ROLE OF BONE MORPHOGENETIC PROTEIN IN THE LIVER

By

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Abstract

Elevated levels of plasma homocysteine (Hcy) are believed to be involved in several health problems, including liver fibrosis. However, the mechanism of Hcy induced liver injury remains to be further investigated. Although all hepatic cell types are involved in fibrogenesis, the activation and proliferation of hepatic stellate cells (HSCs) are considered to be central events. Moreover, it is reported that bone morphogenetic proteins (BMPs) play an important role in cell proliferation, differentiation and apoptosis. In this experiment, we examined the expression of BMPs in rat liver with hyperhomocysteinemia (HHcy) and in HSC cell lines at both mRNA and protein levels. The principal findings suggest that the expression of BMP-13 was significantly reduced in the liver of rats after 4 and 12 weeks of HHcy compared with that in control. Furthermore, there were significant increase in cell proliferation and modification of HSCs after Hcy treatment. In conclusion, a long term high methionine diet can cause a reduction of BMP-13 in the liver. The reduction of BMP-13 in the liver may contribute to liver injury induced by a high methionine diet.

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Abbreviations

%: percent

$\cdot O_2^-$: superoxide anion

·OH: hydroxyl radical

aa: amino acid

AIF: apoptosis induce factor

ALT: aminotransferase

ALK: activin receptor-like kinase

AP: ammonium persulfate

b FGF: basic fibroblast growth factor

BHMT: betaine-homocysteine methyltransferase

bp: basepair(s)

BM MSCs: bone marrow multipotent mesenchymal stromal cells

BSA: Bovine serum albumin

CBS: cystathionine β-synthase

CDMP: cartilage-derived morphogenetic protein

cDNA: complementary DNA

CLD: chronic liver disease

CNS: central nervous sysem

Co-Smad: common Smad

CSCs: cancer stem cells

CTRL: control

CK-19: cytokeratin-19

DCs: dendritic cells

DMEM: Dulbnecco's modified eagle medium

DNA: deoxyribonucleic acid

EB: ethidium bromide

ECs: endothelial cells

ECM: extracellular metrix

EDTA: ethylene diaminetetracetic acid

EGF: epidermal growth factor

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

FDA: Food and Drug administration

FGFs: fibroblast growth factors

G6Pase: glucose-6-phatase

GAA: guanidinoacetate

GAPDH: glyceraldehydes 3-phosphate dehydrogenase

GCL: glutamate-cysteine ligase

GDF: growth differentiation factor

GSH: glutathione

HCC: hepatocellular carcinoma

Hcy: homocysteine

HHcy: hyperhomocysteinemia

HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A

HOCl: hypochlorous acid

hr: hour(s)

HSC: hepatic stellate cell

IFN: interferon

IGF: insulin-like growth factor

IL-8: interleukin-8

M: molar

Mad: mothers against decapentaplegic gene

MAPK: mitogen-activate protein kinase

MAT: methionine adenosyl transferase

ml: milliliter

mM: millimolar

MMPs: matrix metalloproteinases

mRNA: messenger RNA

MS: methionine synthetase

MTHF: methyltetrahydrofolate

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NKs: natural killer cells

NMT: N-methyltransferase

OD: optical density

OLT: ortothopic liver transplantation

ONOO/RNS peroxynitrite

PBS: phosphate buffered saline

PCR: Polymerase Chain Reaction

PCs: parenchymal cells

PDGF: platelet-derived growth factor

PEMT: phosphatidylethanolamine N-methyltransferase

PNS: peripheral nervous system

RNA: ribonucleic acid

RNS: reactive peroxynitrite

RO·/ROO·: Oxygen centre organic radicals

ROOH: organic hydroperoxide

ROS: reactive oxygen species

rpm: rotations per minute

RT-PCR: Reverse transcription PCR

SAM: S-adenosylmethionine

SAH: S-adenosyl homocysteine

SDS: sodium dodecyl sulfate

SECs: sinusoidal endothelial cells

Smad: sma and Mad

SOD: superoxide sismutase

SREBPs: sterol regulatory element-binding proteins

TBS: Tris buffered saline

TBS-T: Tris buffered saline with 0.1% Tween 20

TE: Tris-EDTA

TEMED: N,N,N',N-tetramethylethylenediamine

TGF-β: transforming growth factor beta1

TNF: tumor necrosis factor

TRAFII: to tumor necrosis factor receptor associated facter II

Tris: tris (hydroxymethyl) amino methane

TIMP: tissue inhibitors metalloproteinase

Tween-20: polyxyethylene-sorbitan monolaurate

UPR: unfolded protein response

v/v: volume per volume

w/v: weight per volume

α-SMA: α-Smooth muscle actin

Introduction

1. Background

Methionine is an essential amino acid obtained from daily meals. It is primarily metabolized in the liver with the help of B-vitamins and a series of enzymes (Urist 1965; Purohit, Abdelmalek et al. 2007). Homocysteine (Hcy) is one of the metabolic products in methionine metabolism. In normal livers, Hcy can be converted to glutathione or be recycled to methionine. However, abnormally elevated levels of Hcy called hyperhomocysteinemia (HHcy) is a dysfunction in this metabolism pathway, which is regarded as a crucial factor for several diseases (Remkova and Remko 2009). The causes of HHcy include deficiency of B-vitamins, miscoded genes for essential metabolic enzymes or simply excessive dietary methionine intake (Ueland and Refsum 1989; Hase 1998; Billion, Tribout et al. 2002; Haynes 2002; Troen, Lutgens et al. 2003; Moat, Bao et al. 2004). Moreover, HHcy has been found to be a precondition of liver fibrosis (Bataller and Brenner 2005).

Hepatic stellate cells (HSCs) are non-parenchymal cells, located in the vicinity of adjacent hepatocytes. In normal livers, HSCs are described as being in a quiescent state. The quiescent stellate cells represent 5-8% of the total number of liver cells. Each cell has several long protrusions that extend from the cell body and wrap around the sinusoids. Lipid droplets in stellate cells are usually storage places of retinoid, while the function and role of quiescent HSCs are still unclear. However, in an injured liver, stellate cells go through an activation process characterized as a cell phenotype change to myofibroblasts-like cells mediated by different factors. The fate of activated HSCs in the liver is still under investigation but apoptosis and/or reversion back to quiescent state have been

proposed (Friedman 2000). It is commonly accepted that activated HSCs are the major cell type in the liver responsible for the production of extracellular matrix proteins and the development of liver fibrosis (Gressner 1996; Friedman 2000; Bataller and Brenner 2005). Liver fibrosis is a wound healing process in the liver. It is suggested that HHcy can induce hepatocyte apoptosis and release free radicals, which will stimulate HSCs activation (Ji and Kaplowitz 2004). Moreover, it has been reported that cytokines and growth factors can induce the activation of HSCs (Adachi, Togashi et al. 2005; Galli, Svegliati-Baroni et al. 2005; Friedman 2008).

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGF-β) superfamily, which are a group of important cytokines involved in cell differentiation and proliferation (Gressner, Weiskirchen et al. 2002). These proteins mediate target gene expression through BMP (Smad) signaling pathways (Sekelsky, Newfeld et al. 1995). Smads are pivotal intracellular molecules that transfer signals of TGF-β superfamily members from the cell surface to the nucleus. Previous studies have identified that BMPs could significantly increase α-SMA expression during activation of HSCs, which indicates a role of BMPs in the HSCs activation process (Fan, Shen et al. 2006). At the present time, fourteen BMPs have been found, and some of them have been approved by the Food and Drug Administration (FDA) for clinical application. BMP-13 is known as growth differentiation factor 6 (GDF-6) and was first identified as a component of bovine cartilage. However, some recent reports indicated that BMP-13 could inhibit osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells, indicating that deficiency of BMP-13 may induce excess bone formation (Mahfouz and Kummerow 2004).

2. Liver Injury

The liver places a vital role in the synthesis of proteins, detoxification, storage (e.g. vitamin A, glycogen and minerals) in addition of other chemicals metabolism. Chronic liver disease (CLD) is characterized by repetitive hepatocyte injury, hepatocyte regeneration and/or replacement of liver parenchyma with extracellular matrix protein, which eventually leads to liver fibrosis and cirrhosis, and further to liver failure. Cirrhosis is one of the several most common causes of death in western countries, so it is important to develop more therapeutic strategies especially at gene and molecular level to combat this condition (Pinzani and Rombouts 2004). Furthermore, cirrhosis predisposes to the generation of hepatocellular carcinoma (HCC), which presents the most common cause of cancer mortality world-wide (Parola and Pinzani 2009). The investigation of treatment of advanced CLDs and HCC becomes critical as the number of cohosts who contracted hepatitis C virus during seventies and early eighties reach end-stage liver disease. The causes of liver cirrhosis include hepatotropic viruses such as hepatitis B and C viruses, toxin or drugs, such as alcohol and acetaminophen, induced liver disease, metabolism and autoimmune diseases etc.

To date, the most suitable treatment for end-state liver disease is orthotropic liver transplantation (OLT). However, in the past decades, knowledge of hepatic fibrosis has been increased (Bataller and Brenner 2005) and our understanding of cellular and molecular mechanisms of early-stage liver fibrosis has been significantly improved.

3. Hepatic Cell Types and Pathogenesis

The hepatic lobule is formed by parenchymal cells (PCs) and non-parenchymal

cells. Parenchymal cells (hepatocytes and cholangiocytes) are the main cell types of the liver, making up 60% of total liver cells. Hepatocytes perform the majority of liver functions, including protein synthesis, cholesterol synthesis, detoxification, carbohydrates transformation, and synthesis and secretion of bile (Sasse, Spornitz et al. 1992). Generally, hepatocytes are known to have high oxygen consumption rate both *in vivo* and *in vitro*, and hepatocytes will respond promptly to a decrease in oxygen, especially hepatocytes in zone 3 (Ichikawa, Zhang et al. 2001). In fibrotic condition, hepatocytes within pseudo-lobules are surrounded by collagen and in more hypoxic condition.

Non-parenchymal cells are associated with the sinusoids including: endothelial cells (ECs), Kupffer cells, Pit cells, HSCs and hepatic progenitor cells (HPCs) (Wake 1971; Wake 1980).

Sinusoidal endothelial cells form a large part of the extremely thin lining of the sinusoid and have large fenestra between cells (Kojima, Sato et al. 2001);

Kupffer cells are specialized macrophages, predominantly located in the liver along the walls of the sinusoids. Activated Kupffer cells are a source of several pro-inflammatory cytokines (tumor necrosis factor-alpha (TNF-α)) and superoxide. TNF-α will then activate HSCs leading to collagen accumulation and fibrogenesis (Wheeler 2003).

Pit cells are large granular lymphocytes. Pit cells are functionally defined as liver-specific natural killer (NK) cells. They remain in the liver and depend on Kupffer cells to activate. Once activated, these cells will then translocate into targeted tumor sites. The function of Pit cells depends on their expression of natural cytotoxin (Wisse, Luo et al. 1997).

HSCs are also known as Ito cells, fat storing cells and lipocytes (Stanciu, Cotutiu et al. 2002) and are located in vicinity of adjacent hepatocytes and sinusoidal endothelial cells. In a normal liver, HSCs are described as being in a quiescent state. Quiescent stellate cells represent 5-8% of the total number of liver cells. Each cell has several long protrusions that extend from the cell body and wrap around the sinusoids. Moreover, there are numerous lipid droplets around the nucleus of cells, which are storage places of retinoid in the human body. Furthermore, quiescent stellate cells can also produce certain growth factors and cytokines to remodel ECM in the liver (Sato, Suzuki et al. 2003; Gressner and Weiskirchen 2006). Although the exact function and role of quiescent HSCs are still unclear, it is commonly accepted that activated stellate cells are the major cell type responsible for the development of liver fibrosis (Gressner 1996; Friedman 2008).

Hepatic progenitor cells (HPCs) are like adult stem cells with limited abilities and have the potential to differentiate into more than one specific cell types (Shafritz and Oertel; Potten, Booth et al. 1997). HPCs are scarce in the normal liver, but upon stimulation HPCs could proliferate across hepatic lobule into liver parenchyma (Knight, Matthews et al. 2005). HPCs have been demonstrated to differentiate into the hepatocyte lineage after stimulation with several growth factors such as the epidermal growth factor (EGF), hepatocyte growth factor (HGF) and BMPs (He, Tan et al. 2003; Fan, Shen et al. 2009).

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and is mostly induced by viral hepatitis infection (hepatitis B or C) or cirrhosis. Chronic infections develop HCC by repeatedly causing the body's immune system to attack the

liver cell, while the healing process can lead to mistakes during DNA repair, which in turn leads to carcinogenesis (Chen, Yang et al. 2006).

4. Hepatic Stellate Cells

4.1 Profile

Von Kupffer first identified HSCs in 1876. In 1951, Ito observed lipid-containing perisinusoidal cells in a human liver and described morphological features of these cells under electron microscopy (Ito and Shibasaki 1968). The function of these lipid-containing cells was only known as the storage of vitamin A before the 1980s. Further studies on HSCs demonstrated that these cells were major producers of ECM in the liver (Friedman, Roll et al. 1985). Therefore, HSCs were commonly considered as a critical cell type in liver fibrogenesis. At present, there are several HSC cell lines representing different stages of HSCs activation such as CFSC-3H, CFSC-5H, CFSC-2G and CFSC-8B. These cell lines were originally isolated from CCl_4 induced cirrhotic livers of rats and were widely used as an *in vitro* model for the study of HSCs (Schaefer, Rivas-Estilla et al. 2003).

4.2 Hepatic stellate cells in normal liver

Morphology of HSCs has been well examined by several investigators (Wake 1980). It is known that HSCs have spindle-shape or angular-shape bodies with long branching cytoplasmic processes, through which they connect hepatocytes and sinusoidal endothelial cells (Zou, Ekataksin et al. 1998). Moreover, there are abundant lipid droplets around the nucleus, which contain vitamin A. Vitamin A is known to regulate diverse cellular activities, including cell proliferation and differentiation. Vitamin A also is

important in morphogenesis and tumorgenesis (Blomhoff 1994). In physiological conditions, 80% vitamin A is stored in lipid droplets of HSCs. HSCs are responsible for homeostasis of vitamin A, as they transport and exchange vitamin A with bloodstream (Blomhoff, Senoo et al. 1992).

Quiescent HSCs maintain extracellular matrix homeostasis by expressing balance amounts of ECM, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMP), which then remodel ECM components in the liver (Li, Sato et al. 1999; Wang, Sato et al. 2003; Sarem, Znaidak et al. 2006). In return, ECM also plays an important role in regulating HSCs such as regulation of morphology, proliferation, migration and functions (Donato, Lavano et al. 1992).

4.3 Hepatic stellate cells activation in pathological conditions

Due to chronic or acute liver injury, HSCs undergo a process of activation, which involves two steps of initiation and perpetuation. Initiation refers to early changes in gene expression and phenotype transdifferentiation that render the cells responsive to cytokines and stimuli; initiation is due to paracrine stimulation by hepatocytes and inflammatory cells. During this stage, quiescent stellate cells transdifferentiate from the star-shaped phenotype to myofibroblast phenotype, and they also lose lipid droplets. Perpetuation results from the persistent effects of these stimuli on maintaining the activated phenotype, which results in contraction, proliferation, secretion of excess ECM protein including type I and type III collagens (Burt, Griffiths et al. 1990), and attraction of more stellate cells to the injured site, etc. Increased proliferation, differentiation and excess secretion of collagen are the central pathogenic process during liver fibrogenesis as outlined in Figure 1. The fate of activated HSCs includes selective clearance by

apoptosis and/or reversal to the quiescent phenotype (Friedman 2000; Davis, Chen et al. 1996).

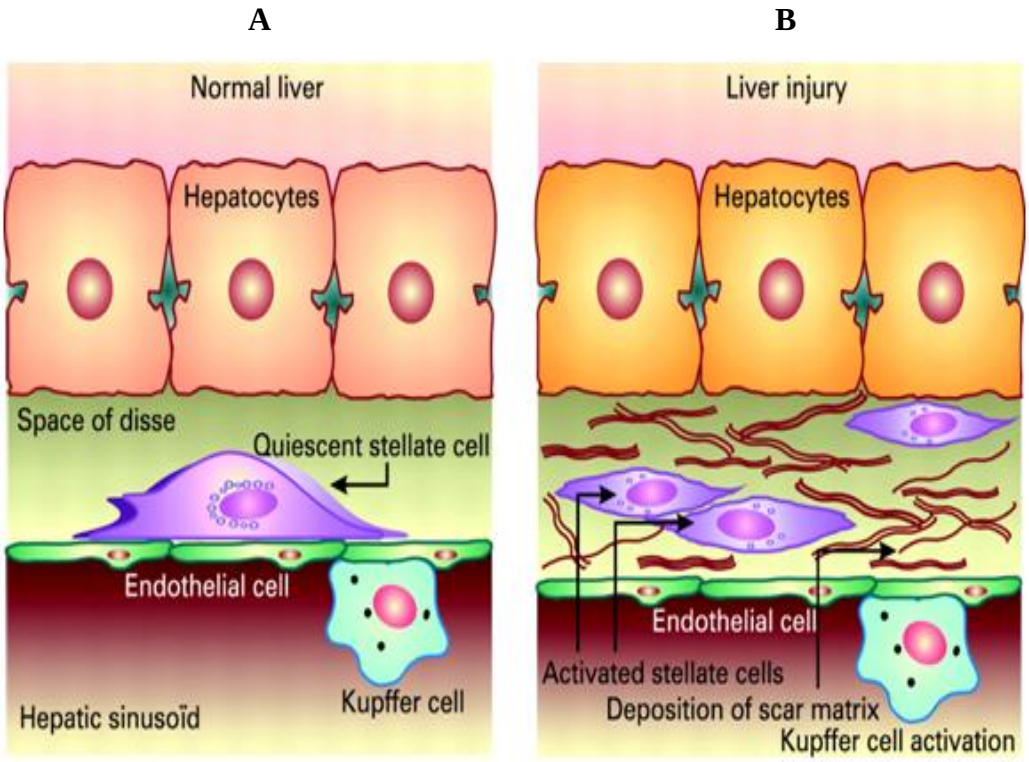


Figure 1: Hepatic architecture. A: Normal liver; B: Injury liver. When the liver is injured, hepatocytes undergo either necrosis or apoptosis, and other hepatocytes may dedifferentiate and regenerate. Injured hepatocytes can release certain factors to activate Kupffer cells and HSCs. When HSCs are activated, they will go through a transdifferentiation change from the star-shaped phenotyped to myofibroblast-like cells. During this process, HSCs lose retinoid droplets, acquire contractile activity, proliferate and secrete large amount of extracellular matrix protein (Copyright © November 18, 2011, by BMJ Publishing Group Ltd and the British Society of Gastroenterolog).

4.4 Mediators involved in hepatic stellate cells activation

It is generally considered that activation of HSCs is involved in both paracrine (initiation stage) and autocrine (perpetuation stage) mechanisms. It is also involved in a variety of interactions among hepatocytes, lymphocytes, Kupffer cells, endothelial cells, hepatic sinusoids, and HSCs themselves (Kmiec 2001). For example, injured hepatocytes due to virus, alcohol, drugs, toxin etc. could release inflammatory cytokines and/or reactive oxygen species (ROS), which then activate Kupffer cells. Kupffer cells play a prominent role in HSCs activation. Interaction of different liver cells and cytokines released from them are listed in Table 1.

In addition, in the perpetuation stage, several growth factors and cytokines also play an important role in sustaining development of liver fibrosis. For example, (i) Proliferation, PDGF (the most potent stellate cell mitogen), TGF-β, epidermal growth factor (EGF) and basic fibroblast growth factor (b-FGF) are key factors regulating stellate cell proliferation (Wong, Wight et al. 1996); (ii) Fibrogenesis, TGF-β, as well as cytokines such as TNF-β and lipid peroxidation products, stimulate accumulation of ECM and induce fibrosis (Whalen, Rockey et al. 1999); (iii) Chemotaxis, certain chemoattractants can recruit HSCs into the injured site (Marra, DeFranco et al. 1999; Maher 2001); (iv) Contractility, HSC contraction is one of the major events contributing to intrahepatic portal hypertension. Endothelin-1 is the main contracting factor while NO is the main relaxing factor (Rockey 2001); (v) Matrix degradation, HSCs can also secret matrix metalloproteinase-2 (MMP-2), which is able to degrade normal ECM in the liver and make space for the deposit of abnormal ECM (Benyon and Arthur 2001); (vi) Retinoid loss, during stellate cell activation, lipid droplets are lost but the exact

mechanism still needs to be investigated.

Table 1 Cells and their role in HSCs activation process

Cell type	Factors	Function
Kuffer cells	TGF-β 1	Matrix synthesis, proliferation, retinoid lose
	TGF-α	Proliferation
	ROS	Enhancing HSCs activation and collagen synthesis
	NO	Limit ROS effect
	Lipid peroxides	Matrix synthesis, proliferation, retinoid lose
	IL-10	Decrease collagen synthesis, increase collagenase formation, and collagen synthesis
Hepatocytes	ROS	Enhancing HSCs activation and collagen synthesis
	TGF- β 1	Collagen synthesis
Platelets	PDGF	Proliferation
	TGF-β 1	Collagen synthesis
	EGF	Proliferation
Leukocytes	ROS	Collagen synthesis
	NO	Limit ROS effect
	IFN-γ	Collagen degradation
	TNF	Collagen degradation

Abbreviations: MMP-9: metalloproteinase 9; PDGF: platelet-derived growth factor; TGF-β 1: transforming growth factor beta 1; TGF-α: transforming growth factor alpha; IFN-γ: interferon gamma; TNF: tumor necrosis factor; IL-10: interleukin 10; NO: nitric oxide

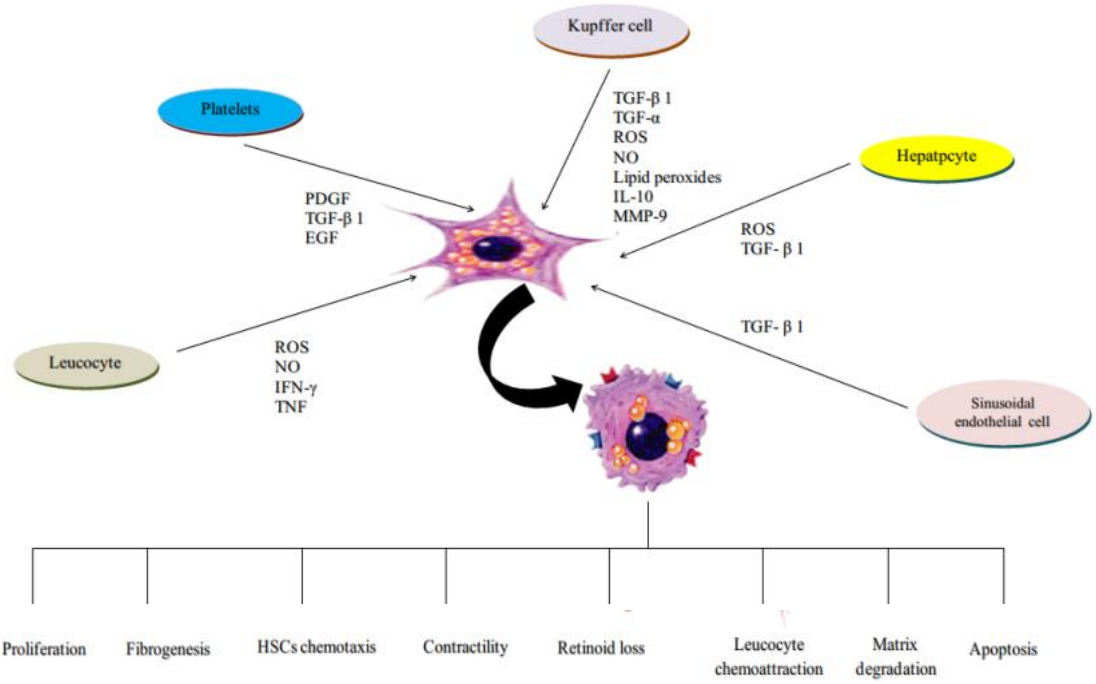


Figure 2: HSC activation. There are several factors that will initiate HSC activation and perpetuate phenotype changes during liver injury, such as TGF-β 1, ROS, IFN, etc. These paracrine effects come from hepatocytes, Kuffer cells, leucocytes, platelets and sinusoid endothelial cells. Typically, they will induce HSCs phenotype changes, such as proliferation, chemotaxis, Contractility, retinoid loss, leucocyte chemoattraction, matrix degradation and cell apoptosis, eventually result in liver fibrosis.

5. Bone Morphogenetic Protein Family

5.1 Growth factors and cytokines

Growth factors are peptides that initiate activation of cellular proliferation and/or differentiation by binding to receptors on certain cell surface (Wikipedia2). Most growth factors are able to stimulate cell division in different cell types through binding to particular membrane receptors. Cytokines are large signaling proteins similar to hormones and growth factors (Wynn 2004). Cytokines are produced by variety of hematopoietic and non-hematopoietic cell types and secreted as autocrine, paracrine and endocrine factors (Dooley and Ten Dijke). Cytokines primarily work for cellular communication, immune regulation and embryogenesis. Currently more than one hundred growth factors and/or cytokines have been discovered and can be classified based on their primary functions

Although liver fibrosis and cirrhosis are mediated by various growth factors and cytokines mentioned as above, TGF-β is believed to be the most significant one in hepatic fibrogenesis and activation of HSCs process (Bedossa, Peltier et al. 1995). In the early stage of liver injury, TGF-β is mainly released by necrotic hepatocytes and Kupffer cells. In the late stage of liver injury, TGF-β is predominantly produced by hypoxic hepatocytes (Ichikawa, Zhang et al. 2001). Therefore, a full understanding of TGF-β and TGF-β inhibition in chronic liver disease might be the key to developing an effective therapeutic strategy for liver fibrosis and cirrhosis.

5.2 Bone morphogenetic proteins

BMPs are the biggest members of the TGF-β family. Although TGF-β and BMPs have different effects in different tissues (Heckman, Ehler et al. 1999), it is reasonable to

think that BMPs may have the ability to represent most of TGF-β functions during liver fibrosis.

BMPs are important cytokines involving in cell proliferation and differentiation, especially in bone morphogenesis. To date, more than twenty members of BMP family have been identified but only 14 members bear the name of BMPs. Among these BMPs, BMP-2 and BMP-7 have been extensively investigated and they have been approved by FDA for human clinical applications. Although BMP-1 has the name of BMP, it does not belong to BMPs family instead it is a metalloprotease (Wozney, Rosen et al. 1988). Originally, BMPs were discovered due to their ability to induce the formation of bone and cartilage, but the expression of BMPs and their receptors are found in numerous cell types which suggests that BMPs may have broader functions than are currently revealed, including morphogenesis, cell proliferation and differentiation, apoptosis, as well as maintenance and differentiation of embryonic stem (ES) cells (Bleuming, He et al. 2007) (Miller, Harvey et al. 2000; Reddi 2000; Xiao, Xiang et al. 2007) (Table 2). BMPs have also been documented to decrease proliferation of human breast cancer cells and prostate cancer cells; to induce apoptosis of cells in the interdigits (Ganan, Macias et al. 1996) and of neural crest-derived cells located in the rhombomeres (Mehler, Mabie et al. 1997); to promote elencephalic neuro-epithelial cells to differentiate into astrocyte in the central nervous system (CNS) and to induce neuronal differentiation in the peripheral nervous system (PNS) (Mehler, Mabie et al. 1997).

In our laboratory, we have focused on investigation of BMP-4 in the liver. We have reported that BMP-4 expression was significantly elevated in the liver of bile duct ligation induced liver fibrosis (Fan, Shen et al. 2006), and that BMP-4 was able to induce

hepatic progenitor cell (WB-F344 cell) differentiation into hepatocyte lineage (Fan, Shen et al. 2009). Moreover, we found that BMP-4 was able to stimulate HSC transdifferentiation and increase the expression of α-smooth muscle actin (α-SMA) in cultured HSCs (Fan, Shen et al. 2006; Fan, Shen et al. 2009). Furthermore, we documented that BMP-4 induced these changes through activation of several intracellular signaling molecules such as Smad1, ERK1/2 and p38. However, the roles of other BMPs in the liver still remain to be investigated.

Table 2 BMPs members and their known function

Name	Alternative name	Known function
BMP-1	See note	
BMP-2	BMP-2 alpha	Bone and cartilage formation; retinoid mediator; osteoblast differentiation
BMP-3	Osteogenin	Bone formation
BMP-4	BMP-2 beta	Hepatic cell proliferation and differentiation
BMP-5		Cartilage development and chondrogenesis
BMP-6	Vegetalising factor related-1 (Vgr-1)	Joint integrity in adults
BMP-7	Osteogenic protein-1 (Op-1)	Osteoblast differentiation; Smad1 production; renal development and repair
BMP-8	BMP-8a/Op-2	Bone and cartilage development
BMP-9	Growth & differentiation factor-2 (GDF-2)	Angiogenesis
BMP-10		Trabeculation of the embryonic heart
BMP-12	GDF-7/ Cartilage-derived morphogenetic protein-3 (CDMP-3)	Ligament and tendon development
BMP-13	GDF-6/CDMP-2	Ligament and tendon repair; cartilage development
BMP-14	GDF-5/CDMP-1	Enhances tendon healing and formation
BMP-15		Oocyte and follicular development

Note: BMP-1 is a metalloprotease that regulates procollagen I, II, and III. It is not considered as the member of BMP family.

5.3 Receptors and signaling transducer

There is increasing evidence indicating that BMP signaling plays an important role during cell differentiation (Zhao, Harris et al. 2002). BMPs mediate target gene expression through the BMP (Smad) signaling pathway. Smads are pivotal intracellular molecules that transfer signals of TGF-β superfamily members from the cell surface to the nucleus. BMPs initiate signaling transduction by binding to type I and type II serine/threonine kinase receptors, which will then phosphorylate Smad proteins (Massague 1998). The first serine/threonine kinase receptor was discovered through identification of the activin receptor and therefore, type I receptors of the TGF-β superfamily are defined as activin receptor-like kinases (ALKs). These ALKs are designated as ALK1 and ALK5 for type I receptors of TGF- β, ALK2 and ALK4 for type I receptors of Activin, ALK3 and ALK6 for type I receptors of BMPs/GDFs, and ALK7 for nodal (ten Dijke, Ichijo et al. 1993). On the other hand, there are five type II receptors, including TGFRII for TGF- β, ActRIIA and ActRIIB for Activins and BMPs, BMPRII for BMPs, and AMHRII for AMH (Massague 1998; Manning, Whyte et al. 2002). Since members of TGF-β superfamily have similar molecular structures, they can interact with the limited set of receptors (Wozney 1992; Wozney and Rosen 1998); for example, BMP-9, BMP-10, BMP-13 and BMP-14 share 51% amino acid sequence identity with each other. Although type I and type II receptors have specific affinity to BMPs ligands, both receptors are required to transduce signal in cells (Massague 1998). It used to be believed that ligand binding in the TGF-β superfamily to their receptor was specific; however, recent studies revealed that there are cross-links between BMPs and other receptors (Yamashita, ten Dijke et al. 1995).

Type I and type II receptors initiate two distinct down-stream Smad-signaling pathways (Figure N): Smad1/5/8 pathway and Smad2/3 pathway (Kretzschmar and Massague 1998; Chen and Massague 1999; Watatani, Ieda et al. 1999). At present, eight Smads have been found in vertebrates and they can be subdivided into three groups according to their functions: (1) Receptor-associated Smad (R-Smad) which include Smad 1,2,3,5 and 8; (2) Common-mediated Smad (Co-Smad) including Smad4; (3) Inhibitory Smads including Smad 6 and 7 (Attisano and Wrana 2000; Massague and Wotton 2000). It is generally considered that Smad 1, 5 and 8 mediate BMPs signaling transduction and Smad 2 and 3 are involved in other TGF-β signal pathways (Miyazono, ten Dijke et al. 2000). However, all these pathways use Smad 4 as a Co-mediator. After binding with specific ligands, the activated type I receptor dimer will activate the type II receptor dimer to become a hetero-tetrameric complex, which will then phosphorylate R-Smads. Phosphorylated R-Smad will then dissociate from the cell membrane and form a complex with common Smad (Co-Smad). Phosphorylated R-Smad and Co-Smad heterotrimer or heterotetramer will then translocate into the nucleus where they bind to a specific DNA sequence called Smad binding element (SBE) and associate with other transcription factors to function as either co-activator or co-repressor to regulate target gene expression as shown in Figure 3 (Attisano and Wrana 2000; Massague, Blain et al. 2000; Miyazono 2000) (Wrana 2000).

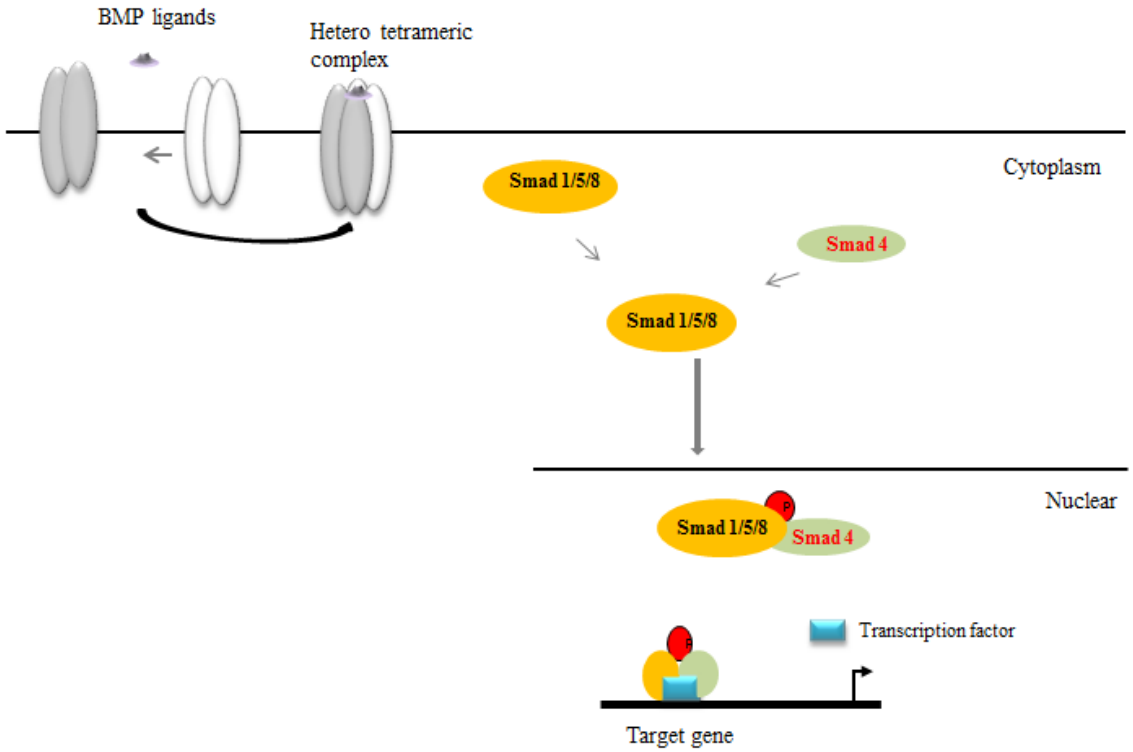


Figure 3: BMP signaling pathway. One of the main signaling pathways of BMPs is mediated by intracellular protein Smads, especially Smad1/5/8. First, BMP ligands will bind to their specific receptors, which initiate a cascade phosphorylation, and recruit Smad1/5/8 and interact with other transcription factors, such as Runx2 in osteoblasts, to initiate target gene expression (Chen, Zhao et al. 2004). As there is limited availability of receptors on the cell membrane, BMPs function could be inhibited by BMP antagonists. P: phosphorylate.

BMPs functions are regulated by BMP antagonists (Massague and Chen 2000; Canalis, Economides et al. 2003), which are proteins; either associated with BMPs or BMP receptors to prevent BMPs interaction with their receptors. BMPs antagonists work as switches of BMP ligands and are subdivided according to cysteine residues in the structure. Since some of BMPs over-perform their functions, blocking certain BMPs at certain times is absolutely necessary. Deregulation of BMPs signaling has been shown to be involved in several pathological processes such as cancer (Kodach, Wiercinska et al. 2008). However, over-activation of BMP signaling can also lead to reflux-induced esophagitis that provokes Barrett's oesophagus and is thus instrumental in the development of adenocarcinoma in proximal portion of the gastrointestinal tract (Kodach, Wiercinska et al. 2008).

5.4 Bone morphogenetic protein-13

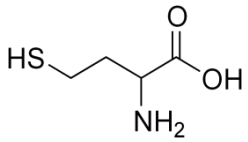
BMP-13, also known as growth differentiation factor 6 (GDF-6) or cartilage-derived morphogenetic protein 2 (CDMP-2), is one of the members of BMPs family and was first identified as a component of bovine cartilage. BMP-13 is the least investigated member of the BMP family and it has been argued whether BMP-13 is involved in bone and cartilage formation (Storm, Huynh et al. 1994; Wozney and Rosen 1998). Although BMP-13 does not regulate chondrocyte and cartilage cell proliferation, it displays exclusively high affinity to BMPRIB (ALK6) and BMPRII. Both BMPR1B and BMPRII are critical important factors for chondrogenesis (Kawakami, Ishikawa et al. 1996). Recent investigations demonstrated that BMP-13 inhibited osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells, indicating that deficiency of BMP-13 may induce unexpected bone formation (Mahfouz and Kummerow 2004).

Therefore, our understanding of BMP-13 is its ability not to induce osteogenesis. Moreover, more recent studies documented that BMP-13 medicated repair of tendon and ligament-like tissues (Forslund and Aspenberg 2001; Helm, Li et al. 2001; Forslund 2003; Wong, Fu et al. 2005). Helm et al. reported that intramuscular injection of adenovirus-expressing BMP-13 could induce type I collagen accumulation in tendon and ligament-like connective tissues in rats (Helm, Li et al. 2001). In addition, Wong et al. also reported that BMP-13 was able to stimulate cell proliferation and expression of type I collagen in human patellar tendons (Wong, Fu et al. 2005). Collagen deposition and extracellular matrix formation are the hallmark of the healing process in tendons. These findings indicate that BMP-13 may trigger cell proliferation, differentiation and collagen synthesis during healing processes of tendon and ligament-like connective tissues. BMP-13 signal transduction pathway was also investigated and it has been found that BMP-13 induced gene expression through the activation of Smad 1/5/8 molecules (Mazerbourg, Sangkuhl et al. 2005).

6. Homocysteine (Hcy)

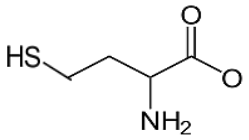
6.1 Chemical structure

Hcy is an amino acid with the formula of HSCH2CH2CH (NH2) CO2H. Hcy is the homologue of the semi-essential amino acid cysteine, different from a methylene group.

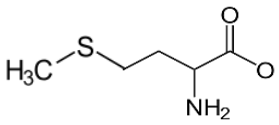


Homocysteine chemical structure

IUPAC name is 2-amino-4-sulfanylbutanoic acid



Cysteine chemical structure



Methionine chemical structure

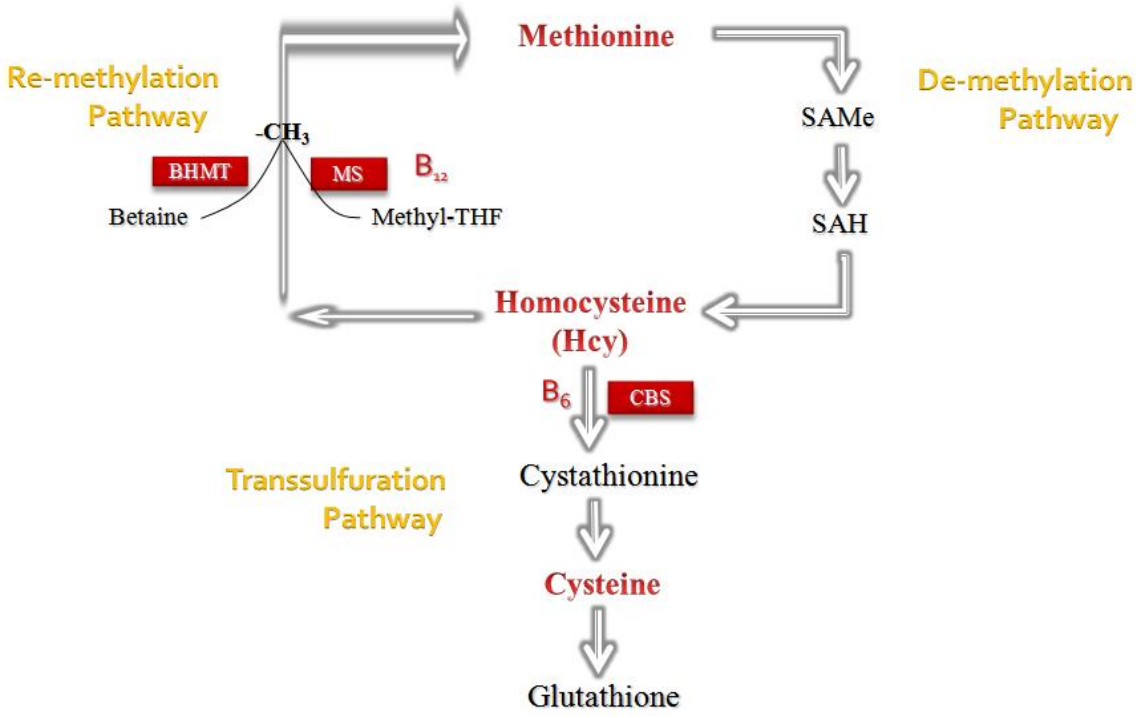
6.2 Methionine metabolism

Methionine is an amino acid and is found primarily in meats, eggs, dairy products, fish, chicken, seeds, nuts and some vegetables. Hcy is a bio-intermediate product in the methionine metabolism pathway, which occurs primarily in the liver (Figure 4) (Purohit, Abdelmalek et al. 2007). A central event of Hcy metabolism is the synthesis of glutathione, which is a powerful detoxifier in the liver. Furthermore, Hcy paves the way to the body's production of some essential hormones in the brain, such as serotonin (a happy hormone), melatonin (a sleep and mood improving hormone), dopamine (a euphoria hormone) and adrenaline (a fight and flight hormone) (Holford et al. 2003).

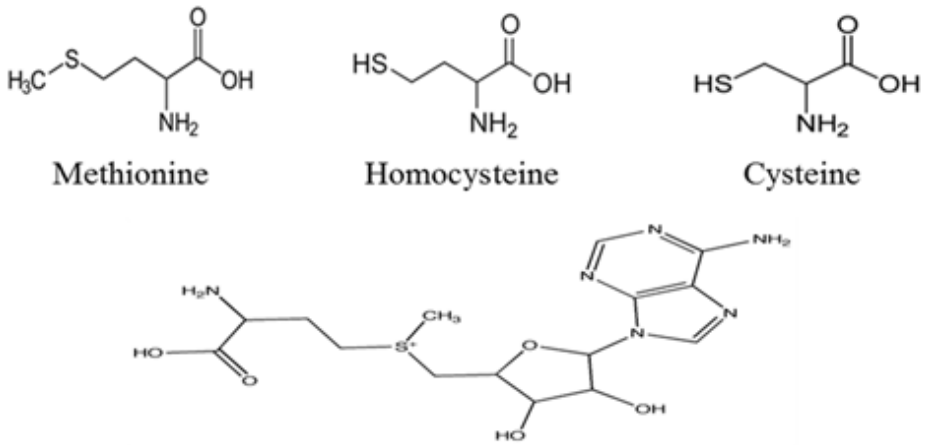
In the liver, methionine metabolism is outlined in Figure 4. An adenosine group from ATP attaches to methionine to form S-adenosylmethionine (SAMe). This process is catalyzed by methionine adenosyl transferase (MAT). SAMe is then converted to S-adenosyl Hcy (SAH) by transferring a methyl group to another receptor. These substrates include nucleic acids, proteins, lipids and secondary metabolites (Cantoni 1952). SAH form Hcy catalyzed by SAH hydrolase. In normal liver, Hcy undergo three metabolism pathways; one of them is transsulferation pathway, while the other two are transmethylation pathways. In transmethylation pathways, Hcy received a methyl group to be re-cycled back to methionine. However, mechanisms involved in these two processes are quite different. One is catalyzed by methionine synthetase (MS) and also

requires normal concentration of folate. The methyl group is transferred from N5-methyltetrahydrofolate (MTHF) to vitamin B12 to form methylcobalamine, which in turn transfers the methyl group to Hcy to produce methionine. In the other transmethylation pathway, the methyl donor is betaine, which is a metabolic of choline. The reaction is catalyzed by betaine-homocysteine methyltransferase (BHMT). Conversion of Hcy back to methionine is important to maintain normal Hcy concentration in the liver, and also prohibits elevated level of Hcy. In the transsulferation pathway, Hcy releases free cysteine and eventually converts to glutathione (GSH). Firstly, Hcy releases serine to form cystathionine by cystathionine β-synthase (CBS) and vitamin B6 as a cofactor. Cystathionine is then cleaved by another vitamin B6 dependent enzyme - γ-cystathionase, which results in the release of free cysteine. Cysteine is a rate-limiting precursor of GSH synthesis in all mammalian cells. GSH is then synthesized via a two-step process, where the first step is catalyzed by glutamate-cysteine ligase (GCL), and the reaction in the second step is catalyzed by glutathione synthetase.

A


Methionine
Re-methylation Pathway
De-methylation Pathway
-CH3
BHMT
MS
B12
Betaine
Methyl-THF
SAMe
SAH
Homocysteine (Hcy)
B6
CBS
Transsulfuration Pathway
Cystathionine
Cysteine
Glutathione

B


Methionine
Homocysteine
Cysteine
S-adenosylmethionine (SAMe)

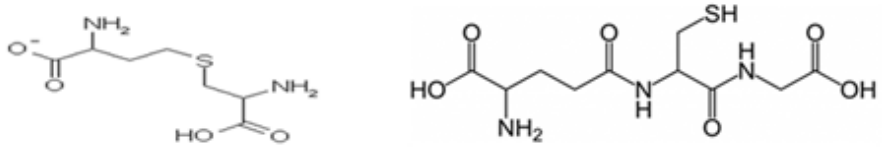

Cystathionine
Glutathione

Figure 4 Methionine metabolism pathways in the liver. A. Hcy undergoes two remethylation pathways and one trans-sulfuration pathway in the liver. In the two remethylation pathways, Hcy receives methyl group from betaine and/or methyl-THF, reactions are catalyzed by different factors in each pathway but both require B-vitamin as cofactor. In transsulfuration pathway, Hcy synthesizes the critical antioxidant, GSH. Any deficiency of either co-factors or enzymes will result in Hcy accumulation in the liver, which was reported as a typical fibro genesis factor (Cheng et al. 2004). Regulation of methionine metabolism, either remethylation or trans-sulfuration, depend on different affinities of MS, BHMT and CBS. B. Structure of chemicals involved in methionine metabolism pathway.

7. Hyperhomocysteinemia

7.1 General introduction

Hyperhomocysteinemia (HHcy) is a result of methionine metabolism disorder and/or dysfunction of Hcy clearance, which induces excess amounts of total Hcy in the blood stream (Remkova and Remko 2009), while healthy normal levels of Hcy in the blood are thought to pave the way for several of the body's essentials production. High levels of Hcy in the blood will contribute to the development of diseases in the heart, and bone, kidney and liver (Table 3).

Clinically, HHcy was first identified in children who had learning difficulties 50-years ago, and since then HHcy has been reported in several general populations (Ji and Kaplowitz 2004). At present, HHcy is a frequent phenomenon, more than 15% of the Caucasian population is suffering from HHcy (Roblin, Pofelski et al. 2007), and the high concentration of Hcy is found in the populations with low B-vitamin intake, such as residents in parts of New Delhi. Hcy concentration goes up as people age. Moreover, women have less Hcy in the blood than men during their reproductive ages, which may help to explain the fact that women suffer from myocardial infarction on an average of 10 to 15 years later than men (Selhub, Jacques et al. 1999).

7.2 Clinical appearance of hyperhomocysteinemia

Early stages of HHcy may have no visible symptom until the concentration reaches extremely high levels. However, when symptoms appear, patients would experience the following feelings: pain, heart uncomfortable, inflammation reaction, recurring blood clots, and vascular damage (Selhub, Jacques et al. 1999). Moreover, once symptoms appear, HHcy may already cause serious health risk (Table 4).

Table 3 Total serum reference ranges of Hcy

Health Risk	**Fasting level in serum (μM)**
Super healthy	< 6
Low risk	6-8.9
Moderate risk	9-12
High risk	12-15
Very high risk	15-20
Extremely high risk	> 20

(Tessa Jupp, 2003)

Table 4 The symptom of HHcy

Super healthy	Primary condition	Terminally ill
Boundless energy	Constant tiredness	Chronic fatigue
Enlivened by life	Feel drained	Feel exhausted
Sharp mind	Poor concentration	Constant pain
Positive outlook	Mood swings	Depression
Full of life	Exercise exhausts	Pessimistic
Physically fit	Unfit	Unable to exercise
Rarely ill	Frequently ill	Incapacitated by ill
Lead full life	Easily overwhelmed	Life is hard work
Well toned	Body flabby	Like a vegetable
Content, happy	Dissatisfied	In despair

(Tessa Jupp, 2003)

7.3 Hyperhomocysteinemia determination

A lot of factors contribute to the elevation of Hcy such as genetic abnormalities, various nutritional and hormonal disorders, gender, age, mood and some chronic disease complications. Among all these factors, genetic abnormalities and nutritional disorders are the two most important factors.

(i) For the genetic abnormalities, polymorphism in the genes coding for the enzymes in methionine metabolism pathways (Hase 1998), such as MTHF, CBS, BHMT, MAT and any catalyzers or enzymes that are involved in methionine metabolic pathways. The most common genetic defect causing HHcy is a point mutation such as a C-T substitution at nucleotide 677 (C677-T) in the open reading frame of MTHFR gene. This point mutation could cause a substitution of valine for alanine resulting in a thermo-labile variant of MTHFR, which decreases enzymatic activity. This is an autosomal recessive mutation, and frequency of C677-T polymorphism varies among different ethnic groups with 13% of T/T homozygous and 50% C/T heterozygous among Caucasian and Asian population respectively, and very low incidence among African-Americans (Frosst, Blom et al. 1995; Ma, Stampfer et al. 1996; Woo, Qiao et al. 2002; Jeng, Wu et al. 2003). Another common genetic cause associated with severe HHcy is homozygous CBS deficiency, which resulted in plasma Hcy concentration up to 400 μmol/L compared to normal plasma level of 10 μmol/L (Ueland and Refsum 1989; Mudd, Levy et al. 2001). (ii) Nutritional disorders that potentially lead to HHcy include deficiencies in vitamin B12, folate and vitamin B6, because de novo synthesis of methionine methyl groups requires both vitamin B12 and folate cofactors, whereas synthesis of cystathionine requires pyridoxal 5-phosphate (vitamin B6). Although it has been shown that

deficiencies of vitamin B12 and folate are related to increased plasma Hcy concentrations (Ueland and Refsum 1989; Billion, Tribout et al. 2002; Haynes 2002; Moat, Bao et al. 2004), the relationship of Hcy levels and vitamin B6 status is less clear (Cravo and Camilo 2000; Lakshmi, Maniprabha et al. 2001). In addition, excess dietary methionine has been shown to induce HHcy in mice (Troen, Lutgens et al. 2003). (iii) The other major cause of HHcy is the activation of hepatic guanidinoacetate (GAA) and N-methyltransferase (NMT). GAA is produced in the kidney and then converted to creatine in the liver by GAA-NMT, which uses SAMe and generates SAH. Creatine is exported to muscle and also inhibits the enzyme in the kidney, which produces GAA. GAA supplementation could also induce HHcy, and creatine feeding could lower Hcy (Stead, Au et al. 2001).

7.4 Hyperhomocysteinemia and diseases

HHcy is related to various diseases including cardiovascular (e.g. atherosclerosis) (Refsum, Ueland et al. 1998) and cerebrovascular diseases, neuro-degenerations (e.g. Alzheimer and Parkinson), renal and venous thrombosis, diabetes, bone disease, cancer and liver fibrosis.

Hcy metabolism primarily occurs intracellularly; however, excessive Hcy can be transported into an extracellular medium such as plasma and body fluid. The kidney is one of the major organs responsible for Hcy clearance (van Guldener 2005). Therefore, patients with chronic renal failure often have higher Hcy concentration than those with normal renal function. Moreover, HHcy frequently occurs after renal transplantation, indicating the kidney's role in clearance of Hcy. However, the mechanism of HHcy in the kidney is still under investigation.

7.5 Hyperhomocysteinemia and liver disease

7.5.1 Mechanism of hyperhomocysteinemia induced liver injury

Hcy/HHcy induces liver injury primarily through three mechanisms. The first and most important one is oxidative stress; the second one is activation of pro-inflammatory factors; and the third one is endoplasmic reticulum stress (Ji and Kaplowitz 2004).

7.5.1.1 Oxidative stress

Reactive oxygen species (ROS) are small molecules that include oxygen ions and peroxides and are highly reactive due to the presence of unpaired valence shell electrons. ROS are a by-product of normal metabolism of oxygen. Normal amounts of ROS are beneficial, because they are used to kill pathogens by immune system. However, excessive ROS can result in severe damage of cell structures. For example, ROS can cause lipid peroxidation, which leads to cell membrane cleavage; ROS can oxidize amino acids in proteins, which inactivate enzymes; and ROS can disrupt energy generators, which damages the mitochondria. Overwhelmed damage of the mitochondria will trigger cell apoptosis or programmed cell death. Severe oxidative stress will result in ATP depletion and necrosis. Moreover, oxidative stress will also lead to DNA damage (Lennon, Martin et al. 1991; Lelli, Becks et al. 1998; Evans and Cooke 2004; Valko, Morris et al. 2005).

ROS include a series of free radicals and peroxides as follows: superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), organic hydroperoxide (ROOH), alkoxy and peroxy radicals ($RO\cdot$, $ROO\cdot$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) (Table 5) (Lee and Shacter 1999). One of the major free radicals is hydrogen peroxide (H_2O_2), which is converted from superoxide. Superoxide is

normally produced in mitochondria. Under normal conditions, H_2O_2 can be converted into oxygen and water by catalase, which will minimize oxidative damage to cells. However, if hydrogen peroxide is not completely converted, it will persist inside the cells and cause cell damage (Liu, Head et al. 2002). Therefore, oxidative stress is caused by an imbalance between production and clearance of ROS. In normal cells, there are several anti-oxidative systems to prevent ROS induced cell damage, such as glutathione, catalase and SOD. HHcy induced oxidative stress is mediated by activation of NADPH oxidases. It is known that HHcy can elicit a transient formation of ROS, which activates extracellular signal-regulate kinase (ERK) and P38 mitogen-activate protein kinase (MAPK), and subsequently initiates NADPH oxidase activation (Zalba, San Jose et al. 2001; Czaja 2005; Zou, Gao et al. 2009). Activated NADPH oxidase leads to the production of more ROS, which can rapidly interact with nitric oxide (NO) to form highly reactive nitrogen species (RNS) such as peroxynitrite. It is reported that these RNS and ROS may result in activation of the MAPK or Akt pathway, which are involved in HSCs proliferation process, thus initiating liver fibrosis (Figure.5). RNS is responsible for irreversible damage to complexes I and II of the respiratory chain, to inhibition of ATP synthesis and eventually to release of cytochrome C from mitochondria. Cytochrome C will activate caspase and induce caspase-dependent apoptosis. Moreover, RNS can also activate the apoptosis induce factor (AIF), which will have the same kind of result. ROS can induce lipid peroxidation and protein oxidation, while RNS can induce protein nitration. All these reactions will inactivate enzymes and cause necrosis (Novo and Parola 2008)

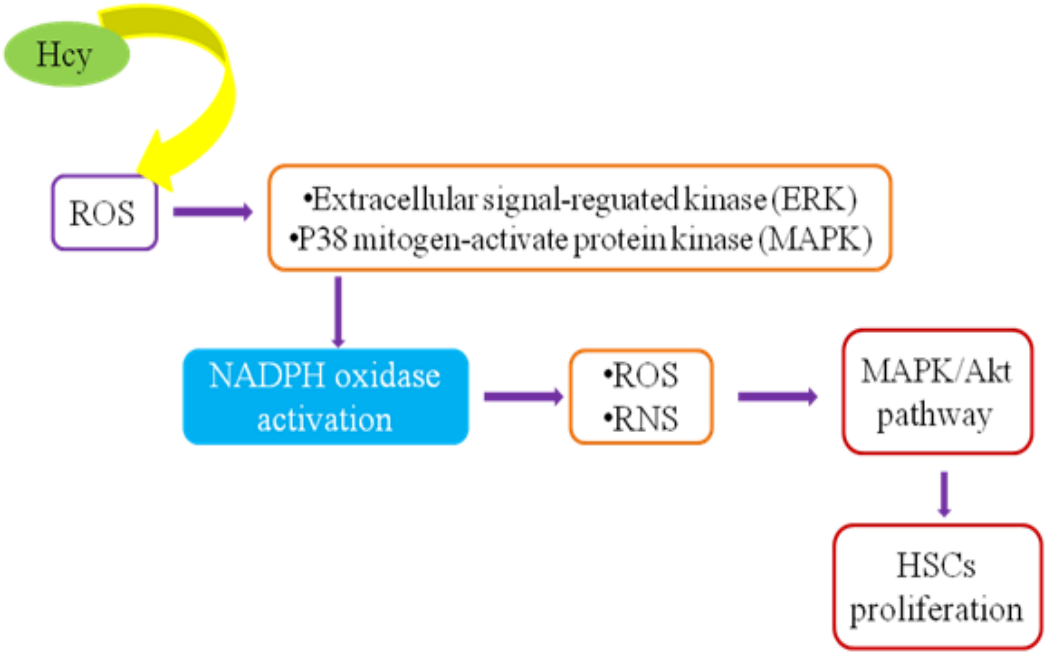


Figure 5 Possible mechanism of Hcy induced HSCs proliferation. Firstly, Hcy initiate small amount of ROS, which are considered involved in extracellular signal-regulated kinase (ERK) and P38 mitogen-activate protein kinase (MAPK) activation process. These enzymes subsequently initiate NADPH oxidase activation. Activated NADPH oxidase helps to form more ROS and RNS. These abnormally elevated cytokines are reported to have the ability to induce HSCs proliferation and further result in liver fibrosis through activation of MAPK or Akt pathway.

Table 5 Free radicals and peroxides

	Description
$\cdot O_2^-$	One electron reduction state of O_2, formed in many autoxidation reactions and by the electron transport chain. Undergoes dismutation to form H_2O_2, or by enzymatic catalysis and is a precursor of metal-catalyzed ·OH.
H_2O_2	Two-electron reduction state, formed by dismutation of $\cdot O_2^-$, or by direct reduction of O^2. Lipid soluble and thus able to diffuse across membranes.
·OH	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components.
ROOH	Formed by radical reactions with cellular components such as lipids and nucleases.
RO· and ROO·	Oxygen centred organic radicals. Lipid forms participate in lipid peroxidation reactions.
HOCl	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
$ONOO^-$	Formed in a rapid reaction between $\cdot O_2^-$and NO•. Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo hemolytic cleavage to form hydroxyl radical and nitrogen dioxide.

7.5.1.2 Activation of pro-inflammatory factors

HHcy increases production of several pro-inflammatory cytokines. Cytokines are small molecules that are used in cellular communication and produced by monocytes, macrophages, and liver specific macrophage - Kupffer cells.

The mechanism of HHcy induced inflammatory reaction is known as the activation of NF-κB (Collins and Cybulsky 2001). NF-κB is a transcriptional factor involved in regulation of the immune response to infections (Gilmore 1999; Tian and Brasier 2003; Brasier 2006; Gilmore 2006; Perkins 2007). NF-κB acts as "first responder" to harmful stimuli, such as stress, free radicals, ultraviolet irradiation, and bacterial or vial pathogens (Gilmore 2006). However, incorrect activation of NF-κB will result in cancer, inflammatory, autoimmune diseases, and improper immune development (Albensi and Mattson 2000). NF-κB is one of the major transcriptional factors that regulate genes responsible for both innate and adaptive immune stimuli (Livolsi, Busuttil et al. 2001). Through a cascade of phosphorylation events, NF-κB enters the nucleus to up-regulate genes involved in T-cell development, maturation, and proliferation (Livolsi, Busuttil et al. 2001). Activated NF-κB could stimulate production of cytokines, chemokines, interferons, leukocyte adhesion molecules and growth factors, which all have the ability to influence inflammation (Collins and Cybulsky 2001; Hofmann, Lalla et al. 2001).

7.5.1.3 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a primary site for protein synthesis and folding, calcium storage and signaling, and a factory of steroids, cholesterols and other lipid productions. Proteins are synthesized and transported in the ER, where they undergo post-translational modifications for specific functions. Correctly folded proteins are

allowed to reach their destiny through a secretory pathway, while unfolded and misfolded proteins are exported or dislocated from the ER, and are degraded by cytoplasmic proteasomes (Kaufman 1999; Welihinda, Tirasophon et al. 1999; Liu, Schroder et al. 2000; Kaufman 2002; Kaufman, Scheuner et al. 2002; Ma and Hendershot 2002; Shen, Chen et al. 2002).

HHcy induced ER stress response has recently been receiving more and more attention. ER stress is described as the accumulation of unfolded and misfolded proteins (Pahl and Baeuerle 1997; Outinen, Sood et al. 1999; Althausen and Paschen 2000; Werstuck, Lentz et al. 2001; Zhang, Cai et al. 2001; Ji and Kaplowitz 2003; Lluis, Colell et al. 2003; Roybal, Yang et al. 2004). Unfolded protein response (UPR) is an intracellular signaling pathway of ER stress. Early stages of UPR will be eliminated in a timely and efficient way by cells. However, prolonged UPR following ER stress has severe consequences, for example the activation of tumor necrosis factor receptor associated factor II (TRAFII). TRAFII will activate caspase-dependent cell apoptosis. ER stress also activates sterol regulatory element-binding proteins (SREBPs), which then transcriptionally regulate HMG-CoA reductase expression and subsequently induce hepatic lipid accumulation due to excessive cholesterol biosynthesis (Beg, Stonik et al. 1985; Nakanishi, Goldstein et al. 1988; Jurevics, Hostettler et al. 2000).

7.5.2 Hyperhomocysteinemia induced hepatic stellate cells activation

Activation of HSCs can be initiated by cytokines, ROS, and growth factors respectively. Moreover, HSC proliferation can be promoted by various growth factors, such as PDGF, TGF-β and epidermal growth factors (Bataller and Brenner 2005). These changes have been identified to contribute to the pathogenesis of liver fibrosis (Friedman

2000; Bataller and Brenner 2005). Furthermore, certain investigations also documented that ROS could be a profibrogenic stimulus for HSCs as a consequence of increased cellular proliferation. In addition, it was found that HHcy induced liver fibrosis could be due to Hcy up-regulate mitogen-activate protein kinase (MAPK) – p38 and/or protein kinase B (Akt), which is responsive for HSCs proliferation (Figure 5) (Adachi, Togashi et al. 2005; Galli, Svegliati-Baroni et al. 2005; Friedman 2008).

7.6 Treatment of patients with hyperhomocysteinemia

To date, there are few options for patients suffering from elevated Hcy concentrations. It is recommended to consume more folic acid products in order to get sufficient B-vitamins intake, which plays an important role in the Hcy metabolism pathway. However, it is still unclear whether reducing Hcy to normal levels will alter the effects of HHcy on organs that are already injured by HHcy. Therefore, this dietary treatment might be just for prevention of HHcy. However, in most conditions, the earlier HHcy is detected, the more effective treatment should be.

8. Introduction Overview

Although all hepatic cell types are involved in fibrogenesis, HSCs are considered to be the central cell type responsible for the development of hepatic fibrosis and cirrhosis (Bataller and Brenner 2005). The fate of activated HSCs includes apoptosis and/or reversion back to a quiescent phenotype, depending on apoptotic and survival signals they received. Apoptotic signals include proteins from membrane receptors, such as death receptors, nerve growth factor receptors and peripheral-type benzodiapepine receptors, as well as proteins, such as BCL-2 family members, from the cytoplasm. The

survival signals are induced by cytokines such as TIMP-1, PDGF, TGF-β1 and insulin-like growth factor-1. Previous studies have demonstrated that Hcy up-regulates the expression of TIMP-1 and procollagen-1 in myofibroblasts-like HSCs, suggesting a novel role of Hcy in hepatic fibrogenesis (Torres, Garcia-Trevijano et al. 1999; Garcia-Tevijano, Berasain et al. 2001). The results of HSC activation involve several discrete changes in cell behaviour, proliferation, and accumulation of ECM.

There are several excellent review articles regarding therapeutic approaches to liver fibrosis and cirrhosis (Bataller and Brenner 2001; Bedossa and Paradis 2003; Lotersztajn, Julien et al. 2005; Roberts and Gores 2005). These therapeutic approaches include removal of causative agents such as ROS, suppression of hepatic inflammation, modulation of collagen synthesis and degradation, and inhibition of HSCs activation. Approaches that specifically initiate HSCs apoptosis are other effective strategies to treat liver fibrosis. Understanding HSCs apoptosis is becoming more and more important to developing specific target treatment for patients with liver fibrosis and cirrhosis.

Hypothesis and Objectives

1. Rationale

The present study was designed to study the role of BMPs in the liver and their involvement in hepatic pathogenesis in rats with elevated levels of Hcy. To achieve this goal, we examined BMPs mRNA and proteins expression in both rat liver tissues and HSC cell line. At the same time, we investigated the effect of Hcy on HSCs proliferation process.

Previous investigations related to BMP-13 have documented its role in tendon cell proliferation, differentiation and signaling pathways, which may indicate that BMP-13 may have diverse physiological activities in other tissues. Similar to the healing process of an injured tendon, hepatocytes also undergo a dedifferentiation process which allows them to proliferate and regenerate after partial hep atectomy. Moreover, if a chemical reagent is used to block hepatocyte replication after partial hepatectomy, hepatic progenitor cells start to differentiate into hepatocytes and proliferate to regenerate the liver. Furthermore, in many kinds of liver injury, there is a transdifferentiation of HSCs to myofibroblasts, which is also considered as a wound healing process in the liver. In addition, previous studies of BMPs function during liver injury mainly focused on BMP-4 and BMP-9. BMP-9 was shown to cause a modest increase in proliferation of primary cultured rat hepatocytes (Miller, Harvey et al. 2000); and BMP-4 was found to mediate bile duct ligation induced liver fibrosis through the activation of Smad1 in liver cells (Fan, Shen et al. 2006).

It is generally accepted that HSCs play crucial role in liver fibrosis process (Friedman 1996). Similar to as tendon fibroblasts, HSCs are the major source of type I

collagen and extracellular matrix production. Upon liver injury, HSCs undergo activation with phenotype changes. Like wound healing processes, the characteristics of liver injury are described as transdifferentiation and proliferation of HSCs. Interestingly, BMPs signaling molecule-Smad 1-expression was found to be increased during HSCs activation (Shen, Huang et al. 2003). Hcy is a metabolic product of essential amino acid - methionine. It has been documented that elevated Hcy may be involved in the development of liver disease. Some studies about Hcy induced hepatic fibrosis through activation of HSCs (Zou, Gao et al. 2009). Therefore, we can hypothesize that BMPs especially BMP-13, play critical roles in Hcy induced liver injury.

2. Hypothesis

An increase in Hcy in the liver can induce liver injury, which may be mediated by BMPs; therefore, BMPs play an important role in the liver healing process.

3. Objectives

(1) To examine BMPs expression in the liver.

(2) To examine BMPs expression in HSCs (CFSC-8B cell line).

(3) To investigate BMPs expression in the liver of HHcy.

Materials and Methods

1. Materials

Table 6 Materials

Product	Company
100×20mm Tissue Culture Dish	SARSTEDT
25cm^2 Cell Culture Flask	Corning
75cm^2 Cell Culture Flask	Corning
15ml Polypropylene Centrifuge Tubes	VWR
50ml Polypropylene Centrifuge Tubes	VWR
100bp DNA Ladder	Invitrogen
10µl Pipette Tips	Fisher Scientific
200µl Pipette Tips	Fisher Scientific
1000µl Pipette Tips	Fisher Scientific
10ml Pipette	Fisher Scientific
96-well Tissue Culture Plate	SARSTEDT
2-Mercaptoethanol	Aldrich
7-AAD viability Dye	Beckman Coulter
Acrylamide	Fisher Scientific
Alcohol	Fisher Scientific
ALT assay kit	Diagnostic Chemical Ltd
Ammonium Persulfate	Bio-Rad
Balance PJ400	Fisher Scientific
BCA Protein Assay Reagent	PIERCE

Blotting Tank	Bio-Rad
Chloroform	Sigma
DL-Homocysteine	Sigma
DMEM	GIBCO
Dry Bath Incubator	Fisher Scientific
ECL Plus Western Blotting Detection System	GE Health Care
Essential Amino Acids	GIBCO/BRL
Formamide	Sigma
GP Centrifuge	Beckman
Horizontal Gel Electrophoresis Apparatus	GIBCO/BRL
Human CD133 antibodies	Miltenyi Biotec
IMx Kit	Abbott
IQ™ SYBR® Green Supermix	Bio-Rad
iScript™ cDNA Synthesis Kit	Bio-Rad
Isopropyl Alcohol	Sigma
Kodak Biomax XAR Film	GE Health Care
L-Glutamine	Invitrogen
MEM-EBSS	HyClone
Methanol	Fisher Scientific
Mini Centrifuge for PCR tubes	ManSci
Nitrocellular membranes	Bio-Rad
PCR Thermowell Tubes	SARSTEDT
Penicillin	Invitrogen

pH meter	Fisher Scientific
Power Pac.	Bio-Rad
Protein Standard	Bio-Rad
Refrigerated Microcentrifuge	Savant
Skim Milk Power	Carnation
Slow Speed Roto Mix	Thermolyne
Stirring & Hotplate	Fisher Scientific
TEMED	GIBCO/BRL
Thermix Stirrer	Fisher Scientific
Trypsin 0.05%	Hyclone
Tris-Base	Fisher Scientific
Tween-20	Sigma
UltraPure™ Agarose	Invitrogen

2. Methods

2.1 Animal model of hyperhomocysteinemia induced liver injury

To create rat models with HHcy, male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) aged 8 weeks were randomly divided into three groups and were sacrificed after 1 week, 4 weeks and 12 weeks respectively. Each group was further divided into two subgroups and fed on the following diets: (1) control diet (regular diet) consisting of Lab Diet Rodent Diet 5001 (PM1 Nutrition International, St, Louis, MO, USA) which contains 0.43% (wt/wt, 4.3g/kg) methionine, 0.00059% (wt/wt, 5.9 mg/kg) folic acid and 0.0006% (wt/wt, 6 mg/kg) vitamin B6; (2) high-methionine diet consisting of regular diet plus 1.7% (wt/wt) methionine (Wang, Woo et al. 2002; Au-Yeung, Woo et al. 2004; Mahfouz and Kummerow 2004; Woo, Siow et al. 2005). At the end of the experiment, serum samples were collected for Hcy and ALT assays. Liver samples were collected and immediately stored at -80℃ for subsequent determination. All procedures were performed in accordance with the "Guide to the Care and Use of Experimental Animals" published by Canadian Council on Animal Care and were approved by the University of Manitoba Protocol Management and Review Committee.

Hcy concentrations in the serum and liver were measured by the Abbott total Hcy IMx system (Abbott Diagnostics Division, Abbott Park, IL, USA) (Wang, Woo et al. 2002; Au-Yeung, Woo et al. 2004; Woo, Siow et al. 2005), which is a fully automated fluorescence polarization immunoassay method based on highly selective enzymatic conversion of Hcy to S-adenosyl-L-homocystene (SAH). SAH was subsequently recognized by a specific monoclonal antibody (Shipchandler and Moore 1995). Samples were free of pre-treatment, all reagents were supplied by Abbott Diagnostics in liquid

form ready to be used and calculations were performed by the IMx system using a machine-stored calibration curve.

Liver injury status was examined by measuring the activity of alanine aminotransferase (ALT) in serum using ALT assay kits (Diagnostic Chemicals Ltd, Charlottetown, PE) followed by the manufacturer's manual. ALT is an enzyme that catalyzes transfer of an amino group from alanine to α-ketoglutarate resulting in synthesis of pyruvate and glutamate. ALT is mostly found in the cytoplasm of hepatocytes. Normally, ALT activity in the liver is over 3000 times that of serum activity (Sohocki, Sullivan et al. 1997). In response to hepatocellular injury or necrosis, ALT was released from damaged cells to the serum. Thus, measurement of ALT levels in the serum was commonly used to diagnose liver injury in clinical practice.

The pyruvate could be detected due to its ability to convert the colorless probe to color that can be detected at a wavelength of 570 nm. The pyruvate standard was first measured by colorimetric assay. Tissue samples were then prepared in an ice-cold ALT assay buffer. After removing insoluble materials, samples were set in 96-well plates for reading. Appropriate sample concentrations were pre-tested to make sure all readings were within the standard curve range. Total volumes of 100 µl samples (Table 7) were mixed well and read at wavelength 570 nm twice with a time interval of 60 min. The OD value generated by oxidation of pyruvate was considered as a difference. ALT activity was then calculated according to the following formulation:

ALT activity (nmol/min/ml = U/L) = B nmol/ 60 (min) × V (L)

(B: pyruvate amount read from pyruvate standard curve; V: original sample volume added in each reaction well). One unit of ALT activity was defined as the amount of ALT

that generates 1.0 μmol of pyruvate per minute at 37 ℃.

Table 7 Content of ALT reaction mix

Content	Volume (μl)
ALT assay buffer	86
OxiRed probe	2
ALT enzyme mix	2
ALT substrate	10

2.2 Tissue section and Hematoxylin and Eosin staining

2.2.1 Tissue section

Liver tissues from control and high methionine groups were employed for histological examination by Hematoxylin and Eosin (H&E) staining. Fresh tissues were embedded in a clear frozen section compound (VWR, West Chester, PA) in cryomolds. Frozen blocks were stored at -80 ℃ for 5 min. Tissues were cut to 20 μm thick cryostat sections at working temperature -14 ℃, and then the sections were mounted onto superfrost slides (Fisherbrand, US). The slides were then stored at -20 ℃ for further staining.

2.2.2 Hematoxylin and Eosin staining

Slides were placed in a slide holder and fixed in 95% ethanol for 1 min. Slides were then dipped in water 10 times to rinse off fixatives. A series of stainings were performed by first immersing in Hematoxalin (Poly Scientific, Bayshore, NY) once for 3 min, rinsing with deionized water and washing in tap water for 5 min; and then second dipping

into acid ethanol (1ml concentrated HCL plus 400 ml 70% ethanol) quickly for 10 times to destain, rinsing in tap water twice for 1 min each time and in deionized water once for 2 min. Samples were then stained with Eosin (Poly Scientific, Bayshore, NY) once for 30 sec, washed 3 times with sequence of 95% ethanol, 100% ethanol and xylene for 5 min once (StatLab, Lewisville, TX) respectively. Slides were then covered with a drop of xylene based Permount (Fisherbrand, US) and coverslips, while avoiding bubbles, and were finally dried overnight in the hood before microscope observation.

2.3 Cell line and cell culture condition

All cell culture procedures were performed at complete sterile conditions with the laminar flow to avoid any possible contamination. All apparatus were pre-autoclaved before use.

2.3.1 Rat hepatic stellate cell line (CFSC-8B)

CFSC-8B cells are one of the cell lines of cirrhotic fat-storing cells (CFSC) that were originally isolated from CCl_4-induced cirrhotic liver. CFSC-8B cells were used as an *in vitro* model for partially activated and differentiated HSCs due to their ability to produce collagen (Greenwel, Schwartz et al. 1991). CFSC-8B cells were kindly provided by Dr. Marcos Rojkind (George Washington University, Washington, DC).

2.3.2 Cell culture condition

Cells were cultured in a 75 cm^2 culture flask at 37℃ with HyClone MEM/EBSS supplemented with 10% FBS, 1% non-essential amino acids (Invitrogen Canada Inc., Burlington, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mmol/l L-glutamine under 5% CO_2 and 95% air humidified atmosphere incubator. The culture medium was changed every two days.

Cell passaging was made with PBS (Table 8) and trypsin (Thermo Fisher Scientific, Nepean, Ontario, Canada). Cells were rinsed twice with pre-warmed PBS (37℃, 5 ml per time for a 75 cm^2 flasks). After washing with PBS, 2 ml of 0.05% trypsin were added in the flask to detach the cells from the flask surface. Cells were then incubated for 3 min at 37℃ for optimal operation of trypsin. After 3 min, the full culture medium was immediately added into the flask to stop trypsin activity and prohibit damage to cells. Appropriate amounts of cells were then transferred and sub-cultured in 10 cm culture dishes for experiments. For every experiment, cells were incubated with serum free culture medium at least 12 hrs before the start of the experiment.

Table 8 Content of 10×PBS

Content	Concentration (g / L)
KCl	2
KH_2PO_4	2
NaCl	80
Na_2HPO_4	11.5

*Adjust pH to 7.4 with NaOH, autoclave and keep in room temperature

2.4 Microphotography

Cell morphology was observed every day. Cells were imaged and photographed on an Olympus inverted-phase microscope (CK-40) using a mounted Olympus 35-mm camera (Carsen Group, Markham, ON, CA) upon necessity.

2.5 RNA isolation

Total RNA was extracted from liver tissues and cells by TRIzol® reagent (Invitrogen Canada Inc, Burlington, Canada) according to manufacturer's manual. Samples were kept on ice during the whole procedure except for the incubation times indicated. Tissues (about 100 mg) were homogenized with 1 ml TRIzol® reagent. Homogenized samples were incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. After adding 0.2 ml chloroform (Sigma, Oakville, Ontario, Canada), tubes were shaken vigorously by hand for 15 sec, then incubated for 2 min at room temperature. Following centrifugation, the mixture was separated into a lower red (phenol-chloroform phase), a white interphase, and a clear upper aqueous phase. RNA remained in the aqueous phase. The upper phases were transferred into fresh Eppendorf tubes and RNA was precipitated with isopropyl alcohol (0.5 ml) (Sigma, Oakville, Ontario, Canada). After adding isopropyl alcohol, samples were let stand for 10 min at room temperature to allow completed reaction, then centrifuged at 12,000 × g for 10 min at 4℃. RNA pellets were washed with 1 ml 75% ice-cold ethanol then centrifuged at 7,500 × g for 5 min at 4℃. RNA pellets were then briefly air-dried before dissolving in RNase-free water, which prohibits RNase degrading RNA and provides a buffered condition for subsequent experiments. RNA pellets should not be dried completely as this may affect RNA solubility.

RNA concentration was assessed by UV spectrophotometer (Bio-Rad SmartSpec 3000 Spectrophotometer, Life Science Research Division, Mississauga, ON). Absorbance of each diluted RNA samples was measured at wavelengths 260 nm and 280 nm. Nucleic acid concentration was calculated using the Beer-Lambert law, $A=\varepsilon Cl$ (A=absorbance at

a particular wavelength, C=concentration of nucleic acid, l=path length of the spectrophotometer cuvette (typically 1cm), ε=the extinction coefficient for RNA). Using this equation, an A260 reading of 1.0 was equivalent to 40 μg/ml of single-stranded RNA. The samples' final concentrations were calculated as A260 × 40 × dilution factors. RNA would be expected to have a higher absorbance at 260 nm than 280 nm, and the ratio OD260/OD280 would indicate if and at what level the sample was contaminated with protein or phenol. A high purity of RNA was considered to have an A260/280 ratio greater than 1.82. RNA was then stored at -80 ℃ until further experimentations.

2.6 Northern blot

RNA quality was examined by the Northern Blot. 1.0 g agarose was dissolved in 72 ml H_2O and heated until boiled. Then the flask was cooled to around 60 ℃ in a fume hood, and then 10 ml of 10×3-(N-morpholino) propanesulfonic acid (MOPS) buffer, which is composed of 0.2 M MOPS sodium salt, 100 mM sodium acetate, 10 mM EDTA-2Na and pH 7.5, followed by 18 ml of 12.3 M formaldehyde were added into the flask to make up 100 ml. While pouring gel was put into a casting tray and allowed to set, RNA samples were loaded into wells and the gel was run with 1×MOPS running buffer. RNA samples were prepared before loading as follows: 5 μg RNA in total volume of 11 μl with 5 μl 10×MOPS, 9 μl formaldehyde (12.3 M) and 25 μl formamide were mixed and centrifuged briefly. Samples were than incubated at 55 ℃ for 15 min and loaded with 10 μl formaldehyde loading buffer (Table 9). The gel was run at 70 V until the bromphenol blue dye migrated one-half to two thirds of the length of gel. Bands were viewed and photographed with the gel imager (Alpha Innotech, San Leandro, US). The bands were then analyzed by ImageJ program. For eukaryotic RNA, there are two distinct ribosomal

bands corresponding to both 18S and 28S ribosome RNA on a denaturing agarose gel. 28S rRNA has 4802 bases, while 18S rRNA has 2000 bases. A good quality RNA sample was expected to have a ratio of 28S to 18S around 2.

Table 9 Content of formaldehyde loading buffer

Content	Volume (μl)
Formaldehyde	100
Formamide	300
10×MOPS	60
Loading buffer	130
Ethidium bromide	2.25

2.7 Reverse transcription polymerase chain reaction (RT-PCR)

The first strand of cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Briefly, a 10 μg RNA template was dissolved in nuclease-free water to achieve a volume of 15 μl. Subsequently, a 1 μl iScript reverse transcriptase and 4 μl 5× iScript reaction mix were added to get a final reaction volume of 20 μl. The reaction mix was then incubated at 25℃ for 5 min, 42℃ for 30 min and 85℃ for 5 min, and then at 4℃ until PCR amplification.

PCR reaction was performed using iScript^{TM} One-Step RT-PCR kit (Bio-Rad, Hercules, CA) and the primers of 14 different BMPs respectively. In order to ensure tissues and cells were loaded properly, expression of glyceraldehyde-3 phosphate dehydrogenase (GAPDH), a housekeeping gene found in all tissues at high level, was used to analyze quality and loading of RNA samples before detection of each BMP.

Primers were designed using Oligo 7 software according to mRNA sequences published at Genbank and synthesized by Invitrogen (Invitrogen, Burlington, ON) (Table 10). PCR was performed with iQ™ SYBR® Green Supermix. Briefly, 1 μl of reverse transcriptase reaction solution was added into each tube and a mixture of RNase-free water, primers (500 nM for each primer) and SYBR Green Supermix was prepared. 24 μl of the mixture solution was added into PCR reaction tubes to make up the final reaction volume of 25 μl (Table 11). After gently mixing the contents, tubes were placed onto a PCR thermal cycler (Bio-Rad S1000TM). The PCR cycle was set as following: initial denaturation at 95℃ for 2 min, followed by 35 cycles of each with denaturation at 95℃ for 30 sec, annealing for 30 sec (according to different primers annealing temperature listed in Table 10), extension for 40 sec at 72℃, a final extension at 72℃ for 5 min and then stored at 4℃.

Table 10: List of primers and PCR conditions

Name	Primer Sequences	Product (bp)	Tm (℃)
BMP-2	Sense: 5' CCCCTATATGCTCGACCTG 3' Antisense: 5' CCTGCATTTGTTCCCGAAA 3'	248	57.3
BMP-3	Sense: 5' CTTCAAAGAAAGGGCTTGCACAC 3' Antisense: 5' GTCTTGCTTGGCCTTCCGTA 3'	309	56.3
BMP-4	Sense: 5' CCGGGAAAAGCAACCCAAC 3' Antisense: 5' GCCCACGTCACTGAAGTCCA 3'	286	59.6
BMP-5	Sense: 5' AGGCATTACAAAGAATTTCGG 3' Antisense: 5' GGCAGATTTTACATTGATGCT 3'	336	53.4
BMP-6	Sense: 5' GTCTCCAGCAGCCTCAATCCC 3' Antisense: 5' ACACCCCATCCTCTTCGTCGTC 3'	156	60.8
BMP-7	Sense: 5' AGGCCGTCTTCAGTACCCAG 3' Antisense: 5' CGCTCCCGGATGTAGTCCTT 3'	229	59.1
BMP-8	Sense: 5' GCCCTCTCCATCCTCCTACCC 3' Antisense: 5' AATGACATGCACCGCTATACCTG 3'	337	57.1
BMP-9	Sense: 5' CCTGCCCTTCTTTGTCGTC 3' Antisense: 5' GCAGCCTCCTTTACACTCGT 3'	337	58.5
BMP-10	Sense: 5' ATGCCATCTGCTAACATCATCCG 3' Antisense: 5' GCTCCTCTCATCCTCGCTAC 3'	252	56.3
BMP-11	Sense: 5' GCACCCCTACCAAGATGTCCCC 3' Antisense: 5' GGAGCAGCCACATCGATCCACC 3'	107	57.4
BMP-12	Sense: 5' CCGCCGAGCCCCTAGACAG 3' Antisense: 5' CCGAGATCCAGCGCCTAGTCC 3'	338	64.7
BMP-13	Sense: 5' ACCGCCCTCAGCAGCCGTCA 3' Antisense: 5' GGCCTTCCTCCGTGGTCCC 3'	407	61.8
BMP-14	Sense: 5' CACGCAGTCATTCAGACCCTA 3' Antisense: 5' GCAGCCACAAGATTCCACGA 3'	165	55.9
BMP-15	Sense: 5' CCCCGTCTCTATACCCCAA 3' Antisense: 5' TGCGATTCACTAGTTCATTGACA 3'	118	53.2
GAPDH	Sense: 5' CAAAGTGGACATTGTGCCAT 3' Antisense: 5' ATACTCAGCACCAGCATCACC 3'	205	56.2
α-SMA	Sense: 5' CACGGCATCATCACCAAC 3' Antisense: 5' GGAAGAAGAGGAAGCAGCAG 3'	370	59.0

Table 11 Components for PCR cycle

Component	Volume (μl)
cDNA template	1.5
iQ™ SYBR® Green Supermix	12.5
RNase-free water	10.0
Forward primer	0.5
Reverse primer	0.5

2.8 DNA gel electrophoresis

PCR products were analyzed by electrophoresis on 1.5% agarose gels. 10 μl of PCR samples were mixed with 1 μl 10×DNA loading buffer (20% Ficoll 400, 0.1 M EDTA-Na_2, 1.0% SDS, 0.25% bromphenol blue and 0.25% xylene cyanol), and then loaded into each well. The gel was run with 1×TAE (Tris-base 2.0 M, glacial acetic acid 1M, EDTA-Na_2 50 mM, adjusted pH to 7.2) for 1 h with a voltage of 100 V at room temperature. Gels were stained with ethidium bromide for 30 mins at room temperature and washed twice (30 min per time) with water to get rid of unexpected staining. Bands were viewed and analyzed by the ImageJ program.

2.9 Protein isolation

2.9.1 Extraction of cellular protein from cells

Cellular protein was isolated from cells with protein extraction buffer, with components of protein extraction buffer listed in Table 12. Cells were cultured in 10 cm culture dish and washed with ice cold PBS twice. After washing, the cells were lysized by the addition of 500 μl ice cold protein extraction buffer with protease inhibitors

(Roche, Laval, CA), which protect proteins against a broad range of proteases. Cells were removed from the culture dish with a scraper and collected into a pre-chilled microcentrifuge tube with pipettes. Cells were then sonicated twice (Branson sonifier 150, Heinemann, Germany) for 10 sec with the tube sitting on ice between each sonication. Tubes were then centrifuged at 12,000 × g for 20 min at 4℃. After centrifugation, supernatant was transferred into a clean pre-chilled tube and kept at -80℃ until analysis.

Cellular protein concentrations were measured by bicinchoninic acid (BCA) assay kits (PIERCE, Rockford, IL) which contain protein standard solution. The principle of protein determination is that Cu^{+} can be reduced from Cu^{2+} catalyzed by protein in an alkaline solution, which can be sensitively and selectively chelated by two molecules of BCA with one Cu^{+} and detected by purple color. This water-soluble BCA and Cu^{+} complex has a broad linear absorbance at 562 nm as protein concentration increases (Smith, Krohn et al. 1985). Throughout the whole procedure, the manufacturer's manual was followed. Appropriate amounts of cellular proteins were subjected to western blot analyses.

Table 12 Component for protein extraction buffer

Content	Volume (ml)	Final Conc.
1M Tris-HCl pH 7.4	0.25	10mM
0.5M EDTA pH 8.0	0.05	1mM
1M NaCl	0.25	10mM
10%SDS	2.5	1%
ddH_2O	21.95	

2.9.2 Extraction of cellular protein from liver tissues

Frozen liver tissues were thawed on ice. Protein extraction buffer was added into each tube containing liver tissue and homogenized with a tissue homogenizer (Kinematica GmbH CH 6005 Luzern, Switzerland). Protein extraction buffer consists of 1 M NaCl, 1 M Tris-HCl, 0.5 M EDTA, 10% SDS and 2 tablets of protease inhibitor cocktail (Roche, Laval, CA) in 10 ml solution. After homogenization, tissues were placed on ice to avoid protein denaturation and degradation. Samples were then centrifuged for 30 min at 15,000 × rpm at 4℃. Protein concentration was measured by BCA protein assay kits as described above.

2.10 Western blot analysis

Sixty micrograms of total protein was employed to detect target BMP by Western blot analysis. 10 ml of each stacking and separating gels were prepared by the following sequence: added in certain amount of ddH_2O, pre-warmed 30% acrylamide mixture, and 10% SDS in a 50 ml flask; mixed the ingredients by holding the neck and swirled it 8 to 10 cycles; added in TEMED and swirled the solution gently but thoroughly; finally added

10% ammonium persulfate (AP) and initiated the gel formation for 45 min. Sodium dodecyl sulfate (SDS) has the ability of dissolve nearly all proteins in the presence of anions. SDS bound to proteins with high affinity which conferred a negative charge to polypeptides and relaxed higher order protein structures. Proteins' tertiary disruption was an essential determinant to protein separation according to molecular weight. Smaller proteins migrate faster through this mesh than bigger ones. The gradient of pore size or gel percentage chosen was based on molecular weight of target proteins (Thermo scientific application protocol) (Table 13).

Table 13 Choice of gel percentage

Protein molecular weight (MW) range (kDa)	Gel percentage (%)
5-50	18
5-60	16
10-80	14
20-150	12
30-200	10
40-250	8
60-300	6
100-400	4

Proteins with 4×gel loading buffer (240 mmol/L Tris-HCl (pH 6.8), 0.8% SDS, 20% glycerol, 0.2 bromphenol blue and 5% β-mercaptoehanol) were boiled for 5 min exactly to denature and unfold proteins. In order to make proteins detectable by

antibodies, they were transferred from the gel to a Nitroplus-2000 membrane (Micron Separations Inc., Westborough, MA) in a setting of sandwich cassette. In order to keep temperature down, the transfer cassette was supplied with plenty of ice and a stir bar. A blocking membrane for 1 h at room temperature with 5% skim milk was required to reduce nonspecific antibody binding, false positive bands and clearer positive bands. 5% skim milk was prepared in fresh TBS-T solution (Tris-buffered-saline-Tween, 2.41 g Tris base, 29.25 g NaCl and 1 ml Tween 20 per liter). After blocking with skim milk, membranes were incubated overnight with 5% skim milk containing primary antibodies at 4 ℃. After briefly washing membranes to remove unbound primary antibodies (Table 14) in TBS-T for 30 min, membranes were incubated with secondary antibodies (donkey anti-rabbit IgG or sheep anti-mouse IgG) at a dilution ratio of 1:1000 and strep Tactin-HRP (Ladder antibody, Bio-Rad) in TBS-T and 2% skim milk at room temperature for 1 h with gentle shaking. After incubation with horseradish peroxidase-linked secondary antibody, a luminol substrate was incubated with the membrane. This can be converted to a light emission substance in proportion to the amount of protein. Then, this protein-antibody complex was detected by an enhanced chemiluminescence kit (Amersham ECL Plus substrate, GE health care, Quebec, CA) according to manufacturer's instruction. Immune-reactive bands were visualized by exposing to Kodak BioXAX film for certain times according to intensity of reaction. Band densities were measured by ImageJ program.

Table 14 Antibody and condition of Western blot

Primary antibodies	Type	Host	Total protein used (μg)	Dilution ratio	Company
GDF-6 (BMP-13)	Polyclonal	Rabbit	60	1:1000	Abcam (San Francisco, US)
β-actin	Monoclonal	Mouse	60	1:1000	Sigma-Aldrich (Okaville, ON)

2.11 Cell proliferation assay

Cell proliferation can be considered as a cell division in the culture medium. There are several methods that can be used to analyze the number of viable cells, such as direct cell counting; however this method can easily bring in personal error. Another way to determine viable cells is to measure DNA synthesis by incorporating [^{3}H]-thymidine into nucleic acid and then determining radioactivity. The third method to determine viable cells is to measure enzymatic activity that cleaves tetrazolium salts. The principle of this method is that mitochondrial dehydrogenases activity remains consistent during cell division, which can cleave stable tetrazolium salt such as WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) to a soluble formazan. Therefore, the amount of formazan dye directly correlates to the number of metabolically active cells in the culture. WST-1 method does not require either washing or harvesting of cells; assay can be performed in the same microplate.

To reveal the role of Hcy on HSCs, 10^5 CFSC-8B cells were seeded into a 96-well

tissue culture plate. Cells were cultured with FBS free culture medium 12 h before treatment with indicated concentrations of Hcy for 24 h. At the end of experiment, 100 µl of culture medium and 10 µl of cell proliferation reagent WST-1 were added into each well. Optical absorbance was measured at 1.5 hrs after incubation of WST-1 reagent at 37℃. The plate was thoroughly shaken for 1 min on a shaker. Absorbance of samples against background control as blank was measured using a microplate (ELISA) reader at absorbance wavelength at 450 nm and reference wavelength at 600 nm.

2.12 Statistical analyses

Each experiment was replicated at least 4 times within and between subjects. Statistical analyses between control and treatment groups were carried out by t-test, Dunnett's test and one way ANOVA repectively using GraphPad Prism 5 software. Gel images were all normalized to their specific loading controls respectively before graph drawing. Differences with P values < 0.05 were considered to be statistically significant.

Results

1. Animal Models with Hyperhomocysteinemia

Below are preliminary results of Nan Wu (Dr. Karmin O's lab, University of Manitoba), data published in *American Journal of Physiology* (Wu, Yao et al. 2009).

1.1 Homocysteine concentration in serum and liver

In this experiment, Hcy concentration in the serum and liver were first measured in rats with either regular diets or high methionine diets for 1 and 4 weeks of different rats, since Hcy cannot be directly taken from food. As the liver is the major organ for methionine metabolism, excess intake of methionine could induce accumulation of Hcy in the liver and this is the model of HHcy in an animal. Rats with high methionine diet after 1 week and 4 weeks showed a significant increase in concentration of Hcy in both serum and the liver (Values are mean ± SEM, n=4, $P<0.05$).

Table 15 Hcy concentration in serum and liver

			1 week	4 weeks
Mean concentration of Hcy	Serum (μmol/L)	Control	5.1	5.0
		High methionine	32.1	32.5
	Liver (nmol/g)	Control	6.2	3.5
		High methionine	17.1	11.5

1.2 Activity of alanine aminotransferase in serum

Activity of ALT in rat serum was a marker of liver injury based on pyruvate level, so liver injury was indicated by determination of ALT level in the serum. After 1 week, serum ALT level in rats with high methionine diet was significantly higher than that of rats with regular diet (22 v 31(U/L), mean ± SEM, n=4, $p<0.05$). It is rationally expected to have the same result in the 4 week and 12 weeks groups.

2. Histological changes

This experiment was designed for investigating the histological changes after treatment with high methionine compared to those treated with the control. Rat liver sections from each 1week, 4 weeks and 12 weeks' time points were stained with H&E method respectively. Slides were then photographed with Olympus inverted-phase microscope using TMAX 400 Kodak camera and observed at 200 times magnification. Although there was no obvious change after the 1 week treatment with high methionine, there were significant histological changes after treatment with high methionine for 4 and 12 weeks, as shown in figure 6.

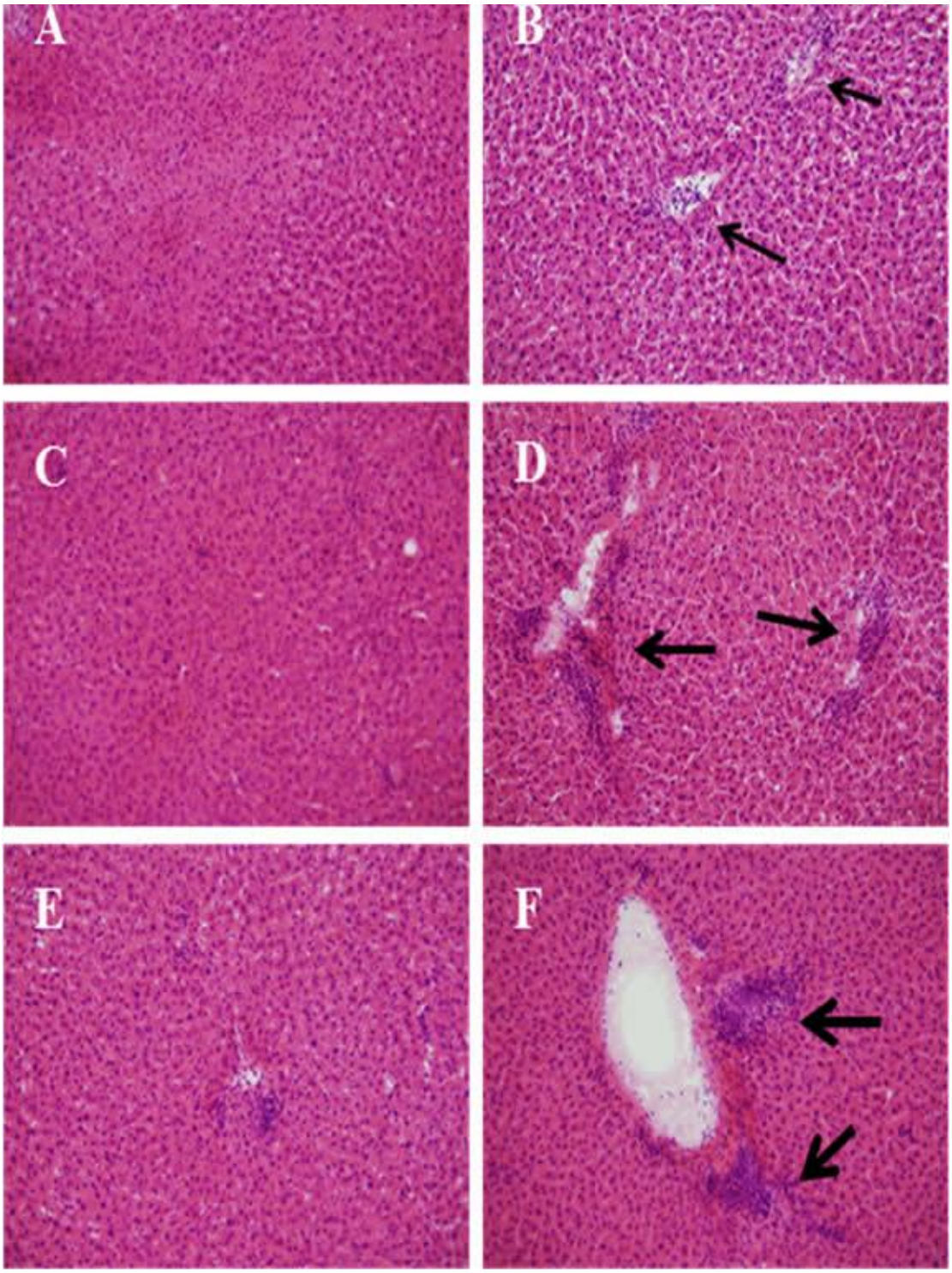

A
B
C
D
E
F

Figure 6 H&E staining of rat liver tissue. Liver samples from control and high methionine diet groups were stained with H&E method. No or nearly no obvious liver damage associated with inflammatory response was observed in livers of control groups at 1 week (A), 4 weeks' (C) and 12 weeks' (E) time point. Liver sections of 1 week high methionine group showed slight inflammatory response as shown in (B) with arrows. Inflammatory responses (right arrow) were obvious and necrotic lesions (left arrow) were shown within parenchyma of the livers after 4 weeks high methionine diet group (D). Critical inflammatory responses and more necrotic lesions existed in liver sections of 12 weeks' high methionine diet group (F), and these were widely distributed.

3. Bone Morphogenetic Proteins Expression in Rat Liver

Gene expression is a process by which the genetic code stored in DNA is interpreted in synthesis of a functional gene product, protein and/or RNA. Through gene expression a genotype gives rise to a phenotype. Gene expression can be modulated in several steps, including transcription (RNAs), RNA splicing, translation (proteins), and post-translational modification of a protein. Gene regulation will result in cell structural and functional control of cellular differentiation, morphogenesis, and so on. Regulation of gene expression may induce gene function in a cell or in a multicellular organism.

This experiment was designed to identify which BMP genes were differentially expressed in the liver of rats treated with high concentration of methionine.

3.1 Total RNA amount and quality

Total RNAs from the regular diet group and high methionine diet group were isolated and evaluated by a UV spectrophotometer (Table 16). About 150 μg to 300 μg of high quality RNA were extracted from each 100 g liver tissue sample respectively. A260/A280 ratio of these RNAs was all above 1.82, which indicated that high quality RNA was isolated and there was no contamination with protein and phenol.

Table 16 Results of UV spectrophotometer reading of rat liver tissue total RNA at wavelength 260 nm and 280 nm.

Sample	Conversion factor	Dilution factor	Concentration (μg/ml)	A260/A280
C1-1	40	100	3815.912	2.25
C1-2	40	100	5842.22	2.19
C1-3	40	100	5510.32	2.20
C1-4	40	100	6957.34	2.23
M1-1	40	100	6140.76	2.17
M1-2	40	100	4091.96	2.06
M1-3	40	100	5887.60	2.12
M1-4	40	100	5150.76	2.11
C4-1	40	100	6505.25	2.13
C4-2	40	100	5553.34	2.12
C4-3	40	100	6369.82	2.13
C4-4	40	100	7856.26	1.96
M4-1	40	100	7415.02	2.00
M4-2	40	100	4948.54	2.07
M4-3	40	100	5823.25	2.07
M4-4	40	100	7663.04	2.01
C12-1	40	100	7408.40	2.36
C12-2	40	100	5432.21	2.23
C12-3	40	100	7467.62	2.23
C12-4	40	100	7559.06	2.21
M12-1	40	100	6565.31	2.32
M12-2	40	100	7350.49	2.51
M12-3	40	100	7350.50	2.27
M12-4	40	100	7528.22	2.30

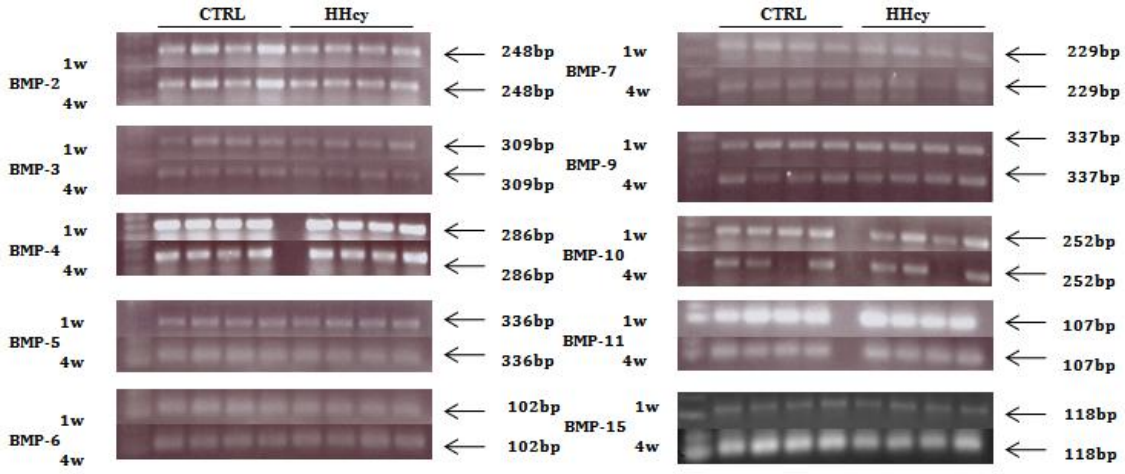
3.2 Bone morphogenetic proteins gene expression in rat liver

RT-PCR was employed to confirm BMPs' gene expression and to examine gene response after Hcy stimulation *in vivo.* Fourteen BMPs and GAPDH mRNA expressions were investigated in the liver. Results from this section revealed that BMP-2, 3, 4, 5, 6, 7, 9, 10, 11, 13 and 15's gene was expressed in rat livers (Table 17 and Figure 7). As shown in Figure 7 section A, abundance of BMPs mRNA was not changed compare to control, except BMP-13. The remarkable finding in this study is that BMP-13 mRNA level was significantly reduced after 4 weeks of feeding with a high methionine diet.

Table 17 BMPs expression on liver tissue

BMP expression positive	BMP expression negative
BMP2	BMP8
BMP3	BMP12
BMP4	BMP14
BMP5	
BMP6	
BMP7	
BMP9	
BMP10	
BMP11	
BMP13	
BMP15	

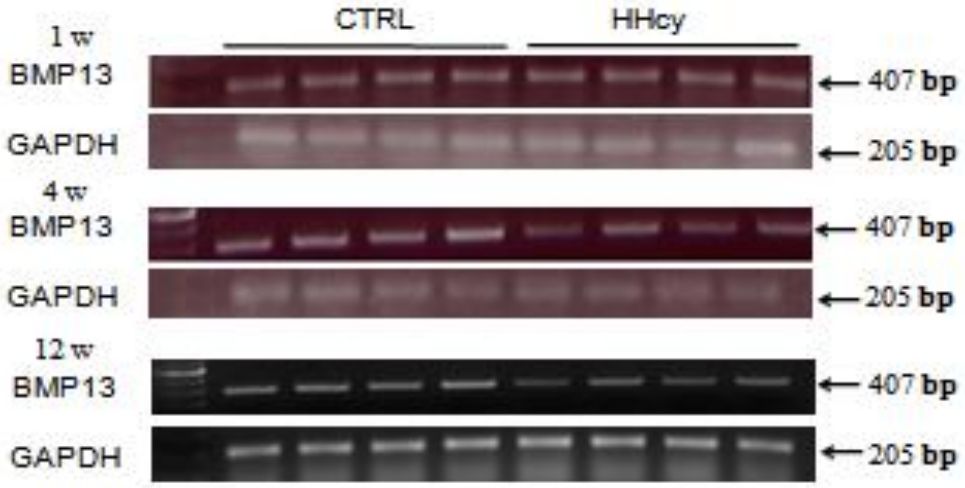
A


CTRL
HHcy
BMP-2
1w
4w
248bp
248bp
BMP-3
309bp
309bp
BMP-4
286bp
286bp
BMP-5
336bp
336bp
BMP-6
102bp
102bp
BMP-7
229bp
229bp
BMP-9
337bp
337bp
BMP-10
252bp
252bp
BMP-11
107bp
107bp
BMP-15
118bp
118bp

B


CTRL
HHcy
BMP-8
1w
4w
337bp
337bp
BMP-12
338bp
338bp
BMP-14
165bp
165bp
GAPDH
205bp
205bp

C


CTRL
HHcy
1 w
BMP13
GAPDH
4 w
BMP13
GAPDH
12 w
BMP13
GAPDH
407 bp
205 bp
407 bp
205 bp
407 bp
205 bp

D

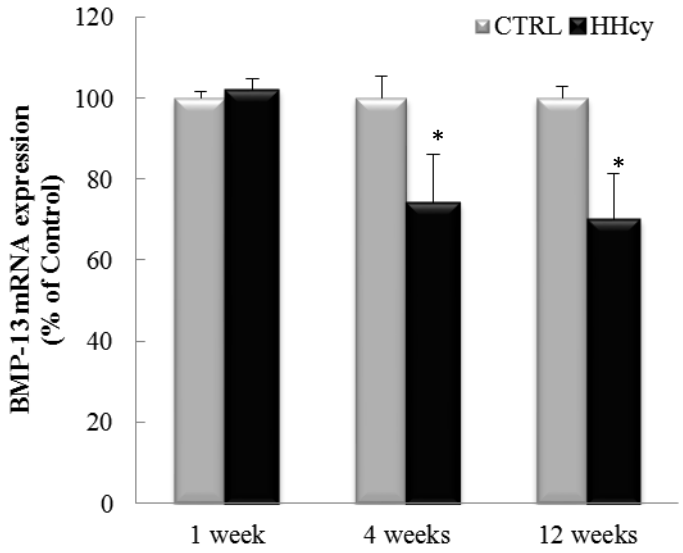

CTRL
HHcy
BMP-13 mRNA expression (% of Control)
120
100
80
60
40
20
0
*
*
1 week
4 weeks
12 weeks

Figure 7 RT-PCR analyses of BMPs and GAPDH expression in rat liver. Panel A and B represent electrophoresis gel picture of BMPs mRNA expression in the liver. GAPDH was used as a loading control. Panel C represents the expression of BMP-13 in the liver of rats with regular diets and high methionine diets for 1, 4, 12 weeks respectively. GAPDH was used as the loading control. Panel D displays the RNA expression of BMP-13 versus GAPDH in the liver of rats after feeding for 1, 4, and 12 weeks. Data represent mean ± SEM from four independent rats. Abundance of BMP-13 mRNA was significantly decreased after 4 weeks of high methionine diet compared with the control group. * indicates $p<0.05$ between regular diet groups and high methionine diet groups.

3.3 Bone morphogenetic proteins protein expression in rat liver

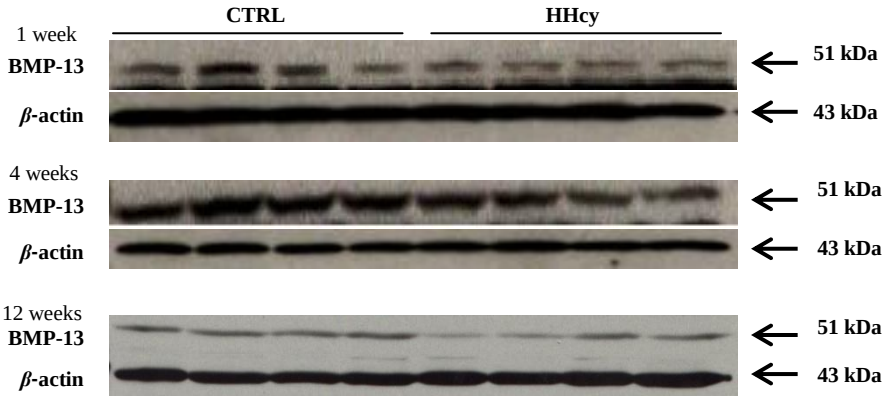
Protein expression is a subcomponent of gene expression, which is widely used in the measurement of the presence and abundance of one or more proteins in a particular cell or tissue. To confirm the reduction result of mRNA expression by RT-PCR, BMP-13 protein expression was evaluated by western blot analysis and β-actin was used as a loading control.

Protein concentrations were firstly evaluated from all liver samples respectively (Table 18). 60 μg proteins from each individual liver sample were used for Western blot analysis. Results showed in figure 10 indicated that BMP-13 protein expression was significantly reduced after 4 and 12 weeks exposure to the high methionine diet. However, there is no statistical difference in the 1 week group. This result is also consistent with the mRNA expression evaluation.

Table 18 Sample component for Western blot

Sample	Protein Conc. (μg/μl)	60 μg/lane protein (μl)	4×Loading buffer (μl)	ddH_2O (μl)
C1-1	18.83	3.2	7	17.8
C1-2	13.44	4.5	7	16.5
C1-3	15.31	3.9	7	17.1
C1-4	9.73	6.2	7	14.8
M1-1	13.00	4.6	7	16.4
M1-2	14.06	4.3	7	16.7
M1-3	16.48	3.6	7	17.4
M1-4	19.05	3.2	7	17.8
C4-1	24.37	2.5	7	18.5
C4-2	20.68	2.9	7	18.1
C4-3	21.87	2.7	7	18.3
C4-4	22.77	2.6	7	18.4
M4-1	29.20	2.1	4	18.9
M4-2	24.13	2.5	4	18.5
M4-3	11.54	5.2	4	15.8
M4-4	13.72	4.4	4	16.6
C12-1	15.04	4.0	4	8.0
C12-2	23.46	2.6	4	9.4
C12-3	25.30	2.4	4	9.6
C12-4	22.33	2.7	4	9.3
M12-1	19.18	3.1	4	8.9
M12-2	21.28	2.8	4	9.2
M12-3	17.84	3.4	4	8.6
M12-4	17.84	3.4	4	8.6

A


CTRL
HHcy
1 week
BMP-13
β-actin
51 kDa
43 kDa
4 weeks
BMP-13
β-actin
51 kDa
43 kDa
12 weeks
BMP-13
β-actin
51 kDa
43 kDa

B

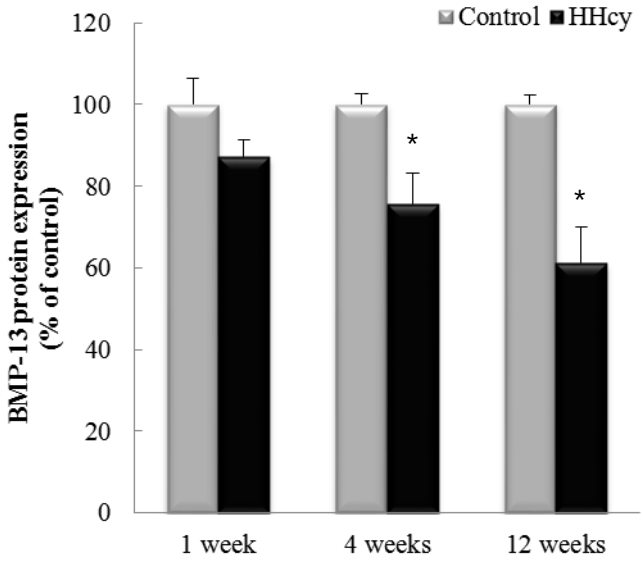

Control
HHcy
BMP-13 protein expression (% of control)
120
100
80
60
40
20
0
*
*
1 week
4 weeks
12 weeks

Figure 8 Western blot analysis of BMP-13 expression in rat liver. Total liver proteins were isolated from each group respectively. 60 μg of total protein from each sample was analyzed by Western blot as described in methodology section. *B-actin* was used as loading control. Panel A represents a typical BMP-13 and β-actin band which was shown on Kodak films from 1 week, 4 week and 12 weeks groups. Panel B displays the bar chart of density data from four independent samples (mean ± SEM), which is a ratio of BMP-13 to *β*-actin. As shown on figure, BMP-13 protein expression was significantly reduced after 4 and 12 weeks of the high methionine diet. *indicates $p<0.05$.

4. Effect of Homocysteine on Cell Proliferation

This experiment was designed to reveal the effect of Hcy on HSCs. About 10^5/well CFSC-8B cells were placed on 96-well plates and treated with 1.0, 2.0, 3.0, 4.0 and 5.0 mM Hcy in the presence of 5% FBS medium for 24 hrs. Control cells were treated with the same culture medium but free of Hcy. The data shown are means of triplicate experiments. Hcy caused a significant increase in absorbance at the wavelength 450 nm with WST-1 reagent at concentration over 2.0 mM of Hcy compared with control group ($p<0.05$). The effect was dose dependent as shown in figure 11. However, there was no obvious change of absorbance at a concentration of 1.0 mM Hcy.

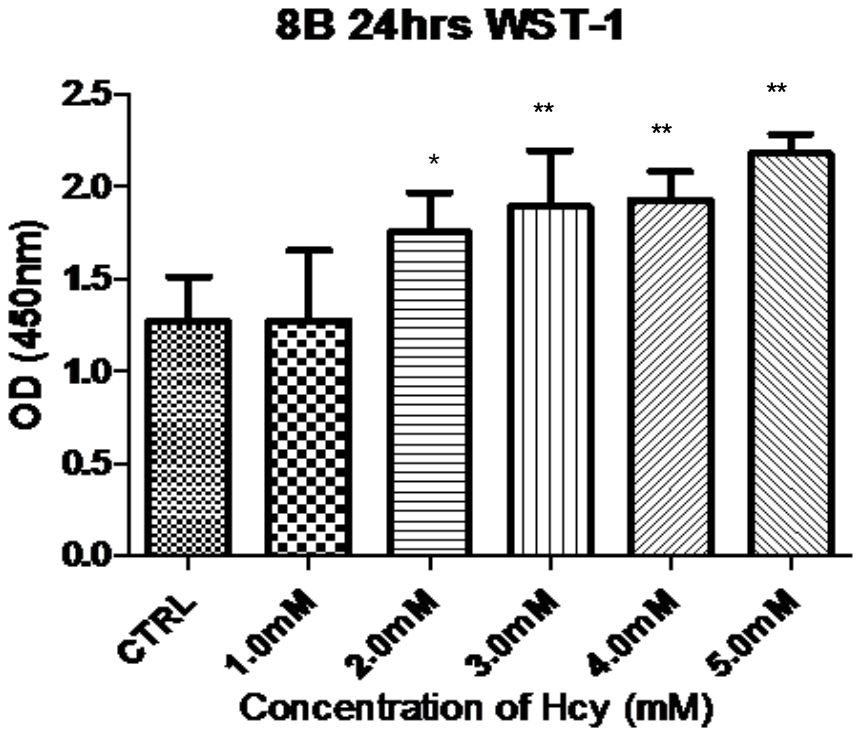


Figure 9 Effect of Hcy on the proliferation of CFSC-cells. CFSC-8B cells were treated with 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mM Hcy in culture medium for 24 hrs. Cell proliferation was determined by WST-1 reagent. Bar graph shows a dose dependent increase in cell proliferation after Hcy treatment. Values are mean ± SEM for 6 wells per dose point. * indicates significant differences (*p<0.05; **p<0.001) from control at Hcy concentration over 2.0 mM.

5. Bone Morphogenetic Proteins Expression in CFSC-8B Cells

This experiment was designed to identify BMP-13 mRNA and protein levels in CFSC-8B cells after treatment of Hcy. Each experiment was repeated at least three times.

5.1 Dose-response of CFSC-8B cells after treatment of homocysteine for 24 hours

CFSC-8B cells were sub-cultured in 10 cm dishes for the experiment and divided into control and Hcy treated groups. Cells were treated with 0.05, 0.1, 0.2 and 0.5 mM of Hcy for 24 hrs respectively. Total RNA and protein from control and Hcy-treated cells was isolated and evaluated for quality using spectrophotometer (Table 19). About 36 to 52 μg of total RNA were extracted respectively. As shown in Figure 10, Hcy did not induce significant change in the BMP-13 mRNA level. 40 μg proteins with loading buffer were loaded (Table 20) and analyzed by Western blot analysis. Results shown in Figure 11 indicate Hcy treatment did not induce significant change in the BMP-13 protein level compared to the control.

Table 19 Result of UV spectrophotometer read of RNA concentration from CFSC-8B cells

Sample	Conversion factor	Dilution factor	Concentration (μg/ml)	A260/A280
Control	40	150	895.19	2.13
Hcy 0.05mM	40	150	636.49	2.32
Hcy 0.1mM	40	150	1048.78	2.44
Hcy 0.2mM	40	150	1305.30	2.37

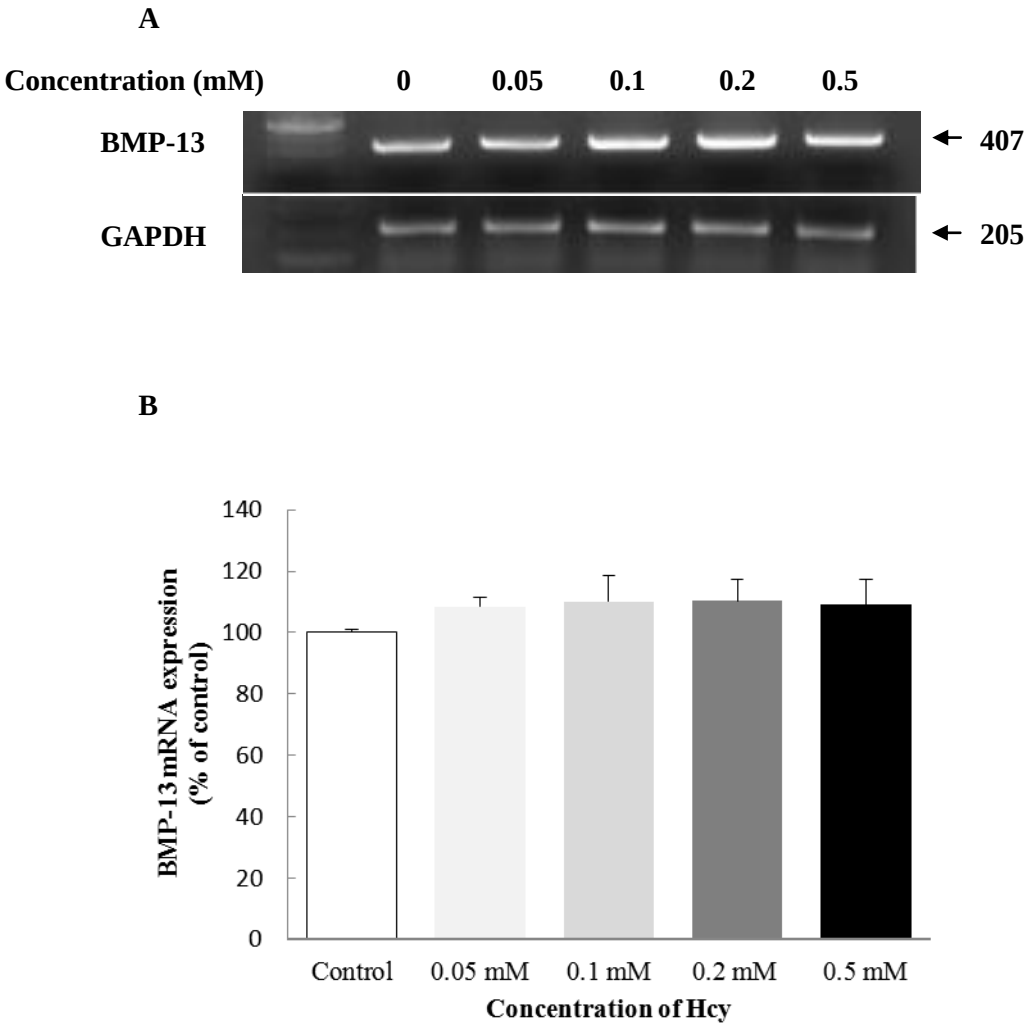


Figure 10 BMP-13 mRNA expression in CFSC-8B cells after treatment of Hcy. CFSC-8B cells were treated with indicated concentrations of Hcy or Hcy free medium for 24 hrs before total RNA was extracted. Panel A represents typical agarose gel pictures and visualized by UV. GAPDH was used as a loading control. Panel B represents the bar chart of density data of BMP-13 versus GAPDH from four independent experiments (mean ± SEM).

Table 20 Content of protein samples for Western blot analysis

Sample	Protein conc. (µg/µl)	40 µg/lane protein (µl)	4×Loading buffer (µl)	ddH_2O (µl)
Control	1.62	25	9	2
Hcy 0.125 mM	2.16	19	9	8
Hcy 0.25 mM	2.10	19	9	8
Hcy 0.5 mM	2.14	19	9	8
Hcy 1.0 mM	2.24	18	9	9
Hcy 2.0 mM	2.41	17	9	10

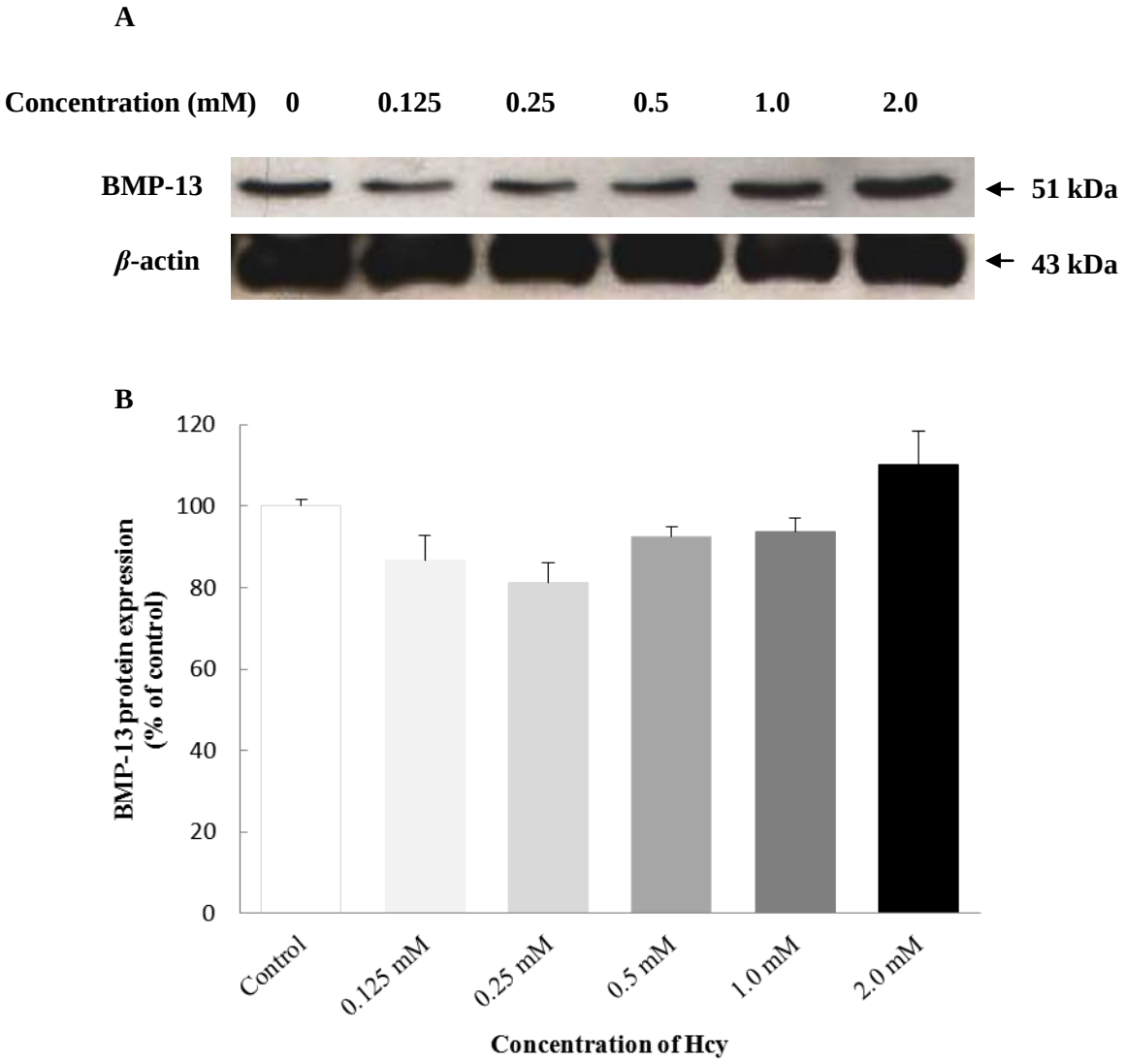


Figure 11 BMP-13 protein expressions in CFSC-8B cells upon treatment with Hcy. Cellular proteins were isolated from each group respectively. 40 μg BMP-13 protein expressions from each sample were analyzed by Western blot as described in the methodology section. *B-actin* was used as a loading control. Panel A represents typical BMP-13 and *β*-actin bands which were shown on Kodak films. Panel B displays the bar chart of density data of BMP-13 to *β*-actin from three independent experiments (mean ± SEM).

5.2 Time-course effect of homocysteine on CFSC-8B cells

CFSC-8B cells were sub-cultured in 10 cm dishes for the experiment. Cells were treated with 0.5 mM of Hcy for 3, 6, 12, 24 and 48 hrs. Total RNA was isolated at different time intervals respectively. RNA was then evaluated for quality using spectrophotometer (Table 21). About 37 to 72 μg of total RNA were extracted respectively. As shown by the data in Figure 12, there was an increasing trend in BMP-13 mRNA in the range of time intervals but there was no statistically significant difference.

Table 21 Result of UV spectrophotometer read of RNA concentration of CFSC-8B cells

Time of Hcy treated	Conversion factor	Dilution factor	Concentration (μg/ml)	A260/A280
3 hrs	40	150	915.40	2.12
6 hrs	40	150	1049.18	2.04
12 hrs	40	150	763.87	2.16
24 hrs	40	150	1237.21	2.20
48 hrs	40	150	1793.10	1.95

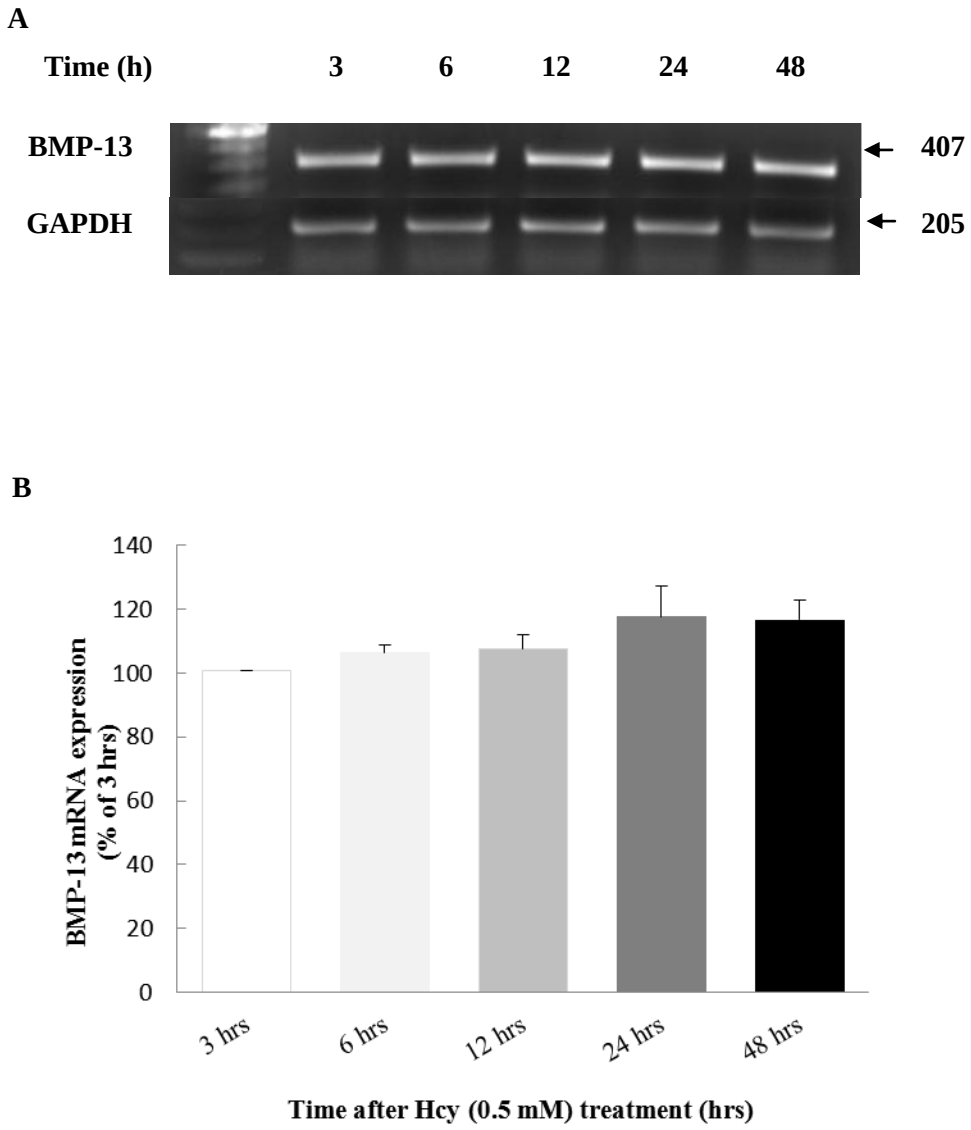


Figure 12 Time-course effect of 0.5 mM Hcy on CFSC-8B cells. Cells were treated with 0.5mM Hcy for 3, 6, 12, 24 and 48 hours respectively before total RNA was extracted. Panel A represents typical agarose gel pictures and visualized by UV. GAPDH was used as a loading control. Panel B represents the histogram of density data of BMP-13 to GAPDH from four independent experiments (mean ± SEM).

Discussion

Elevated levels of plasma Hcy (HHcy) are a critically harmful factor in the body, which have been linked to several health problems including in the liver. For example, patients with MTHFR C677T gene mutation develop hepatic steatosis and fibrosis (Adinolfi, Ingrosso et al. 2005). Moreover, in CBS-deficient mice, there was HHcy and liver fibrosis (Namekata, Enokido et al. 2004; Robert, Nehme et al. 2005).

1. Hyperhomocysteinemia Induced Phenotypic Change of Hepatic Stellate Cells

Although Hcy did not affect BMP-13 gene expression in HSCs, it did induce morphological changes of these cells. HSCs activation is one of the most important pathological modifications during hepatic fibrogenesis (Friedman 2000). Activation of HSCs is associated with change of their morphology from a quiescent phenotype to a myofibroblasts-like phenotype. In this study, we did observe that Hcy stimulated HSC proliferation with morphological changes from fusiform cells to round square cells (figure not shown). These findings may indicate that Hcy can induce HSCs activation, which contributes to the development of liver fibrosis in patients with HHcy. However, we also found that with different concentrations of Hcy, there were different morphological modifications in HSCs. At the higher concentrations of Hcy, there were more round but floating cells, which may indicate a toxic effect of Hcy on HSCs (figure not shown). Whether it is due to Hcy induced cell death or transdifferentiation induced apoptosis remains to be investigated.

2. *In vitro* Study of Liver Cells with Homocysteine

In this study, concentrations of Hcy needed to affect cell morphological changes and BMP-13 expression in CFSC-8B cells are much higher (1000-fold) than reported serum levels of patients with HHcy (Tessa Jupp, 2003). There are several reasons that can explain this 1000-fold concentration for cells, which is mainly because *in vitro* studies are distinct from physiologic conditions. Hcy easily degrades *in vitro*, but is constant for a long time *in vivo*. Wall et al reported rapid loss of Hcy on endothelial cells in vitro within 2 hrs (Wall, Harlan et al. 1980). Another reason is that Hcy is generated intracellularly in the liver and will be transsulfurated by CBS before being secreted to blood circulation (Van and Stehouwer. 2003). Furthermore, to increase two to six-fold intracellular Hcy levels require 1 to 5 mM exogenous Hcy (Werstuck, Lentz et al. 2001).

3. Animal Model

Ideal research models for HHcy are experimental animals including the mouse, rat, pig and woodchuck. Due to ethical consideration, human tissue is not applicable for current study. Animal models of liver fibrosis and HHcy are critical to study fibrogenesis and mechanism involved in HHcy induced liver disease because mammalian animals exhibit similar physiology and pathology to human livers, and diet-induced liver fibrosis in rats is one of the five typical animal models that were established and applied in experiments (Tsukamoto, Matsuoka et al. 1990; Constandinou, Henderson et al. 2005). However, whether HHcy can induce liver fibrosis is still unclear, but our finding indicates that HHcy certainly affects gene expression in the liver of rats.

4. Role of Bone Morphogenetic Proteins in Rat Liver with Hyperhomocysteinemia

With the HHcy model in the liver of rats, we examined the expression of 15 BMP genes. Although 11 out of 15 BMP genes are expressed in the liver, with the exception of BMP-8, BMP-12 and BMP-14, what are their concise functions in liver fibrosis or other liver pathological conditions?

A lot of studies have reported that TGF-β expression was increased during liver fibrogenesis (Jeong, Do et al. 2004; Gressner, Weiskirchen et al. 2002), and one of the BMP members, BMP-4, has been documented to mediate liver injury and fibrosis in bile duct ligation models of liver disease (Fan, Shen et al. 2006). However, whether other BMPs are also involved in liver fibrogenesis still remains to be investigated.

In our study, we gave evidence that Hcy initiated HSC activation with phenotype change and increased cell proliferation, as well as down regulated BMP-13 expression in the liver of rats fed with a high methionine diet. From 1 to 12 week, the mRNA abundance in the liver of rats fed with a high methionine diet gradually and significantly decreased. However, there was no significant modification of other BMP genes. This result goes against our previous study on BMP-4 expression in BDL rats, which demonstrated that the abundance of BMP-4 was significantly increased after one week of BDL (Fan et al. 2006). The difference may be due to either the model established or BMP (Smad) signal transduction. In BMP signaling pathway, BMP ligands typically bind to type I receptors and then recruit Smad 1, 5 and 8. However, they can be further categorized into subgroups, such as the BMP-2/4 group, BMP-5/6/7/8 group, BMP-9/10 group and GDF-5/6/7 group. Some studies indicate that certain BMPs or GDFs may be misnamed because they can neither activate BMP type I receptors nor Smad 1, 5 and 8

(Derynck et al. 2008; Kawabata et al. 1998). The inverse effect may be due to the mediation of BMP-4 and GDF-6 by different BMP downstream molecules.

Our study revealed that HHcy had an effect on the *in vitro* change in phenotype of the HSC cell line; these observations suggest a link between Hcy and HSC activation. However, the results cannot be used *in vivo* directly. Although activated HSCs are considered to be the major cell type in developing liver fibrosis, all liver cells are actually involved in the injury process. Moreover, there is no evidence to prove whether Hcy can immediately activate HSCs by ROS or if the activation is more intermediated by other cell types, such as damaged hepatocytes and Kupffer cells via paracrine methods. Based on our study, we demonstrated that Hcy did not directly regulate BMP-13 gene expression in HSCs, meaning decreased expression of BMP-13 in the liver of rats fed with a high methionine diet may instead be due to down-regulation of BMP-13 expression in other hepatic cells.

Furthermore, we were not able to get a significant increase of α-SMA expression (data not shown) after a diet with high methionine for 1, 4 and 12 weeks, which is commonly accepted as a marker of HSC activation. The increased secretion of growth factor may regulate the activation of HSCs (Whalen, Rockey et al. 1999). From this point, decreased BMP-13 expression in the liver with HHcy may indicate that BMP-13 can inhibit the effect of HHcy in liver (Figure 13). However, this hypothesis should be further investigated by increasing exogenous BMP-13 levels both in *in vivo* and *in vitro* studies. Whether BMP-13 can prevent HHcy induced HSC activation in the liver or has nothing to do with HSCs remains unknown.

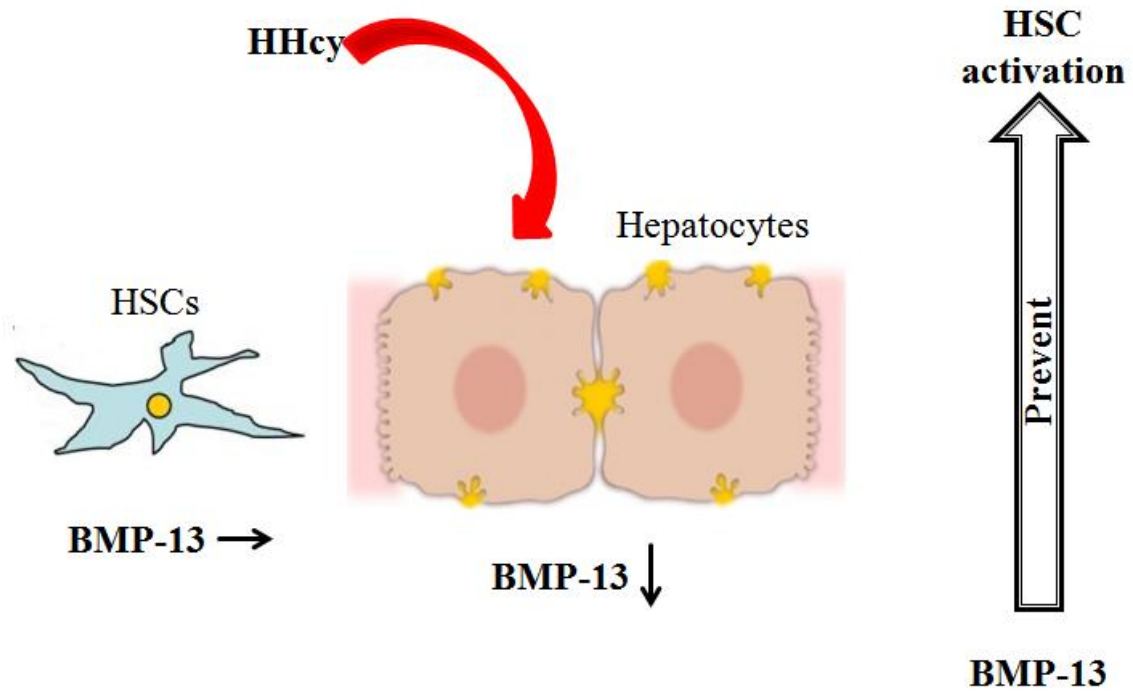


Figure 13 A proposed regulation of BMP-13 inhibits Hcy effects in liver injury. Activation of HSCs can be either by HHcy or by damaged hepatocytes mediated by ROS, cytokines and growth factors through a paracrine method. Our study demonstrated that HHcy directly works on other hepatic cells, such as hepatocyte, instead of HSCs. BMP-13 as a growth factor may be a mediator of the activation of HSCs. As this point, the down-regulation of BMP-13 expression may indicate that BMP-13 can prevent the injury of HHcy in the rat liver.

Summary and Conclusion

(1) There was no difference of BMP-13 mRNA abundance in the liver of rats with the normal control diet and high methionine diet after 1 week. However, the BMP-13 mRNA level was significantly reduced in the liver of rats after 4 and 12 weeks of high methionine diet compared with that in the liver of the normal control diet.

(2) There was no significant change of BMP-13 mRNA level after Hcy treatment in the HSC cell line. However, there were significant increases in cell proliferation and modification of HSC cell morphology after Hcy treatment.

In conclusion, long term high methionine diets can cause a reduction of BMP-13 in the liver. The reduction of BMP-13 in the liver may contribute to liver injury induced by the high methionine diet.

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