Review

Discovery of cytoglobin and its roles in physiology and pathology of hepatic stellate cells

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Abstract: Cytoglobin (CYGB), a new member of the globin family, was discovered in 2001 as a protein associated with stellate cell activation (stellate cell activation-associated protein [STAP]). Knowledge of CYGB, including its crystal, gene, and protein structures as well as its physiological and pathological importance, has increased progressively. We investigated the roles of oxygen (O2)-binding CYGB as STAP in hepatic stellate cells (HSCs) to understand the part played by this protein in their pathophysiological activities. Studies involving CYGB-gene-deleted mice have led us to suppose that CYGB functions as a regulator of O2 homeostasis; when O2 homeostasis is disrupted, HSCs are activated and play a key role(s) in hepatic fibrogenesis. In this review, we discuss the rationale for this hypothesis.

Keywords: cytoglobin, hepatic stellate cells, oxygen homeostasis, fibrosis, bioenergetics, reactive oxygen species

I. Introduction

In 2001, we discovered cytoglobin (CYGB) in hepatic stellate cells (HSC),1) which are important regulators of tissue repair in the damaged liver.

Normal mammalian adult tissues restore their architectural and functional integrity via regenerative or reparative processes. Regeneration is a process in which tissue parenchymal cells play a major role in reconstructing the lost part of the tissue, resulting in both structural and functional restoration. Repair is a process in which non-parenchymal connective tissue cells largely contribute to reconstructing the damaged tissue, resulting in structural but not functional restoration. Most adult tissues have lost their regenerative capacity and, thus, employ the repair process to regain structural integrity when damaged;2) this generally results in the formation of scar tissue composed mainly of a fibroblast-embedded disorganized extracellular matrix (ECM), which comprises largely collagen. Despite its high regenerative capacity throughout life, the liver undergoes restoration in which the reparative activities of the non-parenchymal cells gradually predominate over the regenerative capacity of the parenchymal tissues when exposed to damage that causes prolonged inflammation; as a result, the liver becomes fibrogenic and, in the worst-case scenario, cirrhotic and tumorigenic. HSCs play major roles in both regeneration3) and repair,4) indicating that these cells respond to stresses on the liver by becoming activated.5)

To investigate the biochemical aspects of HSC activation, we performed a comparative proteomic analysis of normal and activated rat HSCs (A-HSCs),6) which led to the discovery of “stellate cell activation-associated protein” (STAP)1) [later renamed cytoglobin (CYGB)]. CYGB is the fourth member of the globin family, which previously consisted of hemoglobin (Hb, first member), myoglobin (Mb, second), and neuroglobin (Ngb, third),
whose common feature is their high affinity for molecular oxygen (O$_2$) through the heme iron located at the center of the porphyrin ring. A fifth member, named androglobin, was identified recently. The discovery of CYGB led us to study its biological and pathophysiological importance in the histological homeostasis of the liver and to rethink the biological features of HSCs as a cell type that harbors O$_2$-bearing globin, especially in relation to their activation upon hepatic insult. Above all, we reconsidered the process of HSC activation as the adaptive and protective reactions of HSCs to the disturbance of intracellular O$_2$ homeostasis caused by insults to the liver. This consideration appeared to be challengeable because, unlike Mb-expressing cells, which are highly contractile (e.g., cardiac and skeletal muscle cells), the hitherto-known functions of HSCs and their activation were not fully discussed from the viewpoint of the intracellular O$_2$ homeostasis of HSCs. However, in this context, it is appropriate to cite the study conducted by Ankoma-Sey et al. in which HSCs were recognized as an “O$_2$-sensing cell type” because these cells are activated under hypoxic conditions. In 2000, the cited authors predicted the presence of an “intracellular” hypoxia-inducible heme-based oxygen sensor responsible for the hypoxic induction of VEGF in HSCs. Our discovery of CYGB fulfilled this prediction. In this review, we consider and discuss the physiological and pathological roles of CYGB in the regulation of HSC activities in relation to their intracellular O$_2$ homeostasis. There is also the possibility that CYGB of HSCs might create the regional gaseous milieu in hepatic parenchymal tissues, which is not discussed in this review, because experimental data relevant to this possibility have not been currently sufficient, and will be considered elsewhere in future.

II. Cell types containing globin

Oxygen is essential to eukaryotes, which acquire free energy by aerobic respiration. Globins are “respiratory” globular proteins containing heme, a coordination complex of the porphyrin ring with an iron ion. O$_2$ is bound between the iron ion and histidine residues of the polypeptide chain of globin. Two types of globin had been identified in vertebrates before the discovery of neuroglobin (Ngb) in 2000: the first globin, Hb, was discovered in 1840 by Hunefeld. About 60 years later, the second globin (Mb) was discovered by Mörner. It should be noted that >100 years separated the discovery of the second and third globins (>150 years since the discovery of Hb). The aforementioned roles of globin proteins in O$_2$ carriage and storage were proposed, tested, validated, and gradually generalized during this time. These initial two globins could be termed “classical” globins. The recent consecutive discovery of intracellular globins, Ngb and CYGB, which we term “new” globins, is biologically important because the presence of globin proteins in “common” cells other than muscle cells resulted in the modification of the above notion regarding O$_2$ utilization by cells. The presence of Ngb in neurons could be rationalized within the classical framework, because neurons are an exceptional cell type in terms of their energy requirements. The brain has a greater requirement for energy compared with other organs. Despite the fact that the brain comprises only ~2% of the weight of the body, it consumes ~20% of the available oxygen, even at rest. Thus, the question of whether HSCs are a high-energy-consumption cell type arises. This may, indeed, be the case, as HSCs exhibit a muscle-cell-type phenotype (contractility), especially when they are activated.

When stressed, HSCs activate themselves and transform into a type of myofibroblast (MFB), which are highly contractile. Based on the relationship between Mb and O$_2$ homeostasis in muscle cells, this aspect of HSCs is one reason we were interested in the function of CYGB in relation to O$_2$ homeostasis. In this review, we reconsider the biological and pathophysiological functions of HSCs from the viewpoint
of CYGB-mediated energy homeostasis and address which function(s) of HSCs are associated with the presence of intracellular globin (CYGB). Additionally, we propose a rationale for the presence of CYGB in HSCs and present evidence that HSCs are a high-energy demanding cell type and are sensitive to disruptions in O₂ homeostasis in their pathophysiological context.

III. Functions of HSCs

HSCs, which are among the liver’s major non-parenchymal cell types, are located between the basolateral surface of hepatocytes and the anti-luminal side of sinusoidal endothelial cells. The HSC population is considerably smaller than that of hepatocytes; these comprise ~4% and ~65% of cells, respectively, in the healthy human liver. It should be noted that HSCs are supplied primarily by portal veins that convey relatively “hypoxic blood,” as the hepatic hypoxic microenvironment is a major contributor to the activation of HSCs, and we assume that the major function of CYGB in HSCs is as a regulator of O₂ homeostasis.

Since the first description of HSCs in 1876 as liver Sternzellen (star-shaped cells), this cell type has been assigned various names, such as perisinusoidal cells, Ito cells, lipocytes, parasinusoidal cells, and fat-storing cells; however, they have been termed HSCs since the agreement in 1996. Normal HSCs have two features. First, they contain large amounts of vitamin A and retinoids as intracellular lipid droplets (LDs), which is why they were once called “lipocytes” and historically identified using the gold chloride method of detecting vitamin-containing droplets. Second, these cells are major synthesizers of hepatic ECM, regulate hepatic fibrogenesis, an important process in wound healing that generally occurs when an organ is damaged, and replace injured tissues with fibrotic ECM. Hepatic fibrosis (the accumulation of largely collagen fibers) occurs when the liver is chronically damaged. Kent et al. first showed that lipocytes with vitamin A fluorescence accumulate in fibrotic regions of CCl₂-treated rat livers and that vitamin A autofluorescence and fat droplets decrease as fibrosis proceeds. They proposed that the lipocyte is a precursor of the fibroblasts responsible for parenchymal fibrogenesis (i.e., HSCs are activated by liver damage and transdifferentiate to collagen-producing fibroblasts). This idea is now widely accepted. The notion that HSCs are a major collagen-producing cell type in the normal liver was experimentally verified in 1984 by Senoo et al. and 1985 by Friedman et al. who also pointed out that normal HSCs exhibit not only fibroblastic characteristics but also smooth muscle cell-like features, such as the production of basement membrane collagen (type VI collagen) and the expression of the intermediate filament protein desmin. These previous studies provide a unique biological image of HSCs, a fibroblastic but also a lipocyte- and smooth-muscle-cell-like type of cell with a high retinol capacity.

IV. Activation of HSCs

HSCs have two faces, a quiescent and an activated face. Under healthy (non-stressed) conditions, HSCs are called “quiescent” (Q-HSCs) to distinguish them from HSCs that have been transformed into activated HSCs (A-HSCs) and to distinguish A-HSCs from myofibroblasts (MFbs), the highly contractile fibroblasts residing in the portal mesenchyme in the liver. A-HSCs are often dubbed “MFb-like cells” because of their morphological resemblance to MFbs. Both A-HSCs and MFbs are highly proliferative. Puche et al. presented direct evidence that Q-HSCs can transform into A-HSCs and that these Q-HSC-derived A-HSCs significantly amplify the response to liver injury. The cited authors also showed that hepatic fibrogenesis depends largely on whether Q-HSCs are able to transdifferentiate to “proliferative Q-HSCs (A-HSCs).” Generally, wound healing begins with inflammation. Although HSCs are the type of cell responsible for inflammation-triggered fibrogenesis, they are less involved in the initial inflammation reaction itself. HSCs likely make only minor contributions to overall inflammation in vivo. It is worth emphasizing the importance of the role of HSCs in human hepatic tumor development, because liver cirrhosis is the most important mechanism of human hepatocarcinogenesis: the incidence of HCC in non-cirrhotic human livers is only 15–20% of all cases; these figures stand in contrast to the comparable values for rodents, in which liver tumorigenesis without fibrosis and cirrhosis is common.

The phenomenon of the spontaneous activation of cultured Q-HSCs should be noted given that we discovered CYGB was associated with HSC activation. Attempts to isolate and cultivate HSCs began in the early 1980s. Since then, HSC culture has been fundamental in determining the characteristics of these cells. Soon after the advent of HSC culture technology, researchers realized that HSCs in primary culture progressively lose lipid/vitamin A
droplets, become proliferative, and increase ECM production, all of which are phenotypic alterations seen in A-HSCs in the fibrogenic liver in vivo.\textsuperscript{23,31–33}\linebreak\vspace{1pt} When HSCs are cultured, they gradually alter their original morphological phenotypes (such as loss of LDs and spines) and take on the appearance of MFBs as culture proceeds. These cells also change function-related phenotypes by increasing the expression levels of various mRNAs/proteins, such as hepatic fibrogenesis-related proteins and \(\alpha\)-smooth muscle action (\(\alpha\)-SMA). This process is accompanied by drastic phenotypic changes of cultured Q-HSCs and is termed “spontaneous activation of Q-HSCs,” because the newly acquired phenotype resembles that of MFBs. Although the mechanism of spontaneous activation remains unclear, Q-HSCs in culture are in an artificial environment and are thus in a “stressed state,” which results in their activation. Indeed, although the spontaneous in vitro activation of HSCs is a simple and useful model for investigating the mechanism underlying their activation following hepatic injury in vivo, it should be remembered that this model likely recapitulates some, but not all, aspects of in vivo activation.\textsuperscript{34}

V. HSCs as a “high-energy-consuming” cell type: the contractile and collagen-producing nature of HSCs

Mitochondria consume \(\sim 90\%\) of the \(O_2\) absorbed by mammalian cells under the following “standard” conditions: the individual is an adult and is awake but is resting, stress-free, not digesting food (prior food intake being at or around maintenance level), and maintained at a temperature that elicits no thermoregulatory effect.\textsuperscript{35} Of the \(90\%\) of \(O_2\) taken up by mitochondria, \(80\%\) is used for ATP synthesis.\textsuperscript{35} Major energy-requiring cellular activities under the standard conditions are protein synthesis, membrane transportation of \(Na^+–K^+\) and \(Ca^{2+}\), and cell motility-related activities, which consume \(\sim 25–30\%, 19–28\%, 4–8\%, \) and \(2–8\%, \) respectively, of the total ATP generated.\textsuperscript{35} HSCs are the sole hepatic cell type with the capacity to actively produce ECM when necessary under normal physiological conditions. Moreover, when activated, HSCs can produce abundant ECM material under pathological conditions.\textsuperscript{36,37} Production of type I collagen, a predominant constituent of fibrous tissues, consumes more energy than production of other common proteins, such as globular proteins, due to its unique chemical and structural features. Type I collagen has a triple-helical structure known as the collagen triple-helix, stabilization of which requires numerous intramolecular hydrogen bonds.\textsuperscript{38} In addition, collagen fibril and fiber formation also requires energy-consuming processes, such as cross-linking of collagen molecules and association with other ECM matrix components. Thus HSCs as the major producer of type I collagen have a relatively high energy requirement. Therefore, the functions of activated HSCs, especially the increase in collagen synthesis and acquisition of robust contractility, require an abundant ATP supply.

Hepatocytes, stellate cells, and sinusoidal endothelial cells are intimately associated and form a biological unit called “the stellate cell unit” or, in brief, “the stellon.”\textsuperscript{39,40} Such anatomical location and histological features suggest that, together with sinusoidal cells such as endothelial cells, HSCs play a role in regulating sinusoidal blood flow due to their contractile capacity.\textsuperscript{41} We reported previously that normal Q-HSCs in rat livers express \(\alpha\)-SMA.\textsuperscript{6} as do those in human livers.\textsuperscript{42} This contractility of quiescent stellate cells might reflect their physiological role in maintaining normal hepatic activity by regulating sinusoidal resistance and, consequently, blood flow, by contracting around sinusoids.\textsuperscript{41}

The contractile nature of HSCs was demonstrated using in vitro experimental models. First, HSCs were cultured in a monolayer on silicon rubber-coated coverslips. In this model, these cells contracted the substrate (silicon rubber), which resulted in the formation of wrinkles. HSC contractility was demonstrated utilizing this wrinkle as a visible surrogate marker.\textsuperscript{43,44} “Collagen culture” has been used to demonstrate the contractility of fibroblasts, in which fibroblasts are cultured in three-dimensional lattices of type I collagen gels placed in bacteriological dishes. The fibroblasts therein adhere to and contract the collagen fibrils, pulling the fibrils toward themselves.\textsuperscript{45,46} It is thought that culture of HSCs within collagen gels is more likely replicate the authentic milieu within the liver with respect to their contractility.\textsuperscript{41} The contractile nature of Q- and A-HSCs was first quantitatively compared using normal rat HSCs cultured in type I collagen gels,\textsuperscript{47} which suggested that Q-HSCs exhibit little or no contractility and that contractility is associated with their activation. These and other studies provide the following picture of HSC contractility. First, HSCs could activate themselves in vivo when the liver undergoes fibrogenesis; second, spontaneous activation of HSCs in culture could be a faithful model of their in vivo activation in the fibrogenic liver; and third, Q-HSCs are considered
not to be contractile or their contractile capacity is weaker than that of A-HSCs. Currently, we consider that activation of Q-HSCs requires a large amount of energy. Moreover, A-HSCs have a high energy demand for synthesis of contractile proteins and collagens, Ca\(^{2+}\) mobilization, and generation of actomyosin-mediated contractile force.

VI. Metabolic reprogramming in relation to the bioenergetics of HSC activation

The activation of Q-HSCs to A-HSCs is a global epigenetic remodeling process in which suppression of adipogenesis and retinoid metabolism and stimulation of fibrogenesis, mitosis, and contractility proceed simultaneously. It seems apparent that these drastic phenotypic changes are energetically costly for the cells.\(^{48}\) Therefore, Q-HSCs likely contain a mechanistic entity required to overcome energy-dependent epigenetic barriers to transdifferentiate to A-HSCs.\(^{49}\)

What is the source of the energy required for HSC activation? And how do HSCs utilize this energy source when they are to be activated to respond to pathophysiological requirements? To our knowledge, no systematic study has been performed; thus, we discuss major aspects of the energetics of HSC activation by introducing several studies that addressed alterations of energy metabolism during HSC activation.

When tissues are damaged, their microenvironment becomes hypoxic in the early phase of “post-damage reactions” due mainly to disturbed vascular circulation, which induces production of a transcription factor, hypoxia-inducible factor (HIF).\(^{50}\) HIF plays an important role in regulating the molecular signaling underlying cellular-level alterations, including inflammatory reactions. HIF-regulated signaling contributes to the transition from Q-HSC to A-HSC, as HSC activation appears to proceed in the hypoxic microenvironments generated in the damaged liver.\(^{51}\) Indeed, upregulation of HIF-1-\(\alpha\) mRNA expression in activating HSCs was reported in cultured rat primary HSCs,\(^{52}\) the peak stimulation (>10-fold compared to day 0) being at day 2 of culture. The cited study also suggested that activating HSCs were intracellularly hypoxic.\(^{52}\)

HIF-1\(\alpha\) reduces mitochondrial respiration\(^{53},^{54}\) by suppressing both the Krebs cycle and oxidative phosphorylation and inducing pyruvate dehydrogenase kinase to shunt pyruvate away from mitochondria.\(^{55}\) Instead, HIF-1\(\alpha\) stimulates glycolytic energy production by inducing genes for glucose transporters, such as GLUT1 and GLUT3; glycolytic enzymes, including lactate dehydrogenase, the enzyme that produces lactate and NAD\(^{+}\) from pyruvate; and monocarboxylate transporter 4, which is responsible for lactate secretion from cells.\(^{53},^{56}\)

Taken together, these studies suggested that HSCs change their glucose metabolism from mitochondrial respiration to glycolysis during their activation in the damaged liver. A recent study supported this prediction by demonstrating that Q-HSCs exhibit altered glucose metabolism during a very early phase of their transition to A-HSCs in both in vitro and in vivo HSC activation models.\(^{52}\) The genes encoding key enzymes that regulate the glycolysis pathway—such as hexokinase 2 (HK2), phosphofructokinase (PFKP), and pyruvate kinase M2 (PKM2)—are markedly upregulated before activation of A-HSC marker proteins, such as \(\alpha\)-SMA. This strongly suggests that energy metabolism of Q-HSCs becomes glycolytic prior to the change in their phenotype to A-HSCs.\(^{52}\)

Indeed, intracellular levels of lactate are rapidly increased in the in vitro HSC spontaneous activation model.\(^{52}\) Thus, Q-HSCs undergoing activation are thought to switch their energy metabolism from mitochondrial respiration to glycolysis despite the presence of sufficient O\(_2\) (the so-called Warburg effect, aerobic glycolysis). These results seem to be consistent with the fact that A-HSCs undergo active growth, because highly proliferative cells (including cancer cells) show high glycolytic activity.\(^{57}\) The Warburg effect (aerobic glycolysis) has been identified in cancer cells as the paradoxical dependence of cancer cell growth on glycolysis.\(^{58}\) The reprogramming toward glycolytic metabolism seems to be a prerequisite for Q-HSC to A-HSC differentiation for the following reasons: first, addition of 2-deoxyglucose, an inhibitor of glycolysis, to HSC cultures resulted in decreased proliferation, repressed expression of A-HSC genes, re-expression of lipogenic genes, and re-accumulation of lipids.\(^{52}\) Second, similar results were observed when FX11, an inhibitor of LDHA (lactate dehydrogenase A), the enzyme that converts pyruvate into lactate, was included in HSC cultures.\(^{52}\)

The metabolic reprogramming from mitochondrial respiration (Q-HSCs) to glycolysis (A-HSCs) is thought to require hedgehog (Hh) signaling, because cyclopamine, which blocks hedgehog signaling by binding to Smoothened (Smo), inhibited the accumulation of the PKM2-positive cells generated when liver repair is provoked by acute partial hepatectomy.\(^{52}\) Hh was originally identified as a hypoxia-tolerance gene in Drosophila as a model system.\(^{59}\)
A study using adult mice showed that hypoxia per se can induce a rapid systemic Hh response, including expression of the pathway ligand, sonic hedgehog (SHH) and its receptor, Patched1, in various organs. In vitro studies have shown that this rapid Hh-response to hypoxia was preceded by the accumulation of HIF-1α, suggesting that hypoxia is translated into an Hh response through HIF-1α.

However, HSCs that had undergone spontaneous self-activation and, thus, been glycolytic in terms of energy metabolism, exhibited dramatically increased numbers of mitochondria: ~8 mitochondria per cell at day 0 (primary Q-HSCs) versus >~175 mitochondria per cell at day 7 (spontaneously A-HSCs). This does not support the above-mentioned prediction (based on the general functions of HIF-1α) that the mitochondrial activities of A-HSCs are suppressed. It is tempting to speculate that HSCs utilize lactate as a material for their growth and mitochondrial ATP for energy-demanding activities, including their contractility-dependent functions, such as migration and connective tissue remodeling; however, this remains to be determined.

There have been studies that support the notion that hypoxia-HIF-1α-Hh signaling is involved in the increased contractility of A-HSCs. The Hh pathway regulates the cellular contractility of carcinoma (cancer)-associated fibroblasts (CAFs), the major cell type in the tumor stroma in which cancer cells reside and the cell type that exhibits myofibroblastic characteristics with a prominent contractile capability.

Recently, it was reported that the activities of CAFs in lung cancer are regulated by Forkhead Box F1 (FoxF1), a member of the FOX transcription factor family. A recent analysis of conserved non-coding sequences surrounding Fox1 and Fox11 identified Gli1-binding sites, one of which was shown to actually bind to Gli proteins, confirming FoxF1 as a direct target of Hh signaling. CAF and other fibroblasts exhibit increased contractility when stimulated by TGF-β and PDGF. FoxF1-expressing fibroblasts showed a significantly increased contractile force compared with controls. These results strongly suggest a role for the Hh signaling pathway in regulating fibroblast contractility through controlling the activity of FoxF1. Another study supported the presence of Hh-FoxF1 signaling in A-HSCs. Foxf1−/− mice treated with CCl4 displayed abnormal liver repair and diminished activation of hepatic stellate cells, which was associated with diminished induction of A-HSC-associated genes, such as type I collagen and α-SMA.

VII. Lipid/retinoid droplets as possible energy sources for activation of HSCs

Generally, LDs are present as intracellular lipid reservoirs in most eukaryotic cells and serve as sources of building blocks of membranes or as substrates for energy metabolism. Most eukaryotic cells utilize lipids as basic substrates for energy homeostasis and possess LDs (also called lipid bodies, fat bodies, or adiposomes) in their cytoplasm. Some mammalian cell types concentrate LDs at relatively higher abundance, among which adipocytes are the most highly specialized for lipid and energy storage. LDs have been recognized as simple cytosolic structures that passively store triglycerides (TGs) and cholesterol, but recent proteomic studies have resulted in these lipid-rich structures being regarded as organelles that perform a variety of biological functions. Identification of LD-specific proteins such as caveolin (cholesterol-binding protein) and perilipins (LD membrane protein) have contributed to our knowledge of their biological and pathophysiological functions. Recently, it was reported that LDs are substrates for autophagy (“macroautophagy”), a lysosomal pathway by which intracellular organelles and proteins are degraded to supply the cell with energy and maintain cellular homeostasis.

Compared with other LD-rich cell types, the roles of LDs in HSCs are poorly understood despite the fact that HSCs are the major source of retinoids in the body and a lipid-rich cell type. Approximately 70% of the total body retinoid is present in the liver, 90–95% of which is stored as retinyl ester (RE) in the LDs of HSCs, indicating that HSCs are the location of the vast majority of the body’s retinoid stores. Most studies of LDs of HSCs have been conducted to characterize the retinoid moiety of LDs but not their lipid moiety. Lipids in HSCs are thought to be mere carriers of retinoids (e.g., vitamin A, which is otherwise metabolically unstable) and not to be used as energy sources. This is in contrast to adipocytes, which utilize LDs as an energy source. However, recently a few studies have reported, suggesting that Q-HSCs under activation and/or A-HSCs break down LDs and liberate free fatty acids as an energy source.

The classical pathway of lipid metabolism is mediated by cytosolic lipases. Autophagy is a process of self-eating (self-cannibalization), in which cells capture their own cytoplasm and organelles, consume them in lysosomes, and utilize the breakdown products as inputs to cellular metabolism for energy.
generation and protein and membrane synthesis. A study of hepatocytes demonstrated a “lipophagy” process, in which LDs are utilized as substrates for autophagy. Thoen et al. first examined whether lipophagy is required for the transformation of Q-HSCs to A-HSCs. LC3, microtubule-associated protein 1 (MAP1) light chain 3, is a mammalian homologue of yeast “autophagy-related gene (ATG) 8 (Atg8).” It is processed to LC3-II through post-translational modification and lipidation by ubiquitination-like reactions mediated by Atg7 and Atg3, which are bound to both the outer and inner membranes of the autophagosome. Thus, LC3-II can be used to monitor autophagosome formation. LC3 fused with monomeric red-fluorescence protein (mRFP-LC3) was found to exhibit a different localization profile from that fused with GFP (GFP-LC3): the former retained fluorescence through fusion with lysosomes, but the latter lost it due to the acidic conditions of the lysosome. Therefore, the autophagosome maturation process can be visualized using mRFP-GFP tandem fluorescence-tagged LC3. Immunoblotting for LC3B, a marker of autophagosomes, in liver samples from CCl4-treated and control mice showed that the number of autophagosomes was significantly increased in fibrotic livers. Then, the cited authors investigated whether autophagic flux is increased during spontaneous HSC activation in vitro using fresh mouse HSCs electroporated with a plasmid harboring DsRed-GFP-LC3B. Autophagic flux was estimated by monitoring red and green fluorescence. The results indicated that autophagic activity significantly increased during the activation process. In addition, they showed that mouse HSCs in primary culture exhibited suppressed activation-associated phenotypic alterations, such as in the morphology and expression of α-SMA, Col1α1, and PDGFR-β, when bafilomycin (which inhibits the fusion between autophagosomes and lysosomes by acting as a suppressor of lysosomal acidification) was included in culture medium. Therefore, this study demonstrated the involvement of LDs in the activation of HSCs.

One year later, another study reported using autophagy-related gene-deleted mice to test the hypothesis that autophagy promotes HSC activation. The Atg7fl/fl,GFAP-cre transgenic mouse line was produced by crossing Atg7fl/fl mice with mice expressing cre recombinase under the control of the promoter of the glial fibrillary acidic protein gene (GFAP, expressed in various cell types, including HSCs). Wild-type and transgenic mice were treated with CCl4 to induce liver fibrosis. Compared with wild-type mice, Atg7fl/fl,GFAP-cre mice showed attenuation of CCl4-induced liver fibrotic changes, as assessed by Sirius Red staining and the total α-SMA protein quantity in liver specimens and the COL 1 quantity in isolated HSCs. The effect of Atg7 on HSC activation was further examined using the JS1 mouse immortalized HSC cell line. These cells in culture were treated with 3-methyladenine (3-MA), an inhibitor of autophagy, or transduced with siRNA for Atg7. As expected, the treated JS1 cells exhibited increased LD accumulation compared with control cells. Importantly, the ATP contents of 3-MA-treated HSCs were significantly lower than those of untreated cells, suggesting not only that HSC activation is an energy-requiring process but also that the energy for activation is supplied by the autophagy of LDs.

These studies on the relationship between HSC activation and LD autophagy support the notion that HSCs require a relatively high level of energy for their physiological and pathological functions. Another study, in which the effect of mitochondrial uncoupling on the process of Q-HSC activation was investigated, supported this. Primary mouse HSCs were cultured in the presence or absence of two chemical uncouplers—carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) or valinomycin. Either of these uncouplers decreased the ATP content per cell to ~70% of that of untreated cells. Interestingly, these uncouplers also inhibited the spontaneous activation of HSCs in terms of the expression of α-SMA, proCol1α1, and PDGFR-β and DNA synthetic activity. The cited authors claimed that mild mitochondrial uncoupling inhibits culture-induced HSC activation. This finding confirms that HSC are a high-energy-demanding cell type, activation of which requires a certain level of activity from energy (ATP)-generation systems, such as those in mitochondria.

**VIII. Roles of mitochondria in HSC activation**

Mitochondria are a cellular energy-producing center in which >90% of the ATP required by cells is produced. In addition, as described above, it has been suggested that mitochondrial activities are involved in the process of HSC activation.

Most mitochondria-related studies of HSCs have addressed the roles of mitochondria in generating reactive oxygen species (ROS), which are thought to be a major causative agent of HSC activation. Damaged tissues generally become hypoxic compared with normal tissues due to the disturbance of local
circulation. A hypoxic microenvironment is considered to be an initiator and promoter of HSC activation. Mitochondria function as a direct O2-sensor through complex IV (cytochrome C oxidase complex) of the electron transport chain (ETC). The cited study showed that O2-sensing mitochondria paradoxically generate more ROS when intracellular O2 tension decreases (1% < P O2 < 5%), largely from ETC complex III (coenzyme Q-cytochrome c reductase), through a currently undetermined mechanism(s). The produced ROS was proposed to inactivate prolyl hydroxylase domain-containing protein (HPD), the enzyme responsible for the ubiquitin–proteasome system-mediated degradation of HIF-1α. HIF-1α is expressed in HSCs and may be stabilized under hypoxic conditions; it forms a heterodimer with the constitutively expressed HIF-1β subunit and functions as a nuclear transcription factor by activating downstream genes, including those related to HSC activation, such as those associated with glycolytic energy production (e.g., lactate dehydrogenase A). Damage-activated HSCs are thought to reduce mitochondrial respiration and biogenesis through hypoxia-induced HIF1.

Because ROS production requires O2 as a substrate, cells under hypoxic conditions are logically expected to decrease their ROS generation. However, studies that do not support this intuitive notion have accumulated. These works demonstrated that cells under hypoxia generate more mitochondrial ROS than those under normoxia. For example, the Hep3B human hepatoma cell line and ρ0-Hep3B cell line (mitochondria-deleted Hep3B cells) were cultured in media containing 1–8% O2, and their ROS generation capacities were evaluated. ROS production was increased by 1.5-, 4-, and 7-fold at O2 concentrations of 5%, 3%, and 1%, respectively. These responses to changes in O2 concentrations took place in mitochondria, because the ρ0-Hep3B cells did not show such responses, even at 1% O2. The hypoxia-dependent generation of mitochondrial ROS was reproduced using a fluorescence resonance energy transfer (FRET) sensor termed HSP (heat shock protein)-FRET to assess oxidant stress in cells subjected to hypoxia. The involvement of mitochondria in hypoxia-induced ROS generation was further validated in a study in which ρ0-Hep3B cells failed to generate hypoxia-induced ROS when their mitochondrial ETC was disrupted by transfection with a plasmid harboring the shRNA sequence of RISP (the Rieske iron-sulfur protein, which normally transfers an electron from ubiquinol to cytochrome c1 in complex III). However, the mechanism underlying the hypoxia-induced generation of ROS in mitochondria remains to be determined.

IX. Discovery of cytoglobin

We prepared two types of A-HSC from rat livers, in vivo- and in vitro-A-HSCs, together with their normal (quiescent) counterparts (in vivo- and in vitro-Q-HSCs) and subjected them to analysis of cellular and secreted proteins. Rats were treated with CCl4 for 8 weeks, and HSCs were isolated. These cells were plated on plastic culture dishes for 3 h, and the attached cells were obtained as in vivo-A-HSCs. Similarly, HSCs were isolated from normal (control) rats for in vivo- and in vitro-Q-HSCs. In parallel experiments, Q-HSCs were cultured for 9 days in fetal bovine serum (FBS)-containing medium, and the cells were collected as in vitro-A-HSCs. For analysis of the secretome of in vivo-A-HSCs, these cells were plated on culture dishes in FBS-containing culture medium and allowed to attach for 24 h; they were then cultured for an additional 2 days in FBS-free medium with PDGF. Culture medium was collected on the last 2 days for analysis of secreted proteins. For secretome analysis of in vitro-activated HSCs, HSCs from normal livers were cultured for 7 days in FBS-containing medium and cultured for an additional 2 days in FBS-free medium. Culture medium from the final 2 days was used for the analysis of secreted proteins. The proteomic analysis of cellular and secreted proteins from in vitro- and in vivo-activated HSCs identified 21 peptides, which included fragments of the same proteins, the expression levels of most of which were >2-fold those in quiescent cells. Among them, 12 and 9 peptides were cellular and secreted peptides, respectively.

Among the protein spots, one unknown spot with a PI of 6 and a molecular mass of 21 kDa was markedly upregulated in both in vivo and in vitro A-HSCs. Analysis of tryptic digests of this protein resulted in its identification as “stellate cell activation-associated protein” (STAP) by quadrupole-time-of-flight mass spectrometry and determination of two amino acid sequences: PGDME(I/L)ER and ANCEDVGVA. Cloning of rat STAP cDNA resulted in determination of its full amino acid and nucleotide sequences, which led us to conclude that STAP is a globin protein with ~40% homology to myoglobin. Immunohistochemical examinations using polyclonal antibodies against rat STAP revealed that it was expressed exclusively in HSCs in the liver. Experiments with recombinant rat STAP indicated that
STAP was a heme protein with peroxidase activity against hydrogen peroxide and lipid hydroperoxides. One year later, Hankeln and Hargrove identified new heme-globins termed cytoglobin and histoglobin, respectively, by mining the publicly available human and mouse express sequence tag (EST) databases using the amino acid sequence of the *Drosophila* globin as query in the former case and the EST and genomic sequencing data of *Mus musculus* and *Homo sapiens* for predicted genes harboring a globin domain in the latter. Sequence data and characteristics showed that these three types of globin were the same protein. This newly identified, but differently named, globin is now commonly called cytoglobin (CYGB) after Hankeln et al.

The crystal structure of CYGB shows many similarities to that of other globins, including the classic three-over-three alpha helical globin fold and a *P*O₂ of ~0.2 Torr, which is similar to that of Mb. However, the biological and physiological roles of CYGB are largely unknown and are the focus of intensive investigations. Aspects of its functions have been proposed based on studies using CYGB gene (CYGB-null mice) and CYGB over-expressing mice. The results suggest that CYGB plays a role in the fibrotic response in various organs, including the liver and kidney, as we predicted in the study that led to the discovery of CYGB (STAP).

Compared with Hb, Mb, and Ngb, the tissue distribution of CYGB is much broader, although seemingly restricted to specific cell types. Immuno-histochemistry and immunoelectron microscopy revealed that CYGB is uniquely localized in fibroblast-like cells in splanchic organs, namely the vitamin A-storing cell lineage, but it is not present in epithelial cells, endothelial cells, muscle cells, blood cells, macrophages, or dermal fibroblasts. However, it was later shown that CYGB is also expressed in esophageal cells, smooth muscle cells of the vasculature, and melanocytes.

**X. Biochemistry of CYGB**

The heme iron of the globin family has six coordination sites, four of which are involved in binding with the porphyrin ring and two of which are for exogenous ligands. Classic globins (Hb and Mb) are penta-coordinate in the deoxy-form: four sites (first to fourth) are coordinated with four N atoms of the porphyrin ring, the fifth site is coordinated with the proximal His residue of the globin protein, and the sixth site is vacant. Exogenous gaseous ligands, such as O₂ molecules, bind to this vacant sixth site. In contrast, the de-oxy form of CYGB (also Ngb) is hexa-coordinate in either its ferrous (Fe²⁺) or ferric (Fe³⁺) form, all six sites being coordinated with “endogenous” ligands, a status of bis-histidyl coordination. His E7 (distal His) is the internal ligand that coordinates with the sixth site of the heme iron. Therefore, CYGB does not have heme iron coordination sites for gaseous ligands, indicating that the binding of an external ligand to heme requires the dissociation of the “internal ligand” (His E7). In other words, O₂, for example, should compete with His E7 for binding to the sixth site.

However, despite the difference in their coordination structures, CYGB exhibits a high O₂ affinity that is comparable to that of Mb under physiological conditions due to CYGB’s conformation-dependency of the binding affinity between the six sites and the distal His. The unique presence of the six sites for internal ligands in hexa-coordinated globins suggests unique biological and physiological functions other than O₂ storage and delivery. This is because structural changes could be induced when an internal ligand is replaced with an external ligand. As a result, the interactions of this type of globin with other proteins may be markedly different, facilitating functions as a signaling and/or O₂-sensing protein. The crystal structure of CYGB suggests this protein is a cysteine-linked homodimer, with each heme pocket facing opposite sides of the dimer surface. In contrast, its quaternary structure in situ is unknown. A study that examined the structure of CYGB in solution at ~5 µM (practically 0.1–20 µM), a concentration that is thought to be comparable to that in situ, showed that CYGB was present mostly as the monomeric form.

CYGB could be a redox-sensitive protein, because it has two cysteine residues [at the B2 (cys38) and E9 (cys83) positions in the case of humans whose SH groups can be free (2 SHs) when the surrounding environment is reductive or form a disulfide bridge (S-S) in an oxidative environment. Moreover, it has heme iron, which is either Fe⁴⁺ in an oxidative environment or Fe³⁺ in a reductive environment. It is most probable that alterations in the redox state induce conformational changes in CYGB, which affects its interactions with gaseous ligands and other chemicals and proteins, a situation in which CYGB functions as a redox-regulated signaling molecule. CYGB with an intramolecular disulfide bridge [CYGB (S-S)] has a higher affinity for O₂ than does that with free cysteine residues [CYGB (SH-SH)]. In other words, O₂
affinity decreases when CYGB is reduced. This affinity alteration can be explained as follows. In CYGB (S-S), the S-S bridge exerts significant stress on the E-helix, which results in the preference of the distal His for O2 as a ligand in the sixth coordinate site of the heme iron. In contrast, after the breaking of the S-S bond of CYGB (S-S), the distal His relaxes toward a stable conformation, which makes the binding of the distal His to the sixth coordinate site easier. In other words, O2 binding to the sixth coordinate site is exposed to intense competition with the distal His. The oxygen affinity \( P_{50} \) of CYGB ranges from \( \sim 0.2 \) for CYGB S-S to \( \sim 2 \) Torr for CYGB 2SH, depending on the extent/strength of the S-S bonding. Based on these published experimental data, we consider that monomeric CYGB is prone to binding O2 when the intracellular microenvironment becomes more oxidative.

Heme iron is also sensitive to the redox condition, taking the \( \text{Fe}^{2+} \) or \( \text{Fe}^{3+} \) form, and is involved in regulating the biological functions of CYGB. Studies have suggested that the distal histidine coordination affinity of CYGB is lowered in \( \text{Fe}^{3+}-\text{CYGB} \) compared with its \( \text{Fe}^{2+} \)-counterpart at physiological pH values, indicating that the former has higher ligand-binding activity than the latter. Thus exogenous ligands can relatively easily access the sixth coordination site of \( \text{Fe}^{3+}-\text{CYGB} \), because the binding of its sixth coordination site to the distal His is “weak,” and the \( \text{Fe}^{3+}-\text{CYGB} \) shows partial penta-coordination (\( \sim 15\% \)).

CYGB binds lipids under appropriate redox conditions for the cysteine residues (Cys 38 and 83) and heme iron. Lipids such as oleate and cardiolipin were able to bind to CYGB (\( \text{Fe}^{3+} \)) but not CYGB (\( \text{Fe}^{2+} \)) with a stoichiometry of 1:1. Thus, under an oxidative environment in which CYGB is oxidized to the \( \text{Fe}^{3+} \) form, CYGB is considered to interact with membrane lipids to form biologically active cell-signaling molecules that might play a unique role in the regulation of cellular activities. For example, cardiolipin is a major constituent of the mitochondrial inner membrane and plays essential roles in regulating the activities of the enzymes involved in energy metabolism. A further study by the same research group demonstrated that monomeric CYGB with an internal disulfide bond between Cys38 (Helix E) and Cys83 (Felix B) can bind lipid molecules, but dimeric CYGB with intermolecular disulfide bonds and monomeric CYGB without an internal disulfide bond cannot. These studies show that CYGB is redox-sensitive through its iron ion and Cys residues. CYGB could regulate lipid metabolism under relatively oxidative conditions. For example, it could enhance lipid peroxidation, one of the major pathways that produce reactive products that exert oxidative stresses on cells. Major products of oxidized lipids are electrophilic in nature and are collectively termed reactive lipid species (RLS). The RLS produced as electrophilic lipids might have profound effects on cellular activities as damage-inducing pro-oxidants or as signaling molecules for physiological processes. Thus, it is conceivable that CYGB contributes to HSC fate determination (remaining quiescent or becoming activated) by changing lipid species (e.g., O2 and lipids) or by altering its affinity for ligands, depending on the intracellular redox status.

Mb and Hb can peroxidize lipids in the ferryl oxidation state by removing hydrogen from the polyunsaturated fatty acids. The aforementioned investigators demonstrated that \( \text{Fe}^{3+}-\text{CYGB} \) (S-S), but not dimeric CYGB (\( \text{Fe}^{3+}-\text{CYGB-S-S} \)), shows higher lipid peroxidase activity levels than does Mb in 0.1 M sodium phosphate buffer (pH 7.4). It was assumed that \( \text{Fe}^{3+}-\text{CYGB} \) is dominant over \( \text{Fe}^{3+}-\text{CYGB} \) in a normal cellular environment, because CYGB is more susceptible to reduction by ascorbate, which is known to rapidly reduce \( \text{Fe}^{3+}-\text{CYGB} \) to the \( \text{Fe}^{2+} \)-form, than are other types of globin, such as Mb and Hb. Taken together, these findings suggest that CYGB plays a role as a redox-sensitive cell-signaling molecule.

CYGB is present in relatively reduced microenvironments predominantly as \( \text{Fe}^{2+}-\text{CYGB} \) (2 SH), whose affinity for O2 is not high. When the microenvironment becomes more oxidative, the O2 affinity of CYGB increases. When the environment becomes more oxidative due to, for example, a shortage of NADPH and NADH, \( \text{Fe}^{3+}-\text{CYGB} \) (S-S) becomes dominant, a form in which the sixth coordination site becomes more easily accessible to exogenous ligands, such as lipids and related substances. CYGB in this situation could exhibit peroxidase activity or generate RLS. At low levels RLS, could function as a “beneficial” stimulant by inducing several physiological pathways, including resolution of inflammation and cellular antioxidants, whereas, at higher concentrations, it could be cytotoxic.

XI. Phenotypic analysis of CYGB-deleted mice

CYGB was discovered as a “stellate cell activation-associated protein” and was expected to be useful as a molecular marker that revealed new
aspects of the biological nature of Q-HSCs and the mechanisms underlying their activation. We generated CYGB-deficient mice by deleting exon 1 of the mouse CYGB gene using the Cre/loxP recombination system and backcrossing on the C57BL/6J background. The phenotypes of CYGB+/+ and CYGB−/− mice were then compared. CYGB may either stimulate or repress Q-HSC activation. We predicted the latter, because this protein showed peroxidase activity against hydrogen and lipid peroxides, which suggests CYGB to be an antioxidant.

The homozygote (CYGB−/− mice) appeared normal 1 month after birth, which is similar to wild-type mice (CYGB+/+ mice). However, tumors developed in several tissues and organs—such as lymphoid tissues and those in the lung, liver, heart, intestine, and kidney—as the CYGB−/− mice became older. The frequency of all abnormalities in 12–24-month-old CYGB−/− mice was 72%, significantly higher than the 6% in wild-type mice (manuscript in preparation). CYGB−/− liver tumors were immuno-histochemically diagnosed as hepatocellular carcinoma (HCC). These carcinoma cells robustly expressed γH2AX, a DNA damage marker. The appearance of the effects of CYGB-deletion on hepatocytes but not on HSCs was unexpected, because HSCs had been considered to be the sole cell type that expresses CYGB in the liver. This finding led us to ask how the absence of CYGB in HSCs induced hepatocytes to transform into HCC cells. Thus, we characterized the phenotypes of CYGB−/− HSCs isolated from young CYGB−/− mice.

HSCs were isolated from the livers of 12-week-old WT and CYGB−/− mice (HSCsCYGB-wild and HSCsCYGB-null, respectively) and cultured for up to 7 days (Fig. 1). Phase-contrast microscopic observations revealed that HSCs CYGB-null transformed to A-HSCs much earlier than did HSCs CYGB-wild (Fig. 1A). The former lost LDs and became dendritic earlier than the latter. HSCs CYGB-null were evidently α-SMA− as early as day 1, whereas HSCs CYGB-wild became faintly α-SMA+ only at day 4 (Fig. 1B). In addition, most HSCs CYGB-null were more weakly positive for Oil-Red-O staining than HSCs CYGB-wild at day 7 (Fig. 1B d, h), because, to our knowledge, few studies had investigated the roles of ROS in Q-HSC activation.

We examined the possibility that HSCs CYGB-null contained higher levels of ROS than the wild-type counterparts. The oxidative fluorescent dye dihydroethidine (DHE) is freely permeable to cells and is oxidized to 2-hydroxyethidium when superoxide anion is available and trapped by intercalating with DNA. DHE was included in the cultures of both types of HSCs and cells were examined under a fluorescence microscope. HSCs CYGB-null showed greater fluorescence compared with HSCs CYGB-wild at all time points of culture (Fig. 1C). We also characterized the HSCs in terms of their activation state. HSCs CYGB-null exhibited marked upregulation of A-HSC-related genes compared with wild-type cells after 1 day in culture. The upregulated genes included those associated with fibrogenesis, such as α-SMA, Col1α1, and Timp-1; cytokines, such as II-6, TNF-α, and II-1β; and chemokines, such as Cxcl1, 2, 5, and 7 and Ccl2, 3, and 4. Immunoblotting indicated increased expression of HO-1 and p-ERK in HSCs CYGB-null at 1 day. These data clearly show that HSCs CYGB-null can be qualified as A-HSCs or at least as HSCs undergoing transformation to A-HSCs. Therefore, we concluded that the absence of CYGB causes HSCs to generate ROS, which induces their activation. The absence of CYGB disturbs O2 homeostasis in HSCs and increases the frequency of the generation of hypoxia. Accordingly, mitochondria become active and generate more ROS. The increased level of ROS acts as a trigger for the activation of HSCs. A correlation between HSC activation and the increased level of ROS has been suggested by several studies. TGF-β has potent HSC-activating effects. Utilizing human LX-2 HSCs, we showed that these cells generate ROS upon exposure to TGF-β. Shah et al. demonstrated that TGF-β1-dependent HSC activation is mediated via ROS. ROS was also shown to participate in TGF-α and collagen type I-induced HSC activation, in which ROS signals were transmitted by c-myb and NF-κB.

An important implication of the scenario shown in Fig. 1 is that CYGB acts as a physiological suppressor of HSC activation. This was supported by an experiment in which HSCs CYGB-wild were transfected with CYGB-siRNA. These HSCs became morphologically enlarged and expressed higher levels of α-SMA mRNA and protein than control cells. Our study suggested that CYGB suppresses HSC activation not only intracellularly but also extracellularly, because HSCs CYGB-null treated with 100 µg/mL recombinant human CYGB for 72 h showed marked reductions in α-SMA mRNA and protein levels and maintained their quiescent morphological features.

Therefore, we speculate that CYGB-free HSCs are under pro-oxidative conditions due to endogenously generated ROS, which causes them to enter a “pre-activated state” in which the cells are sensitive to...
Fig. 1. Deletion of CYGB accelerates spontaneous activation of HSCs in vitro. HSCs were isolated from WT (wild) and CYGB−/− mice (null), both at the age of 12 weeks and cultured for up to 7 days. (A) The cells were observed through a phase contrast microscope at the indicated days. a–c, wild; d–e, null; a, b, d, and e, ×200 magnification; c and f, ×400 magnification. (B) Cells were subjected to double immunofluorescence staining for α-SMA (green) and CYGB (red) at the indicated days. Cells were counterstained by DAPI for nuclear staining (blue). Oil Red O staining was also made on the cells cultured for 7 days. a–d, wild, e–h, null; a–c and e–g, staining for α-SMA (green) and CYGB (red); d and h, Oil Red O staining; a–c and e–g, ×400 magnification. Scale bars in d and h, 100 µm. (C) Cells were subjected to fluorescent detection for ROS at the indicated days. Cells were treated with dihydroethium (DHE) for 1 hr before the indicated day and fixed for observation by a fluorescent microscope. a–c, wild; d–f, null; a–f, ×200 magnification. These figures are reprinted from the research article108) appeared in Am. J. Pathol., Vol. 185, Pages No. 1045–1060, Copyright (2015) with permission from Elsevier.
stresses generated intracellularly or extracellularly. Therefore, the loss of CYGB both in vitro and in vivo was considered to induce priming conditions in which the cells produced ROS and showed high levels of expression of fibroinflammatory genes. Therefore, we hypothesized that CYGB in HSCs functions as an antioxidant protein by suppressing endogenous ROS generation or by "scavenging" endogenously generated ROS, thus preventing HSC activation.

XII. Susceptibility of CYGB-deleted mouse livers to stresses

As described above, HSCs in CYGB-deleted mouse livers were in a "predisposed-to-activation" state. Our studies suggest that mouse livers with such pre-activated HSCs become "sensitized" to alterations that disturb liver homeostasis. The susceptibility to stress of livers harboring HSCs (CYGB-null) was demonstrated in two independent studies using CYGB-/- mice who were subjected to treatment with a carcinogen and consumed a high-fat diet. One-month-old CYGB-/- and wild-type C57BL/6J mice were administered 25-ppm N,N-diethylnitrosamine (DEN), a liver-specific carcinogen. The C57BL/6J mouse line is resistant to DEN-induced liver tumors. Deletion of CYGB (hetero- or homozygotically) was found to increase the sensitivity to the drug, as all mice developed liver tumors (100%) at 6 months after the initiation of DEN treatment; in contrast, the tumor incidence in the wild-type mice was 44%. The DEN susceptibility of CYGB-deficient mice was confirmed when the animals were administered a lower, non-toxic dose of DEN, 0.05 ppm, a concentration that did not induce tumor formation in wild-type mice at 36 weeks post-treatment. CYGB-deficient mice developed tumors, the incidence of which was dependent on the degree of deficiency, ~10% in CYGB+/-- and >40% in CYGB-/-, reinforcing the notion that CYGB has an anti-DEN (carcinogen) effect.

C57BL/6J mice administered a choline-deficient amino acid-defined (CDAA) diet develop fibrosis, systemic insulin resistance, and steatohepatitis, which is similar to the pathophysiology of human NASH. Our study showed that the CYGB-/- mouse liver was sensitive to the high-fat diet stress. WT and CYGB-/- mice at the age of 8 weeks were fed the CDAA diet continuously for 8, 16, or 32 weeks. Histological examination by staining with H&E, Sirius Red, Oil red O showed that, as early as 2 months after CDAA feeding, CYGB-deficient livers showed an accumulation of inflammatory cells, such as F4/80+ macrophages; collagen deposition, particularly along sinusoids; and lipid droplets. In contrast, WT mice exhibited only minor steatosis and no signs of fibrosis. These molecular and histological data indicated earlier HSC activation and liver fibrogenesis in CYGB-/- mice compared with wild-type controls.

XIII. Proposed functions of CYGB

Four possible functions of CYGB have been proposed: (1) O2 storage and transport; (2) ROS/reactive nitrogen species (RNS) scavenging; (3) cytoprotection against hypoxia stress, including detoxification of ROS (hydrogen peroxidase) and NOS (NO oxydgenase); and (4) tumor suppression. Functions 2 and 3 are apparently related. This categorization is closely related to the known exogenous ligands of CYGB: O2 (categories 1 and 2), CO and NO (category 3), nitrates (nitrate reductase, category 3), and lipids. Category 4 might be related to category 3 and 4, and has been known from the studies that showed the relationship between tumorigenesis and the silencing (loss of heterozygosity) of CYGB located at the chromosomal locus (17q25). However, whether a common mechanism(s) underlies these functions or CYGB is multifunctional in nature remains unknown. We discuss the possible functions of CYGB from the viewpoint of HSC energetics based mainly on currently available experimental data obtained from our studies as well as on relevant data from other research groups.

Generally, together with RNS, ROS is associated with the initiation and progression of inflammation, which could progress to fibrogenesis and tumorigenesis. Analyses of markers and indicators of ROS-related agents have supported the notion that CYGB-/-HSCs are exposed to greater oxidative stress compared with their wild-type counterparts. We found that even hepatocytes in the CYGB-/- liver are pimimidazole+ (hypoxic). CYGB-/- livers showed markedly higher levels of iNOS and HO-1 proteins and expression of the myeloperoxidase gene. These results suggest a role for CYGB in regulating O2 homeostasis in HSCs and in liver tissues. It is conceivable that the intracellular redox state of normal cells is locally and temporally changeable depending on the concentrations of pro- and anti-oxidants, which are affected not only by intrinsic cellular activities, such as metabolic demands, but also by environmental variables, such as pH, temperature, and O2 concentration. Under physiologically healthy conditions, pro- and anti-
Intracellular O2 concentrations in Q-HSCs in normal livers vary continuously around the average concentration as cellular metabolic demands change temporally and locally. CYGB could exert a buffering effect against variable O2 concentrations, particularly when HSCs require high levels of O2 due to their higher metabolic activities. CYGB is most likely to be monomeric with Fe$^{3+}$ and without intra-S-S bonding (Fe$^{3+}$-CYGB-2SH), which has a relatively low affinity for O2. Fe$^{3+}$-CYGB-2SH in Q-HSCs facilitates well-balanced and regulated intracellular O2 homeostasis, which suppresses mitochondrial ROS generation under mild hypoxia; thus, they show activation when the liver is exposed to acute severe oxidative stresses or chronic weak stresses. In contrast, in the absence of CYGB, O2 homeostasis may be easily disturbed (i.e., Q-HSCs are susceptible to changes in the O2 level). For example, the intracellular O2 level is insufficient (hypoxic) when the cells become metabolically active. In HSCs with CYGB at a physiological concentration, hypoxia could result from exposure to severe and long-lasting stresses. If hypoxia is prolonged, HSCs experience a chronic O2 shortage due to the demand exceeding the O2 supply from CYGB; under such conditions, mitochondria, which function as “O2-sensors,” become functional and start to generate ROS. In this case, mitochondrial ROS acts as a signaling molecule and initiates a series of downstream biochemical reactions, such as the stabilization of HIF-1, leading to the activation of HIF-inducible pathways. These alterations in the intracellular environment result in Q-HSCs entering a pre-activated state in which HSCs are not transformed to A-HSCs but are preconditioned to A-HSCs. Cytoglobin concentrations are considered to increase under this situation because CYGB is upregulated by HIF-1.122,123

Q-HSCs acquire energy (as ATP) largely through mitochondrial oxidative phosphorylation. However, preconditioned HSCs exhibit glycolytic activity but can still utilize considerable quantities of ATP from mitochondria; this enables them to proliferate and synthesize the ECM components and proteins involved in contractility mechanisms. Continuation of the preconditioned state drives HSCs to transform into A-HSCs. We believe that CYGB has functionally and locally close relationships with mitochondria, which enables this protein to provide mitochondria with appropriate amounts of O2 in a context-dependent manner.

The intracellular environment of A-HSCs is considered to be more oxidative than that of Q-
HSCs due to relatively high levels of oxidants, such as ROS, and to have a relatively acidic pH due to glycolytic energy metabolism. Regarding O2 availability, A-HSCs experience “extracellular hypoxia” as a result of both limited sinusoidal vascularity and “intracellular hypoxia,” which is due to ATP-requiring activities such as contraction, migration, and ECM protein synthesis. All of these processes require mitochondrial respiration. In addition, A-HSCs undergo lipid autophagy for ATP procurement, suggesting active lipid metabolism therein. When chronic hypoxia is prolonged, preconditioned HSCs become fully activated to A-HSCs, in which lipid autophagy is active. This results in generation of sufficient ATP for the activities of myofibroblastic A-HSCs. It is intriguing to speculate that CYGB could participate as a signaling molecule in the initiation of autophagic lipolysis through binding to lipids in the mitochondrial and/or endoplasmic reticulum membrane. We suppose that CYGB could be present as Fe^{+++}-CYGB-S-S in A-HSCs, which have an oxidative intracellular environment. Fe^{+++}-CYGB-S-S exhibits an increased affinity for lipid metabolites. Fe^{+++}-CYGB-S-S binds lipid metabolites from LDs

Fig. 2. A hypothetical process of Q-HSC activation. Q-HSCs transform to A-HSCs via pre A-HSCs. The cytoplasn of Q-HSCs is mildly reductive, moderately alkaline in pH, and normoxic. CYGB is present as CYGB (Fe^{2+}/2SH); its concentration and affinity to O2 are normal and relatively low, respectively. The CYGB concentration increases during HSC activation. The O2 level in normal and hypoxic in Q- and A-HSCs, respectively. The redox state is reductive and oxidative in Q- and A-HSCs, respectively. ROS is present at low levels and acts as a signaling molecule and its levels increase during HSC activation. Intracellular pH is alkaline in Q-HSCs and becomes acidic in A-HSCs due to metabolic reprogramming. HSCs acquire ATP primarily from mitochondrial respiration, a process in which CYGB might play a role as an O2 supplier. Mitochondrial respiration also increases and LD autophagy begins when HSCs are activated, which results in increased ATP generation. ATP consumption by HSCs increases during their activation. When the O2 homeostasis of Q-HSCs is disturbed and they become hypoxic, the CYGB concentration increases and the CYGB (Fe^{2+}/S-S) form becomes detectable. HIF-1 levels also increase. Lipid mobilization is increased in pre-A-HSCs, which results in generation of RLS as a “beneficial” signaling molecule to suppress the further activation of pre-A-HSCs. However, as LD autophagy continues to progress, the pro-activation tendency overcomes the anti-activation tendency because the increased RLS exert oxidant effects. The CYGB concentration increases markedly in A-HSCs, possibly to store O2 for use by mitochondria engaged in ATP production. The intracellular environment in A-HSCs is hypoxic due to the activities of mitochondria, and it is acidic due to the high glycolytic activity necessary to support proliferation. Q-HSCs lose spines and drastically change their morphology. The possibility remains, although not shown in this figure, that CYGB in A-HSCs plays bimodal roles: it could supports their highly ATP-consuming activities as an O2-supplier and also produces microenvironments for “reverting” the A-HSCs to Q-HSCs. The alteration from Q-HSCs to pre A-HSCs is reversible, but that of from pre A-HSCs to A-HSCs irreversible. Receptors for cytokines and growth factors are illustrated on the pre A-HSC’s cell membrane to indicate active auto- and paracrine signaling during HSC activation. The active collagen synthesis in A-HSCs is indicated by several waved thin lines.
and probably also from mitochondrial and endoplasmic reticulum membranes. CYGB in this situation could exhibit peroxidase activity or could generate electrophilic RLS, which might function as signaling molecules. RLS at low levels could function as a “beneficial” stimulant by inducing several physiological pathways, including those leading to the resolution of inflammation and production of cellular antioxidants. In contrast, at higher concentrations, RLS could be cytotoxic. It is possible that CYGB could exhibit peroxidase activity or could generate antioxidants. In contrast, at higher concentrations, CYGB could not only prevent Q-HSC activation but also function thereafter to revert pre A-HSCs to Q-HSCs.

**XIV. Conclusion**

About 15 years have passed since we identified CYGB as a STAP (stellate cell activation-associated protein); during this period, research on the functions of CYGB has accumulated. For example, a PubMed search in October 2015 using the keyword “cytoglobin” returned ~240 publications. These studies have demonstrated that CYGB affects a wide range of biological, physiological, and pathological phenomena (i.e., it is a multifunctional protein). In this review, we aimed to identify the mechanisms by which CYGB exerts its effects; we used HSCs, in which we first identified CYGB, as a model cell type. We hypothesized that CYGB functions as a regulator of intracellular O2 homeostasis by acting as a major redox-sensitive anti-oxidative protein in HSCs. We evaluated the activities of HSCs from an energetics point of view. Through this review, we realized that further detailed characterizations of this protein are required to obtain a realistic picture of its physiological and pathological functions in HSCs, including its interactions with ligands and mitochondria and the temporal and local changes in its concentration within cells in physiological or pathological environments.

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**References**

1) Kawada, N., Kristensen, D.B., Asahina, K., Nakatani, K., Minamiyama, Y., Seki, S. and Yoshizato, K. (2001) Characterization of a stellate cell activation-associated protein (STAP) with peroxidase activity found in rat hepatic stellate cells. J. Biol. Chem. **276**, 25318–25323.

2) Gartner, G.C., Werner, S., Barrandon, Y. and Longaker, M.T. (2008) Wound repair and regeneration. Nature **453**, 314–321.

3) Yin, C., Evasion, K.J., Ashina, K. and Statinek, D.Y. (2013) Hepatic stellate cells in liver development, regeneration, and cancer. J. Clin. Invest. **123**, 1902–1910.

4) Wallace, K., Burt, A.D. and Wright, M.C. (2008) Liver fibrosis. Biochem. J. **411**, 1–18.

5) Friedman, S.L. (2008) Hepatic stellate cells: protein, multifunctional, and enigmatic cells of the liver. Physiol. Rev. **88**, 125–172.

6) Kristensen, D.B., Kawada, N., Imamura, K., Miyamoto, Y., Tateno, C., Seki, S., Kuroki, T. and Yoshizato, K. (2000) Proteome analysis of rat hepatic stellate cells. Hepatology **32**, 268–277.

7) Burmester, T., Ebner, B., Weich, B. and Hankeln, T. (2002) Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. Mol. Biol. Evol. **19**, 416–421.

8) Hoogewijs, D., Ebner, B., Germani, F., Hoffmann, F.G., Fabrizius, A., Moeus, L., Burmester, T., Dewilde, S., Storz, J.F., Vinogradov, S.N. and Hankeln, T. (2012) Androglobin: a chimeric globin in metazoa that is preferentially expressed in mammalian testes. Mol. Biol. Evol. **29**, 1105–1114.

9) Ankoma-Sey, V., Wang, Y. and Dai, Z. (2000) Hypoxic stimulation of vascular endothelial growth factor expression in activated rat hepatic stellate cells. Hepatology **31**, 141–148.

10) Burmester, T., Weich, B., Reinhardt, S. and Hankeln, T. (2000) A vertebrate globin expressed in the brain. Nature **407**, 520–523.

11) Godecke, A., Flögel, U., Zanger, K., Ding, Z., Hirchenhain, J., Decking, U.K. and Schrader, J. (1999) Disruption of myoglobin in mice induces multiple compensatory mechanisms. Proc. Natl. Acad. Sci. U.S.A. **96**, 10495–10500.

12) Bernal, D., Sepulveda, C., Mathieu-Costello, O. and Graham, J.B. (2003) Comparative studies of high performance swimming in sharks I. Red muscle morphometrics, vascularization and ultrastructure. J. Exp. Biol. **206**, 2831–2843.

13) Hunefeld, F.L. (1980) Die Chemismus in der thierischen Organization. Leipzig. 158–163.

14) Mörner, K.A.H. (1897) Beobachtungen über den Muskelfarbstoff. Nord. Med. Ark. **30**, 1–8.

15) Laughlin, S.B., de Ruyter van Steveninck, R.R. and Anderson, J.C. (1998) The metabolic cost of neural information. Nat. Neurosci. **1**, 36–41.

16) Karbowski, J. (2014) Constancy and trade-offs in the neuroanatomical and metabolic design of the cerebral cortex. Front. Neural. Circuits. **8**, 9.

17) Yoshizato, K., Tateno, C. and Utoh, R. (2009) The mechanisms of liver size control in mammals: a novel animal study. Int. J. Des. Nat. Ecodyn. **4**, 123–142.

18) Giampieri, M.P., Jezquel, A.M. and Orlandi, F. (1981) The lipocytes in normal human liver. A
quantitative study. Digestion 22, 165–169.
19) Blauer, W.S., O’Byrne, S.M., Wongsirojoj, N., Kluwe, J., D’Ambrosio, D.M., Jiang, H., Schwabe, R.F., Hillman, E.M., Piantedosi, R. and Libien, J. (2009) Hepatic stellate cell lipid droplets: a specialized lipid droplet for retinoid storage. Biochim. Biophys. Acta 1791, 467–473.
20) Wake, K. (1971) “Sternzellen” in the liver: perisinusoidal cells with special reference to storage of vitamin A. Am. J. Anat. 132, 429–462.
21) Kent, G., Gay, S., Inouye, T., Bahu, R., Minick, O.T. and Popper, H. (1976) Vitamin A-containing lipocytes and formation of type III collagen in liver injury. Proc. Natl. Acad. Sci. U.S.A. 73, 3719–3722.
22) Senoo, H., Hata, R., Nagai, Y. and Wake, K. (1984) Stellate cells (vitamin A-storing cells) are the primary site of collagen synthesis in non-parenchymal cells in the liver. Biomed. Res. 5, 451–458.
23) Friedman, S.L., Roll, F.J., Boyles, J. and Bissell, M.I., Munoz, U., Kraus, T., Lee, T., Yee, H.F. Jr. (2008) Activation-dependent contractility of rat liver stellate cells of the liver: important roles in retinol metabolism and fibrosis. FASEB J. 5, 271–277.
24) Wake, K. (2006) Hepatic stellate cells: Three-dimensional structure, localization, heterogeneity and development. Prog. Jpn. Acad., Ser. B, Phys. Biol. Sci. 82, 155–164.
25) Soon, R.K. Jr. and Yee, H.F. Jr. (2008) Stellate cell contraction: role, regulation, and potential therapeutic target. Clin. Liver Dis. 12, 791–803, viii.
26) Yamaoka, K., Nouchi, T., Marumo, F. and Sato, C. (1993) Alpha-smooth-muscle actin expression in hepatic stellate cells of the liver: important roles in retinol metabolism and fibrosis. Biochim. Biophys. Acta 115, 164–175.
27) Escobedo, G., Arjona-Roman, J.L., Melendez-Perez, R., Suarez-Alvarez, K., Guzman, C., Aguirre-Garcia, J., Gutierrez-Reyes, G., Vivas, O., Varela-Fascinetto, G., Rodriguez-Romero, A., Robles-Diaz, G. and Kershunobich, D. (2013) Liver exhibits thermal variations according to the stage of fibrosis progression: A novel use of modulated-differential scanning calorimetry for research in hepatology. Hepatol. Res. 43, 785–794.
28) Blomhoff, R. and Wake, K. (1991) Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. FASEB J. 5, 271–277.
29) Sohn, R.K. Jr. and Yee, H.F. Jr. (2008) Stellate cell contraction: role, regulation, and potential therapeutic target. Clin. Liver Dis. 12, 791–803, viii.
30) Yamaoka, K., Nouchi, T., Marumo, F. and Sato, C. (1993) Alpha-smooth-muscle actin expression in normal and fibrotic human livers. Dig. Dis. Sci. 38, 1473–1479.
31) Kawada, N., Klein, H. and Decker, K. (1992) Eicosanoid-mediated contractility of hepatic stellate cells. Biochem. J. 285, 367–371.
32) Kawada, N., Seki, S., Kuroki, T. and Kaneda, K. (1999) ROCK inhibitor Y-27632 attenuates stellate cell contraction and portal pressure increase induced by endothelin-1. Biochem. Biophys. Res. Commun. 266, 296–300.
33) Bell, E., Ivarsson, B. and Merrill, C. (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc. Natl. Acad. Sci. U.S.A. 76, 1274–1278.
34) Yoshizato, K., Taira, T., Yamamoto, N. and Sasaki, K. (1985) Remodeling of collagen in an in vitro model of connective tissue. Biomed. Res. 6, 287–296.
35) Rockey, D.C., Housset, C.N. and Friedman, S.L. (1993) Activation-dependent contractility of rat hepatic lipocytes in culture and in vivo. J. Clin.
59) Azad, P., Zhou, D., Zarndt, R. and Haddad, G.G.
58) Vander Heiden, M.G., Cantley, L.C. and
54) Hwang, H.J., Lynn, S.G., Vengellur, A., Saini, Y.,
55) Kim, J.W., Tchernyshyov, I., Semenza, G.L. and
61) Pietras, K. and Ostman, A. (2010) Hallmarks of
57) Hume, D.A., Radik, J.L., Ferber, E. and
50) Lokmic, Z., Musyoka, J., Hewitson, T.D. and
52) Chen, Y., Choi, S.S., Michelotti, G.A., Chan, I.S.,
62) Goetz, J.G., Minguet, S., Navarro-Lerida, I.,
60) Hepatology 296, 139–185.
51) Copple, B.L., Bai, S., Burgoon, L.D. and Moon, J.O.
1984–1985.
56) Schonenberger, M.J. and Kovacs, W.J. (2015)
53) Chen, Y., Choi, S.S., Michelotti, G.A., Chan, I.S.,
54) Schwiderska-Syn, M., Karaca, G.F., Xie, G.,
55) Moylan, C.A., Garibaldi, F., Premont, R.,
56) Suliman, H.B., Piantadosi, C.A. and Diehl, A.M.
57) Hedgeman, M.J. (1978) Aerobic glycolysis and
58) Madison, B.B., McKenna, L.B., Dolson, D., Epstein,
59) Hwang, H.J., Lynn, S.G., Vengellur, A., Saini, Y.,
60) Hwang, H.J., Lynn, S.G., Vengellur, A., Saini, Y.,
61) Pietras, K. and Ostman, A. (2010) Hallmarks of
62) Goetz, J.G., Minguet, S., Navarro-Lerida, I.,
63) Saito, R.A., Micke, P., Paulsson, J., Augsten, M.,
64) Maeda, Y., Dave, V. and Whitsett, J.A. (2007)
65) Costa, R.H., Guimaraes, E.L., Dolle, L., Mannaerts,
66) Madison, B.B., McKenna, L.B., Dolson, D., Epstein,
67) Tingstrom, A., Heldin, C.H. and Rubin, K. (1992)
68) Kalinichenko, V.V., Bhattacharyya, D., Zhou, Y.,
69) Walther, T.C. and Fareese, R.V. Jr. (2012) Lipid
70) Walther, T.C. and Fareese, R.V. Jr. (2012) Lipid
71) Martin, S. and Parton, R.G. (2006) Lipid droplets:
72) Dong, H. and Czaja, M.J. (2011) Regulation of
73) Rabinowitz, J.D. and White, E. (2010) Autophagy
74) Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak,
75) Thoen, L.F., Guijarres, E.L., Dolle, L., Mannerts,
76) Kimura, S., Noda, T. and Yoshimori, T. (2007)
77) K. YOSHIZATO
78) 1984–1985.
79) 1984–1985.
80) 1984–1985.
81) 1984–1985.
82) 1984–1985.
83) 1984–1985.
84) 1984–1985.
85) 1984–1985.
86) 1984–1985.
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198) 1984–1985.
199) 1984–1985.
200) 1984–1985.
201) 1984–1985.
Functions of cytoglobin in O2 homeostasis of hepatic stellate cells

dine environment of cytoglobin and neuroglobin. Biochemistry 44, 13257–13265.
90) Makino, M., Sugimoto, H., Sawai, H., Kawada, N., Yoshizato, K. and Shiryo, Y. (2006) High-resolution structure of human cytoglobin: identification of extra N- and C-termini and a new dimerization mode. Acta Crystallogr. D Biol. Crystallogr. 62, 671–677.
91) Lechauve, C., Chauvierre, C., Dewilde, S., Moens, L., Green, B.N., Marden, M.C., Celier, C. and Kiger, L. (2010) Cytoglobin conformations and disulfide bond formation. FEBS J. 277, 2696–2704.
92) Thuy, Le T.T., Morita, T., Yoshida, K., Wakasa, K., Izuka, M., Ogawa, T., Mori, M., Sekiya, Y., Momen, S., Motoyama, H., Ikeda, K., Yoshizato, K. and Kawada, N. (2011) Promotion of liver and lung tumorigenesis in DEN-treated cytoglobin-deficient mice. Am. J. Pathol. 179, 1050–1060.
93) Nishi, H., Inagi, R., Kawada, N., Yoshizato, K., Mimura, I., Fujita, T. and Nangaku, M. (2011) Cytoglobin, a novel member of the globin family, protects kidney fibroblasts against oxidative stress under ischemic conditions. Am. J. Pathol. 178, 128–139.
94) Nakatani, K., Okuyama, H., Shimahara, Y., Saeki, S., Kim, D.H., Nakajima, Y., Seki, S., Kawada, N. and Yoshizato, K. (2004) Cytoglobin/STAP, its unique localization in splanchic fibroblast-like cells and function in organ fibrogenesis. Lab. Invest. 84, 91–101.
95) McDonald, F.E., Risk, J.M. and Hodges, N.J. (2012) Protection from intracellular oxidative stress by cytoglobin in normal and cancerous oesophageal cells. PLoS One 7, e30587.
96) Halligan, K.E., Jourdeheul, F.L. and Jourdeheul, D. (2009) Cytoglobin is expressed in the vasculature and regulates cell respiration and proliferation via nitric oxide dioxygenation. J. Biol. Chem. 284, 8539–8547.
97) Fujita, Y., Koinuma, S., De Velasco, M.A., Bolz, J., Manel, T., Togashi, Y., Terashima, M., Hayashi, H., Matsuo, T. and Nishio, K. (2014) Melanoma transition is frequently accompanied by a loss of cytoglobin expression in melanocytes: a novel expression site of cytoglobin. PLoS One 9, e94772.
98) Fago, A., Hvidal, C., Dewilde, S., Gilany, K., Moens, L. and Weber, R.E. (2004) Allosteric regulation and temperature dependence of oxygen binding in human neuroglobin and cytoglobin. Molecular mechanisms and physiological significance. J. Biol. Chem. 279, 44417–44426.
99) Ascenzi, P., Marino, M., Polticelli, F., Coletta, M., Gioia, M., Marini, S., Pesce, A., Nardini, M., Bolognesi, M., Reeder, B.J. and Wilson, M.T. (2013) Non-covalent and covalent modifications modulate the reactivity of monomeric mammalian globins. Biochim. Biophys. Acta 1834, 1750–1756.
100) Reeder, B.J., Svitumenko, D.A. and Wilson, M.T. (2011) Lipid binding to cytoglobin leads to a change in haem co-ordination: a role for cytoglo-
bin in lipid signalling of oxidative stress. Biochem. J. 434, 483–492.

101) Hamdan, D., Kiger, L., Dewilde, S., Green, B.N., Pesce, A., Uzan, J., Burmester, T., Hankeln, T., Bolognesi, M., Moens, L. and Marden, M.C. (2003) The redox state of the cell regulates the lipid binding affinity of human neuroglobin and cytoglobin. J. Biol. Chem. 278, 51713–51721.

102) Tsujino, H., Yamashita, T., Nose, A., Kukino, K., Sawai, H., Shiro, Y. and Uno, T. (2014) Disulfide bonds regulate binding of exogenous ligand to human cytoglobin. J. Inorg. Biochem. 135, 20–27.

103) Beckerson, P., Wilson, M.T., Svistunenko, D.A. and Reeder, B.J. (2015) Cytoglobin lipid binding regulated by changing haem-co-ordination in response to intramolecular disulfide bond formation and lipid interaction. Biochem. J. 465, 127–137.

104) Higdon, A., Diers, A.R., Oh, J.Y., Landar, A. and Darley-Usmar, V.M. (2012) Cell signalling by reactive lipid species: new concepts and molecular mechanisms. Biochem. J. 442, 453–464.

105) Levonen, A.L., Landar, A., Ramanchandran, A., Ceaser, E.K., Dickinson, D.A., Zanon, G., Morrow, J.D. and Darley-Usmar, V.M. (2004) Cellular mechanisms of redox cell signallung: role of cysteine modification in controlling antioxidative defenses in response to electrophilic lipid oxidation products. Biochem. J. 378, 373–382.

106) Gutierrez, J., Ballinger, S.W., Darley-Usmar, V.M. and Landar, A. (2006) Free radicals, mitochondria, and oxidized lipids: the emerging role in signal transduction in vascular cells. Circ. Res. 99, 924–932.

107) Halder, P., Trent, J.T. 3rd and Hargrove, M.S. (2007) Influence of the protein matrix on intramolecular histidine ligation in ferric and ferrous hexacoordinate hemoglobins. Proteins 66, 172–182.

108) Thuy, Le T.T., Matsumoto, Y., Thuy, T.T.V., Hai, H., SooH, M., Unrara, Y., Motoyama, H., Fujii, H., Tamori, A., Kubo, S., Takesura, S., Morise, T., Yoshizato, K. and Kawada, N. (2015) Cytoglobin deficiency promotes liver cancer development from hepatosteatosis through activation of the oxidative stress pathway. Am. J. Pathol. 185, 1045–1060.

109) Miller, F.J. Jr., Guttermann, D.D., Rios, C.D., Heistad, D.D. and Davidson, B.L. (1998) Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ. Res. 82, 1298–1305.

110) Ikeda, R., Ishii, K., Hoshikawa, Y., Azumi, J., Arakaki, Y., Yasui, T., Matsuura, S., Matsumi, Y., Kono, Y., Mizuta, Y., Kuimassa, A., Hisatome, I., Friedman, S.L., Kawaoka, H. and Shiota, G. (2011) Reactive oxygen species and NADPH oxidase 4 induced by transforming growth factor beta1 are the therapeutic targets of polyenylphosphatidylcholine in the suppression of human hepatic stellate cell activation. Inflamm. Res. 60, 597–604.

111) Shah, R., Reyes-Gordillo, K., Arellanes-Robledo, J., Lechuga, C.G., Hernandez-Nazara, Z., Cotty, A., Rojkind, M. and Lakshman, M.R. (2013) TGF-beta1 up-regulates the expression of PDGF-beta receptor mRNA and induces a delayed P38, AKT-, and p70(S6K)-dependent proliferative response in activated hepatic stellate cells. Alcohol. Clin. Exp. Res. 37, 1838–1848.

112) Lee, K.S., Buck, M., Hou glamour, K. and Chojkier, M. (1995) Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myc expression. J. Clin. Invest. 96, 2461–2468.

113) Verna, L., Whyssner, J. and Williams, G.M. (1996) N-nitrosodimethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol. Ther. 71, 57–81.

114) Diwan, B.A., Rice, J.M., Ohshima, M. and Ward, J.M. (1986) Interstrain differences in susceptibility to liver carcinogenesis initiation by N-nitrosodimethylamine and its promotion by phenobarbital in C57BL/6NCr, C3H/HeNCrMTV- and DBA/2NCr mice. Carcinogenesis 7, 215–220.

115) Denda, A., Kitayama, W., Kishida, H., Murata, N., Tsutsumi, M., Tsujiuchi, T., Nakae, D. and Konishi, Y. (2002) Development of hepatocellular adenomas and carcinomas associated with fibrosis in C57BL/6J male mice given a choline-deficient L-amino acid-defined diet. Jpn. J. Cancer Res. 93, 125–132.

116) Chakraborty, S., John, R. and Nag, A. (2014) Cytoglobin in tumor hypoxia: novel insights into cancer suppression. Tumour Biol. 35, 6207–6219.

117) Asahina, K., Kawada, N., Kristensen, D.B., Nakatani, K., Seki, S., Shiokawa, M., Tateno, C., Obara, M. and Yoshizato, K. (2002) Characterization of human stellate cell activation-associated protein and its expression in human liver. Biochim. Biophys. Acta (BBA) Gene Struct. Expr. 1577, 471–475.

118) Novo, E., Marra, F., Zamara, E., Valfrè di Bonzo, L., Caligiuri, A., Cannito, S., Antonaci, C., Colombatto, S., Pinzani, M. and Parola, M. (2006) Dose dependent and divergent effects of superoxide anion on cell death, proliferation, and migration of activated human hepatic stellate cells. Gut 55, 90–97.

119) Friedman, S.L., Roll, F.J., Boyles, J., Arenson, D.M. and Bissell, D.M. (1989) Maintenance of differentiated phenotype of cultured rat hepatic lipocytes by basement membrane matrix. J. Biol. Chem. 264, 10756–10762.

120) El Taghdouini, A., Najimi, M., Sancho-Bru, P., Sokal, E. and van Grunsven, L.A. (2015) In vitro reversion of activated primary human hepatic stellate cells. Fibrogenesis Tissue Repair. 8, 14.

121) Kriegl, J.M., Bhattacharyya, A., Nienhaus, K., Deng, P., Minkow, O. and Nienhaus, G.U. (2002) Ligand binding and protein dynamics in neuro-
globin. Proc. Natl. Acad. Sci. U.S.A. 99, 7992–7997.

122) Fordel, E., Geuens, E., Dewilde, S., Rottiers, P., Carmeliet, P., Grooten, J. and Moens, L. (2004) Cytoglobin expression is upregulated in all tissues upon hypoxia: an in vitro and in vivo study by quantitative real-time PCR. Biochem. Biophys. Res. Commun. 319, 342–348.

123) Guo, X., Philipsen, S. and Tan-Un, K.C. (2007) Study of the hypoxia-dependent regulation of human CYGB gene. Biochem. Biophys. Res. Commun. 364, 145–150.

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Profile

Katsutoshi Yoshizato was born in Nagasaki Prefecture in 1943 and graduated from Faculty of Science of University of Tokyo in 1965 and its Graduate School of Science in 1971, when he received Ph.D. After a 4-month-long postdoctoral study granted by the Japanese Association of Promotion of Science, he joined in Florida State University as a research fellow. He stayed there for 2.5 years (1971–1973) and then became a faculty member of Kitasato University Medical School (1973–1987) as a lecturer (1973–1981) and an associate professor (1981–1987). Then, he worked in Tokyo Metropolitan University Faculty of Science as an associate professor from 1987–1990 and thereafter in Hiroshima University Faculty of Science as a professor from 1990 to 2007, in which he once served as a vice president of the University (2003–2005). He organized the Yoshizato MorphoMatrix Project from 1992 to 1997 in Exploratory Research for Advanced Technology (ERATO) of Japan Science and Technology Corporation (JST). His activities in the ERATO project resulted in foundation of biotechnology company, Phoenixbio, in which he is currently serving as an academic advisor. Through his research activities, he has been interested in revealing the mechanisms underlying in the remodeling phenomena seen in organogenesis and metamorphosis, and dedicating to understanding the life system through creating technologies to reconstruct the liver and skin. The discovery of cytoglobin and generation of a chimeric mouse whose hepatocytes are replaced with human hepatocytes were outcomes of the studies on reconstruction of liver and a methodological establishment for hair regeneration therapy a fruit of that of skin reconstruction. The accomplishments in his studies brought him Zoological Society Prize from The Zoological Society of Japan and Biomaterial Society Prize from the Biomaterial Society of Japan (1994), Doctor of Science Honoris Causa from Purdue University Indianapolis (2002) and Cultural Prize from Chugoku Newspaper (2003).