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Review Article

In Vitro and *In Silico* Liver Models: Current Trends, Challenges and Opportunities

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Abstract

Most common drug development failures originate from either bioavailability problems or unexpected toxic effects. The culprit is often the liver, which is responsible for biotransformation of the majority of xenobiotics. Liver may be modeled using liver-on-a-chip devices, which may include established cell lines, primary human cells, and stem cell-derived hepatocyte-like cells. The choice of biological material along with its processing and maintenance greatly influence both the device performance and the resultant toxicity predictions. Impediments to the development of liver-on-a-chip technology include problems with standardization of cells, limitations imposed by culturing, and the necessity to develop more complicated fluidic contours. Recent breakthroughs in the development of cell-based reporters, including ones with fluorescent labels, permit monitoring of the behavior of the cells embedded into the liver-on-a-chip devices. Finally, a set of computational approaches has been developed to model both toxic responses and the homeostasis of human liver as a whole; these approaches pave the way to enhance the *in silico* assessment of potential toxicity.

1 Introduction

Drug development is becoming more and more expensive: It takes 12-15 years and around two billion dollars to bring a single drug into the market. The current paradigm of drug discovery relies on high throughput screening (HTS) of chemical libraries to identify compounds that bind purified target molecules produced by means of genetic engineering (Coussens et al., 2017; Raucy and Lasker, 2010). Lead compounds identified in HTS *in vitro* assays are subjected to secondary screens in established cell models before proceeding into animal testing. Currently, there is a substantial push for the development of standardized cell-based assays compatible with HTS format (Nickischer et al., 2018; Xia and Wong, 2012) or even the whole-animal assays (Delvecchio et al., 2011; O'Reilly et al., 2014; Pandey and Nichols, 2011).

Using human-derived cell line-based assays as the secondary screen has a number of advantages, the most important one being that cultivated cells are very close in their composition and metabolite concentrations to the cells in the human body. This characteristic helps to deprioritize drug candidates that are incapable of competing with endogenous co-substrates or co-ligands. Moreover, compounds that are unable to permeate cell membranes or that display direct toxicities would be also eliminated. However, work with the cell-line based models has a fundamental shortcoming: the human body is built from many hundreds of cell types, while commonly established cell cultures are typically homogenous.

One way to bridge this gap is to perform secondary screens in cell types representing the tissues most commonly contributing to the systemic toxicity that may arise when the whole body is exposed to a novel pharmaceutical. The culprit is often the liver, which is responsible for biotransformation of the majority of xenobiotics. Direct toxicity to the hepatic parenchyma is also quite common. Historically, approximately 20-30% of all drug withdrawals from US and EU markets are due to hepatotoxicity, with drug-induced liver injury (DILI) contributing at least 40% to the

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number of withdrawals in the United States (Olson et al., 2000; Peters, 2005). Ideally, potential drug toxicities should be discovered during preclinical testing in cellular or animal models. Nevertheless, many drugs were found to cause injury to human liver only after marketing and were subsequently either discontinued or received the warning label. Whether the failure of preclinical modeling is due to human genetic variants, immune reactions or disease-related metabolic problems, the animal models are limited in detecting human-specific phenomena. In addition, there is also a strong push to minimize the use of animal models due to ethical concerns (Langley et al., 2015).

Therefore, in this review we concentrate on liver cell-based platforms amenable to standardizing for their eventual use in toxicity screening compatible with HTS mode. This goal can be achieved with the development of liver-on-a-chip devices with embedded human cells. Such platforms may be useful both at the earliest steps of drug development (Fig. 1), which embraces the paradigm of "failing early-failing cheaply", and at later development stages, where they will eventually reduce the use of animals and provide more human-relevant information.

2 Human liver metabolism differs from that of animals

Liver is an intricate factory built by a variety of cooperating cells that perform over 500 distinct functions, including large scale synthesis of various blood components, metabolism of glucose, fatty acids and cholesterol, production of bile, and detoxification/biotransformation of endogenous and exogenous substances. Of all the important liver functions, its metabolism – especially that of pharmaceutical drugs and other xenobiotics – is perhaps the most important in the context of pharmaceutical toxicology. In hepatic parenchyma, xenobiotics undergo three phases of metabolism and transport: (1) Phase I, which is mainly catalyzed by the cytochrome P450 enzymes (CYP450) and results in transformation of lipophilic compounds into water-soluble metabolites; (2) Phase II, where various enzymes conjugate xenobiotics and/or their metabolites to highly polar molecules such as glucose, glucuronic acid, sulfate, or glutathione; (3) Phase III, where specific or non-specific transporters efflux these highly polar metabolites out of hepatocytes into the bile, or release them back to the blood for subsequent excretion with urine. The extreme diversity of the Phase I/II enzymatic system is a root cause for poor predictive performance of cell-based hepatotoxicity assays for possible adverse effects of potential drugs in humans.

Although Phase I reactions may be carried out by any combination of 50 cytochrome P450 (CYP450) monooxygenases, some of these enzymes are more important than others. Six cytochromes, namely CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5, are capable of metabolizing 90% of known drugs (Liddle and Stedman, 2007; Lynch and Price, 2007). Both the isoenzyme profile of these cytochromes and the relative catalytic activity within a particular source of the liver

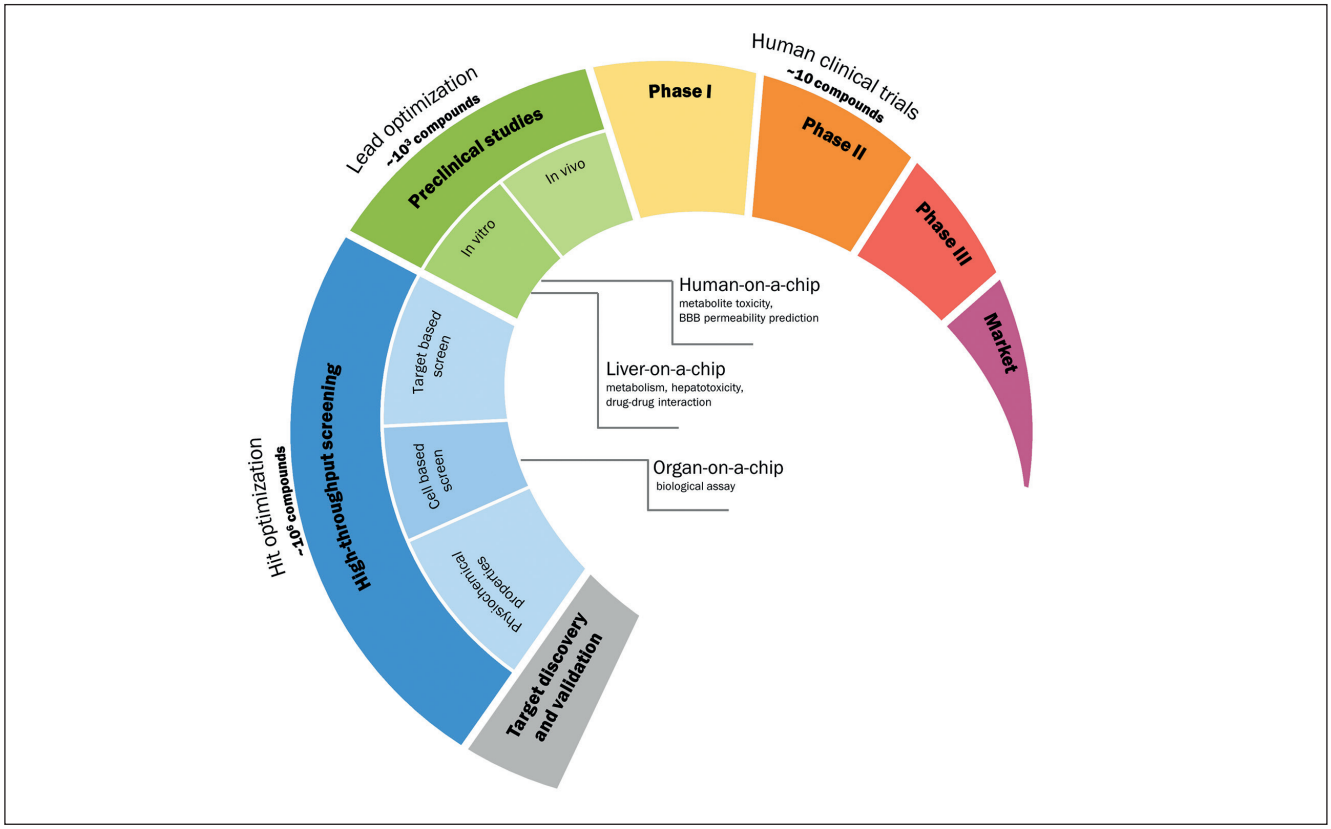

Fig. 1: HTS-based drug discovery paradigm

Tab. 1: Comparison of cell sources for liver-on-a-chip technology

Cell source (relevant references)	**Primary hepatocytes** (Bell et al., 2017; Hart et al., 2010)	**Upcyte®** (Ramachandran et al., 2015b; Herzog et al., 2016; Schaefer et al., 2016, 2018)	**HepG2** (Gerets et al., 2012; Hart et al., 2010)	**Differentiated HepaRG** (Bell et al., 2017; Gerets et al., 2012; Hart et al., 2010; Le Vee M. et al., 2006)	**iPSC derived** (Baxter et al., 2015; Chaudhari et al., 2016)
Genetic similarity to human hepatocytes	Exact (derived from particular individual)	Exact (derived from particular individual)	Medium (derived from hepatoblastoma with abnormal karyotype and highly malignant phenotype)	High (quiescent, differentiated cancer cell line with stable sub-diploid karyotype with minimal alterations)	Exact (derived from particular individual)
Variability	High	Low	High	Low	High
Standardization	Impossible	Possible	Impossible	Possible	Impossible
Use in personalized medicine	Yes	No	No	No	Yes
Supply	Limited	Unlimited	Unlimited	Relatively unlimited	Unlimited
Viability	10-14 days	10-14 days	7 days	30 days	2-4 days
Number of passages	n/a	40	Unlimited	20 to preclude instability	50
Functional stability	Yes	Yes	No	Yes	Unknown
P450 enzymatic profile	1A2, 2A6, 2C8, 2B6, 2C19, 2C8, 2D6, 2E1, 2C9, 3A4, 3A5, 3A7	1A2, 2C8, 2B6, 2C19, 2C9, 2D6, 3A4	1A2, 2C8, 2B6, 2C9, 3A5, 3A7	1A2, 2A6, 2B6, 2C19, 2C8, 2E1, 2D6, 2C9, 3A4, 3A5, 3A7	1A2, 2A6, 2B6, 2C19, 2C8, 2E1, 2D6, 2C9, 3A4, 3A5, 3A7
Total P450 activity	High	Low	Low	Medium	Relatively low, as in fetal hepatocytes
Transporters	ABCBA, ABCB7, ABCF3, ABCB1, ABCC1, ABCC2, ABCC3, ABCE1, ABCF1, ABCF2, GTR1, SLCO1B1, SLC22A1	ABCF3, ABCB1, ABCC1, ABCC2, ABCE1, ABCF1, ABCF2, GTR1, ABCB11, SLCO1B1, SLC22A1, SLC47A1	ABCF3, ABCB1, ABCC1, ABCE1, ABCF1, ABCF2, GTR1	ABCBA, ABCB7, ABCF3, ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCE1, ABCF1, ABCF2, GTR1, SLC22A1, SLCO2B1, SLCO1B1, SLC10A1	ABCBA, ABCB7, ABCF3, ABCB1, ABCB11, ABCC1, ABCC2, ABCE1, ABCF1, ABCF2, GTR1, SLC10A1
Generation of reporter lines	Impossible	Possible	Possible	Possible with limitations	Possible

material are most critical for the standardization and for proper functioning of the liver-on-a-chip device.

Phases I and II are commonly referred to as "metabolic detoxification," which is a misnomer, as many xenobiotics are metabolized into either bioactive or toxic compounds. Recapitulation of the *in vitro* liver drug metabolism is the pivotal aim of a liver-on-a-chip development. Ideally, preclinical assays would require use of highly standardized cellular components; at the end, the potencies and other characteristics of various drugs should be compared one to another across the batches of disposable devices. The easiest way to standardize biological components of the chip without reverting to cell immortalization is to use cells prepared from inbred animals, for example, a particular strain of mice. Unfortunately, the comparison of the content of nine CYP450 enzymes in the microsomes derived from hepatic specimens procured from mouse, rat, rabbit, dog, micropig and monkey revealed that no single model species resembles the enzyme activities in human liver (Bogaards et al., 2000). More-

over, the metabolic abilities of Phase II enzymes also markedly differ among species as was demonstrated for UDP-glucuronosyltransferase (UGT) activity (Hanioka et al., 2016) as well as for sulfation and glutathione conjugation (Miller et al., 1993).

Therefore, we would have to create liver chips using cells of human origin to best predict human liver metabolism. Let us consider how close different preparations of liver cells may resemble human liver (Tab. 1).

3 Liver cells and cell lines: available choices

Liver slices and whole perfused organs preserve an intact tissue structure, thereby being the most physiologically realistic model. However, their standardization and long-term maintenance have proven close to impossible. However, some reliable approximation of the liver could be achieved with the aid of microfabrication and advanced tissue engineering, capable of the generation of "on-a-chip" tissue and organ models suitable for HTS purposes. Of note, these models can be imaging- and analysis-friendly as they allow for real-time monitoring of the state of the living cells and their extracellular environment. A successful platform for mimicking liver physiology and hepatic drug metabolism *in vitro* is expected to replicate all major liver functions by controlling cellular dynamics over a prolonged period of time, which is currently defined as more than 28 days.

3.1 Hepatocytes

Human hepatocytes represent nearly 60% of the total cell population within the liver. These cells, capable of performing a majority of liver functions, can be isolated from the human liver via collagenase perfusion. Primary hepatocytes are commonly accepted as the "gold standard" for constructing liver models for drug testing and other applications. Maintaining an *in vivo*-like phenotype for isolated hepatocytes is challenging, since in a monolayer culture these cells undergo significant changes in Phase I and II metabolism and lose function over 72 hours (Rodriquez-Antona et al., 2002). Moreover, cultured primary hepatocytes may also lose their polarization, which would, in turn, greatly affect their ability to efflux biotransformed compounds (Luttringer et al., 2002; Noel et al., 2013). Luckily, both the widely used well-differentiated human hepatoma cell line HepaRG and primary human hepatocytes retain their polarization even in monolayers, as is evident by differential expression of a proper set of influx and efflux transporters at their sinusoidal and canalicular poles, respectively (Le Vee et al., 2013, 2015).

The functional life of hepatocytes may be prolonged by a variety of techniques. One promising approach is to culture them as spheroids, through inhibiting hepatocyte attachment to vessel walls and, thereby, enforcing their floating as aggregates. Spheroids may be formed by mechanical agitation by rotary shaker or spinner flask, hanging drop or using non-adherent surface chemistry. Hepatocellular spheroids retain a majority of the parenchymal functions, including the secretion of albumin, urea, transferrin and bile, along with Phase I and II biotransformation activity, for at least three weeks after seeding (Arakawa et al., 2017), which make them suitable for cytochrome induction tests. Hepatocyte spheroids are compatible with a serum-free medium and co-culturing with non-parenchymal Kupffer, stellate and biliary cells (Bell et al., 2016). Heterotypic spheroids reflect tissue environments *in vivo*, and, at least theoretically, permit construction of larger tissue models by higher order assembly of individual spheroids. In composite spheroids, i.e., spheroids composed of collagen microparticle scaffolds and cells, the optimal ratio of hepatocytes to type I collagen microparticles is approximately 1:1; the shift towards more of the collagen microparticles compromises hepatocyte functions (Yamada et al., 2015). On the other hand, an optimal co-culturing ratio for hepatocytes supported with endothelial progenitor cells is 5:1, in the presence of alginate-collagen (Chan et al., 2016). Another recent work used a 3D printing technique to combine alginate hydrogels with primary hepatocytes and mesenchymal stem cells (MSCs). This approach improved the viability of isolated hepatocytes to more than 90% and their morphological stability to up to 7 days (Kim et al., 2018).

Another hepatic modeling approach is based on sandwich cultures, also known as overlays. Sandwiching hepatocytes between two layers of ECM leads to formation of "plate" structures similar to the *in vivo* liver anatomy. The planar structure of the sandwich allows easy microscopic imaging. This technique preserves the polarity, including basal surfaces induced by ECM layers and apical surfaces by cell-to-cell contact, and leads to the development of a canalicular network and the secretion of bile (Swift et al., 2010). In sandwich cultures, the viable period may be increased to up to 6-8 weeks. However, sandwiched hepatocytes maintain their biotransformation activities and the ability to induce many Phase I and Phase II enzymes for the first two 2 weeks only.

Culturing primary hepatocytes within 3D structures formed by porous poly(L-lactide-co-glycolide) (PLGA) modified type I collagen nanofibers that mimic the natural liver environment is a promising strategy to improve the synthetic function of the liver cells over time (Brown et al., 2018).

In a recent study, primary human hepatocytes grown in a sandwich overlaid with extracellular matrix were directly compared to 3D spheroids in repeated-dose toxicity studies using 5 different liver toxins. To ensure robustness of the findings, the study was performed in six different laboratories using cryopreserved cells collected from the same set of donors. The study showed superiority of the 3D spheroids in expression of ADME-related proteins, as well as in catalytic activities of five different cytochromes (Bell et al., 2018).

3.2 Upcyte® hepatocytes

The upcyte® ("upregulated") technology involves virus-guided introduction of a unique combination of genes that induce and maintain cell proliferation until the cells reach confluence. This allows the primary cells to be passaged many times with the generation of billions of cells. Human upcyte® hepatocytes[1] are primary human hepatocytes derived by transducing E6 and E7

[1] http://www.upcyte.technologies.com

proteins of human papillomaviruses, which release hepatocytes from cell cycle arrest and allow their proliferation in response to oncostatin M (OSM), a member of the interleukin-6 (IL-6) superfamily involved in liver regeneration. In cultures, upcyte® hepatocytes undergo a finite number of cell divisions without being immortalized or losing adult primary cell phenotype (Burkard et al., 2012). Upon stimulation with OSM, doubling time for these cells is between 33 and 49 hours. After OSM is withdrawn, upcyte® hepatocytes differentiate to generate highly functional cells. This method allows expanding human hepatocytes for 35 population doublings, resulting in 10^{15} (a quadrillion) cells from each liver biopsy. Over 12 billion upcyte® hepatocytes can be generated from one vial of primary human hepatocytes, thus meeting the high demand for standardized cells necessary for HTS studies. In the first-generation upcyte® hepatocytes, cytochromes CYP1A2, CYP2B6, and CYP3A4, but not CYP2B6, were drug-inducible at the mRNA level, suggesting the necessity for additional optimization. Second-generation upcyte® hepatocytes (Levy et al., 2015) form metabolically functional, polarized cultures with functional bile canaliculi and expression profiles for nuclear receptors, Phase I and II enzymes, and drug transporter genes comparable to those in primary human hepatocytes. As with the first-generation upcyte® hepatocytes, second-generation cells lack fetal markers and express cytokeratin 8 and 18, human serum albumin and store glycogen (Levy et al., 2015).

Upcyte® technology opens new horizons in modeling organotypic cultures. In a recent report, functional 3D hepatic structures were generated using a defined mixture of three types of differentiated human upcyte® cells, namely hepatocytes, liver sinusoidal endothelial cells (LSECs) and mesenchymal stem cells (MSCs). When all three types of cells were plated on a thick layer of Matrigel™, they self-organized to form liver organoid-like structures within 24 hours; during a 10 day culturing in a bioreactor, these liver organoids showed typical functional characteristics of liver parenchyma including activity of CYP3A4, CYP2B6 and CYP2C9 as well as mRNA expression of several marker genes and other enzymes (Ramachandran et al., 2015a).

It is also important to note that upcyte® hepatocytes can be transformed with reporter constructs to permit real time monitoring of hepatocyte functions and/or drug effects.

3.3 HepG2 cells

The HepG2 cell line was derived from a hepatocellular carcinoma of a 15-year-old Caucasian male. Due to low endogenous expression of cytochromes, HepG2 cells are a relatively poor choice for detection of hepatotoxicity (Wilkening et al., 2003) (Tab. 1). Even when HepG2 cells are made to express cytochromes forcibly, via adenoviral transfection, these cells do not reach liver model standards, as they also lack activity of aldolase B; several drug transporters such as BSEP, OATP-C, NTCP, and OCT-1; and a range of non-cytochrome Phase II enzymes, such as GSTA 1/2 and GSTM1 (Gripon et al., 2002; Guillouzo et al., 2007; Wilkening et al., 2003).

Historically, HepG2 cells were extensively exploited to examine cytoprotective, antioxidative, hepatoprotective, anti-hepatoma, hypocholesterolemic, anti-steatosis, bioenergetic homeostatic and anti-insulin resistant properties of various bioactive compounds of chemical and botanical origin (Kaur et al., 2018). Due to high content of organelles and mtDNA, HepG2 cells remain a model of choice for investigation of mitochondrial toxicity through evaluations of mitochondrial fragmentation, lysosome content and mitophagy as well as mitochondrial release of cytochrome c, leading to apoptosis and/or necrosis (Paech et al., 2018; Paemanee et al., 2017). Because of that, attempts to improve the overall performance of HepG2 are continued, with the chief strategy to overcome their limitations being the development of three dimensional (3D) models, including co-culturing (He et al., 2018) and generation of the spheroids maintained in the hanging drops or otherwise (Hurrell et al., 2018; Shah et al., 2018).

3.4 HepaRG cells

Although the HepaRG cell line was derived from a hepatoma of a female patient with cirrhosis following hepatitis C virus infection (Gripon et al., 2002), unlike other human liver cell lines, HepaRG cells express many drug processing genes at levels similar to those in primary human hepatocytes. In particular, HepaRG express various nuclear receptors, transporters, and specific markers of adult hepatocytes (albumin, haptoglobins, and aldolase B) (Guillouzo et al., 2007). In confluent cultures, HepaRG cells differentiate from a stem cell/progenitor state to mature hepatocytes and primitive biliary cells and maintain a relatively stable function for several weeks (Jossé et al., 2008). HepaRG cells, including 3D-organotypic HepaRG cultures obtained using a scaffold-free, high-throughput hanging drop system are considered a viable option for evaluating hepatotoxic chemicals with reproducible responses (Gunness et al., 2013).

A high-throughput transcriptional profiling of both differentiated and undifferentiated HepaRG cells found that these cells have much higher resemblance to primary human hepatocytes and biopsied livers that HepG2 (Hart et al., 2010). These transcriptomics data have been recently supported by proteomics: a global proteomic analysis of HepG2, upcyte®, and HepaRG showed that the cytochrome activity levels of both HepG2 and upcyte® were reduced by 90% in comparison to primary hepatocytes, while levels in HepaRG cells were reduced by 60% (Sison-Young et al., 2015). Remarkably, HepaRG cells also retained expression of MRP3 and P-gp (MDR1) transporters.

Molecular profiling data described above indicate that the HepaRG cell line in many ways resembles human primary hepatocytes, which is encouraging for utilization of these cells in the studies of xenobiotic metabolism, hepatotoxicology, and hepatocyte differentiation. It is, however, important to note that HepaRG cells eliminate galactose/sorbitol and produce albumin at rates higher than in primary hepatocytes, while being unable to excrete urea (Lübberstedt et al., 2011). As HepaRG cells are a clone derived from a particular individual, it is not surprising that the levels of cytochrome activities and their relative induc-

ibility in these cells match some primary hepatocyte cultures but not others (Berger et al., 2016; Hart et al., 2010; Lübberstedt et al., 2011; Sison-Young et al., 2015). These differences are most likely intrinsic as they reflect variation in the expression levels of individual cytochromes across healthy humans.

3.5 Induced pluripotent stem cells (iPSC)

Induced pluripotent stem cell (iPSC) technology was introduced in 2006 (Takahashi and Yamanaka, 2006). iPSCs originate from adult cells reprogrammed by the introduction of several genes essential for embryonic stemness, namely Oct3/4, Sox2, c-Myc, and Klf4. Similar to embryonic stem cells, iPSCs can be differentiated into endoderm, mesoderm or ectoderm. This technology has important implications for drug toxicology (Anson et al., 2011). In particular, utilization of iPSCs in drug testing addresses the main problems arising from utilization of primary cells, such as limited quantities, donor to donor variation and relatively short lifespan *in vitro*, and circumvents ethical requirements since these cells do not come from embryos (Shafritz et al., 2009). iPSCs allow construction of surrogate liver panels to represent the most common combinations of Phase II enzyme variants and, therefore, to evaluate the potential of adverse drug reactions in the population and to provide an additional step towards the personalization of medicine. Thus, iPSCs may help to identify the potential for idiosyncratic hepatotoxicity, which may develop in some patients but not others – something which may be missed in the course of typical hepatotoxicity studies.

Since 2009, when the first protocol for the production of iPSC derived hepatocyte-like cells was published (Song et al., 2009), a variety of optimizing modifications to the standard procedure were proposed (Chen et al., 2012; Chin et al., 2009; Huang et al., 2014; Liu et al., 2010; Schwartz et al., 2014; Si-Tayeb et al., 2010; Takayama et al., 2012). Remarkably, some of these modifications simplified the workflow: instead of differentiating the cells in the presence of serum supplemented with growth factors and small metabolites, the protocols shifted towards greater standardization, with elimination of primary feeder cell requirements and introducing serum-free media.

iPSC-derived hepatocyte-like cells maintain a majority of hepatocytic functions, including the production of albumin, expression of cytochromes, and the storage of glycogen, while displaying global expression profiles resembling those of primary human hepatocytes (Gao and Liu, 2017). However, transcriptomic analyses revealed that for certain functional gene sets, the expression patterns of iPSC and of cultured primary hepatocytes differ substantially, with genes related to endocytosis upregulated (Bell et al., 2017), and cytochrome production downregulated (Bell et al., 2017; Si-Tayeb et al., 2010). Another aspect that limits wide utilization of iPSCs in drug testing is the standardization issue. The genetics of each iPSC line reflect the genetics of its donor. Consequently, iPSC lines differ in their epigenetic profiles, miRNA patterns and differentiation properties (Chin et al., 2009; Marchetto et al., 2009; Miura et al., 2009), which greatly contribute to lab-to-lab variations typically observed in toxicological studies. Furthermore, the efficiency of iPSC differentiation into hepatocytes is at 60% and even when best protocols are used (Chistiakov and Chistiakov, 2012) deriving the line requires expensive and time consuming extraction and purification steps.

To overcome the mentioned limitations, Cellular Dynamics International (CDI) has developed 95% pure iPSC derived iCell® Hepatocytes, which are claimed to closely resemble primary cells. However, a recent comparative study of these cells along with HepaRG and human hepatocyte co-cultures showed that their metabolic activity is more than tenfold lower and approximates that of HepG2 (Kratochwil et al., 2017). This regretful outcome stresses the importance of keeping an eye on a "Holy Grail" of iPSC-based cellular modeling: a resemblance between the naturally differentiated cells residing within the body and forcefully differentiated cells produced from iPSCs.

One way to make sure that the produced cells perform adequately is to compare their expression profiles with those of naturally differentiated cells using CellNet, a computational platform which evaluates the extent to which cell- and tissue-specific gene regulatory networks are established under one or another differentiation protocol (Cahan et al., 2014; Radley et al., 2017). When correctly assessed for the resemblance of their target cell type, iPSC-derived hepatocyte-like cells may become a valuable tool for personalized toxicology and metabolism studies.

4 Cell line standardization problem

An induction of liver-on-a-chip technology into the main aisle of toxicology labs is critically dependent on our ability to standardize the cells seeded on to the chip. In principle, cell standardization requires the stability and reproducibility of the four main characteristics: (a) Viability for prolonged periods of time, which may be assessed via fluorescence imaging techniques that rely on a combination of nuclear and cytosolic dyes; (b) the retention of a toxicologically-relevant metabolic profile with stable activity of Phase I and Phase II enzymes; (c) drug transporter activity, which should be taken into consideration during initial vetting of an *in vitro* platform for studies of drug metabolism; (d) secretory capacity, which is approximated by albumin biosynthesis and urea excretion. Notably, a consistently high albumin production at levels of approximately ~1-5 µg per 10^6 cells per hour serves as an indispensable indicator of the overall metabolic health of the cells; urea plays a similar role for evaluating general metabolic capacity. To directly detect albumin secreted by hepatocyte culture, 3D scaffold-based immunoassay chips have been developed recently (Yan et al., 2015).

As can be seen from Table 1, the standardization requirements are fulfilled only by upcyte® and HepaRG cells, with HepaRG being even closer in their cytochrome and transporter profiles to primary hepatocytes than the upcyte® cultures. To fully standardize the cell behavior in the liver-on-a-chip devices, there is a need to collect data to evaluate their response to exposure to a panel of at least 100 drugs, roughly divided into four categories that include safe and efficacious, safe and non-efficacious, non-safe and efficacious, and non-safe and non-efficacious compounds, all with well characterized *in vivo* metabolism. More-

over, each drug has to be studied in a range of concentrations to determine both acute and chronic effects. In other words, both IC_{50}/LD_{50} and a variety of the biomarkers of functional impairment have to be assessed.

5 Limitations imposed by culturing

Until recently, liver cell monocultures were a mainstay of toxicology practice for a number of well-defined "fit-for-purpose" assays. Nowadays, it is widely recognized that single cell type monolayers do not reflect the complexity of a tissue developed within a living organism. The limitations of hepatocyte monoculture are obvious. One of the most promising approaches to overcome the issues with viability of hepatocyte monoculture is the utilization of microfluidic perfusion devices (Knöspel et al., 2016; Shulman and Nahmias, 2013; Tehranirokh et al., 2013; Wagner et al., 2013). Unfortunately, for a majority of cell types, the cultivation period still does not exceed 14 days. Recently, Klein et al. (2014) demonstrated that HepaRG cells can be maintained in optimized serum-free media for 30 days without a decline in their viability. This finding certainly opens up the opportunity for the use of these cells in systems toxicology.

Both viability and functioning of hepatocytes are reduced in the absence of non-parenchymal cells. These supportive cells include fibroblasts, endothelial cells, stellate and Kupffer cells, and biliary epithelial cells (Ries et al., 2000; Soto-Gutierrez et al., 2010). Importantly, the addition of even one type of auxiliary cell often helps. For example, Okamoto et al. (1998) developed a co-culture system of primary human hepatocytes with the hepatic stellate cell line LI90, which retains a substantial activity of P450 cytochromes for at least 2 weeks, however, no rescue of urea excretion was noted. In another study, primary human hepatocytes were co-cultured with human umbilical vein endothelial cells (HUVEC) to achieve marked improvement of albumin production, urea biosynthesis and the rate of diazepam biotransformation (Salerno et al., 2011). Kostadinova et al. (2013) developed a 3D mixed culture of primary hepatocytes with a variety of non-parenchymal cell types. This liver-like culture maintained the production of albumin, fibrinogen, transferrin and urea for up to 3 months, while retaining the ability to induce the synthesis of cytochrome P450 on a drug exposure cue.

From the standpoint of liver biology, non-homotypic cultures have a better chance to correctly predict drug-induced liver injury as its development often depends on the communication between hepatocytes and the resident macrophages, which, upon exposure to certain drug metabolites, may be activated to serve as intrahepatic sources of inflammation (Endo et al., 2012; Kegel et al., 2015). Indeed, previously mentioned 3D liver equivalents containing a variety of non-parenchymal cells, including Kupffer macrophages, already demonstrated their value in the detection of potential inducers of idiosyncratic liver injury (Kostadinova et al., 2013). Similar results were obtained for micropatterned co-cultures containing either primary human hepatocytes or iPSC-derived hepatocytes and murine fibroblasts (Ware et al., 2015).

It is important to note that hepatocyte-produced drug metabolites may be non-toxic for liver cells while exerting adverse effects on other organs and tissues. To address this problem, several heterotypic cell cultures have been developed. For example, a microfluidic-based platform for co-culture of neurospheres and liver equivalents was recently employed in a two-week assay involving repeated exposure to the neurotoxin 2,5-hexanedione. It showed a significantly higher sensitivity compared to either hepatocyte or neurosphere monocultures (Materne et al., 2015). The same group also developed a platform for co-culture of liver organoids with skin (Wagner et al., 2013). On a microfluidic chip for the co-culture of HepaRG and kidney cells (MDCK) created by another group (Choucha-Snouber et al., 2013), a 3-day exposure to ifosfamide led to the detection of apparent nephrotoxic effects, while no toxicity was observed in monocultured MDCKs. In spheroid co-culture of HepaRG with primary human hepatic stellate cells (HSCs), exposure to pro-fibrotic compounds allowed the detection of multiple fibrotic features such as HSC activation, collagen secretion and deposition, thereby providing an avenue for *in vitro* testing of possible contributors to liver fibrosis (Leite et al., 2016).

6 The way to improve hardware

The need for better detection and instrumentation, as well as better materials, stems from the need to pump controlled microliter volumes of medium through the chip circuits and, in particular, to distribute the flow at critical junctions with a high degree of precision. Most scientists agree that devices of 100 μm in height or less are preferable since at that scale they approach the diameter of the liver sinusoids, which is in the order of 5-10 μm. At this height, the problems with the signal and/or metabolite dilution that plague larger-scale platforms are reduced.

Interpretation of the data generated with the aid of a microfluidic device critically depends on correct scaling of the physical and physiological parameters and on the relevance of the selected computational model of drug response and toxicity. One of the most important parameters that control the adsorption, distribution, metabolism, excretion and toxicity (ADMET) in physiological systems is the exposure time of the tissue to drugs and other xenobiotics, which is called the organ-specific transit time. According to an initial model generated in 1963 after experimentation on dogs, liver-specific transit time is between 10 and 20 seconds (Goresky, 1963). However, later studies demonstrated that in humans the hepatic transit time is substantially longer, from minutes to hours (Chiou, 1983). Moreover, hepatic transit time depends on the structure of a compound (Chiou, 1983) and may vary between individuals, being influenced by their genetics and overall state of health (Pedersen et al., 2005). Accordingly, to mimic *in vivo* ADMET characteristics in the fluidic systems, one has to have an ability to adjust fluidic residence times to the required physiological values.

To evaluate drug metabolite-induced toxicity, more complicated fluidic systems are necessary (Fig. 2) (Marx et al., 2016). Multi-parametric evaluation of the drug effects may include the

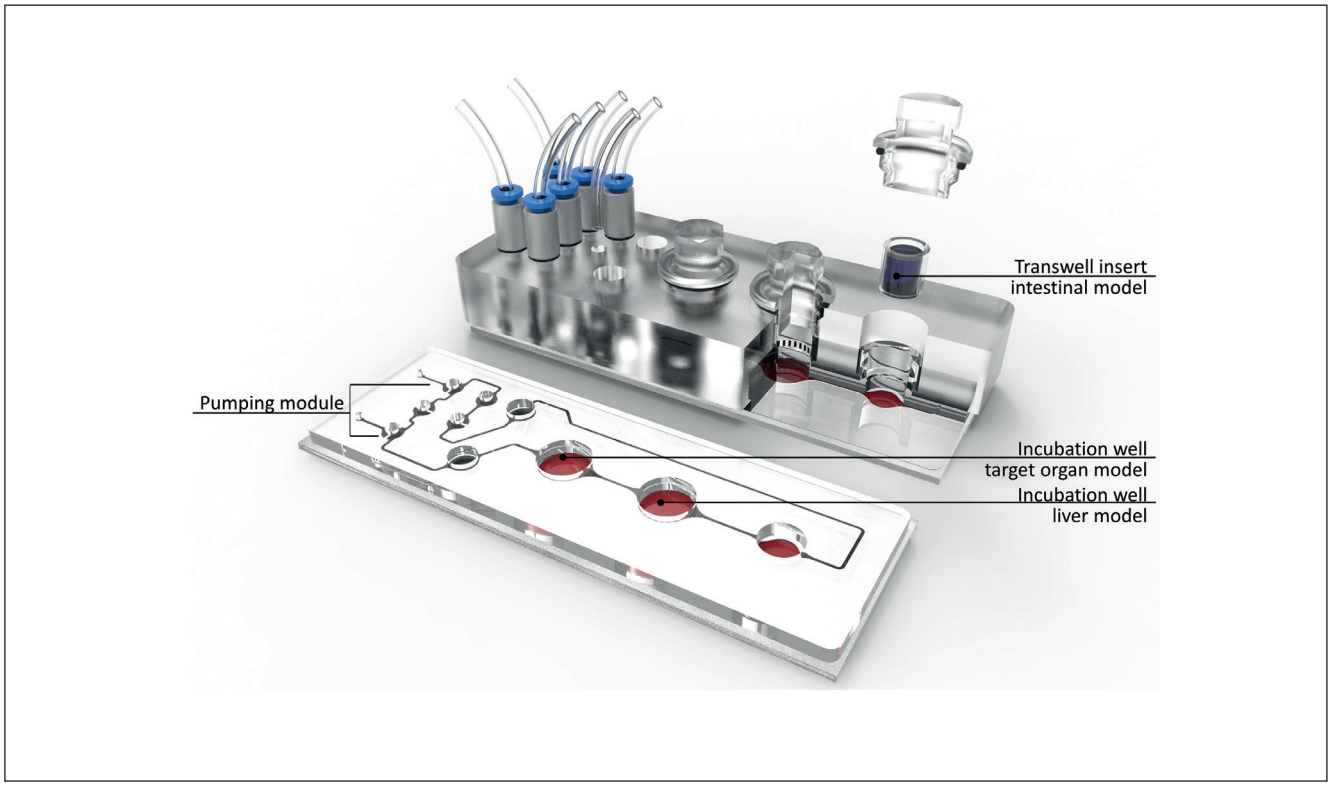

Fig. 2: Microfluidic platform for long-term multi-tissue coculture with closed circuit

studies of intestinal permeability, biotransformation pathways, as well as tests for the toxicity of a drug and its metabolites (Semenova et al., 2016; Zakhariants et al., 2016). Employing a combination of different cell types that reflect tissue-tissue interactions observed in whole organisms could significantly add to the value of the collected data. However, the disadvantages of multi-cell type devices stem from their underlying complexity and include low throughput and questionable scalability.

Detection in the microfluidic platforms is a challenge. In order to capture both acute and chronic effects of exposure to drugs, toxins or environmental factors, successful liver-on-a-chip devices should sum and present the data stream collected in real-time. On top of that, due to cell to cell differences observed in all types of culture, quantitative monitoring of intracellular changes and cell-cell interactions should be performed on a per cell basis, rather than in bulk. Growing trends of single-cell transcriptomics and biochip compatible reporters, are, in part, catering to this need.

One of the approaches to explore intracellular changes might be microRNA level monitoring in the culture medium of the microfluidic platform. MicroRNA (miRNA) is a class of small non-coding RNAs that mediate post-transcriptional gene silencing by sequence-specific inhibition of the target mRNAs' translation and/or lowering their half-lives in the cytoplasm (Turchinovich et al., 2015, 2016). Together with their binding partners, Argonaute proteins, miRNAs form cores of RNA-induced silencing complexes. Finally, the discovery of cell-free miRNAs in all biological fluids suggests that miRNAs might also act as signaling molecules outside the cell, and may be utilized as biomarkers (Makarova et al., 2014, 2016).

To monitor the state of the hepatocyte cell culture, both hepatocyte-specific microRNAs (miR-122) and miRNA species highly expressed in the liver (miR-21, miR-19a/b, miR-106a/b) may be employed. The change in the representation of these microRNAs in the culture medium may be utilized for sensing a physiological change in the hepatocytes under the influence of the studied compound. Also, a number of microRNAs affecting the expression of ADMET genes were discovered. So, for example, miR-27b and miR-378 (Mohri et al., 2010) regulate the expression of cytochromes 1B1, 2E1 and 3A4. The appearance of such microRNAs in the microfluidic system cell culture medium may thus significantly affect the functional capacity of the device.

Metabolomics presents yet another, very interesting alternative for extracting quantitative information about the dynamic metabolic response of the modelled liver to pathophysiological conditions. In two recent studies, the metabolite profiles of HepG2 cells treated with various test substances were analyzed to reveal concentration-response effects mapped to a variety of the response patterns consistent with different liver toxicity mechanisms (García-Cañaveras et al., 2016; Ramirez et al., 2018).

On the other hand, the spatiotemporal dynamics of the multicellular milieu could be monitored with the aid of a small fluorescent molecule (probe) or a protein-based fluorescent biosensor. In this respect, reporter cells have great promise; such

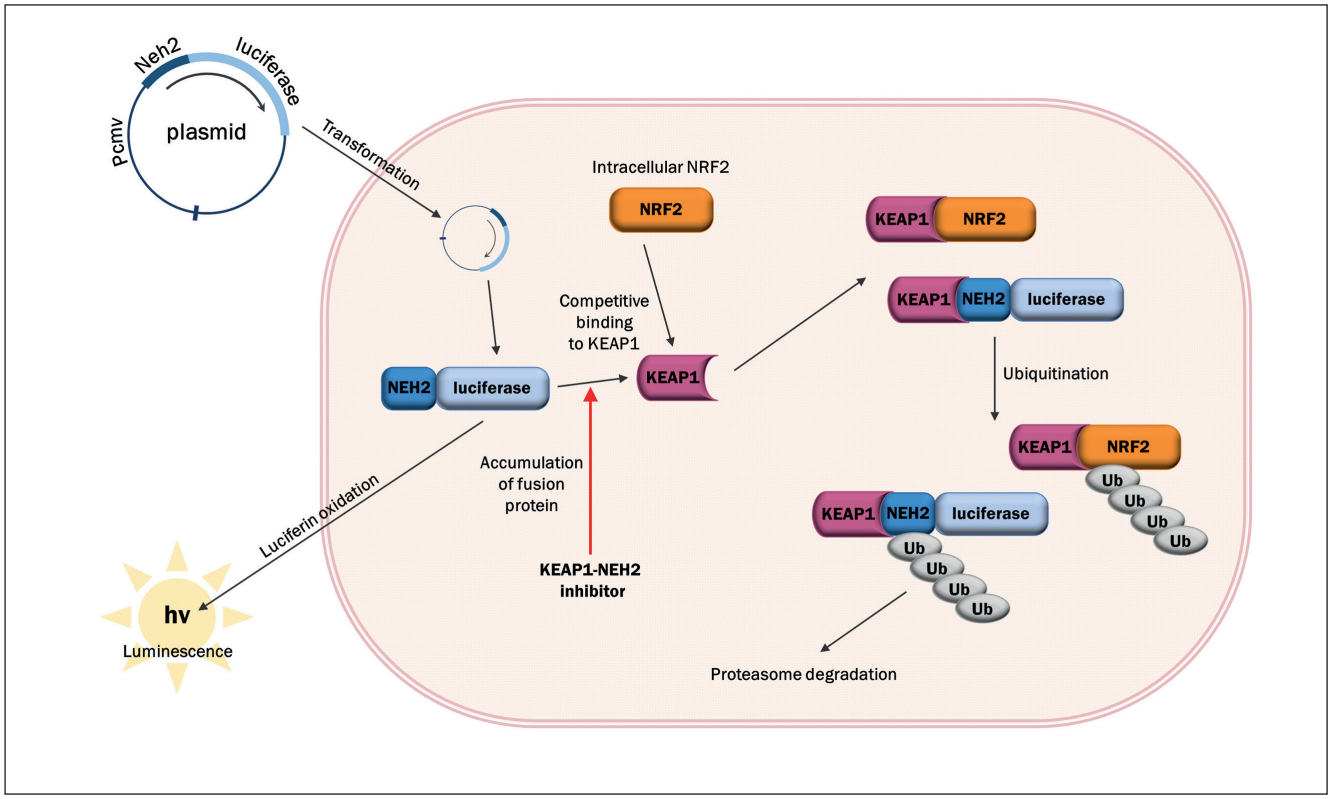

Fig. 3: Luciferase fusion reporter concept in the case of NRF2

cells natively fluoresce upon stimulation or under certain stress conditions, thus revealing specific information about the state of the cell. Optical interrogation of the hepatocyte culture with integrated "sensor" cells engineered to respond to particular signals may provide a way to extract this type of information in a real-time format.

7 Biochip compatible reporter assays

One of the most important modern trends in drug discovery is the switch from the "one disease – one target" mentality to the understanding that diseases are driven by shifts in a homeostatic balance. Even the smallest of these changes may involve many interacting genes and proteins upstream and downstream of a malfunctioning element in a biological puzzle. Hence, the focus of the HTS efforts has also shifted toward a search for various modulators which exert their action either through fine-tuning various transcription factors, or by controlling epigenetics landscapes. Cell-based reporter-enabled biochips are ideally suited for the purpose of HTS for activators and inhibitors of transcription factors. Reporters with fluorescent labels are preferable for use on the liver-on-a-chip devices, since they permit monitoring of the reporter response in intact cells. However, the recent development of a cell-permeable reagent for *Renilla* luciferase (Lindberg et al., 2013) opens the possibility of measuring luminescence within the intact cells.

The choice of reporter constructs for transforming hepatocyte-like cell lines is wide (Tab. 1). In particular, classic promoter-based reporters comprised of a fluorescent or luminescent protein gene under the promoter of a gene of interest, are readily available from commercial sources. Such promoter-based reporters are useful for monitoring of expression, and have been recently employed to monitor activation of CYP genes in HepG2 and HepaRG cells (Tsuji et al., 2014). The chief disadvantage of these reporters is their relatively low level of signal, which is often detected with a substantial delay due to the time necessary for promoter activation, transcription and translation of the reporter protein. Another problem is that many genes require for proper expression either relatively large promoter regions or even the presence of distant enhancers, which cannot be spliced into the plasmid- or virus-based reporter construct due to size limitation. This limitation could be surmounted by using bacterial artificial chromosome (BAC) transgene-based cell lines with very large, locus-wide holding capacity inclusive to all regulatory elements ensuring the normal physiological regulation of a fluorescent or luciferase reporter expression (Poser et al., 2008). The Bscl2-GFP and Srxn1-GFP BAC reporter assays were successfully employed to differentiate between two different types of response to genotoxic agents in many stably transfected cell lines (Hendriks et al., 2012). Recently, the feasibility of the BAC-reporter approach has been evaluated by testing the effect of over 2000 FDA approved drugs and active natural product compounds on the modulation of the Srxn1-GFP reporter in

HepG2 cells (Wink et al., 2014). Integration of BAC-reporters into the cell component of the liver-on-a-chip devices is expected in the nearest future. The most current panel of BAC-GFP modified HepG2 cells, each complete with an upstream sensor, downstream transcription factor and their respective target gene, include reporters for the oxidative stress response pathway (Keap1/Nrf2/Srxn1), the unfolded protein response in the endoplasmic reticulum (Xbp1/Atf4/BiP/Chop), and the DNA damage response (53bp1/p53/p21) (Wink et al., 2017).

Another way to overcome the size limitation obstacle is to use a fusion-based reporter, which, typically, is comprised of either fluorescent or luminescent protein label enjoined with a full-size protein of interest expressed under a constitutive promoter, for example, p_{cmv}. These reporters permit monitoring the half-life and the trafficking of the protein in the living cell. The overwhelming problem with these types of reporters is that the fused protein is fully physiologically active and is often capable of disturbing the intracellular balance, thus triggering an expression of the downstream genes and significantly shifting the transcriptomic and proteomic profile of the cells. In turn, these shifts reflect upon the efficacy and toxicity profiles obtained for the drugs under study. Another drawback of the fusion systems is that the promoter is constitutively active, often leading to overexpression of the construct over the physiological limits and resultant perturbation of cell homeostasis.

Yet another type of reporter has been developed for the monitoring of transcription factors regulated by ubiquitination and proteasomal degradation. The concept for these reporters is shown in Figure 3. In a nutshell, it is a fusion where a luminescent or fluorescent label is added to an isolated ubiquitination machinery recognition domain instead of a complete target protein. As the recognition domain has no affinity for DNA, it does not activate the specific program regulated by a particular transcription factor, even when overexpressed. There is still a possibility that overexpression of the recognition domain may serve as a decoy for ubiquitination machinery, hence, to at least some degree, stabilizing the endogenously expressed transcription factor, but these effects are usually negligible.

Among transcription factors regulated by ubiquitination and proteasomal degradation, there are three which are directly relevant to monitoring performance of hepatocytes embedded into liver-on-a-chip devices. These are the transcription factors Nrf2, HIF (hypoxia inducible factor), and NF-κB.

7.1 Nrf2

Nrf2 (nuclear factor erythroid 2-related factor 2) orchestrates the antioxidant response by inducing the expression of cytoprotective, pro-survival proteins such as thioredoxin reductase, glutathione reductase, glutathione S-transferase (GST), hemeoxygenase-1 (HO1), catalase and others. Under homeostatic conditions, Nrf2 is sequestered by binding to its inhibitory protein, Keap1 (Kelch-like ECH-associated protein-1). Keap1 serves as a bridge between Nrf2 and the Cul3-Rbx1 E3 ubiquitin ligase, which permits polyubiquitination of the lysines positioned within the central α-helix of the Neh2 recognition domain. As a result of oxidative/electrophilic stress, active cysteines of Keap1 are modified, and Nrf2 protein is released for subsequent translocation to the nucleus, where it induces expression from the promoters with the antioxidant response elements (ARE) (Kaspar et al., 2009).

Importantly, Nrf2 is constitutively active in many primary tumors (de la Vega et al., 2016). Hence, it is not surprising that tumor-derived HepG2 and HepaRG cells overexpress Nrf2-regulated genes. Upcyte® cultures do that too, however, to a lesser degree (Sison-Young et al., 2015). When HepaRG were compared to upcyte® cells, peroxiredoxin-1 and -2, thioredoxin reductase and thioredoxin were found to be overexpressed substantially (Sison-Young et al., 2015), indicating that HepaRG cells are protected against effects of the drugs causing glutathione depletion. In other words, drug toxicity estimates obtained while working with HepaRG or HepG2 tend to be a bit more optimistic than they should be.

A reporter for monitoring Nrf2 activation, Neh2-luc (Smirnova et al., 2011), permits the real time monitoring right after addition, with no lag-period, while being 10-fold more sensitive than ARE-luc reporters (Smirnova et al., 2011). This reporter has already found its use in the discovery labs, especially when an estimate of the intracellular alkylating power of a drug is needed. Neh2-luc enabled screening of drug libraries showed that at least 10% of all compounds behave as non-specific Nrf2 activators, meaning that they may alkylate active protein and peptide thiols in general, and glutathione in particular. In other words, administration of these drugs actively shifts the cellular redox balance and triggers the adaptive response. It is expected that Neh2-derived reporter will be fitted with a fluorescent label compatible with liver-on-a-chip devices.

7.2 HIF

HIF, a transcription factor capable of activating a battery of genes involved in glucose uptake and metabolism, extracellular pH control, angiogenesis, erythropoiesis, mitogenesis, and apoptosis, is expressed ubiquitously. It consists of 2 subunits, known as HIF1-α and HIF1-β. The levels of HIF1-α are regulated by hydroxylation of its Pro564 and/or 402 residues. This modification serves as a prerequisite for interaction with the tumor suppressor von Hippel-Lindau (VHL) protein, yielding a complex that provides for a rapid HIF ubiquitination and degradation (see review (Kaelin, 2005) and references therein). HIF hydroxylation is executed by the so-called HIF prolyl hydroxylases represented by 3 isozymes. Upregulation of HIF is an indication of the low oxygen supply and the enactment of the Warburg effect, a metabolic shift towards glycolysis.

A *luc*-reporter with ODD (oxygen degradable domain) of HIF has been developed (Safran et al., 2006) and its variant with *Renilla* luciferase has just become commercially available from Promega. In HTS, employment of HIF1 ODD-luc reporter allowed an identification of the hit with excellent neuroprotective properties later confirmed in hemorrhagic stroke models *in vivo* (Smirnova et al., 2010; Karuppagounder et al., 2016). Such a reporter might also be utilized for evaluation of substrate specificity of HIF prolyl hydroxylase isoforms and structure-activity relationship studies (Osipyants et al., 2017; Poloznikov et al., 2017; Smirnova et al., 2017). This fact clearly demonstrates

superior properties of novel generation of cell-based reporters with respect to drug development. In liver-on-a-chip devices, in addition to drug discovery purposes, the reporter can be used to quantitate hypoxia and HIF activation. In hypoxia (or with HIF1 activation by other means), expression of cytochromes P450 and Phase II enzymes is down-regulated. This metabolic feature principally affects drug toxicity profiles; this is especially true for drugs developed for oncological treatment. Under hypoxia, HepaRG cells have been shown to display metabolic changes similar to those observed in poorly differentiated hepatocarcinomas; therefore, these cells may serve as a suitable *in vitro* model for testing of anticancer agents in hypoxic versus normoxic conditions (Legendre et al., 2009).

7.3 NF-κB

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) controls both the inflammatory cytokine production and the survival of cells. In an inactive state, NF-κB is complexed with the inhibitory protein IκBα. Activation of IκB kinase (IKK) results in phosphorylation of IκBα protein, and its subsequent ubiquitination, which leads to dissociation of IκBα from NF-κB and eventual degradation of IκBα by the proteasome. The activated NF-κB is then translocated into the nucleus. Inhibiting NF-κB signaling has a potential for the treatment of cancers and inflammatory diseases. Importantly, persistant activation of NF-κB, which is known as chronic inflammation, is known to be a component of idiosyncratic hepatotoxicity (Jiang et al., 2017). Currently, there is no cell-based reporter to monitor NF-κB activation directly. However, a reporter monitoring degradation of its inhibitory partner IκBα can be constructed under the same principle as Neh2- and HIF ODD-derived reporters. The IκBα-derived reporter can be used for drug discovery and for assessing drug toxicity, similarly to other reporters of this kind, with a special value for studying idiosyncratic hepatotoxicity in assays of controllably activated NF-κB.

8 *In silico* modelling of liver function

There has been significant progress in developing the liver-on-a-chip and other liver-emulating technologies. However, the field is still somewhat in its infancy in terms of the standards, procedures and methods for translating the data obtained *in vitro* into reliable predictions applicable to human body responses. In parallel to various *in vitro* efforts, the development of predictive computational models of hepatic metabolism is also under way. Although many models perform quite well on the datasets they were developed on, they sometimes suffer from low statistical performance, with imbalanced sensitivity *vs* specificity ratios.

Speaking generally, the predictive power of any computational model heavily depends on the quality of the respective training data set. When machine learning approaches are used, bigger datasets are preferable to smaller ones. The frameworks of large-scale screening programs, e.g., Tox21, already allowed the development of prediction models with an accuracy as high as 86.9%, sensitivity of 82.5%, and specificity of 92.9% (Capuzzi et al., 2016; Chen et al., 2012). On the other hand, the larger the dataset is, the higher the chance of mislabeling either the chemical structures or their toxicity classes. Consequently, manual trimming of large datasets may lead to improvement of model precision. To expand availability of highly confident data, industry-driven collaborative efforts are required. One example of such efforts is the eTOX project, which is comprised of a database filled with unpublished toxicology reports donated by 13 members of the pharmaceutical industry along with public toxicology data, and its customizable interface eTOXsys (Sanz et al., 2015). The models developed in the course of the eTOX project are available as possible augmentations for higher-level predictors. In one recent study, the models for BSEP, BCRP, P-glycoprotein and for OATP1B1 and 1B3 were investigated for their potential to improve the DILI-predicting algorithm. Surprisingly, in this particular case, an integration of the transporter-related data did not significantly improve the performance of the resultant model (Kotsampasakou et al., 2017).

There are some interesting attempts to model the liver "as a whole", with the homeostasis or the "starting state" of the liver being described by a set of differential equations, which can be modified as a reflection of the respective change observed under certain disease conditions and immunological states. This approach treats hepatotoxicity as a complex outcome of the factors at play, which includes the genotype of the patient, the drugs he or she is exposed to, and any underlying diseases, for example, steatosis. An example of this type of systemic model would be a VirtualLiver, developed by Strand Life Sciences, which couples equations describing the kinetics of biochemical pathways involved in liver homeostasis with those obtained after collection of a set of *in vitro* measurements quantifying various drug-induced perturbations (Subramanian et al., 2008). Clearly, this type of approach describes the biological system better than any endpoint analysis of toxicity, as it is able to reflect a steady accumulation of changes which eventually culminate in reaching an irreversible outcome. The development of holistic *in silico* models that simulate the metabolism of the liver is a necessary step towards an adequate and timely assessment of various chemical entities, natural or synthetic.

9 Conclusion

In this review, we describe the current approaches to develop liver-on-a-chip devices for the prediction of the liver toxicity in humans. These devices may include established immortal cell lines, for example, HepG2 – a "workhorse" of liver toxicology – or its less malignant counterpart HepaRG, unmodified or modified primary human cells, and stem cell-derived hepatocyte-like cells, or iPSC. Like many technologies developing on the interface of applied biology and bioengineering, the liver-on-a-chip devices were started in an attempt to produce a "one-fits-all" solution, but eventually ended facing a variety of important dilemmas. In particular, the choice of biological material greatly influences both the performance of the devices and the precision of the toxicity reports. Unfortunately, unavoidable manipulations

with the living component of the chip lead to activation of the Nrf2 and NF-κB-dependent transcriptional programs, which, in turn, reflect upon the cellular response to environmental stressors. Among other impediments to the development of liver-on-a-chip technology are the problems with standardization of cells, limitations imposed by culturing, and the necessity to develop more complicated fluidic contours. Fortunately, recent breakthroughs in the development of cell-based reporters, including ones with fluorescent labels, permit monitoring the behavior of the cells embedded into the liver-on-a-chip devices. Finally, a set of computational approaches has been developed to model both toxic response pathways and the homeostasis of human liver as a whole; these approaches pave the way to enhance the *in silico* stage of assessment of potential toxicity.

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Conflict of interest

The authors declare no conflicts of interest.

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