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Models and methods for *in vitro* testing of hepatic gap junctional communication

Michaël Maes[#], **Sara Crespo Yanguas**[#], **Joost Willebrords**, and **Mathieu Vinken**
Department of *In Vitro* Toxicology and Dermato-Cosmetology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium.

[#] These authors contributed equally to this work.

Abstract

Inherent to their pivotal roles in controlling all aspects of the liver cell life cycle, hepatocellular gap junctions are frequently disrupted upon impairment of the homeostatic balance, as occurs during liver toxicity. Hepatic gap junctions, which are mainly built up by connexin32, are specifically targeted by tumor promoters and epigenetic carcinogens. This renders inhibition of gap junction functionality a suitable indicator for the *in vitro* detection of nongenotoxic hepatocarcinogenicity. The establishment of a reliable liver gap junction inhibition assay for routine *in vitro* testing purposes requires a cellular system in which gap junctions are expressed at an *in vivo*-like level as well as an appropriate technique to probe gap junction activity. Both these models and methods are discussed in the current paper, thereby focusing on connexin32-based gap junctions.

Keywords

gap junction; connexin; liver; *in vitro* model; nongenotoxic carcinogen; intercellular communication

1. Introduction

As in all other multicellular systems and organs, cellular communication is an *absolute conditio sine qua non* for the maintenance of liver homeostasis. Direct intercellular signaling is mediated by gap junctions. These communicating cell junctions arise from the interaction of two hemichannels of neighboring cells that on their turn are hexamers of connexin (Cx) proteins. More than twenty different connexin species have been identified, yet they all share a similar structure consisting of four transmembrane domains, two extracellular loops, one cytoplasmic loop, one cytoplasmic carboxytail and one cytoplasmic aminotail (Figure 1)

Address for correspondence: Prof. Mathieu Vinken (Ph. D., Pharm. D., E. R. T.), Vrije Universiteit Brussel Department of *In Vitro* Toxicology and Dermato-Cosmetology, Laarbeeklaan 103, B-1090 Brussels, Belgium; Tel: +32-2-4774587; Fax: +32-2-4774582; mvinken@vub.ac.be.
michael.mc.maes@vub.ac.be (M. Maes), sara.crespo.yanguas@vub.ac.be (S. Crespo Yanguas), joost.willebrords@vub.ac.be (J. Willebrords).

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(Maes et al., 2014; Vinken et al., 2008; Vinken et al., 2009; Vinken et al., 2011). The predominant connexin species in the liver is Cx32, which is abundantly expressed by hepatocytes and to a lesser extent by sinusoidal endothelial cells. The latter cells as well as stellate cells also produce small quantities of Cx26 (Fischer et al., 2005), while Cx43 is detectable in Kupffer cells, stellate cells, sinusoidal endothelial cells and cholangiocytes (Berthoud et al., 1992; Bode et al., 2002; Fischer et al., 2005). However, the presence of functional gap junctions has only been demonstrated in hepatocytes and stellate cells (Fischer et al., 2005). Gap junctional intercellular communication (GJIC) includes the passive exchange of small and hydrophilic substances, such as second messengers, between adjacent cells (Alexander and Goldberg, 2003; Wang et al., 2013), and is regulated by a vast array of mechanisms, including connexin phosphorylation (Solan and Lampe, 2009). As such, hepatic GJIC, in particular between hepatocytes, has been shown to drive a number of essential liver-specific processes, namely xenobiotic biotransformation (Neveu et al., 1994a; Shoda et al., 2000), bile secretion (Nathanson et al., 1999; Temme et al., 2001) albumin secretion (Yang et al., 2003a), glycogenolysis (Nelles et al., 1996; Stumpel et al., 1998) and ammonia detoxification (Yang et al., 2003a). Moreover, gap junctions are also key players in liver development (Vinken et al., 2012a), liver cell growth (Vinken et al., 2011) and liver cell death (Decrock et al., 2009; Vinken et al., 2010; Vinken et al., 2012b).

Because of their critical roles in supporting liver homeostasis, it is not surprising that these structures are affected in disease. Indeed, hepatic GJIC typically deteriorates in liver cancer, cholestasis, liver fibrosis and cirrhosis, hepatitis and systemic inflammation, and hepatic liver ischemia and reperfusion injury (Vinken, 2012; Vinken et al., 2012a). In addition, gap junctions are also involved in liver toxicity. In this respect, a plethora of chemical and biological toxic compounds are known to suppress hepatocellular GJIC, including environmental pollutants, biological toxins, organic solvents, pesticides, pharmaceuticals, peroxides, metals and phthalates (Table 1). This is usually associated with the gradual disappearance of Cx32, whilst Cx43 production is enhanced in these pathological circumstances (Vinken et al., 2009). The deleterious outcome of these compounds on gap junction production and functioning is frequently manifested in a species-specific and tissue-specific manner. Such specificity in performing detrimental cellular actions as well as the lack of causing direct DNA damage are typical hallmarks of nongenotoxic carcinogenicity. In fact, many of the chemical and biological compounds that suppress hepatic GJIC are tumor promoters or epigenetic carcinogens. Hence, inhibition of GJIC may represent an interesting biomarker for the detection of nongenotoxic carcinogens in general (Budunova and Williams, 1994; Combes, 2000; Cowles et al., 2007; Mally and Chipman, 2002; Mesnil et al., 1995; Ruch and Klaunig, 1986). This may be challenging from an *in vitro* toxicologist's perspective, since no validated *in vitro* assays are currently available for the testing of nongenotoxic carcinogenicity. When developing such *in vitro* screens, care should be taken while selecting the cellular system, which actually defines the scope of the present paper. Besides an overview of *in vitro* systems that appropriately maintain liver gap junctions at an *in vivo*-like level, in particular primary hepatocyte culture models, methods to probe hepatic GJIC are discussed.

2. *In vitro* models to study of liver gap junctions

Cell lines are frequently used tools for studying gap junctions *in vitro*. However, cell lines not always provide an appropriate reflection of the *in vivo* situation. The human hepatoma-derived HepG2 cell line, routinely used in experimental liver research, lacks many liver-specific traits, including functional expression of biotransformation capacity (Wilkening et al., 2003). In addition, HepG2 cells do not exhibit physiological cell junction patterns. In these cells, production of Cx26 is downregulated, whereas Cx32 displays aberrant localization (Yano et al., 2001; Yano and Yamasaki, 2001). Likewise, nontumorigenic rat liver epithelial WB-F344 cells highly express Cx43, but not Cx32 (Neveu et al., 1994b; Rae et al., 1998). The former also holds true for immortal BRL-3A rat liver cells (Wang et al., 2015). As a result, hepatic cell lines can not be used to address specific toxicological issues, such as effects of strain, species, age and gender of nongenotoxic carcinogens on hepatocellular GJIC (Kamendulis et al., 2002; Klaunig and Ruch, 1987). For this reason, the use of primary hepatocyte systems is considered to be a more suitable alternative. Nevertheless, these *in vitro* models suffer from a number of fundamental disadvantages, which mainly result from events that occur during hepatocyte isolation and subsequent cultivation (Elaut et al., 2006; Fraczek et al., 2013). This topic as well as the attempts to overcome this hurdle are discussed in the following sections, with emphasis on the re-establishment of normal liver gap junction patterns *in vitro*.

2.1. Effects of hepatocyte isolation on gap junctions

The two-step collagenase perfusion technique is the most commonly used procedure to isolate hepatocytes from the livers of animals and humans (Lecluyse and Alexandre, 2010; Lee et al., 2013; Papeleu et al., 2006). One of the fundamental principles of this procedure is based on the notion that calcium ions are indispensable for cellular adhesion. Initially, the freshly isolated liver is perfused with a calcium-free medium, often supplemented with a calcium chelator, in order to abandon calcium-dependent cell-cell contacts (*i.e.* adherens junctions). In the second step, the liver is perfused with a collagenase-containing buffer to disrupt cell-extracellular matrix (ECM) interactions (Alpini et al., 1994; Berry et al., 1997; Seglen, 1976). Limited perfusion of the isolated liver with collagenase yields hepatocyte doublets (Gautam et al., 1987), which better retain gap junctions (Coleman et al., 1995; Roma et al., 1997; Yoshizawa et al., 1997). However, in the conventional procedure, gap junctions are fully disrupted by mechanically dispersing the digested liver (Berry et al., 1997). Finally, connective tissue is removed by filtering and further centrifugation separates viable hepatocytes from both dead hepatocytes and nonparenchymal cells (Alpini et al., 1994; Berry et al., 1997; Seglen, 1976). Subsequent cultivation elicits an adaptive response in hepatocytes, as they need to accustom to their new artificial environment. This is associated with a progressive loss of differentiated capacities, the acquisition of a more flattened fibroblast-like morphology and a concomitant return towards a more fetal-like status (Tuschl et al., 2009; Tuschl and Mueller, 2006). Several changes in gene expression profiles accompany this dedifferentiation event. Generally, proliferation-enhancing and survival-promoting genes are upregulated, whereas the expression of differentiation-related genes is lost (Baker et al., 2001). Not surprisingly, drastic modifications also occur at the level of gap junctions. Indeed, Cx43, a connexin species typically found in fetal hepatocytes,

re-appears in cultured adult hepatocytes (Kojima et al., 1995a; Stutenkemper et al., 1992; Vinken et al., 2006a). The functional relevance of this process is unclear, but has been linked to the onset of spontaneous cell death in primary hepatocyte cultures (Vinken et al., 2012b). Possibly, *de novo* Cx43 expression results from altered *cis/trans* regulation of its gene expression. Upon isolation of hepatocytes, *c*-fos and *c*jun are induced (Etienne et al., 1988; Loyer et al., 1996). These proto-oncogenes dimerize to form the transcription factor activator protein-1, which is known to control Cx43 expression (Echetebu et al., 1999). In rat myometrium, activator protein-1 has been shown to induce Cx43 expression under stress conditions (Lefebvre et al., 1995). Although no solid scientific data are presently available, this scenario might also take place in isolated primary hepatocytes in culture.

When seeded under conventional culture conditions, viable hepatocytes adhere to the plastic surface within four hours and aggregate in groups of two to ten cells, thus re-establishing intercellular contacts. After twelve hours of cultivation, hepatocytes start to regain their typical polyhedral morphology, yet this phenotype very rapidly deteriorates (Wanson et al., 1977). In order to counteract this dedifferentiation process and thus to maintain gap junctions, a number of strategies have been followed, which typically aim at mimicking the *in vivo* hepatocyte micro-environment *in vitro*, including (*i*) the addition of differentiation-promoting soluble molecules to the cell culture medium producing improved monolayer cultures, (*ii*) the direct restoration of homotypic and heterotypic intercellular contacts yielding co-cultures and (*iii*) the re-establishment of cell-ECM interactions resulting in tridimensional cultures (Fraczek et al., 2013; Godoy et al., 2013; Hewitt et al., 2007; LeCluyse et al., 1996; Vanhaecke and Rogiers, 2006).

2.2. Improved monolayer cultures

The formulation of the cell culture medium is a crucial parameter for maintaining the hepatocyte-specific phenotype *in vitro*. Historically, hepatocytes have been cultivated on plastic culture dishes covered with serum-supplemented standard media (LeCluyse et al., 1996). Although serum improves cell attachment and survival (Bissell and Guzelian, 1980), many deleterious effects have been reported. Indeed, serum decreases liver-specific functionality, such as phase I xenobiotic biotransformation activity (Enat et al., 1984; Paine and Andreakos, 2004). In addition, it promotes cellular depolarization by inhibiting the re-establishment of gap junctions (Lee et al., 1993; Spray et al., 1987). Therefore, a number of serum-free chemically defined culture media have been developed, including Dulbecco's modified Eagle's medium, William's medium E and Leibovitz's L15 medium. However, when cultivated in these media as such, hepatocytes rapidly undergo functional and morphological deterioration (LeCluyse et al., 1996). Thus, Cx26 and Cx32 levels decrease by 1.6-fold to 2.9-fold, respectively, within two days, regardless of the commercial medium used (Kwiatkowski et al., 1994). For this reason, efforts have been focused on the enrichment of these standard media with both physiological and nonphysiological soluble factors in order to improve the maintenance of the hepatocyte-specific functionality and morphology, all which affect gap junctions.

2.2.1. Dimethylsulfoxide—The beneficial effects of dimethylsulfoxide (DMSO) on the differentiated status of primary cultured hepatocytes have been extensively described. In

general, 2% v/v DMSO is used and promotes liver-specific functionality, such as albumin secretion (Arterburn et al., 1995; Isom et al., 1987). This effect is associated with the long-term (*i.e.* more than four weeks) maintenance of an *in vivo*-like morphology, including the presence of gap junctions. The latter has been shown to result from enhanced expression of Cx26 and Cx32, which thereby promotes GJIC (Kojima et al., 1995a; Kojima et al., 1996a; Stoehr and Isom, 2003; Yoshizawa et al., 1997). Moreover, DMSO inhibits the re-appearance of Cx43 (Kojima et al., 1995a; Stoehr and Isom, 2003). The mechanisms by which DMSO exerts its differentiation-inducing effects are not clear. It has been proposed that DMSO is an oxygen radical scavenger (Kojima et al., 1996b). Alternatively, DMSO can alter intracellular calcium levels and phosphorylation events (Mizuguchi et al., 1998), which might directly affect gap junctions (Arterburn et al., 1995).

2.2.2. Corticosteroids—Glucocorticosteroids are known to retard dedifferentiation in primary cultures of hepatocytes. This has been attributed to their positive effects on cellular functionality (LeCluyse et al., 1996). Glucocorticosteroids, such as dexamethasone, also stabilize the *in vitro* hepatocyte morphology by improving cytoskeleton arrangement and cell-cell contacts (LeCluyse et al., 1996; Ren et al., 1994). In particular, the expression of Cx26 and Cx32 are promoted, resulting in enhanced GJIC (Kwiatkowski et al., 1994; Ren et al., 1994; Siddiqui et al., 1999). In general, glucocorticosteroids affect gene expression by acting at the level of gene transcription, a mechanism that is mediated by the glucocorticoid response element. However, neither Cx26 nor Cx32 contain such a *cis*-acting element within their gene structure (Hennemann et al., 1992). As the presence of adherens junctions is known to be a prerequisite for gap junction formation (Hernandez-Blazquez et al., 2001; Laird, 1996; Lampe et al., 1998) and since the E-cadherin gene is known to contain a glucocorticoid response element (Ringwald et al., 1991), it is thought that the glucocorticosteroid-mediated induction of connexin expression is an indirect result of its positive effects on E-cadherin gene expression (Kwiatkowski et al., 1994).

2.2.3. Nicotinamide and derivatives—Primary cultured hepatocytes rapidly lose their intracellular nicotinamide adenosine dinucleotide (NAD) content. Nevertheless, the presence of this factor is of utmost importance to maintain differentiated features, as NAD serves as a cellular co-enzyme for a number of biochemical reactions (Mitaka et al., 1998). Bearing this in mind, several research groups have explored the possibility of preventing NAD depletion by adding NAD precursors, such as the vitamin nicotinamide and its derivate 3-acetylpyridine, to the cell culture medium of primary hepatocytes. These molecules were shown to stabilize albumin secretion over the cultivation time course (Inoue et al., 1989; Sato et al., 1999) and to preserve Cx26 and Cx32 production for more than two weeks (Higaki et al., 2001; Sato et al., 1999). Nicotinamide not only serves as a NAD precursor, but also inhibits polyadenosine diphosphate ribose polymerase activity, an enzyme that negatively correlates with cellular differentiation (Mitaka et al., 1998).

2.2.4. Cyclic adenosine monophosphate derivatives and modulating agents—Cyclic adenosine monophosphate (cAMP) is a well-known inducer of GJIC in primary cultures of hepatocytes. Upon addition of 8-bromo-cAMP, a membrane-permeant cAMP derivative, to the cell culture medium, the disappearance of GJIC, which generally occurs

soon after the hepatocyte isolation procedure, is delayed, concomitant with a well-preserved cellular morphology. This is thought to be due to positive effects on Cx32 mRNA stability and/or phosphorylation (Saez et al., 1986). Agents known to increase the intracellular cAMP amount in hepatocytes, such as irsogladine (Nakashima et al., 2000) and glucagon (Kojima et al., 1995b; Siddiqui et al., 1999), also enhance the expression of Cx26 and Cx32, and therefore GJIC. Paradoxically, insulin decreases the intracellular cAMP content by stimulating cAMP phosphodiesterase activity (Saez et al., 1986), while inducing connexin production in primary cultures of hepatocytes (Kojima et al., 1995b; Siddiqui et al., 1999). The mechanism behind this observation remains to be elucidated.

2.2.5. S-adenosylmethionine—*S*-adenosylmethionine is a precursor of glutathione and polyamine synthesis, and acts as a methyldonor in cellular transmethylation reactions in the liver. The deteriorative process taking place in cultures of primary hepatocytes, whereby a Cx32-to-Cx43 switch occurs, is associated with decreased *S*-adenosylmethionine levels. Supplementation of the hepatocyte culture medium with this compound results in elevated Cx32 levels and counteracts the appearance of Cx43. The latter is due to reduced accumulation of nuclear β-catenin, which in turn negatively affects Wnt signaling-dependent gene transcription of Cx43 (Yamaji et al., 2011).

2.2.6. Histone deacetylase inhibitors—Chromatin structure undergoes several changes in various physiological situations, such as during cellular differentiation. These effects are partly mediated by histone acetyltransferases and histone deacetylases (HDACs). The former catalyze the acetylation of histones, thereby disrupting DNA-histone interactions, whereas the latter promote the inverse reaction. In general, HDAC activities are associated with gene silencing. Several natural and synthetic molecules are known to inhibit HDAC activity, thereby drastically altering gene expression. This generally results in major modifications in the homeostatic balance in favor of the differentiated status (Papeleu et al., 2005; Vanhaecke et al., 2004; Zhang and Zhong, 2014). Thus, upon addition of sodium butyrate, a naturally occurring HDAC inhibitor, to primary cultured hepatocytes, both functionality and morphology are better maintained in comparison with nontreated cells. The morphological enhancement induced by sodium butyrate is linked to the presence of gap junctions at a level comparable to the *in vivo* situation (Engelmann et al., 1987; Gladhaug et al., 1988; Iwai et al., 2002; Staecker et al., 1988). In agreement with these findings, trichostatin A, a hydroxamate HDAC inhibitor, to the culture medium of primary rat hepatocytes, increases Cx32 expression and GJIC, yet it also enhances Cx43 production and negatively affects Cx26. All these effects are greatly enhanced when trichostatin A treatment is already initiated during the hepatocyte isolation procedure (Vinken et al., 2006a). 4-Me_2N BAVAH, a structural analogue of trichostatin A with a more beneficial metabolic profile, also promotes Cx32 production in primary hepatocyte cultures and suppresses the expression of both Cx26 and Cx43 (Vinken et al., 2007).

2.2.7. Miscellaneous culture medium additives—Isolation and cultivation of primary hepatocytes is associated with oxidative stress (Elaut et al., 2006), which is deleterious for the stability of gap junctions (Morsi et al., 2003; Schmelz et al., 2001). The addition of oxygen radical scavengers was therefore thought to be an efficient strategy to maintain gap

junction integrity *in vitro*. Indeed, upon addition of anti-oxidants, such as vitamin C derivatives (Tateno and Yoshizato, 1999), melatonin (Kojima et al., 1997) and taurine (Fukuda et al., 2000), to the medium of primary cultured hepatocytes, gap junctions are better preserved. Growth factors, like epidermal growth factor, are often used as medium additives, because they increase cell survival (LeCluyse et al., 1996). However, epidermal growth factor negatively affects xenobiotic phase I biotransformation activity (De Smet et al., 2001) and decreases the number of gap junctions in hepatocyte cultures (Berthiaume et al., 1996). Similarly, biotransformation enzyme inducers, such as phenobarbital, are known to retard dedifferentiation of primary cultured hepatocytes (LeCluyse et al., 1996), although they have been shown to inhibit GJIC in these *in vitro* models (Ren and Ruch, 1996).

2.3. Co-cultures

In liver, hepatocytes are in direct contact with each other by means of gap junctions. They also form heterotypic contacts with the surrounding nonparenchymal cells. The presence of both cellular interactions is a prerequisite for normal liver-specific functioning (Bhatia et al., 1999; LeCluyse et al., 2012; Maher and Friedman, 1993). Therefore, the restoration and/or boosting of these contacts was considered an evident strategy to improve liver-specific functionality *in vitro* (LeCluyse et al., 1996). Several research groups have explored co-cultivation of hepatocytes with another cell type. Both hepatic nonparenchymal and nonhepatic cells have been used for this purpose (Coecke et al., 1999; LeCluyse et al., 1996), but the best results have been obtained by co-cultivating hepatocytes with rat liver epithelial cells of primary biliary origin (Corlu et al., 1997; Guguen-Guillouzo and Guillouzo, 1983). In general, co-cultivated hepatocytes display liver-specific functions, including xenobiotic biotransformation capacity and albumin secretion, for long periods (Coecke et al., 1999; LeCluyse et al., 1996). Simultaneously, hepatocyte morphological traits are well preserved and stable Cx32-based gap junctions are present (Bhatia et al., 1999; Mesnil et al., 1993). However, the exact nature of the cellular interaction between hepatocytes and their cultivation partners remains elusive. It has been suggested that heterologous GJIC could account for the improvement of the hepatocyte phenotype. Nevertheless, no gap junction-mediated communication has been observed between hepatocytes and rat liver epithelial cells (Diener et al., 1994; Mesnil et al., 1987; Novikoff et al., 1991). Likewise, heterologous GJIC is absent in co-culture systems consisting of hepatocytes and fibroblasts (Sugimachi et al., 2004) as well as of hepatocytes and stellate cells (Fischer et al., 2005). Hepatocytes and their cultivation partners might be in paracrine contact with each other. A candidate mediator for such communication is epimorphin, a protein that is produced by a number of hepatic cells, such as stellate cells, but not by hepatocytes. Epimorphin plays a role in liver differentiation and its expression pattern, as observed during liver regeneration, is very similar to that of Cx32 in hepatocytes (Spray et al., 1987).

Another strategy to retard dedifferentiation *in vitro* includes the boosting of homotypic hepatocyte interactions. This can be achieved by continuously rotating hepatocytes in suspension or by using cell-repelling substrata. Both methods result in the formation of multicellular hepatocyte aggregates called spheroids (LeCluyse et al., 1996). Within these structures, hepatocytes are in intimate contact with each other. This is associated with the

abundant presence of gap junctions (Abu-Absi et al., 2002; Hou et al., 2001; Koide et al., 1990; Yang et al., 2003a) and results in the long-term maintenance of liver-specific functionality. This approach has been further optimized by including nonparenchymal liver cells in the hepatocyte aggregation process. Such heterospheroids retain global cell-cell contacts and thus closely resemble the *in vivo* situation (LeCluyse et al., 1996).

2.4. Tridimensional cultures

In normal liver, hepatocytes are in contact with a broad range of ECM proteins, including collagens, glycoproteins, proteoglycans and glycosaminoglycans (Amenta and Harrison, 1997; Iredale and Arthur, 1994; LeCluyse et al., 2012; Maher and Bissell, 1993). These ECM components control hepatocellular homeostasis *via* integrin-mediated signaling (LeCluyse et al., 1996; Mousa, 1998). Based on this knowledge, many studies have focused on the re-introduction of a natural scaffold in hepatocyte cultures in order to regain the *in vivo*-like hepatocyte phenotype (Depreter et al., 2002; Hamilton et al., 2001; LeCluyse, 2001). In general, the presence of ECM proteins promotes liver-specific functionality, which is associated with enhanced gap junction formation (Berthiaume et al., 1996; Iredale and Arthur, 1994; Mooney et al., 1992). In this respect, collagens, glycoproteins, proteoglycans and glycosaminoglycans were all shown to induce the expression of connexin proteins and GJIC activity (Fujita et al., 1986; Fujita et al., 1987; Spray et al., 1987). However, seeding cells on a layer of ECM proteins provides *in vitro* systems that retain liver-specific functions for about five days (Knop et al., 1995; Maher and Bissell, 1993), which is only two days more in comparison with conventional monolayer cultures (Vanhaecke and Rogiers, 2006). Improvements of this strategy rely on the application of a second ECM layer (*i.e.* the sandwich technique) (Dunn et al., 1991; Koebe et al., 1994) or the entrapment of hepatocytes in a collagen gel (*i.e.* the immobilization method) (Koebe et al., 1994) in which hepatocyte functionality, such as xenobiotic biotransformation capacity and albumin secretion, can be kept for more than two months. This stabilized functionality is also reflected at the level of cell morphology. Indeed, an *in vivo*-like morphology, including the presence of gap junctions, is re-established in these *in vitro* systems (Berthiaume et al., 1996; Hamilton et al., 2001; LeCluyse, 2001).

Nonphysiological and synthetic ECM scaffolds have also been used to create organotypical hepatocyte cultures. Thus, Matrigel®, a laminin-rich extract from Engelbreth-Holm-Swarm mouse tumor, has been proposed as a cultivation substratum for hepatocytes (Iredale and Arthur, 1994; Maher and Bissell, 1993). Upon cultivation in this nonphysiological matrix, hepatocytes express liver-specific functions for more than four weeks associated with the abundant presence of Cx32-based gap junctions (Hamilton et al., 2001; LeCluyse, 2001). Several laboratories have tested synthetic compounds as hepatocyte scaffolds, such as poly(lactide-co-glycolide) acid (Hasirci et al., 2001) polyvinyl formal resin (Yang et al., 2001) and polyurethane foam (Pahernik et al., 2001). The use of synthetic ECM scaffolds is frequently combined with spheroid formation, yielding hepatocyte aggregates that highly express Cx32 (Seo et al., 2004).

3. *In vitro* methods to study liver gap junctions

Several methods are currently available to test GJIC in cultured cells. They can be divided into three classes, namely (*i*) metabolic coupling assays, (*ii*) electrical coupling assays and (*iii*) dye coupling assays.

3.1. Metabolic coupling assays

The metabolic co-operation approach is based upon the monitoring of the transfer of endogenous and biologically relevant compounds. For this procedure, fluorescently marked donor cells are incubated in the presence of radiolabelled precursors, like nucleotides or glucose, and then co-cultured with unlabeled recipient cells. Subsequently, donor cells are separated from receiver cells through fluorescence-activated cell sorting and the amount of the radio-isotope in the receipt cell population is assessed by chromatography and/or quantitative autoradiography (Goldberg et al., 1999; Goldberg et al., 1998). A more indirect method includes the tracking of calcium waves, which correlates with the presence of functional gap junctions. In this technique, cells are loaded with a calcium-sensitive fluorescent dye and are stimulated electrically, mechanically or chemically in order to generate inositol triphosphate, which triggers the actual calcium wave. A more sophisticated approach is the local liberation of inositol triphosphate from a caged precursor by flash photolysis, which allows the stimulation of single cells (Decrock et al., 2015; Leybaert and Sanderson, 2001). Metabolic coupling assays, in particular transfer of labelled nucleotides, have been used to demonstrate the inhibitory effects of 12-*O*-tetradecanoylphorbol-13-acetate on GJIC in co-cultures of primary chick embryo hepatocytes and Chinese hamster V79 lung fibroblasts (van der Zandt et al., 1990).

3.2. Electrical coupling assays

The dual voltage patch clamp technique envisages the recording of gap junctional electrical conductance, whereby originally two separate micro-electrodes were introduced in each cell of a cell pair, one for current injection and another one for voltage control (Spray et al., 1979, 1981). This technique was later modified to a double whole cell voltage clamp technique, using only one patch pipet *per* cell, which is a very sensitive method that allows the recording of a single gap junction channel (Hamill and Sakmann, 1981; Neyton and Trautmann, 1985). In a more recent method, GJIC is measured using a combination of single cell electrophysiology, large-scale optical recordings and a sensor of plasma membrane potential (Ceriani and Mammano, 2013). Analysis of gap junctional electrical conductance, however, is a labor-intensive, expensive and rather slow technique that requires appropriate expertise and technical skills (Abbaci et al., 2008; Yamasaki, 1997). Electrical coupling assays, in particular voltage patch clamping, have been used to demonstrate the inhibitory effects of carbon tetrachloride on GJIC in cultures of primary rat hepatocytes (Saez et al., 1987).

3.3. Dye coupling assays

Dye coupling methods are by far the most frequently used ones, mainly because of their ease of use. This kind of assays relies on the introduction of small dyes into living cells that are traced in their intercellular movement. A wide variety of tracers, mostly fluorescent, are

used (Abbaci et al., 2008; Meda, 2000), and there are several ways to introduce these reporter dyes into cells, including micro-injection (Kanno and Loewenstein, 1964), mechanical loading by scraping (el-Fouly et al., 1987) and electroporation (De Vuyst et al., 2008; Decrock et al., 2015; Raptis et al., 1994). Recently, a high-throughput GJIC measurement system based on robotic micro-injection has been described (Liu et al., 2015). In addition, a number of noninvasive dye coupling protocols have been established. In fluorescence recovery after photobleaching (FRAP) analysis, cells are loaded with a lipophilic cell plasma membrane permeable dye, such as calcein acetoxymethyl ester. Upon cellular uptake, this dye is hydrolized by cytoplasmic esterases, producing calcein, which is a fluorescent and membrane-impermeable molecule. Fluorescence in a single cell is then irreversibly photobleached using a high-powered laser beam and subsequent transfer of fluorescent dye from neighboring cells into the target cell is monitored (Abbaci et al., 2007; Wade et al., 1986). FRAP can be applied to monolayer culture systems as well as to tridimensional *in vitro* models (Kuzma-Kuzniarska et al., 2014).

Both the preloading assay and the parachute technique also require cell loading with cell plasma membrane-permeable dyes. In the former, loaded cells are suspended together with unloaded counterparts and are then allowed to form a confluent monolayer (Goldberg et al., 1995), whereas in the latter, loaded cells in suspension adhere to a monolayer of unloaded cells (Ziambaras et al., 1998). In both cases, the spread of the dye from donor cells to receiver cells is studied by fluorescence microscopy and is a measure for GJIC. In the local activation of molecular fluorescent probe (LAMP) method, a new generation of caged coumarin-like fluorophores is used. Like in FRAP, these dyes are processed by intracellular esterases, but they only become fluorescent upon subsequent local illumination with a small dose of ultraviolet light. The latter is unlikely to cause photodamage, in contrast to the high-powered laser beam used in the FRAP approach (Dakin et al., 2005). An improvement to the LAMP method has been described, the so-called infrared-LAMP assay, which allows examination of cell-cell coupling in three dimensions (Yang and Li, 2009). Dye coupling assays, such as based on microinjected Lucifer Yellow, have been used to demonstrate the inhibitory effects of the nongenotoxic peroxisome proliferating drug nafenopin in cultures of primary rat hepatocytes (Elcock et al., 1998).

4. Conclusions and perspectives

Because of its unique localization and function in the organism, the liver is a primary target for systemic toxicity. For this reason, a lot of attention has been paid, and it still being paid, to the establishment of liver-based models for *in vitro* toxicity testing purposes. Among those, cultures of primary hepatocytes are considered as the gold standard, as they provide an appropriate reflection of the hepatic *in vivo* situation (Fraczek et al., 2013; Godoy et al., 2013; Lin et al., 2015). In these experimental systems, gap junctions and concomitant physiological connexin expression can be maintained by applying a number of techniques that intend to create an *in vivo*-like environment for hepatocytes (Vinken et al., 2006b). Although promising, these techniques are not able to completely counteract the dedifferentiation process that is triggered during hepatocyte isolation. A major reason for this shortcoming is that these culture configurations act on the consequences of this deteriorative process. In recent years, a number of innovative methodologies has been

introduced that are targeted towards the actual cause of dedifferentiation, such as by directly interfering with the gene transcription of liver-specific proteins (Fraczek et al., 2013; Vinken et al., 2012c). It is conceivable to assume that gap junctions are equally positively affected by these novel strategies, yet this remains to be experimentally confirmed. Furthermore, in the last decade, the field of *in vitro* toxicology has witnessed the introduction of sophisticated liver-based systems in which the *in vivo* functional phenotype can be kept for extended periods of time, such as microfluidic liver bioreactors (Khetani et al., 2015; LeCluyse et al., 2012; Lin et al., 2015). In parallel, stem cells have entered the *in vitro* toxicology area along with several strategies for their differentiation into hepatocyte-like cells (Kia et al., 2013; Sauer et al., 2014). In this context, liver progenitor cells or oval cells mainly express Cx43 (Zhang and Thorgeirsson, 1994), which also holds true for many liver cell lines. As there is increasing evidence that alteration of liver progenitor cells and oval cells by toxic chemicals plays an important role in the development of different chronic liver diseases (Canovas-Jorda et al., 2014), it should be stressed that liver cell lines may still be of great use to study alterations of GJIC by chemicals in toxicologically and pathologically relevant Cx43-expressing cell types, thereby providing information complementary to chemical effects on Cx32-dependent GJIC in differentiated hepatocytes. Obviously, these developments may open new perspectives for the establishment of cutting-edge *in vitro* systems to test hepatic GJIC. The latter is a goalkeeper of liver homeostasis and hence a key determinant of hepatotoxicity. In particular, unlike their genotoxic counterparts (Ruch, 1994; Yamasaki and Naus, 1996), nongenotoxic carcinogens typically inhibit GJIC, *in casu* in liver (Budunova and Williams, 1994; Oyamada et al., 1990; Trosko et al., 1994; Vinken et al., 2009; Yamasaki, 1995). A number of assays are nowadays used for testing gap junction functionality, with the dye-based methods being the most commonly used ones. These assays are featured by many advantages (Abbaci et al., 2008), yet they may not reflect actual GJIC *per se*. Indeed, the reporter dyes used in these methods substantially differ from the natural gap junction permeants. In this regard, the biophysical properties of a given gap junction highly depend on the connexin species that compose the channel. Thus, Cx26-based gap junctions are known to favor cation transfer, whereas gap junctions consisting of Cx32 rather promote anion passage (Bukauskas et al., 1995). In a similar way, adenosine triphosphate is conveyed about three hundred times better through gap junctions formed by Cx43 compared with Cx32-based channels (Goldberg et al., 2002). In the upcoming years, efforts should be focused on the further optimization of gap junction methods that allow (patho)physiologically relevant assessment of GJIC. When combined with appropriate cellular systems, it can be expected that a valuable *in vitro* tool will be generated eligible for the evaluation of the nongenotoxic carcinogenic potential of chemical compounds during the process of risk assessment.

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Abbreviations

cAMP	cyclic adenosine monophosphate
Cx	connexin
DMSO	dimethylsulfoxide
ECM	extracellular matrix
FRAP	fluorescence recovery after photobleaching
GJIC	gap junctional intercellular communication
HDAC(s)	histone deacetylase(s)
LAMP	local activation of molecular fluorescent probe
NAD	nicotinamide adenosine dinucleotide

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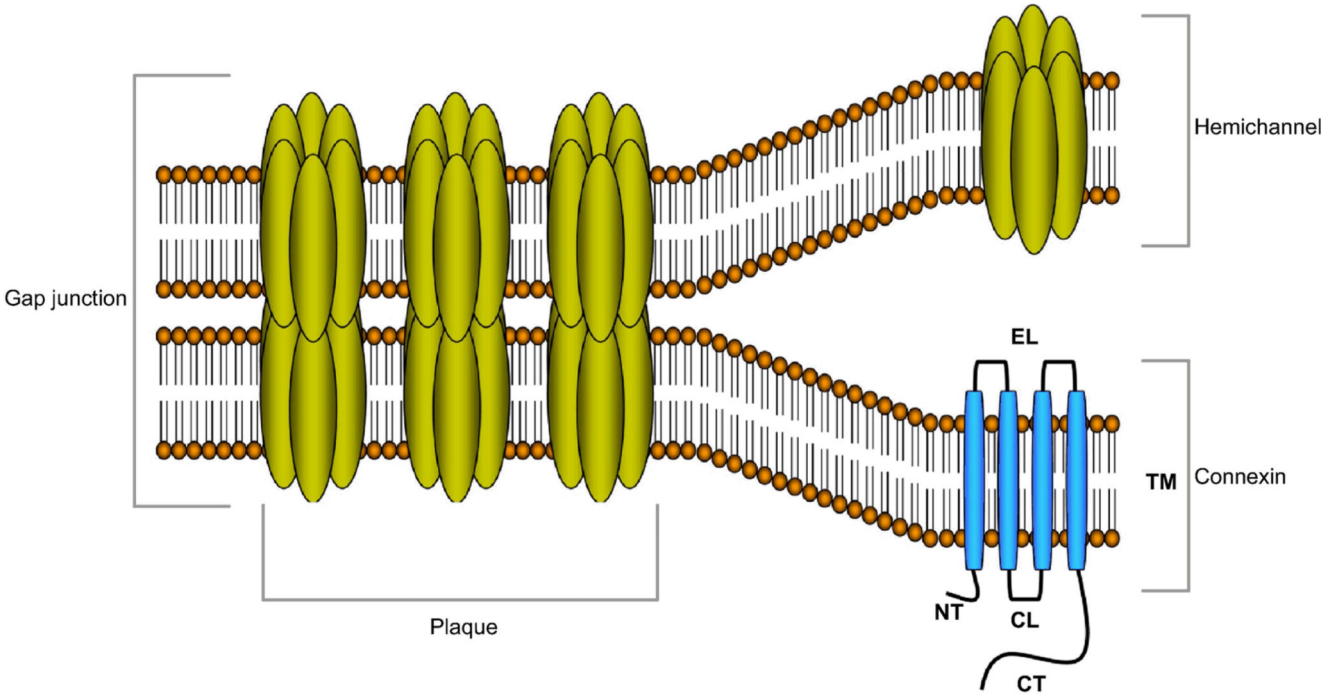

Figure 1. Molecular architecture of gap junctions

Gap junctions are grouped in plaques at the cell plasma membrane surface of two apposed cells and are composed of twelve connexin proteins, organized as two hexameric hemichannels. The connexin protein is organized as four transmembrane domains (TM), two extracellular loops (EL), one cytoplasmic loop (CL), one cytoplasmic aminotail (NT) and one cytoplasmic carboxytail (CT).

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Table 1

Agents that negatively affect hepatic gap junctions (Vinken et al., 2009).

Environmental pollutants
Polycyclic aromatic hydrocarbons
Polychlorinated dibenzodioxins
Polychlorinated biphenyls
Biological toxins
Phorbol esters
Lipopolysaccharide
Ochratoxin A
Patulin
Gossypol
Organic solvents
Ethanol
Carbon tetrachloride
Trichloroethylene
Pesticides
Organophosphorous pesticides
Cyclodiene organochlorine pesticides
Dichlorodiphenyltrichloroethane
Lindane
Hexachlorobenzene
Pentachlorophenol
Pharmaceuticals
Hypolipidemic drugs
Phenobarbital
Methapyrilene
Miscellaneous compounds
Peroxides
Metals
Phthalates