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Review

Pathophysiological role of cytoglobin, the fourth globin in mammals, in liver diseases

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Summary. Cytoglobin (Cygb), a stellate cell-specific globin, has recently drawn attention due to its association with liver fibrosis and cancer. In human and rodent livers, Cygb is expressed only in stellate cells and can be utilized as a marker to distinguish stellate cells from hepatic fibroblast-derived myofibroblasts. Loss of Cygb accelerates liver fibrosis and cancer development despite its etiology in mouse models of chronic liver injury. This review discusses the current perception of the distribution, regulation and function of Cygb with regard to liver diseases, with an emphasis on its role in tumorigenesis. Further investigation of Cygb may shed new light on the biology of organ carcinogenesis.

Keywords: Hepatic stellate cell, ROS/RNS, Oxidative stress, Liver cancer, Fibrosis

Introduction

Almost 15 years have passed since the discovery of cytoglobin (Cygb) using a proteomic approach in activated rat hepatic stellate cells (HSCs). The protein was originally named stellate cell activation-associated protein (STAP) (Kawada et al., 2001). Later, it was classified as a novel member of the globin family in mammals, after myoglobin (Mb), hemoglobin (Hb), and neuroglobin (Ngb), and renamed Cygb (Burmester et al., 2002). Many subsequent studies explored its characteristic structure. Sawai et al. (2003) demonstrated that Cygb is a 21-kDa protein consisting of 190 amino acids that exhibits ~25% identity with vertebrate Mb and Hb and 16% identity with human Ngb (Sawai et al., 2003). Moreover, several key ligand-binding residues are highly conserved among the Ngb, Mb and Hb of different species. On the other hand, both Cygb and Ngb have unusual features, which are quite different from those of traditional pentacoordinated globins such as Mb and Hb. Spectroscopic studies have shown that Cygb and Ngb contain a hexacoordinated heme iron, to which two His imidazole groups in both the deoxy ferrous and ferric states are bound directly (Sugimoto et al., 2004).

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Abbreviations. αSma, α smooth muscle actin; Akt, Serine/threonine-specific protein kinase; AP1, AP2, activator proteins, Cat-1, catalase-1; Ccl, chemokine (C-C motif) ligand; Cygb, cytoglobin; C/EBP, CCAAT/enhancer binding protein; CDAA, choline-deficient amino acid-defined diet; Col1a1, collagen, type I, alpha 1; DEN, diethynitrosamine; DNMT1, DNA methyl transferase 1; DAPI, 4', 6-diamidino-2-phenylindole; Erk, Extracellular signal-regulated kinases; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; Hb, Hemoblogin; IGF2, insulin-like growth factor 2; Il-6, interleukin-6; Il-1β, interleukin-1β; iNos, inducible nitric oxide synthase; MDA, malondialdehyde; Mb, Myoglobin; Mpo, myeloperoxidase; NASH, non-alcoholic steatohepatitis; Ngb, Neuroglobin; NT, nitrotyrosine; NOD, nitric oxide dioxygenase; NF1, NFκB, NFAT, nuclear factors; PRPF40A, pre-mRNA processing factor; ROS, reactive oxygen species; RNS, reactive nitrogen species; Tnfα, tumor necrosis factor α; Tgfβ-1, Tgfβ -3, transforming growth factor β; SiR-FG, Sirius red and fast-green; SP1, stimulatory protein 1; UCP2, uncoupling protein-2.1.

Therefore, exogenous ligands, such as O_2 or carbon monoxide (CO), can bind to the iron after displacement of one of the His imidazole groups from the axial coordination site (Trent et al., 2001; Sawai et al., 2003). However, similarly to Mb, Cygb exhibits high intrinsic affinity to O_2 (Sawai et al., 2003; Sugimoto et al., 2004). Cygb contains two cysteines, which might form an intramolecular or intermolecular disulfide bridge (Hamdane et al., 2003; Lechauve et al., 2010). Their substitution or reduction diminishes the affinity of Cygb for O_2. This indicates that the cellular redox state may influence protein structure due to S-S bond formation or cleavage, thus affecting O_2 binding. It has been proposed that conformational changes alter the E-helix position and, subsequently, the affinity of HisE7 for iron (Hamdane et al., 2003; Lechauve et al., 2010). Amino acids in close spatial vicinity to heme create an apolar environment (Pesce et al., 2002), which renders Cygb stable in the oxy-state (Sawai et al., 2003).

The *Cygb* gene is located on chromosome 17q25.3 in humans and chromosome 11E2 in mice. Mouse and human Cygb share 92.8% of nucleotides and 95.3% of amino acids in the coding region (Burmester et al., 2002). The *Cygb* gene has the lowest mutation rate among vertebrate globins (Burmester et al., 2002; Wystub et al., 2004), which indicates that not only individual residues but also large sections of the protein are crucial for Cygb function (Wystub et al., 2004). As is typical of all globins, the mammalian *Cygb* gene comprises two introns at positions B12-2 and G7-0 (i.e., before the first nucleotide in the seventh codon of helix G). Genes encoding hexacoordinated globins harbor an additional third intron. Although it is usually located at position E11-0, the third intron of *Cygb* uniquely occupies position H36-2 (Trent and Hargrove, 2002).

Not only structural but also functional and pathophysiological characterizations of Cygb have been reported. However, its molecular role remains under investigation. This review will highlight the pathophysiological role of Cygb in the liver and future prospects.

Distribution of Cygb

Multiple studies have shown that Cygb is expressed ubiquitously in all vertebrate organs, including the brain, liver, heart, lung, retina, gut, esophagus and others (Table 1). With regard to its tissue- and cell-specific distribution, Cygb is found primarily in fibroblasts of connective tissue and in fibroblast-like cells such as chrondroblasts, osteoblasts, and HSCs (Kawada et al., 2001; Nakatani et al., 2004; Schmidt et al., 2004; Avivi et al., 2010; Motoyama et al., 2014). Specifically, in the liver, Cygb has been detected in HSCs (Kawada et al., 2001), but not in hepatocytes, Kupffer cells, endothelial cells or myofibroblasts (Motoyama et al., 2014). In the other organs of the digestive system-such as the pancreas, stomach, intestine, and colon-Cygb was found in pancreatic stellate cells around acini, stromal cells, spindle-shaped cells and fibroblasts, respectively. Cygb is also present in the stromal cells of red pulp in the spleen. In the kidney, Cygb was expressed at high levels in the stromal cells along the proximal and distal uriniferous tubules (Nakatani et al., 2004). In the heart, Cygb is present in fibroblasts, but not in myocytes; in bone, Cygb is expressed by both osteoblasts and osteocytes and to a higher level in the former. In the trachea and lung, Cygb is found in chondroblasts in the cartilage but not in mature chondrocytes; it is expressed in stromal cells along the alveolar walls and also in the bronchioles and pulmonary artery. In the wrist and skeletal tissue, Cygb is expressed in fibroblasts in muscle and tendon; in contrast, skeletal muscle cells are negative for Cygb expression. The epidermis does not contain Cygb-positive cells (Nakatani et al., 2004; Schmidt et al., 2004). However, a recent study showed that melanocytes express Cygb (Fujita et al., 2014).

Although the ubiquitous expression of Cygb in all organs has been confirmed, its cellular distribution remains a matter of debate. Some studies have demonstrated that Cygb expression is localized in macrophages, muscle cells (Shigematsu et al., 2008), hepatocytes (Geuens et al., 2003), and epithelial cells (Geuens et al., 2003; Shigematsu et al., 2008; Emara et al., 2010). However, the general view on the distribution of Cygb has been derived from the reproducible results reported by several research groups (Nakatani et al., 2004; Schmidt et al., 2004, 2005; Tateaki et al., 2004; Mammen et al., 2006; Avivi et al., 2010; Motoyama et al., 2014; Thuy le et al., 2015); Cygb is expressed strictly in the cytoplasm of fibroblast-like cells and other mesenchymal cells (Table 1). Cytoplasmic and nuclear Cygb localization was evident mainly in neurons. Specific Cygb expression in neurons suggests that the globin may play a different role in these cells compared with that in mesenchymal cells (Schmidt et al., 2004). Thus, the discrepancies between the cell-type and subcellular localization of Cygb might have arisen due to technical issues related to the specificity of the antibodies used, immunodetection methods applied, and endogenous Cygb expression levels (Oleksiewicz et al., 2011).

Assumed function of Cygb

Oxygen storage, diffusion and sensor for cellular respiration and metabolism

Cygb exhibits intrinsic O_2-binding capacity, because its heme iron has the same affinities for exogenous ligands and the same equilibrium constants for oxygen compared with that of myoglobin (Kawada et al., 2001; Sawai et al., 2003). With regard to its distribution in fibroblast-like cells, which are not generally associated with high metabolic rates and oxygen consumption, Cygb might function as an oxygen sensor and might be involved in cell proliferation and possibly oxygen diffusion for synthesis of healing collagen (Burmester et

al., 2007). Recently, Teranishi et al. (2015) demonstrated that Cygb in HSCs plays a role in augmenting the O_2 supply to hepatocytes for CYP-mediated xenobiotic oxidative metabolism induced by acetaminophen or CCl4 treatment (Teranishi et al., 2015). Since O_2 binding resulted in conformational changes in the disulfide bridge, a shift in Cygb structure and concomitant O_2 release (Hamdane et al., 2003), Cygb may putatively act as a signal transducer in pathways associated with oxygen sensing (Sawai et al., 2003; Hankeln et al., 2005). However, no evidence indicating direct signal transduction by Cygb has been reported; therefore, further studies are warranted.

Nitric oxide scavenger

Globin commonly functions as a nitric oxide dioxygenase (NOD). NOD activity is also proposed for Cygb (Gardner et al., 2006; Vinogradov and Moens, 2008; Gardner et al., 2010). According to Smagghe and

Table 1. Cellular expression and function of CYGB.

Reference	Species	CYGB-positive tissues/organs	Detection method(s)	Specific cell type	Location	Functional description
Kawada et al., 2001	Rat	Activated hepatic stellate cells	Proteomics	Hepatic stellate cells	Cytoplasm	A heme protein exhibiting peroxidase activity catabolizing hydrogen peroxide and linoleic acid hydroperoxide.
Burmester et al., 2002	Human	All normal tissues	Northern Blot	ND	ND	ND
Trent and Hargrove, 2002	Human	All normal tissues	Northern Blot	ND	ND	Oxygen-binding capacity
Asahina et al., 2002	Human	Normal adult brain, heart, kidney, lung, trachea, liver, and placenta	qRT-PCR and IHC	HSCs	Cytoplasm	A heme protein with peroxidase activity
Geuens et al., 2003	Mouse	Normal brain, liver, kidney, and pancreas	IHC	Epithelial cells	Nuclear	Possible function of globin-folded proteins as transcriptional regulator
Schmidt et al., 2004	Rat and mouse	Normal connective tissues	IHC/IF	Fibroblasts and their derivatives and hepatic stellate cells	Cytoplasm	Hypoxia-responsive gene
	Mouse	Normal central nervous system	IHC/IF	Neurons	Cytoplasm and nuclear	
Nakatani et al., 2004	Rat	Normal tissues	IHC/IF	Fibroblast-like cells	Cytoplasm	Potential fibrosis disorder associated gene
Tateaki et al., 2004	Rat	Normal and fibrotic livers	IHC	Myofibroblasts and activated hepatic stellate cells	Cytoplasm	Fibrosis -associated gene
Kugelstadt et al., 2004	Chicken	Normal brain, muscle, liver, spleen, eye, heart tissues	Revert transcription PCR	ND	ND	ND
Fordel et al., 2004	Mouse	Hypoxia skeletal muscle, heart, and brain tissue	qRT-PCR	ND	ND	Hypoxia-responsive gene
Schmidt et al., 2005	Mouse	Eye	IF	Fibroblasts	Cytoplasm	Respiratory protein
Xu et al., 2006	Rat	Primary HSCs	qRT-PCR and IHC	HSCs	ND	Protects HSCs against oxidative stress-induced activation and inhibits tissue fibrosis
Mammen et al., 2006	Mouse	Embryogenesis, normoxic and hypoxic brain	In situ hybridization, RT-PCR, and Northern blot	Distinct regions of brain	Nuclear	Oxygen-responsive tissue, hemoglobin
McRonald et al., 2006	Human	Esophageal biopsy specimens	RT-PCR and bisulphite pyrosequencing	ND	ND	Downregulation of CYGB associated with esophageal tylosis disease
Xinarianos et al., 2006	Human	Human non-small-cell lung carcinoma tissues	mRNA level and promoter methylation analysis	ND	ND	Candidate tumor suppressor gene
Li et al., 2007	Mouse	H_2O_2 treated neuroblastoma cell line	RT-PCR	Epithelial cells	ND	Protect cells from oxidative -stress-mediated injury
Fordel et al., 2007b	Mouse	Hypoxic brain, liver, heart, skeletal muscle, eyes	qRT-PCR	ND	ND	ROS scavenger
Hodges et al., 2008	Human	Neuronal cell line TE671	Transfection of cells with the cytoglobin-GFP fusion protein	Neuronal cell	Cellular and Nuclear	ROS scavenger, survival rate enhancer; free-radical-mediated DNA damage
Man et al., 2008	Mouse	Fibrotic liver	IHC	HSCs and fibroblasts	Cytoplasm and nuclear	Liver-fibrosis-related gene

colleagues, in the oxy-ferrous state, all human Ngb and Cygb, rice nsHb (riceHb1), *Synechocystis* Hb (cyanoglobin, SynHb), and horse heart Mb can rapidly destroy NO *in vitro*; Cygb showed the highest consumption rate (Smagghe et al., 2008). At low O_2 levels (0-50 mM), Cygb together with cellular reductants regulates the NO consumption rate in response to changes in O_2 concentration and is approximately 500-fold more sensitive to changes in O_2 level than is Mb (Liu et al., 2013). The NO-scavenging function of Cygb protects the NO-sensitive aconitase, decreases peroxynitrite formation, and protects cellular respiration (Gardner et al., 2010). One study of Cygb expression patterns in human and rat hippocampus showed co-expression of Cygb and neuronal nitric oxide synthase (nNOS) and their upregulation following chronic restrain stress (Hundahl et al., 2013). The high level of Cygb and nNOS co-expression supports the hypothesized involvement of Cygb in NO metabolism. Accumulation of peroxynitrite and other nitrosative molecules results in nitrosative stress, which might target tyrosine residues, metalloproteins, lipids and nucleic acids (Pacher et al., 2007; Hill et al., 2010). Thus, the NO-scavenging function of Cygb would be crucial for protecting cells/tissues from NO accumulation.

Involvement in hypoxia and oxidative stress

Cygb was first reported as a hypoxia-responsive gene in the heart and liver by an *in vivo* study on rats exposed to hypoxia (9% oxygen for 22 or 44 hours) (Schmidt et al., 2004). Other studies using various tumor cell lines, including sporadic head-and-neck squamous cell carcinoma (Shaw et al., 2009) and human glioblastoma multiform (Emara et al., 2010), animal models, such as that for murine embryogenesis, and

Table 1. (Continuation).

Reference	Species	CYGB-positive tissues/organs	Detection method(s)	Specific cell type	Location	Functional description
Chua et al., 2009	Human	Breast cancer cell line MCF-7	PCR array	Epithelial cells	ND	Oxidative-stress-related gene
Shaw et al., 2009	Human	Samples from oral or oropharyngeal squamous cell carcinoma patients; human sporadic head-and-neck squamous cell carcinoma (HNSCC) cell lines	qRT-PCR, pyrosequencing methylation analysis	Epithelial cells	ND	Promoter methylation and tumor hypoxia regulates CYGB expression
Avivi et al., 2010	Rat and the blind mole rat Spalax	Normoxic and hypoxic brain, heart, and liver	IF and qRT-PCR	Fibroblast-like cells and neurons	Cytoplasm of fibroblast-like cells and nucleus of neurons	Cytoprotective effect under pathological hypoxic/ischemic conditions in mammals
Mimura et al., 2010	Rat	Normal and fibrotic kidney	IHC	Interstitial cells	Cytoplasm	Protects tissues from ROS
Gardner et al., 2010	Rat	Rat hepatocyte expressed human Cygb, and recombinant human CYGB protein	Nitric oxide consumption assay	Rat hepatocyte	ND	NO dioxygenase
Fang et al., 2011	Human	Glioma cells	Fluorescence transfection and measurement assay	Glioma cells	Partial nuclear	Protect cells from oxidative stress induced cell injury; putative tumor suppressor function
Thuy le et al., 2011	Mouse	DEN-treated Cygb-knockout livers	mRNA and protein levels	HSCs	Cytoplasm	Tumor suppressor gene
Basu et al., 2012	Human	Various prostate normal and cancer cell lines	mRNA PCR array, Immunoblot	Epithelial cells	ND	Stress respond gene
Liu et al., 2013	Human	Human recombinant CYGB protein	Model of O_2-dependent NO consumption by Cygb	ND	ND	NO consumption
Hundahl et al., 2013	Human and rat	Hippocampus	IHC and in situ hybridization	Neuronal cell	Cell soma and processes	NO metabolism
Motoyama et al., 2014	Human/ mouse	Human normal, and fibrotic livers; primary mouse HSCs	IHC	HSCs	Cytoplasm	Inversely correlated with fibrosis stage
Thuy le et al., 2015	Human	Normal, NASH and HCC	IHC and IF	HSCs	Cytoplasm	Implicated on pathogenesis of NASH; protective role in the process of fibrosis and cancer development
	Mouse	Primary HSCs; CDAA-treated Cygb-knockout livers	IF	HSCs	Cytoplasm	

ND, not determined; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; DEN, diethylnitrosamine; CDAA, choline-deficient amino acid-defined diet; IHC, immunohistochemistry; IF, immunofluorescence; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species.

adult tissues (Mammen et al., 2006) have also suggested hypoxia-dependent regulation of the *CYGB* gene. Moreover, Wystub et al. (2004) demonstrated that the non-coding sequence of *CYGB* contains multiple conserved regions associated with the cellular response to hypoxia (Wystub et al., 2004). This includes hypoxia-responsive elements (HRE), hypoxia-inducible protein binding sites and recognition sites required for the binding of a number of hypoxia-related transcription factors. Hypoxia-inducible factor 1 (HIF-1) is assumed to be an important transcription factor for *Cygb*, because HREs at positions 141, 144, and 448 are essential for the activation of *CYGB* expression, and the binding of HIF-1 to this area has been confirmed (Fordel et al., 2004; Guo et al., 2007). Recently, Singh et al. (2009) reported that Cygb expression is markedly upregulated in the hypoxia-induced hypertrophic heart due to increased binding of transcription factors-including activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT)-to the putative promoter region of *Cygb* via calcineurin-dependent regulation (Singh et al., 2009).

In addition to hypoxic conditions, *CYGB* is also overexpressed under oxidative stress conditions (Mammen et al., 2006; Li et al., 2007). *CYGB* overexpression protected human neuroblastoma SH-SY5Y cells from H_2O_2-induced death (Fordel et al., 2006, 2007). *CYGB* overexpression also rescued the human neuronal cell line TE671 from DNA damage induced by the pro-oxidant Ro19-8022 (Hodges et al., 2008). Furthermore, it has been reported that *in vitro* and *in vivo* overexpression of Cygb in rat HSCs protected these cells against oxidative stress and inhibited their differentiation into an active phenotype (Xu et al., 2006). In contrast, primary cultured mouse HSCs isolated from *CYGB*-deficient mice showed robust reactive oxygen species (ROS) accumulation, similar to those isolated from wild-type (WT) mice transfected with *CYGB* siRNA (Thuy le et al., 2015). Recently, Latina et al. (2015) reported that *CYGB* is transcriptionally regulated by ΔNp63 in primary epithelial cells (keratinocytes) and in cancer cells (H226, MCF-7) under both normal proliferating conditions (normoxia) and following oxidative stress (Latina et al., 2015). Taken together, these reports suggest that, in addition to functioning as a gas carrier, CYGB may act as a cytoprotective factor under hypoxia and oxidative stress.

Diseases associated with Cygb

It has been demonstrated that Cygb is involved in the pathogenesis of disorders of various organs. Firstly, Cygb was discovered in rat HSCs isolated from fibrotic liver (Kawada et al., 2001). Next, other groups reported that forced overexpression of Cygb played a protective role in both toxic and cholestatic models of rat liver injury (Xu et al., 2006) and in chemical-induced liver fibrosis (Man et al., 2008). Furthermore, the association of Cygb with not only liver fibrosis but also kidney fibrosis was demonstrated using transgenic rats overexpressing Cygb (Mimura et al., 2010). Increased expression of Cygb has been found in glaucoma (Ostojic et al., 2006), gastroesophageal reflux disease (McRonald et al., 2006), putaminal neurons and glia of patients with hereditary ferritinopathy (Powers, 2006), and cytoplasmic inclusions in the neocortex of patients with psychomotor retardation and/or epilepsy (Hedley-Whyte et al., 2009). In contrast, downregulation of *CYGB* has been reported in several human cancerous tissues and human cancer cell lines. Decreased expression of *CYGB* as well as hypermethylation of the *CYGB* promoter has been reported in tylosis patients, non-small cell lung carcinoma tissues, head-and-neck cancers, ovarian cancers, and breast cancers (Presneau et al., 2005; McRonald et al., 2006; Xinarianos et al., 2006; Chua et al., 2009; Shaw et al., 2009; Wojnarowicz et al., 2012; Chen et al., 2014; Hubers et al., 2015; Latina et al., 2015). McRonald et al. (2006) reported that *CYGB* gene expression in tylosis with esophageal cancer was reduced to ~70% of expression in the normal esophagus, which was accompanied by hypermethylation of the promoter (McRonald et al., 2006). This author further evaluated an *in vitro* model of Cygb knockdown in normal esophageal epithelial (NE-1) and CCD-18Co colonic myofibroblasts, as well as Cygb overexpression in TE-8 esophageal squamous cell carcinoma cells. Overexpression of Cygb in TE-8 cells afforded protection from buthionine sulfoximine-induced oxidative stress; however, this was observed only at high, non-physiological concentrations of Cygb. In addition, downregulation of Cygb in NE-1 cells had no effect on their sensitivity to oxidative stress (McRonald et al., 2012). A significant reduction in *CYGB* mRNA expression and hypermethylation of *CYGB* were reported in non-small cell lung carcinoma tissues compared with healthy samples (Xinarianos et al., 2006). Additionally, Shivapurkar et al. (2008) reported high levels of *CYGB* promoter methylation in lung, breast, bladder, and colon cancers and in leukemia in humans. Augmented growth of NCI-H661 lung cancer cells silenced for *CYGB* by RNA interference and suppression of proliferation of NCI-H228 cells stably transfected with plasmids containing *CYGB* cDNA have also been reported (Shivapurkar et al., 2008). Regarding lung cancer, one recent study reported that co-expression of *CYGB* and its potential upstream regulatory gene ΔNp63 negatively affected the survival outcomes of early-stage non-small cell lung carcinoma patients (Latina et al., 2015). Fujita et al. (2014) reported a high level of CYGB expression in several melanoma cell lines and melanocytes, the origin of melanoma. The abrogated CYGB expression in the remaining melanoma cell lines was epigenetically regulated by hypermethylation of the promoter region of *CYGB*. By assessing proliferation of *CYGB*-knockdown cells and those exposed to oxidative stress, these authors suggested that CYGB plays a tumor suppressor role via ROS regulation (Fujita et al., 2014). In the case of ovarian cancer, two groups have reported downregulation of *CYGB* in ovarian cancer compared

with normal specimens (Wojnarowicz et al., 2012; Chen et al., 2014). Low expression of *CYGB* was also found in glioma patients and was significantly associated with a higher histological grade and tumor recurrence (Xu et al., 2013). We created Cygb-deficient ($Cygb^{-/-}$) mice, monitored their phenotype for 2 years and found that 67% of those aged 1-2 years exhibited spontaneous abnormalities and cancer development in multiple organs, including the liver, lung, lymph nodes and heart (manuscript in preparation). Furthermore, $Cygb^{-/-}$ mice developed rapidly, and numerous liver cancers developed in models exposed to chemical and dietary carcinogenic factors (Thuy le et al., 2011, 2015). These reports indicate that *Cygb* has a tumor suppressor function.

Role of Cygb in liver diseases

Liver fibrosis suppression

Liver injury triggers HSC activation, which has been identified as a key event in hepatic fibrogenesis. During the activation process, HSCs acquire proliferating, fibrogenic, and contractile properties (Friedman, 2008). Increased expression of Cygb was initially found in activated HSCs (Kawada et al., 2001) and was suggested to support HSCs in liver injury, during which they are exposed to high levels of endogenous ROS. This suggests a ROS scavenger function of Cygb, as evidenced by its ability to detoxify radicals via reaction with its heme group (Nishi et al., 2011). Similarly, forced overexpression of Cygb significantly increased the total oxy-radical scavenging capacity compared with the control expressing eGFP (Xu et al., 2006). In accordance with these data, Xu et al. (2006) demonstrated that overexpression of Cygb protected primary rat HSCs against oxidative stress, as assessed by reduced production of malondialdehyde and 4-hydroxy-2-noneal, biomarkers of lipid peroxidation. Finally, Cygb overexpression reduced tissue fibrosis in both toxic and cholestatic models of liver injury (Xu et al., 2006). Both acute and chronic liver injury induced by bile duct ligation in $Cygb^{-/-}$ mice resulted in large clusters of hepatocyte death during the acute phase and development of severe fibrosis during the chronic phase (Tuong et al., manuscript in preparation). In our previous studies, both diethynitrosamine (DEN, a well-known carcinogen) (Thuy le et al., 2011) and choline-deficient amino acid-defined (CDAA) diet (Thuy le et al., 2015) treatment induced greater liver fibrosis formation in $Cygb^{-/-}$ mice compared with WT mice (Fig. 1). Cytologically, HSCs in the absence of Cygb ($HSCs^{Cygb\text{-}null}$) became enlarged, developed an αSMA network after 7 days in culture, and lost cellular lipid droplets more rapidly than did $HSCs^{Cygb\text{-}wild}$ (Fig. 2). Moreover, $HSCs^{Cygb\text{-}null}$ demonstrated a pre-activated phenotype, with increased oxidative stress and elevated expression of cytokines and chemokines, such as Il-6, Tnfα, Il-1β, Cxcl-1, and -2, and Ccl-2, -3, and -4 (Thuy le et al., 2015). Taken together, these findings suggest that Cygb plays a role in the prevention of fibrosis development by suppressing HSC activation.

Motoyama et al. (2014) reported that in human liver tissues damaged by hepatitis C virus (HCV) infection, the number of Cygb-positive cells decreased with fibrosis progression (Motoyama et al., 2014). Interestingly, CYGB was abundant in HSCs, but absent in myofibroblasts that were rich in fibrotic septum and positive for α-smooth muscle actin, fubulin-2 and Thy-1. In detail, the densities of CYGB-positive cells were

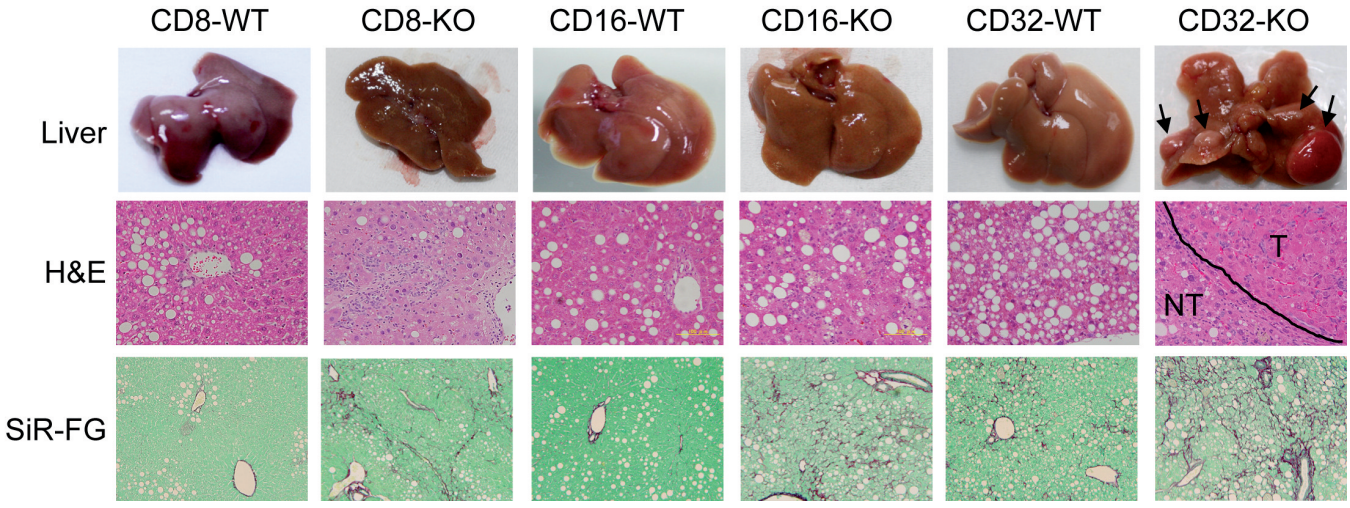

Fig. 1. Promotion of fibrosis and liver tumor development in CDAA-fed Cygb / mice Wild-type (WT) and Cygb / mice (KO) were fed choline-deficient amino acid-defined diets for 8 (CD8), 16 (CD16), or 32 (CD32) weeks. Representative macroscopic and microscopic liver sections stained with H&E, Sirius Red and Fast Green (SiR-FG). Black arrows, tumor nodules; T, tumor; NT, non-tumor area. H&E, × 400; SiR-FG, × 200

17.9±1.29, 19.7±1.01, 16.2±0.82, and 13.8±1.06 cells/mm^2 in fibrosis stages F1, F2, F3, and F4, respectively. With regard to ROS as a key stimulus for myofibroblast development and subsequent development of fibrosis, the ROS scavenger CYGB may function by inhibiting the initiation of HSC activation. Hence, the anti-fibrotic activity of Cygb is a potential target for development of novel fibrosis suppression therapies.

Liver tumor suppression

Besides the various well-known liver tumor suppressor genes, such as p53 (Murakami et al., 1991), p16 *INK4A* (Kita et al., 1996), insulin-like growth factor 2 (*IGF2*) (De Souza et al., 1995), *PTEN* (Horie et al., 2004), *CYGB* might also be an interesting tumor suppressor gene candidate not only in the liver but also in other organs. Numerous investigations of the tumor suppressor activity of Cygb have been reported since 2005, and these have shown that most cancer cells have reduced Cygb expression and/or loss of heterozygosity (LOH), and promoter hypermethylation both *in vitro* and *in vivo* (Table 1) (Presneau et al., 2005; McRonald et al., 2006; Xinarianos et al., 2006; Shivapurkar et al., 2008; Chua et al., 2009; Shaw et al., 2009; Fang et al., 2011; Thuy le et al., 2011). Recently, John et al. (2014) examined the response of Cygb to DNA-damaging agents such as Adriamycin and etoposide in human osteosarcoma U20S cells (John et al., 2014). The results revealed a dramatic increase in the level of Cygb expressed from a vector following a few hours of DNA damage induction; indeed, the expression pattern paralleled that of cellular p53. Since Cygb stabilizes p53 and enhances the expression of the p53 target gene p21, this study demonstrated that Cygb inhibits cell proliferation and induces G1 arrest during genotoxic stress, supporting a tumor suppressor function of Cygb (John et al., 2014). The anti-tumor mechanism of Cygb in the liver was investigated using a carcinogenesis model in Cygb$^{-/-}$ mice (Thuy le et al., 2011). The frequency of liver cancer development was significantly higher in Cygb$^{-/-}$ mice treated with 25 ppm DEN for 25 weeks in comparison with WT mice. At a very low dose of DEN (0.05 ppm for 9 months), WT mice showed no tumor formation, while Cygb$^{-/-}$ mice showed development of liver cancer. Moreover, pericellular fibrosis developed in DEN-treated Cygb$^{-/-}$ mice. Furthermore, reactive nitrogen species (RNS) (including nitrotyrosine) was abundant in liver tissues derived from Cygb$^{-/-}$ mice. These results indicate high-level production of NO, an endogenous molecule that causes cellular and DNA damage (Xu et al., 2002; Ying and Hofseth 2007; Halligan et al., 2009), together with superoxide, in Cygb$^{-/-}$ mice. Interestingly, Cygb gene disruption further altered the expression of cancer-related genes, including upregulation of p53, cyclin D2, p21-activated kinase (Pak 1), Src, and Cdkn2a and downregulation of Cebpa, a tumor suppressor that inhibits cell proliferation (Thuy le et al., 2011).

Similar tumor suppressor activity of Cygb has also been reported in the mouse model of non-alcoholic steatohepatitis (NASH) (Thuy le et al., 2015). CDAA treatment for 8 weeks induced prominent inflammation and fibrosis in *Cygb*$^{-/-}$ mice. Surprisingly, at 32 weeks, WT mice showed no tumor formation, while all *Cygb*$^{-/-}$ mice developed liver cancer (Fig. 1), which was ameliorated by treatment with the antioxidant N-acetylcysteine. PCR array analysis of the expression of 84 genes related to oxidative stress and antioxidant defenses revealed increased expression of pro-oxidant genes such as myeloperoxidase (39-fold, p=0.02) and prostaglandin-endoperoxide synthase 2 (4.3-fold, p=0.007) and downregulation of almost all antioxidant genes-such as Gpx-6, Cat-1, Sod-1, Sod-2-which are involved in ROS scavenging. Furthermore, markers of DNA double-stranded breaks, including 53BP-1 and γH2AX, were expressed at high levels in both non-tumor and tumor tissues of the livers of CDAA-fed *Cygb*$^{-/-}$ mice. HSCs isolated from both *Cygb*$^{-/-}$ mice and *Cygb* siRNA-transfected-HSCs exhibited the pre-activation condition. These data suggest that Cygb functions as an ROS/RNS scavenger and that it may contribute to HSC activation, development of liver fibrosis, and cancer cell growth (Thuy le et al., 2015).

The molecular mechanisms associated with the tumor suppressor function of Cygb remain to be determined. Regarding its scavenging capacity, it has been speculated that Cygb reduces the injury induced by oxidative and nitrosative stresses, thereby protecting against damage to DNA, proteins and membranes. Cygb may also alleviate the upregulation of redox-sensitive signaling pathways implicated in carcinogenesis (Klaunig et al., 2010). Alternatively, loss of Cygb in HSCs in the liver and fibroblast-like cells in other organs may impair their function in tissue exposed to multiple microinjuries and environmental insults. This might lead to activation of these cells, which is frequently associated with an inflammatory response and aberrant epithelial-mesenchymal interactions. Inflammatory stimulation results in increased NO synthesis (and peroxynitrite formation), which affects p53 and mitogen-activated protein kinase pathways and promotes angiogenesis, migration, invasion and DNA damage in a manner dependent on NO concentration, cell type and genetic background (Pacher et al., 2007; Yang et al., 2009; Oleksiewicz et al., 2011).

Summary and future directions

Understanding the presence and impact of Cygb in HSC, a special cell with lots of unfolding mysteries related to its protean features, remains challenging. Moreover, it is speculated that Cygb-dependent effects on HSCs may indirectly impact the function and phenotype of hepatocytes. Regarding the Cygb signaling pathway, several molecules have been found to be upstream regulators of Cygb; these include HIF1, stimulatory protein 1, activator proteins (AP1, AP2),

nuclear factors (NF1, NFjB, NFAT), CCAAT/enhancer binding protein, and cellular erythroblastosis virus E26 oncogene homolog 1 (Wystub et al., 2004; Guo et al., 2007). The downstream targets of Cygb include several genes that were immediately downregulated upon Cygb overexpression, such as pre-mRNA processing factor, uncoupling protein-2, collagen, type I, alpha 1 (COL1A1), and DNA methyl transferase 1 (Shivapurkar et al., 2008). These molecules, particularly Col1a1, and others-such as Timp-1, Tgfβ-1, Tgfβ-3, Il1β, Erk, Akt,

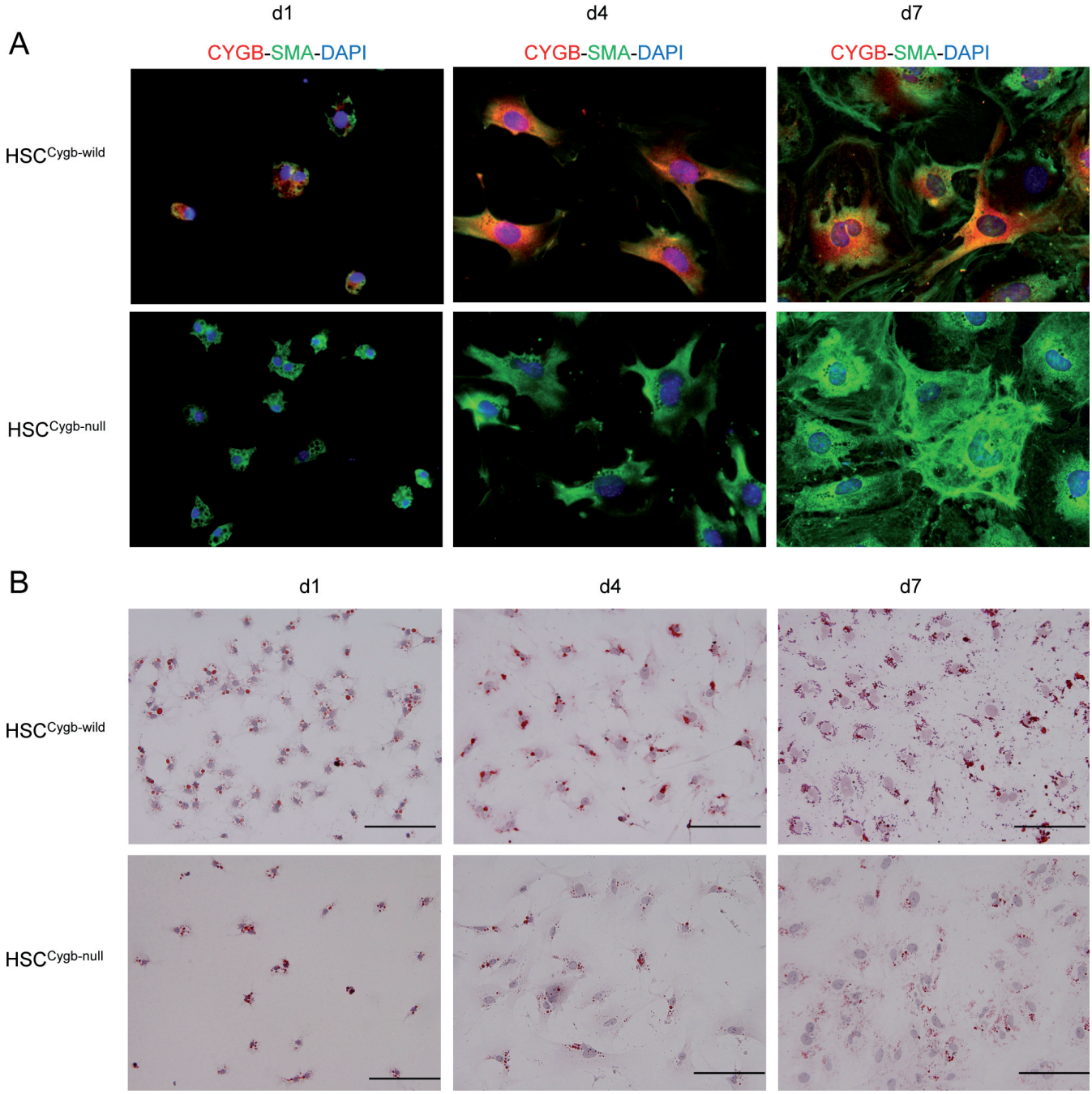

Fig. 2. Cygb deficiency induced priming of HSCs. Primary HSCs isolated from wild-type (HSCsCygb-wild) and Cygb / mice (HSCsCygb-null) were cultured for 1 (d1), 4 (d4), and 7 (d7) days. **A.** Representative confocal images of αSMA (green) and CYGB (red) double staining. Nuclei were stained with DAPI (blue). Original magnification ×400. **B.** Oil Red O staining. Bar: 100 μm.

Cyclin D1, cJun, cFos, myeloperoxidase (Mpo), and nitrotyrosine-exhibited increased expression following the loss of Cygb (Thuy le et al., 2011, 2015). Thus, the question of which molecule is the main target of Cygb and how it affects the function of HSCs and their neighboring cells has been raised. The most relevant mechanism, in our view, is likely related to the radical-scavenging function of Cygb.

Continued elucidation of the function of Cygb is crucial, particularly its contribution to disorders of the liver, the largest exocrine and endocrine gland, as well as its role in preventing fibrosis and tumorigenesis. Further evidence of penetrating and complex cross-talk between HSCs expressing Cygb and epithelial and or inflammatory cells subsets is sure to emerge. It would also be of interest to determine whether Cygb overexpression leads to improved fibrogenesis in a mouse model.

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