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

Le Thi Thanh Thuy, Nguyen Thi Thanh Hai, Hoang Hai and Norifumi Kawada

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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).

Review

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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

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, diethylnitrosamine; DNMT1, DNA methyl transferase 1; DAPI, 4′, 6-diamidino-2-phenylindole; Erk, Extracellular signal-regulated kinases; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; Hb, Hemoglobin; 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; Tnf α, tumor necrosis factor α; Tgf β-1, Tgf β -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 \( \text{O}_2 \) 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 \( \text{O}_2 \) (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; Lechaude et al., 2010). Their substitution or reduction diminishes the affinity of Cygb for \( \text{O}_2 \). This indicates that the cellular redox state may influence protein structure due to S-S bond formation or cleavage, thus affecting \( \text{O}_2 \) 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; Lechaude 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 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 pathophysiologically characterized differences of Cygb have been reported. However, its molecular role remains under investigation. This review will highlight the pathophysiologically relevant 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 chondroblasts, 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 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; Mammel 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 \( \text{O}_2 \)-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
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 CCl\(_4\) 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.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>CYGB-positive tissues/organisms</th>
<th>Detection method(s)</th>
<th>Specific cell type</th>
<th>Location</th>
<th>Functional description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kawada et al., 2001</td>
<td>Rat</td>
<td>Activated hepatic stellate cells</td>
<td>Proteomics</td>
<td>Hepatic stellate cells</td>
<td>Cytoplasm</td>
<td>A heme protein exhibiting peroxidase activity catalyzing hydrogen peroxide and linoleic acid hydroperoxide.</td>
</tr>
<tr>
<td>Burmester et al., 2002</td>
<td>Human</td>
<td>All normal tissues</td>
<td>Northern Blot</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trent and Hargrove, 2002</td>
<td>Human</td>
<td>All normal tissues</td>
<td>Northern Blot</td>
<td>ND</td>
<td>ND</td>
<td>Oxygen-binding capacity</td>
</tr>
<tr>
<td>Asahina et al., 2002</td>
<td>Human</td>
<td>Normal adult brain, heart, kidney, lung, trachea, liver, and placenta</td>
<td>qRT-PCR and IHC-PCR and IHC</td>
<td>HSCs</td>
<td>Cytoplasm</td>
<td>A heme protein with peroxidase activity</td>
</tr>
<tr>
<td>Geuens et al., 2003</td>
<td>Mouse</td>
<td>Normal brain, liver, kidney, and pancreas</td>
<td>IHC</td>
<td>Epithelial cells</td>
<td>Nuclear</td>
<td>Possible function of globin-folded proteins as transcriptional regulator</td>
</tr>
<tr>
<td>Schmidt et al., 2004</td>
<td>Rat and mouse</td>
<td>Normal connective tissues</td>
<td>IHC/IF</td>
<td>Fibroblasts and their derivatives and hepatic stellate cells</td>
<td>Cytoplasm</td>
<td>Hypoxia-responsive gene</td>
</tr>
<tr>
<td>Schmidt et al., 2004</td>
<td>Mouse</td>
<td>Normal central nervous system</td>
<td>IHC/IF</td>
<td>Neurons</td>
<td>Cytoplasm and nuclear</td>
<td>Hypoxia-responsive gene</td>
</tr>
<tr>
<td>Nakatani et al., 2004</td>
<td>Rat</td>
<td>Normal tissues</td>
<td>IHC/IF</td>
<td>Fibroblast-like cells</td>
<td>Cytoplasm</td>
<td>Potential fibrosis disorder associated gene</td>
</tr>
<tr>
<td>Tateaki et al., 2004</td>
<td>Rat</td>
<td>Normal and fibrotic livers</td>
<td>IHC</td>
<td>Myofibroblasts and activated hepatic stellate cells</td>
<td>Cytoplasm</td>
<td>Fibrosis-associated gene</td>
</tr>
<tr>
<td>Kugelstadt et al., 2004</td>
<td>Chicken</td>
<td>Normal brain, muscle, liver, spleen, eye, heart tissues</td>
<td>Revert transcription PCR</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fordel et al., 2004</td>
<td>Mouse</td>
<td>Hypoxia skeletal muscle, heart, and brain tissue</td>
<td>qRT-PCR</td>
<td>ND</td>
<td>ND</td>
<td>Hypoxia-responsive gene</td>
</tr>
<tr>
<td>Schmidt et al., 2005</td>
<td>Mouse</td>
<td>Eye</td>
<td>IF</td>
<td>Fibroblasts</td>
<td>Cytoplasm</td>
<td>Respiratory protein</td>
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<tr>
<td>Xu et al., 2006</td>
<td>Rat</td>
<td>Primary HSCs</td>
<td>qRT-PCR and IHC-PCR</td>
<td>HSCs</td>
<td>ND</td>
<td>Protects HSCs against oxidative stress-induced activation and inhibits tissue fibrosis</td>
</tr>
<tr>
<td>Mammen et al., 2006</td>
<td>Mouse</td>
<td>Embryogenesis, normoxic and hypoxic brain</td>
<td>In situ hybridization, RT-PCR and Northern blot</td>
<td>Distinct regions of brain</td>
<td>Nuclear</td>
<td>Oxygen-responsive tissue, hemoglobin</td>
</tr>
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<td>McDonald et al., 2006</td>
<td>Human</td>
<td>Esophageal biopsy specimens</td>
<td>RT-PCR and bisulphite pyrosequencing</td>
<td>ND</td>
<td>ND</td>
<td>Downregulation of CYGB associated with esophageal tylosis disease</td>
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<td>Xinarianos et al., 2006</td>
<td>Human</td>
<td>Human non-small-cell lung carcinoma tissues</td>
<td>mRNA level and promoter methylation analysis</td>
<td>ND</td>
<td>ND</td>
<td>Candidate tumor suppressor gene</td>
</tr>
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<td>Li et al., 2007</td>
<td>Mouse</td>
<td>H(_2)O(_2) treated neuroblastoma cell line</td>
<td>RT-PCR</td>
<td>Epithelial cells</td>
<td>ND</td>
<td>Protect cells from oxidative -stress-mediated injury</td>
</tr>
<tr>
<td>Fordel et al., 2007b</td>
<td>Mouse</td>
<td>Hypoxic brain, liver, heart, skeletal muscle, eyes</td>
<td>qRT-PCR</td>
<td>ND</td>
<td>ND</td>
<td>ROS scavenger</td>
</tr>
<tr>
<td>Hodges et al., 2008</td>
<td>Human</td>
<td>Neuronal cell line TE671</td>
<td>Transfection of cells with the cytoglobin-GFP fusion protein</td>
<td>Neuronal cell</td>
<td>Cellular and Nuclear</td>
<td>ROS scavenger, survival rate enhancer; free-radical-mediated DNA damage</td>
</tr>
<tr>
<td>Man et al., 2008</td>
<td>Mouse</td>
<td>Fibrotic liver</td>
<td>IHC</td>
<td>HSCs and fibroblasts</td>
<td>Cytoplasm and nuclear</td>
<td>Liver-fibrosis-related gene</td>
</tr>
</tbody>
</table>
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.

**Role of Cygb in liver diseases**

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).

<table>
<thead>
<tr>
<th>Reference</th>
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<th>Location</th>
<th>Functional description</th>
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<td>Chua et al., 2009</td>
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<td>Breast cancer cell line MCF-7</td>
<td>PCR array</td>
<td>Epithelial cells</td>
<td>ND</td>
<td>Oxidative-stress-related gene</td>
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<tr>
<td>Shaw et al., 2009</td>
<td>Human</td>
<td>Samples from oral or oropharyngeal squamous cell carcinoma patients; human sporadic head-and-neck squamous cell carcinoma (HNSCC) cell lines</td>
<td>qRT-PCR, pyrosequencing analysis</td>
<td>Epithelial cells</td>
<td>ND</td>
<td>Promoter methylation and tumor hypoxia regulates CYGB expression</td>
</tr>
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<td>Avivi et al., 2010</td>
<td>Rat and the blind mole rat Spalax</td>
<td>Normoxic and hypoxic brain, heart, and liver</td>
<td>IF and qRT-PCR</td>
<td>Fibroblast-like cells and neurons</td>
<td>Cytoplasm of fibroblast-like cells and nucleus of neurons</td>
<td>Cytoprotective effect under pathological hypoxic/ischemic conditions in mammals</td>
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<td>Mimura et al., 2010</td>
<td>Rat</td>
<td>Normal and fibrotic kidney</td>
<td>IHC</td>
<td>Interstitial cells</td>
<td>Cytoplasm</td>
<td>Protects tissues from ROS</td>
</tr>
<tr>
<td>Gardner et al., 2010</td>
<td>Rat</td>
<td>Rat hepatocyte expressed human Cygb, and recombinant human CYGB protein</td>
<td>Nitric oxide consumption assay</td>
<td>Rat hepatocyte</td>
<td>ND</td>
<td>NO dioxygenase</td>
</tr>
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<td>Fang et al., 2011</td>
<td>Human</td>
<td>Glioma cells</td>
<td>Fluorescence transfection and measurement assay</td>
<td>Glioma cells</td>
<td>Partial nuclear</td>
<td>Protect cells from oxidative stress induced cell injury; putative tumor suppressor function</td>
</tr>
<tr>
<td>Thuy le et al., 2011</td>
<td>Mouse</td>
<td>UEN-treated Cygb-knockout livers</td>
<td>mRNA and protein levels</td>
<td>HSCs</td>
<td>Cytoplasm</td>
<td>Tumor suppressor gene</td>
</tr>
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<td>Basu et al., 2012</td>
<td>Human</td>
<td>Various prostate normal and cancer cell lines</td>
<td>mRNA PCR array, Immunoblot</td>
<td>Epithelial cells</td>
<td>ND</td>
<td>Stress respond gene</td>
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<td>Liu et al., 2013</td>
<td>Human</td>
<td>Human recombinant CYGB protein</td>
<td>Model of O$_2$-dependent NO consumption by Cygb</td>
<td>ND</td>
<td>ND</td>
<td>NO consumption</td>
</tr>
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<td>Hundahl et al., 2013</td>
<td>Human and rat</td>
<td>Hippocampus</td>
<td>IHC and in situ hybridization</td>
<td>Neuronal cell</td>
<td>Cell soma and processes</td>
<td>NO metabolism</td>
</tr>
<tr>
<td>Motoyama et al., 2014</td>
<td>Human/mouse</td>
<td>Human normal, and fibrotic livers; primary mouse HSCs</td>
<td>IHC</td>
<td>HSCs</td>
<td>Cytoplast</td>
<td>Inversely correlated with fibrosis stage</td>
</tr>
<tr>
<td>Thuy le et al., 2015</td>
<td>Human</td>
<td>Normal, NASH and HCC</td>
<td>IHC and IF</td>
<td>HSCs</td>
<td>Cytoplast</td>
<td>Implicated on pathogenesis of NASH; protective role in the process of fibrosis and cancer development</td>
</tr>
</tbody>
</table>

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.
Role of Cygb in liver diseases

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 H₂O₂-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 rats 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; McDonald 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). McDonald 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 C-18Co colon 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-nonenal, 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 diethylnitrosamine (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-null) became enlarged, developed an αSMA network after 7 days in culture, and lost cellular lipid droplets more rapidly than did HSCs-Cygb-wild (Fig. 2). Moreover, HSCs-Cygb-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

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**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/mm² 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; McDonald 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 U2OS 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 nitrosyrosine) 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 protein 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, NFκB, NFAT), CCAAT/enhancer binding protein, and cellular erythroblastosis virus E26 oncoprotein 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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Role of Cygb in liver diseases


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