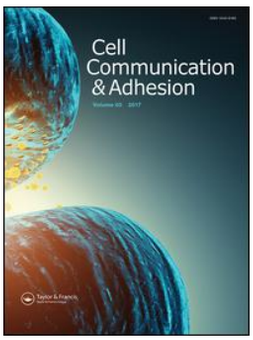

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REVIEW ARTICLE

Structure, Regulation and Function of Gap Junctions in Liver

Joost Willebrords[a]*, Sara Crespo Yanguas[a]*, Michaël Maes[a], Elke Decrock[b], Nan Wang[b], Luc Leybaert[b], Tereza Cristina da Silva[c], Isabel Veloso Alves Pereira[c], Hartmut Jaeschke[d], Bruno Cogliati[c] and Mathieu Vinken[a]

[a]Department of in Vitro Toxicology and Dermato-Cosmetology, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Brussels, Belgium; [b]Department of Basic Medical Sciences, Physiology Group, Ghent University, Ghent, Belgium; [c]Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil; [d]Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

ABSTRACT

Gap junctions are a specialized group of cell-to-cell junctions that mediate direct intercellular communication between cells. They arise from the interaction of two hemichannels of adjacent cells, which in turn are composed of six connexin proteins. In liver, gap junctions are predominantly found in hepatocytes and play critical roles in virtually all phases of the hepatic life cycle, including cell growth, differentiation, liver-specific functionality and cell death. Liver gap junctions are directed through a broad variety of mechanisms ranging from epigenetic control of connexin expression to post-translational regulation of gap junction activity. This paper reviews established and novel aspects regarding the architecture, control and functional relevance of liver gap junctions.

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connexin; gap junction; liver

INTRODUCTION

As holds for all multicellular systems, homeostasis in liver is controlled by the entangled network of extracellular, intracellular and intercellular communication mechanisms. Typically, extracellular signals trigger intracellular signal transduction cascades. Several of these intracellular signals are subsequently propagated to adjacent cells. Such intercellular connections are formed by arrays of gap junctions (Vinken et al., 2006b, 2008, 2009b). The liver was among the first organs in which gap junctions were studied (Loewenstein & Kanno, 1967; Revel & Karnovsky, 1967). In 1974, Goodenough isolated two gap junction proteins from mouse liver, which were designated connexins (Goodenough, 1974). Today, more than 20 different connexin species have been identified. They are all named based upon their molecular weight as predicted by cDNA sequencing and are expressed in a cell type-specific way (Bai & Wang, 2014). Nevertheless, they all share a similar structure consisting of four transmembrane domains, two extracellular loops, one cytosolic loop, one cytosolic carboxy-terminal tail and one cytosolic aminotail. Following their synthesis, six connexins form a hemichannel or connexon at the plasma membrane surface, which then docks with another hemichannel from a neighboring cell to generate a gap junction (Figure 1) (Vinken et al., 2006b, 2008, 2009b). Gap junctions mediate the passive diffusion of small, *i.e.* below 1.5 kDa, and hydrophilic molecules, such as glucose, glutamate, glutathione, adenosine triphosphate, cyclic adenosine monophosphate, inositol triphosphate and ions, including calcium, sodium and potassium (Alexander & Goldberg, 2003; Decrock et al., 2009). Numerous physiological processes are regulated by substances that are intercellularly exchanged via gap junctions and hence gap junctional intercellular communication (GJIC) is considered as a key mechanism in the control of tissue homeostasis (Vinken et al., 2006b, 2008, 2009b). Because of this critical task, GJIC is strictly regulated at multiple levels by a myriad of mechanisms. In this paper, a state-of-the-art overview of the structure, regulation and function of gap junctions in liver is provided.

STRUCTURE OF LIVER GAP JUNCTIONS

The major connexin species in liver is Cx32, which represents 90% of the total hepatic connexin content (Cascio et al., 1995; Neveu et al., 1995). Cx32 is expressed by hepatocytes and to a lesser extent by sinusoidal

CONTACT Mathieu Vinken mvinken@vub.ac.be Department of In Vitro Toxicology and Dermato-Cosmetology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium
*Equally contributing first authors.

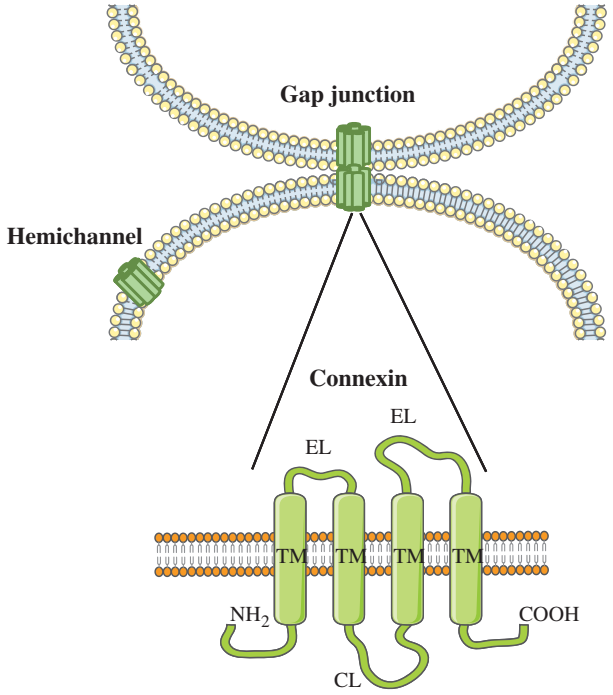

Figure 1. Structure of gap junctions. Gap junctions are formed by the docking of two hemichannels or connexons from neighboring cells, which in turn are built up by six connexins. Connexins share a similar structure consisting of four transmembrane domains (TM), two extracellular loops (EL), one cytosolic loop (CL), one cytosolic carboxyterminal tail (COOH) and one cytosolic aminotail (NH_2).

endothelial cells. These cells as well as stellate cells and Kupffer cells also produce small quantities of Cx26 (Fischer et al., 2005), while Cx43 is detectable in Kupffer cells, stellate cells, sinusoidal endothelial cells, cells of Glisson's capsule and cholangiocytes (Berthoud et al., 1992; Bode et al., 2002; Fischer et al., 2005; Greenwel et al., 1993; Sáez, 1997). Furthermore, Cx40 and Cx37 are present in liver vascular cells (Table 1) (Shiojiri et al., 2006). Despite the wide repertoire of connexins expressed in liver, the presence of functional gap junctions has only been demonstrated in hepatocytes and stellate cells (Fischer et al., 2005). Unlike Cx32, which is uniformly distributed in liver tissue, Cx26 is preferentially expressed in the periportal area (Spray et al., 1994). Hence, most gap junctions in the pericentral and periportal regions are Cx32 homotypic and Cx32–Cx26 heterotypic channels, respectively (Iwai et al., 2000). Gap junctions occupy about 3% of the hepatocyte membrane surface (Spray et al., 1994) and are organized in plaques of 0.2–1 μm in diameter (Kojima et al., 1996) that contains 10–10,000 channels (Musil et al., 2000). Connexin proteins usually display rapid turnover rates in comparison with other plasma membrane proteins. Both *in vitro, i.e.* primary cultured hepatocytes (Traub et al., 1987), and *in vivo, i.e.* regenerating liver (Traub et al., 1983), the half-lives of Cx26 and Cx32 have been found to be 2 and 3 h, respectively, whereas the turnover times of other integral membrane proteins in primary hepatocyte cultures generally range from 17 to 100 h (Chu & Doyle, 1985). Upon degradation, gap junctional channels are internalized by one of the two opposing cells, resulting in the formation of so-called annular gap junction. These structures are further degraded by both lysosomes and proteasomes, although the precise degradation pathway depends on the identity of the connexin and cell type (Laird, 2005, 2006). Degradation of Cx32-based gap junctions in rat liver mainly occurs via the lysosomal pathway (Rahman et al., 1993).

REGULATION OF LIVER GAP JUNCTIONS

Short-term regulation

Short-term control of GJIC, so-called gating, is driven by a number of factors, including pH, transmembrane voltage and calcium concentration (Cottrell & Burt, 2005). Post-translational modifications, such as *S*-nitrosylation, sumoylation and phosphorylation, also directly regulate gap junction opening (Johnstone et al., 2012). Phosphorylation mainly occurs at the cytoplasmic carboxyterminal connexin tail. In fact, all connexins are phosphoproteins with the notable exception of Cx26. The regulation of gap junction opening by phosphorylation is complex, as the outcome of this post-translational modification depends on the nature of the kinase and connexin as well as on the cellular context (Laird, 2005; Solan & Lampe, 2005). Cx43 has been most extensively studied in this regard. Cx43 is a substrate for many kinases, including protein kinases A and C, members of the mitogen-activated protein kinase family, casein kinase 1, the cyclin-dependent kinase 1/cyclin B complex and *v*-Src (Solan & Lampe, 2005, 2009). Different from other connexins, shifts in electrophoretic mobility occur upon phosphorylation of Cx43. Usually, three bands appear during sodium dodecylsulfate–polyacrylamide gel electrophoresis analysis, representing the fast-migrating nonphosphorylated Cx43 isoform, referred to as NP-Cx43, and two slow-migrating phosphorylated Cx43 isoforms, namely P1-Cx43 and P2-Cx43 (Cooper et al., 2000; Solan & Lampe, 2005, 2009). In liver, Cx43 is predominantly presented in its NP-Cx43 isoform (Vinken et al., 2006a, 2012b). Cx32 can be phosphorylated by protein kinases A and C, the epidermal growth factor receptor and calcium-calmodulin-dependent protein kinase II (Lampe & Lau, 2004). While phosphorylation by protein kinase A promotes hepatocellular GJIC (Saez et al., 1986), phosphorylation by protein kinase C

Table 1. Expression of connexins in liver.

Connexin species	Cell type	References
Cx26	Hepatocytes	Kuraoka et al. (1993), Nicholson et al. (1987) and Zhang and Nicholson (1989)
	Stellate cells	Fischer et al. (2005)
	Sinusoidal endothelial cells	Fischer et al. (2005)
	Kupffer cells	Fischer et al. (2005)
Cx32	Hepatocytes	Fischer et al. (2005), Fowler et al. (2013), Kumar and Gilula (1986), Kuraoka et al. (1993), Nakashima et al. (2004), Nicholson et al. (1987), Paul (1986) and Temme et al. (1998)
	Biliary endothelial cells	Bode et al. (2002)
	Sinusoidal endothelial cells	Fischer et al. (2005)
Cx37	Hepatic artery endothelial cells	Chaytor et al. (2001), Hernández-Guerra et al. (2014) and Shiojiri et al. (2006)
	Portal vein endothelial cells	Chaytor et al. (2001), Hernández-Guerra et al. (2014) and Shiojiri et al. (2006)
Cx40	Hepatic artery endothelial cells	Chaytor et al. (2001), Hernández-Guerra et al. (2014) and Shiojiri et al. (2006)
	Portal vein eindothelial cells	Chaytor et al. (2001), Hernández-Guerra et al. (2014) and Shiojiri et al. (2006)
Cx43	Biliary epithelial cells	Balasubramaniyan et al. (2013), Bode et al. (2002), Cogliati et al. (2011), Nathanson et al. (1999), Oyamada et al. (1990), Wang et al. (2013) and Zhang et al. (2007)
	Kupffer cells	Eugenin et al. (2007), Fischer et al. (2005), Gonzalez et al. (2002) and Saez et al. (1997)
	Stellate cells	Fischer et al. (2005) and Hernández-Guerra et al. (2014)
	Sinusoidal enothelial cells	Fischer et al. (2005) and Hernández-Guerra et al. (2014)
	Hepatic artery endothelial cells	Chaytor et al. (2001), Hernández-Guerra et al. (2014) and Shiojiri et al. (2006)
	Portal vein endothelials cells	Chaytor et al. (2001), Hernández-Guerra et al. (2014) and Shiojiri et al. (2006)

prevents calpain-mediated proteolysis (Elvira et al., 1993). Gap junction activity can also be affected by connexin binding partners. Connexins indeed interact with a plethora of proteins, referred to as the interactome. For instance, assembly of adherens junctions composed of E-cadherin and α-catenin at the hepatocyte cell plasma membrane surface is a prerequisite for the formation of Cx32-based gap junctions (Fujimoto et al., 1997).

Long-term regulation

Long-term control of GJIC involves regulation at the transcriptional level of connexin expression (Oyamada et al., 2005). The architecture of most connexin genes is simple, consisting of a first exon (E1) that harbors the 5′-untranslated region (UTR), which is separated by an intron of varying length from a second exon (E2), bearing the complete coding sequence and the 3′-UTR. The Cx32 gene is exceptional because of differential splicing of the 5′-UTR (Oyamada et al., 2005; Söhl & Willecke, 2004). The human and rat Cx32 genes comprise three exons, *i.e.* E1, E1B and E2, whereas their mouse and bovine counterparts contain four exons, *i.e.* E1, E1A, E1B and E2 (Duga et al., 1999; Neuhaus et al., 1996; Söhl et al., 2001). Cx32 gene transcription can be initiated through two tissue-specific promoters. The P1 promoter, located upstream of E1, is active in hepatocytes and pancreatic secretory acinar cells, generating the E1–E2 transcript, whereas the P2 promoter, which is located upstream of E1B, is active in peripheral nerves and yields the E1B–E2 transcript (Neuhaus et al., 1996; Söhl et al., 1996). In mice and cows, a third transcript, *i.e.* E1A–E2, is found in embryonic cells, oocytes and adult liver (Duga et al., 1999; Neuhaus et al., 1996). Such differential promoter usage and alternative splicing have also been reported for the murine and rat Cx43 genes (Pfeifer et al., 2004). Connexin gene promoters show binding affinity for several general transcription factors, including activator protein 1, yin yang 1 and specificity protein 1 (Oyamada et al., 2005). In parallel, a number of cell type-specific transcription factors control connexin gene transcription. Thus, liver-specific expression of Cx32 relies on the binding of hepatocyte nuclear factor 1α at the P1 promoter (Field et al., 2003; Koffler et al., 2002). In the last decade, epigenetic mechanisms, including histone acetylation and DNA methylation, have also joined in as master regulators of connexin expression (Oyamada et al., 2005; Vinken et al., 2009a). In this respect, the absence of Cx43 and Cx32 expression in rat MH1C1 hepatoma cells and rat WB-F344 liver epithelial cells, respectively, is causally linked to DNA methylation in the corresponding gene promoters (Piechocki et al., 1999). Furthermore, inhibition of histone deacetylation positively affects GJIC in primary hepatocyte cultures, which is associated with differential effects on the expression of Cx26, Cx32 and Cx43 (Vinken et al., 2006a, 2007).

FUNCTION OF LIVER GAP JUNCTIONS

Role in proliferation

The adult liver displays very low proliferative activity. However, upon partial hepatectomy, the remaining hepatic lobes start to grow and the original size becomes restored within 7–10 days (Taub, 2004). During this cell cycling event, GJIC transiently increases in the G1 phase, followed by a dramatic decrease upon initiation of the S phase (Dermietzel et al., 1987; Fladmark et al., 1997; Koenig et al., 2006; Kojima et al., 2003; Kren et al., 1993; Meyer et al., 1981; Miyashita et al., 1991; Sugiyama &

Ohta, 1990; Temme et al., 2000; Traub et al., 1983; Yee & Revel, 1978). Similar alterations are seen in Cx32 expression, whereas Cx43 production remains unchanged (Kren et al., 1993; Temme et al., 2000; Traub et al., 1983). These findings can be reproduced in an *in vitro* model of hepatocyte proliferation, namely mitogen-stimulated primary hepatocytes (Fladmark et al., 1997; Kojima et al., 1997, 2004). In this system, mitogen-activated protein kinase phosphorylates Cx32, resulting in its decreased expression (Kojima et al., 2004). In fact, connexin phosphorylation seems to be a major mechanism underlying GJIC alterations in liver cell cycling. In serum-stimulated rat liver cells, progression from the G0 state to the S phase is related to protein kinase C-dependent phosphorylation of Cx43 and disruption of GJIC (Koo et al., 1997). The relevance of altered GJIC during cell cycling is unclear. In the regenerating liver of rats treated with an inhibitor of mitogen-activated protein kinase, the disappearance of Cx32 is inhibited without affecting hepatocyte proliferative activity (Kojima et al., 2003). This suggests that downregulation of gap junctions can occur independently of proliferation and hence may be considered as a fortuitous side effect of the growth response. On the other hand, in the regenerating liver of Cx32 knock-out mice, the G0/S transition of the cell cycle and thereby the proliferative activity of the hepatocytes are not promoted, but the extent of synchronous initiation and termination of DNA synthesis is decreased (Dagli et al., 2004; Sugiyama & Ohta, 1990). In this view, reduction of GJIC does not provide a direct signal for cells to divide per se, but rather permits cell cycle progression upon mitogenic stimulation. GJIC hereby seems to be coordinated with cell growth and serves a purpose other than triggering proliferation. Such a purpose may include the functional segregation of the metabolic pools in dividing cells from their quiescent neighbors in order to avoid homeostatic imbalance (Chipman et al., 2003; Dermietzel et al., 1987; Fladmark et al., 1997). Others strongly believe that gap junctions fulfill a determinate function in cell proliferation control rather than merely an assisting role in growth progression. Gap junctions indeed provide a pathway for the direct exchange of essential growth mediators, such as cyclic adenosine monophosphate (Vinken et al., 2012a). Interfering with connexin gene expression often reveals additional mechanisms involved in gap junction-mediated control of cell proliferation. In this context, transfection of liver-derived cell lines with connexin genes can directly alter gene expression patterns. Forced expression of Cx32 and Cx26 in rat liver epithelial cells and human hepatoma cells triggers the production of p27 and E-cadherin, respectively, which, in turn, negatively affect proliferation (Paul, 1986). Furthermore, connexins can physically interact with cell growth regulators. The scaffolding protein Discs large homolog 1 (Dlgh1) acts as a tumor suppressor protein and its presence at the cell plasma membrane surface, bound to Cx32, is associated with cell cycle arrest. Upon its release, occurring upon downregulation of Cx32 expression, Dlgh1 translocates to the cell nucleus, ultimately resulting in induction of proliferation. Therefore, maintaining Dlgh1 at the cell plasma membrane surface may be a regulatory mechanism by which Cx32 controls hepatocyte proliferation (Duffy et al., 2007).

Role in differentiation and liver-specific functionality

Early hepatic progenitor cells switch from Cx43 to Cx26 production, but especially to a Cx32 modus upon differentiation into hepatocytes (Naves et al., 2001; Neveu et al., 1995; Paku et al., 2004; Zhang & Thorgeirsson, 1994). A similar phenomenon has been observed in *in vitro* settings of liver differentiation, such as in liver epithelial cell line models (Rosenberg et al., 1996; Zhang & Thorgeirsson, 1994). The generation of the Cx26 zonated pattern during liver development parallels the establishment of metabolic heterogeneity in hepatocytes. In this respect, glucagon receptors are mainly found in the pericentral area, whereas the inverse is observed for their ligands (Berthoud et al., 1992). Glucagon is known to perform a much more pronounced inductive effect on the transcription of Cx26 genes compared with Cx32 genes (Kojima et al., 1995). It is therefore thought that the hepatic Cx26 zonation pattern is regulated at the transcriptional level and that glucagon is a master regulator of this event (Iwai et al., 2000; Kojima et al., 1995). In adult liver, the establishment of GJIC is critical for the maintenance of several liver-specific functions, including glycogenolysis (Stümpel et al., 1998), ammonia removal (Yang et al., 2003), albumin secretion (Yang et al., 2003), bile secretion (Temme et al., 2001) and xenobiotic biotransformation (Neveu et al., 1994; Shoda et al., 1999et al., 2000). Regarding the latter, both the constitutive and drug-induced expression of cytochrome P450 isoenzymes, in particular cytochrome P450 2B6 and 3A4, require the presence of Cx32-based gap junctions (Hamilton et al., 2001). Induction of cytochrome P450 1A1/2 and 2B1/2 simultaneously occurs with downregulation of pericentral Cx32 protein amounts in rat (Neveu et al., 1994; Shoda et al., 1999et al., 2000). These concomitant changes in the expression of Cx32 and cytochrome P450 isoenzymes could reflect a defense mechanism to restrict the intercellular diffusion of reactive intermediates produced through xenobiotic biotransformation (Neveu et al., 1994). The messengers that

are conveyed through gap junctions and that affect liver functionality largely remain obscure, but an exception exists for glycogenolysis. This metabolic process is initiated by hormonal and neuronal stimuli and mainly takes place in the periportal region. Pericentral hepatocytes also show glycogenolytic activity, albeit to a much lesser extent (Saez et al., 2003; Stümpel et al., 1998). Cx32-based gap junctions hereby propagate the glycogenolytic response from the periportal to the pericentral areas. More specifically, they control the intercellular trafficking of inositol triphosphate, which activates calcium release from endoplasmic reticulum stores, in turn evoking calcium waves throughout the acinar tract (Gaspers & Thomas, 2005; Saez et al., 2003). In line with this observation is the finding that Cx32 knock-out mice show lowered blood glucose levels upon glycogenolytic stimulation (Stümpel et al., 1998). A similar scenario is seen in bile formation and flow, whereby ductular secretion from cholangiocytes depends on the spread of calcium waves through Cx43-containing gap junctions (Bode et al., 2002; Nathanson et al., 1999).

Role in cell death

During the early stages of apoptosis in serum-deprived cultured rat liver cells, GJIC is enhanced. This goes hand in hand with increased Cx43 expression and phosphorylation. The latter is likely to be mediated by the cyclin-dependent kinase 1/cyclin B complex, which also controls the G2/M transition of the cell cycle. Upon further progression of cell death, GJIC activity deteriorates, as evidenced by the absence of communication between apoptotic bodies (Contreras et al., 2004). The temporary induction of GJIC in the early phases of apoptosis could point to a role for gap junctions in the initial spread of a death wave from cell to cell. In this light, calcium ions are thought to be the killing messengers. The onset of apoptosis is generally associated with drastic alterations in the concentration of calcium, an ion that is intercellularly exchanged via gap junctions. The subsequent downregulation of GJIC could serve the reduction of the flux of toxic metabolites, such as nitric oxide and superoxide ions, and hence the protection of living cells (Contreras et al., 2004; Krutovskikh et al., 2002). Of note, compelling evidence shows that connexin hemichannels, rather than gap junctions, are involved in liver cell death. Following induction of apoptosis in primary hepatocytes, GJIC rapidly deteriorates, which is accompanied by a decay of the gap junctional Cx32 protein pool. Concomitantly, Cx32 is *de novo* synthesized and gathers in a hemichannel configuration. This becomes particularly evident toward the final stages of the cell death process (Vinken et al., 2010). Likewise, Cx43 signaling, also involving hemichannels, was found to facilitate the onset of spontaneous apoptosis in cultures of primary hepatocytes (Vinken et al., 2012b). Cx43 hereby interacts with mitochondrial proteins (Vinken et al., 2013), as equally holds true for hepatocellular Cx32 (Fowler et al., 2013). In fact, connexin hemichannels can be located at other subcellular compartments in addition to the plasma membrane, such as mitochondria, where they have been linked to apoptosis (Goubaeva et al., 2007). Furthermore, connexin signaling also plays a role in other cell death modes. Thus, gap junctions consisting of Cx26 and Cx32 have been found to synchronize chemical-induced necrosis in primary hepatocytes (Saito et al., 2014). Recently, GJIC was found to be involved in experimentally triggered autophagy in rat liver cells (Zou et al., 2015).

CONCLUSIONS AND PERSPECTIVES

The liver is a vital organ endowed with several critical tasks. In order to do so, communication between the different liver cell types is a prerequisite. Hepatocytes, the major work horses of the liver, directly communicate with each other through gap junctions. Hepatocellular gap junctions, consisting of Cx32 and Cx26, have been studied for more than half a century (Loewenstein & Kanno, 1967; Revel & Karnovsky, 1967). As a result, many roles have been attributed to these structures, including those related to liver cell growth, differentiation and cell death. In the past decade, it has become clear that the structural precursors of gap junctions, namely connexin hemichannels, may also contribute to the latter process by mediating paracrine signaling (Chandrasekhar & Bera, 2012; Decrock et al., 2009). Furthermore, a novel class of connexin-like proteins has been identified, namely the pannexins, which gather in a configuration identical to connexin hemichannels and that provide an additional pathway for communication between the cytosol of individual cells and their extracellular environment (Penuela et al., 2014). Of those, pannexin1 is expressed by hepatocytes (Csak et al., 2011; Ganz et al., 2011) and has been linked to apoptosis (Xiao et al., 2012). A major challenge now lies in the further exploration of the role of connexin and pannexin hemichannels in other aspects of the liver life cycle. The regulation of connexin and pannexin signaling also deserves further scrutiny. In particular, connexins are known to be subjected to several other post-translational modifications in addition to the currently studied ones, including glycosylation, *N*-acetylation, ubiquitination, lipidation, hydroxylation, methylation and deamidation (D'hondt et al., 2013), yet the functional relevance of these changes, *in casu* in the liver, is not

clear. Similarly, microRNAs have been found to control connexin production at the post-transcriptional level in recent years (Vinken et al., 2009a), but specific information in relation to liver is currently lacking. Most importantly, the role of connexin signaling in liver pathology should be thoroughly investigated. Indeed, because of its key function as a gatekeeper of hepatic homeostasis, it is not surprising that connexin signaling is essentially involved in liver disease. Thus far, connexins and their channels have been found to underlie drug-induced acute liver failure, hepatitis, cholestasis, nonalcoholic fatty liver disease, fibrosis, cirrhosis and hepatocellular carcinoma (Maes et al., 2015; Vinken et al., 2013). Upon introduction of drugs that modulate connexin expression or channel activity, this may open new perspectives for the establishment of new therapies in the hepatology field in the upcoming years.

Disclosure statement

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