DEVELOPMENT OF NEW HEPATIC CELL LINES FOR GENOTOXICITY AND CARCINOGENICITY STUDIES

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Doctoral Dissertation
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JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL

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RAZVOJ NOVIH JETRNIH CELIČNIH LINIJ ZA ŠTUDIJE GENOTOKSIČNOSTI IN KANCEROGENOSTI

Doktorska disertacija

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Ljubljana, Slovenia, January 2019

To my family.

Acknowledgments

This doctoral dissertation covers a very extensive research work in different areas, which would not have been completed without several important people.

First, I would like to thank Prof. Dr. Metka Filipič, who allowed me to implement this very interesting work in her research group at the Department of Genetic Toxicology and Cancer Biology, National Institute of Biology in Ljubljana, for her supervision, guidance, help, and professional support during these years, and for all her efforts and contributions in the last months to finalise this dissertation.

I would like to thank Asst. Prof. Dr. Bojana Žegura for her teaching, assistance, management and patience with my PhD experiments, and Dr. Katja Kološa for introducing me to the world of stem cells.

My gratitude also goes to Monika Waldherr, Dr. Miroslav Mišík, Prof. Dr. Siegfried Knasmüller and other scientists from the Institute of Cancer Research at the Medical University of Vienna for their professional collaboration, and to Dr. Urška Kamenšek from the Institute of Oncology Ljubljana for her participation in cell transfections.

I would also like to thank Dr. Nataša Toplak and Doris Pavlin from Omega d.o.o. for their technical support in qPCR experiments, and Dr. Ana Rotter for her assistance in statistical analysis of gene expression data.

I am grateful to Mitja Lasič from the Jožef Stefan Institute for his help and technical revision of this dissertation.

I would like to thank the members of the evaluation board: Prof. Dr. Marina Dermastia, Prof. Dr. Maja Čemažar and Prof. Dr. Siegfried Knasmüller for the revision of this doctoral dissertation.

I am grateful for all my co-workers at the National Institute of Biology. Especially I am thankful for Mateja V. and Barbara, not only for their assistance and knowledge they gave me, but also for being a friend outside of the laboratory and office.

I appreciate all the encouragement and help of my friends.

Most of all, I am truly grateful to my family: my mother, my father, Rok, Anja, Maša, Mei and Miki, for their endless help, support, patience, love and understanding and for numerous walks in the nature.

This doctoral dissertation was financially supported by the Slovenian Research Agency: the research core funding P1-0254, project J6730, and Young Researcher Grant MR-JTomc.

Abstract

The main problem of the current routine *in vitro* genotoxicity testing for *in vivo* genotoxicity and carcinogenicity assessment, which is in the EU required for nearly all chemicals prior to marketing, is relatively low predictivity of rodent cell-based models used for this purpose. The consequence is unnecessary *in vivo* follow-up testing, or the discontinued development of promising chemicals. Unacceptably low predictivity is ascribed to a number of deficiencies that the commonly used cell lines possess, wherein their abnormal metabolic capacity is certainly the substantially contributing factor. The most advantageous approach to overcome the problem of poor predictivity of the *in vitro* genotoxicity test systems would be a cell line of human origin, which reflects *in vivo* xenobiotic metabolism, has a stable karyotype, is p53 and DNA-repair proficient, and is able to respond to direct and indirect acting chemicals. The use of several human hepatic tumor-derived cell lines with preserved activity of certain metabolic enzymes *in vitro* has been shown to be promising, but a much larger number of other human cell lines derived from hepatic tumors is available on the market that have never been used in genotoxicity studies, and some of them may be even more suitable for this purpose. However, some cancerous features of such cells might significantly affect their sensitivity and specificity compared to normal cells. In that respect, new cell-based models with metabolic capacities similar to primary hepatocytes are needed.

In the first part of this work, the characteristics relevant for the potential use in genotoxicity testing have been investigated in a panel of human liver-derived cell lines. The findings suggested HepG2 and HuH6 hepatic cancer lines as potentially the best cell models. The two cell lines have comparable doubling times, express functional wild type p53, their chromosome number is in narrow range and they both showed similar sensitivity to detect DNA damage induced by all tested model pro-carcinogens. The basal expression of selected liver-specific transcription factors and genes involved in the metabolism of xenobiotics has been analysed in both lines and compared to that of primary human hepatocytes (pHep) from two donors, to get insight into their metabolic profiles. Furthermore, the transcriptional regulation of the selected genes was determined after the 24 h exposure to non-cytotoxic concentrations of three model pro-carcinogens. The comparison of metabolic profiles of hepatic tumor-derived cells to that of primary hepatocytes elucidated several limitations of metabolic capacity of HepG2 and HuH6 cells and none of them has been shown to be more comparable to pHep. However, the findings represent valuable information that can be used for improvements of both cancer lines to be more convenient for *in vitro* genotoxicity studies and for the better understanding and interpretation of toxicological results obtained with HepG2 or HuH6 line.

In the second part, the development of metabolically active differentiated hepatic progenies (hDHP) from human adipose tissue-derived mesenchymal stem cells (hASC) using a three-step hepatic differentiation procedure has been performed. Except morphology, the hASC-derived hepatic progenies expressed typical hepatic functional characteristics, such as glycogen storage and albumin secretion. The results of the comet assay revealed comparable sensitivity of hASC and hDHP towards direct acting

genotoxic agent tert-butyl hydroperoxide. The exposure to model indirect acting genotoxins did not induce DNA damage in hASC, while hDHP cells have responded to B(a)P and AFB_1 exposure indicating capability of metabolic activation of the two genotoxic agents. The gene and protein expression analysis confirmed the presence of several phase I and phase II xenobiotic metabolic enzymes in an inducible form in hDHP cells. The generated hDHP cells have been further immortalized with transfection with *hTERT*. The transfected cells lost metabolic capacity, but can be easily matured to metabolically active cells with sensitivity comparable to that of non-immortalized hDHP, providing limitless supply of hDHP cells.

The findings suggest many advantages of hASC-derived immortalized hDHP cells and several limitations of HepG2 and HuH6 cell lines. All three cell models are worth further investigations in terms of development of a test model, which could contribute to the reduction of the use of laboratory animals and to more efficient safety evaluation of new chemical products needed for better human health protection.

Povzetek

Glavni problem rutinskega *in vitro* testiranja genotoksičnosti kemikalij za oceno tveganja genotoksičnosti in kancerogenosti *in vivo*, ki jo mednarodna zakonodaja v EU zahteva za skoraj vse kemikalije na tržišču, je sorazmerno nizka napovedna moč eksperimentalnih sesalčjih celičnih modelov, ki se v ta namen uporabljajo. To posledično vodi do nepotrebnega *in vivo* testiranja na živalih ali pa prekinjenega razvoja obetavnih kemičnih izdelkov. Razlog za nizko napovedno moč predstavljajo številne pomanjkljivosti trenutno uporabljanih celičnih modelov, pri čemer je njihova nenormalna presnova ksenobiotikov eden bistvenih dejavnikov. Kot najbolj obetaven pristop pri premagovanju tega problema se je izkazala uporaba celičnih modelov, ki so človeškega izvora, odražajo *in vivo* metabolizem ksenobiotikov, imajo stabilen kariotip in nespremenjene mehanizme popravljanja DNA poškodb ter se odzovejo na kemikalije z neposrednim in posrednim mehanizmom delovanja. Nekaj človeških celičnih linij, ki izvirajo iz jetrnih tumorjev in so ohranile aktivnost pomembnih presnovnih encimov, se sicer uporablja v *in vitro* študijah genotoksičnosti, vendar je na trgu na voljo veliko več humanih jetrnih linij, ki se niso nikoli uporabljaje v ta namen in so morda bolj primerne. Ker je večina teh linij rakavega izvora, lahko določene kancerogene lastnosti pomembno vplivajo na njihovo občutljivost in specifičnost v primerjavi z normalnimi celicami in zato je za *in vitro* testiranje kemikalij potrebno vzpostaviti nove celične modele, ki bodo odražali biotransformacijo kemikalij, primerljivo s primarnimi jetrnimi celicami, in bodo zaznali genotoksične lastnosti kemikalij bolje od celičnih modelov, ki se trenutno uporabljajo.

V prvem sklopu doktorskega dela smo na podlagi celičnih lastnosti, ki so pomembne pri testiranju genotoksičnosti, izmed trenutno razpoložljivih jetrnih celičnih linij človeškega izvora identificirali najbolj primerne. Izbrani sta bili dve rakavi liniji, HepG2 in HuH6, ki imata primerljiv čas proliferacije, izražata nemutirani tumor zavirajoči p53 protein in sta genomsko dokaj stabilni. Obe liniji sta s podobno občutljivostjo zaznali poškodbe DNA, povzročene z modelnimi genotoksini s posrednim delovanjem. Da bi dobili vpogled v metabolne profile obeh celičnih linij, smo izvedli analizo izražanja izbranih jetrno specifičnih transkripcijskih faktorjev in genov, ki sodelujejo pri presnovi ksenobiotikov. Njune metabolne profile smo primerjali z metabolnimi profili primarnih človeških hepatocit dveh darovalcev. Poleg bazalnega izražanja teh genov smo analizirali tudi njihovo inducibilnost po 24-urni izpostavitvi ne-citotoksičnim koncentracijam treh modelnih genotoksičnih kemikalij. S primerjavo presnovnih genskih profilov smo ugotovili, da nobena od rakavih linij ni bolj primerljiva s primarnimi celicami. Z analizo pa smo pojasnili nekatere omejitve presnovnih kapacitet HepG2 in HuH6 celic, kar predstavlja dragocene informacije. Novo pridobljeno znanje je mogoče uporabiti za izboljšavo obeh linij, da bi bili bolj primerni za uporabo v *in vitro* študijah genotoksičnosti, ali pa za boljše razumevanje in interpretacijo dobljenih rezultatov.

Druga polovica dela je bila namenjena razvoju metabolno aktivnih jetrnim celicam podobnih celic z diferenciacijo mezenhimskih matičnih celic iz človeškega maščobnega tkiva (hASC). Razen morfologije so diferencirane celice izražale značilne jetrne lastnosti, kot je shranjevanje glikogena in izločanje albumina. Nediferencirane hASC in

diferencirane jetrnim celicam podobne celice so se s primerljivo občutljivostjo odzvale na izpostavljenost modelni genotoksični kemikaliji z neposrednim delovanjem (tert-butil hidroperoksid). Izpostavljenost modelnim genotoksičnim kemikalijam s posrednim delovanjem pa pri hASC ni povzročila DNA poškodb, medtem ko so se diferencirane celice odzvale na izpostavljenost kemikalijam B(a)P in AFB_1. To pomeni, da smo z diferenciacijo uspeli dobiti metabolno aktivne celice, kar smo potrdili tudi z analizo izražanja metabolnih genov in encimov. Diferencirane celice so izražale ključne encime, ki so bili tudi inducibilni. Diferencirane celice smo takoj po diferenciaciji imortalizirali s transfekcijo s *hTERT*. Imortalizirane celice so sicer izgubile metabolno kapaciteto, vendar se jih zlahka ponovno diferencira v metabolno aktivne celice, ki se odzovejo na genotoksično aktivnost kemikalij B(a)P in AFB_1 s primerljivo občutljivostjo kot neimortalizirane diferencirane celice. Imortalizirane celice tako zagotavljajo neomejeno zalogo jetrnim celicam podobnih celic, ki so primerne za rutinsko *in vitro* testiranje kemikalij.

V doktorski disertaciji smo pokazali, da imajo imortalizirane diferencirane celice številne prednosti, pa tudi, da imata celični liniji HepG2 in HuH6 nekaj omejitev. Vsekakor so vsi trije celični modeli vredni nadaljnjih raziskav v smislu razvoja *in vitro* testnega modela, ki bi lahko prispeval k zmanjšanju uporabe poskusnih živali in boljši oceni varnosti novih kemičnih izdelkov za učinkovitejše varovanje zdravja ljudi.

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Abbreviations

3D	...	Three-dimensional
3R	...	Replacement, Reduction and Refinement
AFB_1	...	Aflatoxin B_1
B(a)P	...	Benzo(a)pyrene
cDNA	...	Complementary DNA
Ct	...	Cycle threshold
CYP	...	Cytochrome P450
DNA	...	Deoxyribonucleic acid
ESC	...	Embryonic stem cells
EU	...	European Union
hASC	...	Human adipose tissue-derived mesenchymal stem cells
hDHP	...	Human differentiated hepatic progenies
HepLC	...	Hepatocyte-like cells
HNF	...	Hepatocyte nuclear factor
ICC	...	Immunocytochemistry
iPSC	...	Induced pluripotent stem cells
IQ	...	2-amino-3-methyl-3Himidazo[4,5-f]quinolone
LETF	...	Liver-enriched transcription factors
mRNA	...	Messenger ribonucleic acid
MSC	...	Mesenchymal stem cells
MUW	...	Medical University of Vienna
NDMA	...	N-nitrosodimethylamine
p53	...	Tumor protein p53
PAHs	...	Polycyclic aromatic hydrocarbons
PAS	...	Periodic acid - Schiff staining
pHep	...	Primary hepatocytes
PhIP	...	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
Pro-carcinogen	...	Proximate carcinogen
ROS	...	Reactive oxygen species
Rq	...	Relative expression
SC	...	Stem cells
TBHP	...	Tert-butyl hydroperoxide
TC	...	Tissue-culture
TF	...	Transcription factor
WHO	...	World Health Organization

Chapter 1

Introduction

People have always been exposed to natural chemicals and, as a consequence of the industrial and technological evolution over the last century, millions of man-made chemicals have been introduced into the environment and thousands of them have entered the market. The global output of chemicals has increased approximately 10-fold between 1970 and 2010 and it still continues to grow, which leads to serious environmental and human health concerns. It has been estimated by the World Health Organization (WHO) that more than 25 % of diseases is linked to environmental factors, such as exposures to toxic chemicals (WHO 2010). Exposure to genotoxic chemicals is of particular concern as they induce DNA-damage that is considered to play a crucial role in the etiology of accelerated ageing, cancer, infertility, offspring malformation, arthritis, immune deficiencies, and other human disorders (Altindag et al., 2007; Hales, 2005; Hoeijmakers, 2009; Slatter & Gennery, 2010). In the context of EU and international legislation aimed at the protection of human health, the genotoxicity assessment for prediction of carcinogenicity represents an essential component of the safety evaluation of nearly all types of substances on the market (Corvi, Madia, Worth, & Whelan, 2013).

1.1 Current Limitations in Genotoxicity Assessment

The genotoxicity testing follows a step-wise approach, beginning with a basic battery of *in vitro* tests with bacteria and mammalian cells and, if positive results are obtained, *in vivo* tests with rodents are performed. The disadvantage of this strategy, which cannot be ignored, is a relatively high rate of misleading positive and negative results of *in vitro* testing, obtained particularly when using rodent cell lines (Kirkland, Aardema, Henderson, & Muller, 2005). The consequence is unnecessary *in vivo* testing, which opposes the implementation of the Animal Welfare Guideline (2010/63/EU) and the 3R principles focused on reducing the use of animas for experimental purposes, or the discontinued development of promising chemicals, if regulatory requirements do not permit *in vivo* follow-up experiments (Pfuhler et al., 2010).

Unacceptable low predictivity of conventional *in vitro* tests is ascribed to a number of deficiencies that the commonly used cell lines possess. Their abnormal metabolic capacity and missing crucial biotransformation enzymes are certainly the substantially contributing factor. To mimic the biotransformation of the tested chemical in cell-based *in vitro* genotoxicity tests, rodent liver S9 fraction is commonly used as an exogenous source of metabolic activation. However, these enzyme fractions are far away from reflecting the complete human xenobiotic metabolism and metabolites produced may be quite different (Kirkland et al., 2007; Pfuhler et al., 2011). Further characteristics of the

established rodent cell lines, namely karyotypic instability and deficiencies in p53 function or DNA-repair mechanisms, are additional limitations potentially leading to false results of *in vitro* tests for the *in vivo* genotoxicity prediction (Fowler et al., 2012; Kirkland et al., 2007; Lilienblum et al., 2008). To overcome these problems, it has been generally accepted that the use of currently established cell-based models should be replaced with new mammalian cell lines, which will provide more reliable *in vitro* results (Kirkland et al., 2007; Pfuhler et al., 2011).

1.2 Human Hepatic Cell-Based Models for *in vitro* Genotoxicity Studies

Due to species-dependent differences, the cells of human origin appear to be a better model for *in vitro* detection of chemicals, which might pose health hazards to human. The most advantageous approach to overcome the problem of poor predictivity of the *in vitro* genotoxicity test systems would be a human cell line, which reflects complete *in vivo* xenobiotic metabolism, has a stable karyotype, is p53 and DNA-repair proficient, and is able to respond to direct and indirect acting chemicals (Kirkland et al., 2007; Pfuhler et al., 2011). It should be noted that no individual cell type can reflect the full human biotransformation capacity and that hepatocytes represent the closest model since the liver is the primary site of xenobiotic metabolism (Ioannides, 2002).

Several human hepatic *in vitro* cell-based models have been adopted for the use in different scientific fields, including genotoxicity testing. These models include primary hepatocyte culture models; liver-derived cell lines, either established from isolated tumors or from laboratory-immortalized hepatocytes; genetically engineered cells expressing specific metabolic enzymes; and stem cells-derived hepatocytes (Castell, Jover, Martínez-Jiménez, & Gómez-lechón, 2006; Gomez-Lechon, Tolosa, Conde, & Donato, 2014; Zeilinger, Freyer, Damm, Seehofer, & Knöspel, 2016). Recently, efforts are focused on the development of 3D hepatic culture models, such as spheroids and scaffold systems, which provide long-term maintenance of liver-specific functions and expression of biotransformation enzymes. However, these models are costly and time consuming and thus not suitable for routine testing of a large number of chemicals.

1.2.1 Primary hepatocyte culture models

Primary hepatocytes, obtained from human liver, have been considered as one of the best *in vitro* models for the use in drug metabolism and toxicological investigations since they can reflect a complete *in vivo* hepatic functionality. The expression profiles of phase I and phase II biotransformation genes and enzymes in primary hepatocytes have been shown to be comparable to that of human liver tissue (Jennen, Gaj, et al., 2010; Vildhede, Wiśniewski, Norén, Karlgren, & Artursson, 2015). Even if cryopreserved, they retain the expression and inducibility of xenobiotic metabolizing enzymes after thawing (Gomez-Lechon, Donato, Castell, & Jover, 2003). They were shown to be very useful as indicator cells for detection of direct and indirect acting carcinogens in genotoxicity studies (Schehrer, Regan, & Westendorf, 2000). A major drawback of primary hepatocytes is their short life span and inability to replicate. This requires a new hepatocyte preparation for each experiment which is incompatible with a limited availability of primary cells, while their immortalization turned out to be inappropriate due to reduced

biotransformation capability (Donato, Jover, & Gómez-Lechón, 2013; Gomez-Lechon et al., 2003). Furthermore, their phenotypic instability over longer culture periods, high inter-variability between different donors and cell alterations due to isolation procedures hinder the use of primary human hepatocytes in standardized routine studies (Gomez-Lechon et al., 2014).

1.2.2 Cell lines derived from hepatic tumors

As an alternative to primary human hepatocytes, several cell lines derived from hepatic tumors have been proposed to be used as cell-based models for *in vitro* studies. Lower costs, high availability, easy handling, high proliferation capacity and stable phenotype make them very convenient for screening purposes (Gomez-Lechon et al., 2014).

In the early 1990s, Natarajan and Durroudi (1991) showed that HepG2 cancer cell line detects representatives of different classes of genotoxic pro-carcinogens without the addition of exogenous source of metabolic activation, and has the capability to discriminate between structurally related carcinogenic and non-carcinogenic compounds. Their findings were confirmed in many subsequent experiments and it was shown that these cells are able to identify DNA reactive carcinogens which give false negative results in conventional *in vitro* tests (Knasmüller et al., 1998). Today, this line remains the most characterized of the currently available hepatic cancer lines. The cells synthesize and secrete many of the plasma proteins resembling the characteristics of normal human liver cells (Knowles, Howe, & Aden, 1980). Their unaltered p53 expression (Bressac et al., 1990) and the expression and inducibility of a variety of phase I and II biotransformation enzymes, which play a role in activation and detoxification of chemical carcinogens, made them to be used in a large number of genotoxicity studies (Knasmüller et al., 1999; Majer, Mersch-Sundermann, et al., 2004; Mersch-Sundermann, Knasmüller, Wu, Darroudi, & Kassie, 2004; Pezdirc, Žegura, & Filipič, 2013; Štraser, Filipič, & Žegura, 2013). However, pronounced differences in sensitivity and strong variations in activities of xenobiotic metabolizing enzymes were reported when using clones from different sources of HepG2 cell line (Hewitt & Hewitt, 2004; Knasmüller et al., 2004). Poor reproducibility between experiments was reported even when using the same clones of HepG2 cells (Fowler et al., 2012). Besides, compared to human hepatocytes or lymphocytes, several studies demonstrated much lower sensitivity of HepG2 cells to detect well characterised genotoxic agents with different modes of action (Fowler et al., 2014; Wilkening, Stahl, & Bader, 2003), suggesting that the predictions based on HepG2 *in vitro* studies can lead to misdetection of effects caused by reactive compounds.

A number of other cell lines have been isolated from hepatic human tissue in the following years. Some with relatively well-retained expression of xenobiotic metabolizing enzymes and ability to detect genotoxins, such as Hep3B, HCC1.2 and HepaRG cell lines. The sensitivity of Hep3B cell line to detect genotoxic chemicals was soon demonstrated to be generally much lower (Khoury, Zalko, & Audebert, 2016; Majer, Kassie, et al., 2004; Majer, Mersch-Sundermann, et al., 2004). The HCC1.2 cell line was shown to be useful or even more suitable for routine testing than HepG2 and Hep3B cells (Sagmeister et al., 2008; Winter et al., 2008), but it is more of particular interest for cancer studies (Gauglhofer et al., 2014; Sagmeister et al., 2008; van Zijl et al., 2011). Recently, it was postulated that the HepaRG cell line has an excellent metabolic competency and is thus very promising for use in the field of genotoxicity testing (Doktorova et al., 2013; Le Hegarat et al., 2010; Le Hégarat et al., 2014). HepaRG cells exhibit a set of characteristics that define the human hepatic bipotent progenitor

signature, and are able to differentiate to mature functional hepatocyte- and biliary-like cells *in vitro* (Cerec et al., 2007). However, this hepatic tumor-derived line is only commercially available, has demanding culture requirements to maintain the differentiated phenotype, and its characterization is insuficient, which limits the widespread use of HepaRG cells (Gomez-Lechon et al., 2014). Other isolated cell lines have been mainly used for studies of hepatocellular functions, cancer development and therapy, and occasionally in acute toxicity experiments, but very rarely or never in genotoxicity studies. Yet, they have not been characterized regarding their predictivity as experimental models for *in vitro* genotoxicity testing.

Despite the expression of key metabolic enzymes in currently used cell lines derived from hepatic tumors this has been shown to be much different, generally lower, from normal human hepatocytes (Gerets et al., 2012; Guo, Dial, Shi, Branham, & Liu, 2011; Jennen, Magkoufopoulou, et al., 2010; Lin et al., 2012; Wilkening, Stahl, & Bader, 2003). Besides, the cancerous features of these cells, including intrinsic genomic instability, aberrant expression of DNA damage response mechanisms, and the expression of drug resistance proteins may significantly affect their sensitivity and specificity compared to normal hepatocytes, and preclude their suitability for such mechanistic studies (Kirkland et al., 2007; Pfuhler et al., 2011).

1.2.3 Stem cells-derived hepatocytes

To replace the use of primary hepatocytes and cell lines derived from hepatic tumors, *in vitro* development of metabolically active hepatocyte-like cells (HepLC) obtained by differentiation of stem cells indicates a great potential. Stem cells (SC) are clonogenic cells capable of both self-renewal and differentiation to more specialized cell types. They are responsible for the development and regeneration of tissues and organ systems (Weissman, 2000), and comprise of two main classes: embryonic stem cells (ESC) present during the early phases of embryonic development and can give rise to all types of cells (pluripotent); and more specialized adult stem cells with diverse differentiation capacities (multi-, oligo-, bi- or unipotent) that are found in all kinds of tissues (Uzbas et al., 2015).

Several reports have already indicated that human ESC-derived hepatocytes could be the applicable *in vitro* model for drug metabolism studies and toxicological investigations (Ek et al., 2007; Ulvestad et al., 2013; Yildirimman et al., 2011), yet their usage also raises sharp ethical and political controversies (Lo & Parham, 2009). Besides, broad variability of the key biotransformation enzymes in HepLC derived from ESC has been reported by distinct laboratories (Gomez-Lechon et al., 2014). The epigenetic reprogramming of somatic cells, which results in cells similar to ESC in their capacity of self-renewal and differentiation (Takahashi et al., 2006), has been proposed to potentially offer new opportunities avoiding the ethical problems (Gao & Liu, 2017; Kang et al., 2016; Lu et al., 2015). However, aberrant or incomplete reprogramming and questionable genomic stability of induced pluripotent stem cells (iPSC) (Ullah, Subbarao, & Rho, 2015; Vinoth et al., 2014), as well phenotypic variability of the iPSC-derived HepLC, have been reported (Gomez-Lechon et al., 2014).

A group of adult SC that gained a lot of interest in regenerative medicine are fibroblast-like mesenchymal stem cells (MSC). MSC contribute to the regeneration of mesenchymal tissues, including bone, cartilage, muscle and adipose (Pittenger et al., 1999), as well they can give rise to ectodermal (Kopen, Prockop, & Phinney, 1999) and endodermal tissues (Petersen et al., 1999). The existence of MSC was first described in mouse bone marrow (Friedenstein, Chailakhyan, Latsinik, Panasyuk, & Keiliss-Borok,

1974). Lately it was shown that cells with MSC characteristics can be found in many other tissues, including brain, spleen, liver, kidney, lung, muscle, thymus, pancreas (da Silva Meirelles, 2006), adipose tissue (Zuk et al., 2002) and umbilical cord blood (Bieback, Kern, Klüter, & Eichler, 2004). The earliest report on MSC-derived hepatocytes was published by Petersen et al. (1999) who transplanted bone marrow cells in rat liver to demonstrate that these cells contribute to hepatic lineage. A number of following studies has confirmed the ability of different extrahepatic MSC to differentiate into HepLC *in vitro* (Al Ghrbawy, Afify, Dyaa, & El Sayed, 2016; Lee et al., 2004; Lee, Min, Bak, Hwang, & Kim, 2015; Schwartz et al., 2002; Snykers et al., 2007). According to isolation procedures and ethical considerations, the adipose tissue-derived MSC (ASC) became the most widely exploited in this manner (Banas et al., 2007; Lee et al., 2015; Seo, Suh, Bae, & Jung, 2005; Taléns-Visconti et al., 2006; Yin et al., 2014). They represent an easily accessible cell population with great differentiation capability (Seo et al., 2005; Timper et al., 2006; Zuk et al., 2002) which is not affected by different harvest sites (Choudhery, Badowski, Muise, Pierce, & Harris, 2015). However, much less efforts have been focused on metabolic characterization of ASC-derived hepatocytes and their usefulness as *in vitro* toxicological models.

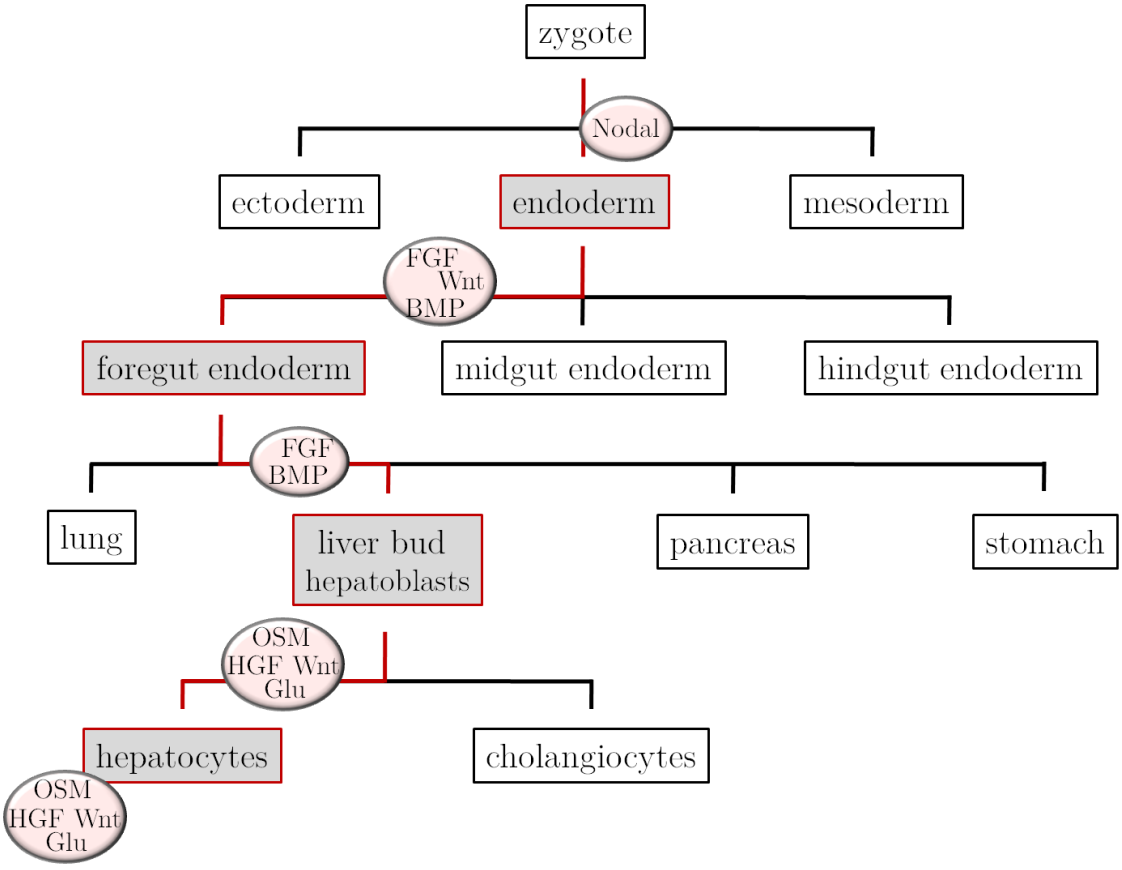

Figure 1.1: Hepatogenesis and signaling. The onset of hepatogenesis requires signalling of Nodal, which initiates endoderm and mesoderm formation in a concentration-dependent manner. FGFs, BMPs and Wnt from the surrounding mesoderm probably act cooperatively to induce hepatic fate from endoderm lineage. Following the hepatic specification, the expansion and proliferation of bipotential hepatoblasts is regulated by FGFs and HGF secreted from liver stromal cells, while HGF, Wnt, Glu, and OSM

secreted by hematopoetic cells control the differentiation of hepatoblasts into hepatocytes and their final maturation (Gordillo et al., 2015; Si-Tayeb, Lemaigre, & Duncan, 2010; Zhao & Duncan, 2005; Zorn, 2008).

Various protocols have been designed to direct hepatic differentiation of MSC *in vitro*. In principle, they are based on mimicking the liver microenvironment and developmental signalling patterns through step-wise addition of soluble liver-specific factors to the culture medium in a concentration-dependent manner. The most important signalling molecules of hepatogenesis have been identified (Figure 1.1), namely Nodal, which is a member of transforming growth factor β (TGFβ) superfamily, fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), Wnt proteins, hepatocyte growth factor (HGF), glucocorticoids (Glu) and oncostatin M (OSM) (Gordillo et al., 2015; Zhao & Duncan, 2005; Zorn, 2008), and their application in differentiation protocols is now well established.

To improve the success of differentiation procedures, various factors have been revealed through the years of research, from the importance of tight intercellular communications to the use of serum-free conditions since the serum might contribute to random cell differentiation. A minor role has been ascribed to the type of coating used (Snykers et al., 2009). However, several factors still need to be addressed to overcome the limitation of this approach for the application in routine *in vitro* testing, including low reproducibility between experiments due to donor inter-variability, isolation procedures, long differentiation protocols and replicative senescence of MSC (Kim et al., 2009). Besides, stability of the differentiated HepLC *in vitro* is questionable (Guguen-Guillouzo, Corlu, & Guillouzo, 2010; Snykers et al., 2009).

1.3 Hepatic Metabolism of Xenobiotics: *in vivo* vs *in vitro*

"Xenobiotic biotransformation is a series of enzyme-catalyzed processes that alter the physiochemical properties of xenobiotic from those that favor absorption across biological membranes to those favoring elimination in urine or bile" (Parkinson & Ogilvie, 2008, p. 161). It generally involves two phases, namely phase I where more polar metabolites are formed, and phase II where conjugation with endogenous substrates modifies phase I metabolites to highly hydrophilic non-toxic molecules (Ioannides, 2002). Regulation of crossing of xenobiotics and conjugated metabolites into and out of cells by transporter proteins that stretch over the cell membranes is sometimes referred to as phase III (Croom, 2012; Klaasen & Aleksunes, 2010). Some phase I metabolites can also be excreted prior to conjugation, while some xenobiotics can be conjugated, especially glucuronidated, without undergoing phase I reactions (Sanchez & Kauffman, 2010; Testa, 2008). The xenobiotic metabolizing enzymes and transporters are localized in every tissue of the body, although their highest concentrations are found in the liver which consequently functions as the primary site of xenobiotic metabolism (Ioannides, 2002; Parkinson & Ogilvie, 2008). Some of them are inducible in response to high levels of xenobiotics to enhance the rate of biotransformation and elimination. However, the paradox of xenobiotic biotransformation is that this process can also activate the compound to more reactive molecules instead of detoxify it, and such molecules may interact with cellular DNA, RNA, lipids and proteins, resulting in cell toxicity or DNA damage (Ioannides, 2002). Many xenobiotics with genotoxic properties require such activation to electrophilic molecules to exert their ultimate effects. They are called pro-carcinogens (proximate carcinogens). On the other hand, the biotransformation of some

xenobiotics can result in the production of toxic by-products, e.g. reactive oxygen species (ROS), which cause damage through oxidative stress and lipid peroxidation (Parkinson & Ogilvie, 2008).

The roles of individual human enzymes in the metabolism of xenobiotics and particular carcinogenic compounds have been extensively studied in the last three decades (Rendic & Guengerich, 2012), which has provided valuable information that needs to be considered when establishing new cell-based models for routine *in vitro* genotoxicity testing. As described previously, most currently used hepatic alternatives show different expression patterns compared to primary hepatocytes (Baxter et al., 2015; Harris, Dial, & Casciano, 2004; Jennen, Magkoufopoulou, et al., 2010) with insufficient levels of key xenobiotic metabolic enzymes and transporters (Gerets et al., 2012; Guo et al., 2011; Lin et al., 2012; Ulvestad et al., 2013). Inadequate expression of bioactivation enzymes or excessive expression of detoxifying enzymes in cell-based models can result in falsely negative outcomes and vice versa (Hewitt et al., 2007). Likewise, inhibited expression of efflux transporters influences the amount of metabolized xenobiotic, which can result in hypersensitivity to the tested compound and vice versa (Chen et al., 2012).

1.3.1 Bioactivation and detoxification enzymes

Cytochromes P450 (CYPs) represent the primary group of enzymes involved in xenobiotic metabolism (Parkinson & Ogilvie, 2008). From 57 isoforms identified in humans, 15 possess a capacity to metabolize a vast number of structurally diverse xenobiotics with CYP1A1, 1A2, 1B1, 2C9, 2C19, 2D6, 2E1 and 3A4 having a dominant role in this respect (Figure 1.2 A) (Guengerich, 2006; Rendic & Guengerich, 2012). Others are characterized for their roles in the metabolism of endogenous compounds. Although less specific than originally thought, they can interact also with exogenous substances (Guengerich, 2017; Pelkonen et al., 2008). CYPs are considered as a double-edged sword concerning the metabolic activation, and most of the above-mentioned are responsible for the majority of CYP-mediated bioactivation reactions of pro-carcinogens (Figure 1.2 B) (Rendic & Guengerich, 2012). Nevertheless, CYPs still remain the significant enzymes of the detoxification of the majority of xenobiotic compounds, including carcinogens (Guengerich, 2017).

Flavin containing monooxygenases (FMOs), another important enzyme family of phase I metabolism, are normally responsible for detoxification, but sometimes their substrates can be activated to electrophilic reactive metabolites which covalently bind and inactivate neighbour proteins, including CYPs. Many of the FMO-mediated reactions can be catalysed also by CYPs (Parkinson & Ogilvie, 2008). Other important enzymes of phase I metabolism, highly represented in xenobiotic inactivation, are carbonyl reductases. This group includes aldo–keto reductases (AKRs) and short-chain dehydrogenases/reductases (SDRs) (Sanchez & Kauffman, 2010). Besides, AKRs have been found to highly contribute in bioactivation of certain pro-carcinogens (Rendic & Guengerich, 2012). Epoxide hydrolases (EHs), namely microsomal (mEH) and soluble (sEH) isoforms, are crucial in metabolizing highly reactive electrophilic epoxides formed by CYP-mediated biotransformation. They are both present in tissues and cells that also contain cytochromes P450, which probably ensures the rapid detoxification of reactive metabolites. Sometimes, the EH-metabolites can undergo further oxidation by CYPs, resulting in DNA reactive diol epoxide derivatives (Parkinson & Ogilvie, 2008; Stiborová et al., 2014). Other phase I enzymes involved in detoxification and sometimes contributing to bioactivation of exogenous compounds include alcohol (ADHs) and

aldehyde (ALDHs) dehydrogenases (Jelski & Szmitkowski, 2008), carboxylesterases (CESs) (Hosokawa, 2008), NADPH quinone oxidoreductases (NQOs) (Sanchez & Kauffman, 2010) and glutathione peroxidases (GPXs) (Brigelius-Flohé & Maiorino, 2013).

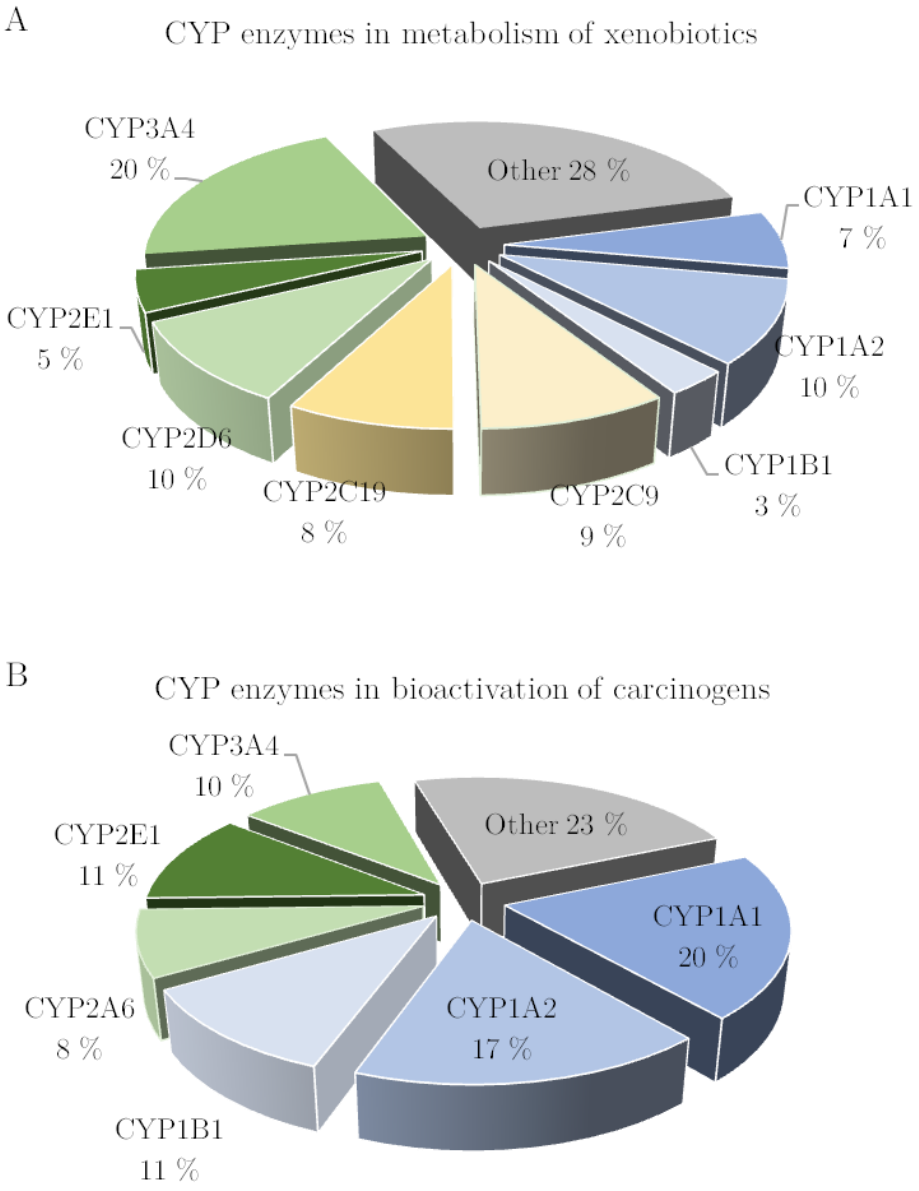

Figure 1.2: Contribution of CYP enzymes in the metabolism of xenobiotics (A) and bioactivation of carcinogens (B) (modified from Rendic & Guengerich (2012)).

Among phase II conjugation enzymes, UDP-glucuronosyltransferases (UGTs) and glutathione S-transferases (GSTs) are the major detoxifying enzymes (Jancova, Anzenbacher, & Anzenbacherova, 2010; Rendic & Guengerich, 2012). GSTs are especially important in detoxification of certain carcinogens and reactive metabolites formed by other metabolizing enzymes as well as in cellular protection against reactive oxygen species formed in normal metabolic processes. N-acetyltransferases (NATs) are another double-edged sword in the metabolism of several known carcinogenic compounds.

Depending on the type of acetylation reaction they catalyse, the compound can be detoxified (N-acetylation) or activated (O-acetylation) (Jančová & Šiller, 2012). Various methyltransferases, such as thiopurine S-methyltransferases (TPMTs) and catechol O-methyltransferases (COMTs), and sulfotransferases (SULTs) also have a prominent role in the detoxification perspective. However, SULTs can activate a number of pro-carcinogens into highly reactive intermediates (Jancova et al., 2010; Rendic & Guengerich, 2012).

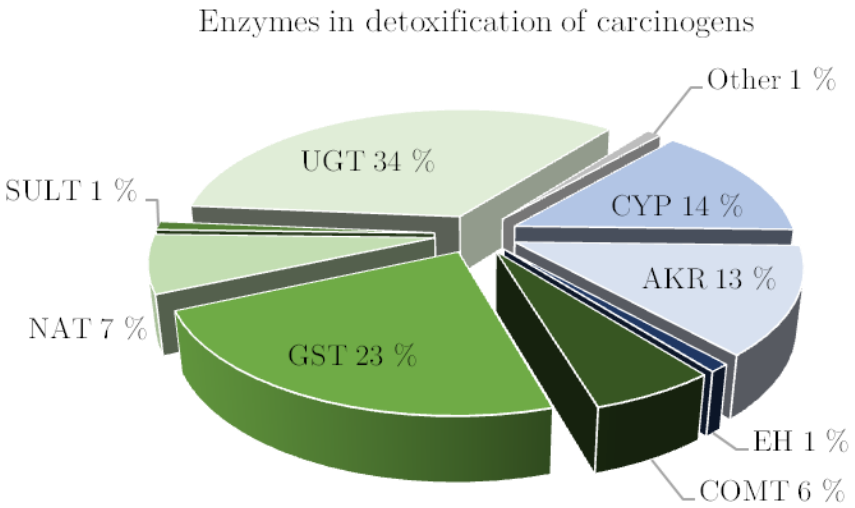

Figure 1.3: Enzyme contributions to detoxification of carcinogens (modified from Rendic & Guengerich (2012)).

Rendic and Guengerich (2012) analysed the existing literature about the biotransformation of carcinogens by human enzymes. They reported the dominant role of CYP enzymes in the metabolism of carcinogenic compounds, especially in their bioactivation, followed by SULT, AKR, NAT and FMO enzymes in the bioactivation respect. On the other hand, UGT and GST enzymes were found to be prevalent in the detoxification of carcinogens, followed by AKRs, NATs and COMTs, and EHs and SULTs in minority (Figure 1.3).

1.3.2 Transporter proteins

The role of transporters in xenobiotic metabolism has been relatively ignored until recently. However, these proteins are important in biotransformation and disposition of xenobiotics, elimination of metabolic waste, as well as in non-metabolic removal of xenobiotics. A number of transporters is highly expressed in the liver (Klaasen & Aleksunes, 2010; Parkinson & Ogilvie, 2008), mainly members of two families: ATP-binding cassette (ABC) transporters, which are constitutively expressed and function as efflux pumps for a wide range of xenobiotics; and solute carrier (SLC) transporters which play a significant role in xenobiotic disposition (Figure 1.4) (Lehman-McKeeman, 2008).

Multidrug resistance proteins (MDRs), multidrug resistance-related proteins (MRPs), breast cancer resistance protein (BCRP), and bile salt export pump (BSEP) are the most

relevant ABC transporters in human hepatocytes (Hewitt et al., 2007). Some of them also confer cell resistance to multiple drugs (Lehman-McKeeman, 2008).

From SLC family, particularly organic anion transporting polypeptides (OATP/*SLCO*), and organic cation and anion transporters (*SLC22*), namely poly-specific organic cation transporters (OCT) and organic anion transporters (OATs), play significant roles in the hepatic uptake of a wide range of compounds. However, the latter three function as bidirectional transporters, and sometimes act as excretion carriers, depending on the conditions (Hewitt et al., 2007; Lehman-McKeeman, 2008). Sodium-bile salt co-transporters (*SLC10*), also part of the SLC family, are influx transporters and participate in the transport of sulphated solutes and many other xenobiotics (Klaasen & Aleksunes, 2010).

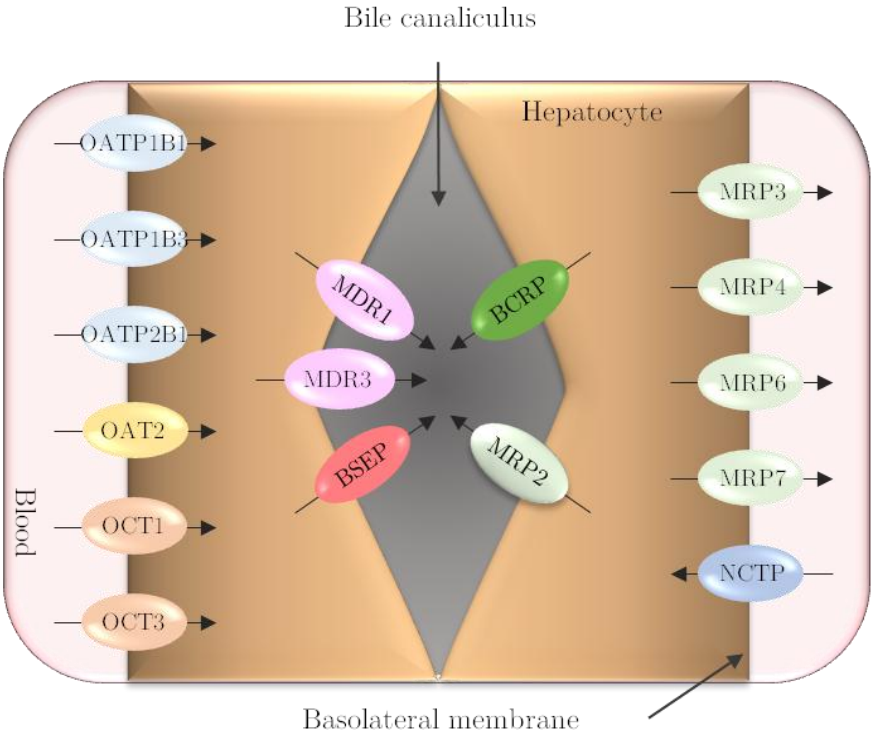

Figure 1.4: Uptake and efflux transporters in hepatocytes (modified from Klaasen & Aleksunes (2010)). NTCP – Sodium taurocholate cotransporting polypeptide.

1.3.3 Transcriptional regulation of xenobiotic metabolism

Over the last decades, the modulation of expression of a number of metabolizing enzymes and transporter proteins by a complicated network of transcription factors (TFs) has been identified as an important phenomenon of xenobiotic biotransformation. As the components of intercellular signaling mechanisms, the TFs play an important role in overall cell development, homeostasis and metabolism (Akiyama & Gonzalez, 2003; Brivanlou & Darnell, 2002; Chen et al., 2012; Tirona & Kim, 2005).

Different classes of xenobiotics can induce or repress the expression of genes encoding biotransformation enzymes and transporters through the activation of specific TFs,

"xenobiotic receptors", together with other classes of regulatory proteins (coactivators and corepressors). The structure and mechanism of action of most xenobiotic receptors is quite similar. They have the ability to recognize a variety of structurally diverse compounds, and act as metabolic sensors that respond to such compounds or these compounds bind directly to the receptors. However, many of them require heterodimerization with retinoic acid receptor (RXRα) to regulate the transcription of target genes (Chen et al., 2012; Omiecinski, Vanden Heuvel, Perdew, & Peters, 2011). The xenobiotic receptors belong to several families of transcription factors: basic helix-loop-helix, basic leucine zipper, forkhead box, homeobox, and nuclear hormone receptor. The latter is the master family with its members, such as constitutive androstane receptor (CAR), pregnane X receptor (PXR), farnesoid X receptor (FXR), liver X receptor (LXR), vitamin D receptor (VDR), hepatocyte nuclear factor 4 alpha (HNF4α), peroxisome proliferator-activated receptor (PPAR), glucocorticoid receptor (GR) and RAR-related orphan receptor (ROR) that are considered as the key regulators of the xenobiotic biotransformation processes (Chen et al., 2012; Omiecinski et al., 2011; Prakash et al., 2015; Wallace & Redinbo, 2013).

PXR and CAR as primary transcriptional modulators are highly expressed in tissues at the entry points for xenobiotic metabolism, including liver (Omiecinski, Vanden Heuvel, Perdew, & Peters, 2011; Prakash et al., 2015; Wallace & Redinbo, 2013), and regulate the expressions of a wide spectrum of genes involved in this process. They exhibit overlapping ligand specificity as well as recognize similar response elements and therefore share a significant number of target genes, not to mention their extensive cross-talking during the transcriptional activation of their targets (Chen et al., 2012; Omiecinski et al., 2011). Their transcriptional expression is often regulated by other xenobiotic receptors: PXR by FXR, PPARα and HNF4α, and CAR by GR, PPARα and HNF4α (Prakash et al., 2015). Unlike other receptors, CAR is constitutively active, but can be more activated through direct ligand binding or through indirect activation mechanisms, which are poorly understood (Omiecinski et al., 2011).

Another receptor that masters the xenobiotic metabolism is aryl hydrocarbon receptor (AHR), which cross-talks with PXR and CAR and resembles the two in cellular localization, ligand binding and activation, nuclear localization and target gene transcriptional activation, except heterodimerization with AHR nuclear translocator (ARNT). Its transcriptional regulation of CYPs that bioactivate many carcinogens, such as polycyclic aromatic hydrocarbons (PAHs) and aflatoxins, has placed the AHR as a central player in the initiation of chemical carcinogenesis (Chen et al., 2012; Omiecinski et al., 2011).

Other relevant xenobiotic receptors that form a heterodimer with RXRα after ligand binding are LXR, FXR, PPARs and VDR. This makes the RXRα a crucial player in metabolic regulation, since it enables other receptors to recognize and bind to specific response elements in DNA and regulate the transcription of different and common metabolic genes and TFs. It is expressed in tissues where such receptors are present, including liver (Chen et al., 2012; Wallace & Redinbo, 2013).

A unique, but very important xenobiotic receptor is small heterodimer partner (SHP), which lacks the DNA binding domain and its primary function is the repression of many nuclear receptors, including GR, FXR, LXR, HNF4α, PPARγ, PXR, and CAR (Wallace & Redinbo, 2013). Crucial in metabolism is also the oxidative stress sensor, nuclear factor erythroid 2-related factor 2 (NRF2, NFE2L2), the "master regulator" of antioxidant response. It is activated by ROS and electrophiles, which are often associated with xenobiotic biotransformation (Gallorini et al., 2015; Wallace & Redinbo, 2013).

Another important group, contributing to transcriptional regulation of xenobiotic metabolism, are constitutively active liver-enriched transcription factors (LETFs), a

heterogeneous class of TFs that is distinctly more expressed in liver compared to other tissues. So far, HNF1, HNF3, HNF4, HNF6, CCAAT/enhancer-binding protein (C/EBP), and D site-binding protein (DBP) were characterized as members of this class. They are necessary for organ development and maintenance of hepatic phenotype. Besides, LETFs can cooperate with ligand-activated xenobiotic receptors and thus contribute to tissue-specific transcriptional regulation of their target genes (Schrem, Klempnauer, & Borlak, 2002). From these, nuclear receptor HNF4α has the most prominent role in xenobiotic metabolism. It forms a homodimer to regulate gene transcription and it does not need a ligand to be activated (Wallace & Redinbo, 2013). Its expression and transcriptional activity are modulated by diverse signaling pathways, and various transcription factors can interact with HNF4α to regulate hepatic gene transcription. However, peroxisome proliferator-activated receptor gamma coactivator (PGC1α) is its key coactivator. HNF4α is considered to be necessary for the constitutive expression of xenobiotic metabolizing genes (Lu, 2016). From HNF1 family, α member appears to be involved in xenobiotic metabolism as well as carcinogenesis, since it regulates the transcription of several important CYPs (Akiyama & Gonzalez, 2003) and the majority of UGTs (MacKenzie et al., 2010). HNF1 and HNF3 members mainly act as coactivators of other LETFs and particular xenobiotic receptors to enhance their binding to DNA response elements of target genes (Lay & Kaestner, 2010; MacKenzie et al., 2010).

One of the reasons for the differences in xenobiotic metabolism between primary hepatocytes and their surrogates is considered to be significantly different expression of major transcription factors, including their cooperative interactions that stimulate specific gene transcription. In human hepatic cancer cells, genes encoding TFs have been shown to be mainly expressed at much lower levels compared to normal hepatocytes, while mRNA expression of a few transcription factors was found to be close or even higher from that in hepatocytes (Jover, Bort, Gómez-Lechón, & Castell, 2001; Martínez-Jiménez, Gómez-Lechón, Castell, & Jover, 2006; Moya, Gómez-Lechón, Castell, & Jover, 2010; Olsavsky et al., 2007; Rodrigues-Antona et al., 2002). Low expression of transcription cofactors has also been demonstrated as a common feature of cell lines derived from human hepatic tumors, which can contribute to repression of important metabolic genes (Martínez-Jiménez, Gómez-Lechón, et al., 2006). However, many studies have reported that the transfection of hepatic cancer cells or SC-derived hepatocytes with adenoviral vectors encoding for specific regulatory transcription factors, such as C/EBPα (Jover, Bort, Goèmez-Lechoèn, & Castell, 1998), HNF3γ (Bort, Gómez-Lechón, Castell, & Jover, 2004), HNF3β (Li, Li, Heyward, & Wang, 2016), HNF4α (Naiki et al., 2002), HNF6 (Sasaki et al., 2013), or combinations of a few of the relevant transcription factors (Benet, Lahoz, Guzmán, Castell, & Jover, 2010; Riffel, Schuenemann, & Vyhlidal, 2009), or vectors encoding transcription coactivators (Martínez-Jiménez, Gómez-Lechón, et al., 2006) can be used to regulate the expression of important metabolic genes in these *in vitro* cell-based models.

Chapter 2

Aim of Study, Objectives and Research Hypotheses

2.1 Aims and Specific Objective

The dissertation is divided into two parts with the final objective to obtain a stable metabolically active *in vitro* cell model of human origin, which would reflect *in vivo* biotransformation of chemicals with genotoxic properties as close as possible, would be able to reliably detect direct and indirect acting genotoxins and could be used for routine testing.

Several hepatic cancer cell lines with a relatively retained expression of xenobiotic metabolizing enzymes and the ability to detect genotoxins have been identified in the past years, namely HepG2 cells (Natarajan & Durroudi, 1991), Hep3B cells (Majer, Mersch-Sundermann, et al., 2004), HCC1.2 cells (Winter et al., 2008) and HepaRG cell line (Le Hegarat et al., 2010). Since tumor-derived cells are of particular interest for cancer development and therapy studies, a large number of different hepatic cancer lines is available on the market, but they have never been used in genotoxicity studies and some of them may be even more suitable for this purpose. Therefore, a comprehensive comparative analysis of a panel of currently available human liver-derived cell lines was carried out in the first part of this research work. The criteria to choose the most suitable candidate were the cell characteristics, which are relevant for the potential use in genotoxicity testing, including the morphological similarity to primary hepatocytes; the chromosomal stability; the doubling time and the p53 status of the cells. Their sensitivity towards representatives of different classes of environmental and dietary genotoxic carcinogens, which are all of human relevance and require metabolic activation by different phase I and phase II enzymes, was evaluated and compared. Gene expression analysis of liver-specific transcription factors and genes involved in the metabolism of xenobiotics was performed with the two most promising cell lines to get insight into their metabolic profiles. The comparison of their metabolic profiles to the metabolic profiles of primary human hepatocytes revealed how closely the two cell lines reflect *in vivo* hepatic metabolism of xenobiotics.

In the second part of the work, the hepatic differentiation of human adipose tissue-derived mesenchymal stem cells (hASC) was performed with the aim to obtain metabolically active differentiated hepatic progenies convenient for the use in genotoxicity testing. Adipose tissue represents an abundant and easily accessible source of stem cells, and the methods for the isolation and protocol for *in vitro* propagation of hASC have been previously developed in our laboratory (Kološa, 2016). Several reports indicate that human ESC- and iPSC-derived hepatocytes can be the applicable liver

model for drug metabolism studies and toxicological investigations, expressing important xenobiotic metabolizing enzymes and transporter proteins (Gao & Liu, 2017; Lu et al., 2015; Ulvestad et al., 2013; Yildirimman et al., 2011). A number of studies have been conducted regarding *in vitro* hepatic differentiation of hASC, but mainly for therapeutic purposes of liver injury (Banas et al., 2007; Lee et al., 2015; Seo, Suh, Bae, & Jung, 2005; Taléns-Visconti et al., 2006; Yin et al., 2014). However, much less efforts have been focused on metabolic characterization of ASC-derived hepatocytes and their applicability in *in vitro* toxicological testing. Therefore, besides the evaluation of specific hepatic characteristics and functionality of the generated hepatic progenies in this study, their predictive potential was demonstrated through the response of cells to the exposure to different classes of model genotoxic pro-carcinogens. Additionally, the expression analysis of selected enzymes involved in xenobiotic metabolism in generated cells was performed, and the transcriptional response of these enzymes after the exposure of cells to the model pro-carcinogens investigated. In order to get a permanent cell line suitable for the use in routine testing, immortalization of the generated hepatic progenies with stable transfection with *hTERT* was performed to prolong their life span and consequently provide limitless supply of human differentiated hepatic progeny (hDHP) cells.

2.2 Research Hypotheses

Hypothesis I

Among the available human liver-derived cell lines, such cell lines exist that are equally or even more suitable for the detection of DNA damage induced by genotoxic carcinogens than the cell lines currently used for *in vitro* genotoxicity testing.

Hypothesis II

The expression levels and inducibility of liver-specific transcription factors and genes involved in the metabolism of xenobiotics in the selected candidate cell lines differ between the cell lines as well as from that of primary human hepatocytes.

Hypothesis III

Human adipose tissue-derived mesenchymal stem cells can be differentiated into metabolically competent hepatic progenies that can be exploited for *in vitro* genotoxicity testing.

Hypothesis IV

The immortalization of the differentiated hepatic progenies from human adipose tissue-derived mesenchymal stem cells extends their replicative life span and the generated immortal cells retain the sensitivity to detect DNA damage induced by genotoxic pro-carcinogens.

Chapter 3

Materials and Methods

3.1 Chemicals

All primary antibodies were purchased from Abcam (Cambridge, UK). SuperScript™ IV VILO™ Master Mix with ezDNase™, TaqMan High-Capacity cDNA Reverse Transcription Kit, TaqMan Gene Expression Assays, TaqMan PreAmp Master Mix Kit and TaqMan Universal Master Mix II with UNG were from Applied Biosystems (Carlsbad, CA, USA). Blotting grade blocker, Bradford assay and PVDF membrane were purchased Bio-Rad Laboratories (California, US), GelRed Nucleic Acid Stain was from Biotium (Fremont, CA, USA), and Amersham ECL detection system from GE Healthcare Life Sciences (Buckinghamshire, UK). Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), foetal bovine serum (FBS), geneticin, L-glutamine and penicillin/streptomycin were obtained from Gibco, Life technologies (Carlsbad, CA, USA). Alexa Fluor 488®-conjugated secondary antibodies, low melting point (LMP) agarose, normal melting point (NMP) agarose and Prolong® Gold anti-fade reagent were purchased from Invitrogen (Carlsbad, CA, USA), and sucrose, potassium phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and magnesium chloride ($MgCl_2$) from Kemika d.d. (Zagreb, Croatia). Trypsin-EDTA was from Life Technologies (Karlsruhe, Germany). Hepatocyte Maintenance medium, Hepatocyte Maintenance Supplement, Hepatocyte Plating medium, Hepatocyte Plating Supplement and Hepatocyte Thawing medium were obtained from Lonza (Basel, Switzerland). Citric acid and disodium hydrogen phosphate dihydrate for McIlvaine buffer, Tris and Tween®20 were from Merck (Darmstadt, Germany). Heat-inactivated FBS was from PAA Laboratories GmbH (Pasching, Austria). Activin A (ActA), bone morphogenic protein 2 (BMP-2), hepatocyte growth factor (HGF) and oncostatin M (OSM) were obtained from PeproTech (Rocky Hill, NJ, USA). CellTiter 96® AQueous cell proliferation assay and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Promega Corporation (Madison, WI, USA). EndoFree Plasmid Mega Kit and endotoxin-free water were from Qiagen (Hilden, Germany), and Collagen R solution from Serva Electrophoresis GmbH (Heidelberg, Germany). Acetic acid, aflatoxin B1 (AFB_1), benzo(a)pyrene (B(a)P), bovine serum albumin (BSA), Brij 35, collagen type I solution from rat tail, dexamethasone (DXM), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), Eagle's Minimum Essential Medium (EMEM), EDTA disodium salt (EDTA-Na_2), glutaraldehyde, insulin - transferrin - selenium (ITS), Iscove's modified Dulbecco's medium (IMDM), methanol, Minimal essential Medium Eagle (MEME), nicotinamide (vitamin B3; VIT B3), N-nitrosodimethylamine (NDMA), non-essential amino acids (NEAA), paraformaldehyde, pepstatin A, periodic acid solution, phenylmethylsulfonyl fluoride (PMSF), potassium

chloride (KCl), propidium iodide, quinacrine dihydrochloride and thymol for quinacrine staining solution, Roswell Park Memorial Institute Medium (RPMI-1640), Schiff's solution, sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium pyruvate ($C_3H_3NaO_3$), tert-butylhydroperoxide (TBHP), Triton X-100 and Trizma® base were all obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Dithiothreitol (DTT), HepaRG™ Thaw, Plate & General Purpose Medium Supplement and HepaRG™ ToxMed Supplement, PageRuler™ Plus Prestained Protein Ladder, TRIzol® reagent, William's E medium and XbaI restriction enzyme came from Thermo Fisher Scientific (Waltham, Massachusetts, USA). 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methyl-3Himidazo[4,5-f]quinolone (IQ) were purchased from Toronto Research Chemicals (North York, Canada), and colcemid from VWR (Radnor, Pennsylvania, USA).

3.2 Selection of the Most Suitable Candidates Among a Panel of Hepatic Cell Lines for the Application in Genotoxicity Testing

3.2.1 Origin and cultivation of cell lines

HCC1.2 cell line was kindly provided by Dr. Eisenbauer (Institute of Cancer Research, Medical University of Vienna (MUW), Vienna, Austria). Hep3B, HepG2, PLC/PRF5 and SK-Hep1 cell lines were obtained from the American Type Tissue Culture (ATCC; Manassas, VA, USA), and HepaRG™ cells from Thermo Fisher Scientific (Waltham, Massachusetts, USA). HuH6, HuH7, SNU-398, SNU-449 and WRL68 cell lines were kindly provided by Dr. Fabregat (Bellvitge Biomedical Research Institute, Barcelona, Spain) and JHH6 cells by Dr. Grassi (Department of Life Sciences, University Hospital of Cattinara, Trieste, Italy). Information about the origin of the cell lines and their cultivation conditions is listed in Table 3.1.

All lines, except HepaRG™, were grown in TC-treated plates (Sigma-Aldrich, St Louis, Missouri, USA). HepaRG™ cells were cultivated according to the instructions of the supplier. Briefly, the cells were thawed in medium (Table 3.1.) complemented with HepaRG™ Thaw, Plate & General Purpose Medium Supplement, and plated on collagen-coated plates (0.2 mg/ml Collagen R solution). For passaging and toxicological experiments, the medium (Table 3.1.) complemented with HepaRG™ ToxMed Supplement was used. HuH6 cells were grown in 4.0 % FBS, all other lines were grown in 10.0 % FBS, and HCC1.2 and JHH6 were cultivated in 10.0 % heat-inactivated FBS. All lines were incubated at 37 °C and 5.0 % CO_2, except HuH7 and WRL68, both at 37 °C and 8.0 % CO_2.

Table 3.1: Origin and cultivation of the cell lines.

Cell line	Origin	Donor details			Cultivation	Reference
		Sex	Age /yrs	Ethnic group		
HCC1.2	Hepatocellular carcinoma	M	56	Caucasian	RPMI-1640 2.0 g/l $NaHCO_3$	Sagmeister et al., 2008
Hep3B	Hepatocellular carcinoma	M	8	Black	EMEM 2.2 g/l $NaHCO_3$	Aden et al., 1979
HepaRG™	Cholangio - carcinoma	F	NS	Caucasian	William's E	Gripon et al., 2002
HepG2	Hepatocellular carcinoma	M	15	Caucasian	MEME 2.2 g/l $NaHCO_3$ 1.0 % NEAA 1.0 mM $C_3H_3NaO_3$	Aden et al., 1979
HuH6	Hepatoblastoma	M	1	Asian (Japanese)	RPMI-1640 2.0 g/l $NaHCO_3$	Doi, 1976
HuH7	Hepatocellular carcinoma	M	57	Asian (Japanese)	DMEM 3.7 g/l $NaHCO_3$	Nakabayashi et al., 1982
JHH6	Hepatocellular carcinoma	F	57	Asian (Japanese)	William's E 1.0 % glutamine	Fujise et al., 1990
PLC/PRF5	Hepatocellular carcinoma	M	24	South African	MEME 2.2 g/l $NaHCO_3$	Alexander et al., 1976
SK-Hep1	Adenocarcinoma of the liver	M	52	Caucasian	EMEM 2.2 g/l $NaHCO_3$	Heffelfinger et al., 1992
SNU-398	Hepatocellular carcinoma	M	42	Asian (Korean)	RPMI-1640 2.0 g/l $NaHCO_3$	Park et al., 1995
SNU-449	Hepatocellular carcinoma	M	52	Asian (Korean)	RPMI-1640 2.0 g/l $NaHCO_3$	Park et al., 1995
WRL68	Normal human liver tissue		E		DMEM 3.7 g/l $NaHCO_3$	Gutiérrez-Ruiz et al., 1994

yrs – years. M – male. F – female. NS – not specified. E – embryo.

3.2.2 Proliferation kinetics

The proliferation kinetics measurements were conducted in cooperation with M. Waldherr, Dr. Mišík, and Prof. Dr. Knasmüller (Institute of Cancer Research, MUW).

0.5×10^6 cells of each line were seeded to Petri dishes (Sigma-Aldrich) in a corresponding medium. After 24 h, the cells were detached with trypsin-EDTA, re-suspended in medium, and 50.0 µl of cell suspension was transferred to CASY-cup (OLS OMNI Life Science GmbH & Co. KG, Bremen, Germany). The proliferation kinetics was monitored using CASY® Cell Counter and Analyzer System (TTC-2EA-1087, Schärfe System GmbH, Reutlingen, Germany) according to the manufacturer's instructions. Three measurements were performed per experimental point, and the average number of viable cells/ml and % viability were calculated. The process was performed every 24 h over a period of 7 days. Doubling times were determined using GraphPad Prism 5 software (San Diego, California) and exponential growth equation.

3.2.3 Karyotyping

Preparation and visualization of chromosomes in the mitotic phase for karyotyping was conducted in cooperation with Dr. Haas and Dr. Mai (Labdia Labordiagnostik GmbH, Vienna).

When reaching 70 % confluence, the cells were treated with 10.0 µg/ml colcemid for 90 min. After washing with PBS, the cells were trypsinized and centrifuged. The pellets were re-suspended and incubated in KCl (10 min at 37 °C), centrifuged, and fixed in methanol and acetic acid containing fixative. A few drops of fixed cells were placed to microscopic slides, and dried in the air. Optimal cell density was controlled under the microscope before analysis.

Slides were stained with quinacrine solution and incubated in McIlvaine solution, and analysed using Applied Spectral Imaging Case Data Manager (Version 5.5.2.2, Applied Spectral Imaging, Carlsbad, CA, USA). At least 20 metaphases per cell line were karyotyped.

3.2.4 Real-time quantitative PCR analysis (qPCR)

The expression and inducibility of *p53* gene in cell lines was analysed after the 24 h exposure of cells to genotoxic pro-carcinogen B(a)P (30.0 µM; for details see Chapter 3.2.6) using the qPCR method. At the end of the exposure, the cells were washed with PBS and total RNA was isolated using TRIzol® reagent according to the manufacturer's protocol. RNA quantity and quality were assessed by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from 1.0 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit following the manufacturer's instructions. The cDNA samples were stored at - 20 °C until qPCR analysis.

Gene expression was quantified using ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA), TaqMan Universal Master Mix II and the following TaqMan Gene Expression Assays: *TP53* (tumor protein p53 Hs00153349_m1 and Hs01034249_m1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Human Endogenous Controls 4310884E). Each cDNA sample was diluted 1:10. Final PCR reaction volume was 10.0 µl and the thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min, following 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The experiments

were repeated three times independently, each with two technical replicates per experimental point.

The amplification plots were analysed with ABI 7900HT Sequence Detection System 2.4.1 software (Applied Biosystems). The threshold values of mRNAs were adjusted to 0.2 for each reaction plate. mRNA was considered as not expressed if the cycle threshold (Ct) value was undetermined or above 37 in two out of three experiments. Each Ct of *p53* gene was normalized to the Ct of a reference gene *GAPDH*, and a comparative $\Delta\Delta$Ct algorithm was applied to determine the relative expression (Rq) of *p53* gene in B(a)P exposed cells according to the vehicle control cells. The criterion for deregulation of *p53* gene was Rq $\geq$ 1.5. The data were statistically analysed using GraphPad Prism 6 software (San Diego, California) and Student's t test was applied to calculate the significant difference (p values $\leq$ 0.05 were considered as statistically significant).

3.2.5 Western blot analysis

The expression and inducibility of p53 protein was quantified by Western blot analysis after the 24 h exposure of cells to genotoxic pro-carcinogen B(a)P (for details see Chapter 3.2.6). At the end of the exposure, the cells were washed with PBS and proteins isolated using lysis buffer solution (50.0 mM Tris, 30.0 % Brij 35, 20.0 mM DTT, 5.0 mM EDTA-Na_2; pH 6.9) supplemented with protease inhibitors PMSF and pepstatin A. Total protein concentrations were determined using Bradford assay according to the manufacturer's instructions. The protein samples were stored at - 80 °C until the analysis was performed.

Ten µg of each protein sample was loaded to 12.0 % SDS-PAGE gel along with the molecular weight marker (PageRuler™ Plus Prestained Protein Ladder). Electrophoresis was run at 12 mA (1 h at RT) and 30 mA (1.5 h at RT), followed by protein transfer to the PVDF membrane (overnight at 4 °C). The membrane was blocked with 5.0 % non-fat milk (Blotting grade blocker) (3 h at RT) and incubated with selected primary antibody (overnight at 4 °C). After washing with 0.1 % Tween®20/PBS (PBST), the membrane was incubated with an appropriate HRP-conjugated secondary antibody (1:2500, HRP-conjugated anti-rabbit or anti-mouse) (1 h at RT) and washed again with PBST. The chemiluminescent signal was developed using the Amersham ECL detection system according to the manufacturer's instructions, and western blot images were obtained with the darkroom chemiluminescence development technique. The following primary antibodies were used: mouse antibody against p53 (1:500, ab1101), rabbit antibody against mutant p53 (1:1000, ab32049) and rabbit antibody against GAPDH (1:2000, ab9485). The experiments were repeated two times independently.

3.2.6 Treatment with model genotoxic agents

The cell lines were exposed to graded concentrations of model genotoxic pro-carcinogens: B(a)P (2.0 µM, 10.0 µM, 30.0 µM, 50.0 µM and 90.0 µM), AFB_1 (1.0 µM, 2.5 µM, 5.0 µM, 10.0 µM, 20.0 µM and 30.0 µM) and NDMA (50.0 mM, 100.0 mM and 200.0 mM) for 24 h, and PhIP (25.0 µM, 50.0 µM, 100.0 µM and 300.0 µM) and IQ (25.0 µM, 50.0 µM, 100.0 µM and 250.0 µM) for 48 h.

The stock solutions of pro-carcinogens B(a)P (10.0 mM), AFB_1 (10.0 mM), PhIP (10.0 mM) and IQ (1.0 mM) were prepared in 100.0 % DMSO. The stock solution of NDMA (200.0 mM) was prepared in PBS. The medium containing DMSO (adjusted to 1.0 %) or PBS was used as the vehicle control.

3.2.7 Comet assay

Sensitivity of hepatic cell lines was evaluated through determination of DNA damage induced by B(a)P, AFB_1, PhIP, IQ and NMDA. The comet assay was performed as described in an international guideline (Azqueta & Collins, 2013; Tice et al., 2000) in cooperation with M. Waldherr, Dr. Mišík, and Prof. Dr. Knasmüller (Institute of Cancer Research, MUW).

Prior to the conduction of the comet assay, cell viability after the exposure to the highest concentration of tested compounds was determined with CASY® Cell Counter and Analyzer System (as described in Chapter 3.2.2) to confirm that the cytotoxicity of tested compounds is ≤ 30 % as recommended (Tice et al., 2000).

For the comet assay, the cells were washed with PBS at the end of the exposure, trypsinized, centrifuged, and diluted in cold PBS. 15.0×10^3 cells were embedded in 0.5 % LMP agarose and added to microscopic slides pre-coated with 1.5 % NMP agarose. One slide with two gels was prepared per culture. The cells in agarose were lysed (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Trizma® base, 1.0 % Triton X-100, 10.0 % DMSO; pH 10.0) (1 h at 4 °C), incubated in alkaline electrophoresis buffer (300.0 mM NaOH, 1.0 mM Na_2EDTA; pH > 13.0) (40 min at 4 °C) and electrophoresed at 0.75 V/cm and ~300.0 mA (30 min at 4 °C). The slides were then washed twice with cold ddH_2O (8 min) and air-dried. The experiments were repeated two times.

For the DNA damage analysis, the gels were stained with propidium iodide (10.0 µg/ml), and the images observed using the fluorescence microscope (Eclipse E600; Nikon, Tokyo, Japan) and image analysis software Comet Assay IV (Perceptive Instruments, Bury St Edmunds, UK). 50 randomly selected nuclei were evaluated per experimental point. The DNA damage was expressed as average % of DNA in the comet tail. The results were statistically analysed using GraphPad Prism 5 software (San Diego, California). Ordinary one-way analysis of variance (one-way ANOVA) followed by Dunnett's multiple comparisons was performed to determine the significant difference between DNA damage of treated groups and the DNA damage of the vehicle control group (p values ≤ 0.05 were considered as statistically significant).

3.3 Gene Expression Analysis of Liver-Specific Transcription Factors and Genes Involved in the Metabolism of Xenobiotics

3.3.1 Cultivation of selected cell lines and human primary hepatocytes

HepG2 and HuH6 cell lines were cultivated as described in Chapter 3.2.1. Cryopreserved human primary hepatocytes from two donors (Lonza, Basel, Switzerland; for characteristics see Supplementary Material Table A.1) were cultivated according to the supplier's instructions. Briefly, the cells were thawed in Hepatocyte thawing medium, and plated on collagen-coated plates (0.05 mg/ml collagen type I solution from rat tail) in Hepatocyte plating medium complemented with Plating Supplement. 4–6 h post-seeding, the plating medium was replaced with Hepatocyte maintenance medium complemented with Maintenance Supplement. The cells were incubated at 37 °C and 5.0 % CO_2.

3.3.2 Treatment with model genotoxic agents

HepG2 cells, HuH6 cells and human primary hepatocytes were exposed to non-cytotoxic concentrations of model genotoxic pro-carcinogens for 24 h: B(a)P (10.0 µM), AFB_1 (3.2 µM) and PhIP (20.0 µM).

The stock solutions were prepared in 100.0 % DMSO: B(a)P 9.9 mM, AFB_1 3.2 mM and PhIP 20.0 mM. The medium containing DMSO (adjusted to 0.1 %) was used as the vehicle control.

3.3.3 Gene selection

The selection of genes for expression analysis based on the literature data (Akiyama & Gonzalez, 2003; Guo et al., 2011; Hilgendorf et al., 2007; Martínez-Jiménez, Castell, Gómez-Lechón, & Jover, 2006; Martínez-Jiménez, Gómez-Lechón, et al., 2006; Schrem et al., 2002; Tirona & Kim, 2005; Wallace & Redinbo, 2013), and the information from available databases (GeneCards, 2016; NCBI gene, 2016; PhosphoSitePlus®, 2016; UniProt, 2016). 90 probes for liver-specific genes involved in the metabolism of xenobiotics were selected (listed in Supplementary Material Table A.2): 32 probes for phase I metabolic genes, 21 probes for phase II metabolic genes, 11 probes for metabolic transporter genes, and 26 probes for transcription factor genes. If possible, probes that span exon boundaries were chosen. 4 probes for endogenous control genes were included in the analysis (Supplementary Material Table A.2).

3.3.4 qPCR analysis

At the end of the exposure to genotoxic agents, the cells were washed with PBS and total RNA was isolated using TRIzol® reagent, according to the manufacturer's instructions. The quantity and quality of purified RNA were assessed by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from 1.2 µg of total RNA with SuperScript™ IV VILO™ Master Mix following the protocol provided by the manufacturer. The thermo labile DNase enzyme was added to each reverse transcription reaction to remove the possible genomic DNA (gDNA) contamination. To confirm the absence of gDNA, negative control reactions without reverse transcriptase enzyme were performed in parallel for each sample. The cDNA samples and negative control samples were used immediately or they were stored at - 80 °C until qPCR analysis.

Gene expression analysis was performed by ViiA™7 Real-Time PCR System (Applied Biosystems). TaqMan assays (Supplementary Material Table A.2) were pre-loaded to 384–well reaction plates using Janus Integrator (PerkinElmer, Waltham, MA, USA). Each cDNA sample was mixed with TaqMan Universal Master Mix II (cDNA dilution 1:10), and applied to the specified wells. The final PCR reaction volume was 10.0 µL and the thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min, following 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The experiments were repeated three times independently.

3.3.5 Data analysis

The amplification plots were analysed using QuantStudio™ Real-Time PCR System 1.3 software (Applied Biosystems). Threshold values of all target mRNAs were adjusted to 0.2 for all reaction plates. Genes with two or more undetermined Ct values or Ct above 37 within the three replicates for each exposure condition and vehicle control were considered as not expressed. Ct of each target gene was normalized to the average Ct of four reference genes, *ACTB, GAPDH, HPRT1* and *PGK1* respectively. A comparative $\Delta\Delta$Ct algorithm was applied to determine the relative gene expression (Rq) in: vehicle control primary hepatocytes of donor II (pHepII), HepG2 cells and HuH6 cells relative to the vehicle control primary hepatocytes of donor I (pHepI); B(a)P, AFB_1 or PhIP exposed cells relative to the vehicle control cells for each cell model. To determine deregulated genes in each experimental group, the following criteria were used: $Rq \leq 0.67$ and $Rq \geq 1.5$ for the average of the three replicates within the experimental group; $Rq \leq 0.83$ and $Rq \geq 1.2$ for at least two replicates within the experimental group; the same direction of Rq for all the replicates within the experimental group. Genes that were not expressed in vehicle control group and were expressed after the exposure to B(a)P, AFB_1 or PhIP and vice versa were also considered as deregulated.

The statistical analysis and assessment of gene expression differences between primary hepatocytes and selected cell lines was performed in cooperation with Dr. Ana Rotter using the Limma software package R version 3.4.3 (Bioconductor, 2019) that provides an integrated solution for analysing data from gene expression experiments. Limma uses empirical Bayes moderated t-statistics and their associated p-values to moderate the standard errors of the estimated log-fold changes, which results in a more stable assessment of the significance of the observed expression changes, especially for experiments with small numbers of arrays (Ritchie et al., 2015; Smyth, 2004). Statistical outputs were presented as Venn diagrams. To visualize the differences in metabolic gene expression profiles among the selected cell lines and primary hepatocytes, hierarchical clustering and heatmap analysis were performed.

3.4 Development of Metabolically Active Differentiated Hepatic Progenies from Human Adipose Tissue Stem Cells for the Application in Genotoxicity Testing

3.4.1 Hepatic differentiation of hASC

Isolation, culturing and characterisation of hASC, obtained from subcutaneous adipose tissue of a healthy female donor, has been performed in our laboratory by Dr. Kološa (Kološa, 2016).

hASC were seeded on collagen-coated plates (0.05 mg/ml collagen type I solution from rat tail) at a density of 6.0×10^3 cells/cm^2, and cultured in DMEM supplemented with 10.0 % FBS, 100.0 IU/ml penicillin, 0.1 mg/ml streptomycin and 2.0 mM L-glutamine. When reaching 80 % confluence, the cells were serum-deprived in IMDM medium with 100.0 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2.0 mM L-glutamine for 24 h, to inhibit the proliferation prior to induction of hepatic differentiation. The hepatic differentiation was performed using a three-step differentiation protocol according to Lee et al. (2015) with minor modifications (Table 3.2). The control hASC group was cultivated in parallel in IMDM medium with 0.5 % BSA, 100.0 IU/ml penicillin, 0.1

mg/ml streptomycin, and 2.0 mM L-glutamine and without liver-specific growth factors and cytokines. The cells were incubated at 37 °C and 5.0 % CO_2.

Table 3.2: Three-step differentiation protocol for hepatic differentiation of hASC.

Endoderm induction	Hepatic induction		Hepatic maturation
3 days	3 days	4 days	14 days
IMDM*	IMDM*	IMDM*	IMDM*
100.0 ng/ml ActA	30.0 ng/ml bFGF	50.0 ng/ml HGF	20.0 ng/ml OSM
10.0 ng/ml bFGf	20.0 ng/ml BMP-2	1.0 % ITS	1.0 µM DXM
10.0 ng/ml EGF	1.0 % ITS	0.61 g/l VIT B3	0.1 % DMSO
0.5 % BSA	0.61 g/l VIT B3	0.5 % BSA	
	0.5 % BSA		

(*) IMDM medium supplemented with 100.0 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2.0 mM L-glutamine.

3.4.2 Periodic acid - Schiff (PAS) staining

On the last day of hepatic differentiation, the presence of glycogen in the cells was determined using the PAS staining method. After washing with PBS, the cells were fixed with 0.5 % glutaraldehyde (15 min at RT), rinsed with ddH_2O, and incubated in 0.5 % periodic acid solution (5 min at RT), in ddH_2O (3 min at RT), and in Schiff's solution (15 min at RT). The cells were rinsed with ddH_2O one more time and the staining was visualized using the light microscope (Eclipse Ti, Nikon) and NIS-Elements Advanced Research software 4.13.04 (Nikon). The experiments were repeated three times independently.

3.4.3 Immunocytochemistry (ICC)

The ICC technique was used to detect the expression of albumin (ALB) at the end of differentiation process. The cells were fixed with 4.0 % paraformaldehyde (15 min at RT), and incubated in 0.1 % Triton X-100/PBS (10 min at RT) for membrane permeabilization. The non-specific protein interactions were blocked with 10.0 % BSA (30 min at RT). The cells were incubated with mouse primary antibody against human serum albumin (1:100, ab10241) (overnight at 4 °C), followed by incubation with Alexa Fluor 488®-conjugated secondary antibody (1:300, Alexa Fluor 488® goat anti-mouse) (1.5 h at RT, in dark). Each individual step was completed by rinsing the cells with PBS. Finally, the cells were mounted in Prolong® Gold anti-fade reagent and the fluorescence of secondary antibodies was visualized using the inverted fluorescence microscope (Eclipse Ti, Nikon) and NIS-Elements Advanced Research software 4.13.04 (Nikon). The experiments were repeated three times independently.

3.4.4 Treatment with model genotoxic agents

At the end of the differentiation process, hDHP and hASC cells were exposed to graded concentrations of model genotoxic pro-carcinogens: B(a)P (7.5 µM, 15.0 µM and 30.0 µM), AFB_1 (1.9 µM, 3.8 µM, 7.5 µM and 15.0 µM) and PhIP (50.0 µM, 100.0 µM and 200.0 µM) for 24 h, and to direct acting genotoxin TBHP (100.0 µM, 200.0 µM and 400.0 µM) for 20 min. Human hepatic cancer cells HepG2 were cultured as described in Chapter 3.2.1, and exposed to the highest tested concentrations of model genotoxic pro-carcinogens B(a)P, AFB_1 and PhIP for 24 h, and to the highest tested concentration of direct acting TBHP for 20 min.

The stock solutions of pro-carcinogens were prepared as described in Chapter 3.3.2. The stock solution of TBHP (5.0 mM) was prepared in PBS. The medium containing DMSO (adjusted to 1.0 %) or PBS was used as the vehicle control.

3.4.5 MTS cytotoxicity assay

Cytotoxicity of B(a)P, AFB_1 and PhIP was assessed through determination of hDHP and hASC cell viability using the colorimetric CellTiter 96® AQueous cell proliferation assay according to the manufacturer's protocol. At the end of the exposure, the medium containing genotoxic compounds was replaced with fresh cell medium. A mixture of tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and electron coupling reagent PMS (phenazine methosulfate) was added to the medium. After 3 h, the absorbance was measured at 490 nm using a microplate reader (Sinergy Mx, BioTek, Bad Friedrichshall, Germany). One experiment with five replicates per experimental point was performed.

The optical density of the wells containing exposed cells was compared to optical density of the wells containing vehicle control cells and the results were expressed as average % of cell viability. The data were analysed using GraphPad Prism 6 software (San Diego, California).

3.4.6 Comet assay

To determine DNA damage induced by B(a)P, AFB_1, PhIP and TBHP, the alkaline comet assay was performed as described by Žegura and Filipič (2004). At the end of the exposure, the cells were washed with PBS, trypsinized and centrifuged. 30.0 µL of cell suspension was mixed with 1.0 % LMP agarose, added to fully frosted slides pre-coated with 1.0 % NMP agarose, and lysed (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris, 1.0 % Triton X-100; pH 10.0) (1 h at 4 °C). After the lysis, the slides were placed in an electrophoresis buffer (300.0 mM NaOH, 1.0 mM Na_2EDTA; pH > 13.0) (20 min at 4 °C) for DNA unwinding, and electrophoresed in the same buffer at 1 V/cm (20 min at 4 °C). Finally, the slides were neutralized (0.4 M Tris; pH 7.5) (15 min at 4 °C) and air-dried. The experiments were repeated three times independently.

For DNA damage analysis, the gels on the slides were stained with GelRed Nucleic Acid Stain (1:3333), and the images observed using the fluorescence microscope (Eclipse 800, Nikon) and image analysis software Comet Assay IV (Perceptive Instruments). 50 randomly selected nuclei were analysed per each experimental point and the DNA damage was expressed as average % of DNA in the comet tail. The data were statistically analysed using GraphPad Prism 6 software (San Diego, California). One-way ANOVA with Dunett's multiple comparisons test was applied to determine the significant

difference between DNA damage of treated groups and the DNA damage of the vehicle control group (p values ≤ 0.05 were considered as statistically significant).

3.4.7 qPCR analysis

At the end of the differentiation process mRNA expression of *ALB* was determined in hDHP and hASC cells and gene expression and inducibility of selected xenobiotic metabolic enzymes was determined in hDHP cells exposed to the highest concentrations of model genotoxic pro-carcinogens B(a)P, AFB_1 or PhIP for 24 h (for details see Chapter 3.4.4). The cells were washed with PBS and total RNA was isolated using TRIzol® reagent according to the manufacturer's protocol. RNA quantity and quality were assessed by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from 1.0 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit following the manufacturer's instructions. Due to the low expression of selected genes (Ct > 38), the cDNA was pre-amplified prior to the conduction of qPCR reactions, using TaqMan PreAmp Master Mix Kit according to the protocol provided with the kit. The pre-amplified cDNA samples were stored at - 20 °C until qPCR analysis.

Gene expression was quantified using ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA), TaqMan Universal Master Mix II and the following TaqMan Gene Expression Assays: *ALB* (albumin, Hs00910225_m1), *CYP3A4* (cytochrome P450 3A4, Hs02514989_s1), *CYP1A1* (cytochrome P450 1A1, Hs01054797_g1), *CYP1A2* (cytochrome P450 1A2, Hs00167927_m1), *NAT2* (N-acetyltransferase 2, Hs01854954_s1), *SULT1A1* (sulfotransferase 1A1, Hs00419411_m19), *GSTA1* (glutathione-S-transferase alpha 1, Hs00275575_m1), *UGT1A1* (UDP-glucuronosyltransferase 1A1, Hs02511055_s1), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Human Endogenous Controls 4310884E). Each pre-amplified cDNA sample was diluted 1:5. Final PCR reaction volume was 10.0 µl and the thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min, following 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The experiments were repeated three times independently, each with two technical replicates per experimental point.

The amplification plots were analysed with ABI 7900HT Sequence Detection System 2.4.1 software (Applied Biosystems). The threshold values of mRNAs were adjusted to 0.2 for each reaction plate. mRNA was considered as not expressed if the Ct value was undetermined or above 37 in two out of three experiments. Ct of each target gene was normalized to the Ct of a reference gene *GAPDH*. A comparative $\Delta\Delta$Ct algorithm was applied to determine: the Rq of *ALB* in hDHP cells according to hASC cells; the Rq of target genes in B(a)P, AFB_1 or PhIP exposed hDHP cells according to the vehicle control hDHP cells. The criteria for deregulation of expressed genes were Rq ≤ 0.67 and Rq ≥ 1.5. The data were statistically analysed using GraphPad Prism 6 software (San Diego, California) and Student's t test was applied to calculate the significant difference (p values ≤ 0.05 were considered as statistically significant).

3.4.8 Western blot analysis

At the end of differentiation, the basal expression of metabolic enzymes CYP3A4, CYP1A1, NAT2, SULT1A1 and UGT1A1 was evaluated in hDHP cells using Western blot analysis as described in Chapter 3.2.5.

The following primary antibodies were used: rabbit antibody against CYP3A4 (1:500, ab3572), rabbit antibody against CYP1A1 (1:500, ab79819), rabbit antibody against UGT1A1 (1:500; ab194697), rabbit antibody against SULT1A1 (1:500, ab38411), mouse antibody against NAT2 (1:500, ab88443) and rabbit antibody against GAPDH (1:2000, ab9485). The experiments were repeated three times independently.

3.5 Immortalization of the Differentiated Hepatic Progenies of Human Adipose Tissue Stem Cells

3.5.1 Immortalization of hDHP

The immortalization procedure was performed in cooperation with Dr. U. Kamenšek (Institute of Oncology Ljubljana). The hDHP were immortalized by transfection with human telomerase reverse transcriptase (*hTERT*) using pBABE-neo-*hTERT* plasmid, which was kindly provided by Bob Weinberg (Addgene plasmid # 1774; Counter et al., 1998).

Plasmid DNA was isolated and purified using an EndoFree Plasmid Mega Kit according to the instructions provided with the kit, and eluted in endotoxin-free water to the concentration of 1.0 mg/ml. The purity and yields were spectrophotometrically determined (Epoch Microplate Spectrophotometer, Take3™ Micro-Volume Plate, BioTek). Additionally, the concentration and identity were confirmed by restriction analysis on an electrophoretic gel. For the preparation of stable cell line, the isolated plasmid DNA was cut with XbaI restriction enzyme, and expression cassette encoding the *hTERT* and eukaryotic selection marker (neomycin, selectable by geneticin) was isolated from the electrophoretic gel.

Electroporation was used for the introduction of plasmid DNA into hDHP cells. The cells in the exponential growth phase were trypsinized, washed and centrifuged. After centrifugation, the cells were re-suspended in ice-cold electroporation buffer (125.0 mM sucrose, 10.0 mM K_2HPO_4, 2.5 mM KH_2PO_4, and 2.0 mM $MgCl_2$) and prepared at the concentration of 25.0×10^6 cells/ml. 10.0 µl of linearized plasmid DNA was added to the cell suspension per each 1.0×10^6 of cells. 50.0 µl of the suspension (containing 10.0 µg of plasmid DNA and 1.0×10^6 cells) was pipetted between stainless steel parallel plate electrodes. The suspension was electroporated with eight square-wave electric pulses with amplitude per distance ratio 600 V/cm, 5 ms duration time, and 1 Hz repetition frequency, delivered through electrodes connected to a generator of electric pulses (Faculty of Electric Engineering, University of Ljubljana, Ljubljana, Slovenia). The electroporation procedure was repeated three times (3.0×10^6 cells were electroporated in total). After electroporation, the cells were plated into Petri dishes and cultured for 1 month. The increasing concentrations (500.0 µg/ml – 1000.0 µg/ml) of the selection agent geneticin were used to obtain resistant clones.

The starting concentration of the selection agent geneticin was determined in a separate experiment, where un-transfected cells were exposed to increasing concentrations of geneticin (100.0 µg/ml – 1000.0 µg/ml) and the first concentration that killed all the cells, i.e. 500.0 µg/ml, was selected for the preparation of a stable cell line.

3.5.2 Treatment with model genotoxic agents

Immortalized hDHP cells were exposed to non-cytotoxic concentrations of model genotoxic pro-carcinogens for 24 h: B(a)P (30.0 µM), AFB_1 (15.0 µM) and PhIP (200.0 µM). The concentrations of pro-carcinogens were selected based on the results of MTS cytotoxicity assay (for details see Chapter 3.4.5).

The stock solutions of pro-carcinogens were prepared as described in Chapter 3.3.2. The medium containing DMSO (adjusted to 1.0 %) was used as the vehicle control.

3.5.3 Comet assay

To determine DNA damage induced by model genotoxic compounds B(a)P, AFB_1 or PhIP, the comet assay was performed as described in Chapter 3.4.6.

3.5.4 Hepatic differentiation of immortalized hDHP

Immortalized hDHP cells were cultured in either hepatic induction medium followed by hepatic maturation medium or only in the maturation medium as described in Chapter 3.4.1 (Table 3.2).

At the end of differentiation, the cells were exposed to non-cytotoxic concentrations of model genotoxic pro-carcinogens for 24 h: B(a)P (30.0 µM), AFB_1 (15.0 µM) and PhIP (200.0 µM). The concentrations of pro-carcinogens were selected based on the results of MTS cytotoxicity assay (for details see Chapter 3.4.5). The stock solutions of pro-carcinogens were prepared as described in Chapter 3.3.2., and the medium containing DMSO (adjusted to 1.0 %) was used as the vehicle control.

DNA damage induced by model genotoxic compounds B(a)P, AFB_1 or PhIP was determined using the comet assay as described in Chapter 3.4.6.

Chapter 4

Results and Discussion

4.1 Selection of the Most Suitable Candidates Among a Panel of Hepatic Cell Lines for the Application in Genotoxicity Testing

HepG2, Hep3B, HCC1.2 and HepaRG cell lines are currently the most frequently employed experimental models in *in vitro* genotoxicity tests. Despite relatively promising results obtained with these hepatic tumor-derived lines, their suitability for genotoxicity studies is questionable due to much lower sensitivity and specificity compared to normal hepatocytes (Fowler et al., 2014; Gerets et al., 2012; Wilkening et al., 2003). However, there is a number of human liver cells available on the market, which have so far not been applied in genotoxicity studies and some of them may be equally or even more suitable for this purpose. Therefore, a comprehensive analysis of characteristics, relevant for the potential use in genotoxicity testing, has been performed in twelve human liver-derived cell lines. In addition to HepG2, Hep3B, HCC1.2 and HepaRG cells, eight hepatic lines which have been mainly used in cancer studies and occasionally in acute toxicity experiments were included in the analysis. With the exception of WRL68, all the lines were of carcinogenic origin.

4.1.1 Characterization of hepatic cell lines

The general characteristics of liver-derived cell lines, namely morphology, proliferation kinetics and chromosome number, are presented in Table 4.1. According to morphological similarity to primary hepatocytes, the HCC1.2, Hep3B, HepaRG, HepG2, HuH6, HuH7, JHH6 and PLC/PRF5 cell lines exhibited typical epithelial cell morphology, while SK-Hep1, SNU-398, SNU-449 and WRL68 displayed mesenchymal features, but mainly with polygonal appearance (Table 4.1). The morphological characteristics of the cell lines can be found in Supplementary material of our paper Waldherr et al. (2018).

The doubling time of cells can provide information about the cell cycle duration, which should be known when designing the protocols for genotoxicity experiments that require cell division, for example *in vitro* micronucleus test. This information is the guidance to define exposure time and culture duration in order to allow interaction of the test compound with all relevant cellular components for induction of genotoxic effects (Kirsch-Volders et al., 2011), and DNA damage response mechanisms to repair the induced damage. Relatively short doubling times were found in HuH7, WRL68 and SNU

cell lines, with HuH7 having the fastest proliferation kinetics from 23–27 h (Table 4.1). The HCC1.2, Hep3B, HepaRG, HepG2 and HuH6 exhibited slow mitotic activities, wherein HepaRG had the longest doubling time from 48–69 h (Table 4.1).

Table 4.1: Morphology, growth kinetics and karyotype of hepatic cell lines.

Cell line	Morphology	Doubling time/h	Chromosome No
HCC1.2	Epithelial	46-53	111-127
Hep3B	Epithelial	41-53	62-67
HepaRG™	Epithelial	48-69	46-47
HepG2	Epithelial	41-57	49-53
HuH6	Epithelial	45-50	82-86
HuH7	Epithelial	23-27	65-111
JHH6	Epithelial	33-47	53-70
PLC/PRF5	Epithelial	36-43	39-58
SK-Hep1	Mesenchymal	35-54	59-61
SNU-398	Mesenchymal (polygonal)	30-33	59-64
SNU-449	Mesenchymal (polygonal)	28-33	52-55
WRL68	Mesenchymal (polygonal)	25-30	61-254

The variation in chromosome number is a commonly known feature of cancer cells, which also frequently display structural chromosomal aberrations. Each gain or loss of a single chromosome induces changes in the expression levels of multiple genes (Nicholson & Cimini, 2013). Consistent with previous publications (Gripon et al., 2002; Hwang et al., 2003; Wong et al., 2000), none of the analysed cell line possessed a normal karyotype. The majority of lines showed numerically and structurally complex karyotypes (HCC1.2, HepG2, HuH6, HuH7, JHH6, SNU-398, SNU-449 and WRL68 cells), with the exception of Hep3B, HepaRG and SK-Hep1 cells in which relatively uniform karyotype was found. The numbers of chromosomes in HepaRG and HepG2 lines were the closest to that found in normal human diploid somatic cells (Table 4.1) which consist of 23 pairs of chromosomes (Nicholson & Cimini, 2013). However, aneuploidy has been demonstrated as a frequent occurrence of human hepatocytes as well (Duncan et al., 2012). A broad range in chromosomal numbers was revealed in HCC1.2, HuH7, JHH6, PLC/PRF5 and WRL68 cell lines (Table 4.1), indicating higher chromosomal instability, while a more narrow range was found in Hep3B, HepaRG, HepG2, HuH6, Sk-Hep1 cells and both SNU

cell lines (Table 4.1). The chromosomal instability over long-term culturing has been assumed as potential factor contributing to misleading results in genotoxicity assays with some of the frequently used rodent cells before (Kirkland et al., 2007).

The p53 is a transcription factor of which classic characteristic is tumor suppression by regulation of gene networks implicated in the induction of cell cycle arrest, allowing DNA repair or apoptosis after DNA damage (Ozaki & Nakagawara, 2011). Cells with impaired p53 function may be more susceptible to give misleading positive results *in vitro* that are not confirmed *in vivo* in some cases. Fowler et al. (2012) compared the predictivity of p53-deficient rodent cell lines with p53-competent human cells using the *in vitro* micronucleus (MN) test after the exposure of cells to the compounds that were shown to produce misleading positive results in *in vitro* mammalian cell assays. Much higher susceptibility to cytotoxicity and MN induction was found in rodent p53 deficient cells than in p53-competent cells. *p53* gene is among the most commonly mutated genes identified in human cancer cells (Ozaki & Nakagawara, 2011). Therefore, the p53 analysis was included in this study as one of the important selection factors considering the convenience of the cell lines for *in vitro* genotoxicity studies. The qPCR and Western blot analyses revealed the absence of p53 expression at transcriptional and protein level in Hep3B line (Figure 4.1; Figure 4.2). Mutated p53 protein was determined in HCC1.2 and SNU-398 cells by the Western blot method (Figure 4.2). After the 24 h exposure to model genotoxic pro-carcinogen B(a)P, the induced *p53* mRNA expression was found in HCC1.2 and HepG2 cell lines (Figure 4.1), and induced p53 protein was revealed in HepG2 and HuH6 cells (Figure 4.2).

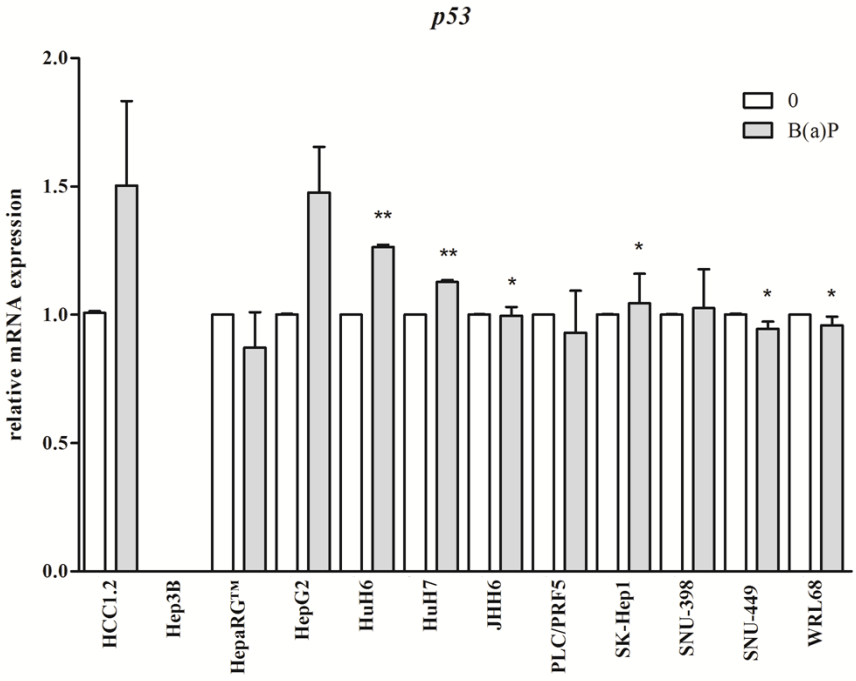

Figure 4.1: The basal expression and inducibility of *p53* gene in cell lines, determined with the qPCR method. The cell lines were exposed to genotoxic pro-carcinogen B(a)P (30.0 µM) for 24 h. The results are presented as relative mRNA expression in treated cells normalized to the mRNA expression in vehicle control cells. Data are means ± SD of three independent experiments. Significant difference (Student's t test) between treated cells and the vehicle control cells (0) is indicated by (*) ($p < 0.05$) and (**) ($p < 0.01$). More than 1.5-fold change indicates up-regulation of gene.

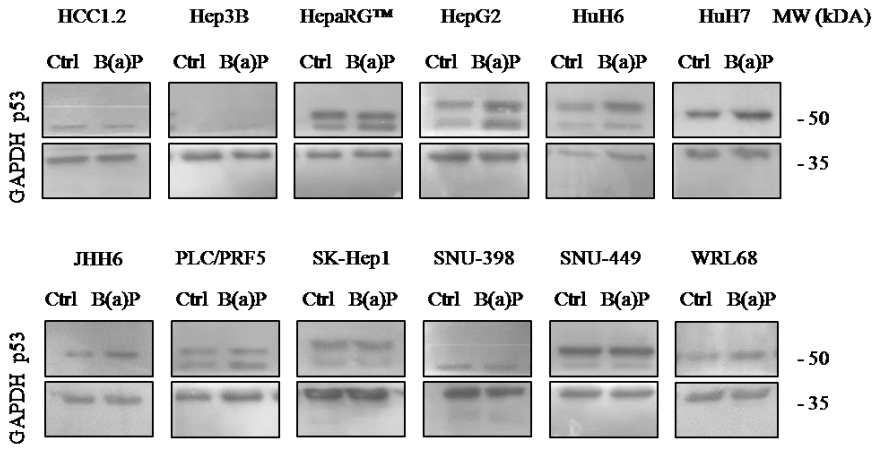

Figure 4.2: The basal expression (Ctrl) and inducibility (B(a)P) of p53 protein, quantified by Western blot analysis in vehicle control cells and cells exposed to genotoxic pro-carcinogen B(a)P (30.0 µM) for 24 h. In HCC1.2 and SNU-398 mutated p53 was detected.

4.1.2 Evaluation of sensitivity of hepatic cell lines towards model pro-carcinogens

The sensitivity to detect the true genotoxins is an additional factor that requires consideration when choosing the cell-based model for *in vitro* genotoxicity assays. In this study, the sensitivity of hepatic cell lines was evaluated using a comet assay after the exposure to representatives of different classes of genotoxic carcinogens that require metabolic activation: polycyclic aromatic hydrocarbon B(a)P, mycotoxin AFB_1, heterocyclic aromatic amines PhIP and IQ, and nitrosamine NDMA.

The representatives B(a)P, AFB_1, PhIP, IQ and NDMA were selected because their metabolic pathways are well established and characterised, especially their bioactivation to genotoxic metabolites. The polycyclic aromatic hydrocarbon B(a)P is mainly activated by CYP1A1 in combination with microsomal EH to B(a)P-diols which are further oxidised by CYP1A1 or 1B1 to highly DNA reactive metabolites. A minor role in the activation of B(a)P-diols is also played by CYP1A2 and 3A4, and AKR enzymes. B(a)P can also be converted by peroxidases to radical cations which can react with DNA to form unstable adducts. The DNA reactive metabolites formed are usually detoxified by UGT, GST, SULT, and NQO1 enzymes (Shimada, 2006; Stiborová et al., 2014). A number of human CYPs are involved in biotransformation of the mycotoxin AFB_1. Predominantly it requires CYP3A4-mediated bioactivation to genotoxic AFB_1 exo-epoxides, and a less important role in its activation has been ascribed to CYP1A2. In addition, CYP1A2 and 3A4 also oxidize AFB_1 to deactivated products that are generally poor substrates for epoxidation or, after epoxidation, do not interact with DNA. Glutathione transferases, α and especially µ class, catalyze exo-epoxide conjugation with glutathione in humans (Ayed-Boussema, Pascussi, Maurel, Bacha, & Hassen, 2012; Guengerich et al., 1998; Nwankwo, Tahnteng, & Emerole, 2000; Oda et al., 2001). The representatives of heterocyclic aromatic amines, PhIP and IQ, are metabolically activated predominantly by CYP1A2 and in part by CYP1A1 and 1B1 to already DNA reactive N-hydroxy-amino metabolites that are further metabolized by sulfotransferases and by N-

acetyltransferases (primarily NAT2), to produce highly DNA reactive nitrenium ion. For bioactivation of PhIP N-hydroxy metabolites, SULT enzymes appear to be more important. PhIP and its N-hydroxy metabolites can be conjugated specifically by UGT1A enzymes or GSTs to non-reactive species, while IQ metabolites are primarily detoxified by SULTs (Crofts, Sutter, & Strickland, 1998; Rendic & Guengerich, 2012; Turesky, 2010). The nitrosamine N-Nitrosodimethylamine (NDMA) is bioactivated through hydroxylation of methyl group primarily catalysed by CYP2E1 (Glatt, Schneider, & Liu, 2005; Guengerich & Shimada, 1991). There is not much information about its detoxification. However, the majority of nitrosamines is detoxified by UGT catalysed glucuronidation (Rendic & Guengerich, 2012).

Prior to the genotoxicity testing, the cytotoxicity of B(a)P, AFB_1, PhIP, IQ and NDMA was determined at their highest tested concentrations that were selected based on the available literature data. None of the model compounds reduced the viability of cells by more than 30 %, which is in accordance with the suggested criteria for cytotoxicity limit in comet assay experiments (Tice et al., 2000).

HCC1.2, HepG2 and Huh6 lines detected all five model pro-carcinogens, but with a different sensitivity. In HepG2 and HuH6 lines an increase in DNA damage was observed after the exposure to all tested concentrations of B(a)P, while the sensitivity of HCC1.2 to B(a)P exposure was much lower. HepG2 and HuH6 cells responded to AFB_1 in a dose-dependent manner at the two highest concentrations tested. However, the AFB_1 concentration range differed between experiments with HCC1.2 and HepG2 cells, and HuH6 cell line. HepG2 cells showed higher sensitivity to detect AFB_1 induced DNA damage than HCC1.2 or HuH6 cells. The response to PhIP and IQ exposure was similar in all three cell lines. In HuH6 cell line NDMA induced DNA damage in a dose-dependent manner at the two highest concentrations, while HCC1.2 cell line responded to all tested NDMA concentrations, indicating higher sensitivity to NDMA exposure compared to HepG2 and HuH6 cells (Table 4.2).

HepaRG cells responded to four of the tested pro-carcinogens, B(a)P, AFB_1, PhIP and NDMA. AFB_1 induced DNA damage at all tested concentrations, and response of cells after the exposure to the highest two B(a)P concentrations was dose dependent. The sensitivity of this cell line to the exposure to B(a)P, AFB_1 and NDMA was comparable to that of HepG2 cell line (Table 4.2).

Hep3B and HuH7 cell lines detected two of the tested pro-carcinogens. In Hep3B cells DNA damage was observed after exposure to 10.0 µM B(a)P but not to higher B(a)P concentrations, and the AFB_1 induced DNA damage was dose dependent at the highest two concentrations. HuH7 cell line responded to the exposure to the highest two B(a)P concentrations in a dose-dependent manner, and to the highest NDMA concentration.

JHH6 and PLC/PRF5 cells were responsive only to AFB_1 exposure, but with a higher sensitivity than HCC1.2 and HuH6 cell lines.

SK-Hep1, SNU-398, SNU-449 and WRL68 lines did not respond to any of the tested model compounds (Table 4.2).

Table 4.2: Induced DNA damage in human hepatic cell lines after the exposure to model genotoxic pro-carcinogens.

Cell line	B(a)P				AFB_1				PhIP				IQ				NDMA			
	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /mM	% tail DNA	±	SD
HCC1.2	0.0	0.68	±	0.50	0.0	1.38	±	0.48	0.0	6.61	±	2.14	0.0	2.93	±	1.40	0.0	1.77	±	1.64
	2.0	2.28	±	1.77	1.0	3.74	±	2.69	50.0	**17.50**	±	**4.84**	25.0	1.39	±	0.94	50.0	**16.30**	±	**4.52**
	10.0	3.07	±	0.97	5.0	4.02	±	1.22	100.0	**15.10**	±	**2.32**	50.0	1.86	±	0.44	100.0	**17.10**	±	**3.64**
	50.0	**5.69**	±	**2.10**	20.0	**8.75**	±	**1.48**	300.0	**14.80**	±	**2.63**	100.0	**7.29**	±	**1.87**	200.0	**8.87**	±	**0.30**
Hep3B	0.0	0.79	±	0.66	0.0	0.93	±	0.86	0.0	1.56	±	0.18	0.0	0.92	±	0.58	0.0	0.57	±	0.21
	10.0	**3.97**	±	**1.51**	1.0	1.35	±	0.81	25.0	1.18	±	0.39	50.0	0.88	±	0.71	50.0	0.62	±	0.45
	30.0	2.47	±	1.77	5.0	**4.03**	±	**1.93**	50.0	1.43	±	0.24	100.0	1.75	±	1.45	100.0	0.92	±	0.45
	90.0	2.80	±	1.30	20.0	**4.31**	±	**0.92**	100.0	0.82	±	0.53	250.0	2.09	±	1.38	200.0	0.61	±	0.47
HepaRG™	0.0	1.43	±	1.54	0.0	4.16	±	1.40	0.0	0.53	±	0.66	0.0	2.34	±	2.92	0.0	0.96	±	0.59
	2.0	0.79	±	0.36	5.0	**11.60**	±	**1.50**	25.0	2.56	±	1.95	25.0	3.34	±	3.31	50.0	1.47	±	0.10
	10.0	**5.72**	±	**0.96**	10.0	**9.67**	±	**2.17**	50.0	**4.82**	±	**3.57**	50.0	2.39	±	4.10	100.0	1.41	±	1.04
	50.0	**6.71**	±	**2.24**	20.0	**7.50**	±	**1.76**	100.0	2.86	±	1.73	100.0	2.38	±	1.97	200.0	**5.09**	±	**0.52**
HepG2	0.0	0.83	±	0.62	0.0	1.02	±	0.28	0.0	2.90	±	1.03	0.0	2.16	±	0.54	0.0	0.35	±	0.21
	10.0	**14.30**	±	**3.03**	1.0	0.85	±	0.41	25.0	1.33	±	0.83	50.0	1.32	±	0.91	50.0	0.83	±	0.26
	30.0	**15.40**	±	**2.53**	5.0	**2.96**	±	**0.66**	50.0	**6.92**	±	**2.00**	100.0	**5.38**	±	**2.02**	100.0	1.12	±	0.72
	90.0	**13.50**	±	**2.79**	20.0	**5.17**	±	**1.57**	100.0	**6.78**	±	**1.37**	250.0	**5.39**	±	**1.94**	200.0	**1.55**	±	**0.49**
HuH6	0.0	0.91	±	0.49	0.0	1.50	±	1.09	0.0	1.81	±	1.15	0.0	4.01	±	1.68	0.0	1.73	±	**2.62**
	10.0	**3.54**	±	**0.88**	10.0	3.35	±	2.75	50.0	**11.51**	±	**3.64**	25.0	5.49	±	2.24	50.0	2.91	±	1.45
	30.0	**7.44**	±	**1.58**	20.0	**7.20**	±	**3.1**	100.0	**8.67**	±	**2.67**	50.0	5.91	±	3.05	100.0	**6.86**	±	**1.74**
	90.0	**5.16**	±	**1.91**	30.0	**11.90**	±	**4.30**	300.0	**24.70**	±	**2.15**	100.0	**10.82**	±	**5.71**	200.0	**13.10**	±	**4.41**

Table 4.2 (Continued)

Cell line	B(a)P				AFB_1				PhIP				IQ				NDMA			
	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /mM	% tail DNA	±	SD
HuH7	0.0	1.08	±	0.47	0.0	6.29	±	1.57	0.0	5.12	±	1.05	0.0	1.29	±	1.30	0.0	0.61	±	0.35
	10.0	2.00	±	1.14	1.0	2.76	±	0.52	50.0	5.35	±	2.84	50.0	2.16	±	0.91	50.0	0.69	±	0.48
	30.0	**4.79**	±	**1.25**	2.5	3.12	±	0.70	100.0	9.21	±	2.26	100.0	1.11	±	0.60	100.0	0.77	±	0.60
	90.0	**5.36**	±	**1.03**	5.0	5.81	±	4.64	300.0	3.55	±	2.08	250.0	1.88	±	1.68	200.0	**3.01**	±	**2.11**
JHH6	0.0	1.67	±	0.99	0.0	1.67	±	0.99	0.0	0.59	±	0.21	0.0	0.59	±	0.21	0.0	1.17	±	1.11
	10.0	2.33	±	1.18	1.0	1.70	±	0.75	25.0	0.25	±	0.15	25.0	0.76	±	0.56	50.0	1.33	±	0.66
	50.0	3.78	±	3.42	2.5	ND	±		50.0	0.93	±	1.09	50.0	1.29	±	1.55	100.0	1.69	±	0.81
	90.0	3.37	±	1.96	5.0	**4.52**	±	**1.43**	100.0	1.44	±	0.53	100.0	0.93	±	0.67	200.0	1.16	±	0.53
PLC/PRF5	0.0	1.09	±	0.81	0.0	1.84	±	1.44	0.0	3.82	±	1.22	0.0	2.47	±	2.08	0.0	1.15	±	1.25
	10.0	1.39	±	0.55	1.0	4.57	±	1.83	50.0	2.51	±	0.56	50.0	5.17	±	4.50	50.0	1.04	±	0.33
	30.0	1.31	±	1.72	2.5	4.77	±	1.75	100.0	4.00	±	3.72	100.0	2.84	±	2.65	100.0	1.65	±	1.55
	90.0	1.54	±	0.87	5.0	**6.68**	±	**2.94**	300.0	2.43	±	2.03	250.0	4.61	±	1.54	200.0	1.09	±	0.33
SK-Hep1	0.0	0.46	±	0.29	0.0	0.60	±	0.41	0.0	2.84	±	2.06	0.0	0.59	±	0.24	0.0	0.46	±	0.16
	10.0	0.87	±	0.86	1.0	1.30	±	0.37	50.0	5.18	±	1.19	50.0	0.62	±	0.47	50.0	0.70	±	0.42
	30.0	2.20	±	2.36	5.0	1.13	±	0.72	100.0	5.83	±	1.06	100.0	1.43	±	0.95	100.0	0.59	±	0.24
	90.0	1.17	±	0.80	20.0	1.24	±	0.88	300.0	5.77	±	4.30	250.0	0.68	±	0.70	200.0	0.37	±	0.34
SNU-398	0.0	0.64	±	0.27	0.0	2.50	±	1.08	0.0	5.60	±	3.26	0.0	1.02	±	0.88	0.0	0.40	±	0.14
	10.0	0.73	±	0.45	1.0	2.53	±	1.68	50.0	2.92	±	1.18	50.0	1.42	±	0.72	50.0	0.65	±	0.50
	30.0	0.56	±	0.45	5.0	3.06	±	0.28	100.0	7.04	±	5.97	100.0	1.20	±	0.81	100.0	0.83	±	0.53
	90.0	2.28	±	2.73	10.0	2.83	±	0.61	300.0	10.30	±	1.06	250.0	1.63	±	1.25	200.0	0.28	±	0.11

Table 4.2 (Continued)

Cell line	B(a)P				AFB_1				PhIP				IQ				NDMA			
	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /µM	% tail DNA	±	SD	Conc /mM	% tail DNA	±	SD
SNU-449	0.0	0.79	±	0.41	0.0	2.87	±	2.05	0.0	2.12	±	0.59	0.0	1.20	±	1.18	0.0	0.23	±	0.16
	10.0	1.26	±	0.62	1.0	2.04	±	2.18	50.0	2.02	±	0.81	50.0	3.67	±	2.95	50.0	0.52	±	0.24
	30.0	0.79	±	0.21	2.5	1.14	±	1.95	100.0	3.06	±	1.08	100.0	1.46	±	1.21	100.0	0.54	±	0.11
	90.0	0.74	±	0.64	5.0	3.37	±	3.14	300.0	1.17	±	0.83	250.0	0.18	±	0.09	200.0	0.15	±	0.03
WRL68	0.0	2.66	±	1.17	0.0	3.14	±	1.66	0.0	2.89	±	1.16	0.0	0.76	±	0.77	0.0	0.43	±	0.22
	10.0	3.10	±	3.43	1.0	2.32	±	0.89	50.0	5.17	±	3.21	50.0	1.15	±	1.44	50.0	1.03	±	0.74
	30.0	4.30	±	0.34	2.5	1.98	±	0.41	100.0	3.77	±	1.36	100.0	0.56	±	0.67	100.0	0.93	±	0.05
	90.0	6.10	±	3.26	5.0	1.72	±	0.78	300.0	3.39	±	3.35	250.0	1.32	±	1.64	200.0	0.12	±	0.01

The cells were exposed to graded concentrations of B(a)P, AFB_1, and NDMA for 24 h, and to PhIP and IQ for 48 h. DNA damage was assessed using the comet assay and is expressed as % DNA in the comet tail. 50 nuclei were analysed per experimental point in each of the two independent experiments. Results are presented as average % DNA in the comet tail ± SD. Bold font denotes significant difference ($p < 0.05$; One-way ANOVA, Dunett's test) between treated cells and the vehicle control cels. ND – not determined.

To summarize, out of 12 hepatic cell lines included in this analysis, only HCC1.2, HepG2 and HuH6 cell lines were sensitive to detect DNA damage induced by all five tested model pro-carcinogens. This information greatly narrowed the panel of cell lines, convenient for *in vitro* genotoxicity studies. To make the selection from these three hepatic cancer lines, HCC1.2 showed better sensitivity from HepG2 and HuH6 only to NDMA exposure. Besides, numerically and structurally complex and unstable karyotype, and mutated p53 protein are not characteristics of cell-based model that meets the criteria for better *in vitro* predictivity (Table 4.3) (Kirkland et al., 2007). HepG2 and HuH6 cells similarly responded to the tested compounds (Table 4.3). They both have a comparable doubling time, express functional wild type p53, and their chromosome number is in a narrow range (Table 4.3). However, the number of chromosomes in the HepG2 cell line is closer to that found in normal human cells. In the HuH6 line, the number of chromosomes is twice as high as in normal cells.

Table 4.3: Summary of general characteristics and sensitivity evaluation of the most suitable candidates among a panel of hepatic cell lines.

Cell line	Morphology	Doubling time/h	Chromosome No	p53 expression		Sensitivity
				Gene	Protein	
HCC1.2	Epithelial	46-53	111-127	+	+*	+
HepG2	Epithelial	41-57	49-53	+	+	++
HuH6	Epithelial	45-50	82-86	+	+	++

(*) mutated form.

The ability of HepG2 cell line to detect different classes of genotoxic pro-carcinogens without the use of exogenous metabolic system has been confirmed many times before (Knasmüller et al., 1998; Uhl, Helma, & Knasmuller, 1999; Winter et al., 2008). However, the main problem when using this cell line is poor reproducibility between experiments (Fowler et al., 2012; Knasmüller et al., 2004). Especially with heterocyclic aromatic amines positive (Knasmüller, Schwab, Land, Wang, Sanyal, Kundi, Parzefall, & Darroudi, 1999; Pezdirc, Žegura, & Filipič, 2013; Viegas et al., 2012) and negative results (Khoury et al., 2016; Wilkening et al., 2003; Winter et al., 2008) have been reported.

HuH6 cell line has been mainly employed in studies of hepatocellular functions in cancer development and therapy (Canal et al., 2015; Goldstein et al., 2013; Kawai, Kaneko, Honda, Shirota, & Kobayashi, 2001; Soini et al., 2017), but never in genotoxicity investigations. The findings of this study suggest a good potential of HuH6 cancer line in this respect, since it was able to detect all tested representatives of different classes of genotoxic carcinogens with similar sensitivity than the commonly used cell line HepG2, and thus confirm the first research hypothesis of the dissertation.

At this point, it is difficult to draw the conclusions about which cell line is more applicable for *in vitro* genotoxicity studies. The observations in the responses of HepG2 and HuH6 cells towards the exposure of the model compounds indirectly suggest the expression of xenobiotic metabolizing enzymes involved in bioactivation of the used pro-carcinogens in both cell lines. Therefore, both lines were selected as the most promising

candidates and subjected to further comparative analysis concerning their usefulness for routine *in vitro* testing of chemicals.

4.2 Comparative Gene Expression Analysis of Liver-Specific Transcription Factors and Genes Involved in the Metabolism of Xenobiotics in the Selected Hepatic Tumor-Derived Cell Lines and Primary Human Hepatocytes

One of the major factors substantially contributing to the high rate of misleading results is inadequate representation of xenobiotic metabolizing enzymes in cell-based experimental models used for routine *in vitro* testing. An appropriate model should reflect a complete hepatic xenobiotic metabolizing profile, including the ability to respond to xenobiotic exposure by induction of biotransformation enzymes. The findings of the screening of 12 hepatic cell lines suggested that HepG2 and HuH6 lines are potentially the best for the use in genotoxicity testing (see Chapter 4.1). However, the availability of data about xenobiotic metabolic gene expression profiles of the selected cell lines is relatively limited. Many reports exist that are focused on the comparison of HepG2 cell line to primary hepatocytes, but are mainly addressed to their whole genome expression profiles (Harris et al., 2004; Hart et al., 2010; Jennen, Magkoufopoulou, et al., 2010; Jetten, Kleinjans, Claessen, Chesné, & van Delft, 2013; Olsavsky et al., 2007) or transcriptomic signatures (Doktorova et al., 2013; Tyakht et al., 2014). Studies investigating the metabolic capacity of HepG2 relative to primary hepatocytes cover the mRNA expression of several phase I or phase II xenobiotic metabolizing enzymes, transporters or xenobiotic receptors (Martin, Riley, Back, & Owen, 2008; Rodrigues-Antona et al., 2002; Westerink & Schoonen, 2007a, 2007b; Wilkening et al., 2003), while the detailed profiling of multiple genes involved in xenobiotic biotransformation has been reported only few times (Gerets et al., 2012; Guo et al., 2011). The metabolic gene profile of HuH6 cell line remains rather unexplored, with few studies demonstrating mRNA expression of several biotransformation genes and one transcription factor in these cells (Sugatani et al., 2010; Goldstein et al., 2013).

To get insight into the metabolic profiles of the two hepatic tumor-derived cell lines, a comprehensive gene expression analysis of carefully selected liver-specific transcription factors and genes involved in the metabolism of xenobiotics was performed in this study. In addition to the basal expression of selected genes, their transcriptional deregulation was determined after the 24 h exposure of cells to non-cytotoxic concentrations of model pro-carcinogens B(a)P, AFB_1 and PhIP. To investigate how closely the two hepatic cancer cell lines reflect *in vivo* hepatic xenobiotic metabolism, the metabolic profiles of HepG2 and HuH6 cells were compared to that of primary human hepatocytes from two different donors (pHepI and pHepII), which were considered as fully functional hepatocytes.

4.2.1 Comparison of metabolic profiles of HepG2 cells, HuH6 cells and primary hepatocytes: basal expression and deregulation after exposure to model pro-carcinogens

The expression analysis of transcription factors and xenobiotic metabolising genes in HepG2 cells, HuH6 cells and primary hepatocytes, was performed with the high-throughput quantitative PCR technique. Each custom-made qPCR array contained 32 probes for phase I metabolic enzymes, 21 probes for phase II enzymes, 11 probes for transporters and 26 probes for transcription factors and cofactors. To visualize the differences and similarities in gene expression profiles between hepatic cancer cell lines and primary hepatocytes, hierarchical clustering and heatmap analyses were performed.

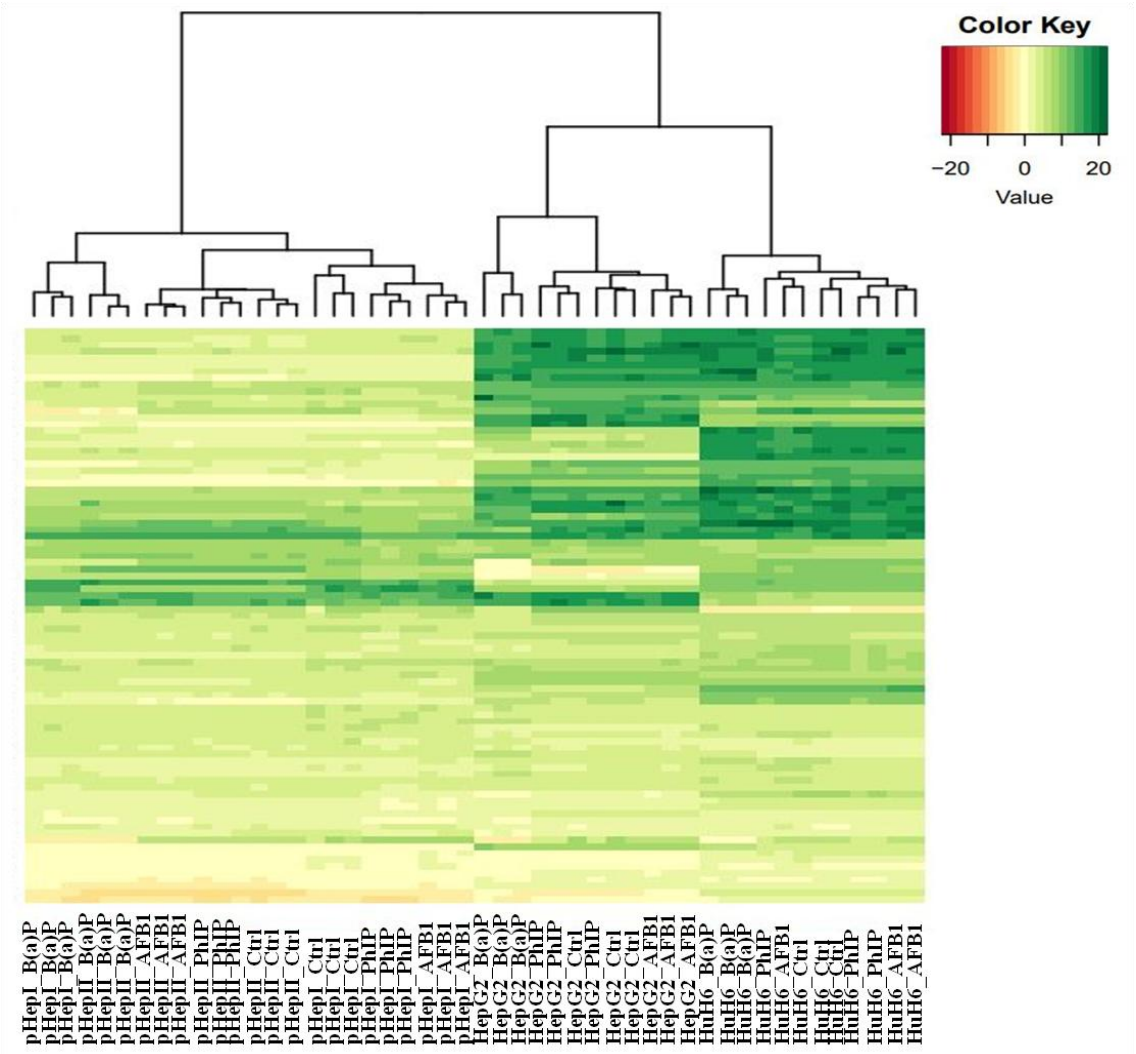

Figure 4.3: Hierarchical clustering and heatmap analyses of basal gene expression profiles and gene expression profiles after 24 h exposure to B(a)P (10.0 µM), AFB_1 (3.2 µM) and PhIP (20.0 µM) in primary hepatocytes of two donors (pHepI and pHepII) and HepG2 and HuH6 cell lines. The results are presented as ΔCt values of target genes normalized to the average Ct of four reference genes (*ACTB, GAPDH, HPRT1* and *PGK1*). 90 genes were included in the analysis and the experiments were performed three times independently for each cell sample.

Clear differences in metabolic expression profiles between primary hepatocytes and hepatic cancer cell lines can be observed in Figure 4.3, where one distinct cluster represents the primary hepatocytes and the other cluster represents cell lines derived from hepatic tumors. Down the hierarchy of primary cells cluster, two larger clusters demonstrate that the transcriptional response of primary hepatocytes from both donors after B(a)P exposure was similar and differed from basal expression profiles as well as from expression profiles after AFB_1 and PhIP exposure. In the cluster of hepatic cancer lines, HepG2 and HuH6 lines form two distinct clusters demonstrating obvious difference in their gene expression profiles. Despite the variation between primary hepatocytes of the two donors, the expressions of metabolic genes and transcription factors differ much more between the two cancer cell lines than between primary hepatocytes (Figure 4.3).

4.2.2 Basal expression of liver-specific genes involved in the metabolism of xenobiotics

From the 53 analysed genes encoding phase I or phase II metabolic enzymes, 41 genes were expressed in HepG2 cells, 36 in HuH6 cell line, 50 in pHepI and 51 in pHepII. All of the 11 analysed genes encoding transporter proteins were expressed in primary hepatocytes from both donors. In HepG2 and HuH6 cells, 4 genes were not expressed among which two were common to both cell lines. In primary human hepatocytes and HepG2 cells all of the 26 analysed genes encoding transcription factors and cofactors were expressed, while in Huh6 cells one was not expressed. The non-expressed genes in primary hepatocytes and hepatic cancer cell lines are summarized in Table 4.4.

As expected, considerably lower mRNA levels of the majority of the expressed genes were observed in HepG2 and HuH6 lines compared to those in primary hepatocytes. The differences in expression levels of the analysed genes were evident also between primary hepatocytes. Mainly the basal gene expression levels were lower in pHepI compared to the expression levels of pHepII, which can also be seen in Figure 4.3 (pHepI_ctrl; pHepII_ctrl) and in Supplementary Material Table A.3.

It has been reported that hepatic cancer cell lines frequently express non-hepatic enzymes (Castell et al., 2006). *CYP2W1*, which has been shown to be tumor-specific (Karlgren et al., 2006), was the only gene expressed in both hepatic tumor-derived cell lines and not expressed in primary hepatocytes in this study (Table 4.4). *GPX5* that belongs to glutathione peroxidase family was expressed neither in primary hepatocytes nor in hepatic cancer cell lines. This is not unexpected, as this enzyme is specifically expressed in the epididymis in the male reproductive tract where its function is connected to the protection of spermatozoa membranes from damaging effects of lipid peroxidation (Brigelius-Flohé & Maiorino, 2013). *SULT1C2* was expressed in pHepII and in both cancer lines, but not in pHepI (Table 4.4). SULT1C subfamily is found predominantly in developing human fetus as the major detoxification enzyme group (Jancova et al., 2010).

ADHC1 encoding for alcohol dehydrogenase that plays a major role in alcohol catabolism was not expressed in hepatic cancer cell lines. The basal expressions of *CYP2C* family members (*2C8*, *2C9*, *2C18* and *2C19*), which predominate in detoxification of xenobiotics (Rendic & Guengerich, 2012), were mainly not presented in hepatic cancer cell lines (Table 4.4). However, the variability of xenobiotic-metabolizing CYPs in humans is more the rule than the exception, and the most important CYP polymorphisms are found within the CYP2 family (Pelkonen et al., 2008). Importantly, the gene encoding the most abundant CYP in human liver, CYP3A4 that metabolizes the greatest number of xenobiotics from all members of this enzyme system (Pelkonen et

al., 2008), was expressed at Ct value above 37 in HuH6 cell line and at very low levels in HepG2 cells. *FMO3*, a major hepatic member of FMOs (Parkinson & Ogilvie, 2008), was not detected in any of the hepatic tumor-derived lines (Table 4.4).

Table 4.4: The non-expressed genes in vehicle-treated primary hepatocytes (pHep) and hepatic cancer cell lines HepG2 and HuH6.

Gene symbol	pHep I	pHep II	HepG2	Huh6
	Rq	Rq	Rq	Rq
Phase I metabolism				
ADH1C	1.00	1.00	N.E.	N.E.
CES5A	1.00	1.00	N.E.	N.E.
CYP2C18	1.00	1.00	N.E.	N.E.
CYP2C19	1.00	1.00	N.E.	N.E.
CYP2C8	1.00	1.00	1.00	N.E.
CYP2C9	1.00	1.00	N.E.	N.E.
CYP2W1	N.E.	N.E.	1.00	1.00
CYP3A4	1.00	1.00	1.00	N.E.
FMO1	1.00	1.00	N.E.	1.00
FMO3	1.00	1.00	N.E.	N.E.
GPX5	N.E.	N.E.	N.E.	N.E.
SRD5A2	1.00	1.00	N.E.	N.E.
Phase II metabolism				
CHST1	1.00	1.00	N.E.	N.E.
GSTT1	1.00	1.00	1.00	N.E.
NAT2	1.00	1.00	1.00	N.E.
NNMT	1.00	1.00	N.E.	1.00
SULT1A1	1.00	1.00	N.E.	N.E.
SULT1B1	1.00	1.00	1.00	N.E.
SULT1C2	N.E.	1.00	1.00	1.00
UGT2B4	1.00	1.00	1.00	N.E.
UGT2B7	1.00	1.00	1.00	N.E.
Transporter proteins				
AQP9	1.00	1.00	N.E.	N.E.
SLC22A3	1.00	1.00	1.00	N.E.
SLC22A7	1.00	1.00	1.00	N.E.
SLCO1A2	1.00	1.00	N.E.	1.00
SLCO1B1	1.00	1.00	N.E.	1.00
SLCO1B3	1.00	1.00	N.E.	N.E.
Transcription factors/cofactors				
PPARAGC1A	1.00	1.00	1.00	N.E.

Genes with two or more undetermined Ct values or Ct above 37 within the three replicates were considered as not expressed. The Ct of each expressed target gene was

normalized to the average Ct of four reference genes (*ACTB, GAPDH, HPRT1* and *PGK1*), and the relative expression (Rq) of a target gene in solvent control cells was determined by using the comparative ΔΔCt algorithm. N.E. – not expressed.

Important observation is also that both hepatic cancer cell lines lacked *SULT1A1* expression. SULT1A1 is the main form of all SULT1 enzymes in adult human liver, involved in detoxification and activation of a number of pro-carcinogens (Jancova et al., 2010; Rendic & Guengerich, 2012). Genetic polymorphisms are well known for SULT enzymes, especially for SULT1A1 and 1C2 (Jancova et al., 2010). Also non-expressed genes *NAT2*, *SULT1B1* and *UGT2B7* in HuH6 cell line have to be emphasised (Table 4.4). SULT1B1 is an important player in the activation of several PAHs and its metabolites, and UGT2B7 is involved in the detoxification of many foreign compounds, including carcinogenic nitrosamines, PAH and HAA metabolites. NAT2 is an especially important double-edged sword, participating in detoxification and activation of several carcinogenic compounds, such as arylamines, HAA, nitroarenes and their metabolites (Rendic & Guengerich, 2012).

The majority of genes encoding uptake transporters, which have a significant role in the disposition of xenobiotic compounds, were not expressed in HepG2 and HuH6 cells (Table 4.4), while the basal expression of genes encoding efflux transporters from ABC family was well presented in both hepatic tumor-derived lines (Supplementary Material Table A.3).

The qPCR analysis revealed a well-presented basal expression of selected transcription factors in primary hepatocytes and HepG2 cell line (all 26 analysed genes; Supplementary Material Table A.3). In HuH6 line, *PPARAGC1A* encoding PGC1α transcription cofactor was not expressed (Table 4.4). PGC1α is one of the most important HNF4α coactivators necessary for the constitutive basal expression of xenobiotic metabolizing genes (Lu, 2016), and for proper HNF4α-mediated regulation of multiple hepatic genes involved in the metabolism and homeostasis. However, low expression of this cofactor is a common event in human hepatic cancer lines (Martínez-Jiménez, Gómez-Lechón, et al., 2006), and it could be speculated that genes encoding several xenobiotic-metabolizing enzymes and transporters could not be detected in HuH6 cell line due to the missing expression of this transcription cofactor (Table 4.4) (Kamiyama et al., 2007). Like already demonstrated by other studies, CAR, PXR, FXR, PPARα and RXRα mRNAs have been expressed at much lower levels in both liver cancer cell lines compared to primary human hepatocytes in this study, and the mRNA expressions of HNF4α and NFE2L2 were higher from that in cultured hepatocytes (Supplementary Material Table A.3) (Jover et al., 2001; Moya et al., 2010; Olsavsky et al., 2007). On the contrary to the study of Jover and co-workers (2001), HNF1α, HNF3α and C/EBPα gene expressions were observed at much higher levels in HepG2 and HuH6 cells, compared to primary hepatocytes (Supplementary Material Table A.3).

4.2.3 Transcriptional deregulation of liver-specific genes involved in the metabolism of xenobiotics after exposure to model pro-carcinogens

When comparing the gene expression profiles of primary hepatocytes from both donors after exposure to model pro-carcinogens, comparable transcriptional responses were observed after B(a)P and AFB_1 exposure, while the expression of 21 genes differed significantly after 24 h exposure to PhIP (Figure 4.4 A). This made the comparison of

metabolic profiles of hepatic cancer lines to profiles of primary hepatocytes after PhIP exposure more complex. The comparison of metabolic profiles of hepatic cancer cell lines after exposure to the three pro-carcinogens revealed the highest variability in response to B(a)P with 31 genes that significantly differed in their expression levels among HepG2 and HuH6 lines (Figure 4.4 B).

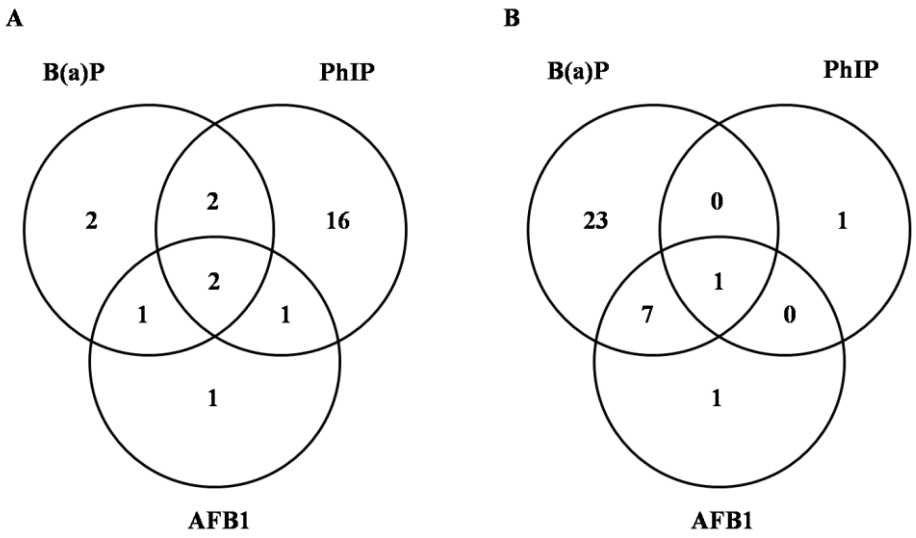

Figure 4.4: The number of genes that differed in their expression between primary hepatocytes from both donors (A), and between Huh6 and HepG2 cell line (B) after 24 h exposure to B(a)P (10.0 µM), AFB_1 (3.2 µM) and PhIP (20.0 µM). Genes with Ct value under 37 were included in the analysis.

4.2.3.1 Differences in expression of liver-specific genes involved in the metabolism of xenobiotics in hepatic tumor-derived cell lines and primary hepatocytes after the exposure to B(a)P

Expression of metabolic genes after 24 h exposure to 10.0 µM B(a)P was far more deregulated in HepG2 cell line than it was in HuH6 line or primary hepatocytes. 31 genes with significantly different expression levels compared to pHep of both donors were found in HepG2 cells. Of these, 10 genes are encoding phase I metabolic enzymes, 9 genes phase II metabolic enzymes, 4 genes transporter proteins and 8 genes transcription factors and cofactors. In HuH6 cells the expression of 6 phase I, 4 phase II, 2 transporter and 4 transcription factor genes statistically differed from that in primary hepatocytes of both donors (Figure 4.5; Supplementary Material Table A.4).

Among genes encoding enzymes involved in bioactivation and detoxification of B(a)P, *CYP1A1*, *EPHX1*, *NQOs*, *GSTs* and *UGTs*, were mainly induced to much higher levels in HepG2 cells or were in HepG2 cell line induced and not affected in primary hepatocytes. High variability was also observed in the deregulation of *CYP1A2*, which was induced to a lower level of expression in HepG2 cells than in pHep. In HuH6 cells the majority of genes involved in B(a)P biotransformation showed the same pattern of expression than in pHep, with statistically significant differences in *CYP1A1*, *1A2* and *UGT1A1* expressions, which were less up-regulated in the HuH6 line. Few B(a)P specific detoxification genes, *GSTA1* and *UGT1A6*, showed different expression patterns between

HuH6 cells and primary hepatocytes. HuH6 cell line lacked expression of many important genes for B(a)P metabolism, which were deregulated in pHep, including *CYP3A4* and *2C9*, *UGT2B4* and *2B7*, and *SULTs*. Of these genes, HepG2 line lacked expression of *CYP2C9*. *CYP3A4* that showed weak basal expression in HepG2 cells was not detected after exposure to B(a)P.

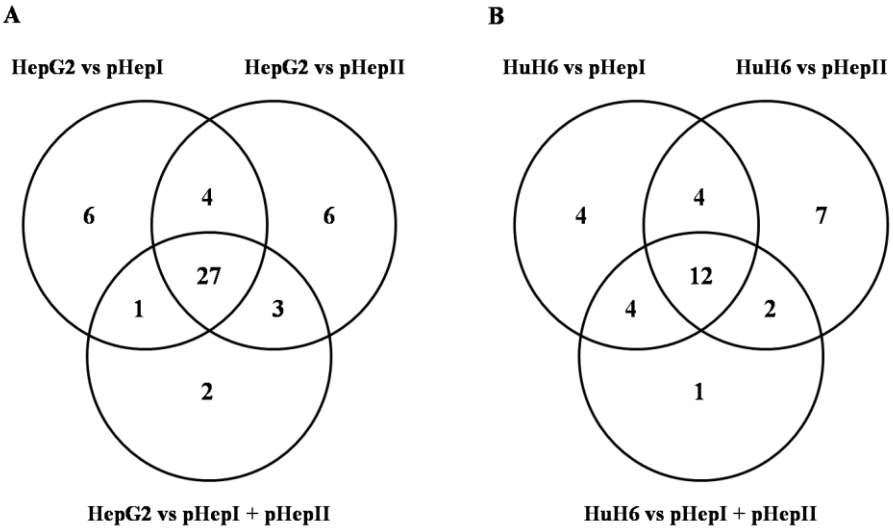

Figure 4.5: The number of genes that differed in their expression in hepatic cancer cell lines HepG2 (A) and HuH6 (B) versus primary hepatocytes of donor I (pHepI), versus primary hepatocytes of donor II (pHepII), and versus primary hepatocytes of both donors (pHepI+pHepII) after 24 h exposure to 10.0 µM B(a)P. Genes with Ct value under 37 were included in the analysis.

One of the phase I genes, the expression of which significantly differed among the primary hepatocytes and both hepatic cancer cell lines, was *ALDH3A1*, even though up-regulated trend of its expression was observed in all three cell types. ALDHs mainly detoxify toxic biogenic and xenobiotic aldehydes, and it has been reported that their activity is significantly higher in cancer cells compared to normal tissue (Jelski & Szmitkowski, 2008). However, in HuH6 line it was induced to a lower level of expression compared to pHep. Another gene with statistically different expression was *CYPE21*, which is involved in ethanol oxidizing system, and was down-regulated in hepatic cancer cell lines but not affected in primary hepatocytes. *DHRS2*, with very high basal expression in HepG2 cells compared pHep and Huh6, was after B(a)P exposure in HepG2 down-regulated, while in primary hepatocytes and in Huh6 cells it was up-regulated. *DHRS2* is a member of short-chain alcohol dehydrogenase/reductase (SDR) family that plays a role in detoxification of dicarbonyl compounds (Shafqat et al., 2006). Later, it has been shown that *DHRS2* is also involved in MDM2-p53 stress response pathway and p53 stabilization (Deisenroth, Thorner, Enomoto, Perou, & Zhang, 2010). Interestingly, the non-expressed *NAT2* was detected after B(a)P exposure in HuH6 line, while *CES5A* and *CHST1* genes were expressed after exposure to B(a)P in both cancer lines. CES enzymes are involved in detoxification or activation of various xenobiotics, including carcinogens, while CHSTs belong to phase II sulfotransferases which seem to be related more to carbohydrate metabolism and play a fundamental role in extracellular signalling (Iida et al., 2002). B(a)P-induced *CHST1* expression was observed in primary cells as well.

On the contrary to pHep, in both hepatic tumor-derived cell lines B(a)P induced expression of *ABCC2* gene encoding transporter MRP2 that belongs to the family of the multidrug resistance proteins. Among the genes encoding organic anion and poly-specific organic cation transporters, which play a role in hepatic uptake or elimination of endogenous small organic anions and cations as well as a wide array of drugs and environmental toxins (Hewitt et al., 2007; Lehman-McKeeman, 2008), expressions of *SLC22A1* and *SLC22A7* were deregulated in HepG2 cells and statistically differed from their expressions in pHep. In Huh6 line the expression of *SLC22A1* was, like in HepG2 cells, up-regulated, while *SLC22A7* was not expressed.

Several genes encoding TFs and their cofactors were expressed at statistically significant lower levels in hepatic cancer cells compared to pHep. Yet, fewer differences were observed in HuH6 line. One of such genes was *NFE2L2* encoding for oxidative stress sensor nuclear factor erythroid 2-related factor 2 that regulates the response to oxidative stress (Gallorini et al., 2015). *NFE2L2* was up-regulated in pHep and not affected in HepG2 and Huh6 cells. *NR1I3* encoding for constitutive androstane receptor (CAR) was induced in both pHep and to a lesser extent in HuH6 line, while in HepG2 cells it was not affected. CAR is known as the master transcription regulator of phase II metabolic enzymes, and it also contributes to the regulation of several *CYP2C*, and *ABC* and *SLCO* transporter genes (Chen et al., 2012; Omiecinski et al., 2011). Significant variability in *NR1I3* expression was found also between primary hepatocytes. The differences in the expressions of metabolic genes between HepG2 cell line and primary hepatocytes correlate well with the difference in the expression of *AHR*, the aryl hydrocarbon receptor gene, which was induced in HepG2 line but not affected in pHep. AHR receptor is capable of binding a broad spectrum of carcinogens, including B(a)P, and is a major regulator of the *CYP1A* and *UGT1A* genes (Chen et al., 2012; Wallace & Redinbo, 2013). *ALDH3A1*, *NQO1* and *GSTA1* are included in the AHR target gene battery as well (Nebert et al., 2000).

4.2.3.2 Differences in expression of liver-specific genes involved in the metabolism of xenobiotics in hepatic tumor-derived cell lines and primary hepatocytes after the exposure to AFB_1

The transcriptional responses of metabolic genes after 24 h exposure to 3.2 µM AFB_1 in HepG2 and HuH6 cell lines were closer to that of primary hepatocytes compared to the exposure to B(a)P. Statistical analysis revealed 9 genes with significantly different expression levels compared to pHep in both hepatic cancer lines. In HepG2 cells these differences were ascribed to 4 genes encoding phase I enzymes, 1 gene encoding phase II enzyme, 2 genes encoding transporter proteins and 2 genes encoding transcription factors. Huh6 cell line differed from primary cells in the expressions of 5 phase I genes, 2 phase II genes, 1 transporter gene and 1 transcription factor gene (Figure 4.6, Supplementary Material Table A.5).

Regarding the AFB_1 related metabolic enzymes, a number of human CYPs are involved in its biotransformation. Although the predominant roles in its activation and detoxification are ascribed to CYP3A4 and 1A2, AFB_1 induces also *CYP1A1*, *2C9* and *2B6* expression in primary hepatocytes (Ayed-Boussema et al., 2012). In this study, AFB_1 induced expression of *CYP1A2*, *2B6* and *2D6* in primary hepatocytes, while variations were observed in *CYP1A1* and *3A4* expression patterns among donors (repressed or not affected). In both cancer lines, the expression of *CYP1A1* significantly differed from that in pHep. Besides, in HepG2 line the difference in *CYP2B6* mRNA expression, and in HuH6 line the difference in *CYP2D6* expression were observed in comparison to primary

cells. *CYP2C9*, which lacked basal expression in both cancer lines, has been detected in HepG2 and HuH6 cells after exposure to AFB_1 indicating its induction. In pHep, *CYP2C9* did not show any transcriptional deregulation after AFB_1 exposure. Importantly, the gene encoding for the major enzyme of AFB_1 activation, CYP3A4, was not expressed in HuH6 cell line. In this line also the expression of *EPHX2* was detected at significantly higher levels compared to pHep. Epoxide hydrolases detoxify highly reactive electrophilic epoxides formed by CYP-mediated biotransformation of xenobiotics, although mEH (*EPHX1*) has been specifically proposed to be involved in the detoxification of AFB_1- epoxides (McGlynn et al., 1995). Among phase II enzymes which catalyze conjugation of reactive AFB_1 metabolites with glutathione, namely GSTA and especially GSTM (Guengerich et al., 1998), HepG2 differed from pHep in *GSTA1* expression and HuH6 line in *GSTA1* and *GSTM4* expression.

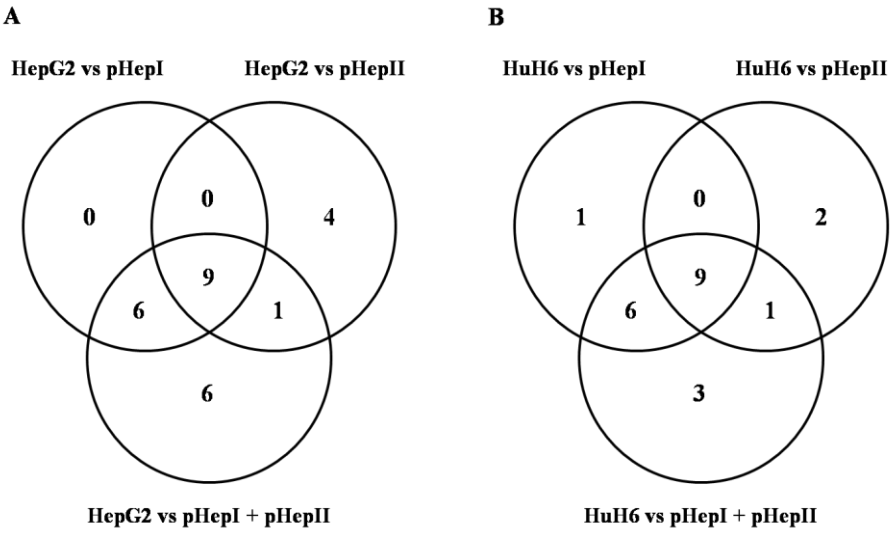

Figure 4.6: The number of genes that differed in their expression in hepatic cancer cell lines HepG2 (A) and HuH6 (B) versus primary hepatocytes of donor I (pHepI), versus primary hepatocytes of donor II (pHepII), and versus primary hepatocytes of both donors (pHepI+pHepII) after 24 h exposure to 3.2 µM AFB_1. Genes with Ct value under 37 were included in the analysis.

The highest significant differences compared to primary hepatocytes were observed in expressions of *FMO4*, *MAO* and *GPX3* genes in both cancer lines. FMO and MAO are phase I metabolic enzymes, normally responsible for detoxification and sometimes for the production of potentially toxic metabolites (Parkinson & Ogilvie, 2008; Tipton & Strolin-Benedetti, 2002). GPXs are important due to their antioxidant activity and regulation of ROS from xenobiotics or formed as by-products of metabolism of foreign compounds (Brigelius-Flohé & Maiorino, 2013; Parkinson & Ogilvie, 2008). In HepG2 cells, the expression of *DHRS2* gene was also after the exposure to AFB_1 differentially deregulated compared to primary hepatocytes.

AFB_1 induced expression of *ABCC2* gene encoding MRP2 transporter in both hepatic tumor-derived cell lines but not in pHep. Statistical difference between pHep and HepG2 line was observed also in deregulated expression of *SLC22A7* gene. In Huh6 line, the *SLC22A7* was not expressed.

AFB_1 has been suggested as a ligand for PXR, CAR and AHR receptors, inducing the expression of their mRNAs in human hepatocytes (Ayed-Boussema et al., 2012). These

receptors are considered as the major transcriptional regulators of crucial xenobiotic metabolizing enzymes and transporters, including CYPs, CES enzymes, ADHs and ALDHs, UGTs, GSTs, SULTs, and ABC and SLCO transporters (Chen et al., 2012; Tirona & Kim, 2005; Wallace & Redinbo, 2013). In this study, the mRNA expression of the three receptors was mainly unaffected by AFB_1 in primary hepatocytes and correlated with PXR (*NR1I2*), CAR (*NR1I3*) and *AHR* expressions in HuH6 cell line. In HepG2 cells, a difference compared to pHep was observed in *NR1I3* expression. Contrary to its expression in pHep, *AHR* was induced in HepG2 line although this difference was not statistically confirmed. Besides, in HepG2 line the expression of *CEBPA* significantly differed from its expression in pHep. As reported by Jover et al. (1998), C/EBPα transcription factor is an important transcriptional regulator of hepatic expression of the CYP2 family members (2B6, 2C9 and 2D6).

4.2.3.3 Differences in expression of liver-specific genes involved in the metabolism of xenobiotics in hepatic tumor-derived cell lines and primary hepatocytes after the exposure to PhIP

The 24 h exposure to 20.0 µM PhIP caused a weak effect on transcriptional response in HepG2 cell line. Less pronounced response in the expression of metabolic genes was observed also in HuH6 cells and primary hepatocytes of one donor. Inter-individual variability between primary cells (Figure 4.4 A) exceeded the differences between primary and cancer cells. Expression of 4 genes statistically differed in HepG2 line compared to primary hepatocytes of both donors, encoding phase I metabolic genes and transcription factors. In HuH6 cells, 9 genes were found to significantly differ in their expression from pHep. Of these genes, 5 belonged to phase I metabolism, 2 to phase II metabolism, 1 to transporter proteins and 1 to transcription factors (Figure 4.7, Supplementary Material Table A.6).

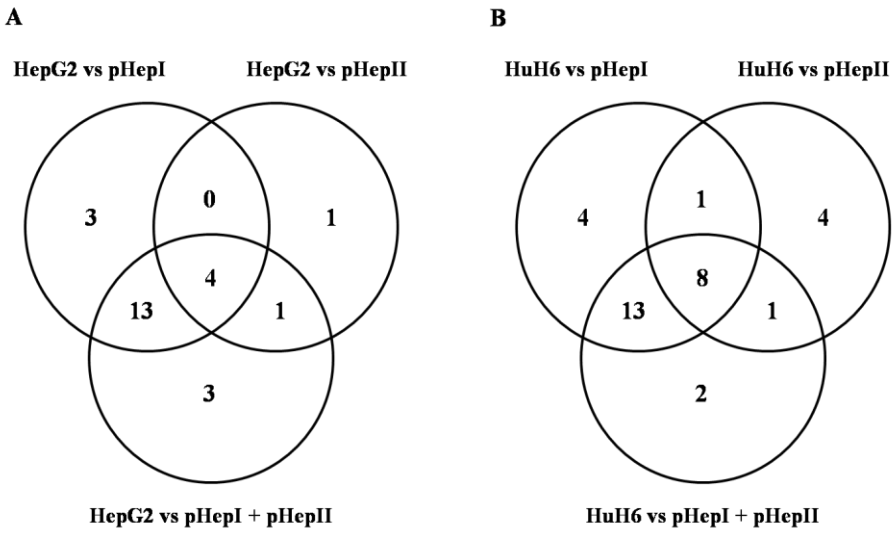

Figure 4.7: The number of genes that differed in their expression in hepatic cancer cell lines HepG2 (A) and HuH6 (B) versus primary hepatocytes of donor I (pHepI), versus primary hepatocytes of donor II (pHepII), and versus primary hepatocytes of both donors (pHepI+pHepII) after 24 h exposure to 20.0 µM PhIP. Genes with Ct value under 37 were included in the analysis.

Statistically significant differences in expression of genes involved in bioactivation of PhIP were observed in both hepatic cancer lines compared to primary cells. In HepG2 and Huh6 cells the expression of *CYP1A1* was up-regulated while in pHep it was down-regulated. The up-regulation of *CYP1A2* was observed in hepatocytes form one donor and was significantly higher compared to *CYP1A2* up-regulation in HepG2 and Huh6 cells. The expression of *NAT2* was up-regulated in pHep of one donor and obviously induced in Huh6 cells (not expressed in non-treated control cells, and detected after exposure to PhIP). The expression of *SULT1A1* was not detected in any of the cancer lines. However, it was also not deregulated in primary hepatocytes. Importantly, the expressions of two out of three analysed *SULTs* were not detected in HuH6 line. The PhIP-related detoxification genes, namely *GST* and *UGT1A*, have mainly shown a comparable expression between primary hepatocytes and hepatic tumor-derived cell lines, except the *GSTA1* expression which differed between HuH6 and primary cells. Another observation in HuH6 cell line was PhIP-induced expression of *UGT2B7* and *SLC22A7* genes, which were not expressed in non-treated control cells. The expression of *SLC22A7* gene was not affected in pHep, and *UGT2B7* was induced in pHep of one donor.

Significant differences between primary cells and both hepatic cancer lines were observed also in the expression of the *ALDH1A1* gene, which was repressed in pHep and not affected in HepG2 and HuH6 cells. Besides, in HuH6 line the expression levels of *MAO* genes statistically differed from their expression levels in both primary hepatocytes.

From the group of genes encoding transcription factors, the expressions of *CEBPA, DBP, NR0B2* and *NR1I3* were induced in primary hepatocytes of both donors. Expressions of *CEBPA* and *DBP* in HuH6 cells differed from their expressions in both primary hepatocytes. In HepG2 line the expressions of all four transcription factors differed from their expressions in primary hepatocytes. Dumont et al. (2010) demonstrated that induction of *CYP1A1* and *1A2* by PhIP occurs through the activation of the AHR receptor. However, in this study, *AHR* expression remained unaffected in all cell models investigated.

Taken together, the results of this part of the study confirmed the inter-variability in basal metabolic gene expression profiles between primary hepatocytes as well as the variability between basal profiles of hepatic cancer cell lines and primary cells. The hepatic tumor-derived lines showed the absence or lower expression of the majority of genes, while the expression levels of certain genes, such as *CYP2W1*, *DHRS2*, *FMO1*, *NQO1*, *GSTP1*, and several transcription factor genes were much higher compared to primary hepatocytes. Similar observations have been reported by other studies that characterized the basal metabolic gene expression profiles in comparison to primary hepatocytes, although they include only the HepG2 cell line (Gerets et al., 2012; Guo et al., 2011; Hart et al., 2010; Jennen, Magkoufopoulou, et al., 2010; Jetten et al., 2013). Regarding HuH6 cells, only few reports about the expression of several metabolic genes are available. One of them demonstrated a well-presented expression of *CYP3A4* in this cell line (Goldstein et al., 2013) which is inconsistent with our results. However, it has not been compared to primary cells. After the exposure to model pro-carcinogens, the transcriptional response to B(a)P observed in HuH6 cell line was closer to the response of primary hepatocytes. In HepG2 cells the B(a)P-induced gene expression was far more pronounced than in pHep. The transcriptional responses of both cancer lines were similar after AFB_1 exposure and also close to that of primary hepatocytes, although the mRNA expression differences in HuH6 line were more relevant, since the line lacked *CYP3A4*, the predominant enzyme of AFB_1 biotransformation. Like in this study, also Jennen et al. (2010) reported less pronounced gene expression changes in HepG2 cells after exposure to AFB_1 than after exposure to B(a)P. Jetten et al. (2013) investigated the whole genome

gene expression responses to B(a)P and AFB_1 exposure in HepG2 line and primary hepatocytes of 13 donors. Unlike in our study, they observed comparable B(a)P-induced gene expression profiles of HepG2 and primary hepatocytes, and different, more restricted ability of HepG2 line to respond to AFB_1 exposure in comparison to primary hepatocytes. But, the concentrations they used were lower than in our study. In their study AFB-response in HepG2 cells included fewer biotransformation-related genes compared to primary hepatocytes while in this study the number of deregulated metabolic genes in HepG2 cells and pHep after AFB_1 exposure was generally close. Also the inter-individual variation between donors exceeded the variation between the different cell types after B(a)P exposure in their study, while in ours, the response of pHep of both donors was the closest after B(a)P exposure and differed the most from HepG2 or HuH6 cell line. However, Jetten and co-workers (2013) found in HepG2 cell line a much higher number of B(a)P-deregulated genes compared to AFB_1 exposure in whole genome expression analysis and biotransformation related genes, and B(a)P strongly up-regulated *CYP1A1* and *CYP1A2* expression in both cell models, which is in line with our results. In our study, both hepatic cancer lines also similarly responded to PhIP exposure at the transcriptional level. Their response was close to that of primary hepatocytes of one donor while the inter-individual variability between primary cells exceeded the differences between primary and cancer cells in this case. Yet, HepG2 line showed a slightly closer expression pattern to pHep. Importantly, HuH6 line lacked the expression of the majority of *SULTs*, which appear to be very important for the bioactivation of PhIP N-hydroxy metabolites (Turesky, 2010). However, also HepG2 cell line lacked *SULT1A1* expression. Comparison of metabolic gene regulation in HepG2 line and primary hepatocytes after exposure to PhIP has been previously described in the literature, where PhIP did not induce significant changes in the expression of the examined genes in the analysis that included 10 phase I and 4 phase II genes, including *CYP1A1, 1A2, SULT1A1*, *NAT1* and *UGT1A6* (Wilkening et al., 2003). However, the results are not in line with ours.

The differences between primary hepatocytes and HepG2 and HuH6 cell lines in biotransformation gene expression and regulation have been elucidated and thus the second research hypothesis of the dissertation has been confirmed. Based on the current observations, no conclusions can be made regarding which hepatic cancer cell line is more comparable to primary hepatocytes. HepG2 line was closer to primary hepatocytes in responses to AFB_1 and PhIP, yet it showed higher possibility to give false positive results after exposure to compounds that require metabolic pathways similar to B(a)P. Also the number of genes encoding transcription factors, which differed in their expression and regulation compared to primary hepatocytes was higher in HepG2 line, with C/EBPα mRNA being consistently expressed at much lower levels after the exposure to the three carcinogens. Even though it seems that HuH6 cells can express certain genes after exposure to pro-carcinogens, the basal expression of which has not been detected, this cell line still showed a higher number of genes that remained unexpressed at any experimental conditions. Such disadvantage cannot be ignored since any of these genes can play a relevant role in the activation or detoxification of unknown compounds in genotoxicity testing. The expression and deregulation of transcription factors genes differed less from primary cells in HuH6 cell line, but the line consistently lacked the expression of cofactor PGC1α mRNA, an important coactivator of HNF4α transcription factor. These results could present valuable information for further improvement of existing test systems. It could be hypothesized that the transfection of HepG2 cells with C/EBPα mRNA, or HuH6 cell line with PGC1α might result in closer gene expression patterns to primary hepatocytes and better responsiveness of the cell lines at the transcriptional level to the exposure to model pro-carcinogens. It should be noted that in

this study primary hepatocytes from only two donors were used to compare their expression profiles with the expression profiles of HepG2 and Huh6 cancer cell lines. While HepG2 and HuH6 cultures consist of a homogenous cell population with a relatively stable genome and little environmental influences on their gene expression, the primary human hepatocytes are derived from different donors with intrinsic genetic polymorphisms and exposed to many environmental factors, which all contribute to large inter-individual differences in response to xenobiotic exposure. Thus, more donors should be included in the future research. Besides, the qPCR analysis is only a snap-shot of multiple long- and short-term events which take place on the transcriptional level in the cell, and the comparison of selected cancer cell lines with pHep in DNA damage response induced by model compounds would be an additional step to determine which of the hepatic cancer lines represents a better cell model for *in vitro* genotoxicity studies. In this study the comet assay with primary hepatocytes was not possible to perform, whereas the concentrations of B(a)P, AFB_1 and PhIP compounds used in the previous part of the study, where the induction of DNA damage was observed in HepG2 and HuH6 cell lines, were higher (see Chapter 4.1.2).

4.3 Development of Metabolically Active Differentiated Hepatic Progenies from Human Adipose Tissue Stem Cells for the Application in Genotoxicity Testing

Stem cell-derived hepatocytes indicate a great potential for the application in the *in vitro* genotoxicity testing as the experimental model. However, only hiPSC or hESC-derived hepatocytes have been shown to be suitable surrogates to primary hepatocytes in drug metabolism and toxicity studies so far (Gao & Liu, 2017; Kang et al., 2016; Lu et al., 2015; Ulvestad et al., 2013; Yildirimman et al., 2011). The hASC-derived hepatocytes have been mostly exploited in regenerative medicine for therapeutic purposes of liver injury (Banas et al., 2007; Lee et al., 2015; Taléns-Visconti et al., 2006) and fewer efforts have been focused on their metabolic characterization and usefulness as *in vitro* toxicological models. The hASC used in this study have been isolated and protocol for their *in vitro* propagation has been previously developed in our laboratory by Dr. Kološa (2016). In this study, attempts have been made to differentiate hASC to metabolically active differentiated hepatic progenies (hDHP) that are sensitive to detect DNA damage after the exposure genotoxic carcinogens.

4.3.1 Determination of hepatic phenotype of the generated hDHP cells

The sequential addition of lineage-specific factors is crucial to obtain functional SC-derived hepatocytes *in vitro* (Snykers et al., 2006). In differentiating hASC towards hepatic lineage, liver-specific factors FGFs, HGF, DXM (synthetic glucocorticoid), and OSM have been proven to be the most advantageous (Banas et al., 2007; Lee et al., 2015; Li et al., 2014). Also, the direction towards endoderm lineage in the initial step of differentiation has been demonstrated to be a prerequisite for successful differentiation into mature endoderm progenies. In this manner Activin A (ActA), a member of the TGFβ superfamily, mimics the signalling of Nodal (Zorn, 2008), and pre-treatment of hASC with high concentrations of ActA can be applied to redirect the cells towards

endodermal fate (Lee et al., 2015; Li et al., 2014). Some studies emphasized the need of supplementary medium factors, such as insulin - transferrin - selenium (ITS) and nicotinamide (VIT B3), for enhanced hepatic differentiation of hMSC, and the epigenetic modification with very low concentrations of DMSO has been shown as well to improve the differentiation of hASC at the final step of the process (Seo et al., 2005).

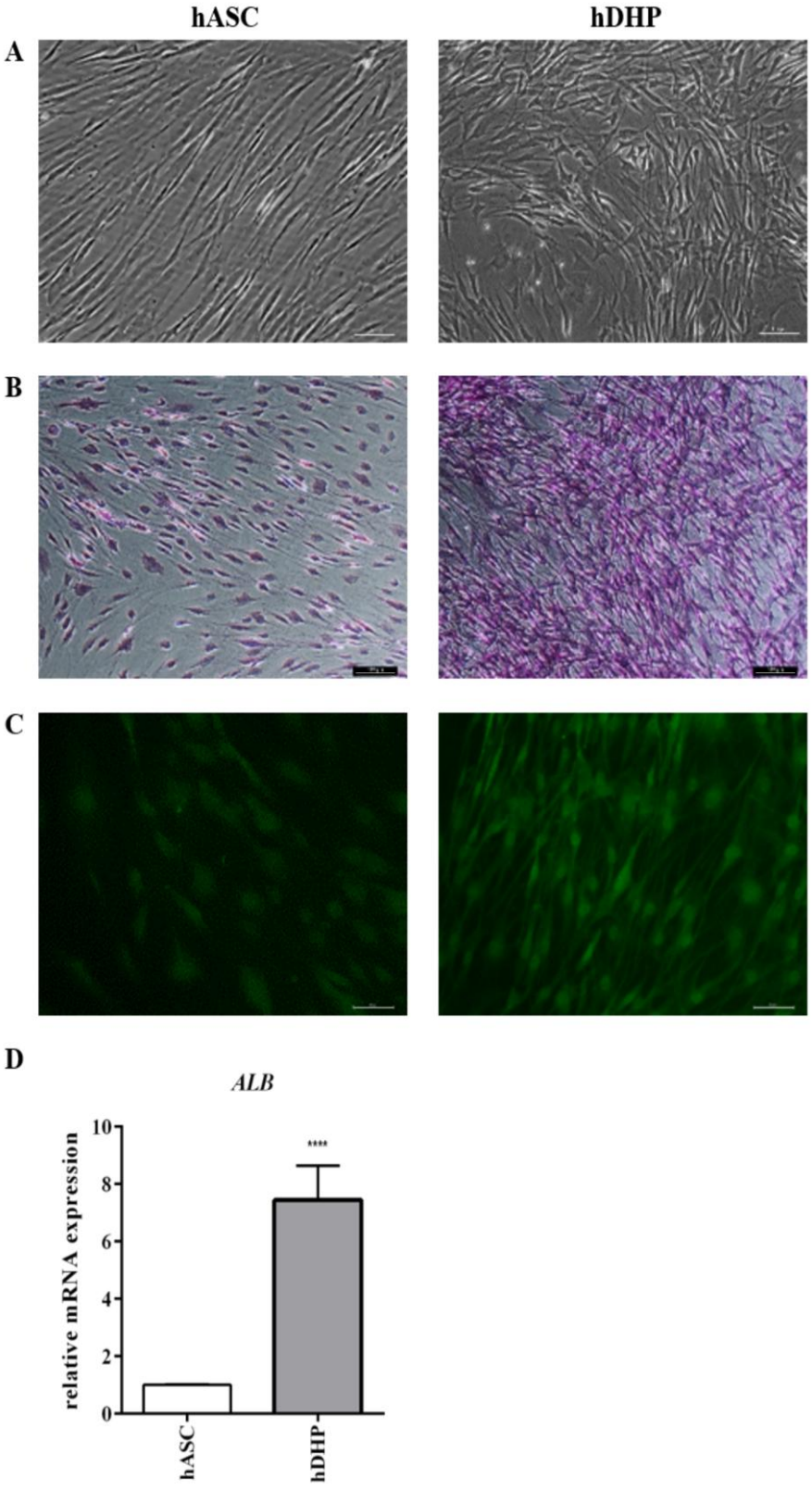

Figure 4.8: Determination of hepatic phenotype of hDHP cells: changes in morphology from day 1 (hASC) to day 24 (hDHP) visualized by light-microscopy (A; scale bar 100 µm); glycogen storage determined by PAS staining (B; scale bar 100 µm); albumin expression determined by ICC (C; scale bar 50 µm); relative mRNA expression of *ALB* determined by qPCR (D). The results of qPCR are presented as relative *ALB* expression in hDHP cells normalized to the *ALB* expression in hASC cells. Data are means ± SD of three independent experiments. Significant difference (Student's t test) between hDHP and hASC is indicated by (****) ($p < 0.0001$).

A three-step hepatic differentiation protocol was applied in this study to generate hepatic cells from hASC (see Chapter 3.4.1; Table 3.2). The protocol was designed according to Lee et al. (2015) with minor modifications, mimicking the hepatic developmental signalling patterns. The differentiated cells were morphologically not typically hepatocyte-like, but they expressed hepatic markers, therefore they represent differentiated hepatic progeny cells. Figure 4.8 depicts the characteristics of hepatic phenotype of the generated hDHP, namely morphology, glycogen storage and ALB expression. At the beginning, the hASC exhibited typical spindle-shaped fibroblast-like morphology of stem cells. After 24 days of differentiation, the cells became wider and flatter, and appeared as mixed population of fibroblast-like cells and cells with assumed cuboid shape (Figure 4.8 A).

The hepatocytes mainly perform liver-specific functions from the synthesis of plasma proteins; intermediary metabolism of amino acids, lipids, and carbohydrates; and detoxification of xenobiotic compounds (Duncan, Dorrell, & Grompe, 2009). The characterization of a distinct hepatic phenotype includes, among others, the ability of cells to store glycogen and secrete albumin (Snykers et al., 2009), which were both confirmed in the generated hDHP cells in this study. PAS and ICC techniques revealed more intensive staining in the differentiated hepatic progenies, indicating higher glycogen (Figure 4.8 B) and ALB (Figure 4.8 C) content compared to undifferentiated hASC. Significantly higher expression of *ALB* in generated progenies was detected also at the mRNA level (Figure 4.8 D).

4.3.2 Metabolic characterization of the generated hDHP cells and their suitability for genotoxicity testing

Most of the studies regarding hASC-derived hepatocytes have been focused on classical functional characterisation (glycogen uptake, urea metabolism, ALB secretion), while only a little attention has been paid to the expression and inducibility of xenobiotic metabolizing enzymes, which is a key feature of the functional hepatic phenotype and of cell-based models for toxicological investigations. Even if the hDHP cells in this study were morphologically not fully differentiated hepatocytes, the aim was to generate metabolically active cells which can be applicable in genotoxicity testing. Therefore, the sensitivity of hDHP cells to detect model genotoxic pro-carcinogens was evaluated and the expression of selected enzymes involved in the metabolism of corresponding genotoxins was determined in generated progenies, including the transcriptional deregulation of metabolic enzymes after the exposure of cells to the same model genotoxic compounds.

4.3.2.1 Response of hDHP and hASC cells to model genotoxic compounds

To investigate the sensitivity of hASC and hDHP cells to detect genotoxic compounds, the induction of DNA damage was determined after their exposure to model indirect acting carcinogens B(a)P, AFB_1, or PhIP, using a comet assay. Their intrinsic sensitivity was explored in parallel with exposure of cells to direct acting genotoxic compound TBHP, which does not require metabolic activation and readily induces DNA strand breaks. Induction of DNA damage in both cell types was compared to the induced DNA damage in HepG2 cells exposed to the highest tested concentrations of selected genotoxins.

Prior to the genotoxicity testing and gene expression analyses, the effects of the highest test concentrations of B(a)P, AFB_1 and PhIP on the viability of cells were determined using MTS assay. At these concentrations, none of the model compounds reduced the viability by more than 30 % (Figure 4.9).

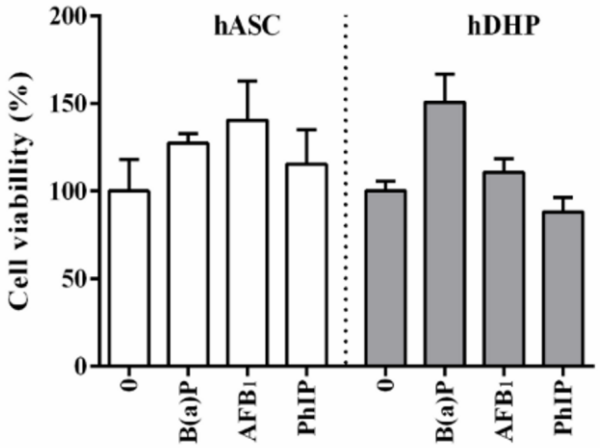

Figure 4.9: The effects of B(a)P, AFB_1, and PhIP on the viability of the undifferentiated hASC and generated hDHP cells. The viability was determined with the MTS assay after 24 h exposure to B(a)P (30.0 µM), AFB_1 (15.0 µM), or PhIP (200.0 µM). The results are expressed as % of cell viability ± SD. One experiment with five replicates per treatment point was performed.

The results of the comet assay are presented in Figure 4.10. B(a)P and AFB_1 induced a significant increase in DNA damage at all tested concentrations in hDHP cells, which is comparable to that observed in metabolically active HepG2 cells (Figure 4.10 A, B). The hDHP cells were, similarly to the HepG2 cell line, irresponsive to the exposure to PhIP (Figure 4.10 C). In hASC, none of the three compounds induced DNA damage (Figure 4.10 A, B, C). Similar responses to B(a)P and AFB_1 exposure have been found also in HepG2, HuH6 and HepaRG cell lines in the previous part of the dissertation, while the response to PhIP does not correlate with the response of HepG2 cells in the previous sensitivity evaluation (see Chapter 4.1.2). Regarding PhIP-induced genotoxicity, the results in the literature reports are inconclusive. As already mentioned, positive (Knasmüller et al., 1999; Le Hegarat et al., 2010; Pezdirc et al., 2013) and negative results (Khoury et al., 2016; Wilkening et al., 2003; Winter et al., 2008) have been reported with HepG2 and HepaRG cell lines. Ghaderi et al. (2011) compared the sensitivity of hMSC-derived hepatocytes, hematopoietic stem cell-derived hepatocytes, and their mesenchymal and hematopoetic ($CD34^+$) progenitors from umbilical cord blood

towards AFB_1-induced DNA damage. The hepatocyte-like cells differentiated from MSC were found to be more sensitive than the ones differentiated from $CD34^+$ cells, and their sensitivity correlated with *CYP3A4* mRNA expression. However, on the contrary to this study, they also observed elevated DNA damage in undifferentiated mesenchymal and hematopoetic SC after AFB_1 exposure, although these cells did not express *CYP3A4*.

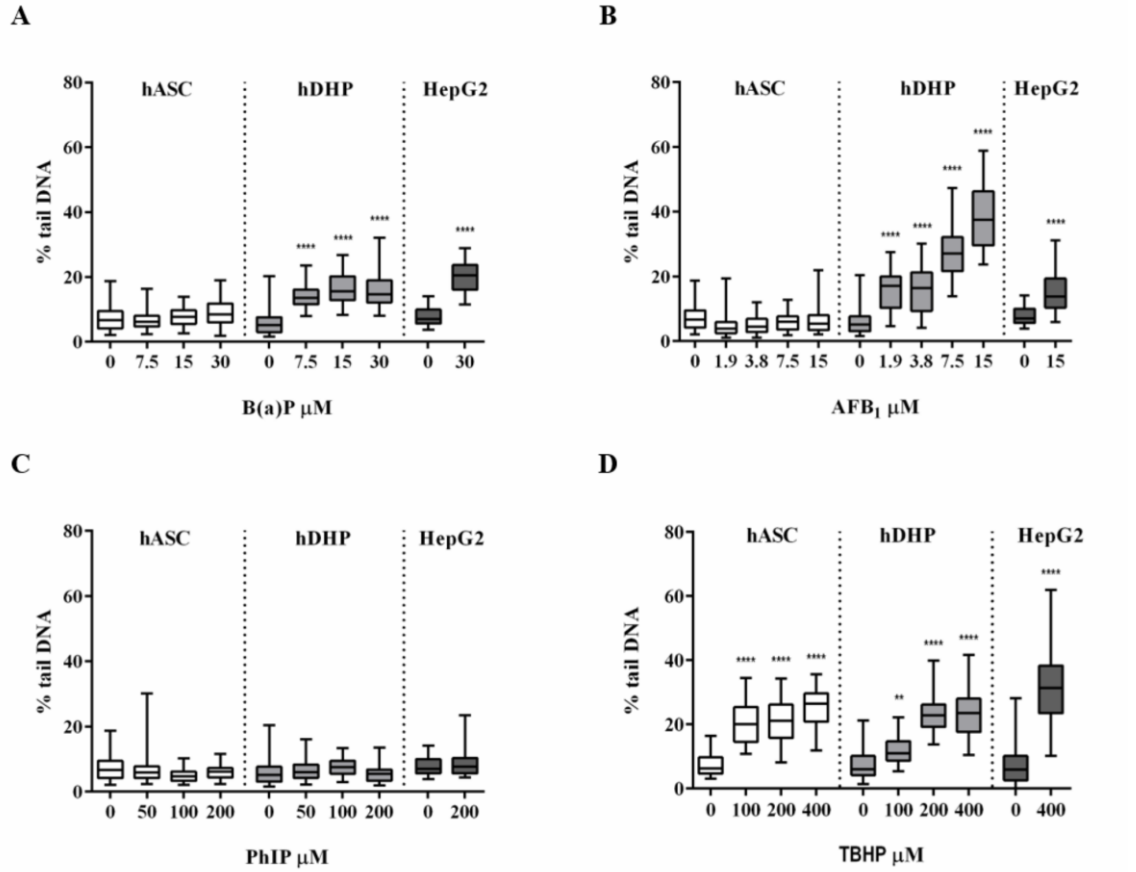

Figure 4.10: Induced DNA damage in hASC and hDHP cells after the exposure to model indirect acting genotoxins B(a)P (A), AFB_1 (B), and PhIP (C), and direct acting genotoxin TBHP (D). The cells were exposed to graded concentrations of B(a)P, AFB_1, or PhIP for 24 h and to TBHP for 20 min. HepG2 cells, exposed to the selected genotoxic agents at their highest tested concentration, were applied as control cells. DNA damage was assessed using the comet assay and is expressed as % DNA in the comet tail. 50 nuclei were analysed per experimental point in each of the three independent experiments. Significant difference (One-way ANOVA, Dunett's test) between the treated cells and the vehicle control cells (0) is indicated by (**) ($p < 0.01$) and (****) ($p < 0.0001$). The % tail DNA of the vehicle control cells did not differ significantly from that of non-treated control cells (data not shown).

It has been reported that hESC are intrinsically more resistant to DNA damage induced by direct acting genotoxins compared to their differentiated fibroblastic progenies (George, Heng, Vinoth, Kishen, & Cao, 2009; Vinoth et al., 2014). However, not much is known about hASC response to direct genotoxic stress. Interestingly, the hASC and hDHP cells in this study showed comparable response towards the direct acting TBHP. Significant increase in DNA damage was observed in both cell types and was also consistent with DNA damage induced by TBHP in HepG2 cells (Figure 4.10 D),

and with results of the study of Johnson et al. (2017) who observed significantly high DNA tail lengths in hASC following the treatment with direct acting genotoxins.

The metabolism of the three model pro-carcinogens B(a)P, AFB_1 and PhIP is well-characterised, and their bioactivation pathways to DNA reactive metabolites well known. The observed differences in the response of hDHP and hASC towards B(a)P and AFB_1 exposure indirectly suggest that hDHP cells express xenobiotic metabolizing enzymes involved in bioactivation of the model pro-carcinogens, and indicate their metabolic capacity required for the application in genotoxicity testing.

4.3.2.2 Expression and inducibility of xenobiotic metabolizing enzymes in hDHP cells

To confirm the assumption about metabolic capacity of differentiated hepatic progenies, the expression of selected enzymes involved in the metabolism of the three genotoxins B(a)P, AFB_1 and PhIP was determined at transcriptional and protein level in hDHP cells.

qPCR and Western blot analyses confirmed the basal expression of CYP3A4, 1A1, NAT2, SULT1A1, and UGT1A1 at the transcriptional and protein level in hDHP cells (Figure 4.11). The expression of GSTA1 was confirmed at the mRNA level, but it was not included in the Western blot analysis (Figure 4.11 A).

Since the xenobiotic metabolizing enzymes are mostly inducible, the effect of the 24 h exposure of hDHP cells to B(a)P, AFB_1, or PhIP on the expression of selected xenobiotic metabolizing genes was determined with qPCR analysis. The expression of *CYP3A4* was up-regulated after the exposure to AFB_1 and PhIP, and the exposure to B(a)P repressed its expression. The exposure to B(a)P and AFB_1 induced the expression of *CYP1A1* (Figure 4.11 A). However, the expression of *CYP1A2* mRNA was not detected in hDHP (data not shown). Expression of *SULT1A1* was induced only after exposure to PhIP while the exposure to B(a)P and AFB_1 significantly down-regulated the *SULT1A1* expression. *NAT2* expression was significantly deregulated after PhIP exposure and *GSTA1* expression after AFB_1 exposure. The exposure to AFB_1 and PhIP induced the expression of *UGT1A1* in hDHP cells (Figure 4.11 A).

As previously described, CYP1A1, 1A2, and 3A4 are involved in bioactivation of the three pro-carcinogens, NAT2 and SULT1A1 participate in the activation of PhIP, and GSTA1 and UGT1A1 play an important role in detoxification of the used compounds (Guengerich et al., 1998; Shimada, 2006; Turesky, 2010). The deregulated expressions of analysed genes after exposure to B(a)P and AFB_1 (Figure 4.11 A) correlate to their involvement in the metabolic activation of the two model pro-carcinogens as well as with the observed induction of DNA damage (Figure 4.10 A, B). Regarding PhIP, its exposure induced expression of *SULT1A1* and *NAT2* (Figure 4.11 A), but the absence of *CYP1A2* mRNA that is predominant in the bioactivation of this compound (Crofts et al., 1998) may explain the lack of PhIP-induced DNA damage in hDHP cells (Figure 4.10 C). These results confirmed our assumption about the ability of hASC to differentiate into hDHP cells that express important phase I and phase II metabolic enzymes. Furthermore, the induction and repression of genes encoding biotransformation enzymes is mainly regulated through the activation of various xenobiotic receptors after exposure to specific compounds (Wallace & Redinbo, 2013), and the observed transcriptional deregulation of metabolic genes by the exposures to B(a)P, AFB_1 and PhIP implies that hDHP cells also possess certain xenobiotic receptors.

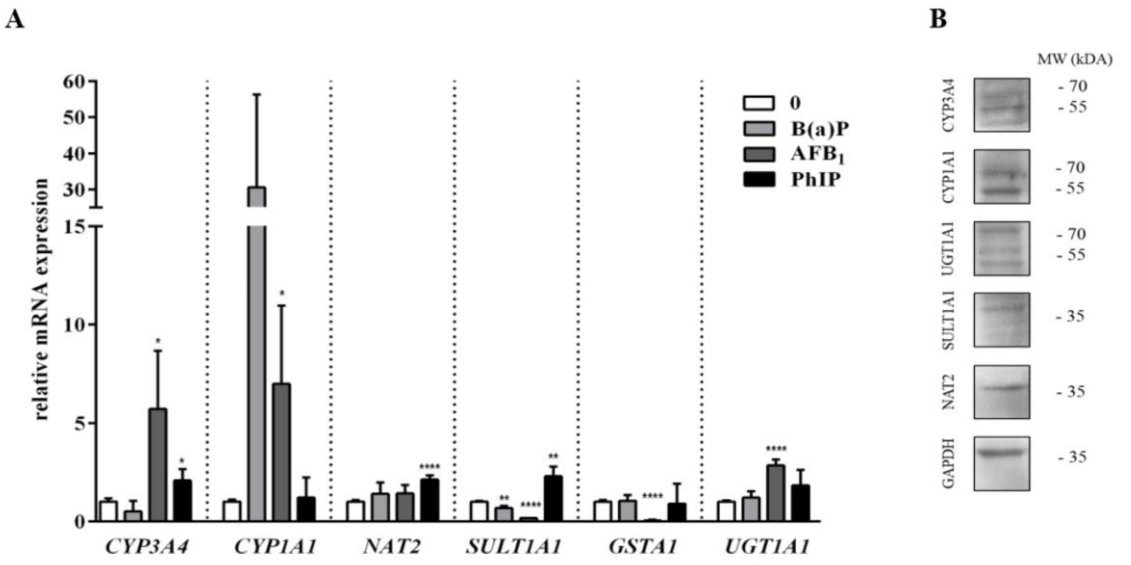

Figure 4.11: Gene (A) and protein (B) expression of metabolic enzymes involved in the activation and detoxification of indirect acting genotoxic compounds in hDHP cells determined with the qPCR and western blot, respectively. For gene expression analysis, the cells were exposed to B(a)P (30.0 µM), AFB_1 (15.0 µM), or PhIP (200.0 µM) for 24 h. The results of qPCR are presented as relative mRNA expression in treated cells normalized to the mRNA expression in vehicle control cells. Data are means ± SD of three independent experiments. Significant difference (Student's t test) between the treated cells and the vehicle control cells (0) is indicated by (*) ($p < 0.05$), (**) ($p < 0.01$), and (****) ($p < 0.0001$). Less than 0.67- and more than 1.5-fold change indicates deregulation of gene.

Taken together, the potential of hASC to differentiate into metabolically active hepatic progenies has been confirmed. The hDHP cells have expressed several phase I and phase II xenobiotic metabolic enzymes in an inducible form and have been capable of metabolic activation of indirect acting model genotoxic carcinogens B(a)P and AFB_1. They have shown a great potential to be exploited in *in vitro* genotoxicity testing and thus the third research hypothesis of the dissertation has been confirmed.

4.4 Immortalization of the Differentiated Hepatic Progenies of Human Adipose Tissue Stem Cells

One of the limitations of the stem-cell hepatic differentiation approach for the application in routine testing is questionable stability of the differentiated HepLC *in vitro* (Guguen-Guillouzo et al., 2010; Snykers et al., 2009). Also, in this study the generated hepatic progenies (see Chapter 4.3) rapidly de-differentiated after re-plating and for each genotoxicity experiment, the whole differentiation procedure has been performed from the beginning. This increases the possibility for lower reproducibility between experiments, especially due to the long differentiation protocol, which in this case took 24 days. Besides, the supplements are rather expensive, which makes the approach less convenient for a routine use. In addition, the limitations of primary cells apply here. MSC undergo replicative senescence and their ability to differentiate is altered as they age (Kim et al.

, 2009). The availability of a sufficient quantity of hASC needed for the differentiation to hDHP cells is thus limited, and obtaining fresh hASC is time consuming and again associated to deviations in reproducibility of final experiments due to donor inter-variability. In order to overcome these limitations, the attempts have been made in this study to immortalize the generated hDHP cells to get a permanent cell line, which is metabolically competent and thus applicable in routine genotoxicity testing.

4.4.1 Response of immortalized hDHP cells to model genotoxic compounds

The hDHP cells were immortalized by stable transfection with human telomerase reverse transcriptase (*hTERT*) and their sensitivity to detect genotoxic pro-carcinogens was investigated through the determination of DNA damage after exposure of cells to model indirect acting carcinogens B(a)P, AFB_1, or PhIP, using a comet assay. Several studies reported that transduction with *hTERT* extends the life span of MSC, while it does not affect their typical properties (Liang et al., 2012; Mihara et al., 2003). However, the metabolic activity of immortalized hDHP cells has been apparently repressed or the cells de-differentiated during the process of immortalization and selection of clones, which stably express *hTERT*, since none of the three compounds induced DNA damage (Figure 4.12).

4.4.2 Hepatic differentiation of immortalized hDHP cells and their response to model genotoxic compounds

To restore the metabolic activity of immortalized hDHP cells, the hepatic differentiation protocol, established in a previous part of the study, has been applied as described in Chapter 3.4.1; Table 3.2. The *hTERT*-transfected cells were divided in two groups. One group was cultured in the hepatic induction medium followed by the hepatic maturation medium (21 days), and the other group was cultured only in the maturation medium (14 days). At the end of the differentiation, their sensitivity to detect genotoxic pro-carcinogens was investigated using a comet assay after the exposure of cells to model indirect acting carcinogens B(a)P, AFB_1, or PhIP.

The results of the comet assay indicated re-differentiation of both groups of immortalised hDHP cells to metabolically active hDHP cells. Both cell groups comparably responded to B(a)P and AFB_1 exposure, as observed in significantly increased DNA damage. PhIP did not induce DNA strand breaks in any of the groups of re-differentiated cells (Figure 4.12).

Overall, the stable transfection with *hTERT* has been shown to be a convenient approach to obtain an unlimited source of cells, and the hepatic maturation step has been proven to be sufficient to generate metabolically active differentiated hepatic progenies applicable for routine genotoxicity testing in a short time period.

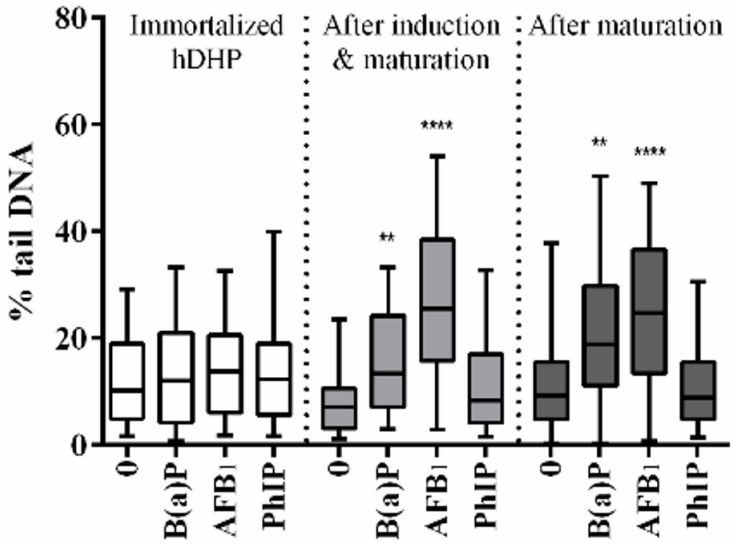

Figure 4.12: B(a)P-, AFB_1-, and PhIP-induced DNA damage in immortalized hDHP, and immortalized hDHP after re-differentiation by hepatic induction and maturation or only the maturation step of the hepatic differentiation protocol (Table 3.2). The cells were exposed to B(a)P (30.0 µM), AFB_1 (15.0 µM), or PhIP (200.0 µM) for 24 h. DNA damage was assessed using the comet assay and is expressed as % DNA in the comet tail. 50 nuclei were analysed per experimental point in each of the three independent experiments. Significant difference (One-way ANOVA, Dunett's test) between the treated cells and the vehicle control cells (0) is indicated by (**) ($p < 0.01$) and (****) ($p < 0.0001$). The % tail DNA of the vehicle control cells did not differ significantly from that of non- treated control cells (data not shown).

To summarize, the fourth research hypothesis of the dissertation has only been partially confirmed. The differentiated hepatic progenies, which have been shown to be sensitive to detect certain types of genotoxic pro-carcinogens, became unresponsive after the immortalization process, probably due to the lost metabolic activity during the selection of stably transfected clones. However, immortalized hDHP have been easily re-differentiated into metabolically active cells that respond to indirect acting genotoxic compounds comparably to non-immortalized hDHP cells, which potentially offers a new opportunity to replace the use of established cell lines that are currently applied in routine *in vitro* testing.

Chapter 5

Conclusions

The overall goal of this study was to obtain a stable metabolically active human-derived *in vitro* cell model, which would reflect *in vivo* biotransformation of chemicals as close as possible, would be able to detect direct and indirect acting genotoxins reliably, and could be used in standardized routine genotoxicity studies.

In the first part of the study, a comprehensive analysis of characteristics, relevant for the potential use in genotoxicity testing, has been performed in a series of available human hepatic cell lines. In addition to the hepatic cancer cell lines that have already been used in genotoxicity studies, other cell lines, mainly derived from hepatic tumors, which have been occasionally used in specific hepatotoxicity studies, were included in the analysis. The findings of the screening suggested HepG2 and HuH6 cancer lines as potentially the best cell models for the use in genotoxicity testing. The two cell lines have comparable doubling times, express functional wild type p53, their chromosome number is in narrow range and they both showed similar sensitivity to detect DNA damage induced by all tested model pro-carcinogens. To get insight into their metabolic capacities, the two hepatic cancer cell lines were subjected to gene expression analysis of carefully selected genes involved in the metabolism of xenobiotics and genes encoding for liver-specific transcription factors, and metabolic profiles of the two cancer lines were compared to those of primary human hepatocytes from two different donors. The findings confirmed the expected absence or much lower basal expression levels of the majority of genes in cancer lines compared to primary hepatocytes with the exception of certain genes that were expressed at significantly higher levels. Furthermore, the transcriptional regulation of these genes was determined after the 24 h exposure to non-cytotoxic concentrations of three model pro-carcinogens. The gene expression profile of xenobiotic metabolic enzymes of HepG2 line was closer to primary hepatocytes in responses to AFB_1 and PhIP, yet it showed higher possibility to give false positive results after exposure to compounds that require metabolic pathways similar to B(a)P. The transcriptional response of HuH6 cells was close to that of primary hepatocytes after B(a)P and AFB_1 exposure, however this line showed a higher number of unexpressed metabolic genes. Such disadvantage cannot be ignored since any of these genes can play a relevant role in the activation or detoxification of unknown compounds in genotoxicity testing. The number of genes encoding transcription factors, which differed in their expression and regulation compared to primary hepatocytes, was higher in HepG2 than in HuH6 cell line. Based on the current observations, no conclusions can be made regarding which hepatic cancer line is more comparable to primary hepatocytes. However, the findings represent valuable information that can be used for improvements of HepG2 and HuH6 cells to be more convenient for *in vitro* genotoxicity studies, such as the transfection of cells with specific genes encoding transcription factors or cofactors. The obtained findings

also contribute to better understanding and interpretation of toxicological results relevant for the regulatory toxicology. However, to confirm the credibility of the present results, the metabolic profiles of hepatocytes of more donors should be compared with the expression profiles of HepG2 and Huh6 cell lines. The main problem when using HepG2 cell line in genotoxicity studies has been shown to be poor reproducibility of the results between experiments. In this respect, HuH6 cell line demonstrated more reproducible results that remain to be further confirmed. Therefore, experimental work with reference genotoxic and non-genotoxic compounds recommended by the European Centre for Validation of Alternative Methods (ECVAM) is in progress.

Of particular scientific relevance is the second part of this work, wherein the potential of hASC to differentiate into metabolically active hepatic progenies (hDHP) has been demonstrated. The generated cells are not of carcinogenic origin, meaning that some limitations which can affect the predictivity of *in vitro* hepatic cancer cell-based models are avoided. The hDHP cells have been capable of metabolic activation of indirect acting model genotoxic carcinogens B(a)P and AFB_1, and expressed several phase I and phase II xenobiotic metabolic enzymes in an inducible form. To make the use of hDHP cells more convenient for routine *in vitro* genotoxicity testing, the attempts have been made to immortalize the generated hDHP cells with a stable transfection with *hTERT*. Although the *hTERT*-transfected hDHP were unresponsive, they have been easily matured to metabolically active cells that respond to indirect acting genotoxic compounds comparably to non-immortalized hDHP cells. The immortalized cells have a prolonged replicative life span and represent a stable source of cells from which an almost limitless supply of hDHP cells can be obtained and are amenable for the production of large quantities of standardized cells for genotoxicity testing. However, more research is needed for a wider use of hDHP cells. Since they lacked the mRNA expression of important liver-specific *CYP1A2*, which is responsible for the bioactivation of different pro-carcinogens, the maturation conditions should be optimized to improve the expression of xenobiotic metabolic enzymes. Besides, like HepG2 and HuH6 cell lines, hDHP cells should also be analysed regarding their metabolic gene expression profiles and compared to that of primary human hepatocytes to elucidate their potential limitations.

The findings of the study suggest many advantages of hASC-derived hDHP cells and several limitations of HepG2 and HuH6 cell lines. All three cell models are worth further investigations in terms of development of a test model, which could contribute to the reduction of the use of laboratory animals and to more efficient safety evaluation of new chemical products needed for better human health protection.

Appendix A

Supplementary Material

A.1 Human Primary Hepatocytes Donor Report

Table A.1: Human primary hepatocytes donor demographics and gene induction report provided by supplier.

Human cryopreserved hepatocytes	Demographics report									Induction report (fold induction of mRNA expression)[1]		
	Sex	Age/yrs	Ethnic group	BMI	Tobacco use	Alcohol use	Drug use	Serological data	Cause of death	CYP1A2[a)]	CYP2B6[b)]	CYP3A4[c)]
Donor I	Female	54	Caucasian	26.2	No	1 wine / day	No	CMV + EBV+	Stroke	14.2	6.7	9.6
Donor II	Male	27	Caucasian	30.5	Yes	Yes	No	Negative	Gunshot wound to head	496.0	15.9	77.6

[1] Induction report provided by supplier: Cryopreserved human primary hepatocytes were thawed and plated on 24-well collagen-coated plates, overlaid with Matrigel©, then dosed in triplicate with vehicle control (0.1 % DMSO) or control inducers for 72 h. The fold induction was calculated by dividing the induced level by the vehicle control level. Control inducers: [a)] 50.0 µM Omeprazole; [b)] 1.0 mM Phenobarbital; [c)] 10.0 µM Rifampicin. yrs – years. BMI – body mass index.

A.2 List of Metabolic Genes and Genes Encoding for Liver-Specific Transcription Factors Selected for Gene Expression Analysis

Table A.2: List of metabolic genes and genes encoding for liver-specific transcription factors selected for gene expression analysis.

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Phase I metabolism					
Hs02383872_s1	*ADH1C*	Alcohol dehydrogenase 1C (class I), gamma polypeptide	Alcohol dehydrogenase 1C	Oxidoreductase	Dehydrogenase
Hs00923466_m1	*ADH4*	Alcohol dehydrogenase 4 (class II), pi polypeptide	Alcohol dehydrogenase 4	Oxidoreductase	Dehydrogenase
Hs00946916_m1	*ALDH1A1*	Aldehyde dehydrogenase 1 family member A1	Retinal dehydrogenase 1	Oxidoreductase	Dehydrogenase
Hs00964880_m1	*ALDH3A1*	Aldehyde dehydrogenase 3 family memberA1	Aldehyde dehydrogenase	Oxidoreductase	Dehydrogenase
Hs00275607_m1	*CES1*	Carboxylesterase 1	Liver carboxylesterase 1	Hydrolase	Esterase
Hs01101510_m1	*CES5A*	Carboxylesterase 5A	Carboxylesterase 5A	Hydrolase	Esterase
Hs01054797_g1	*CYP1A1*	Cytochrome P450 family 1 subfamily A polypeptide 1	Cytochrome P450 1A1	Oxidoreductase	Oxygenase
Hs00167927_m1	*CYP1A2*	Cytochrome P450 family 1 subfamily A polypeptide 2	Cytochrome P450 1A2	Oxidoreductase	Oxygenase
Hs04183483_g1	*CYP2B6*	Cytochrome P450 family 2 subfamily B polypeptide 6	Cytochrome P450 2B6	Oxidoreductase	Oxygenase
Hs02383413_s1	*CYP2C18*	Cytochrome P450 family 2 subfamily C polypeptide 18	Cytochrome P450 2C18	Oxidoreductase	Oxygenase
Hs00426380_m1	*CYP2C19*	Cytochrome P450 family 2 subfamily C polypeptide 19	Cytochrome P450 2C19	Oxidoreductase	Oxygenase

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Hs00258314_m1	*CYP2C8*	Cytochrome P450 family 2 subfamily C polypeptide 8	Cytochrome P450 2C8	Oxidoreductase	Oxygenase
Hs04260376_m1	*CYP2C9*	Cytochrome P450 family 2 subfamily C polypeptide 9	Cytochrome P450 2C9	Oxidoreductase	Oxygenase
Hs02576168_g1	*CYP2D6*	Cytochrome P450 family 2 subfamily D polypeptide 6	Cytochrome P450 2D6	Oxidoreductase	Oxygenase
Hs00559368_m1	*CYP2E1*	Cytochrome P450 family 2 subfamily E polypeptide 1	Cytochrome P450 2E1	Oxidoreductase	Oxygenase
Hs00908623_m1	*CYP2W1*	Cytochrome P450 family 2, subfamily W polypeptide 1	Cytochrome P450 2W1	Oxidoreductase	Oxygenase
Hs02514989_s1	*CYP3A4*	Cytochrome P450 family 3 subfamily A polypeptide 4	Cytochrome P450 3A4	Oxidoreductase	Oxygenase
Hs01061576_m1	*DHRS2*	Dehydrogenase/reductase 2	Dehydrogenase/reductase SDR family member 2, mitochondrial	Oxidoreductase	Dehydrogenase
Hs01116806_m1	*EPHX1*	Epoxide hydrolase 1, microsomal	Epoxide hydrolase 1	Hydrolase	Esterase
Hs00932305_m1	*EPHX2*	Epoxide hydrolase 2, cytoplasmic	Bifunctional epoxide hydrolase 2	Hydrolase	Esterase
Hs01032912_m1	*FMO1*	Flavin containing monooxygenase 1	Dimethylaniline monooxygenase [N-oxide-forming] 1	Oxidoreductase	Oxygenase
Hs01053455_m1	*FMO3*	Flavin containing monooxygenase 3	Dimethylaniline monooxygenase [N-oxide-forming] 3	Oxidoreductase	Oxygenase

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Hs00157614_m1	*FMO4*	Flavin containing monooxygenase 4	Dimethylaniline monooxygenase [N-oxide-forming] 4	Oxidoreductase	Oxygenase
Hs00173566_m1	*GPX3*	Glutathione peroxidase 3	Glutathione peroxidase 3	Oxidoreductase	Peroxidase
Hs00989769_g1	*GPX4*	Glutathione peroxidase 4	Phospholipid hydroperoxide glutathione peroxidase	Oxidoreductase	Peroxidase
Hs00559733_m1	*GPX5*	Glutathione peroxidase 5	Epididymal secretory glutathione peroxidase	Oxidoreductase	Peroxidase
Hs00167317_m1	*GSR*	Glutathione S-reductase	Glutathione reductase, mitochondrial	Oxidoreductase	Reductase
Hs00165140_m1	*MAOA*	Monoamine oxidase A	Amine oxidase [flavin-containing] A	Oxidoreductase	Oxidase
Hs01106246_m1	*MAOB*	Monoamine oxidase B	Amine oxidase [flavin-containing] B	Oxidoreductase	Oxidase
Hs02512143_s1	*NQO1*	NAD(P)H quinone dehydrogenase 1	NAD(P)H dehydrogenase [quinone] 1	Oxidoreductase	Dehydrogenase
Hs01056948_m1	*NQO2*	NAD(P)H quinone dehydrogenase 2	Ribosyldihydronicotinamide dehydrogenase [quinone]	Oxidoreductase	Dehydrogenase
Hs00165843_m1	*SRD5A2*	Steroid 5 alpha-reductase 2	3-oxo-5-alpha-steroid 4-dehydrogenase 2	Oxidoreductase	Dehydrogenase
Phase II metabolism					
Hs01870672_s1	*CHST1*	Carbohydrate sulfotransferase 1	Carbohydrate sulfotransferase 1	Transferase	Sulfotransferase

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Hs02511558_s1	*COMT*	Catechol-O-methyltransferase	Catechol O-methyltransferase	Transferase	Methyltransferase
Hs00275575_m1	*GSTA1*	Glutathione S-transferase alpha 1	Glutathione S-transferase A1	Transferase	Glutathione S-Transferase
Hs01114170_m1	*GSTK1*	Glutathione S-transferase kappa 1	Glutathione S-transferase kappa 1	Transferase	Glutathione S-Transferase
Hs03045408_m1	*GSTM4*	Glutathione S-transferase mu 4	Glutathione S-transferase Mu 4	Transferase	Glutathione S-Transferase
Hs02383465_s1	*GSTO1*	Glutathione S-transferase omega 1	Glutathione S-transferase omega-1	Transferase	Glutathione S-Transferase
Hs04419827_g1	*GSTP1*	Glutathione S-transferase pi 1	Glutathione S-transferase P	Transferase	Glutathione S-Transferase
Hs01091675_g1	*GSTT1*	Glutathione S-transferase theta 1	Glutathione S-transferase theta-1	Transferase	Glutathione S-Transferase
Hs01041668_m1	*GSTZ1*	Glutathione S-transferase zeta 1	Maleylacetoacetate isomerase	Transferase	Glutathione S-Transferase
Hs00220393_m1	*MGST1*	Microsomal glutathione S-transferase 1	Microsomal glutathione S-transferase 1	Transferase	Glutathione S-Transferase
Hs02511243_s1	*NAT1*	N-acetyltransferase 1	Arylamine N-acetyltransferase 1	Transferase	Acetyltransferase
Hs04194721_s1	*NAT2*	N-acetyltransferase 2	Arylamine N-acetyltransferase 2	Transferase	Acetyltransferase
Hs00196287_m1	*NNMT*	Nicotinamide N-methyltransferase	Nicotinamide N-methyltransferase	Transferase	Methyltransferase

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Hs00738644_m1	*SULT1A1*	Sulfotransferase family 1A member 1	Sulfotransferase 1A1	Transferase	Sulfotransferase
Hs00234899_m1	*SULT1B1*	Sulfotransferase family 1B member 1	Sulfotransferase family cytosolic 1B member 1	Transferase	Sulfotransferase
Hs00602560_m1	*SULT1C2*	Sulfotransferase family 1C member 2	Sulfotransferase 1C2	Transferase	Sulfotransferase
Hs02511055_s1	*UGT1A1*	UDP glucuronosyltransferase family 1 member A1	UDP-glucuronosyltransferase 1A1	Transferase	Glycosyltransferase
Hs04194492_g1	*UGT1A3*	UDP glucuronosyltransferase family 1 member A3	UDP-glucuronosyltransferase 1A3	Transferase	Glycosyltransferase
Hs02512712_s1	*UGT1A6*	UDP glucuronosyltransferase family 1 member A6	UDP-glucuronosyltransferase 1A6	Transferase	Glycosyltransferase
Hs02383831_s1	*UGT2B4*	UDP glucuronosyltransferase family 2 member B4	UDP-glucuronosyltransferase 2B4	Transferase	Glycosyltransferase
Hs00426592_m1	*UGT2B7*	UDP glucuronosyltransferase family 2 member B7	UDP-glucuronosyltransferase 2B7	Transferase	Glycosyltransferase
Transporter proteins					
Hs00240956_m1	*ABCB4 (MDR3)*	ATP binding cassette subfamily B member 4	Multidrug resistance protein 3	Transporter	ABC transporter
Hs00375701_m1	*ABCC10 (MRP7)*	ATP binding cassette subfamily C member 10	Multidrug resistance-associated protein 7	Transporter	ABC transporter
Hs00166123_m1	*ABCC2 (MRP2)*	ATP binding cassette subfamily C member 2	Canalicular multispecific organic anion transporter 1	Transporter	ABC transporter

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Hs01033361_m1	*AQP9*	Aquaporin 9	Aquaporin-9	Transporter	Fluid and solute channel
Hs00161820_m1	*SLC10A1* (NTCP)	Solute carrier family 10 member 1	Sodium/bile acid cotransporter	Transporter	Cation transporter
Hs00427552_m1	*SLC22A1* (OCT1)	Solute carrier family 22 member 1	Solute carrier family 22 member 1	Transporter	Cation transporter
Hs01009568_m1	*SLC22A3* (OCT3)	Solute carrier family 22 member 3	Solute carrier family 22 member 3	Transporter	Cation transporter
Hs00198527_m1	*SLC22A7* (OAT2)	Solute carrier family 22 member 7	Solute carrier family 22 member 7	Transporter	Organic anion transporter
Hs00245360_m1	*SLCO1A2* (OATP1A2)	Solute carrier organic anion transporter family member 1A2	Solute carrier organic anion transporter family member 1A2	Transporter	Organic anion transporter
Hs00272374_m1	*SLCO1B1* (OATP1B1)	Solute carrier organic anion transporter family member 1B1	Solute carrier organic anion transporter family member 1B1	Transporter	Organic anion transporter
Hs00251986_m1	*SLCO1B3* (OATP1B3)	Solute carrier organic anion transporter family member 1B3	Solute carrier organic anion transporter family member 1B3	Transporter	Organic anion transporter
Transcription factors / cofactors					
Hs00907314_m1	*AHR*	Aryl hydrocarbon receptor	Aryl hydrocarbon receptor	Transcription factor	Basic helix-loop-helix transcription factor
Hs01123657_m1	*ARNT*	Aryl hydrocarbon receptor nuclear translocator	Aryl hydrocarbon receptor nuclear translocator	Transcription factor	Basic helix-loop-helix transcription factor

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Hs00269972_s1	*CEBPA*	CCAAT enhancer binding protein alpha	CCAAT/enhancer-binding protein alpha	Transcription factor	CCAAT/enhancer binding proteins
Hs00231733_m1	*CREBBP*	CREB binding protein	CREB-binding protein	Transcription cofactor	Lysine acetyltransferases
Hs00609747_m1	*DBP*	D-box binding PAR bZIP transcription factor	D site-binding protein	Transcription factor	Basic leucine zipper transcription factors
Hs00914223_m1	*EP300*	E1A binding protein p300	Histone acetyltransferase p300	Transcription cofactor	Lysine acetyltransferases
Hs04187555_m1	*FOXA1*	Forkhead box A1	Hepatocyte nuclear factor 3-alpha	Transcription factor	Forkhead box transcription factors
Hs00232764_m1	*FOXA2*	Forkhead box A2	Hepatocyte nuclear factor 3-beta	Transcription factor	Forkhead box transcription factors
Hs00270130_m1	*FOXA3*	Forkhead box A3	Hepatocyte nuclear factor 3-gamma	Transcription factor	Forkhead box transcription factors
Hs00167041_m1	*HNF1A*	HNF1 homeobox A	Hepatocyte nuclear factor 1-alpha	Transcription factor	Homeoboxes (HNF class)
Hs00230853_m1	*HNF4A*	Hepatocyte nuclear factor 4 alpha	Hepatocyte nuclear factor 4-alpha	Transcription factor	Nuclear hormone receptor
Hs00186661_m1	*NCOA1*	Nuclear receptor coactivator 1	Nuclear receptor coactivator 1	Transcription cofactor	Lysine acetyltransferases
Hs00975961_g1	*NFE2L2*	Nuclear factor, erythroid 2 like 2	Nuclear factor erythroid 2-related factor 2	Transcription factor	Basic leucine zipper transcription factors
Hs00222677_m1	*NR0B2*	Nuclear receptor subfamily 0 group B member 2	Small Heterodimer Partner	Transcription factor	Nuclear hormone receptor

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Hs01045844_m1	*VDR*	Vitamin D receptor	Vitamin D3 receptor	Transcription factor	Nuclear hormone receptor
Hs01114267_m1	*NR1I2*	Nuclear receptor subfamily 1 group I member 2	Pregnane X receptor	Transcription factor	Nuclear hormone receptor
Hs00901571_m1	*NR1I3*	Nuclear receptor subfamily 1 group I member 3	Constitutive androstane receptor	Transcription factor	Nuclear hormone receptor
Hs00172885_m1	*NR1H3*	Nuclear receptor subfamily 1 group H member 3	Liver X receptor-alpha	Transcription factor	Nuclear hormone receptor
Hs01026590_m1	*NR1H4*	Nuclear receptor subfamily 1 group H member 4	Farnesoid X receptor	Transcription factor	Nuclear hormone receptor
Hs00353740_m1	*NR3C1*	Nuclear receptor subfamily 3 group C member 1	Glucocorticoid receptor	Transcription factor	Nuclear hormone receptor
Hs00892378_m1	*NR5A2*	Nuclear receptor subfamily 5 group A member 2	Liver Receptor Homolog-1	Transcription factor	Nuclear hormone receptor
Hs00191477_m1	*ONECUT2*	One cut homeobox 2	Hepatocyte nuclear factor 6-beta	Transcription factor	Homeoboxes (CUT class)
Hs00947536_m1	*PPARA*	Peroxisome proliferator activated receptor alpha	Peroxisome proliferator-activated receptor alpha	Transcription factor	Nuclear hormone receptor
Hs01016719_m1	*PPARGC1A*	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha	Transcription cofactor	RNA binding motif containing
Hs00536545_m1	*RORA*	RAR related orphan receptor A	Nuclear receptor ROR-alpha	Transcription factor	Nuclear hormone receptor
Hs01067636_m1	*RXRA*	Retinoid X receptor alpha	Retinoic acid receptor RXR-alpha	Transcription factor	Nuclear hormone receptor

Table A.2 (Continued)

Gene Expression Assays ID	Gene Symbol	Gene Name	Protein name	Class	Group
Endogenous controls					
Hs99999903_m1	*ACTB*	Actin beta	Actin, cytoplasmic 1	Cytoskeletal protein	Actin family cytoskeletal protein
Hs99999905_m1	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	Dehydrogenase
Hs99999909_m1	*HPRT1*	Hypoxanthine phosphoribosyltransferase 1	Hypoxanthine-guanine phosphoribosyltransferase	Transferase	Glycosyltransferase
Hs99999906_m1	*PGK1*	Phosphoglycerate kinase 1	Phosphoglycerate kinase 1	Kinase	Carbohydrate kinase

A.3 Results of Gene Expression Analyses

A.3.1 Basal expression of liver-specific genes involved in the metabolism of xenobiotics in hepatic tumor-derived cell lines and primary hepatocytes

Table A.3: The basal expression levels of selected liver-specific genes involved in the metabolism of xenobiotics in primary human hepatocytes of donor II and human hepatic cancer cell lines HepG2 and HuH6 relative to primary human hepatocytes of donor I.

Gene symbol	pHep I	pHep II			HepG2			HuH6		
	Rq	Rq	±	SD	Rq	±	SD	Rq	±	SD
Phase I metabolism										
ADH1C	1.00	**0.65**	±	0.08	N.E.	±		N.E.	±	
ADH4	1.00	1.16	±	0.14	**0.62**	±	0.06	**0.00**	±	0.00
ALDH1A1	1.00	**2.66**	±	0.25	**0.43**	±	0.04	**0.01**	±	0.00
ALDH3A1	1.00	1.39	±	0.32	1.23	±	0.48	**0.55**	±	0.10
CES1	1.00	**1.53**	±	0.18	**0.10**	±	0.01	**0.07**	±	0.01
CES5A	1.00	**1.61**	±	0.40	N.E.	±		N.E.	±	
CYP1A1	1.00	2.82	±	2.68	0.87	±	0.80	1.96	±	1.82
CYP1A2	1.00	**3.71**	±	1.53	**0.01**	±	0.01	**0.01**	±	0.01
CYP2B6	1.00	**1.75**	±	0.61	**0.00**	±	0.00	**0.25**	±	0.08
CYP2C18	1.00	0.82	±	0.19	N.E.	±		N.E.	±	
CYP2C19	1.00	**3.01**	±	1.52	N.E.	±		N.E.	±	
CYP2C8	1.00	**0.64**	±	0.07	**0.01**	±	0.00	N.E.	±	
CYP2C9	1.00	1.32	±	0.11	N.E.	±		N.E.	±	
CYP2D6	1.00	**2.06**	±	0.17	**0.05**	±	0.01	**0.06**	±	0.02
CYP2E1	1.00	0.70	±	0.13	**0.00**	±	0.00	**0.00**	±	0.00
CYP2W1	N.E.	N.E.	±		↑	±		↑	±	
CYP3A4	1.00	**0.14**	±	0.05	**0.02**	±	0.01	N.E.	±	
DHRS2	1.00	**0.04**	±	0.02	**1027.89**	±	106.98	**0.28**	±	0.05
EPHX1	1.00	1.18	±	0.30	**0.34**	±	0.07	**0.20**	±	0.03
EPHX2	1.00	1.14	±	0.10	0.97	±	0.05	1.23	±	0.03
FMO1	1.00	**0.54**	±	0.09	N.E.	±		**23.61**	±	4.55
FMO3	1.00	0.76	±	0.25	N.E.	±		N.E.	±	
FMO4	1.00	**1.81**	±	0.41	**0.06**	±	0.01	0.91	±	0.12
GPX3	1.00	0.81	±	0.13	**0.54**	±	0.04	**0.23**	±	0.02
GPX4	1.00	0.80	±	0.01	**0.65**	±	0.14	**0.31**	±	0.10
GPX5	N.E.	N.E.	±		N.E.	±		N.E.	±	
GSR	1.00	**2.21**	±	0.24	**1.53**	±	0.37	**0.31**	±	0.04
MAOA	1.00	**1.93**	±	0.06	**0.07**	±	0.01	**0.00**	±	0.00
MAOB	1.00	**2.06**	±	0.64	**0.45**	±	0.11	**0.03**	±	0.00

Table A.3. (Continued)

Gene symbol	pHep I	pHep II			HepG2			HuH6		
	Rq	Rq	±	SD	Rq	±	SD	Rq	±	SD
NQO1	1.00	**2.03**	±	0.46	**120.62**	±	23.13	**0.12**	±	0.03
NQO2	1.00	0.73	±	0.22	**0.27**	±	0.06	0.80	±	0.10
SRD5A2	1.000	**3.06**	±	1.39	N.E.	±		N.E.	±	
Phase II metabolism										
CHST1	1.00	**0.44**	±	0.18	N.E.	±		N.E.	±	
COMT	1.00	0.78	±	0.05	**0.62**	±	0.07	**0.10**	±	0.04
GSTA1	1.00	1.47	±	0.17	**0.00**	±	0.00	**0.03**	±	0.00
GSTK1	1.00	**2.04**	±	0.69	**0.39**	±	0.11	**0.51**	±	0.09
GSTM4	1.00	**1.55**	±	0.13	**0.46**	±	0.09	**0.27**	±	0.06
GSTO1	1.00	0.95	±	0.06	0.73	±	0.09	**0.53**	±	0.08
GSTP1	1.00	2.56	±	2.50	**0.11**	±	0.10	**413.91**	±	351.09
GSTT1	1.00	0.88	±	0.26	1.48	±	0.31	N.E.	±	
GSTZ1	1.00	1.11	±	0.31	**0.47**	±	0.12	0.71	±	0.05
MGST1	1.00	1.12	±	0.19	**0.09**	±	0.02	**0.04**	±	0.01
NAT1	1.00	1.11	±	0.13	**0.60**	±	0.03	**0.23**	±	0.01
NAT2	1.00	1.05	±	0.21	**0.00**	±	0.00	N.E.	±	
NNMT	1.00	0.81	±	0.22	N.E.	±		**0.00**	±	0.00
SULT1A1	1.00	0.90	±	0.40	N.E.	±		N.E.	±	
SULT1B1	1.00	0.87	±	0.23	**0.00**	±	0.00	N.E.	±	
SULT1C2	N.E.	↑	±		↑	±		↑	±	
UGT1A1	1.00	1.18	±	0.22	**0.00**	±	0.00	**0.00**	±	0.00
UGT1A3	1.00	1.27	±	0.71	**0.00**	±	0.00	**0.00**	±	0.00
UGT1A6	1.00	1.44	±	0.25	**0.00**	±	0.00	**0.00**	±	0.00
UGT2B4	1.00	**0.35**	±	0.01	**0.00**	±	0.00	N.E.	±	
UGT2B7	1.00	1.22	±	0.12	**0.03**	±	0.00	N.E.	±	
Transporter proteins										
ABCB4	1.00	**3.08**	±	1.18	**0.37**	±	0.16	**0.19**	±	0.04
ABCC10	1.00	**2.23**	±	1.04	**2.88**	±	1.41	0.77	±	0.36
ABCC2	1.00	**2.24**	±	0.21	0.96	±	0.10	**0.11**	±	0.01
AQP9	1.00	0.75	±	0.13	N.E.	±		N.E.	±	
SLC10A1	1.00	**1.52**	±	0.21	**0.00**	±	0.00	**0.00**	±	0.00
SLC22A1	1.00	**0.56**	±	0.13	**0.01**	±	0.00	**0.00**	±	0.00
SLC22A3	1.00	**2.35**	±	0.29	**0.31**	±	0.04	N.E.	±	
SLC22A7	1.00	**2.13**	±	0.14	**0.29**	±	0.04	N.E.	±	
SLCO1A2	1.00	**0.31**	±	0.22	N.E.	±		**53.35**	±	54.44
SLCO1B1	1.00	1.33	±	0.06	N.E.	±		**0.01**	±	0.00
SLCO1B3	1.00	**0.10**	±	0.01	N.E.	±		N.E.	±	
Transcription factors / cofactors										
AHR	1.00	0.71	±	0.39	1.01	±	0.45	**0.31**	±	0.07

Table A.3. (Continued)

Gene symbol	pHep I	pHep II			HepG2			HuH6		
	Rq	Rq	±	SD	Rq	±	SD	Rq	±	SD
ARNT	1.00	**1.66**	±	0.42	0.77	±	0.27	**0.53**	±	0.12
CEBPA	1.00	**1.62**	±	0.72	**7.54**	±	2.99	**4.37**	±	1.94
CREBBP	1.00	**0.40**	±	0.19	**1.71**	±	0.84	**1.69**	±	0.57
DBP	1.00	**0.49**	±	0.11	**3.61**	±	1.17	**4.03**	±	0.54
EP300	1.00	1.39	±	0.59	**2.24**	±	1.03	**1.75**	±	0.46
FOXA1	1.00	1.48	±	0.29	**7.36**	±	1.20	**1.80**	±	0.22
FOXA2	1.00	**1.53**	±	0.49	1.41	±	0.67	**3.29**	±	0.93
FOXA3	1.00	**0.64**	±	0.23	1.29	±	0.64	**1.59**	±	0.54
HNF1A	1.00	2.23	±	1.29	**2.75**	±	2.20	**2.43**	±	1.77
HNF4A	1.00	**2.63**	±	1.33	**3.08**	±	2.17	1.10	±	0.60
NCOA1	1.00	0.86	±	0.20	**0.60**	±	0.03	1.39	±	0.02
NFE2L2	1.00	0.90	±	0.14	**3.86**	±	0.10	**1.81**	±	0.28
NR0B2	1.00	1.14	±	0.10	**4.37**	±	0.26	**0.12**	±	0.02
VDR	1.00	1.53	±	1.17	0.70	±	0.57	**2.06**	±	1.61
NR1I2	1.00	**1.81**	±	0.49	**0.36**	±	0.11	**0.01**	±	0.00
NR1I3	1.00	**2.01**	±	0.42	**0.03**	±	0.01	**0.02**	±	0.00
NR1H3	1.00	1.08	±	0.38	0.72	±	0.08	**0.41**	±	0.03
NR1H4	1.00	**0.63**	±	0.19	**0.10**	±	0.03	**0.00**	±	0.00
NR3C1	1.00	0.88	±	0.27	0.86	±	0.14	**0.07**	±	0.00
NR5A2	1.00	1.06	±	0.13	**0.08**	±	0.01	**0.28**	±	0.03
ONECUT2	1.00	**1.68**	±	0.56	0.92	±	0.38	**0.22**	±	0.06
PPARA	1.00	**2.11**	±	0.79	**0.34**	±	0.14	**0.17**	±	0.04
PPARAGC1A	1.00	0.76	±	0.17	**0.35**	±	0.10	N.E.	±	
RORA	1.00	0.86	±	0.15	**0.10**	±	0.03	**0.20**	±	0.07
RXRA	1.00	1.62	±	0.83	**0.48**	±	0.30	**0.52**	±	0.22

DMSO-treated control cells were used to calculate basal gene expression levels. The results are presented as relative mRNA expression (Rq) in pHepII, HepG2 cells or HuH6 cells normalized to the mRNA expression in pHepI. Data are means ± SD of three independent experiments. Bold font denotes gene expressed in higher levels (Rq ≥ 1.5) or lower levels (Rq ≤ 0.67). N.E. – not expressed. ↑ – N.E. in pHepI and expressed in pHepII/HepG2/HuH6.

A.3.2 Differential expression of liver-specific genes involved in the metabolism of xenobiotics in hepatic tumor-derived cell lines and primary hepatocytes after the exposure to B(a)P

Table A.4: Relative expression of selected liver-specific genes involved in the metabolism of xenobiotics after the 24 h exposure to B(a)P (10.0 µM) in primary human hepatocytes (donor I and donor II) and human hepatic cancer cell lines HepG2 and HuH6.

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
Phase I metabolism												
ADH1C	**0.63**	±	0.16	**0.58**	±	0.02	N.E.	±		N.E.	±	
ADH4	1.33	±	0.24	0.96	±	0.07	0.68	±	0.10	0.81	±	0.15
ALDH1A1	0.90	±	0.09	**0.65**	±	0.06	0.76	±	0.10	0.89	±	0.02
ALDH3A1	**72.13**	±	25.01	**58.70**	±	10.42	**879.94**	±	47.06	**8.75**	±	1.23
CES1	1.21	±	0.07	1.20	±	0.19	**2.04**	±	0.11	**1.76**	±	0.10
CES5A	1.39	±	0.49	0.91	±	0.11	↑	±		↑	±	
CYP1A1	**712.85**	±	647.10	**233.72**	±	21.01	**929.63**	±	129.16	**84.03**	±	4.84
CYP1A2	**1088.56**	±	356.19	**176.96**	±	17.67	**85.65**	±	90.17	**30.75**	±	22.19
CYP2B6	**7.03**	±	1.39	**1.98**	±	0.16	**4.02**	±	0.98	1.44	±	0.14
CYP2C18	1.23	±	0.25	1.22	±	0.12	N.E.	±		N.E.	±	
CYP2C19	1.31	±	0.15	0.93	±	0.05	N.E.	±		N.E.	±	
CYP2C8	1.11	±	0.24	0.79	±	0.07	↓	±		N.E.	±	
CYP2C9	**1.79**	±	0.17	1.16	±	0.19	N.E.	±		N.E.	±	
CYP2D6	1.45	±	0.12	1.34	±	0.16	1.19	±	0.02	0.82	±	0.15
CYP2E1	0.93	±	0.01	1.06	±	0.15	**0.34**	±	0.03	**0.41**	±	0.26
CYP2W1	N.E.	±		N.E.	±		**0.46**	±	0.09	**1.53**	±	0.36
CYP3A4	**2.06**	±	0.87	**1.94**	±	0.36	↓	±		N.E.	±	
DHRS2	**2.75**	±	0.19	**2.28**	±	0.55	**0.46**	±	0.04	**3.48**	±	0.47
EPHX1	1.11	±	0.16	1.43	±	0.10	**4.05**	±	0.20	1.42	±	0.06
EPHX2	1.07	±	0.11	0.97	±	0.09	**0.51**	±	0.02	1.04	±	0.15
FMO1	**0.62**	±	0.17	**0.57**	±	0.06	N.E.	±		**0.30**	±	0.03
FMO3	1.02	±	0.14	0.88	±	0.13	N.E.	±		N.E.	±	
FMO4	1.13	±	0.21	0.73	±	0.04	**0.49**	±	0.05	0.95	±	0.11
GPX3	**1.60**	±	0.09	**1.94**	±	0.13	**1.85**	±	0.13	1.18	±	0.10
GPX4	0.90	±	0.33	1.22	±	0.11	0.99	±	0.12	1.17	±	0.10
GPX5	N.E.	±		N.E.	±		N.E.	±		N.E.	±	
GSR	**1.56**	±	0.11	1.24	±	0.15	**2.01**	±	0.05	**2.09**	±	0.21
MAOA	0.77	±	0.03	**0.55**	±	0.03	0.86	±	0.09	0.69	±	0.06
MAOB	1.10	±	0.11	0.81	±	0.07	1.37	±	0.10	**1.55**	±	0.13
NQO1	**2.47**	±	0.21	**1.71**	±	0.48	**4.15**	±	0.38	**2.85**	±	0.62
NQO2	1.32	±	0.13	1.11	±	0.10	**2.80**	±	0.00	1.14	±	0.03
SRD5A2	0.78	±	0.05	0.71	±	0.05	N.E.	±		N.E.	±	
Phase II metabolism												
CHST1	**2.60**	±	0.37	1.46	±	0.44	↑	±		↑	±	
COMT	0.88	±	0.26	1.04	±	0.10	1.19	±	0.17	1.40	±	0.22
GSTA1	1.28	±	0.18	1.17	±	0.02	**0.36**	±	0.01	**0.59**	±	0.08
GSTK1	0.96	±	0.07	0.89	±	0.08	1.35	±	0.04	1.07	±	0.07

Table A.4. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
GSTM4	1.08	±	0.14	0.82	±	0.08	0.83	±	0.11	1.04	±	0.05
GSTO1	1.10	±	0.20	1.27	±	0.11	**1.76**	±	0.34	1.37	±	0.08
GSTP1	0.87	±	0.62	0.97	±	0.21	**73.02**	±	6.71	1.49	±	0.16
GSTT1	0.94	±	0.12	1.06	±	0.04	**2.35**	±	0.26	N.E.	±	
GSTZ1	1.22	±	0.13	0.89	±	0.03	1.49	±	0.09	1.02	±	0.04
MGST1	1.15	±	0.08	1.08	±	0.04	**3.57**	±	0.13	**1.62**	±	0.10
NAT1	1.36	±	0.12	1.18	±	0.14	**4.05**	±	0.28	1.27	±	0.12
NAT2	1.48	±	0.24	1.37	±	0.23	1.27	±	0.05	↑	±	
NNMT	1.01	±	0.15	0.72	±	0.06	N.E.	±		**5.95**	±	0.79
SULT1A1	1.11	±	0.37	**0.57**	±	0.06	N.E.	±		N.E.	±	
SULT1B1	0.87	±	0.16	**0.66**	±	0.08	↓	±		N.E.	±	
SULT1C2	N.E.	±		1.04	±	0.18	**0.53**	±	0.11	1.39	±	0.26
UGT1A1	**5.91**	±	0.69	**5.88**	±	0.28	**13.47**	±	0.49	**2.05**	±	0.25
UGT1A3	**1.57**	±	0.30	1.48	±	0.11	**12.06**	±	0.83	1.16	±	0.22
UGT1A6	0.90	±	0.06	0.84	±	0.09	1.34	±	0.16	**2.39**	±	0.96
UGT2B4	1.04	±	0.24	**2.83**	±	0.15	↓	±		N.E.	±	
UGT2B7	1.33	±	0.11	**1.68**	±	0.20	**4.36**	±	0.42	N.E.	±	
Transporter proteins												
ABCB4	1.18	±	0.14	1.05	±	0.03	0.91	±	0.04	0.92	±	0.12
ABCC10	1.39	±	0.16	1.03	±	0.07	**1.54**	±	0.15	1.25	±	0.16
ABCC2	1.25	±	0.05	0.88	±	0.09	**1.53**	±	0.12	**2.54**	±	0.28
AQP9	0.99	±	0.07	0.82	±	0.06	N.E.	±		N.E.	±	
SLC10A1	0.80	±	0.13	**0.53**	±	0.06	↓	±		**0.14**	±	0.07
SLC22A1	0.89	±	0.13	0.87	±	0.03	**3.02**	±	0.60	**1.86**	±	1.44
SLC22A3	0.84	±	0.03	0.80	±	0.03	**0.17**	±	0.01	N.E.	±	
SLC22A7	0.83	±	0.06	0.71	±	0.10	**0.07**	±	0.03	N.E.	±	
SLCO1A2	0.74	±	0.70	↓	±		N.E.	±		**0.57**	±	0.01
SLCO1B1	0.74	±	0.05	0.74	±	0.04	N.E.	±		1.06	±	0.17
SLCO1B3	**0.60**	±	0.06	**0.48**	±	0.34	N.E.	±		N.E.	±	
Transcription factors / cofactors												
AHR	0.91	±	0.17	1.04	±	0.16	**2.27**	±	0.18	**1.98**	±	0.55
ARNT	1.25	±	0.09	0.90	±	0.03	1.20	±	0.04	0.97	±	0.13
CEBPA	**2.03**	±	0.54	1.07	±	0.04	**0.41**	±	0.04	1.05	±	0.10
CREBBP	**0.33**	±	0.05	1.11	±	0.16	**0.31**	±	0.02	**0.33**	±	0.04
DBP	1.01	±	0.06	0.77	±	0.18	**0.13**	±	0.01	**0.43**	±	0.07
EP300	1.15	±	0.13	1.18	±	0.04	1.23	±	0.06	1.21	±	0.15
FOXA1	1.08	±	0.05	1.04	±	0.07	0.78	±	0.02	**0.65**	±	0.05
FOXA2	1.14	±	0.14	1.05	±	0.06	**1.70**	±	0.08	0.99	±	0.13
FOXA3	0.84	±	0.15	0.99	±	0.17	1.34	±	0.14	1.09	±	0.14
HNF1A	**1.85**	±	0.16	1.27	±	0.20	0.69	±	0.10	1.21	±	0.23

Table A.4. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
HNF4A	**1.92**	±	0.02	0.93	±	0.09	0.75	±	0.04	1.05	±	0.11
NCOA1	0.78	±	0.05	**0.62**	±	0.05	**0.22**	±	0.02	**0.52**	±	0.02
NFE2L2	**1.76**	±	0.24	**1.95**	±	0.10	1.34	±	0.12	1.12	±	0.09
NR0B2	**4.09**	±	0.35	**5.27**	±	0.43	**7.72**	±	0.67	**2.43**	±	0.25
VDR	**2.97**	±	1.05	1.30	±	0.11	**3.13**	±	0.33	**4.05**	±	0.23
NR1I2	**1.99**	±	0.09	1.36	±	0.20	**2.06**	±	0.14	0.92	±	0.24
NR1I3	**10.13**	±	2.16	**3.57**	±	0.34	0.90	±	0.22	**2.01**	±	0.58
NR1H3	**1.66**	±	0.21	1.22	±	0.19	**1.71**	±	0.04	1.30	±	0.09
NR1H4	**0.58**	±	0.03	0.73	±	0.06	**0.03**	±	0.00	↓	±	
NR3C1	**1.52**	±	0.24	**1.68**	±	0.62	**2.44**	±	0.19	**2.77**	±	0.11
NR5A2	1.04	±	0.21	1.24	±	0.06	**1.75**	±	0.07	0.94	±	0.03
ONECUT2	1.20	±	0.17	1.01	±	0.13	1.03	±	0.10	1.40	±	0.13
PPARA	1.37	±	0.18	0.87	±	0.11	0.79	±	0.09	0.97	±	0.21
PPARAGC1A	0.94	±	0.08	**0.65**	±	0.02	1.26	±	0.09	N.E.	±	
RORA	0.86	±	0.21	0.92	±	0.04	1.02	±	0.03	**1.60**	±	0.35
RXRA	1.11	±	0.15	**0.57**	±	0.12	**0.29**	±	0.06	**0.67**	±	0.13

The results are presented as relative mRNA expression (Rq) in B(a)P exposed cells normalized to the mRNA expression in vehicle control cells. Data are means ± SD of three independent experiments. Bold font denotes deregulated gene: Rq ≥ 1.5 indicates up-regulation of gene, Rq ≤ 0.67 indicates down-regulation of gene. N.E. – not expressed. ↑ – up-regulated gene (N.E. in vehicle control cells and expressed in cells after exposure to B(a)P). ↓ – down-regulated gene (expressed in vehicle control cells and N.E. in cells after exposure to B(a)P).

A.3.3 Differential expression of liver-specific genes involved in the metabolism of xenobiotics in hepatic tumor-derived cell lines and primary hepatocytes after the exposure to AFB_1

Table A.5: Relative expression of selected liver-specific genes involved in the metabolism of xenobiotics after the 24 h exposure to AFB_1 (3.2 µM) in primary human hepatocytes (donor I and donor II) and human hepatic cancer cell lines HepG2 and HuH6.

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
Phase I metabolism												
ADH1C	**0.59**	±	0.18	0.73	±	0.13	N.E	±		N.E	±	
ADH4	0.85	±	0.07	1.17	±	0.09	0.72	±	0.06	**0.60**	±	0.25

Table A.5. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
ALDH1A1	**0.47**	±	0.05	**0.65**	±	0.02	**0.64**	±	0.07	0.88	±	0.04
ALDH3A1	0.97	±	0.20	1.33	±	0.15	**1.64**	±	0.39	0.76	±	0.07
CES1	1.30	±	0.07	1.22	±	0.17	1.32	±	0.10	1.22	±	0.10
CES5A	1.11	±	0.31	1.08	±	0.16	↑	±		N.E	±	
CYP1A1	**0.44**	±	0.39	0.68	±	0.11	**1.88**	±	0.08	1.45	±	0.18
CYP1A2	**4.08**	±	0.23	**1.52**	±	0.25	**4.25**	±	4.85	1.41	±	0.90
CYP2B6	**1.62**	±	0.36	0.71	±	0.16	**2.55**	±	1.40	1.49	±	0.11
CYP2C18	1.41	±	0.30	**2.07**	±	0.44	N.E	±		N.E	±	
CYP2C19	1.31	±	0.58	1.25	±	0.36	N.E	±		N.E	±	
CYP2C8	1.15	±	0.07	0.86	±	0.14	0.73	±	0.14	N.E	±	
CYP2C9	0.92	±	0.12	1.12	±	0.10	↑	±		↑	±	
CYP2D6	**2.91**	±	0.26	**1.62**	±	0.17	**1.83**	±	0.19	1.28	±	0.55
CYP2E1	**1.78**	±	0.15	1.11	±	0.13	0.73	±	0.14	**0.59**	±	0.12
CYP2W1	N.E	±		N.E	±		0.70	±	0.16	1.33	±	0.35
CYP3A4	**0.54**	±	0.19	1.01	±	0.45	1.16	±	0.55	N.E	±	
DHRS2	**3.18**	±	0.54	**2.03**	±	0.49	**0.61**	±	0.11	**2.53**	±	0.10
EPHX1	1.05	±	0.13	1.24	±	0.08	1.22	±	0.22	1.15	±	0.06
EPHX2	0.88	±	0.13	1.03	±	0.13	1.04	±	0.09	**1.53**	±	0.15
FMO1	**0.45**	±	0.43	↓	±		N.E	±		1.24	±	0.24
FMO3	0.80	±	0.18	1.07	±	0.20	N.E	±		N.E	±	
FMO4	0.77	±	0.09	0.85	±	0.05	1.42	±	0.29	**1.71**	±	0.19
GPX3	**3.01**	±	0.45	**2.07**	±	0.44	1.12	±	0.06	1.28	±	0.08
GPX4	1.49	±	0.54	1.35	±	0.37	0.90	±	0.24	1.14	±	0.14
GPX5	N.E	±		N.E	±		N.E	±		N.E	±	
GSR	1.40	±	0.11	1.23	±	0.07	0.95	±	0.12	1.30	±	0.12
MAOA	0.73	±	0.04	0.81	±	0.07	1.26	±	0.12	1.12	±	0.03
MAOB	0.67	±	0.13	0.77	±	0.04	0.95	±	0.16	1.32	±	0.09
NQO1	1.22	±	0.13	0.99	±	0.30	0.81	±	0.17	1.08	±	0.19
NQO2	0.78	±	0.12	0.86	±	0.10	0.94	±	0.17	0.98	±	0.02
SRD5A2	0.78	±	0.05	0.94	±	0.25	N.E.	±		N.E	±	
Phase II metabolism												
CHST1	**5.35**	±	0.73	**1.98**	±	0.30	↑	±		N.E	±	
COMT	1.19	±	0.40	1.34	±	0.12	1.08	±	0.21	1.40	±	0.11
GSTA1	0.79	±	0.10	0.82	±	0.03	**0.41**	±	0.06	1.09	±	0.18
GSTK1	1.21	±	0.20	1.04	±	0.19	1.03	±	0.21	1.05	±	0.04
GSTM4	**0.42**	±	0.09	**0.51**	±	0.02	0.68	±	0.18	0.92	±	0.04
GSTO1	1.22	±	0.27	1.29	±	0.20	1.18	±	0.26	1.34	±	0.09
GSTP1	1.24	±	1.07	0.80	±	0.13	1.18	±	0.34	1.06	±	0.07
GSTT1	1.14	±	0.09	1.13	±	0.08	1.43	±	0.11	N.E	±	
GSTZ1	0.94	±	0.10	1.00	±	0.09	1.08	±	0.24	1.08	±	0.02

Table A.5. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
MGST1	1.04	±	0.09	0.98	±	0.08	0.87	±	0.18	1.16	±	0.11
NAT1	**1.66**	±	0.21	1.38	±	0.15	1.16	±	0.03	1.20	±	0.12
NAT2	1.12	±	0.18	1.25	±	0.07	1.25	±	0.32	N.E	±	
NNMT	0.80	±	0.01	**1.57**	±	0.21	N.E	±		**1.67**	±	0.15
SULT1A1	1.33	±	0.70	**0.60**	±	0.10	N.E	±		N.E	±	
SULT1B1	**0.33**	±	0.10	0.76	±	0.22	1.38	±	0.44	N.E	±	
SULT1C2	N.E	±		1.22	±	0.28	0.69	±	0.13	0.92	±	0.10
UGT1A1	1.15	±	0.20	1.20	±	0.07	1.19	±	0.33	0.96	±	0.36
UGT1A3	1.19	±	0.43	1.10	±	0.26	1.34	±	0.23	1.33	±	0.15
UGT1A6	0.70	±	0.11	0.86	±	0.20	**0.63**	±	0.09	0.84	±	0.16
UGT2B4	**0.59**	±	0.15	**1.52**	±	0.48	**1.52**	±	0.41	N.E	±	
UGT2B7	**2.63**	±	0.17	**2.19**	±	0.25	**3.09**	±	0.13	N.E	±	
Transporter proteins												
ABCB4	**1.60**	±	0.43	1.06	±	0.25	1.05	±	0.33	1.20	±	0.05
ABCC10	1.38	±	0.36	1.27	±	0.33	1.20	±	0.37	1.36	±	0.24
ABCC2	0.75	±	0.08	0.74	±	0.05	1.23	±	0.10	**1.67**	±	0.08
AQP9	1.16	±	0.13	1.09	±	0.05	N.E	±		N.E	±	
SLC10A1	**0.39**	±	0.08	**0.53**	±	0.07	0.85	±	0.75	**0.60**	±	0.08
SLC22A1	1.10	±	0.11	0.99	±	0.15	**1.58**	±	0.42	**2.27**	±	1.80
SLC22A3	0.85	±	0.10	0.95	±	0.09	**0.55**	±	0.02	N.E	±	
SLC22A7	**1.54**	±	0.19	0.84	±	0.06	**0.19**	±	0.02	N.E	±	
SLCO1A2	**0.55**	±	0.40	0.75	±	0.19	N.E	±		1.22	±	0.13
SLCO1B1	**0.51**	±	0.04	0.74	±	0.11	N.E	±		1.12	±	0.14
SLCO1B3	0.69	±	0.14	0.77	±	0.14	N.E	±		N.E	±	
Transcription factors / cofactors												
AHR	1.39	±	0.45	1.18	±	0.47	**2.02**	±	0.81	1.41	±	0.14
ARNT	1.20	±	0.14	1.11	±	0.18	0.94	±	0.25	1.08	±	0.02
CEBPA	1.37	±	0.20	1.43	±	0.27	0.75	±	0.09	1.26	±	0.13
CREBBP	**0.32**	±	0.08	1.28	±	0.26	**0.38**	±	0.17	**0.36**	±	0.06
DBP	**0.54**	±	0.14	0.94	±	0.24	**0.60**	±	0.13	0.89	±	0.11
EP300	1.09	±	0.28	1.28	±	0.25	1.06	±	0.39	1.05	±	0.11
FOXA1	1.22	±	0.20	1.38	±	0.10	0.90	±	0.15	1.11	±	0.13
FOXA2	1.05	±	0.09	1.27	±	0.23	1.27	±	0.35	1.17	±	0.15
FOXA3	**1.60**	±	0.43	**1.75**	±	0.60	0.96	±	0.32	1.19	±	0.17
HNF1A	**1.54**	±	0.43	1.32	±	0.58	1.16	±	0.49	**1.54**	±	0.39
HNF4A	0.85	±	0.17	0.89	±	0.21	0.74	±	0.31	1.01	±	0.14
NCOA1	**0.65**	±	0.03	0.68	±	0.06	0.85	±	0.06	0.96	±	0.05
NFE2L2	1.15	±	0.05	1.49	±	0.15	1.24	±	0.12	1.14	±	0.13
NR0B2	**3.76**	±	0.10	**3.86**	±	0.23	**3.31**	±	0.48	**2.57**	±	0.43
VDR	**2.46**	±	0.64	0.99	±	0.35	**2.86**	±	0.86	**2.06**	±	0.33

Table A.5. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
NR1I2	0.98	±	0.18	1.24	±	0.29	1.02	±	0.12	0.87	±	0.13
NR1I3	**1.61**	±	0.29	1.33	±	0.18	0.80	±	0.08	1.41	±	0.24
NR1H3	0.80	±	0.11	0.93	±	0.28	0.84	±	0.16	0.86	±	0.08
NR1H4	**0.47**	±	0.04	0.95	±	0.11	**0.32**	±	0.06	↓	±	
NR3C1	1.46	±	0.25	**1.60**	±	0.78	1.48	±	0.12	**1.55**	±	0.06
NR5A2	0.77	±	0.13	1.07	±	0.23	0.79	±	0.15	1.19	±	0.13
ONECUT2	**1.58**	±	0.22	1.39	±	0.05	0.96	±	0.31	1.21	±	0.13
PPARA	0.73	±	0.17	0.75	±	0.10	1.01	±	0.27	1.16	±	0.08
PPARAGC1A	1.14	±	0.24	1.17	±	0.23	1.09	±	0.31	N.E		
RORA	0.99	±	0.23	1.30	±	0.22	1.24	±	0.32	1.35	±	0.08
RXRA	1.04	±	0.34	0.99	±	0.36	0.72	±	0.26	1.02	±	0.17

The results are presented as relative mRNA expression (Rq) in AFB_1 exposed cells normalized to the mRNA expression in vehicle control cells. Data are means ± SD of three independent experiments. Bold font denotes deregulated gene: Rq ≥ 1.5 indicates up-regulation of gene, Rq ≤ 0.67 indicates down-regulation of gene. N.E. – not expressed. ↑ – up-regulated gene (N.E. in vehicle control cells and expressed in cells after exposure to AFB_1). ↓ – down-regulated gene (expressed in vehicle control cells and N.E. in cells after exposure to AFB_1).

A.3.4 Differential expression of liver-specific genes involved in the metabolism of xenobiotics in hepatic tumor-derived cell lines and primary hepatocytes after the exposure to PhIP

Table A.6: Relative expression of selected liver-specific genes involved in the metabolism of xenobiotics after the 24 h exposure to PhIP (20.0 µM) in primary human hepatocytes (donor I and donor II) and human hepatic cancer cell lines HepG2 and HuH6.

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
Phase I metabolism												
ADH1C	0.74	±	0.17	0.79	±	0.20	N.E.	±		N.E.	±	
ADH4	1.17	±	0.13	0.78	±	0.04	0.98	±	0.04	**1.83**	±	0.45
ALDH1A1	**0.60**	±	0.16	**0.61**	±	0.04	0.94	±	0.08	1.13	±	0.08
ALDH3A1	**3.03**	±	1.02	1.31	±	0.25	1.13	±	0.15	1.01	±	0.09
CES1	1.16	±	0.16	1.33	±	0.29	1.13	±	0.06	1.33	±	0.12
CES5A	**1.56**	±	0.52	0.78	±	0.19	N.E.	±		N.E.	±	
CYP1A1	**0.48**	±	0.40	**0.54**	±	0.09	**4.73**	±	0.68	**2.28**	±	0.16
CYP1A2	**13.08**	±	2.52	1.03	±	0.11	**2.76**	±	1.37	**2.37**	±	1.34

Table A.6. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
CYP2B6	0.99	±	0.27	0.89	±	0.14	0.80	±	0.16	1.44	±	0.18
CYP2C18	1.35	±	0.23	1.47	±	0.21	N.E.	±		N.E.	±	
CYP2C19	0.97	±	0.29	0.95	±	0.07	N.E.	±		N.E.	±	
CYP2C8	0.81	±	0.14	0.89	±	0.16	1.07	±	0.48	N.E.	±	
CYP2C9	1.21	±	0.19	1.04	±	0.16	N.E.	±		N.E.	±	
CYP2D6	**2.07**	±	0.31	1.45	±	0.14	1.33	±	0.05	1.31	±	0.41
CYP2E1	**1.61**	±	0.09	1.38	±	0.10	1.03	±	0.31	0.86	±	0.56
CYP2W1	N.E.	±		N.E.	±		1.14	±	0.10	0.98	±	0.43
CYP3A4	1.10	±	0.27	1.38	±	0.29	**1.95**	±	0.71	N.E.	±	
DHRS2	**5.32**	±	0.54	1.29	±	0.35	1.07	±	0.11	**1.51**	±	0.31
EPHX1	1.48	±	0.10	1.30	±	0.16	1.00	±	0.13	1.13	±	0.04
EPHX2	0.78	±	0.07	1.24	±	0.15	1.28	±	0.04	1.39	±	0.16
FMO1	↓	±		0.68	±	0.09	N.E.	±		**1.70**	±	0.38
FMO3	1.18	±	0.20	0.92	±	0.03	N.E.	±		N.E.	±	
FMO4	1.38	±	0.26	0.97	±	0.10	1.00	±	0.22	**1.54**	±	0.22
GPX3	**2.17**	±	0.51	1.10	±	0.10	1.10	±	0.06	1.12	±	0.18
GPX4	**2.20**	±	0.92	1.40	±	0.25	1.05	±	0.10	1.10	±	0.14
GPX5	N.E.	±		N.E.	±		N.E.	±		N.E.	±	
GSR	**1.76**	±	0.16	1.19	±	0.16	1.20	±	0.15	1.28	±	0.15
MAOA	0.75	±	0.07	0.84	±	0.08	1.02	±	0.06	1.34	±	0.03
MAOB	**0.52**	±	0.14	0.81	±	0.06	1.00	±	0.09	1.39	±	0.14
NQO1	**3.10**	±	1.00	1.11	±	0.42	1.05	±	0.15	1.22	±	0.22
NQO2	1.38	±	0.10	1.02	±	0.10	0.92	±	0.13	0.92	±	0.04
SRD5A2	0.68	±	0.12	1.34	±	0.09	N.E.	±		N.E.	±	
Phase II metabolism												
CHST1	**10.53**	±	3.20	**1.88**	±	1.23	N.E.	±		N.E.	±	
COMT	1.37	±	0.52	1.26	±	0.07	1.18	±	0.08	1.12	±	0.12
GSTA1	0.90	±	0.06	1.12	±	0.03	1.15	±	0.16	**1.68**	±	0.30
GSTK1	1.35	±	0.13	1.02	±	0.10	1.09	±	0.11	1.17	±	0.06
GSTM4	**0.65**	±	0.14	0.83	±	0.07	1.01	±	0.05	1.00	±	0.12
GSTO1	**2.00**	±	0.30	1.21	±	0.01	1.14	±	0.06	1.27	±	0.11
GSTP1	1.28	±	0.05	1.34	±	0.14	1.40	±	0.34	1.05	±	0.11
GSTT1	1.28	±	0.05	0.99	±	0.04	1.01	±	0.17	N.E.	±	
GSTZ1	1.29	±	0.07	0.91	±	0.00	0.97	±	0.15	1.12	±	0.09
MGST1	1.45	±	0.13	1.14	±	0.12	1.22	±	0.11	1.21	±	0.10
NAT1	**1.64**	±	0.11	0.84	±	0.09	1.08	±	0.04	1.17	±	0.14
NAT2	**1.66**	±	0.16	0.79	±	0.12	0.97	±	0.15	↑	±	
NNMT	**1.77**	±	0.08	1.06	±	0.03	N.E.	±		1.24	±	0.10
SULT1A1	1.35	±	0.81	1.10	±	0.43	N.E.	±		N.E.	±	
SULT1B1	**0.39**	±	0.11	**0.56**	±	0.05	0.99	±	0.46	N.E.	±	

Table A.6. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
SULT1C2	N.E.	±		1.38	±	0.22	0.97	±	0.13	1.44	±	0.28
UGT1A1	1.29	±	0.21	1.02	±	0.29	1.28	±	0.18	1.19	±	0.18
UGT1A3	0.99	±	0.62	1.42	±	0.08	1.86	±	1.07	**2.41**	±	0.42
UGT1A6	0.80	±	0.14	1.27	±	0.07	1.06	±	0.14	1.15	±	0.33
UGT2B4	1.11	±	0.29	1.00	±	0.08	1.06	±	0.21	N.E.	±	
UGT2B7	**2.83**	±	0.46	0.93	±	0.08	1.12	±	0.18	↑	±	
Transporter proteins												
ABCB4	1.48	±	0.43	**0.64**	±	0.09	1.08	±	0.22	1.32	±	0.20
ABCC10	1.26	±	0.36	1.30	±	0.02	0.95	±	0.18	1.12	±	0.11
ABCC2	0.91	±	0.09	1.02	±	0.08	1.06	±	0.06	1.16	±	0.06
AQP9	**1.59**	±	0.05	**1.62**	±	0.33	N.E.	±		N.E.	±	
SLC10A1	0.97	±	0.33	0.79	±	0.17	1.89	±	2.15	1.25	±	0.44
SLC22A1	1.07	±	0.09	1.21	±	0.19	1.02	±	0.19	**2.15**	±	1.69
SLC22A3	0.86	±	0.06	1.18	±	0.08	1.01	±	0.03	N.E.	±	
SLC22A7	0.89	±	0.08	0.82	±	0.12	1.39	±	0.31	↑	±	
SLCO1A2	2.76	±	3.96	**2.49**	±	1.58	N.E.	±		**1.55**	±	0.17
SLCO1B1	**0.67**	±	0.02	0.94	±	0.06	N.E.	±		1.20	±	0.23
SLCO1B3	1.44	±	0.11	1.22	±	0.16	N.E.	±		N.E.	±	
Transcription factors / cofactors												
AHR	1.16	±	0.66	1.11	±	0.14	1.22	±	0.35	1.34	±	0.45
ARNT	1.44	±	0.26	1.05	±	0.04	1.07	±	0.20	1.21	±	0.16
CEBPA	**4.55**	±	0.98	**2.49**	±	0.99	1.30	±	0.15	1.38	±	0.15
CREBBP	**0.32**	±	0.11	1.23	±	0.05	**0.36**	±	0.12	**0.37**	±	0.04
DBP	**1.93**	±	0.43	**2.90**	±	0.10	1.26	±	0.13	1.30	±	0.20
EP300	0.92	±	0.26	1.23	±	0.06	1.14	±	0.28	1.17	±	0.05
FOXA1	1.26	±	0.40	1.13	±	0.07	1.04	±	0.11	1.16	±	0.10
FOXA2	1.43	±	0.47	1.20	±	0.04	1.20	±	0.21	1.20	±	0.17
FOXA3	**1.51**	±	0.39	0.87	±	0.09	1.12	±	0.25	1.18	±	0.25
HNF1A	**1.52**	±	0.53	1.42	±	0.08	1.21	±	0.34	1.28	±	0.32
HNF4A	1.18	±	0.41	1.21	±	0.04	1.22	±	0.33	1.33	±	0.16
NCOA1	0.90	±	0.09	1.00	±	0.10	0.92	±	0.04	1.08	±	0.04
NFE2L2	**2.15**	±	0.41	1.32	±	0.18	1.18	±	0.17	1.15	±	0.15
NR0B2	**9.47**	±	1.16	**1.97**	±	0.30	1.21	±	0.09	**1.67**	±	0.37
VDR	**2.77**	±	0.81	1.18	±	0.09	1.18	±	0.15	1.31	±	0.17
NR1I2	**2.35**	±	0.39	1.09	±	0.14	0.93	±	0.11	1.39	±	0.34
NR1I3	**5.70**	±	1.21	**1.79**	±	0.14	1.27	±	0.20	**2.06**	±	0.23
NR1H3	**1.51**	±	0.17	1.10	±	0.17	0.99	±	0.11	1.08	±	0.09
NR1H4	0.96	±	0.19	1.03	±	0.07	0.96	±	0.08	1.01	±	0.30
NR3C1	**2.14**	±	0.53	0.79	±	0.25	1.21	±	0.13	1.40	±	0.20
NR5A2	0.77	±	0.15	1.08	±	0.06	1.05	±	0.16	1.27	±	0.10

Table A.6. (Continued)

Gene symbol	pHep I			pHep II			HepG2			HuH6		
	Rq	±	SD	Rq	±	SD	Rq	±	SD	Rq	±	SD
ONECUT2	1.45	±	0.37	1.22	±	0.14	1.17	±	0.23	1.16	±	0.14
PPARA	1.11	±	0.38	1.26	±	0.15	1.29	±	0.28	1.43	±	0.28
PPARAGC1A	0.91	±	0.19	0.99	±	0.13	1.32	±	0.28	N.E.	±	
RORA	1.44	±	0.30	1.18	±	0.15	1.06	±	0.18	1.45	±	0.27
RXRA	1.28	±	0.40	1.47	±	0.11	1.18	±	0.30	1.18	±	0.21

The results are presented as relative mRNA expression (Rq) in PhIP exposed cells normalized to the mRNA expression in vehicle control cells. Data are means ± SD of three independent experiments. Bold font denotes deregulated gene: Rq ≥ 1.5 indicates up-regulation of gene, Rq ≤ 0.67 indicates down-regulation of gene. N.E. – not expressed. ↑ – up-regulated gene (N.E. in vehicle control cells and expressed in cells after exposure to PhIP). ↓ – down-regulated gene (expressed in vehicle control cells and N.E. in cells after exposure to PhIP).

Appendix B

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Conference contribution abstracts

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Biography

The author of this thesis, Jana Tomc, was born on September 25th, 1984 in Ljubljana, Slovenia. After graduating with praise at the High School of Pharmacy, Cosmetology and Health in Ljubljana as a Pharmaceutical Technician, she continued her study of Laboratory Biomedicine at the Faculty of Pharmacy, University of Ljubljana, and today she holds a Bachelor degree in Engineering in Laboratory Biomedicine and a Master degree in Laboratory Biomedicine. The research work for her master's thesis was performed in Spain within the frame of research projects of the GRET-CERETOX group at the Department of Toxicology, Faculty of Pharmacy, University of Barcelona. Her work was focused on neurotoxicity studies of psychoactive substances using alternative zebrafish larvae models. Jana has applied and been accepted for the position of a Young Researcher at the National Institute of Biology in Ljubljana, Department of Genetic Toxicology and Cancer Biology in 2014. Her PhD research activity was carried out under the supervision of Prof. Dr. Metka Filipič and Asst. Prof. Dr. Bojana Žegura, and was a part of an international project HOPE, implemented with the participation of scientists from two Institutes in Slovenia and scientists from the Medical University Vienna in Austria. With the results of her work, she contributed at several scientific conferences, namely the 42nd Annual Conference of Environmental Mutagen Society of India (EMSI) and National Conference on Environmental Mutagenesis, Mumbai; 8th Conference on Experimental and Translational Oncology, Portorož; 1st International Conference Molecular Life of STEM Cells, Ljubljana; 17th annual Congress of European Society For Alternatives To Animal Testing (EUSAAT), Linz; 5th Croatian Toxicology Congress CROTOX, Poreč; 7th Congress of the Genetic Society of Slovenia, Rogaška Slatina. Her conference presentations have been awarded with the Young Scientist Travel Award, EUSAAT 2016 Congress and the best poster award at the Crotox 2016 Congress. She attended several workshops on the topics of qPCR, scientific writing and toxicology.

Among her private activities, Jana is dedicated to yoga study and practice, from which she holds a degree as a certified teacher. Besides, she actively participates in a non-profit humanitarian society, which is based in Slovenia, and helps Piali Ashar Alo School in Kolkata with international promotion and fundraising. For this purpose, she spent a year in India after finishing her master degree, which is also one of the important milestones in her life.