as suggested by the statistically insignificant rise in AUC of plasma residual $^{125}$I-insulin 90 sec following tail injection (Fig. 8A), insulin clearance in HF-fed L-CC1 remained comparable to that in RD-fed WT mice (Fig. 8A,B). Moreover, $^{125}$I-insulin internalization in primary hepatocytes derived from HF-fed L-CC1 was intact, even after HF intake for 4 months (Fig. 9A), in parallel to preserved rat transgenic CEACAM1 protein level (not shown). Because exogenous insulin is mainly extracted in kidney, it is likely that the slight adverse effect of HF on injected insulin clearance in L-CC1 mice is due to compromised renal CEACAM1-dependent insulin clearance, as suggested by reduced $^{125}$I-insulin internalization in primary proximal tubular cells from mice with null deletion of Ceacam1 (Fig. 9B). Nevertheless, HF intake did not alter steady-state plasma insulin levels in L-CC1 after 30 days (Table 2) and only slightly altered it (by ~1.3-fold) after 4
months of high fat intake, as opposed to WT mice in which HF elevated insulin levels by about 2.5-fold after 1-4 months (Table 3), consistent with protected insulin clearance in the liver, the major site of endogenous insulin clearance, in HF-fed L-CC1 mice.

HF intake did not adversely affect Akt activation in the liver of L-CC1 mice in response to the 4-fold rise in insulin levels upon refeeding (Fig. 10). Moreover, both glucose and insulin tolerance were maintained in these mice even after 5 months of HF feeding (not shown). To further assess insulin sensitivity \textit{in vivo}, a 2-hour hyperinsulinemic-euglycemic clamp was performed on overnight fasted awake mice fed a HF diet for 4 months. Basal glucose at the beginning of the clamp was similar in both groups of mice fed either RD or HF (Fig. 11). During the clamp, which supported comparable glucose levels (Fig. 11), HF reduced glucose infusion rate required to maintain euglycemia in L-CC1 to a much lesser extent than WT mice, leading to a ~2-fold higher glucose infusion rate in the HF-fed transgenics (Fig. 11). Consistently, HF reduced the ability of insulin to suppress hepatic glucose production (by ~ 50%) and overall glucose turnover (Rd) in WT, but not L-CC1 mice in which these parameters remained normal (Fig. 11). Moreover, HF induced whole body glycolysis in L-CC1, but not WT mice (Fig. 11), contributing to higher glucose turnover in these as compared to HF-fed WT. Also contributing to higher glucose turnover in HF-fed L-CC1, is the higher glucose uptake in skeletal muscle, as demonstrated by the higher rise in the rate of glucose uptake in response to submaximal concentrations of insulin (1.2 nM) in the oxidative soleus muscle isolated from L-CC1 than WT mice, in which HF treatment virtually blunted the positive effect of insulin on glucose uptake (Fig. 12). Thus, by preventing impairment in insulin clearance and limiting ensuing hyperinsulinemia, liver-
specific CEACAM1 overexpression protected significantly against insulin resistance in response to a long-period of HF intake. Together, this provides in vivo evidence that hyperinsulinemia, resulting from impairment of CEACAM1-mediated insulin clearance pathways, plays a causative role in diet-induced insulin resistance.

Cause-effect relationship between reduced hepatic CEACAM1 levels and visceral adiposity

HF feeding for 1-4 months caused an increase in hepatic triacylglycerol content in WT, but not L-CC1 mice (Tables 2-3). Histological evaluation of H&E-stained liver sections of mice fed HF for 4 months revealed a diffuse fat infiltration with predominantly microvesicular steatosis and a mix of macrosteatosis in livers from HF-fed WT mice (Fig. 13). In contrast, HF-fed L-CC1 mice showed more fat-free parenchyma alternating with affected areas. Moreover, HF-fed WT, but not L-CC1 livers exhibited occasional foci of inflammatory cells.

qRT-PCR analysis revealed that HF intake elevated the liver mRNA of Fatp1, important in fat transport, and of Srebp1c, the master regulator of lipogenic enzyme transcription, in the liver of WT, but not L-CC1 mice (Table 4), owing to hyperinsulinemia in the wild type animal (Horton et al., 2002). Despite a potential increase in fatty acid β-oxidation, as suggested by elevated mRNA levels of Cpt1 that is involved in fatty acid transport to mitochondria (Table 4), HF induced re-esterification and lipogenesis, as supported by enhanced hepatic triacylglycerol level (Tables 2 & 3). Consequently, HF induced hepatic triacylglycerol output, as suggested by elevated serum
triacylglycerol level (Table 2,3) and ApoB48/100 protein content (Fig. 14), followed by redistribution to white adipose tissue and consequently, increase in visceral adipose mass and lipolysis (Tables 2 & 3). In L-CC1 mice, however, HF intake even for 4 months did not significantly increase visceral mass or elevate serum NEFA levels (Table 3). Consistently, HF failed to increase the mRNA levels of *Lpl* and *Fatp-4*, enzymes involved in triacylglycerol hydrolysis and adipocyte uptake; of *Srebp1c*, a nuclear factor involved in lipogenesis, and of *Hsl*, a key enzyme in lipolysis (Fig. 14, Table 5). Absence of hepatic steatosis and the insignificant gain in visceral fat mass in HF-fed L-CC1 mice support the notion that altered regulation of hepatic insulin and lipid metabolism by CEACAM1 mediates the early events involved in diet-induced visceral obesity.

Moreover, histological analysis of H&E stained sections from gonadal white adipose tissue showed a markedly smaller size of adipocytes in L-CC1 than WT mice under normal feeding conditions (Fig. 15), consistent with enhanced insulin sensitivity (as assessed by insulin-stimulated Akt activation-Fig. 10). HF intake for 4 months elevated the adipocyte size in both strains, restoring its normal size in L-CC1 (as compared to RD-fed WT) and exerting a more robust effect in WT mice, with a parallel concomitant effect on macrophage recruitment, as assessed by the mRNA levels of F4/80 (Table 5).

Moreover, qRT-PCR analysis showed higher mRNA level of *Ucp1*, a key gene in brown adipogenesis (de Jesus et al., 2001), in WAT derived from L-CC1 relative to WT mice (Fig. 16B) that was markedly induced by HF feeding for 4 months. Consistently, immunohistochemical analysis (Fig. 16A) revealed elevated UCP1 protein levels (brown staining) and discrete multilocalulated brown adipocytic foci dispersed between white
adipocytes in gonadal WAT derived from HF-fed L-CC1 mice.

Furthermore, mRNA levels of Ucp1 was slightly reduced in brown adipose tissue (BAT) derived from RD-fed L-CC1 mice (Table 6), consistent with reduced overall adiposity in L-CC1 mice. Following 4 months of HF feeding, Ucp1 mRNA levels underwent a marked 2-fold increase in L-CC1, but not WT mice where they remain unchanged (Table 6). The comparable level of Prdm16 mRNA levels in BAT of WT and L-CC1 mice under both feeding conditions (Table 6), suggests that HF feeding does not affect the programming of brown adipogenesis in either mouse (Seale et al., 2007), and that the rise in Ucp1 mRNA in BAT of L-CC1 is likely to reflect a higher sympathetic outflow to BAT in these mice.

Protected energy balance against the effect of HF diet in L-CC1 mice.

Compared to WT, L-CC1 displayed normal daily food intake when fed either RD (4.12±0.09g/day in WT vs 4.35±0.37 in L-CC1) or HF (2.11±0.14g/day in WT vs 2.60±0.13 in L-CC1). Indirect calorimetry analysis over a 24 hour period revealed a normal overall energy expenditure (heat generation) in L-CC1 mice with comparable O₂ consumption (VO₂), CO₂ production (VCO₂) and spontaneous locomotor activity to WT mice under normal feeding conditions (Fig. 16). Owing to normal VO₂ and VCO₂ levels, the Respiratory Exchange Ratio (RER) was also normal in these animals (0.899±0.004 in L-CC1 vs 0.875±0.008 in WT). HF feeding for 4 months reduced RER to the same extent in both groups of mice (0.801±0.008 in HF-LCC1 and 0.780±0.013 in HF-WT mice), but
reduced VO₂, VCO₂, energy expenditure and locomotor activity to a lesser extent in L-CC1, resulting in a higher basal metabolic rate in HF-fed L-CC1 than HF-fed WT mice.

**Cause-effect relationship between reduced hepatic CEACAM1 levels and obesity**

HF feeding caused an increase in body weight at the time of initiation of feeding in WT mice, but at 4-5 weeks in L-CC1 mice, at which point, the gain of body weight gain became comparable in both groups of mice (Fig. 17A). NMR analysis revealed an increase in fat mass at 3 weeks of HF feeding in WT, but at 7 weeks in L-CC1 mice (Fig. 17B), at which point, lean mass started to drop in these mice (Fig. 17B). Nevertheless, the increase in fat mass did not include visceral fat in L-CC1 mice, as HF feeding for 4 months did not significantly elevate visceral adiposity in these mice, as it did in WT animals (Table 3). Together this indicates that overexpressing CEACAM1 in liver plays an important regulatory role in the pathogenesis of obesity and fat distribution in response to HF.
DISCUSSION

The current studies show that hyperinsulinemia resulting from altered CEACAM1-dependent insulin clearance causes insulin resistance and visceral obesity in response to short-term fat intake before a robust pro-inflammatory state develops.

High-fat intake for 30 days reduces hepatic CEACAM1 levels by >50% in BL6 mice. Consistent with Cc1⁻/⁻ phenotype (DeAngelis et al., 2008b), this loss-of-function of Ceacam1 impairs insulin clearance and leads to hyperinsulinemia and hepatic insulin resistance. Conversely, gain-of-function of hepatic Ceacam1 protects against hyperinsulinemia, insulin resistance and visceral obesity in response to a 30 day-fat intake. These observations suggest that hyperinsulinemia caused by impaired insulin clearance, plays a causative role in the early pathogenesis of diet-induced obesity and insulin resistance.

Our finding of a progressive increase in fat mass beginning at ~ 9 days, followed by hyperinsulinemia and insulin resistance (30 days) is in agreement with other recent reports on dietary obesity in BL6 mice (Kim et al., 2008; Park et al., 2005). The Schwartz laboratory (Kim et al., 2008) showed that 60% fat diet induces hyperinsulinemia on BL6 mice before the development of cellular insulin resistance in liver and skeletal muscle (4-8 weeks), and especially in WAT, which takes ~14 weeks to develop in correlation with systemic and WAT-associated inflammatory state. While this differs slightly from our findings and those of the Kim laboratory (Park et al., 2005) of a concurrent development of hyperinsulinemia with whole body and hepatic insulin resistance, it provides a proof-of-principle that hyperinsulinemia can precede altered
insulin signaling in peripheral insulin target tissues, including liver, and supports our finding of intact insulin signaling in WAT after 30 days of fat intake. Given that inducible expression of CEACAM1 in liver protects against insulin resistance, hyperinsulinemia and visceral obesity, we propose that high-fat diet initially reduces hepatic CEACAM1 and subsequently, impairs insulin clearance to cause hepatic insulin resistance and lipogenesis. This leads to lipid redistribution to WAT to give rise to visceral adiposity, and to the skeletal muscle where it reduces glucose uptake and induces lipotoxicity (Muoio and Koves, 2007).

Of interest, inducible expression of CEACAM1 in liver protects against the hypometabolic state induced by HF intake by preventing a decrease in spontaneous locomotor activity and energy expenditure by HF diet without affecting food intake. Absence of a detectable effect of CEACAM1 expression in liver on food intake is consistent with no effect of Ceacam1 global deletion on the feeding behavior (unpublished observation). Protected energy expenditure and spontaneous locomotor activity is likely related to induced brown adipogenesis in white adipose depot and brown adipose tissue mass in HF-fed L-CC1 mice. It is of interest that programming of brown adipose tissue (BAT) is induced to a comparable extent in both mice by HF feeding, suggesting that HF feeding induces this compensatory mechanism in both groups of mice. Given that CEACAM1 is not endogenously produced in adipocytes, it is likely that cell non-autonomous effect brought about by CEACAM1 overexpression in liver drove the changes in adipose depots. Thus, the preferential induction of Ucp1 in BAT derived from HF-fed L-CC1 mice could imply that CEACAM1 induction in liver stimulates an increase in sympathetic outflow to BAT in response to HF feeding, protecting against
disturbed energy expenditure, visceral obesity and metabolic abnormalities in response to prolonged HF feeding. Thus, protected energy expenditure and spontaneous locomotor activity by HF intake, together with increased brown adipogenesis in BAT and WAT (Rosenbaum and Leibel, 2010), and possible increase in sympathetic traffic to adipose tissue, is likely to contribute to preventing hypometabolism from developing in L-CC1 mice in response to HF diet. Although future studies are necessary to dissect the mechanisms involved in the regulation of energy expenditure by hepatic CEACAM1 induction, the phenotype of L-CC1 mice on HF diet uncovers a putative novel role for hepatic CEACAM1 in regulating brown adipogenesis and energy dissipation through changes in the expression of key genes.

The data provide an in vivo demonstration that reduction of hepatic CEACAM1 levels by fat intake causes hyperinsulinemia, which in turn, triggers various metabolic derangements and promotes visceral obesity. This provides a proof-of-principle that hyperinsulinemia causes insulin resistance, and is not just a marker thereof. Because inducible expression of CEACAM1 in liver prevents insulin resistance and visceral obesity by high-fat diet, not only by preserving insulin clearance and insulin metabolism, but also by inducing brown adipogenesis in the white adipose depot and inducing energy dissipation, the data identify CEACAM1 as a tractable drug target against these metabolic conditions.
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Table 1:
Real Time PCR Primer Sequences

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<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tr>
<td>m PPARα</td>
<td>AGATCGGCCCTGGCCTTCTAAACAT</td>
<td>AGCTTTGGGAAGAGGAAGGTGTCAG</td>
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<tr>
<td>m CPT-1</td>
<td>TCCCATAAGAAACAAGACCTCC</td>
<td>GCTCCAGGGTTCAGAAAGGTAC</td>
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<td>m CD36</td>
<td>TCTTGCTACAGCAAGGCCAGATA</td>
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<tr>
<td>m FATP-1</td>
<td>TCACTGGCGCTGTTTGGTT</td>
<td>GGACGGTGCTGTTATGG</td>
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<tr>
<td>m SREBP-1c</td>
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<td>m AOX</td>
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<td>m IL-6</td>
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<td>m IL-1b</td>
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<td>m F4/80</td>
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<td>m UCP-1</td>
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<td>m FATP-4</td>
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<td>m LPL</td>
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<td>m HSL</td>
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<td>m PDRM</td>
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<td>m DIO2</td>
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Table 2:

Effect of 30 days of a high-fat intake on serum and tissue biochemistry of L-CC1 mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L-CC1</th>
<th></th>
<th>WT</th>
<th>L-CC1</th>
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<tr>
<td></td>
<td>RD</td>
<td>HF</td>
<td>RD</td>
<td>HF</td>
<td></td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>28. ± 0.5</td>
<td>34. ± 0.7 ^a</td>
<td>28. ± 0.8</td>
<td>29. ± 1.2</td>
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</tr>
<tr>
<td>% Visceral Fat/BWt</td>
<td>.67 ± .04</td>
<td>2.0 ± .49 ^a</td>
<td>.75 ± .39</td>
<td>.81 ± .22</td>
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<tr>
<td>Fasting Serum Insulin (pM)</td>
<td>53. ± 5.8</td>
<td>112. ± 19.0 ^a</td>
<td>58. ± 7.1</td>
<td>47. ± 4.5</td>
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<tr>
<td>Fasting Serum C-peptide (pM)</td>
<td>403. ± 84.0</td>
<td>568. ± 148. ^a</td>
<td>361. ± 112.</td>
<td>423. ± 210.</td>
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<tr>
<td>Steady State C/I</td>
<td>7.6 ± 0.6</td>
<td>5.1 ± 0.6 ^a</td>
<td>6.2 ± 1.2</td>
<td>9.0 ± 0.9</td>
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<tr>
<td>Hepatic Triglyceride (μg/mg protein)</td>
<td>151. ± 4.3</td>
<td>219. ± 27.3 ^a</td>
<td>138. ± 12.2</td>
<td>163. ± 18.4</td>
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<tr>
<td>Fasting Serum Triglyceride (mg/ml)</td>
<td>61. ± 11.</td>
<td>105. ± 7.4 ^a</td>
<td>73. ± 27.</td>
<td>74. ± 15.</td>
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<tr>
<td>Fasting Serum FFA (mEq/L)</td>
<td>0.63 ± 0.06</td>
<td>0.82 ± 0.05 ^a</td>
<td>0.6 ± .08</td>
<td>0.68 ± 0.05</td>
<td></td>
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</table>

Male mice (n>8) were fed a HF diet for 30 days starting at 2 months of age. ^a P<0.05 HF versus RD. Values are expressed as mean ± SEM.
Table 3
The effect of 4-6 months of high-fat diet on the metabolic phenotype in L-CC1 mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th>L-CC1</th>
<th></th>
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</thead>
<tbody>
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<td></td>
<td>RD</td>
<td>HF</td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>% Visceral fat weight</td>
<td>2.26 ± 0.122</td>
<td>5.16 ± 0.496&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.54 ± 0.348</td>
<td>3.57 ± 0.374</td>
</tr>
<tr>
<td>Fasting blood Glucose (mg/dl)</td>
<td>144. ± 11.6</td>
<td>191. ± 14.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>167. ± 9.1</td>
<td>163. ± 9.6</td>
</tr>
<tr>
<td>Hepatic triglyceride</td>
<td>34.49 ± 5.548</td>
<td>99.16 ± 13.41&lt;sup&gt;A&lt;/sup&gt;</td>
<td>46.38 ± 7.343</td>
<td>54.87 ±</td>
</tr>
<tr>
<td>Serum triglyceride (mg/dl)</td>
<td>75.56 ± 9.34</td>
<td>91.56 ± 15.62</td>
<td>85.74 ± 9.31</td>
<td>74.59 ± 7.43</td>
</tr>
<tr>
<td>Serum FFA (mEq/l)</td>
<td>0.623 ± 0.044</td>
<td>0.817 ± 0.062&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.681 ± 0.039</td>
<td>0.653 ±</td>
</tr>
<tr>
<td>Serum insulin (pM)</td>
<td>66.94 ± 4.215</td>
<td>149.13 ± 14.63</td>
<td>55.37 ± 3.36</td>
<td>82.53 ± 5.68</td>
</tr>
<tr>
<td>Serum C-peptide/insulin</td>
<td>11.07 ± 0.712</td>
<td>8.428 ± 0.209&lt;sup&gt;A&lt;/sup&gt;</td>
<td>10.85 ± 0.365</td>
<td>10.71 ±</td>
</tr>
<tr>
<td>Serum C-peptide</td>
<td>1313. ± 90.56</td>
<td>2432. ± 460.</td>
<td>572.5 ± 66.14</td>
<td>1835. ±</td>
</tr>
<tr>
<td>Glucose 3 h-post insulin (mg/dl)</td>
<td>93.63 ± 4.162</td>
<td>55.29 ± 8.314&lt;sup&gt;A&lt;/sup&gt;</td>
<td>86.43 ± 4.294</td>
<td>87.53 ±</td>
</tr>
</tbody>
</table>

<sup>A</sup> P<0.05 HF versus RD.  <sup>B</sup> P<0.05 LCC1 versus WT. Values are expressed as mean ± S.E. n ≥ 6 per group.
### Table 4
Hepatic Oxidation Markers following 30 days of HF feeding

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L-CC1</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
<td>RD</td>
<td>HF</td>
<td>RD</td>
<td>HF</td>
<td></td>
</tr>
<tr>
<td><strong>PPAR alpha</strong></td>
<td>1.16 ± 0.11</td>
<td>1.6 ± 0.08(^A)</td>
<td>1.10 ± 0.23</td>
<td>1.08 ± 0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CPT-1</strong></td>
<td>1.137 ± 0.07</td>
<td>1.66 ± 0.02(^A)</td>
<td>0.81 ± 0.06</td>
<td>0.46 ± 0.05(^B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD36</strong></td>
<td>1.227 ± 0.11</td>
<td>1.977 ± 0.27</td>
<td>0.67 ± 0.12</td>
<td>0.86 ± 0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FATP-1</strong></td>
<td>1.190 ± 0.15</td>
<td>2.330 ± 0.10(^A)</td>
<td>1.18 ± 0.05</td>
<td>0.87 ± 0.08(^B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SREBP-1c</strong></td>
<td>0.933 ± 0.07</td>
<td>1.932 ± 0.25(^A)</td>
<td>0.90 ± 0.15</td>
<td>1.23 ± 0.10(^B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AOX</strong></td>
<td>0.845 ± 0.06</td>
<td>0.984 ± 0.23</td>
<td>1.33 ± 0.11</td>
<td>1.06 ± 0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^A\) P<0.05 HF versus RD.  \(^B\) P<0.05 Lcc1 versus WT. Values are expressed as mean ± S.E. n ≥ 6 per
Table 5:
Effect of 4 months of high-fat intake on white adipose tissue (WAT) of L-CC1 mice

<table>
<thead>
<tr>
<th></th>
<th>WT RD</th>
<th></th>
<th>WT HF</th>
<th></th>
<th>LCc1 RD</th>
<th></th>
<th>LCc1 HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>3.69 ± 1.2</td>
<td></td>
<td>4.56 ± 1.2</td>
<td></td>
<td>0.83 ± 0.2</td>
<td></td>
<td>0.78 ± 0.1 B</td>
</tr>
<tr>
<td>F4/80</td>
<td>8.85 ± 4.5</td>
<td></td>
<td>21.8 ± 2.6 A</td>
<td></td>
<td>0.45 ± 0.2</td>
<td></td>
<td>9.08 ± 3.0 A B</td>
</tr>
<tr>
<td>UCP-1</td>
<td>1.03 ± 0.0</td>
<td></td>
<td>1.24 ± 0.2</td>
<td></td>
<td>0.29 ± 0.1 B</td>
<td></td>
<td>1.94 ± 0.5 A</td>
</tr>
<tr>
<td>SREBP1c</td>
<td>1.09 ± 0.1</td>
<td></td>
<td>4.03 ± 0.7 A</td>
<td></td>
<td>1.26 ± 0.5</td>
<td></td>
<td>0.94 ± 0.3 B</td>
</tr>
<tr>
<td>FATP-4</td>
<td>0.80 ± 0.1</td>
<td></td>
<td>1.80 ± 0.2 A</td>
<td></td>
<td>0.96 ± 0.2</td>
<td></td>
<td>0.55 ± 0.1 B</td>
</tr>
<tr>
<td>LPL</td>
<td>1.20 ± 0.2</td>
<td></td>
<td>3.51 ± 0.3 A</td>
<td></td>
<td>1.12 ± 0.1</td>
<td></td>
<td>1.25 ± 0.4 B</td>
</tr>
<tr>
<td>HSL</td>
<td>0.49 ± 0.1</td>
<td></td>
<td>0.80 ± 0.1</td>
<td></td>
<td>0.51 ± 0.1</td>
<td></td>
<td>0.27 ± 0.1 B</td>
</tr>
</tbody>
</table>

Male mice (n>5) were fed a HF diet for 4 months starting at 2 months of age. A P<0.05 HF versus RD. Values are expressed as mean ± SEM.
Table 6:

Effect of 4 months of a high-fat intake on brown adipose tissue (BAT) of L-CC1 mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th>L-CC1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>PDRM</td>
<td>0.92 ± 0.09</td>
<td>1.44 ± 0.10 (^A)</td>
<td>0.6 ± 0.04</td>
<td>1.63 ± 0.19 (^A)</td>
</tr>
<tr>
<td>UCP-1</td>
<td>0.71 ± 0.05</td>
<td>0.80 ± 0.01</td>
<td>0.54 ± 0.02 (^B)</td>
<td>1.11 ± 0.28 (^A)</td>
</tr>
<tr>
<td>Dio2</td>
<td>0.54 ± 0.10</td>
<td>2.10 ± 0.41 (^A)</td>
<td>1.60 ± 0.20 (^B)</td>
<td>0.54 ± 0.10 (^B)</td>
</tr>
</tbody>
</table>

Male mice (n>5) were fed a HF diet for 4 months starting at 2 months of age. \(^A\) \(P<0.05\) HF versus RD. Values are expressed as mean ± SEM.
A. Glucose Tolerance Test

B. Insulin Tolerance Test

Figure 1: 30 days of high fat diet was sufficient to cause both glucose and insulin intolerance in WT mice.
Figure 2: 30 days of HF diet results in aberrant insulin signaling in WT animals
Figure 3: Compensatory insulin secretion in response to 30 days HFD, in addition to reduced insulin clearance, results in hyperinsulinemia leading to insulin resistance.
A. mRNA

9 Days

21-30 Days

B. Protein

9 Days

21-30 Days

Figure 4: 30 days of HF diet decreases CEACAM1 by at both the mRNA and protein levels.
<table>
<thead>
<tr>
<th>Mouse:</th>
<th>RD</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ib: α-CC1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>relb: α-Tubulin</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 5: 30 days of HF diet significantly reduces CEACAM1 levels in kidney
Figure 6: Free fatty acids decrease CEACAM1 levels in primary hepatocytes.
Liver-specific expression of rat CEACAM1 transgene in L-CC1 mice

<table>
<thead>
<tr>
<th></th>
<th>L-CC1</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liv</td>
<td>Int</td>
</tr>
<tr>
<td>lb:α-rCC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relb:α-Actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lb:α-Flag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relb:α-Actin</td>
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</tbody>
</table>

Transgenic CEACAM1 protein levels in liver lysates

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L-CC1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>lb:α-rCC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lb:α-mCC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relb:α-actin</td>
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</tbody>
</table>

Total CC1 mRNA (Gapdh)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L-CC1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
</tbody>
</table>

Figure 7: Expression of rat CEACAM1 is specific to the liver in transgenic mice, and is not decreased with HF feeding.
Figure 8: In vivo insulin clearance is more efficient in L-CC1 mice than in WT after 1 month of HF feeding.
Figure 9: Insulin clearance remains intact in primary hepatocytes extracted from L-CC1 mice but not in proximal tubule cells.
Figure 10: 30 days of HF diet causes aberrant insulin signaling in WT mice, but is protected in L-CC1 mice.
Figure 11: L-CC1 mice are protected against HF-induced insulin resistance demonstrated through hyperinsulinemic-euglycemic clamp analysis.
Figure 12: L-CC1 mice maintain normal glucose uptake in soleus muscle after 4 months of HF intake.
Figure 13: L-CC1 mice are protected against hepatic steatosis and inflammatory infiltration.
**WT** | **L-CC1**
---|---
RD | HF | RD | HF

IB: α-ApoB

<table>
<thead>
<tr>
<th><strong>Lpl mRNA</strong></th>
<th><strong>Fatp-4 mRNA</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Lpl mRNA graph" /></td>
<td><img src="image" alt="Fatp-4 mRNA graph" /></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Srebp-1c mRNA</strong></th>
<th><strong>Hsl mRNA</strong></th>
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<tbody>
<tr>
<td><img src="image" alt="Srebp-1c mRNA graph" /></td>
<td><img src="image" alt="Hsl mRNA graph" /></td>
</tr>
</tbody>
</table>

* vs. RD of same genotype; ^ vs. WT mice of the same feeding group

Figure 14: Lipid homeostasis remains normal in HF-fed L-CC1 mice
Figure 15: Adipocyte size in L-CC1 HF-fed mice remains in the normal range and comparable to that of WT RD-fed mice.
Figure 16: HF-fed WT mice exhibit dampened energy expenditure while HF appears to have no effect energy expenditure in L-CC1 mice.
Figure 17: L-CC1 mice exhibit a delay in onset of increased body weight in response to HF diet in comparison to WT animals.
4 months of HF Diet

Lean Mass (g)

<table>
<thead>
<tr>
<th></th>
<th>RD</th>
<th>HF</th>
<th>RD</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-CC1</td>
<td></td>
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</tbody>
</table>

Fat Mass (g)

<table>
<thead>
<tr>
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<th>RD</th>
<th>HF</th>
<th>RD</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-CC1</td>
<td></td>
<td></td>
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</table>

6 months of HF Diet

Visceral Obesity (% BWT)

<table>
<thead>
<tr>
<th></th>
<th>RD</th>
<th>HF</th>
<th>RD</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-CC1</td>
<td></td>
<td></td>
<td>*</td>
<td>†</td>
</tr>
</tbody>
</table>

Figure 18: L-CC1 mice are protected against an increase in fat mass in response to HF diet compared to WT mice.
REFERENCES


Mice with null mutation of Ceacam1 develop non-alcoholic steatohepatitis

Sumona Ghosh1,§,*; Meenakshi Kaw1,§,*; Payal R. Patel1,§; Kelly J. Ledford1,§;
Thomas A. Bowman1,§; Marcia F. McInerney2,§; Sandra K. Erickson3; Raymond E. Bourey4,§; and Sonia M. Najjar1,§,†

* Authors contributed equally to the work

§ Center for Diabetes and Endocrine Research and the 1 Departments of Physiology and Pharmacology, and 4 of Medicine at the University of Toledo College of Medicine, Health Science Campus, Toledo, Ohio, USA, 2 Department of Medicinal and Biological Chemistry at the College of Pharmacy, the University of Toledo, Main Campus, Toledo, OH USA and 3 Department of Medicine, University of California, and Veterans Affairs Medical Center, San Francisco, USA.

† Address correspondence to:

Sonia M. Najjar, Ph.D.
College of Medicine
University of Toledo
Health Science Campus
3000 Arlington Avenue, Mail stop 1008
Toledo, Ohio, 43614
Tel: (419) 383-4059
FAX: (419) 383-2871
e-mail: sonia.najjar@utoledo.edu
Nonstandard abbreviations used: NASH, nonalcoholic steatohepatitis; BL6, C57BL/6 genetic background; CEACAM1, CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 1; Ceacam1, gene encoding CEACAM1 protein; L-SACC1, transgenic mouse with Liver-specific overexpression of the dominant-negative S503A CEACAM1 phosphorylation-defective mutant; Cc−/−, global Ceacam1 null mouse; Cc+/+, wild type mouse from the same genetic background as Cc−/− null mice; RD, regular diet; HF, high fat diet; TG, triglyceride; FFA, free fatty acids; FAS, fatty acid synthase; PPARα, peroxisome proliferator-activated receptor α; NPC1, Niemann Pick type C1; CYP2E1, cytochrome p450 enzyme; GSH, glutathione; TNFα, tumor necrosis factor-α; TGFβ, transforming growth factor β; IFNγ, interferon gamma; IL, Interleukins; and H&E staining, hematoxylin-eosin staining.
Abstract: Transgenic liver-specific inactivation of the Carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) impairs hepatic insulin clearance and causes hyperinsulinemia, insulin resistance, elevation in hepatic and serum triglyceride levels, and visceral obesity. It also predisposes to non-alcoholic steatohepatitis (NASH) in response to high fat diet. To discern whether this phenotype reflects a physiological function of CEACAM1 rather than the effect of the dominant-negative transgene, we investigated whether Ceacam1 null mice with impaired insulin clearance also develop a NASH-like phenotype on a prolonged high fat diet. 3 month-old male null mice were fed a high fat diet for 3 months and their NASH phenotype was examined. While high fat feeding elevated hepatic triglyceride content in both strains of mice, it exacerbated macrosteatosis and caused NASH-characteristic fibrogenic changes and inflammatory responses more intensely in the null mouse. This demonstrates that CEACAM1-dependent insulin clearance pathways are linked with NASH pathogenesis.

Keywords: Non-alcoholic steatohepatitis, CEACAM1, high-fat diet, inflammation, apoptosis, fibrosis
Introduction

Non-alcoholic fatty liver disease (NAFLD) constitutes a spectrum of diseases ranging from benign hepatic steatosis to chronic nonalcoholic steatohepatitis (NASH) and cirrhosis. The incidence of non-alcoholic fatty liver disease (NAFLD) is on the rise worldwide, in part due to the increase in obesity (Erickson, 2009). The prevalence of NAFLD in the United States is currently estimated at ~ 20-25%, and in the morbidly obese population, at ~75-90%. NASH, its progressive form, is estimated to be present in ~3-5% of the general population (Lazo and Clark, 2008; Ong and Younossi, 2007). Of these, ~3-5% have progressed to cirrhosis, including those with end-stage liver disease and/or primary liver cancer (Bullock et al., 2004; Marrero et al., 2002).

Despite intense research efforts, mechanisms of the etiology and progression of NAFLD remain poorly understood. This could be due in part, to the limitation of the experimental animal models, which have been used to study the pathogenesis of the disease (Anstee and Goldin, 2006; Ariz et al., 2010). Clearly, NAFLD is multi-faceted and includes both genetic and environmental factors (Erickson, 2009). It is generally agreed that visceral obesity and dysregulation in lipid metabolism and hepatic immune response are involved in progression to NASH (Day and James, 1998). Although it is likely that NAFLD likely shares common pathophysiology with type 2 diabetes and metabolic syndrome, the role of insulin resistance in its pathogenesis remains controversial (Farrell, 2009; Green, 2003).

Upon phosphorylation by the insulin receptor, CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1) promotes receptor-mediated insulin endocytosis and degradation in the hepatocyte, the main mechanism of insulin clearance in liver (Formisano et al., 1995). L-SACC1 mice with liver-specific dominant-negative
transgenic inactivation of CEACAM1 and mice with global null mutation of the Ceacam1 gene (Cc1\(^{-/-}\)) develop insulin resistance due to impaired hepatic insulin extraction and hyperinsulinemia. (Poy et al., 2002b) Chronic hyperinsulinemia results in increased hepatic lipid production and output, followed by substrate redistribution to white adipose tissue and visceral obesity.

We have recently shown that L-SACC1 mice develop spontaneous hepatic fibrosis, which becomes more intense when the mice are fed a high fat diet for 3 months. (Lee et al., 2008a) Moreover, other key features of NASH (inflammation, oxidative stress and apoptosis) are induced by sustained high fat intake. (Lee et al., 2008a) This could be due to complete loss of function of CEACAM1 or to the inability of the transgene-encoded protein to become phosphorylated. To gain further insight into the role of CEACAM1 in the etiology and progression of NAFLD, we have assessed the effect of whole-body Ceacam1 gene deletion on the pathogenesis of NASH in response to high fat feeding. We herein report that Ceacam1 null mice (Cc1\(^{-/-}\)) exhibit key features of obesity-related NASH when fed a high fat diet for three months.
Materials and methods

Cell culture

Ceacam1 null mice (Cc1^−/−) null mice were backcrossed twelve times onto the C57BL/6 (BL6) genetic background,(Leung et al., 2006b) kept in a 12-hour dark/light cycle and fed a standard chow ad libitum. All procedures were approved by the Institutional Animal Care and Utilization Committee. Three month-old male mice were fed ad libitum either a standard chow (Teklad 2016) with low 12% fat content (RD) or a 45% high fat diet (HF) diet (Research Diets, Catalog # D12451) for 3 months prior to sacrifice and phenotypic characterization.

Metabolic analysis

Following an overnight fast, mice were anesthetized with sodium pentobarbital at 1100h. Whole venous blood was drawn from the retro-orbital sinuses to measure serum insulin by radioimmunoassays (Linco Research), serum free fatty acids (FFA) by NEFA C kit (Wako) and serum triglycerides by Infinity Triglycerides (Sigma). Hepatic total cholesterol and free cholesterol were measured using Infinity cholesterol reagent (Thermo Electron) and free cholesterol reagent (Wako), respectively,(Erickson et al., 2003) and triglyceride content, as described previously.(Park et al., 2006b) Visceral adipose tissue was excised, weighed, and visceral adiposity expressed as percentage of total body weight.

Liver histology

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin-eosin (H&E) and assessed for the degree of steatosis and lobular inflammation according to
the NASH scoring system recently proposed by the NIDDK-NASH Clinical Research Network. (Bondini et al., 2007) Fibrosis was assessed on deparaffinized and rehydrated slides in 0.1% solution of Sirius Red (Sigma, Direct Red 80). TUNEL assay was performed using the ApopTag Plus Peroxidase Apoptosis Detection Kit (Chemicon International). Tissue sections on the other half of the same slide served as controls without TDT enzyme following digestion with Proteinase K to account for nonspecific binding of enzyme conjugate.

GSH assay

Liver concentrations of reduced glutathione were assayed using the Bioxytech GSH-400 kit (OXISResearch), as previously done. (Lee et al., 2008a)

Western analysis

Tissue lysates or serum (for ApoB) were analyzed by 4-12% gradient SDS-PAGE (Invitrogen) prior to Western analysis with polyclonal antibodies against Caspase 3 (Cell Signaling Technology-Cat#9662), ApoB48/100 (Chemicon International), fatty acid synthase (FAS), (Najjar et al., 2005b) NPC1 (Abcam), p65 NF-kB phosphoserine (Ser 536) and p65 NF-kB (Cell Signaling Technology), as previously done. (Lee et al., 2008a) For normalization, membranes were reprobed with monoclonal antibodies against RPS3 (ProteinTech Group, Inc) and α-actin (Sigma). All blots were incubated with horseradish peroxidase-conjugated anti-IgG antibody prior to protein detection by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and quantification by densitometry and Image J software (v. 1.40, NIH).

Semi-quantitative real-time polymerase chain reaction-based gene expression
Total RNA in the liver was isolated with PerfectPure RNA Tissue Kit (5 Prime) and total RNA in the adipose tissue was isolated with RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using ImProm-II™ Reverse Transcriptase (Promega) using 1μg of total RNA and primers for lipid catabolism, fibrosis and inflammatory markers, and as reference control, GAPDH (Table 1). cDNA was evaluated with real time quantitative polymerase chain reaction (Step One Plus, Applied Biosystems). The relative amounts of mRNA were calculated by comparison to the corresponding standards and normalized relative to GAPDH. Results are expressed in fold change as mean ± SEM.

Statistical analysis

Data were analyzed with SPSS software using one-factor ANOVA analysis and graphed with GraphPad Prism 4 software. P<0.05 was statistically significant.
Results

High fat diet alters hepatic insulin and lipid metabolism in Cc1<sup>−/−</sup> null mice

As previously reported,(DeAngelis et al., 2008b; Xu et al., 2009) 6 month-old male Cc1<sup>−/−</sup> mice exhibited visceral obesity and hyperinsulinemia (Table 2). Consistent with increased visceral obesity, they also developed elevated serum levels of leptin and free fatty acids (FFA) (Table 2). High fat (HF) feeding for 3 months induced serum insulin levels by ~2-fold in both strains of mice (Table 2).

Western analysis revealed hepatic fatty acid synthase (FAS) protein was ~ 2-fold higher in null than wild type mice, regardless of diet (Figure 1a). This could contribute to the significantly higher hepatic triglyceride content in RD-fed null mice (Table 2). Sustained HF intake induced hepatic triglyceride level in both mouse strains, but decreased serum triglyceride in Cc1<sup>−/−</sup> null mice (Table 2). The lower gain in adiposity in Cc1<sup>−/−</sup> null mice in response to HF diet [1.5- versus 3-fold increase in Cc1<sup>+/+</sup> wild type mice (Table 2)] suggests that the lower serum triglyceride in Cc1<sup>−/−</sup> null mice was due to a reduction in hepatic triglyceride output. This notion is in part, supported by the lower serum ApoB100/ApoB48 protein levels in HF-fed null than wild type mice (Figure 1b).

Liver total and free cholesterol were comparable in both mouse strains on the low fat regular diet (RD). Total cholesterol was increased ~ 4-fold in both on the HF diet, with the increase due to increased cholesterol esters (Table 2). The protein level of hepatic NPC1, a late endosomal cholesterol traffic protein, was decreased ~50% by HF diet in null but not wild type mice (Figure 1a). Consistently, hepatic GSH content in Cc1<sup>−/−</sup> was almost half the level in Cc1<sup>+/+</sup> mice on the HF diet (Figure 1c).
Hepatic steatosis was increased in both strains on the HF diet (Figure 2). Histological evaluation from different fields revealed that fat infiltration was more diffuse in livers from $Cc1^{-/-}$ as compared to $Cc1^{+/+}$ wild type mice (panel 4 versus 2), which showed fat-free parenchyma alternating with affected areas. In HF-fed $Cc1^{-/-}$ null mice, the steatosis appeared to be predominantly macrovesicular in addition to microvesicular hepatocyte fat accumulation. However, the HF-fed wild type $Cc1^{+/+}$ livers exhibited predominantly microvesicular steatosis with a mix of macrosteatosis. No ballooning injury of the hepatocyte was noted.

High fat diet induces an inflammatory response in liver and adipose tissue of $Cc1^{-/-}$ null mice

H&E staining of liver sections revealed few inflammatory islands with no significant change in hepatocellular architecture in both strains of mice on the regular diet (Figure 2, panels 1 and 3). Upon HF feeding, $Cc1^{+/+}$ wild type mice showed occasional foci of inflammatory cells mostly periportal (Figure 2, panel 2 and Figure 3a, left panel), as opposed to $Cc1^{-/-}$ null mice, which exhibited multiple foci of inflammatory cell infiltrates in the hepatic lobules as well as in the periportal areas (Figure 2, panel 4 and Figure 3a, right panel). Consistently, HF activated NF-κB to a higher extent in $Cc1^{-/-}$ than $Cc1^{+/+}$ livers, as indicated by a ~10-fold versus ~1.5-fold increase in NF-κB phosphorylation (Figure 3b).

Consistent with macrophage recruitment to white adipose tissue (WAT) in visceral obesity, (Hotamisligil et al., 1993b; Weisberg et al., 2003) RD-fed $Cc1^{-/-}$ null mice exhibited higher F4/80 and TNFα mRNA levels in their WAT than their wild type counterparts, as assessed by semi-quantitative RT-PCR analysis (Table 3). Similarly, hepatic TNFα mRNA content is elevated in the $Cc1^{-/-}$ null mouse. This could derive from
resident hepatic macrophages, the population of which appeared to be elevated based on the higher F4/80 mRNA content in Cc1<sup>−/−</sup> mouse liver (Table 3), in addition to hepatic WAT infiltrates. Sustained HF diet induced a further increase in the macrophage pool (F4/80) and TNF<sub>α</sub> mRNA content in WAT and liver of both groups of mice (Table 3).

mRNA content of CD4 was elevated in RD- fed Cc1<sup>−/−</sup> null mice and remained elevated without further increase in response to HF diet (Table 3). This suggests basal elevation in CD4<sup>+</sup> T cell pool in null mice. Hepatic mRNA content of TGFβ and IFNγ followed a similar change. Elevation in basal IFNγ, but not IL-4/IL-13 mRNA levels, suggests a CD4<sup>+</sup> Th1, but not CD4<sup>+</sup> Th2 response, in RD-fed Cc1<sup>−/−</sup> mouse livers.

In agreement with the small degree of periportal inflammation in HF-fed Cc1<sup>+</sup><sup>+/+</sup> wild type mice (Figure 3a, left panel), HF diet induced hepatic mRNA levels of pro-inflammatory TNF<sub>α</sub> and IFNγ cytokines (Table 3).

High fat diet causes apoptosis in Cc1<sup>−/−</sup> null mice

Excessive fatty acid β-oxidation and lipid ω-peroxidation promote oxidative stress in obesity.(Chavin et al., 1999; Surwit et al., 1998) Consistently, semi-quantitative RT-PCR analysis revealed higher hepatic mRNA levels of PPARα in RD-fed Cc1<sup>−/−</sup> null than Cc1<sup>+</sup><sup>+/+</sup> wild type mice, and a further increase by HF intake in both strains of mice (Table 4). This suggests that fatty acid β-oxidation was elevated in Cc1<sup>−/−</sup> mouse liver. The mRNA levels of hepatic CYP2E1, a member of the microsomal cytochrome p450 family, involved in metabolism of long chain fatty acids (lipoxygenation) and microsomal lipid ω-peroxidation, were comparable in both RD-fed mouse strains, but underwent ~2-fold increase by HF diet only in Cc1<sup>−/−</sup> null mice (Table 4). Thus, it is likely that HF induced oxidative changes in the null mouse,(Robertson et al., 2001) which in the presence of high TNFα levels, predispose to cell death.(McClain et al., 2004) Consistently, Western
analysis (Figure 4a) revealed higher Caspase 3 cleavage in HF-fed Cc1−/− liver than other groups of mice. In addition to TUNEL stain (Figure 4b, panel 2 versus 1), this reveals enhanced apoptosis in HF-fed null but not wild type mice.

Evaluating total NAS score from H&E liver sections based on three histological features (steatosis, inflammation and ballooning) following the Kleiner’s scoring system,(Kleiner et al., 2005) indicated that HF-fed Cc1−/− mice developed NASH (NAS score of ~5), as opposed to the other groups, which scored <2.75 (Table 5).

Development of early fibrosis in Cc1−/− null mice

In NASH, fibrosis usually starts in zone 3 as perivenular and/or pericellular fibrosis, a pattern referred to as "chicken-wire" fibrosis. Sirius red stain revealed a "chicken-wire" pattern of collagen deposition in Cc1−/− (stage 2),(Brunt et al., 1999; Lewis and Mohanty, 2010) but not Cc1+/+ wild type mice on the regular diet (Figure 5, panel 3 versus 1), in spite of elevated levels of the pro-inflammatory IFNγ cytokine, which decreases collagen synthesis.(Bhogal and Bona, 2005) Consistent with the pro-fibrogenic effect of IL-6 cytokine and TGFβ,(Bhogal and Bona, 2005) their mRNA content was higher in the liver of Cc1−/− than Cc1+/+ mice on the regular diet (Table 3). Sustained HF feeding exacerbated these fibrogenic changes in Cc1−/− mice (panel 4 vs 3) without causing further changes in IL-6 and TGFβ mRNA levels. Instead, HF induced serum leptin (Table 2) and hepatic TNFα mRNA levels (Table 3), which together could mediate progression of fibrosis and inflammation in NASH.(Carter-Kent et al., 2008)

In Cc1+/+ wild type mice, HF elevated leptin (Table 2) and hepatic TGFβ and TNFα (Table 3), without altering hepatic IL-6 mRNA levels (Table 3). HF induced a small increase in collagen production, as shown by Sirius red staining (Figure 5, panel 2 versus 1).
Discussion

We and others (Formisano et al., 1995) have shown that similar to L-SACC1 mice with liver-specific inactivation of CEACAM1, mice homozygous for null mutation of the Ceacam1 gene on the C57BL/6 (BL6) genetic background manifest impairment of insulin clearance and hyperinsulinemia. This leads to insulin resistance and increases transcription of lipogenic enzymes (including FAS) to promote de novo hepatic lipid production. (DeAngelis et al., 2008b) On the BL6 genetic background, Ceacam1 null mutation causes preferential lipid redistribution to white adipose tissue and an increase in visceral obesity. 10

The current studies show that sustained high fat feeding of Cc1−/− mice caused lipid accumulation in hepatocytes despite increase in fatty acid β-oxidation. Similar changes are seen in L-SACC1 mice (Lee et al., 2008a) and human NASH. (Abdelmalek and Diehl, 2007; Charlton et al., 2002b) Cc1−/− mice also developed NASH-characteristic fibrosis when fed a high fat diet; even on a regular chow diet, they exhibited low levels of pericellular fibrosis. In response to a high fat diet, which triggers inflammation, (Chiang et al., 2009; Kim et al., 2008b; Shi et al., 2006) Cc1−/− mice exhibited other key features of progressive NAFLD, including diffuse macrosteatosis, inflammation and apoptosis, and possibly oxidative changes, as suggested by increased hepatic CYP2E1 protein levels. These data provide further in vivo evidence that altered CEACAM1-dependent insulin clearance pathways can provide a link between insulin resistance and NASH development, at least in mice with a BL6 genetic component background. (Hill-Baskin et al., 2009) They also confirm that male BL6 mice are susceptible to developing a NASH-like phenotype. (Kirsch et al., 2003)

Ceacam1 null mutation did not affect total and free cholesterol content in liver.
However, reduction of NPC1 protein level in HF-fed Cc1−/− mice suggests increased partitioning of free cholesterol from cytosolic lipid droplets to mitochondria. (Shen et al., 2003) This could reduce mitochondrial GSH stores (Mari et al., 2006a) and increase sensitivity to the cytotoxic effect of the pro-inflammatory cytokine, TNFα, the level of which was high in Cc1−/− livers, resulting partly from steatosis-induced changes in the inflammatory milieu. (Bigorgne et al., 2008; Sheth et al., 1997) Consistent with a role for TNFα–dependent activation of IKK-β in oxidative stress and inflammation, (Crespo et al., 2001) high fat diet activated NF-κB pathways to elicit a more robust inflammatory response in Cc1−/− than Cc1+/+ mice.

The population of CD4+ T cell was basally higher in Cc1−/− than Cc1+/+ mice fed a regular diet. This could at least in part, be due to Ceacam1 deletion in T cells, as suggested by increased CD4+ T pool in mice with conditional deletion of Ceacam1 in T cells, (Nagaishi et al., 2006b) but not in regular chow-fed L-SACC1 mice with functional inactivation of Ceacam1 specifically in hepatocytes. (Lee et al., 2008a) The increase in CD4+ T pool was accompanied by elevation in hepatic mRNA content of IFNγ without changes in IL-4/IL-13 levels, suggesting increased production of CD4+ Th1, but not CD4+ Th2 cytokine in Cc1−/− livers. (Aarsland et al., 1996) Elevated basal leptin level and its inducing effect on TNFα could contribute to the increase in CD4+ Th1 response. (Lord et al., 1998) Given that Cc1−/− mice lack the anti-inflammatory effect of CEACAM1 in T (Gray-Owen and Blumberg, 2006a) and B cells, (Lobo et al., 2009) it is likely that deletion of Ceacam1 in lymphocytes contributed to the more robust inflammatory response to high fat diet in mutant mice.

Consistent with insulin resistance being an independent predictor for fibrosis in NASH, (Angulo et al., 1999) chow-fed Cc1−/− null mice developed a low level of pericellular fibrosis. This could be attributed to elevated hepatic content of the pro-
fibrogenic factors, IL-6 and TGFβ.(Bhogal and Bona, 2005; Syn et al., 2009) In addition to IL-6, leptin increases transcription of TNFα,(Lord et al., 1998; Tilg and Moschen, 2006) which together with IFNγ, reduces collagen synthesis.(Bhogal and Bona, 2005) The combined effect of TNFα and IFNγ inflammatory cytokines could limit the pro-fibrogenic effect of IL-6 and TGFβ in RD-fed null mice. High fat feeding caused progressive fibrosis in parallel to preferentially inducing TNFα without changing IFNγ levels. TNFα triggers apoptosis, which in turn, leads to fibrosis and inflammation.(Canbay et al., 2002; Lewis and Mohanty; Syn et al., 2009) Thus, it is possible that further induction in TNFα by high fat diet activated caspase 3 to cause apoptosis and subsequently, promote fibrosis more preferentially in the null mouse. The increase in serum leptin levels could exacerbate the fibrogenic effect of TNFα.(Carter-Kent et al., 2008; Manco et al., 2007)

In contrast to the null mouse, fat feeding elevated hepatic TGFβ, but not IL-6 mRNA content in Cc1<sup>+/+</sup> wild type mice. It also failed to produce NASH-like fibrogenic changes in these mice. IL-6 could be secreted basally from macrophages, but also from B cells, as part of the adaptive immune response.(Bhogal and Bona, 2005) Given that IL-6 release from B cells induces differentiation of hepatic stellate cells to myofibroblasts to mediate hepatic fibrosis in response to CCl4 in mice, independently of T-cell or antibody stimulation,(Novobrantseva et al., 2005) it is possible that changes in hepatic IL-6 levels contributed to the differential development of hepatic fibrosis in Cc1<sup>−/−</sup>, but not wild type mice. This is in concordance with the positive correlation between the severity of the disease in NASH patients and hepatic IL-6 levels.(Lemoine et al., 2009; Wieckowska et al., 2008)

Our data emphasize that loss of CEACAM1 causes insulin resistance, hepatic
steatosis and visceral obesity, with a subsequent increase in leptin production and release from white adipose tissue. This in turn, promotes production of IL-6 and TNF\(\alpha\), which in addition to other adipokines trigger a pro-inflammatory state. In the absence of CEACAM1, sustained high fat feeding exacerbates the inflammatory response and elevates hepatic TNF\(\alpha\) content and its pro-apoptotic and fibrogenic effect. This model illustrates that null mutation of Ceacam1 integrates metabolic, apoptotic, inflammatory and fibrogenic signals leading to progressive NAFLD. Given that murine models rarely develop spontaneous fibrosis,(Erickson, 2009) the \(Cc1^{-/}\) mouse provides a unique tool to investigate the effect of environmental factors on genetic predisposition to NAFLD progression, and sheds light on the pathogenesis of NASH. The significance of this finding to human disease is highlighted by the strong conservation of CEACAM1 functional domains in humans and rodents, and by the reduction of hepatic Ceacam1 mRNA levels in low capacity running rats that exhibit features of metabolic syndrome(Wisloff et al., 2005) and NASH.(Thyfault et al., 2009)

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**Disclosures**

Authors declare that no conflicts of interest exist.
References


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<th>Reverse Sequence</th>
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<td>TCCTGCCACTTGCTCACTAC</td>
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<td>CYP2E1</td>
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<td>GCCGGAAGCGCTTTGCAAC</td>
</tr>
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<td>CAAGGAGGATGAGTTATCGTG</td>
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<td>GGAATAAAGCATCAAACTCG</td>
</tr>
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<td>CGGACTCCGCAAAGTCTAAG</td>
</tr>
<tr>
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<td>GGCCTTCCCCTACTTCACAAG</td>
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</tr>
<tr>
<td>TGFβ</td>
<td>CAACAATTCCTGCGCCCTTGG</td>
<td>GAAAGCCCTGTATCCGCTT</td>
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**TABLE 1**

Primer sequences used for qRT-PCR
TABLE 2 Effect of high fat intake on serum and tissue biochemistry of 6 month-old male \( Cc1^{+/+} \) and \( Cc1^{-/-} \) mice

<table>
<thead>
<tr>
<th></th>
<th>( Cc1^{+/+} )</th>
<th>( Cc1^{-/-} )</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>26.2 ± 0.35</td>
<td>42.4 ± 0.49</td>
</tr>
<tr>
<td>Visceral fat, % Bwt</td>
<td>1.33 ± 0.15</td>
<td>3.87 ± 0.39</td>
</tr>
<tr>
<td>Serum Insulin, pM</td>
<td>50.0 ± 2.00</td>
<td>123. ± 37.0</td>
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<tr>
<td>Serum FFA, mEq/L</td>
<td>0.50 ± 0.10</td>
<td>1.09 ± 0.11</td>
</tr>
<tr>
<td>Serum TG, mg/dL</td>
<td>46.9 ± 7.00</td>
<td>46. 4 ± 4.84</td>
</tr>
<tr>
<td>Serum Leptin, ng/mL</td>
<td>2.46 ± 0.24</td>
<td>67.2 ± 1.39</td>
</tr>
<tr>
<td>Hepatic TG, mg/g protein</td>
<td>97.9 ± 13.2</td>
<td>224. ± 37.9</td>
</tr>
<tr>
<td>Hepatic Total Cholesterol (mg/g protein)</td>
<td>14.6 ± 0.88</td>
<td>59.6 ± 11.1</td>
</tr>
<tr>
<td>Hepatic Free Cholesterol (mg/g protein)</td>
<td>10.7 ± 0.68</td>
<td>12.1 ± 1.01</td>
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<tr>
<td>Hepatic Cholesterol Esters (mg/g protein)</td>
<td>3.86 ± 0.68</td>
<td>47.5 ± 10.1</td>
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Male mice (n>7; 3 months of age) were fed RD or HF for 3 months. Mice were fasted overnight, and the serum and tissues removed and analyzed. Values are expressed as mean ± SEM \( a P< 0.05 \) HF vs. RD; \( b P < 0.05 \) \( Cc1^{-/-} \) vs. \( Cc1^{+/+} \) in the same feeding group.

**Abbreviations:** \( Cc^{-/-} \), global Ceacam1 null mouse; \( Cc^{+/+} \), wild type mouse from the same genetic background as \( Cc^{-/-} \) mice; RD, regular diet; HF, high fat diet; TG, triglyceride; FFA, free fatty acids.
TABLE 3 Effect of high fat intake on the expression of selected genes related to inflammation in Cc1<sup>+/+</sup> and Cc1<sup>−/−</sup> mouse livers and white adipose tissue (WAT)

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<th>Cc1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>WAT</td>
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<tr>
<td>F4/80</td>
<td>42.2 ± 5.55</td>
<td>262. ± 57.8</td>
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<td>TNF-α</td>
<td>0.03 ± 0.00</td>
<td>0.06 ± 0.00</td>
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<td>Liver</td>
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<tr>
<td>F4/80</td>
<td>0.22 ± 0.08</td>
<td>0.42 ± 0.06</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.04 ± 0.01</td>
<td>0.14 ± 0.02</td>
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<tr>
<td>IL-6 (x10-1)</td>
<td>0.06 ± 0.02</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>CD3</td>
<td>0.01 ± 0.00</td>
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<tr>
<td>CD8</td>
<td>0.26 ± 0.09</td>
<td>0.42 ± 0.06</td>
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<td>IL-4</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
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<td>IL-13</td>
<td>0.18 ± 0.01</td>
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<td>IFN-γ (x10-2)</td>
<td>0.07 ± 0.02</td>
<td>0.20 ± 0.04</td>
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<td>TGFβ</td>
<td>0.29 ± 0.07</td>
<td>0.56 ± 0.05</td>
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Male mice (n≥7; 3 months of age) were fed RD or HF for 3 months. Mice were fasted overnight, the tissues removed and gene expression assessed by semi-quantitative real time-PCR analysis. Values are normalized to Gapdh and expressed as mean units ± SEM

<sup>a</sup>P < 0.05 HF vs. RD; <sup>b</sup>P < 0.05 Cc1<sup>−/−</sup> vs. Cc1<sup>+/+</sup> in the same feeding group.

**Abbreviations:** Cc<sup>−/−</sup>, global Ceacam1 null mouse; Cc<sup>+/+</sup>, wild type mouse from the same genetic background as Cc<sup>−/−</sup> mice; RD, regular diet; HF, high fat diet; WAT, white adipose tissue; TNFα, tumor necrosis factor-a; TGFβ, transforming growth factor b; IFNg, interferon gamma; IL, Interleukins.
TABLE 4

Effect of high fat intake on hepatic expression of selected genes related to lipid catabolism in Cc1+/+ and Cc1−/− mice

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<tr>
<td>PPARα</td>
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<td>CYP2E1</td>
<td>555. ± 77.0</td>
<td>559. ± 70.0</td>
<td>500. ± 38.4</td>
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Male mice (n>7; 3 months of age) were fed RD or HF for 3 months. Mice were fasted overnight, the tissues removed and gene expression assessed by semi-quantitative real time-PCR analysis. Values are normalized to Gapdh and expressed as mean units ± SEM.

<sup>a</sup>P< 0.05 HF vs. RD; <sup>b</sup>P< 0.05 Cc1−/− vs. Cc1+/+ in the same feeding group.

**Abbreviations:** Cc−/−, global Ceacam1 null mouse; Cc+/+, wild type mouse from the same genetic background as Cc−/− mice; RD, regular diet; HF, high fat diet; PPARα, peroxisome proliferator-activated receptor α; CYP2E1, cytochrome p450 enzyme.
### TABLE 5 NAS Score of $Cc1^{+/+}$ and $Cc1^{-/-}$ mice

Male mice (n>4; 3 months of age) were fed RD or HF for 3 months. Scoring for NAS from H&E sections of liver tissues was evaluated following Kleiner’s scoring system. The criteria included 3 major histological features, which include micro- and macrosteatosis (0-3), inflammation involving the hepatic lobules as well as portal triads (0-3) and hepatocyte ballooning (0-2). The ~4.6 score in HF-fed $Cc1^{-/-}$ mice supported the diagnosis of NASH in this group of mice. The other groups of a total NAS score of <3 do not fit into the criteria of NASH.

**Abbreviations:** $Cc^{-/-}$, global Ceacam1 null mouse; $Cc^{+/+}$, wild type mouse from the same genetic background as $Cc^{-/-}$ mice; RD, regular diet; HF, high fat diet.

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<th>Designation</th>
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a. Liver

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<tr>
<td>RelB: α-Actin</td>
<td>Actin</td>
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<tr>
<td>IB: α-NPC1</td>
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<td>RelB: α-RPS3</td>
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b. Serum

<table>
<thead>
<tr>
<th>Cc1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Cc1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>IB: α-ApoB</td>
<td>+100</td>
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<td>+48</td>
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C. Hepatic GSH content

\[
\text{GSH content (µmol/g wet wt)}
\]

\[
\begin{array}{c|c|c|c|c}
\text{RD} & \text{HF} & \text{RD} & \text{HF} \\
\hline
\text{Cc1<sup>+/+</sup>} & & & \\
\text{Cc1<sup>−/−</sup>} & & & \\
\end{array}
\]

\(\text{Ghosh et al., Figure 1}\)
Ghosh et al., Figure 2
Ghosh et al., Figure 3
a. Western analysis

b. TUNEL stain

Ghosh et al., Figure 4
Ghosh et al., Figure 5
FIGURE LEGENDS

**Figure 1** Effect of high fat diet on proteins related to hepatic lipid metabolism and on hepatic GSH levels in male $Cc1^{+/+}$ and $Cc1^{-/-}$ mice. Male wild type $Cc1^{+/+}$ and $Cc1^{-/-}$ mice were fed a regular chow (RD) or a high fat diet (HF) for 3 months, starting at 3 months of age. (A) Liver were then removed and analyzed by immunoblotting (IB) with $\alpha$-FAS and $\alpha$-NPC1, followed by reimmunoblotting (Gomez et al.) with $\alpha$-actin and $\alpha$-RPS3 antibodies, respectively, to normalize for the amount of proteins loaded, as described in Experimental Procedures. The gel represents at least 3 mice per feeding group. (B) Serum (10 µg) was analyzed, as above, by immunoblotting using $\alpha$-ApoB antibody to detect ApoB100 and ApoB48 proteins. Although 2 mice are included in the figure, these experiments were performed on at least 5 mice per feeding group. (C) Effect on hepatic GSH levels. $n>7$ mice per feeding group. Values are mean ± SEM. $^aP<0.005$ HF vs. RD. $^bP<0.05$ $Cc1^{-/-}$ vs. $Cc1^{+/+}$ in the same feeding group.

**Abbreviations:** $Cc^{-/-}$, global Ceacam1 null mouse; $Cc^{+/+}$, wild type mouse from the same genetic background as $Cc^{-/-}$ mice; RD, regular diet; HF, high fat diet; FAS, fatty acid synthase; NPC1, Niemann Pick type C1; ApoB, apolipoprotein B; GSH, glutathione; IB: immunoblotting; reIB: reimmunoblotting.

**Figure 2** Effect of high fat diet on liver histology in male wild type $Cc1^{+/+}$ and $Cc1^{-/-}$ mice. Liver histology was assessed in H&E stained sections ($n>4$ mice per feeding group), as described in Experimental Procedures. Panel 1: $Cc1^{+/+}$, regular diet; Panel 2: $Cc1^{+/+}$, high fat diet; Panel 3: $Cc1^{-/-}$, regular diet; Panel 4: $Cc1^{-/-}$, high fat diet. Representative
sections are shown. In HF-fed Cc1+/+ (panel 2), the lipid infiltration alternates with normal liver parenchyma and is predominantly microvesicular. In contrast, in HF-fed Cc1−/− (panel 4), the lipid infiltration appears more diffuse and predominantly macrovesicular.

**Abbreviations:** Cc−/−, global Ceacam1 null mouse; Cc+/+, wild type mouse from the same genetic background as Cc−/− mice; RD, regular diet; HF, high fat diet; H&E staining, hematoxylin-eosin staining.

**Figure 3** Effect of high fat diet and genotype on the presence of liver inflammatory infiltrate. (A) Representative liver sections from n>4 mice stained with H&E. HF-fed Cc1−/− mouse livers (Right panel) reveal increased perivascular and lobular inflammatory cell infiltrate relative to livers from HF-fed Cc1+/+ mice (left panel) that had fewer and mostly portal inflammatory cell foci. (B) Liver lysates were analyzed by sequential immunoblotting (IB) with α-phospho-NF-κB (upper gel), followed by α-NF-κB (lower gel) antibodies as an index of inflammation. The gel represents at least 3 mice from each feeding group.

**Abbreviations:** Cc−/−, global Ceacam1 null mouse; Cc+/+, wild type mouse from the same genetic background as Cc−/− mice; RD, regular diet; HF, high fat diet; H&E staining, hematoxylin-eosin staining; IB: immunoblotting; reIB: reimmunoblotting.

**Figure 4** Effect of high fat diet and genotype on liver cell apoptosis. (A) Liver lysates were analyzed by sequential immunoblotting (IB) with antibodies against Caspase3 (upper gel), followed by α-actin (lower gel) to detect cleaved caspase 3 (17kdaaltons) as an index of apoptosis. The gel represents at least 3 mice from each feeding group. (B) Liver sections from n>4 mice per feeding group were analyzed by TUNEL staining for apoptotic cells, as described in Experimental Procedures. Representative sections are
shown. Panel 1: HF-fed Cc1<sup>+/+</sup>; Panel 2, HF-Fed Cc1<sup>−/−</sup>. Apoptotic cells are observed only in Cc1<sup>−/−</sup> livers. Panels 1-c and 2-c are negative controls without TDT enzyme following digestion with Proteinase K to account for nonspecific binding of enzyme conjugate. Sections from mice on the regular diet (RD) are not shown because there was little evidence of apoptosis in either genotype.

**Abbreviations:** Cc<sup>−/−</sup>, global Ceacam1 null mouse; Cc<sup>+/+</sup>, wild type mouse from the same genetic background as Cc<sup>−/−</sup> mice; RD, regular diet; HF, high fat diet; IB: immunoblotting; reIB: reimmunoblotting.

**Figure 5** Effect of high fat diet and genotype on liver fibrosis. Liver sections from n>4 mice per feeding group were stained with Sirius red. Panel 2: RD-fed Cc1<sup>−/−</sup> mice show mild pericellular fibrosis. Panel 4: HF-fed Cc1<sup>−/−</sup> mice, show extensive pericellular and perivascular fibrosis.

**Abbreviations:** Cc<sup>−/−</sup>, global Ceacam1 null mouse; Cc<sup>+/+</sup>, wild type mouse from the same genetic background as Cc<sup>−/−</sup> mice; RD, regular diet; HF, high fat diet.
A Role for CEACAM1 in Hepatic Stellate Cell Activation in the Development of Non-alcoholic Steatohepatitis

Sumona Ghosh
Introduction

The pathogenesis of non-alcoholic steatohepatitis (NASH) generally follows a “two-hit” hypothesis. Insulin resistance leading to hepatic triglyceride accumulation comprises the “first hit”, followed by oxidative stress, inflammation and hepatocyte apoptosis, which constitute the “second hit” (James and Day, 1998; Lewis and Mohanty, 2010). Fibrosis and subsequent cirrhosis generally develop at the end stage of the disease.

Hepatic stellate cells (HSCs) are located in the subendothelial space of the liver, between the hepatocytes and sinusoidal endothelial cells (Friedman, 2008). These cells comprise about 5-8% of the total liver cell population. HSCs are spindle-shaped cells which possess subendothelial processes that wrap around neighboring hepatocytes and endothelial cells, serving to sense chemotactic signals to initiate contractile force to move the cells to the site of injury (Friedman, 2008; Sauvant et al., 2011).

Under normal conditions, quiescent HSCs store about 80% of the body’s ingested Vitamin A primarily in the form of retinyl palmitate, contained in lipid droplets (Sauvant et al., 2011). The composition of these lipid droplets vary by dietary consumption and also harbor a significant amount of triglycerides, phospholipids, cholesterol and free fatty acids (Friedman, 2008). Dietary retinol is initially esterified with long-chain fatty acids and packaged into chylomicrons before being taken up by the hepatocyte to be hydrolyzed to retinol and finally transported to the stellate cell for storage (Blomhoff et al., 1982; Friedman, 2008). Quiescent HSCs also proliferate slowly and are thought to
mediate cell-cell communication between neighboring hepatocytes and endothelial cells (Sauvant et al., 2011).

Conversely, in a diseased liver, such as alcoholic or non-alcoholic steatohepatitis, HSCs become activated. Upon activation in liver injury, stellate cells lose their Vitamin A-rich lipid droplets as their rough endoplasmic reticulum enlarges, perhaps indicative of increased protein synthesis (Friedman, 2008). Additionally, these activated HSCs begin to proliferate excessively and start to secrete a higher amount of extracellular matrix (ECM) proteins including alpha-smooth muscle actin (alpha-SMA) and collagen type I (Friedman, 2008; Sauvant et al., 2011).

Stellate cell activation is comprised of two phases: initiation and perpetuation. The initiation phase involves early changes in gene expression and phenotype of HSCs, allowing these cells to become more susceptible to further stimulation. Initiation occurs primarily in response to paracrine stimulation from neighboring platelets, Kupffer cells and endothelial cells, in addition to exposure to lipid peroxides and apoptotic products from damaged hepatocytes (Friedman, 2008; Kisseleva and Brenner, 2007). The second phase, perpetuation, occurs from persisting inflammatory stimulation to maintain the active phenotype needed to produce fibrosis. Severe phenotypic changes are observed during this phase of activation, including proliferation, chemotaxis, fibrogenesis, contractility, retinoid/lipid loss and chemokine/cytokine release (Friedman, 2008). This degree of HSC activation is maintained both through paracrine and autocrine pathways. and HSCs serve to amplify immune response as activated HSCs produce mononuclear and neutrophil chemokines and cytokines to further induce inflammatory infiltration.
(Friedman, 2008). Not only do stellate cells produce cytokines, they also secrete a wide variety of growth factors.

Resident macrophages of the liver, Kupffer cells, are generally found in close proximity of activated hepatic stellate cells in diseased liver (Wynn and Barron, 2010). Newly activated HSCs secrete numerous chemoattractants, including macrophage colony stimulating factors (M-CSF), which attract and stimulate macrophages. Upon recruitment, these macrophages produce a plethora of proinflammatory and profibrogenic cytokines, such as Transforming Growth Factor beta (TGF\(\beta\)), to further promote hepatic stellate cell activation needed to drive fibrogenesis, and platelet-derived growth factor (PDGF) to promote hepatic stellate cell proliferation needed to maintain a fibrogenic state (Wynn and Barron, 2010).

Macrophages also serve as phagocytes, to engulf and digest cellular debris. Liver injury in NASH results in damage of hepatocytes and subsequent hepatocyte apoptosis. Upon ingestion of dying hepatocytes, secretion of TGF\(\beta\) increases to further induce stellate cell activation and fibrogenesis (Kisseleva and Brenner, 2007; Wynn and Barron, 2010).

In the previous chapter, we observed that mice with Ceacam1 null mutation (\(Cc1^{-/-}\)) exhibited a basal level of hepatic fibrosis, a rarely detectable phenotype in mice, and that high-fat feeding for 3-4 months amplified markedly the fibrogenic deposition in the liver of these mice (Ghosh et al., 2010). Consistent with steatosis, we have also observed a high basal macrophage recruitment to the liver of these mice, as demonstrated by increased F4/80 mRNA levels, with a further induction by HF intake (Ghosh et al., 2010).
Together, these data provided the impetus to investigate: 1) whether CEACAM1 is involved in stellate cell activation and 2) the role of macrophage recruitment in the development of fibrosis in $Ccl^{-/-}$ mice.
**Methods**

*Animal Husbandry*—Male and female mice were kept in a 12h-dark/light cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee.

*Ccl-/-* mice were generated as described (Leung et al., 2006) Like the L-SACC1 mouse (Poy et al., 2002), L-CC1 transgenic mice with liver-specific over-expression of rat CEACAM1 were generated using the proximal 490 bp-fragment 5' of the translation start site in the human apolipoprotein (Apo) A-I promoter/enhancer element. The ApoAI/WT Ceacam1 minigene, containing intron 1, the BGH polyadenylation signal and a Flag-tag at the 3' end, was excised and injected in the pronuclei of single-cell fertilized mouse embryos from SJLXC57Bl/6J matings (Yale Transgenic Facility). 11 F₀ founders were identified by PCR analysis of tail gDNA and liver-specific transgene expression was confirmed by Western analysis using the rat-specific CEACAM1 polyclonal antibody (Poy et al., 2002) and a monoclonal anti-FLAG M2 antibody (SIGMA cat# F1804). Two lines were identified and backcrossed onto FVB background and then twice onto BL6 (~75% BL6).

Male mice (2 months of age) were fed *ad libitum* either a standard chow (Teklad 2016) with low 12% fat content (RD) or a 45% high fat (HF) diet (Research Diets, Catalog# D12451). Female mice (2 months of age) were fed *ad libitum* either a standard chow (Teklad 2016) (RD) or Methionine-Choline Deficient (MCD) diet.
**Clodronate Administration** - We obtained clodronate solution from Dr. Van Rooijen, prepared as described (Van Rooijen and Sanders, 1994). Clodronate was administered intraperitoneally once a week for 3 months (1.4g/mL), depleting macrophage population in some lymph nodes, spleen, liver and bone marrow.

**Intraperitoneal Glucose Tolerance Test** - Animals fed with either RD of HF diet were fasted overnight from 5 pm to 9 am. Fasted blood glucose was taken from the tail vein by snipping the tail. Glucose @ 1.5 g/kg body wt (50 % dextrose solution) was administered intraperitoneally to conscious animals. Blood glucose was measured by exsanguination from the tail vein at 15, 30, 45, 60 and 120 min post-glucose injection.

**Histological Analysis:** H&E Sections of formalin-fixed, paraffin-embedded livers were stained with: 1) hematoxylin and eosin to assess for histological features of steatohepatitis or 2) picrosirius red stain to evaluate for hepatic collagen deposition. Liver sections were also subject to immunohistochemical staining for macrophages with monoclonal F4/80 antibody (Abcam, Cambridge, MA) and α-smooth muscle actin (α-SMA) with a monoclonal antibody against α-SMA (Sigma Aldrich) St. Louis, MO and CD31 (Abcam, Cambridge, MA). Vectstain HRP- PEROXIDASE kits were used for IHC.

**Generation of human CEACAM1 shRNA Lentiviral Construct** - To identify a siRNA to knockdown human CEACAM1 (hCEACAM1) a free Web-based tool (http://www.genelink.com/sirna/shRNAi.asp) was used to design a putative siRNA against the hCEACAM1 and to design oligonucleotides that encode a corresponding small hairpin RNA (shRNA). Origene was utilized to construct the shRNA plasmid with oligonucleotides: TGAATCCATGCCATTCAATGTGCAGAGG and the homologous
sequence. The hCEACAM1 shRNA construct was co-transfected together with vectors expressing gag-pol, REV and VSV-G into 293FT cells (Invitrogen) to generate a third generation lentiviral construct. Transfection was achieved using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) using 100 ng total DNA per cm² of the growth plate or well. The supernatants were harvested and the cell debris was removed by centrifugation at 2000 g. The supernatant was used to infect human LX2 stellate cells after addition of polybrene (5 ng/ml, Sigma Chemical Co., St. Louis, MO) to establish a cell line that has hCEACAM1 stably down regulated (hCC1 KD) and scramble shRNA control (Ctrl). After 72 hours the cells were selected by puromycin.

**MTT assay for proliferation** – Transfected LX2 cells (Scr-LX2 and ShCc1-LX2) were cultured in DMEM (w/o sodium pyruvate) containing 10%FBS. Transfected cells were seeded in quadruplicate into 96-well plate (5 plates for daily growth assessment) allowed to attach in medium for 24 hours. Basal cell growth was calculated as a percent of day 1 measurement.

**Real Time PCR** – Total RNA was extracted using PerfectPure RNA Tissue Kit (5 Prime) following the manufacturer’s protocol. RNA concentration was measured using Nanodrop, Thermoscientific. cDNA was synthesized using 1 ug of total RNA with iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Herculus). Relative gene expression was analysed using quantitative real-time PCR (Step One Plus, Applied Biosystems®). Relative amount of mRNA of individual gene was calculated after normalizing to their corresponding GAPDH and the results are expressed as fold change in gene expression.
Free Glycerol Measurement - Free glycerol in cell culture media was measured using the Free Glycerol Assay Kit from BioVision (Cat#K630). Upon reaching equal confluency, 30μl of cell culture media from both Scr-LX2 (n=3) and shCc1 LX2 (n=3) cells were assayed in duplicate. Glycerol concentrations (nmol/μl) were calculated from the glycerol standard curve.

Western Blot Analysis - Protein lysates were prepared by homogenizing the tissues in the lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.6) containing protease and phosphatase inhibitor. Protein concentrations were estimated by BCA method (Thermo Fisher Scientific Inc., Rockford, IL). 30μg of protein was separated by SDS-PAGE (Invitrogen Inc.,) and probed with polyclonal antibody against hCEACAM1, PCNA, pAKT, AKT, pSMAD2/3, SMAD2/3, pMAPK 42/44, MAPK 42/44 and then re-probed with monoclonal β-actin or GAPDH antibody for normalization of protein loading.

Primary Hepatic Stellate Cell Isolation – Whole livers were dissected out of anesthetized mice and pooled together, according to genotype and diet. Excised livers were minced and placed in enzymatic solution containing pronase (Roche), collagenase H (Roche) and DNAase (Sigma) in Hank’s Balanced Salt Solution (HBSS w/o Ca, Mg), for digestion. Hepatic stellate cells were separated from whole digest by gradient centrifugation in Histodenz (Sigma). Isolated stellate cells were plated in Dulbecco’s modified eagle medium (DMEM) with 10%FBS. Media was changed daily for 3 days before lysis and subsequent analysis.

Liver Triglyceride Measurements – Hepatic triglyceride content was assayed as previously described (Park et al., 2006).
Results

Macrophage depletion by clodronate restored glucose tolerance, but failed to protect against steatohepatitis and fibrosis

As demonstrated in the preceding chapter, Cc1−/− mice fed a HF diet developed a more severe NASH phenotype than WT mice, including macrosteatosis, fibrosis and inflammation (Ghosh et al., 2010). The higher level of F4/80 and TNFα mRNA in liver-derived from HF-fed Cc1−/−, compared to HF-fed WT mice, suggested an increase in the population and activation of the resident macrophages (Kupffer cells) of the liver of the null mice. To investigate whether activation of macrophages contributed to the exacerbated phenotype upon HF feeding, we initially tested whether depletion of the macrophage population reverses or prevents progression of the disease.

To this end, mice were given a weekly dose of clodronate (1.4mg/mL) by intraperitoneal injection for 3 months starting at weaning, at the same time as HF feeding also began. Depletion of macrophages was confirmed by loss of F4/80 levels in these mice, as shown by immunohistochemical analysis (IHC) in liver sections (Figure 1 A,B).

Glucose tolerance test demonstrated that clodronate treatment restored glucose disposal after 3, but not 1 month (Figure 2 A,B) of treatment in HF-fed relative to RD-fed WT mice. In concordance with other studies (Kim et al., 2008), this suggests that macrophage recruitment and activation plays an important role in the late stages of diet-induced metabolic abnormalities in WT mice on the C57BL/6 (BL6) genetic background. In Cc1−/− null mice; however, clodronate treatment failed to restore glucose tolerance even after 3 months of treatment (Figure 2 B). This suggests that glucose intolerance in
response to HF diet is primarily caused by factors related to the absence of CEACAM1 in cells other than macrophages, for example, hepatocytes, the site of insulin extraction and clearance; as demonstrated by impaired insulin clearance resulting from Ceacam1 deletion in hepatocytes (DeAngelis et al., 2008).

Because 3 months of clodronate treatment was required to modulate glucose tolerance, we next analyzed its effect on liver histology. As we have previously shown, H&E histological analysis revealed a more diffuse fat infiltration in $\text{Cc1}^{-/-}$ than wild type (WT) livers (Ghosh et al., 2010). Moreover, prolonged HF feeding caused predominantly an increase in macrovesicular fat deposition in $\text{Cc1}^{-/-}$, as opposed to WT livers, where fat deposition was predominantly microvesicular (Ghosh et al., 2010). 3 months of clodronate administration had no significant effect on steatosis in RD- or HF-fed $\text{Cc1}^{+/+}$ wild type (Figure 3 A) or $\text{Cc1}^{-/-}$ animals (Figure 3 B). Biochemical analysis showed a statistically insignificant lower hepatic triglyceride content in both WT and $\text{Cc1}^{-/-}$ by clodronate treatment (WTHFs: 412.0 ± 116.8 vs WTHFc: 242.6 ± 92.3; $\text{Cc1}^{-/-}$HF: 483.0 ± 266.0 vs $\text{Cc1}^{-/-}$HFc: 369.7 ± 18.32).

Additionally, H&E analysis also revealed inflammatory infiltration despite macrophage depletion in both, HF-fed $\text{Cc1}^{+/+}$ and $\text{Cc1}^{-/-}$ animals. Thus, clodronate administration did not prevent the inflammatory response to HF feeding for 3 months (Figure 3A, B). Steatohepatitis involves a Th1 cytokine response, which is characterized by increased release of cytokines from intrahepatic CD4+ T cells, including conventional and invariant Natural Killer T (NKT) cells (Kremer et al., 2006; Li et al., 2005; Tiegs, 2007). Moreover, CEACAM1 inhibits production of Th1 cytokines more strongly than Th2 (Nagaishi et al., 2006). In fact, $\text{Cc1}^{-/-}$ liver exhibited a CD4+ Th1 response (Ghosh...
et al., 2010), which was largely attributed to deletion of Ceacam1 in T-cells, as supported by mice with conditional deletion of Ceacam1 in T-cells (Nagaishi et al., 2006). Thus, it is likely that persistent inflammation in clodronate-treated $CcI^{+/−}$ mice is related to the deletion of Ceacam1 in T-cells, independent of macrophage. Lending further support to this hypothesis is the fact that, unlike $CcI^{+/−}$ mice, L-SACC1 mice with hepatocyte-specific inactivation of CEACAM1 do not exhibit an increase in CD4+T population under normal feeding conditions (Sang Jun Lee, Gastroenterology 2008).

Hepatic fibrosis, often regarded as an end stage phenotype of non-alcoholic steatohepatitis, develops in $CcI^{+/−}$ mice under normal feeding conditions, but is severely exacerbated in response to high fat feeding, as demonstrated in the previous chapter (Ghosh et al., 2010). As shown Sirius Red staining (Figure 4 A,B), clodronate treatment for 3 months failed to ameliorate fibrosis in $CcI^{+/−}$ mice under both feeding conditions. This indicates that macrophage-derived factors do not play a significant role in the pathogenesis of basal fibrosis and in its progression in response to HF feeding in $CcI^{+/−}$ mice. Together, the data show that macrophages do not play a major role in the steatohepatitis and fibrosis caused by Ceacam1 null deletion. This points to different cells playing more major role in the phenotype, most likely the hepatocytes and stellate cells with known integrated role in the regulation of the metabolic and fibrotic aspects of the disease.
Loss of CEACAM1 Causes Activation of Stellate Cells in a Cell Autonomous Manner

Stellate cells become activated within 7 days after being isolated from liver and grown in regular medium (Friedman et al., 1992). In collaboration with the Totonoz laboratory, we have shown a progressive decrease in Ceacam1 mRNA in stellate cells isolated from wild type mice, reaching their lowest expression level on the 7th day post-isolation (Fig. 5). This indicates a marked reduction in Ceacam1 levels in activated stellate cells.

To investigate a potential role for CEACAM1 in stellate cell activation, we stably knocked down Ceacam1 expression by a lentiviral sh-RNA approach in cultured LX2 human stellate cells (kindly obtained from the Friedman lab at Mount Sinai School of Medicine). As Quantitative Real time-PCR (qRT-PCR) and Western analyses reveals, Ceacam1 mRNA expression was markedly reduced (by>80-90%) in these shRNA-Ceacam1 LX2 (shCc1-LX2) relative to shRNA-scrambled cells (Scr-LX2) (Figure 6 A,B).

Quiescent hepatic stellate cells are generally lipid laden and constitute a major storage site for retinol and Vitamin A (Friedman, 2008; Sauvant et al., 2011; Wake, 1971). Upon activation, stellate cells shrink rapidly and lose their lipid and Vitamin A content. Stellate cell activation results in the release of α-smooth muscle actin, the most reliable marker of stellate cell activation as it is absent from other resident liver cells (Friedman, 2008). This leads to the development of fibrosis in liver (Friedman, 2008). Accordingly, Nile Red staining revealed a marked loss of the lipid content of shCc1-LX2
relative to Scr-LX2 cells (Figure 7B). Consistently, the mRNA levels of adipose
differentiation related protein (ADRP), a marker for lipid accumulation and a stimulant of
lipid droplet formation (Londos et al., 1999; Londos et al., 2005), was decreased (Table 2). Consistent with the transcriptional upregulation of hormone sensitive lipase (HSL),
the major enzyme responsible for the hydrolysis of stored triglycerides and the
mobilization of free fatty acid (Haemmerle et al., 2003; Kraemer and Shen, 2002), by
PPARγ (Deng et al., 2006), the mRNA levels of both PPARγ and HSL were elevated in
shRNA Ceacam1 LX2 cells by ~3-4-fold (Table 2). Not only do these data indicate a
mechanism for loss of fat laden droplets via lipolysis, further supported by increased free
glycerol in the cell culture media (Figure 8), but the marked increase in PPARα mRNA
levels (Table 2), suggests that fat oxidation further contributes to the loss of lipid stores
observed in hepatic stellate cells upon losing the majority of their CEACAM1 content.

Activated stellate cells exhibit high proliferation and resistance to apoptosis
(Friedman, 2008; Wobser et al., 2009). Consistently, MTT assay showed an intense
increase in the proliferation of shCc1-LX2 cells by comparison to Scr-LX2 cells (Figure 9 A,B). Moreover, shCc1-LX2 cells exhibited a higher basal phosphorylation (and activation) of Akt than Scr-LX2 cells (Figure 10A). Anti-apoptotic Akt-dependent pathways are mediated by the phosphorylation and inactivation of a number of proapoptotic proteins including Bad (Datta et al., 1997) and caspase-9 (Cardone et al., 1998). Moreover, mRNA expression of the survival molecule, cylin-dependent kinase inhibitor p21 (Figure 10B), downstream of Akt (Lawlor and Rotwein, 2000) was also increased in shCc1-LX2 cells. Together, these data demonstrate that Ceacam1 deletion
activates Akt-dependent pathways to confer resistance to apoptosis and contribute to stellate cells activation in a cell autonomous manner.

Smad2/3 signaling via TGFβ1 is the canonical pathway that results in increased collagen deposition and fibrosis (Inagaki et al., 2005; Inagaki et al., 2001; Lee and Friedman, 2011). Consistently, basal phosphorylation of Smad2/3 was elevated in shCc1-LX2 relative to Scr-LX2 cells (Figure 11) despite a 50% reduction in its mRNA level (Table 2). This provides another indication of an activated state of stellate cells brought about by the loss of Ceacam1.

**Gain of CEACAM1 Blunts activation of Stellate Cells in a Cell Autonomous Manner**

To further investigate what bearing CEACAM1 may have on the development of fibrosis, we fed mice with liver-specific CEACAM1 overexpression (L-CC1) a methionine choline-deficient (MCD) diet that causes experimental NASH, including fibrosis, steatosis and inflammation without causing insulin resistance (Green, 2003). At the end of a 3 month-feeding regiment, stellate cells were isolated from livers to test stellate cell activation and consequently, development of fibrosis. qRT-PCR analysis revealed a decrease in Ceacam1 mRNA levels in WT, but not L-CC1 mice (WTRD: 1.70 ± 0.22 vs WTMCD: 0.88 ± 0.02; L-CC1RD: 1.14 ± 0.08 vs L-CC1 MCD: 10.7 ± 1.23) by MCD (Table 3), most likely resulting from the inducing effect of MCD on Apolipoprotein A1 mRNA levels (data not shown). Reciprocally, MCD elevated the mRNA levels of collagen I and α-SMA in WT, but suppressed them in L-CC1 mice (Table 2). With respect to collagen IV, the increase by MCD in WT mice was not
statistically significant, but like α-SMA and collagen I, MCD reduced its mRNA level in stellate cells derived from L-CC1 mice (Table 3). Moreover, MCD treatment reduced the mRNA of fatty acid synthase (FAS), an important player in lipogenesis, in WT mice, while it induced it in L-CC1. This suggests that CEACAM1 plays a cell autonomous role in stellate cell activation. The cause-effect relationship between increase in CEACAM1 level and lipogenesis in stellate cells is unclear, but it is likely that CEACAM1 promotes insulin action in stellate cells by promoting lipid storage and preventing lipolysis and consequently, activation of stellate cells.

Of interest, Sirius Red staining and immunostaining with αSMA showed substantial rise in fibrotic deposition by MCD in both WT and L-CC1 mice (Figure 12). This suggests that MCD could also cause fibrosis by additional cell non-autonomous mechanisms.
Discussion

The liver specific inactivation of CEACAM1 (L-SACC1) and the whole body knockout of CEACAM1 (Cc1\textsuperscript{−/−}) in mice have been shown to exhibit insulin resistance due, in part, to impairment of insulin clearance. In addition to metabolic abnormalities observed, these mouse lines have also demonstrated basal hepatic fibrosis which is further exacerbated with HF feeding (Ghosh et al., 2010; Lee et al., 2008). Elevated levels of F4/80 mRNA implicate macrophage infiltration to play a role in the spontaneous hepatic fibrosis seen (Ghosh et al., 2010). Though numerous studies have shown macrophage-derived factors to be a primary source of stellate cell activation in the pathogenesis of hepatic fibrosis (Bilzer et al., 2006; Gressner et al., 1993; Matsuoka and Tsukamoto, 1990), we found fibrosis to persist despite macrophage depletion via administration of clodronate. Thus, fibrosis development in Cc1\textsuperscript{−/−} mice may be independent of macrophage derived inflammation.

Our current studies demonstrate a role for CEACAM1 in the activation of the hepatic stellate cells, the driving force behind induction and persistence of fibrogenesis in the pathogenesis of liver disease. Activation of hepatic stellate cells results in loss of lipid content, increase in proliferation, and induction of ECM synthesis (Friedman, 2008; Kisseleva and Brenner, 2007; Sauvant et al., 2011). We find the primary marker for fibrosis (Friedman, 2008), α-SMA mRNA, is dramatically increased by the stable knockdown of Cc1 in LX2 human stellate cells consistent with other models of hepatic fibrosis (Lee et al., 2010; Lin and Chen, 2011; Wobser et al., 2009). Additionally, knocking down Ceacam1 in LX2 cells induced extreme proliferation, another primary characteristic of activated stellate cells (Friedman, 2008; Kisseleva and Brenner, 2007),
as demonstrated by the MTT assay and increase in PCNA protein expression. These markers for activation are seen in the absence of external factors normally found to induce a state of activation, such as inflammatory cytokines or mitogenic factors (Kisseleva and Brenner, 2007). This suggests an intrinsic role for CEACAM1 in the stellate cell itself.

In addition to α-SMA mRNA being increased in response to the knockdown of Cc1 in LX2 cells, we found decreased mRNA levels of ADRP, with increased levels of HSL, indicative of depleted lipid content. Though we find PPARγ levels to be increased in shCc1-LX2 cells, contrary to what others have shown in states of activation (Friedman, 2003; Marra et al., 2000), PPARα levels are increased by nearly 100 times more than PPARγ. As the mechanism through which lipid loss in stellate cell activation occurs remains elusive, our data suggest PPARα activity to be a key player. This data implicate loss of CEACAM1 to play a major role in excessive beta oxidation in the depletion of lipid stores in activated stellate cells.

Resistance to apoptosis and cell survival is yet another characteristic of activated stellate cells (Wobser et al., 2009). The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal (Kennedy et al., 1997). The induction of Akt phosphorylation coupled with increase in p21 mRNA expression in shCc1-LX2 cells, indicates an increase in cell survival, as Lawlor and Rotwein have previously demonstrated Akt to be an intermediate and p21 as an effector of a growth-factor controlled myoblast survival pathway (Lawlor and Rotwein, 2000). Growth factors, including PDGF and EGF are known to activate stellate cells (Bachem et al., 1989; Friedman, 2008), thus, this is a
potential mechanism by which hepatic stellate cells in $Cc1^{−/−}$ mice are activated to drive fibrogenesis.

Generally, TGFβ is the primary cytokine to induce fibrogenic changes via SMAD2/3 signaling. Upon binding of ligand, the activated receptor complex phosphorylates receptor Smads with subsequent translocation to the nucleus to positively or negatively regulate expression of target genes (Miyazawa et al., 2002). However, we find TGFβ mRNA levels to be significantly reduced in shCc1-LX2 cells compared to that of Scr-LX2 cells but with increased Smad2/3 phosphorylation. These data suggest fibrogenic induction via Smad signaling independently of TGFβ as seen can occur through p38 MAPK signaling in human gingival fibroblasts (Leivonen et al., 2002). In addition to playing a role in fibrotic induction, TGFβ has been shown to inhibit proliferation of activated stellate cells (Saile et al., 1999), thus perhaps significantly decreased TGFβ mRNA levels observed in our shCc1-LX2 cells contribute to their proliferative phenotype. Our studies suggest that the extreme proliferation, observed in our shCc1-LX2 cell model, may be the major force, overriding matrix production, behind sustaining hepatic stellate cell activation to induce fibrogenesis in Cc1 null mice.

Leptin levels are generally directly proportional to adipose mass, thus obese people frequently have higher leptin levels. $Cc1^{−/−}$ mice possess basally higher levels of circulating leptin compared to WT mice (Ghosh et al., 2010). Hepatic stellate cells have been shown not only to be activated by leptin (Choi et al., 2010) but also to secrete leptin upon activation (Friedman, 2008). This serves as yet another mechanism by which basal fibrosis in Ceacam1 null mice may persist.
Fibrosis and stellate cell activation persist with the absence of Ceacam1 expression. To test whether an overexpression of CEACAM1 may prevent fibrosis, we fed L-CC1 mice with MCD diet. Indeed, we see MCD diet induced expression of key fibrogenic markers, including αSMA and Collagen I, in hepatic stellate cells isolated from WT mice but decreased expression in cells isolated from L-CC1 mice. Thus, it seems that overexpression of hepatic Cc1 may, likely through mediation of balance between lipogenic and lipolytic pathways, play a protective role against the activation of stellate cells. However, histological evidence shows that fibrosis indeed persists even in L-CC1 mice, despite what we see in isolated stellate cells. This suggests externally derived factors brought on by MCD feeding contributed to the induction and persistence of fibrosis observed.

In conclusion, our data demonstrate a cell-autonomous protective role for CEACAM1 against the activation of hepatic stellate cells. Further experiments need to be performed to elucidate the specific role for CEACAM1 in mediating both lipid accumulation and proliferation.
Figure Legends

Figure 1: Weekly administration of i.p. injected clodronate diminished hepatic macrophage population as evidenced by F4/80 immunohistochemical staining. Dark brown staining, representing macrophages, are seen in Saline treated animals but are absent in Clodronate treated mice.

Figure 2: Glucose tolerance in both, WT and Cc1⁻/⁻ mice, are unaffected by clodronate administration and HF feeding after one month (A). However, macrophage depletion restores glucose tolerance in high fat fed WT mice with no apparent change Cc1⁻/⁻ mice (B), n>5.

Figure 3: Three months of clodronate treatment does not significantly decrease steatosis development with HF feeding in either, WT or Cc1⁻/⁻ mice (A, B). Additionally, HF diet induces inflammatory infiltration despite macrophage depletion in both WT and Cc1⁻/⁻ groups (A, B).

Figure 4: Three months of clodronate treatment does not protect against HF induced fibrosis in either, WT or Cc1⁻/⁻ mice, demonstrated by both Sirius Red staining for collagen (A), and immunohistochemical staining for smooth muscle actin (B).

Figure 5: Hepatic stellate cells isolated from WT mice become activated within 7 days after isolation. This activation coincides with a proportional decrease in both long and short forms of CEACAM1.

Figure 6: Stable transfection to achieve a CEACAM1 knockdown in LX2 cells, a human stellate cell line, is confirmed at both the mRNA (A) and protein levels (B).
Figure 7: Knockdown of CEACAM1 in cultured LX2 cells induces a state of activation as seen through a significant increase in αSMA mRNA levels (A) and a dramatic decrease in intracellular lipid stores (B).

Figure 8: Knockdown of CEACAM1 in cultured LX2 cells results in a dramatic increase in free glycerol levels in cell culture media suggesting lipolysis is increased in CEACAM1 deficient cells.

Figure 9: Knockdown of CEACAM1 in cultured LX2 cells induces a state of activation as shown by drastic increase in proliferation in MTT assay (A) and increased expression of PCNA protein (B).

Figure 10: Knockdown of CEACAM1 in cultured LX2 cells induces activation of pro-survival pathway with increased phosphorylation of Akt (A) with subsequent increase in downstream effector, p21 mRNA (B).

Figure 11: Knockdown of CEACAM1 in cultured LX2 cells induces activation of canonical profibrogenic SMAD2/3 signaling via phosphorylation of SMAD2/3.

Figure 12: Overexpression of CEACAM1 does not protect against the development of fibrosis in the face of MCD diet as we see both increased collagen staining by Sirius Red staining.
Table 1

Real time PCR primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tr>
<td>hGAPDH</td>
<td>ATCCATGACAACTTTGGTATCGT</td>
<td>ATGACCTTGCCCACAGCCTT</td>
</tr>
<tr>
<td>hCc1</td>
<td>TCTACCCTGAACCTTTGAAGCCA</td>
<td>TGAGAGACTTGGAAATACATCAGCAGCCTG</td>
</tr>
<tr>
<td>h α-SMA</td>
<td>CGTGGCTATTCCTCGTTAC</td>
<td>TGCCAGCAGACTCCATCC</td>
</tr>
<tr>
<td>hTGFβ</td>
<td>CCCAGCATCTGCAAAGCTC</td>
<td>GTCAATGTACAGCTGCGCA</td>
</tr>
<tr>
<td>hADRP</td>
<td>AGCAGGCTTCACAGCAGG</td>
<td>GTACACCTTGAGATTTG</td>
</tr>
<tr>
<td>hPPARγ</td>
<td>AGAGCAGCTGGCAAAGGTGGTGTCT</td>
<td>CAGCCATGGTGCCCAGTCTAGC</td>
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<tr>
<td>hHSL</td>
<td>CCACGAGCCCTACCTCAAGA</td>
<td>CCAGGGAGTAGTGCGATGGA</td>
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<td>hPPARα</td>
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<td>GCCCTTGCACGCAGATT</td>
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<tr>
<td>mGAPDH</td>
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<td>ATACCAGGAAATGAGCTTGACAAAGT</td>
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<td>m α-SMA</td>
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<td>TGCCAGCAGACTCCATCC</td>
</tr>
<tr>
<td>mCollagen a1 (I)</td>
<td>TAGGCCATTGTGTATGCAGC</td>
<td>ACATGTTCAGCTTTGTGGACC</td>
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<tr>
<td>mCollagen(IV)</td>
<td>CACATTTTCACAGCAGAGAG</td>
<td>GTCTGGCTTCTGCTGCTTT</td>
</tr>
<tr>
<td>mFAS</td>
<td>ACTGTAAGAAGATGTCTCTTGAAGA</td>
<td>AAGCAACCTCAGCTCATGGCTATA</td>
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<tr>
<td>mTGFβ</td>
<td>CGCCATCTATGAGAAAACCC</td>
<td>GTAACGCCAGGAATTGT</td>
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Table 2
Stellate Cell Markers – Liver – human LX2 cells

<table>
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<tr>
<th></th>
<th>Scr</th>
<th>shRNA-Cc1</th>
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<tbody>
<tr>
<td>α – Smooth Muscle Actin</td>
<td>1.02 ± 0.01</td>
<td>1.91 ± 0.04 A</td>
</tr>
<tr>
<td>TGFβ</td>
<td>0.92 ± 0.09</td>
<td>8.2×10^{-5} ± 3.8×10^{-5} A</td>
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<tr>
<td>ADRP</td>
<td>1.00 ± 0.02</td>
<td>0.63 ± 0.03 A</td>
</tr>
<tr>
<td>PPARγ</td>
<td>1.06 ± 0.09</td>
<td>2.75 ± 0.13 A</td>
</tr>
<tr>
<td>HSL</td>
<td>1.09 ± 0.05</td>
<td>4.29 ± 0.22 A</td>
</tr>
<tr>
<td>PPARα</td>
<td>0.89 ± 0.06</td>
<td>121. ± 32.1 A</td>
</tr>
</tbody>
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\(^{A} P<0.05\) versus Scr-LX2. Values are expressed as mean ± SEM n ≥ 3 per group
Table 3:

Effect of 80 days of a methionine-choline deficient diet intake on mRNA of isolated hepatic stellate cells

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>L-CC1</th>
<th>WT</th>
<th>L-CC1</th>
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<tr>
<td></td>
<td>RD</td>
<td>MCD</td>
<td>RD</td>
<td>MCD</td>
</tr>
<tr>
<td>α-smooth muscle actin</td>
<td>1.03 ± 0.02</td>
<td>1.18 ± 0.02 (^A)</td>
<td>1.31 ± 0.07</td>
<td>0.57 ± 0.01 (^A, B)</td>
</tr>
<tr>
<td>Collagen I</td>
<td>1.22 ± .17</td>
<td>2.35 ± 0.16 (^A)</td>
<td>1.55 ± .22</td>
<td>.69 ± .01 (^A, B)</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>1.04 ± 0.07</td>
<td>1.24 ± 0.09</td>
<td>1.07 ± 0.08</td>
<td>0.75 ± 0.09 (^A)</td>
</tr>
<tr>
<td>FAS</td>
<td>0.76 ± 0.02</td>
<td>0.55 ± 0.01 (^A)</td>
<td>0.79 ± 0.03</td>
<td>0.98 ± 0.03 (^A, B)</td>
</tr>
<tr>
<td>TGFβ</td>
<td>1.01 ± 0.01</td>
<td>0.45 ± 0.03 (^A)</td>
<td>0.79 ± 0.07</td>
<td>0.77 ± 0.04 (^B)</td>
</tr>
</tbody>
</table>

Female mice (n>5) were fed a MCD diet for 80 days starting at 2 months of age. \(^A\) \(P<0.05\) MCD versus RD. \(^B\) \(P<0.05\) L-Cc1 versus WT of the same diet.

Values are expressed as mean ± SEM.
1A. F4/80 Immunohistochemistry

Figure 1A: F4/80 Immunohistochemistry reveals a depletion of macrophage content by clodronate administration in WT mice.
Figure 1B: F4/80 Immunohistochemistry reveals a depletion of macrophage content by clodronate administration in Cc1-/- mice.
2A. 1 month HFD IPGTT (1.5g/kg)

WT

Cc1 KO

2B. 3 month HFD IPGTT (1.5g/kg)

WT

Cc1 KO

Figure 2: (A) 1 month clodronate administration shows no effect on glucose tolerance in either HF-fed WT or HF-fed Cc1KO mice. (B) 3 months clodronate administration protected HF-fed WT mice against diet-induced glucose intolerance, but had no effect on HF-fed Cc1KO mice.
3A. H&E Staining

Figure 3A: H&E staining in HF-fed WT mice reveal both fat and inflammatory infiltration despite macrophage depletion by clodronate administration.
3B. H&E Staining

Figure 3B: H&E staining in HFD-fed Cc1⁻/⁻ mice reveal both fat and inflammatory infiltration despite macrophage depletion by clodronate administration.
4A. Sirius Red Staining

Figure 4A: Sirius Red staining demonstrates that 3 months of clodronate treatment does not protect against fibrosis in HF-fed WT mice.
4B. Sirius Red Staining

Figure 4B: Sirius Red staining demonstrates that 3 months of clodronate treatment does not protect against extensive bridging fibrosis in either RD-fed or HF-fed Cc1 Ry mice.
Figure 5: Primary hepatic stellate cells from WT mice become activated within 7 days after isolation along with a progressive decrease in Ceacam1 mRNA levels.
Figure 6: hCEACAM1 expression is found to be significantly decreased by both mRNA (A) and protein (B) after stable transfection using lentivirus.
Figure 7: Hepatic stellate cell activation by knocking down CEACAM1 in LX2 cells results in a significant increase of α-SMA mRNA expression (A) and a dramatic decrease in intracellular lipid stores assessed by Nile Red staining (B).
Figure 8: Hepatic stellate cell activation by knocking down CEACAM1 in LX2 cells results in a significant increase free glycerol levels in the cell culture media.
Figure 9: Hepatic stellate cell activation by knocking down CEACAM1 results in a dramatic increase in proliferation as assessed by MTT assay [A] and an increase PCNA protein expression [B].
Figure 10: Hepatic stellate cell activation by knocking down CEACAM1 induces activation of cell survival signaling pathway as shown by increased phosphorylation of Akt (A) and increased mRNA levels of downstream effector p21(B).
Figure 11: Hepatic stellate cell activation by knocking down CEACAM1 induces activation of primary fibrogenic pathway, SMAD2/3.
12. Sirius Red Staining

**Figure 12:** Overexpression of hepatic CEACAM1 is not sufficient to protect against development of hepatic fibrosis in either MCD-fed L-CC1 mice, as assessed by Sirius Red staining.
References


