In vitro toxicity testing using human pluripotent stem cell derivatives

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Cover illustration: Human pluripotent stem cell-derived cardiomyocytes

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To Karin
- my everyday sensation,
my love and inspiration

"Alle Dinge sind Gift, und nichts ist ohne Gift; allein die

dosis machts, das sein Ding kein Gift sei"

[Paracelsus, 1493 – 1541]

"Problems worthy of attack prove their worth by fighting back."

[Piet Hein, 1905 – 1996]

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ABSTRACT

Toxicity testing of chemicals, drug candidates, and food additives is in need of a change. The present methods, mainly consisting of animal models with their associated ethical concerns, are expensive, time-consuming, and importantly they are often poor predictors of the human *in vivo* toxicity. With the rapid biotechnology development, a paradigm shift for toxicity testing is emerging, focusing on bioinformatics, computational toxicity, systems biology, and cell-based *in vitro* models.

The aim of this thesis was to investigate the utility of using cells, i.e. hepatocytes and cardiomyocytes, derived from human pluripotent stem cells (hPSC) as *in vitro* models for toxicity testing. The first part explored the feasibility of using hPSC-derived hepatocytes to study toxic drug exposure, and in addition investigated the relevancy of the cellular response. The second and major part of this thesis focused on hPSC-derived cardiomyocytes and the in-depth study of doxorubicin-induced toxicity.

The studies revealed that the differentiation processes and culturing of hPSC-derivatives are stable and reproducible to form the basis for *in vitro* models for toxicity testing, even for longer studies over two weeks. The hepatocytes and the cardiomyocytes showed sensitivity towards the toxic compounds and both cell models displayed a relevant cellular response to the toxic exposure. For example, the hepatocytes showed evidence of steatosis and phospholipidosis when incubated with hepatotoxic compounds over time. Besides an evident effect of doxorubicin on the cardiomyocyte function, the cells also proved to be useful for more in-depth mechanistic evaluations, as these studies gave insight, on multiple biological levels, in plausible mechanisms and identified potential biomarkers for doxorubicin-induced cardiotoxicity.

In conclusion, this thesis presents findings that supports the vision and strategy of using *in vitro* models based on hPSC-derivatives together with advanced omics technologies for toxicity testing and risk assessment of drugs, food additives, and chemicals.

Keywords: toxicity testing, human pluripotent stem cells, cardiomyocytes, hepatocytes, microarray, quantitative proteomics, bioinformatics, transcriptomics, microRNA

SAMMANFATTNING PÅ SVENSKA

Riskbedömning och toxicitetstestning av läkemedel, kemikalier, och ingredienser i mat och kosmetika står inför ett regimskifte när det gäller vilka metoder man använder. De traditionella metoderna för att testa toxicitet (ämnens giftighet) innefattar framför allt försöksdjur och utvärdering av systemiska effekter och patologiska förändringar i dessa försöksdjur. Detta innebär ofta kostsamma, tidskrävande försök, lidande för djuren i fråga, och en låg korrelation till humana *in vivo* situationen, det vill säga det verkliga förhållandet i människokroppen. Med resultat från de senast årens utveckling inom cellbiologi och bioteknologi har en vision om en ny strategi för toxicitetstestning skapats. Denna strategi grundar sig i att man vill bygga en robust kunskapsplattform för bedömning av ämnens toxiska påverkan, baserat på mekanistiska studier av de processer som ett visst ämne påverkar i mänskliga celler, vävnader och individer.

Syftet med denna avhandling var att utvärdera användningen av cell-baserade modeller, så kallade *in vitro* modeller, från human pluripotenta stamceller (hPSC) för att studera ämnens toxicitet. Stamceller är celler med obegränsad förmåga att föröka sig och med en potential att mogna ut till flera eller alla celltyper den mänskliga kroppen är uppbyggd av. Dessa celler utgör därför en optimal källa för cell-baserade modellsystem.

Avhandlingens första delprojekt syftar till att utvärdera hur hPSC-deriverade hepatocyter (leverceller) svarar vid exponering för kända levertoxiska substanser. Det andra delprojektet syftar till att utvärdera användningen av hPSC-deriverade kardiomyocyter (hjärtmuskelceller) för att i detalj studera toxicitet orsakad av läkemedlet doxorubicin vars biverkningar inkluderar hjärttoxicitet.

I dessa forskningsprojekt har vi kunnat visa på att celltyperna från hPSC kan genereras och odlas på ett robust och reproducerbart vis, vilket möjliggör toxicitetsstudier, även under så lång tid som två veckor. Hepatocyterna svarar på ett relevant sätt och man ser en ökad känslighet hos cellerna om de utsätts för toxiska substanser under längre tid. På liknande sätt svarar kardiomyocyterna på ett relevant sätt vid exponering för doxorubicin. En tydlig påverkan ses på cellerna i form av försämrad funktion och förändrat utseende. Förutom detta visar studierna även på att man, med hjälp av avancerade tekniker som mäter cellers totala gen- och proteinuttryck (microarray och kvantitativ proteomik), kan studera ingående mekanistiska effekter av doxorubicin i cellerna. Resultaten visar på påverkade processer både på gennivå och proteinnivå och flertalet potentiella

biomarkörer, det vill säga gener eller proteiner som möjligen kan användas för att förutspå toxiciteten, har identifierats.

Sammanfattningsvis visar den här avhandlingen på att cellmodeller baserade på hPSC kan användas för toxicitetstestning. Avhandlingen styrker visionen om att använda cellbaserade modellsystem för att i framtiden bättre kunna förutse ämnens toxicitet och riskbedöma substanser och därmed minska eller eventuellt helt utesluta djurförsök.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Holmgren, G., Sjögren A. K., Barragan I., Sabirsh A., Sartipy P., Synnergren J., Bjorquist P., Ingelman-Sundberg M., Andersson T. B., and Edsbagge J.
Long-term chronic toxicity testing using human pluripotent stem cell-derived hepatocytes
Drug Metab Dispos 42(9): 1401-1406. 2014

II. Holmgren, G., Synnergren J., Bogestal Y., Améen C., Akesson K., Holmgren S., Lindahl A., and Sartipy P.
Identification of novel biomarkers for doxorubicin-induced toxicity in human cardiomyocytes derived from pluripotent stem cells
Toxicology 328: 102-111. 2015

III. Holmgren, G., Synnergren, J., Andersson, C.X., Lindahl, A., and Sartipy, P.
MicroRNAs as potential biomarkers for doxorubicin-induced cardiotoxicity
Toxicol *In vitro* 34, 26-34. 2016

IV. Holmgren, G., Sartipy, P., Andersson, C.X., Lindahl, A., and Synnergren, J.
Expression profiling of human pluripotent stem cell-derived cardiomyocytes exposed to doxorubicin – integration and visualization of multi omics data
Manuscript

CONTENT

ABBREVIATIONS

AMPK	AMP-activated protein kinase
cDNA	Complementary DNA
CiPA	Comprehensive *In vitro* Proarrhythmia Assay
cTnT	cardiac specific troponin T
CYP	Cytochrome P450
DILI	Drug induced liver injury
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
fCI	F-divergence cutoff index
FDA	Food and Drug Administration
GADD45A	Growth Arrest and DNA Damage Inducible Alpha
GDF15	Growth differentiation factor 15
HAMP	Hepcidin Antimicrobial Peptide
hERG	human Ether-a-go-go-related gene
hESC	human embryonic stem cells
HIF1A	Hypoxia Inducible Factor 1 Alpha Subunit
hiPSC	human induced pluripotent stem cells
hiPS-HEP	hiPSC-derived hepatocytes
hPSC	human pluripotent stem cells
InCroMAP	Integrated analysis of Cross-platform MicroArray and Pathway data

KEGG	Kyoto encyclopedia of Genes and Genomes
LC	Liquid chromatography
LD50	Lethal dose 50%
LDH	Lactate dehydrogenase
miEAA	MicroRNAs enrichment analysis and annotation
mRNA	Messenger RNA
MS	Mass spectrometry
MYH	Myosin heavy chain
MYL	Myosin light chain
ORA	Over-representation analysis
PCA	Principal Component analysis
PHH	Primary human hepatocytes
RNA	Ribonucleic acid
SAM	Significance analysis of microarray data
TMT	Tandem mass tags
TP53I3	Tumor Protein P53 Inducible Protein 3
TPM	Tropomyosin

1 INTRODUCTION

1.1 General Toxicology

The study of poison and poisonous effects to living organisms has an ancient history. From the animal and plant toxins used by African Bushmen and South American Indian tribes to the Egyptian's interest in plant toxins, such as opium, and mineral toxins, such as lead and copper, over 1000 years BC. The ancient Greeks moved the development further with observational and experimental studies of toxins and their antidotes. The term toxicology originates from the Greek word for poison: toxicon. Studies of poisons continued during the middle ages and the renaissance, not least with the recurrent use of poisons in the frequent events of homicides. Paracelsus (1493 – 1541), sometimes referred to as the 'father' of toxicology stated in the late 1530's the famous phrase: 'Alle Ding sind Gift und nichts ohn' Gift; allein die Dosis macht, das ein Ding kein Gift ist' ('All things are poison and nothing (is) without poison; only the dose makes that a thing is no poison')[1]. The founder of modern toxicology is often considered to be Mathieu J B Orfila, who in 1813 published his 'Traité de toxicologie', which describes symptoms and adverse effects of poisons and chemicals.

Toxicology is in modern time generally referred to as the study of adverse effects of chemicals in living organisms and is a segment within biology, chemistry, and medicine (or pharmacology). In the pharmaceutical industry, toxicology, to the far most, associates with the safety assessment of drug candidates often consisting of synthetic chemicals. The term does not only include the study of synthetic chemicals but also refers to studies of harmful effects by agents of biological or physical origin.

Early safety assessment during drug development in general has been focused on animal models. However, there are numerous cases in the history that stress that non-human mammals are poor predictors of human toxicities. The disaster with Thalidomide (with the brand name Neurosedyn) in the late 1950's and early 1960's, resulting in a huge number (>10000) of severe birth defects in children, is often referred to as the biggest man-made medical disaster in history[2]. The thalidomide case demonstrated for the first time that species differences exist in drug reactions/responses and the disaster completely changed the way drugs are tested. Nevertheless, despite comprehensive toxicity testing there are still today

many events of unexpected human toxicity of drugs leading to late attrition of drug candidates and already marketed drugs.

The attrition rates of drug candidates are a major concern for the pharmaceutical industry, and in the late 1990s only approximately one out of nine candidates made it through development and were approved by regulatory authorities as the European Medicines Agency (EMA) and/or the US Food and Drug Administration (FDA). Besides efficacy, safety reasons were the major contributor to late-stage attrition rates during drug development[3]. A more recent study by Hay et al. reports that only approximately 10% of the candidates entering clinical phase I trials is expected to advance to FDA approval[4]. One explanation of the continuous increase in late-stage attrition rates is that the study by Hay et al. included small biotech companies, with the tendency of developing less validated drug classes and targets, and having less experience and fewer resources than large pharmaceutical corporations. This report suggests amongst other, more predictive test models, earlier toxicology evaluation, and biomarker identification as potential improvements to increase the success rate in the future[4].

Due to the poor prediction of human toxicities by non-human mammals, one cannot expect that *in vitro* systems based on cells derived from such animals would do any better. The way to move forward in that sense therefore lays in the development of *in vitro* systems based on human cells.

Over 40 years ago, Russel and Burch elaborated the term of the Three R's (replace, reduce, refine) in the context of live animal experiment. Since then, much have been done in the field of replacement, reduction, and refinement of animal studies, but there is still a lot of work to be done[5]. In 2007, the U.S. National Academy of Science presented a new strategy, illustrated in figure 1, for toxicity testing in their report "Toxicity Testing in the 21st Century: A Vision and a Strategy"[6]. The vision for the strategy is an increased efficiency in toxicity testing and a reduced animal use, by a transition from animal *in vivo* testing to *in vitro* toxicity pathway assays with human cells or cell lines using high-throughput screening with mechanistic quantitative parameters. Risk assessment with this strategy would focus on avoiding significant disturbance in the key identified toxicity pathways[7]. As an example, the Swedish national academic research center Swetox implements this new vision of safety assessment as the three M's (mechanisms, markers, models), where the focus is to use *in vitro*, *in silico*, and chemical analysis, to retrieve knowledge of the toxic mechanisms and identify specific biomarkers to develop new models for safety assessment.

Biomarkers can be defined as a quantifiable object that allows for the detection of a biological event, representing either normal physiology or a pathological state. A biomarker would ideally provide mechanistic information and be translatable to the clinic.

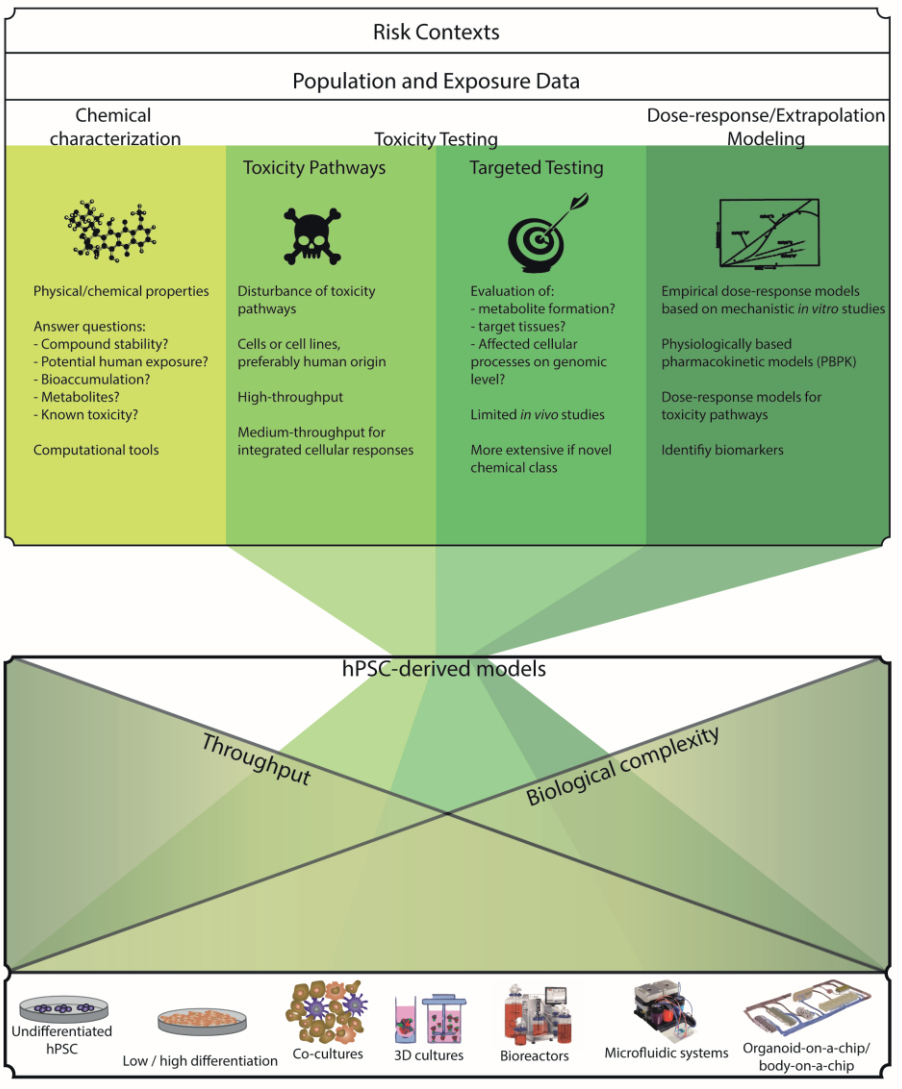

Figure 1. New vision of toxicity testing. *Illustration of the vision of a new strategy for toxicity testing and the potential contribution of hPSC-derivatives.*

1.2 Hepatotoxicity

The term hepatotoxicity refers to the damage of the liver often induced by a chemical agent. The liver is one of the most, if not the most, important target organ for drug-induced toxicity. This vulnerability is mainly associated with the function of the liver and its role in detoxification of various metabolites, including most drugs. The compounds generating the most interest from the pharmaceutical industry are the ones causing drug-induced liver injury (DILI). The list of compounds that are considered potentially toxic to the liver is extensive and DILI has been associated with over one-third of the acute liver failure cases in the USA[8, 9]. Negative effects on the hepatic function is the leading cause of drug failure in clinical trials and retraction of post-market drugs[10].

Preclinical animal models have a weak potential of predicting human DILI, mainly due to large species differences in drug metabolism and toxicity targets[11, 12]. Therefore, during the last decades, large efforts have been made to replace laboratory animals with alternative human cell-based systems. The gold standard of today for hepatotoxicity studies are primary human hepatocytes (PHH). However, the shortage of appropriate human liver samples and the fact that, despite improved *in vitro* culturing systems, the cells rapidly lose key enzymes involved in metabolizing and transporter functions in culture[13-15], hinders the widespread use of PHH. Furthermore, alternative human liver cell lines, especially human hepatoma cell lines, can in some aspects replace PHH but, on the other hand, they show inadequate expression of important metabolizing and transporting enzymes[16, 17].

HepG2 is one of the most frequently used hepatoma cell lines for hepatotoxic evaluations and is, because of its widespread use, also the best characterized. HepG2 cells, like other available hepatoma cell lines, offer some key advantages, such as unlimited life span, reproducibility, easy handling, and accessibility. Using HepG2 cells cultured in small scale formats (e.g., 96- or 386-well plates) together with automated imaging techniques and high content screening assays is considered a very valuable platform for hepatotoxic predication during preclinical development[18-20]. However, because of the very poor expression of key drug-metabolizing enzymes in HepG2 cells, their use to detect metabolism-induced toxicity is limited[21, 22]. Using HepG2 cells for this type of studies can lead to inaccurate detection of cell toxicity as well as over-estimation of toxicity from normally highly-metabolized parent drugs[17].

HepaRG cells are often considered the most promising alternative to PHH for *in vitro* studies. HepaRG are proliferating hepatic progenitor cells with the capacity of becoming both hepatocyte-like and biliary-like cells[23]. The great advantage of these cells are their liver-characteristics and especially the fact that they express higher phase I and phase II metabolizing enzymes than any other hepatoma cell line[22]. The ability to culture HepaRG cells for long time without loss of the metabolic features opens up for potential long term studies of chronic toxicity[24]. Due to these features, the usage of HepaRG cells for hepatotoxic assessment is growing. Several studies show supporting results for HepaRG as a tool for hepatotoxic assessment, however, the number of toxicity screening studies involving larger sets of compounds are few.

A recent study on four hepatic *in vitro* cell models (including PHH, HepG2, Upcyte hepatocytes, and HepaRG) for the assessment of compounds causing DILI, reported that none of the tested cell models fully could distinguish between DILI and non-DILI compounds, using a simple read-out as the determination of effective concentration 50% (EC50) values[25]. The report stresses the fact that current *in vitro* cell models need further development, both regarding cellular features as well as in the readouts or endpoints to measure.

1.3 Cardiotoxicity

One event that has underscored the need to focus on safety assessment during clinical development is the incident in 2004 when Vioxx® (rofecoxib) was withdrawn from the market by FDA. Vioxx® is a nonsteroidal anti-inflammatory drug (selective Cyclooxygenase-2 inhibitor) that was found to be associated with an unforeseen increased risk of cardiovascular side effects. Besides the suffering of those individuals affected, the withdrawal resulted in a tremendous cost due to lost revenues and patient lawsuits and illustrate the need for better and more predictive pre-clinical models[26].

The heart is a complex organ consisting of many different cell types, where the most crucial function is to pump blood through the rest of the body. Dysregulation of the cardiovascular system is a major health concern and cardiovascular disease is the leading cause of death worldwide[27].

Cardiotoxicity is a term that describes the damage of cardiomyocytes resulting in decreased cardiac function. The reduced cardiac function can be caused by impaired electrical properties of the heart due to interactions with ion channels

or alterations in the potassium currents within the cardiomyocytes[28, 29]. Other compounds, often associated with anti-cancer treatment, can result in cardiotoxicity independent of the cardiomyocyte's electrical activities. The mechanisms of toxicity for these compounds vary a lot and include for instance, mitochondrial dysfunction, DNA damage, reactive oxygen species formation, and apoptosis[30].

A variety of model systems are used to evaluate the efficacy and safety of thousands of compounds during the early phases of drug discovery. The current standard for preclinical drug testing is animal models, primarily rodent models. For cell-based assays, cell lines such as H9c2, derived from embryonic rat ventricle[31], or the HL-1 cell line derived from the AT-1 mouse atrial CM tumor lineage[32] have been used for many decades. With increased knowledge in genetic engineering and the human genome, cell lines (commonly from Chinese hamster ovary or human embryonic kidney) with artificially over-expressed ion channels (especially the human ether-a-go-go-related gene (hERG)) have been a preferred choice from the pharmaceutical industry[33]. However, these assays typically only provide information on drug effects on single ion channels, and for a more complex analysis isolated tissue or cardiomyocytes from animals are used. These models commonly originate from rabbit or dog, and the advantages are that the cardiomyocytes are mature and that tissue models constitute a multicellular system. The main concerns with these models are the species differences; no animal model can fully recapitulate the human cardiomyocyte[33].

During safety assessment of new candidate drugs, screening for drugs affecting the hERG plays a critical role. The hERG channel is an important potassium channel responsible for the repolarization of the cardiac action potential. A diverse set of drugs have been observed to increase the tendency of blockage of this potassium current, resulting in a prolonged action potential depolarization and an increased risk of a potentially lethal ventricular arrhythmia called Torsade de Pointes[34, 35]. A model system consisting of mammalian cells that express hERG channels has been, and still provides, a valuable tool for the evaluation of acute impact of new test compounds on this channel in high-throughput screening. However, a major concern with this method is the strong focus on one single ion channel, with the risk of false negatives and false positives due to the lack of other ion channels and cell surface receptors. During the decade of testing this model system, it has become clear that this is a conservative approach that might have resulted in the attrition of potentially useful drugs based solely on their effect on the hERG rather than an evident induction of arrhythmia[36].

In the light of the acquired experience with hERG screening, in 2013, representatives from the Cardiac Safety Research Consortium (CSRC), Health and Environmental Sciences Institute (HESI), and the US FDA organized a workshop with the focus to reconsider approaches for the evaluation of drug-induced arrhythmia. The group ended up with a proposal of a new paradigm named Comprehensive *In vitro* Proarrhythmia Assay (CiPA), a new approach for the early evaluation of the proarrhythmic risk of compounds[37, 38]. The CiPA-initiative consists of two series of tests: First, the *in vitro* study of drug effects on multiple ion channels as input data for an *in silico* model of the adult human ventricular myocyte to assess the proarrhythmic risk. Secondly, the use of human ventricular myocytes, likely derived from human induced pluripotent stem cells (hiPSC), to confirm the ion currents and the *in silico* results[36]. Training, calibration, and subsequent validation of the CiPA initiative is currently ongoing, and if successful, it will most likely have a major impact on the cardiac safety assessment in the pharmaceutical industry in the future.

1.3.1 Anthracycline-induced cardiotoxicity

As mentioned above, many chemotherapeutic agents are associated with cardiotoxic side effects, and chemotherapeutic-induced cardiomyopathy is one of the leading causes of disease and mortality in cancer survivors. Among the most cardiotoxic chemotherapeutic drugs present on the market today are anthracyclines, such as doxorubicin, which are highly efficient agents for a variety of cancers, including hematologic malignancies, soft tissue sarcomas, and solid tumors in both children and adults[39-41].

The anti-tumor effects of anthracyclines manifest primarily as inhibition of DNA replication and transcription, since the main cellular target of anthracyclines is believed to be topoisomerase II[42]. The role of anthracyclines as 'topoisomerase poisons' is due to the blockage of DNA resealing during replication and transcription. Anthracyclines stabilizes the intermediate where the DNA strands are cut and covalently bound to topoisomerase II[43]. This results in impaired DNA replication and transcription as well as the DNA strand break may trigger apoptosis in the cells. Anthracyclines thereby leads to the killing of rapidly dividing cells and in turn slows down tumor progression, features of the anthracyclines that has been known for more than 30 years. The wide-spread success of these compounds as chemotherapeutic agents is, however, hampered due to toxicity to non-cancerous cells, especially cardiomyocytes[44, 45]. Over the years a substantial number of anthracycline analogs have been synthesized and tested. Despite these efforts, few have made it to clinical use, the compounds

have not shown stronger anti-tumor effects than their forerunners, and importantly, none of the newer analogs has substantially improved the cardiac safety profile[42].

The anthracycline-induced cardiotoxicity often manifests in the adult patient within one year after administration and is commonly associated with a dilated and hypokinetic cardiomyopathy leading to heart failure[46]. The cardiotoxicity is classified into three subtypes according to the time of onset: (1) Acute cardiac toxicity occurs during or immediately after initiation of doxorubicin treatment resulting in tachyarrhythmia's, including sinus tachycardia, premature ventricular contractions, and ventricular tachycardia, as well as bradycardia. (2) Early cardiotoxic events develop within one year of exposure and is usually presenting as a dilated and hypokinetic cardiomyopathy leading to heart failure. (3) The late cardiac toxicity may develop one or several years after initial exposure, and can result in a life-threatening cardiomyopathy[46-48]. Anthracycline-induced cardiomyopathy is exponentially dose-dependent[49] and children and adolescent seem to be more susceptible to the cardiotoxic effects[50]. These circumstances indicate that there are several mechanisms responsible for anthracycline-induced cardiotoxicity.

Despite intense research, the exact mechanisms behind anthracycline-induced cardiotoxicity is not fully understood. As illustrated in figure 2, oxidative stress, due to excessive production of reactive oxygen species and an altered iron metabolism have been described as the main contributors to the cardiotoxicity, but also modulation of signaling networks involving DNA damage responses, cardiomyocyte survival, cardiac inflammation, and energetic stress are suggested as potential explanations[51] (see figure 3). Further understanding of the mechanisms causing the anthracycline-induced cardiotoxicity is crucial in order to improve the therapeutic use of anthracyclines and to enable earlier detection of the cardiotoxicity to reduce the damage.

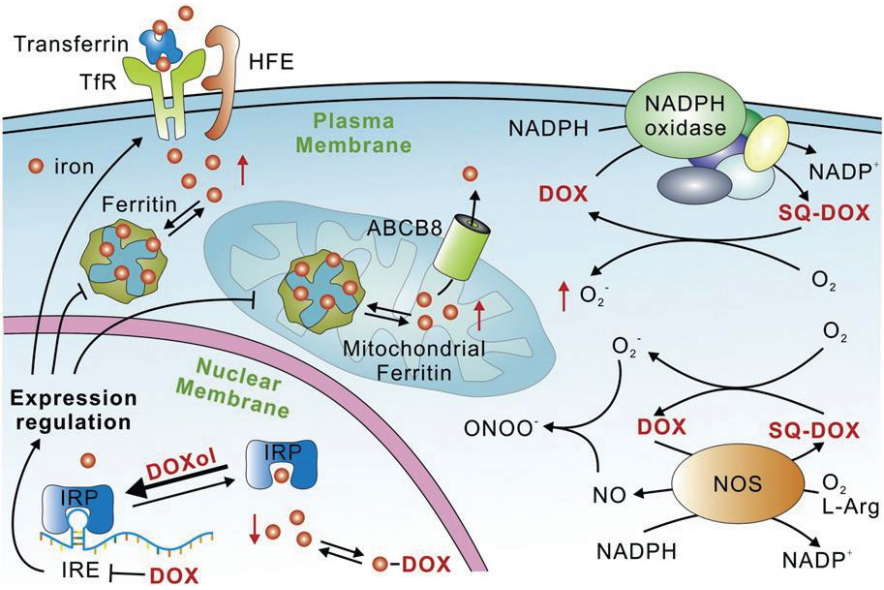

Figure 2. Traditional mechanistic view of anthracycline-dependent oxidative stress and cardiotoxicity. *Anthracyclines, such as doxorubicin, affects iron homeostasis in cells on many levels. Doxorubicin and its metabolites interact with iron regulatory protein (IRP) affecting iron-responsive elements (IREs) in the mRNA of genes involved in iron metabolism, ultimately controlling their expression level. Consequently, the level of free iron increases in both cytosol and mitochondria leading to a doxorubicin-induced iron overload. On the other hand, ATP-binding cassette sub-family B member 8 (ABCB8), a mitochondrial iron export protein, reduces mitochondrial iron accumulation and prevents cardiotoxicity resulting from the iron overload. Another mechanism underlying doxorubicin-dependent oxidative stress is linked to the ability of the drug to directly interfere with the activity of NADPH oxidase and nitric oxide synthase (NOS), resulting in the production of reactive oxygen species. Reprinted from[51], with permission from Elsevier.*

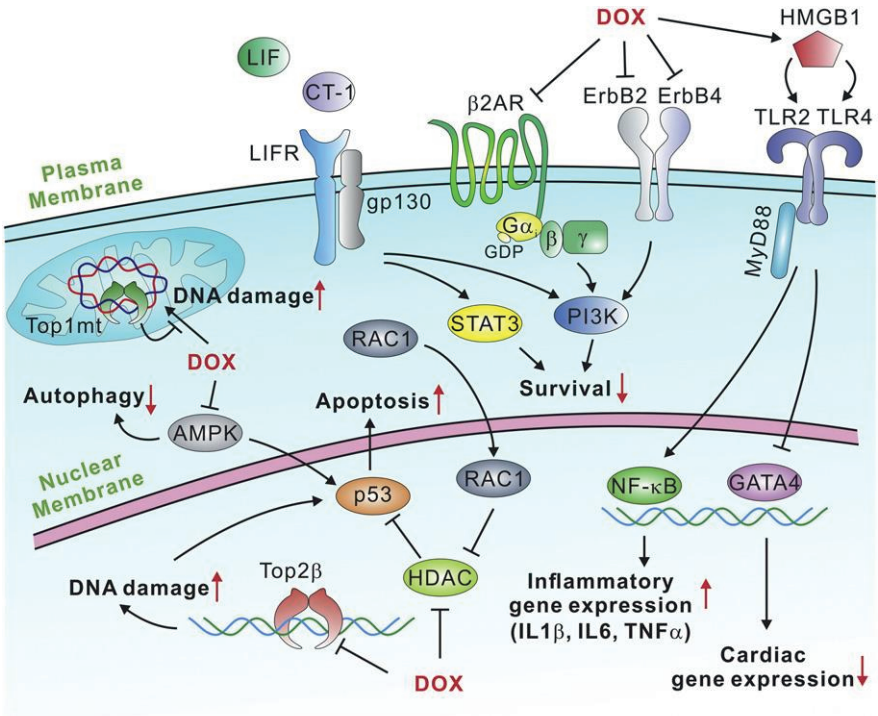

Figure 3. New players in anthracycline-induced cardiotoxicity. *Doxorubicin induces the release of high-mobility group protein B1 (HMGB1) which targets Toll-like receptor 2 and 4 (TLR2 and TLR4). These membrane receptors, influences the transcription factors NF-κ β and GATA4, which promotes immune responses and inhibits cardiac gene expression. At the plasma membrane, doxorubicin also represses the activity of growth factor receptors, ErbB2 and ErbB4, as well as the β 2 adrenergic receptor (β 2AR), further inhibiting PI3K-dependent survival signaling. Interleukin-6-related cytokines, like leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1), protect cardiomyocytes against doxorubicin-induced apoptosis, through both PI3K and STAT3 signaling pathways. On the other hand, doxorubicin directly binds to topoisomerase 2β Top2β) in the nucleus and by stabilizing this enzyme, causes continuous DNA break and prevents the DNA double helix from being resealed. Doxorubicin-dependent DNA damage activates p53 pathway thus resulting in cardiomyocyte apoptosis. Notably, doxorubicin can influence p53-dependent apoptosis through additional signals, e.g. by inhibition of histone deacetylase (HDAC) and inhibition of AMP-activated protein kinase (AMPK) activity. Reprinted from[51], with permission from Elsevier.*

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1.4 Human pluripotent stem cells

Stem cells are defined as immature, unspecialized cells with the ability to divide and differentiate into specialized cell types. Human pluripotent stem cells (hPSC) have the ability of indefinite self-renewal and the potential of differentiate into virtually any cell type in the human body. Human PSC can be of either embryonic origin[52] or be derived from the reprogramming of somatic cells[53, 54]. There is great potential in hPSC as a cell source for a variety of applications. They can provide model systems for the study of developmental biology and they can constitute a platform for various *in vitro* applications, such as disease specific models, for targeted drug studies, and as a tool for toxicological assessments.

1.4.1 Human embryonic stem cells

Human embryonic stem cells (hESC) were first isolated about two decades ago and are pluripotent stem cells derived from a fertilized egg[52, 55] (see figure 4). The fertilized egg is cultured until the blastocyst stage (4-6 days old) which then possesses an outer layer called trophoblast, which during normal embryonic development constitutes the placenta, and an inner cell mass, that subsequently give rise to the embryo. Using immunosurgery and/or enzymatic removal of the trophoblast, the inner cell mass is isolated and seeded into culture dishes coated with either feeder cells, e.g. fibroblast cells of human or animal origin, or extracellular matrix in a specific, defined cell culture medium[52, 56]. This procedure enables the cells to start propagate in their undifferentiated state. To ensure that the cells remain pluripotent and to avoid spontaneous differentiation, the cells need to be passaged, i.e. the cells are dissociated, diluted, and seeded into new culture dishes. When cultured on feeder cells, the hESC traditionally are passaged using mechanical passaging, where the colonies are cut and separated by hand and seeded into new culture dishes. This process can also be done enzymatically, which ensures a higher throughput and enables automated culture.

To ensure that the cells truly are hESC, they undergo extensive characterization, including morphological assessment, telomerase activity, karyotyping, analysis of pluripotency status, and the presence of pluripotency-specific cell surface markers (e.g. Stage specific embryonic antigen 3 (SSEA-3), Stage specific embryonic antigen 4 (SSEA-4), TRA-1-60, TRA-1-81) and transcription factors (e.g. Octamer-binding transcription factor 3/4 (OCT3/4), NANOG, SRY (sex determining region Y)-box 2 (SOX2))[56]. To verify the pluripotent status of the cells, the cells undergo *in vitro* differentiation through the formation of embryonic bodies. The embryonic body formation stimulates spontaneous differentiation of

the hESC and the pluripotency is verified by the presence of cell types originating from all three germ layers. Pluripotency is also analyzed *in vivo* through xenografting of hESC into animal hosts, such as severe combined immunodeficient (SCID) mice[57]. To confirm presence of human tissue from all three germ layers, the xenograft can be analyzed by histopathology or immunohistochemistry.

The great potential and widespread use of hESC has, however, been hampered by the ethical concerns that follows with the origin and the derivation process of the cells. The destruction of an embryo during the derivation of hESC has led to the prohibition of work with hESC in some countries, and strictly regulated work in countries where the work is allowed. The ethical issues surrounding hESC resulted in a focus to derive hESC lines without the destruction of embryos[58, 59]. The promising technique was published in 2007[60] but did not result in a widespread use, most likely due to the coinciding derivation of the first hiPSC.

1.4.2 Human induced pluripotent stem cells

Human iPSC were first derived by several independent groups in 2007[53, 61, 62]. Human iPSC, free of the ethical issues surrounding hESC, are derived from adult somatic cells by genetic reprogramming. The genetic reprogramming, more precisely the over-expression of 3-4 transcription factors, results in a reversal of the cell fate into cells with pluripotent stem cell features, see figure 4.

The hiPSC possess the same characteristics as hESC when it comes to morphology and functionality, propagation potential, telomerase activity, presence of pluripotency markers, and differentiation capacity[53, 63]. The obvious difference between the two are the origin sources: hESC are derived from a blastocyst, while hiPSC are derived from adult cells. As hiPSC originate from adult cells it can result in a potentially modified genetic and epigenetic state of the hiPSC, which might influence the phenotype of the cells and their derivatives. This feature is appealing when it comes to the study of specific diseases. The generation of hiPSC from differentiated somatic cells of healthy donors and patients of inherited and acquired diseases, allows for the creation of 'disease model' cell lines, and enables relevant studies of specific diseases on a cellular and molecular level. Even though this highlights the great advantage with hiPSC, on the contrary the inherited genetic and epigenetic footprints from the donors might lead to misinterpretations of the results in e.g. a general screening of drug candidates[64, 65]. Furthermore, the derivation of hiPSC using viral vectors that integrate the genome could potentially influence the outcome of studies in hiPSC.

This, together with the potential baggage of mutations accumulated in the donor cell, constitutes the major disadvantages of hiPSC for various *in vitro* studies. However, the weakness of hiPSC related to the viral integration has to a large extent been eluded with the intense research and development of non-viral transfection methods[66-70].

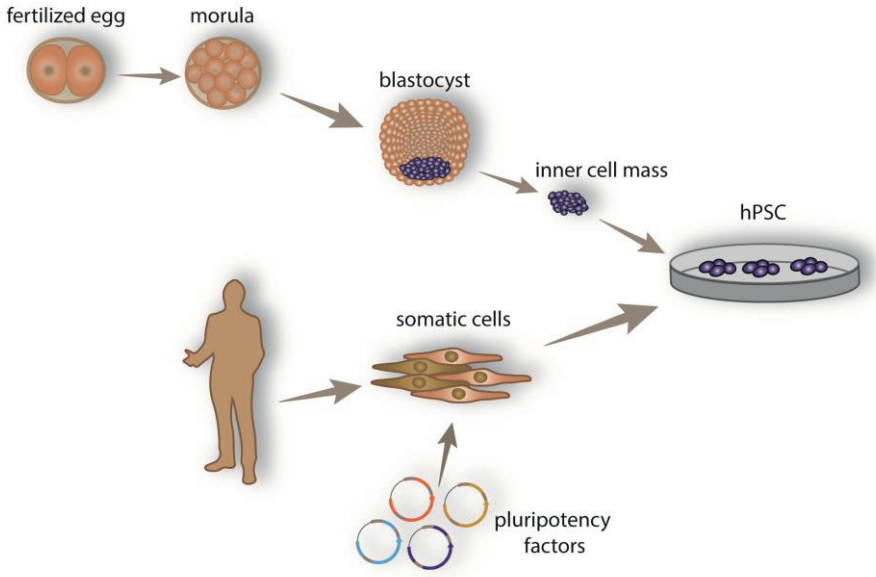

Figure 4. Derivation of hESC and hiPSC. *Human ESCs derived from the inner cell mass of a blastocyst and hiPSCs derived from somatic cells from a donor individual by genetic reprogramming.*

1.4.3 Differentiation of human pluripotent stem cells

One of the most attractive features of hPSC is their ability to differentiate into in principally all specialized cell types in the human body, as illustrated in figure 5.

The differentiation is a complicated process and a successful approach has proved to be to try to mimic the normal embryonic development as close as possible. Culturing the cells on specific matrices in the presence of key growth factors and small molecules steer the cells to differentiate towards the cell type of interest. The complex differentiation protocols are influenced by many cell culturing parameters, and are dependent on precise timing, concentrations and combinations of growth factors and inhibitors to ensure an efficient differentiation.

Recent years of intense research within the stem cell field has significantly increased the knowledge of cellular differentiation and numerous protocols that reports on the differentiation of hESC and hiPSC to e.g. cardiomyocytes, hepatocytes, beta-cells, and neurons have been published[71-79].

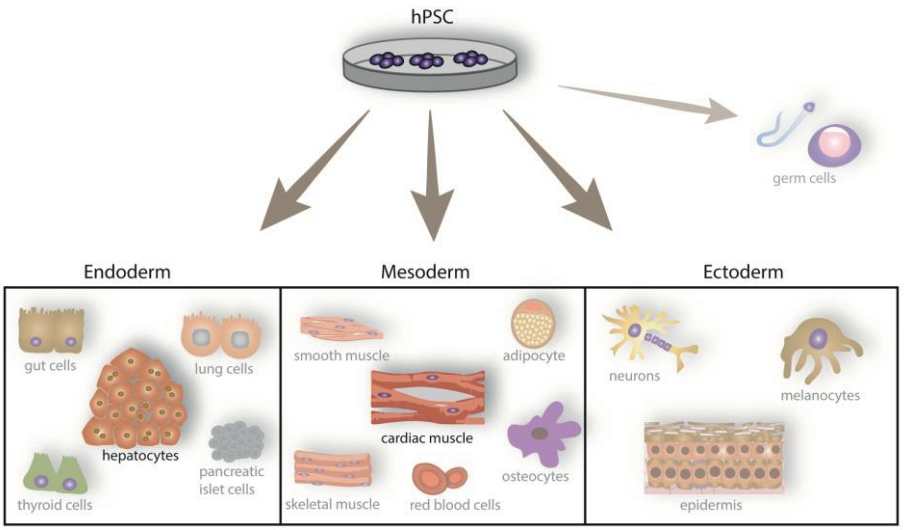

Figure 5. Pluripotency of hPSC. *Differentiation of hPSCs towards cells from all three germ layers; endoderm, mesoderm, and ectoderm. Hepatocytes and cardiomyocytes have been the focus for the investigations in this thesis.*

1.5 Systems biology – omics technologies

Systems biology is often referred to as computational and mathematical modelling of complex biological systems. In contrast to the traditional reductionist research approach, where researchers try to identify important individual components of biological systems, systems biology deals with the integration of many individual components to create a holistic view of the biological system under study[80].

As a result of recent year's dramatic increase in high-throughput experimental techniques and omics data, the field of systems biology has emerged tremendously. Today, systems biology covers methods to obtain, analyze, and integrate large datasets from multiple experimental sources including genomics, epigenetics, transcriptomics, and proteomics.

Within the scope of this thesis, the major work has been focusing on transcriptomics and proteomics and the two major technologies used are microarray analysis and quantitative proteomics using liquid chromatography and tandem mass spectrometry (LC-MS/MS).

1.5.1 Microarray

The microarray technology is a high-throughput technology, introduced in the mid 1990's[81-83], that enables examination of more or less the complete gene expression in a sample at a specific time point. The microarray technology is based on the base paring between DNA and RNA. When complementary strands of DNA and RNA meet, they will join together in a hybridized state. The microarray consists of a surface, usually plastic or glass, with spots of short immobilized DNA strands (cDNA or oligonucleotides) attached to it[84]. Each spot, or probe, corresponds to a specific transcript and consists of a large number of copies of the same DNA strand. Due to the large number of probes on an array, one microarray or a set of microarrays can be used to evaluate the complete mRNA (or microRNA) expression in a cell- or tissue sample[84].

The initial process of a microarray analysis is to extract the total RNA from a biological sample, a critical step of the process since the microarray analysis is dependent on high quality RNA. The extracted RNA is subjected to rigorous quality controls before entering the general processing for microarray analysis.

The standard procedures for microarray analysis differ slightly depending on platform used, but most often include the following series of steps[85]. It starts with

amplification of the material, sometimes including a conversion of the RNA to more stable cDNA, and the amplified RNA or cDNA is labelled with a fluorescent dye. There are two main types of arrays, one-channel arrays and two channel arrays. For one-channel arrays one fluorescent dye is used for labelling and only one sample can be hybridized on each array. For two-channel arrays, two fluorescent dyes (with two different emission wavelengths) are used and two samples are labelled and hybridized to the same array. The microarray is then exposed to the fluorescently labelled RNA or cDNA and if the transcripts find complementary DNA probes it will hybridize. After a number of subsequent washing and drying procedures of the array to remove non hybridized RNA/cDNA, the array is scanned using a scanning system equipped with a laser device.

The scanning of the array measures the fluorescent intensities in each spot, which correspond to the number of bound transcripts, and represent the expression level of that particular gene. Subsequently, the scanned images are transformed to expression values. This process is supported by software included in the scanning device. Next, it is time for low level analysis including handling of missing values, background correction, normalization, and log transformation of the data. The background correction is essential to acquire noise-reduced values, and the normalization is needed to reduce the systematic variation and to separate true biological variation from experimental bias. The normalization process can be done between arrays, within individual arrays, and for the dye-labelled channels on two-channel arrays. The resulting pre-processed dataset is now ready for the high level analysis, where the biologically meaningful information will be extracted from the dataset. This part is discussed in detail in the following method section.

1.5.2 Quantitative proteomics

In recent years, there has been great advances in mass spectrometry-based proteomics and it is now possible to measure several properties, such as the protein abundance, turnover rate, subcellular localization, and post-translational modification, for thousands of proteins. For the studies in this thesis the abundance of proteins has been measure using LC-MS/MS together with labeling of isobaric tags (Tandem mass tags – TMT™, Thermo Fischer Scientific)[86]. The technology is based on the use of LC as a separation tool for proteins (or peptides) with subsequent repeated steps of mass spectrometry to identify and quantify the separated proteins or peptides. The strategy used is a so called "bottom-up" approach, where the proteins, in a first step, are cleaved by the endopeptidase trypsin into peptides, which solely constitute the subjects for the MS/MS

analysis[87]. The digested peptides are separated using LC, before subsequent analysis by MS. In direct connection to the LC instrument, is a mass spectrometer, which measures the mass-to-charge ratio of charged particles, in order to determine the mass of the particles. The separated peptides are loaded into the MS instrument and vaporized before being ionized by an ion source, in this case electrospray ionization. In the mass analyzer the ions, i.e. the charged peptides, are separated by their mass-to-charge ratio using electromagnetic fields, before the detector quantifies the ion signals and convert it to a mass spectra. The information from a mass spectra is usually not sufficient for the identification of a peptide since these signals are not unique for a peptide[87]. Therefore, it is necessary to perform a tandem MS. The first MS step is performed to determine the mass of the intact peptide, the precursor ion. In the second MS step the precursor ion is isolated from other peptides ions, dissociated into fragments and the masses of the fragments are determined. In the case of TMT™ labelled peptides, one additional fragmentation, and a third MS is performed to dissociate and quantify the TMT™ reporter ions.

To determine the abundance of proteins in a sample, either as absolute values or relative the amount of the protein in another sample, different techniques have been developed. Many of these methods involve the labelling of proteins with stable isotopes. The general procedure is to tag samples containing proteins or peptides with reagents with various tag variants. The samples are mixed prior to the MS analysis and the resulting peaks in the mass spectra reveal the ratio of the different tag variants and can be used to determine the relative abundance of each protein or peptide. The TMT™ reagent from Thermo Scientific constitute one method of isotopic labelling for relative quantification[86]. The TMT™ allows for the multiplexing of up to ten samples in a single run, and consist of different isobaric (same molecular weight) tags that are based on the same chemical structure. The tags contain a tag-specific reporter group and a mass normalizer group that leads to an equal overall mass for the different tags. During the fragmentation for the MS3 the reporter groups are cleaved and creates up to ten different signals, the relative intensities of these signals represent the ratios of the corresponding ten samples. Accurate, high-resolution mass spectrometer analyzers allow for the separation between these reporter ion isotopes and enable increased multiplexing for relative quantification and increased sample throughput[88]. In addition, using one of the tags for a reference sample allows for the comparison between TMT™ sets, increasing the throughput even further.

2 AIMS OF THE THESIS

The overall aim of this thesis is to investigate if hepatocytes and cardiomyocytes derived from hPSCs can be used as *in vitro* models for drug-induced toxicity studies and assessment.

2.1 Specific aims

- To explore the feasibility of using hPSC-derived hepatocytes (addressed in paper I) and cardiomyocytes (addressed in paper II) to study toxic drug exposure for up to two weeks.
- To investigate if large scale omics data and bioinformatics tools can be used to evaluate the cellular responses to doxorubicin (addressed in paper II-IV).
- To further explore the cellular responses to doxorubicin and identify potential biomarkers of the compound-induced toxicity (addressed in paper II-III).
- To investigate underlying mechanisms involved in doxorubicin-induced cardiotoxicity by integration of data from mRNA, microRNA, and protein expression (addressed in paper IV).

3 METHODS

3.1 Differentiation of hepatocytes and cardiomyocytes

The work in this thesis was performed in collaboration with Takara Bio Europe AB (former Cellartis AB), a biotechnology company with long standing experience in deriving, culturing, and differentiating hPSC. The thesis work has not included the development of differentiation protocols for the different cell types studied and has, as far as possible, been conducted with established, published protocols or commercially available cell types and kits for differentiation. However, the handling of the cells, related to timing, seeding densities, and culture formats, has been adapted, to ensure optimal conditions for the toxic drug exposure and subsequent analyses. The hepatocytes used were generated from hiPSCs, and the cardiomyocytes were derived from a hESC line. The details about the cell cultures can be found in papers I-IV.

3.2 Cell characterization

The hepatocytes and cardiomyocytes used in this thesis are extensively characterized during the establishment of the differentiation protocols, and this has been reported elsewhere[15, 89, 90]. The main aim of this thesis was not to characterize the cells as such, but rather to investigate potential applications of the cells. However, some basic characterization, consisting of assessment of important cell type-specific proteins and functions, to ensure relevant phenotypes of the cells has been performed. For the hepatocytes, morphological evaluation of the cells, their ability to store glycogen (by Periodic acid Schiff staining), and the presence of cytokeratin 18 and hepatocyte nuclear factor 4a, was reported (Paper I).

Correspondingly, for the cardiomyocytes, morphology evaluation together with immunocytochemistry of cardiac specific troponin (cTnT), NK2 Homeobox 5 (NKX2.5), and Myosin regulatory light chain 2, atrial isoform (MLC2a), was reported (Paper II).

3.3 Incubations with toxicants

The study described in paper I, was designed to investigate the toxic exposure of drugs in hiPSC-derived hepatocytes during a two week time period. Four compounds known to be toxic to hepatocytes were chosen; Amiodarone, Aflatoxin B1, Troglitazone, and Ximelagatran, which all possess different modes-of-actions for their toxicity. All compounds were dissolved in dimethyl sulfoxide (DMSO) and were subjected to half-logarithmic serial dilutions (in DMSO), to ensure precise dilutions over large concentration spans. A second dilution of the concentration series, prior to the exposure to the cells, were performed in cell culture medium to reduce the final concentration of DMSO to 0.4% and to be able to monitor any potential precipitation of the compounds. The highest assay concentrations were chosen based on the properties of the compounds and relevance to the clinical situation. Stem cell derived hepatocytes, differentiated for approximately three weeks, were exposed to the compounds, in a repeated-dose regime, over a two week time period, with medium changes every second day. Appropriate untreated control cells were cultured in parallel.

For the studies reported in paper II – IV, stem cell derived cardiomyocytes were exposed to doxorubicin at various concentrations during two days, followed by a recovery period of 12 days without doxorubicin. This regime was chosen with the rational to study both the acute toxic phase of doxorubicin as well as potentially remaining effects of the exposure. Initial pilot studies, conducted prior to the first study reported in paper II, indicated that a doxorubicin concentration up to 450 nM affects the cardiomyocytes (based on measurements of cTnT release from the cells) without causing substantial general cytotoxicity (judged by viability assessment). A concentration of 450 nM correlates well to the peak plasma concentrations seen during clinical use of doxorubicin[91, 92]. Two additional concentrations of doxorubicin (50 nM and 150 nM) were included, in order to study dose-dependent effects. Doxorubicin was dissolved in DMSO followed by a dilution step in cell culture medium to a final DMSO concentration of 0.05%.

3.4 Viability and cytotoxicity

3.4.1 EZ4U

There are several ways to measure the number of cells in cultures. Direct measurements include e.g. microscopic inspection or the use of an electronic particle counter, and there are indirect means such as the measurement of

incorporated radioactive markers, quantification of total protein content, or measurement of metabolic activity of cellular enzymes.

The proliferation and cytotoxicity assay used in paper I, EZ4U (Biomedica immunoassays), is based on the findings that viable cells are capable to reduce weakly colored tetrazolium salts into intensely colored formazan derivatives. This process requires functional mitochondria, which are inactivated within a few minutes in dead cells. The EZ4U assay contains a non-toxic tetrazolium salt that can be reduced to soluble, colored derivate that indirectly correlates to the number of viable cells in the sample. The assay is recorded in a microplate-reader by measuring the absorbance at a specific wavelength (450 or 492 nm) in each well.

3.4.2 Steatosis and Phospholipidosis

To assess the induction of steatosis and phospholipidosis in the hepatocytes following compound exposure, the cells were stained with fluorescent probes for neutral lipids (HCS LipidTOX Green Neutral Lipid, www.thermofischer.com) and phospholipids (HCS LipidTOX Red Phospholipidosis, www.thermofischer.com) and analyzed in an automated manner using ImageXpress Micro (Molecular Devices, Sunnyvale, CA). The fluorescent probe works as a signaling flag for the target of interest. One part of the probe has a high affinity to bind to the target, in this case neutral lipids and phospholipids, and another part of the probe acts as a fluorophore. The fluorophore works by absorbing light from an external light source, the excitation light, and send out light of another wavelength. The emitted light is detected in a fluorescent microscope or an automated high-content screening device. Using image analysis software, the labelling of the cells can be quantified and reported as e.g. a percentage of positive cells within the cultures.

3.4.3 Lactate Dehydrogenase

Cytotoxic effects in cells are usually associated with damage to the cell membrane. This can be assessed by monitoring the extracellular leakage of intracellular substances that are normally seized inside cells. Lactate dehydrogenase (LDH) is an intracellular enzyme that catalyzes the conversion of pyruvate and lactate and is present in all cell types. Since LDH is a relatively stable enzyme, it has been widely used to evaluate the presence of damage and toxicity of tissue and cells[93, 94].

For the work in this thesis, LDH leakage was measured in conditioned culture medium after the cardiomyocytes had been exposed to doxorubicin, as an indicator of general cytotoxicity within the cultures.

3.4.4 Cardiac-specific troponin

Troponins are regulatory protein involved in Ca2+-mediated processes of contraction and relaxation of muscles. Troponin consists of three subunits C, T, and I, which interact with different parts of the actin/tropomyosin filaments. Each subunit exists in several isoforms that are encoded by separate genes and they can be distinguished by immunological techniques. Troponin T and I both have isoforms that are specific for cardiomyocytes, and are therefore suitable as markers for cardiac injury[95]. Cardiac troponin is also a widely accepted and clinically used biomarker in humans for cardiac injury due to ischemic injury and drug toxicity. It is highly correlated to extent of injury, deficiency of cardiac function, and prognosis[96]. It is also considered a highly translational biomarker and its usage to detect myocardial damage in pre-clinical pharmacology, toxicological studies, and animal studies is quickly growing[95].

For the studies in this thesis (paper II), cTnT was measured in conditioned culture medium, after the cells were incubation with doxorubicin, as a sign of cardiac-specific toxicity. The cTnT was measured using an automated immune-chemiluminiscence instrument and the high sensitive Troponin T assay (Elecsys, Roche).

3.5 Microarray analysis

A key element for the work in this thesis was to use large scale omics data to evaluate the stem cell-derived *in vitro* models. Global microarray experiments have been conducted in order to generate extensive transcriptional datasets. For the project two different microarray platforms were used:

- GeneChip® Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA)
- miRCURY LNA™ microRNA Array 7th Gen (Exiqon, Denmark)

3.5.1 Microarray experiment in paper II

The first study using hESC-derived cardiomyocytes, described in paper II, was designed to evaluate the cells' response to doxorubicin at the transcriptional level. As described in 3.3, the cardiomyocytes were exposed to doxorubicin in three different concentrations for up to two days, followed by a recovery period of an additional 12 days. At selected time points during the study, day 0, 1, 2, 7, and 14, cells were harvested and total RNA was isolated using Qiagen's RNeasy Plus Micro Kit (www.qiagen.com) according to the manual. The experiment, including untreated controls, was run in triplicates resulting in a total of 51 samples collected and subjected for microarray analysis with GeneChip® Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA). Following RNA quality assessment, the samples were hybridized to the microarrays. Using the Expression Console v.1.1.2 (Affymetrix), the expression signals were normalized using the robust multichip average (RMA) normalization method resulting in log2 transformed data, which was subjected to succeeding high-level data analysis. SCIBLU Genomics Core Facility at Lund University performed all the low-level analysis until the normalization step.

3.5.2 Microarray experiment in paper III

The study described in paper III aimed to evaluate the microRNA expression in hESC-derived cardiomyocytes following doxorubicin exposure. The same experimental setup as the mRNA experiment described in paper II was applied here, using the same concentration and sampling parameters. However, total RNA was isolated using GenElute RNA/DNA/Protein plus Purification Kit (E5163, Sigma-Aldrich, Sweden, http://www.sigmaaldrich.com), for simultaneous isolation of protein from the same samples. This isolation kit also allows the preservation of all RNA, including the small microRNA. After assessment of RNA quality, samples were hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon, Denmark). This array is a two channel array, hence the samples are labeled with one fluorescent label (Hy3™) and a RNA reference is labeled with the second fluorescent label (Hy5™) prior to hybridization. After scanning and image analysis of the microarrays, the quantified signals were extracted, background corrected, and normalized using the global locally weighted scatterplot smoothing (LOWESS) regression algorithm. Detected microRNAs with low intensities (below the threshold for each array) in more than 80% of the samples were filtered prior to subsequent expression analysis. All microarray analysis, from RNA quality control up until expression analysis, was conducted at Exiqon Services, Denmark.

3.6 Quantitative proteomics

A major focus for paper IV in this thesis was to investigate the responses to doxorubicin in the hESC-derived cardiomyocytes on the protein level. The microRNA experiment (described in paper III) was designed to enable for the simultaneous acquiring of total protein samples. The protein samples were subjected for a relative quantification using isobaric tag-labeling and LC-MS/MS analysis.

All protein samples together with a reference pool (containing aliquots from all sample groups) were processed for protein reduction, alkylation, and digestion. The resulting peptides were subjected to isobaric mass tag labelling with TMT™ reagent according to the manufacturer's instructions (Thermo Scientific). The TMT™-labeled samples were analyzed using LC-MS/MS and the subsequent raw data files were entered into Proteome Discoverer version 1.4 (Thermo Fisher Scientific) for protein identification and relative quantification. Only peptides unique for a given protein were considered for relative quantitation, and only proteins that were detected in all samples by all three replicates were considered for further analysis. All described work after the isolation of protein samples, until the analysis of the proteomics data, was conducted at the Proteomics Core Facility at Sahlgrenska Academy, Gothenburg University.

3.7 Bioinformatics and statistical analysis

The analysis of the data generated by microarray analysis and protein quantification can be divided into low-level analysis and high-level analysis. Low-level analysis can vary depending on analysis platform but can be described as the pre-processing of the raw data to ensure a valid dataset for the subsequent analysis. The pre-processing often includes: filtering procedures, i.e. removal of bad quality data e.g. bad spots on a microarray; handling of missing values; normalization to remove systemic, non-biological variation; and log transformation of the data.

For the high-level analysis, in order to extract relevant biological information from the extensive data, advanced bioinformatics algorithms are required. The important bioinformatics analyses used for this thesis are described below.

The majority of the analyses within this thesis was carried out using the free R software environment (www.r-project.org), a programming language and

environment for statistical computation and graphics. The R environment provides a wide range of statistical and graphical techniques, has a large collection of tools for data analysis, and is highly extensible via the concept of different 'packages'.

3.7.1 Identification of differential expression

One important part when analyzing large scale omics data is to identify differential expression, i.e. genes or proteins that are induced or repressed under a specific condition or in a particular biological process. This was of relevance in the study of doxorubicin-induced toxicity in the hESC-derived cardiomyocytes, where a major goal was to identify significantly affected mRNAs, microRNAs, and proteins.

There are many different methods to identify differential expression. Simple methods, such as average fold change, where genes are considered to have differential expression if the fold change between two samples, e.g. between case and control samples, are above a certain threshold. One common method is to use statistical significance to decide on differential expression, e.g. perform T-tests and use a p-value threshold for significant differences. When analyzing many genes with these types of methods it is important to consider the multiple testing problem, and the risk of identification of many false positives. These methods therefore require correction of the p-values for multiple testing.

There are also more specialized statistical tests developed for certain applications. For the analyses of the microarray data in paper II and III, the Significance Analysis of Microarray Data (SAM)[97] have been applied. SAM is included in the Siggenes package available in R (http://www.bioconductor.org), and identifies differentially expressed genes in microarray data, by controlling the false discovery rate, i.e. the percentage of genes identified by chance. Briefly, the method assigns a score to each gene based on the difference between samples, in relation to the standard deviation of repeated measurements. The false discovery rate is determined using permutations of the repeated measurements.

The SAM was developed for microarray data, but for the study in paper IV the intent was to integrate the results from the different omics data, and it is desirable to identify differential expression in the diverse sets of data using the same method. Thus, the f-divergence cutoff index (fCI) model was used, since it was developed with the purpose to identify differential expression across different omics data types and multi-dimensional data[98].

Briefly, the fCI model creates control-control and control-treatment combinations and compares the similarity of the ratio distributions for the different combinations. If the treatment causes upregulation or downregulation of certain genes, the distribution of the ratios from control-treatment pairs compared to the control-control (empirical null) distribution will appear different. To identify differentially expressed objects (genes or proteins), the difference between the distributions is quantified by a function called f-divergence. The fCI model uses a mathematical function called the Hellinger distance to calculate the f-divergence, and objects are removed from both tails of the control-treatment distribution until the remaining distribution is similar or identical to the empirical null distribution. The removed objects are classified as dysregulated and the analysis output contains a list of the dysregulated objects for all the pairwise analysis results, including the total number of times an object is found to be dysregulated and the coverage percentage.

3.7.2 Cluster analysis

Data from microarray and quantitative proteomic experiments consists of loads of disperse information and in order to reduce the number of dimensions and facilitate the data interpretation there are many different clustering techniques available. The purpose of clustering is to organize the data, to highlight what contributes to the variation seen in the data, and to identify co-expressed and related genes/proteins. For this project, several different methods have been used, depending on the intention of the clustering.

Principal component analysis (PCA)[99, 100] and agglomerative hierarchical clustering[101] have, in this project, mainly been performed on a global scale, on whole data sets, with the aim to assess that the samples cluster in a biologically relevant way. One purpose was to identify outliers and to assure that the experiment worked out as anticipated.

PCA is a statistical technique to reduce the dimensionality of large data sets and make the data more interpretable. Large data set consists of many related variables and PCA transforms the data into uncorrelated variables, so called principal components, which are ordered in such a way that the first component accounts for as much variability in the data as possible[99]. A PCA plot indicates if your samples are separated in a biologically relevant manner and elucidate relationships in your samples depending on how they cluster.

Agglomerative hierarchical clustering[101] also investigates relationships in the data. The method starts with each object as a separate cluster and gradually link and merge objects into clusters based on a distance measurement. The result is a dendrogram, showing the relationship between the samples, where the branches represent similarity.

As the name suggests, the hierarchical clustering has a hierarchical approach to group objects into larger clusters. The alternative is to use partitional clustering, where the most common method is the K-means algorithm. K-means was developed over 50 years ago, and due to its ease of implementation, simplicity, and efficiency, it is still one of the most widely used algorithms for clustering[102].

There are many different methods to measure the distance, or similarity, between to objects (genes/proteins) or clusters. The main method used for this project is the Pearson correlation, or Pearson Product Moment Correlation. Pearson correlation considers the 'shapes' of expression profiles to calculate the similarity between objects. One advantage of this method is that genes with similar expression profiles but large differences in expression level will still be judged as similar.

3.7.3 Pathway analysis

Pathway over-representation analysis (ORA) has been performed within the scope of this thesis. The aim of a pathway ORA is to analyze whether lists of differentially expressed genes and/or proteins are significantly associated with a particular signaling pathway or set of pathways, and to further interpret the biological meaning of identified differentially expressed genes and/or proteins.

For the ORA of differentially expressed mRNA and proteins found in this project, the database ConsensusPathDB[103-105] have been used. ConsensusPathDB is a database that integrates different types of functional interaction data of currently 32 resources and databases (Release 31, 01. Sept. 2015). Over-represented sets of genes are searched for in predefined gene sets, of e.g. pathway or Gene Ontology category. For each predefined set a p-value is calculated with a hypergeometric test based on the number of physical entities (i.e. genes or proteins) in the predefined set and the user-specified input list. The p-values are corrected for multiple testing using the false discovery rate method (q-value) and the output of the analysis is a list of all sets whose hypergeometric tests p-value passes the threshold set by the user.

The ConsensusPathDB described above is developed for physical entities, such as lists of genes or proteins, however, with the increasing interest and knowledge about microRNAs the need for similar tools for microRNAs is increasing rapidly. In this aspect, the software tool microRNAs enrichment analysis and annotation: miEAA, was developed[106]. Like many similar web-based application, miEAA offers the common statistical tests, such as ORA and Gene Set Enrichment Analysis (GSEA), but with the exception that it is tailored for microRNAs or microRNA precursors as input. The miEAA application calculates significance for a test set to be over- or underrepresented in respect to a reference set and uses Fischer's exact test to compute p-values. Similar to the ORA in ConsensusPathDB, the p-values are corrected for multiple testing and the user can define specific thresholds for significance.

3.7.4 Integration of omics data

With the advances in technologies to generate large scale data on various molecular levels there is a growing demand for cross-platform dataset analysis. The application: Integrated analysis of Cross-platform MicroArray and Pathway data (InCroMAP), was developed for this purpose, and offers a powerful, easy-to-use high-level cross-platform microarray dataset analysis tool[107, 108]. InCroMAP, which is freely available as a Java application, can be used for pathway-based analysis and visualization of heterogeneous, cross-platform datasets.

The user inputs processed datasets, i.e. calculated fold change values or p-values, of any or all of the currently supported platforms: mRNA, microRNA, DNA methylation, and protein modification, and a variety of different methods for the integrated analysis of data from these platforms are available.

For the study described in paper IV, InCroMAP was used to visualize the expression of the various datasets in several selected pathways. The basis for the visualization are imported from the Kyoto encyclopedia of Genes and Genomes (KEGG) database, and consists of a background image of the selected pathway. This background image is overlaid with nodes representing mRNAs, proteins, and microRNAs involved in the pathways. To visualize the expression values, the nodes are colored according to a color gradient ranging from blue through white to red to illustrate down- and upregulation. Non-differentially expressed genes are shown in white, and pathway nodes for which no input data are available are displayed in grey.

4 RESULTS IN SUMMARY

4.1 Paper I: Long-Term Chronic Toxicity Testing Using Human Pluripotent Stem Cell–Derived Hepatocytes

In paper I, the utility of hPSC-derived hepatocytes for hepatotoxicity studies was assessed. Existing hepatic *in vivo* and *in vitro* models show clear limitations when it comes to accessibility, functionality, resemblance to *in vivo* situation, and particularly possibility to be used for longer time periods. For these reasons, the aim of the first study was to assess long term exposure of hepatotoxic drugs in the hPSC-derived hepatocytes. The hiPSC-derived hepatocytes (hiPS-HEP) were exposed to various concentrations of known hepatotoxic compounds for up to 14 days, following a repeated dose regime. Besides monitoring the cell morphology, the cells sensitivity to the compounds was assessed using the EZ4U cell proliferation and cytotoxicity assay (Biomedica/Biotech-IgG, Copenhagen, Denmark). In addition, specific toxicity was assessed by quantification of the induction of steatosis and phospholipidosis within the cells using automated imaging and analysis. The results demonstrate that the cells are sensitive to several of the compounds, since a clear dose-dependent decrease in viability was observed for amiodarone, aflatoxin B1, and troglitazone. The cells also demonstrated an increased sensitivity to these compounds during the long term exposure, as indicated by a significant shift in the dose-response curves for the 7- and 14-days exposures compared to the single dose 2-day exposure. To assess whether the cells responded to the compounds in a hepatocyte-relevant manner, the cells were exposed to sub-cytotoxic concentrations (10% effective concentration at 14 days exposure) of the compounds, and stained for steatosis and phospholipidosis. The results reveal an induction of steatosis and phospholipidosis in the cells when exposed to amiodarone and troglitazone, respectively. Most importantly, this study demonstrates that the hiPS-HEP can be derived in a robust manner and that the cells are stable enough to constitute an *in vitro* cell model that enables a two-week exposure study of hepatotoxic compounds.

4.2 Paper II: Identification of novel biomarkers for doxorubicin-induced toxicity in human cardiomyocytes derived from pluripotent stem cells

The study described in paper II was designed to assess the utility of hPSC-derived cardiomyocytes for cardiotoxicity studies over a two week time period. Doxorubicin, a chemotherapeutic drug with well-known cardiotoxic effects, was chosen as the toxic model compound to study. Doxorubicin demonstrates a dose-dependent cardiotoxicity with a multifactorial etiology, and despite being intensely studied for several decades, the exact mechanisms behind the toxicity remain unrevealed. The hESC-derived cardiomyocytes were exposed to a single dose of doxorubicin at various concentrations (50 nM, 150 nM, and 450 nM) for up to two days, followed by an additional study period of 12 days without doxorubicin. This experimental design enables the study of both the acute toxic response as well as the study of sustained effects of the drug. At selected time points throughout the study (at day 0, 1, 2, 7, and 14), the morphology of the cardiomyocytes were monitored, in connection to the measurement of LDH and cTnT release from the cells, as indications of general and cardio-specific responses to the toxic insult. To get an in-depth understanding of the mechanisms involved in the doxorubicin-induced toxicity, the global transcription within the cells were analyzed using microarray technology. The experiments show that the hESC-derived cardiomyocytes are robust and stable enough to enable a two-week study of drug-induced toxicity. The results of the study further demonstrate a clear effect of doxorubicin already after one day of exposure. The cells displayed an altered morphology and reduced contractility, and these effects were sustained throughout the entire study. A general cytotoxicity could be observed during the acute exposure by elevated leakage of LDH at day two in treated cells compared to controls. A similar dose-dependent pattern could be observed for the cTnT release from the cells during day one and day two of exposure. However, despite a clear morphological and functional effect of doxorubicin during the recovery period, no elevated release of neither LDH nor cTnT could be observed during this period, suggesting that these markers might not be suitable as biomarkers for the late onset of doxorubicin-induced cardiotoxicity. The global transcription analysis reveal an evident effect of doxorubicin on the transcriptional level. Hierarchical clustering of the global mRNA expression shows a clear separation of the samples into six distinct groups, which correlated to the time factor and treatment of the groups. Investigations of dysregulated genes upon doxorubicin

exposure were carried out with the statistical method SAM and the simplified approach of gene expression profile search, and revealed lists of dysregulated genes with high concordance between the two approaches. Further analysis of the differentially expressed genes shows connection of cellular defense and the p53-signaling pathway. Some of the differentially expressed genes, such as Growth Differentiation Factor 15 (GDF15), Neurofilament, Light Polypeptide (NEFL), LDL Receptor Related Protein Associated Protein 1 (LRPAP1), Family with Sequence Similarity 198 Member B (FAM198B), RAD15 paralog C (RAD51C), and Growth Arrest and DNA Damage Inducible Alpha (GADD45A), demonstrate elevated expression for the treated samples compared to the controls throughout the entire experiment, which might indicate a potential role for these genes as biomarkers for doxorubicin-induced cardiotoxicity.

4.3 Paper III: MicroRNAs as potential biomarkers for doxorubicin-induced cardiotoxicity

As a follow-up to the study reported in paper II, the subsequent experiment was designed to elucidate the involvement of the regulatory network of microRNAs in the doxorubicin-induced cardiotoxicity. Additional aims of this study were to identify microRNAs as potential biomarkers for the toxicity, to further strengthen the utility of hPSC-derived cardiomyocytes as a relevant *in vitro* model, as well as to evaluate the use of large-scale data and bioinformatics for these purposes. The cardiomyocytes were subject to the same experimental procedure as in paper II and isolation of total RNA for microRNA profiling was performed. Using microarray technology, the global expression of microRNAs was analyzed. MicroRNAs have in recent years been shown to have a great potential as clinical biomarkers, due to their high evolutionary conservation, stability in various body fluids, and in general a high cell type or tissue specificity. The results from this study reveal a clear effect of doxorubicin on the global microRNA expression. Principal component analysis and hierarchical clustering of the global microRNA expression show a separation of the samples by both the time and the treatment factor, indicating that the cells respond to the doxorubicin treatment in a biologically relevant way. Using SAM, we identified several microRNAs, including miR-34a, miR-34b, miR-187, miR-199a, miR- 199b, miR-146a, miR-15b, miR-130a, miR-214, and miR-424, which are differentially expressed upon, and after, treatment with doxorubicin. The majority of the microRNAs that show a sustained effect during the wash-out period are dose-dependently downregulated. To investigate the biological relevance of the dysregulated microRNAs, pathway

ORA was performed and revealed a connection to pathways related to cardiomyocytes and heart function, apoptosis, inflammatory responses, and cellular defense mechanisms. Three microRNAs show a sustained upregulation upon doxorubicin treatment throughout the whole experiment, which make them very interesting as potential biomarkers for the toxicity. Both miR-34a and miR-34b have previously been shown to be associated with pathological situations in the heart, while miR-187 has been associated with cancer and inflammatory pathways. In summary, the results reported in paper III identify a clear effect of doxorubicin on the microRNA expression with a high correlation to the impaired cardiomyocyte function. Several microRNAs are identified as potential biomarkers for the doxorubicin-induced cardiotoxicity.

4.4 Paper IV: Expression profiling of human pluripotent stem cell-derived cardiomyocytes exposed to doxorubicin – integration and visualization of multi omics data

Protein expression represents the functional counterpart of the mRNA expression and in that sense might illustrate the events taking place in the cells in a more precise way. Recent advances in the mass spectrometry-based proteomics, using for instance covalently bound chemical tags such as TMT™s allow for the relative quantification of many proteins in biological samples simultaneously.

The microRNA experiment reported in paper III was designed for the parallel harvest of proteins from the same samples, thus 51 samples were subjected for relative quantification using LC-MS/MS with isobaric TMT™ labelling. The results were assessed on a global scale and PCA of all samples demonstrate a relevant separation of the samples both by the experimental time factor and the treatment. Differentially expressed proteins were identified with the fCI model, which revealed a clear dose dependent increase in the number of differentially expressed proteins at each time point. In total, the fCI method identified 199 unique proteins that showed differential expression in at least one treatment group compared to the controls during at least one time point. Amongst the identified differentially expressed proteins, we observed proteins related to myofibrillar function such as the myosin light chain family (MYL2, MYL3, MYL4, MYL6, MYL7, and MYL12A), myosin heavy chain (MYH7, MYH9, MYH10), troponins (Troponin C1 (TNNC1), Troponin I3 (TNNI3), and Troponin T2 (TNNT2)), and tropomyosin (TPM1, TPM2, and TPM4); ribosomal proteins;

mitochondrial associated protein such as the cytochrome C oxidase (COX) family, and adenosine triphosphate (ATP) family, and proteins linked to reactive oxygen species formation such as Tumor Protein P53 Inducible Protein 3 (TP53I3). KEGG pathway ORA of the identified differentially expressed proteins revealed a clear connection to cardiomyocyte related pathways, such as cardiac muscle contraction, hypertrophic cardiomyopathy, and dilated cardiomyopathy. K-means clustering was used to identify clusters of differentially expressed proteins with different expression profiles. There are proteins connected to cell communication and mitochondrial apoptosis signaling that show an early upregulation due to the doxorubicin treatment. Clusters that are downregulated upon doxorubicin exposure are found to be related to cardiomyocyte function and contraction as well as there are clusters, also connected to the cardiomyocyte function, that display an upregulation over time.

An additional aim with this study was to, in a more in-depth manner, investigate how doxorubicin influences the cardiomyocyte functionality by integrating the findings from the proteomics data with the previously generated transcriptomics data reported in paper II and III. To enable an integration of the different levels of data, the mRNA and microRNA data were re-analyzed for differential expression using the fCI model, thus using the same approach as for the protein data. This resulted in 7437 mRNA and 387 microRNA that showed differential expression in at least one treatment group during at least one time point.

InCroMAP is an enrichment analysis tool with the ability for pathway-based visualization of multi omics data. The differentially expressed mRNAs, microRNAs, and proteins from the 450 nM treatment group were used as input and KEGG pathways that showed over-representation at all time points were subjected for integrated visualization of the multi omics expression values. The visualization revealed several interesting expression patterns that show a linkage between the proteome, transcriptome, and the regulatory microRNA network. In brief, there is a clear effect on the myofibrils (the unit responsible for the contractility), on important ion channels, on the mitochondrial associated mRNA and proteins, as well as the ribosomal function, especially during the acute toxic phase. For many of the areas where mRNAs are affected, doxorubicin also affects the associated microRNAs. In summary, the identified differentially expressed proteins support results from paper II and III, and the integration of data from multiple omics domains strengthens important findings of dysregulated genes and microRNAs and identifies potential biomarkers for doxorubicin-induced cardiotoxicity.

5 DISCUSSION

In this section, the results presented in this thesis are placed in a general context and discussed with regard to related research performed by others and potential implications for the reported results.

5.1 Derivatives from hPSC show stability and robustness

The studies that constitute this thesis demonstrate the potential of using specialized cell types derived from hPSC as *in vitro* models for toxicity studies, also for longer time periods. The differentiated hepatocytes used in paper I exhibit a stable phenotype that enables a two week study of compound exposure. The model shows robustness for the three replicates, both with the measurements of viability and also for the more specific induction of steatosis and phospholipidosis. Likewise, the hESC-derived cardiomyocytes used in paper II-IV show a stability in culture throughout the whole two week experiment period and the different omics data extracted from the experiments show that the model is stable across all replicates.

5.2 Cellular responses to toxic compound exposure

Equally important as the displayed stability and robustness, of the two investigated hPSC-derived cell types, are the cells' adequate responses to the toxic compound exposure. In paper I, the hepatocytes show sensitivity towards the known hepatotoxic compounds amiodarone, aflatoxin, and troglitazone, a sensitivity that is increased with prolonged exposure. These results reflect the functionality of the cells as the high sensitivity to amiodarone and aflatoxin correlates to the high CYP3A activity seen in these cells, since both compounds are dependent on CYP3A activity to give toxic metabolites[109, 110]. The hiPS-HEP also show sensitivity to troglitazone, at least at higher concentrations, although the dose-response curve is more ambiguous as compared to the results on amiodarone and aflatoxin. This might be a reflection of the hypothesis that troglitazone is toxic both in its original form and as a metabolite[111], and that the idiosyncratic toxicity associated with troglitazone involves bile salt accumulation and bile acid toxicity[112]. In addition to these findings, a time-dependent increase in amiodarone-induced phospholipidosis and troglitazone-induced steatosis is observed, suggesting a hepatocyte-specific toxic response.

The cardiomyocytes used in paper II-IV are able to recapitulate many of the cellular events taking place in cardiomyocytes during doxorubicin-induced toxicity. The cells display a clear acute toxic response to doxorubicin, detected by a dose-dependent increase in the release of the general cytotoxicity marker LDH and the specific cardiotoxicity marker cTnT, in addition to a clear effect of doxorubicin on the cells morphological appearance and contractility. Global analysis of the transcriptomics data identifies several clusters of genes that show differential expression between the treatment groups. ORA of the dysregulated genes showed a clear association to cellular defense mechanisms and the p53-signaling pathway. Genes like Cycline dependent kinase inhibitor 1A (CDKN1A), GADD45A, Ribonucleotide Reductase Regulatory TP53 Inducible Subunit M2B (RRM2B), Ring Finger Protein 144B (RNF144B), and MDM2 Proto-Oncogene (MDM2) have been reported to play important roles during DNA damage and repair and p53 induced apoptosis and cell cycle arrest[113-117]. Among the altered genes, we also detected genes related to cytoskeleton remodeling, such as Plexin A2 (PLXNA2) and Dihydropyrimidinase Like 4 (DPYSL4)[118, 119], and the cardiac specific genes Potassium Voltage-Gated Channel Subfamily J Member 2 (KCNJ2) and Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 2 (HCN2), encoding for two important potassium channels[120, 121]. Further, we report in paper III the effects of doxorubicin on the cardiomyocytes' microRNA expression. Sets of microRNAs that were significantly dysregulated by doxorubicin were identified, some of which are affected throughout the entire experiment. Several of the identified microRNAs might have roles in mechanisms underlying the cardiotoxicity. The microRNA miR-146a, is suggested to, via the Fos/Activator protein 1 (Fos/AP-1) pathway, have an inhibitory effect on matrix metalloproteinases[122, 123], whose expression show a correlation to heart failure[124]. The complex of miR-199a, miR-199b, and miR-214, is downregulated in dilated cardiomyopathy[125] and this has been reported to result in morphological changes in cardiomyocytes[126-128]. In addition, miR-424 downregulation might cause increased cell death due to reduced inhibition of the tumor suppressor Programmed Cell Death 4 (PDCD4)[129]. Over-representation analysis of the identified microRNAs supports the idea that these microRNAs are involved in, for this study, relevant biological processes, such as Toll/Toll-like receptor signaling; ErbB signaling; apoptosis; and inflammatory responses. The putative effects of the identified microRNAs correlate well with the different functions, such as DNA damage response; cardiomyocyte survival; inflammation; and energetic stress, which currently attracts much interest as key players for explaining anthracycline-induced cardiotoxicity[51]. The study in paper IV, with the proteomic analysis of the cardiomyocytes, provides one additional level of

complexity to previous studies. The result from the protein analysis showed a clear effect of doxorubicin in the cells, with a dose-dependent increase of the number of differentially expressed proteins in all four time points. Groups of differentially expressed proteins with different expression profiles and involved in different biological processes were identified. There is an early triggering of apoptotic signaling with upregulation of e.g. TP53I3 and BCL2 Associated Athanogene 3 (BAG3). An early downregulation of AMP-activated protein kinases (AMPKs) which are involved in p53-dependent DNA damage response and apoptosis[130] was also detected. AMPKs does have a key role in energy and nutrient signaling and the subsequent shift towards upregulation of these proteins correlates with reports of a metabolic shift in cardiomyocytes during heart failure[131, 132]. A large portion of the cluster of differentially expressed proteins that show an upregulation over time is associated with oxidative phosphorylation, fatty acid metabolism, and Krebs cycle. Doxorubicin also affects proteins related to the regulation of the free iron within the cell, something that has been reported to cause cellular damage[133]. In general, doxorubicin seems to cause effects on key proteins related to the cardiomyocyte function and energy production, which correlates well with the events taking place on the transcriptional level.

5.3 Comparison to other models for toxicity study

The results in paper I show a similar compound sensitivity for hiPS-HEP as for the widely used HepG2 model during the acute exposure. But since HepG2 is impracticable for long term exposure due to extensive proliferation, this emphasizes the advantage of the hiPS-HEP model for long term studies. The hiPS-HEP demonstrated a similar ranking of the sensitivity to the tested compounds as for PHH[78, 134, 135]. At the same time, the general lack of long-term toxicity studies using PHH illustrates the challenges of maintaining these cells in traditional 2D culture for extended time periods. A recent study assessed four different 2D cultured hepatic cell models (including HEPG2, PHH, Upcyte hepatocytes, and HepaRG) for their ability to detect DILI-compounds[25]. This study revealed that PHH were the most sensitive cell type, identifying eight out of nine hepatotoxic test compounds, followed by HepG2 cells (6/9), whereas HepaRG cells were relatively insensitive (3/9), but none of the cell models could fully distinguish between DILI and non-DILI compounds. The authors concluded that basic cell health endpoints will not predict hepatotoxic risk in simple hepatic cells in the absence of pharmacokinetic data and that more sophisticated signals of molecular initiating events is required to potentially include these cell model in early safety assessment in the pharmaceutical industry.

The study emphasizes the need for more predictive *in vitro* models and much focus is directed towards various 3D culture strategies in an attempt to preserve liver properties *in vitro*. The development of 3D liver models has so far been rewarding and systems are available whose sensitivity and specificity in detecting hepatotoxicity are superior to those of classical 2D culture systems[136]. The hiPS-HEP model assessed in paper I will most likely also benefit from 3D culture conditions to improve functionality and sensitivity to hepatotoxic compounds. The 3D cultures, together with the development of co-culture model systems, are most likely essential for future detection of problematic DILI compounds such as Ximelagatran, studied in paper I. Ximelagatran, a direct thrombin inhibitor, was withdrawn from the market due to elevated levels of alanine aminotransferase in patients. Short term use of Ximelagatran has not indicated a hepatotoxic potential in clinical trials, and increased rates of elevated liver enzymes was first observed after long-term (>35 days) use of ximelagatran. Investigations with PHH did not result in a decrease in cell viability, not even with concentrations of ximelagatran considerably higher than peak plasma concentrations[137]. Standard preclinical toxicological studies showed no indications of the hepatotoxic effect of ximelagatran, nor has any human-based *in vitro* model been able to define mechanisms observed in the long-term clinical trials[138]. In line with other existing human-based *in vitro* models for hepatotoxicity, the hiPS-HEP model did not detect a toxic response to Ximelagatran. The lack of predictive findings for the toxicity of ximelagatran is probably a reflection of the need for long-term exposure to the compound as well as the probable involvement of class II human leukocyte antigens[139].

When turning to cardiomyocyte models to study anthracycline-induced cardiotoxicity, the traditional cardiac models suffer from the common shortcomings of available models, i.e. species differences for the animal models and limited focus for the commonly used cell lines with artificially over expressed single ion channels. Therefore, during recent years, much interest has been focused on the use of hPSC-derived cardiomyocytes to study anthracycline-induced cardiotoxicity. Two recently published papers report similar studies as the ones conducted in this thesis[140, 141], both showing a considerable overlap of the findings of differentially expressed mRNAs and microRNAs reported here. The high correlation between the studies using a similar cell model, together with the high similarity to the responses seen in the human heart *in vivo*, strengthen the hPSC-derived cardiomyocytes as a relevant *in vitro* model for this type of toxicity studies. Furthermore, one additional research group studying doxorubicin-induced cardiotoxicity in hPSC-derived cardiomyocytes highlights a key feature

with hPSC-derived cell models. In a recent paper, they demonstrate that patient-specific hiPSC-derived cardiomyocytes can recapitulate the susceptibility to doxorubicin-induced cardiotoxicity of individual patients at the cellular level[142]. They show that hPSC-derived cardiomyocytes from patients who experienced doxorubicin-induced cardiotoxicity were consistently more sensitive to doxorubicin toxicity *in vitro* compared to cardiomyocytes derived from patients who did not experience doxorubicin-induced cardiotoxicity. Taken together, these studies indicate that hPSC-derived cardiomyocytes are suitable as a platform for the study and characterization of the molecular mechanisms of doxorubicin-induced cardiotoxicity.

5.4 Mechanistic studies of the doxorubicin-induced cardiotoxicity

One major advantage of using cell culture for toxicological investigations, which is highlighted in this thesis, is the suitability for detailed mechanistic studies. Cells derived from hPSC can in combination with omics analysis constitute the formation of complex *in vitro* models for mechanistic studies of e.g. drug-induced toxicity. Papers II-IV in this thesis confirm that hPSC constitute a relevant platform for mechanistic studies of drug-induced toxicity. The studies identify mRNAs, proteins, and microRNAs that are dysregulated by doxorubicin and the integration of the three omics datasets strengthen the findings from the three individual studies on the effects of doxorubicin. It seems that many of the key processes affected by doxorubicin exposure are identified on all three biological levels. There are several examples of markers that show a correlated dysregulation on both mRNA and protein level, linked to a negative correlation to associated microRNA.

For the mechanistic investigation of responses to toxic stimuli, one should bear in mind that differential expression of specific genes not per se results in activation of a toxicity pathway. There are numerous stress response pathways that have evolved to allow cells and tissues to handle and cope with different kinds of stress and maintain or restore homeostasis. Perhaps the most well-known stress response pathway is the p53 signaling pathway that is initiated in cells with damaged DNA, activating gene transcription that, depending on the context, can lead to apoptosis in order to protect the tissue. Stress response pathways are not the cause of the disturbed biological process but rather the response to the disturbed biological process. For the mechanistic study of toxicity one therefore

often also refer to toxicity pathways and adverse outcome pathways as the events causing dysfunction of a cell, tissue, or organ where whole body homeostasis no longer can be maintained[143]. Having that said, stress response pathways will still be of high relevance in the field of toxicology and further identification and interpretation of these pathways is critical for deeper mechanistic understanding of cells' responses to toxic stimuli.

5.5 Potential biomarkers of doxorubicin-induced cardiotoxicity

Thorough mechanistic studies are important for detailing biological events, but for *in vitro* models to be successfully implemented in regular safety assessment during drug development screening, scalable and inexpensive model systems are needed. Consequently, biomarker discovery and validation is a very important task. In that perspective, the studies conducted in paper II-IV were designed to interrogate the hPSC-derived cardiomyocytes' utility to identify potential (novel) biomarkers for doxorubicin-induced cardiotoxicity.

One interesting finding in the study reported in paper II was the dysregulation of the gene GDF15 during doxorubicin exposure. Many studies in recent years have associated GDF15 with heart disease and suggests GDF15 as a potential biomarker for cardiovascular events[144-148]. However, to our knowledge only one study has reported an association between GDF15 and anthracycline-induced cardiotoxicity[149]. GDF15 is activated by e.g. Tumor protein P53 (TP53) and Tumor necrosis factor (TNF) and resides in the periphery of the p53-signaling network. The protein-protein interaction network reported in paper II show a link between GDF15 and proteins like Hepcidin Antimicrobial Peptide (HAMP) and Hypoxia Inducible Factor 1 Alpha Subunit (HIF1A). HAMP has a role in iron homeostasis[150, 151] with a probable importance for doxorubicin-induced cardiotoxicity, while HIF1A is thought to be involved in dexarazoxane-mediated protection against doxorubicin-induced toxicity[152]. In addition, we identified several other genes with expression profiles similar to GDF15, possibly co-regulated or involved in similar processes, and these are interesting for further investigations. In paper III we also identified three microRNAs, miR-34a; miR-34b; and miR-187, which showed a dose-dependent upregulation upon doxorubicin exposure, an upregulation that was sustained throughout the whole experiment and therefore makes them very interesting as potential biomarkers. Of these, the miR-34 family are the microRNAs that historically has generated

most interest due to the association with cardiac function and disease. Several reports suggest that miR-34a has a key role in cardiac injury and repair[153], and it has been shown in mice that miR-34a is upregulated upon doxorubicin prior to the detection of elevated plasma levels of cardiac troponin[154]. MicroRNA miR-34b has been associated with myo-pathological disease[155], while miR-187 mostly has been mentioned as a cancer biomarker but it has also been reported to have an effect on inflammatory pathways[156]. In our data, all three microRNAs exhibit very attracting expression profiles as potential biomarkers for the doxorubicin-induced toxicity. They show an early response to the exposure, especially miR-34a and miR-34b, and a clear dose-response to doxorubicin that is sustained throughout the wash-out period.

The future of *in vitro* toxicology will most likely rely on sets of biomarkers that gives information about which pathways that are active due to the compound exposure. These molecular initiating events conducts the basis of the pathways resulting in adverse outcome. Identification of which pathways are activated by a specific compound tells us about the biological process which has been interfered.

5.6 Advantages of hPSC-based *in vitro* models

The use of cells derived from hPSC as *in vitro* models for toxicity studies offers several advantages, many of which have been addressed in this thesis. The most apparent advantages are the unlimited cell supply due to the self-renewal capacity of hPSC, together with their differentiation ability into virtually any cell type. The latter offers the possibility to study fully functional cells as well as cells at different developmental or maturation stages. The cells can be derived from a stable genetic background and with the possibility to generate cells from individuals of a desired genetic background. The cells can be up scaled to enable high-throughput screening and analysis, a feature that is very appealing for regular safety assessment in the pharmaceutical industry.

One disadvantage of *in vitro* models in comparison to whole animal studies are their lack of the complete organism perspective, the *in vivo* perspective. However, this can to some extent be addressed by hPSC with their potential to generate complex *in vitro* models of multiple cell types and tissue-like cultures. They also possess the possibility for sophisticated co-culturing with e.g. immune cells, to mimic immunological influences of toxic events. Furthermore, as this thesis demonstrate, an hPSC-based *in vitro* model is highly suitable for mechanistic

studies on several biological levels, especially if combined with advanced omics technologies.

5.7 Limitations of this work

The apparent successful use of hPSC-derived hepatocytes for toxicity assessment is somewhat limited by the fact that these cells are not functionally equivalent to PHH. Cultures are not completely homogenous and typically display population of cells exhibiting immature and mature markers simultaneously. However, there are recent reports on the clear improvements of the differentiation procedures of hPSC-derived hepatocytes, both in the dynamic use of the same protocol for several stem cell lines, as well as the fact that the hepatic phenotypes of the differentiated cells tangent to functional levels of PHH[71, 157].

The same applies to the cardiomyocytes as these cultures possess a population of cardiomyocytes with mixed phenotypes, expressing both immature and mature markers and ventricular-, atrial, and nodal phenotypes (reviewed by Barbuti A. et al.[158]).

Regarding the statement by Paracelsus that "the dose makes the poison", a limitation in the type of chemical safety assessment studies performed here, is that the applied concentration most likely is not the 'free' concentration of the compounds in the cell cultures, and therefore might not be representative for the plasma concentrations *in vivo*. Despite this, for decades researchers have performed toxicity experiments, both in cell culture and in animals, with compound concentrations of lethal dose 50% (LD50), often resulting in unreasonably high concentrations, especially for less harmful compounds. We have in some aspects tried to address this issue during the conducted studies, by exposing the cells to concentration well below LD50, aiming for a concentration range around lethal dose 10% (LD10). However, in these studies we have not investigated the pharmacokinetics or biokinetics of the compounds and therefore cannot be certain that the applied concentrations are the effective concentrations, and if they are clinically or biologically relevant or not.

For some of the key genes affected by doxorubicin-induced toxicity where we demonstrate a clear effect on the mRNA level, there is no differential expression detected on the protein level. For example, the main targets for doxorubicin, Topoisomerase (DNA) II alpha (TOP2A) and Topoisomerase (DNA) II beta (TOP2B) show a clear effect on the mRNA expression but no significant

differential expression on the protein level. This discrepancy highlights one possible limitation of this study, since the measurement of protein abundance might not reflect the dysfunctional proteins caused by doxorubicin. Much of the effects of doxorubicin involves activation or inactivation of proteins, phosphorylation, disruption of protein complexes, and in other ways inhibition of protein functions, effects that are not detected when simply analyzing the abundance of the proteins. Another possible limitation in the proteomic technique used for this study is the 'bottom up' approach to identify and measure the abundance of proteins. The approach uses the peptides for identification and quantification and there is a risk that not all comes from a single protein species, the same peptide might originate from several protein isoforms. This might lead to a risk of incorrect conclusions if the results are interpreted as the behavior of a single polypeptide in the cell, when in fact it can be the average of several polypeptides with different characteristics. However, measures are taken against this problem during the analysis of the raw data and only peptides that are unique to a given protein are considered for relative quantification, which in turn substantially reduces the number of proteins identified and quantified. In addition, the method is 'data-dependent', meaning that it randomly samples the population of peptides that is injected into the mass spectrometer, which influences the range of peptides identified and quantified. These limitations might be reflected in the case with the identified potential biomarker GDF15 reported in paper II, which in the mRNA data show high expression level and a clear differential expression upon doxorubicin exposure but despite that, it is not detected in any sample at the protein level.

6 CONCLUSIONS AND FUTURE PERSPECTIVE

This thesis shows the potential of using hPSC derivatives as *in vitro* models for toxicity studies. The results demonstrate the diverse applicability of hPSC as a source for representative cells, and they also illustrate how robust and reproducible the differentiation of hPSC has become. The thesis also display the cells' relevant responses to toxic stimuli and their ability for deep mechanistic exploration of the toxic response.

The studies in this thesis are well in line with the strategy of the three R's (reduce, refine, and replace) in the use of animal models. *In vitro* models based on cells from hPSC offer a promising alternative to animal models, with the potential of being more predictive for the human *in vivo* situation. In conjunction with omics technologies, that provide a system view of cellular events, the development of new mechanistic biomarker sets will be easier achieved. The thesis demonstrates that the integration of several omics branches can provide a deeper understanding of the mechanisms on how compounds disturb normal biological processes and functions. The thesis lends additional support to that all necessary tools for the implementation of the vision of the new toxicity strategy are available. The studies performed follow this new strategy by using hPSC derivatives together with integration of omics data from multiple levels, to investigate the mechanisms behind the specific doxorubicin-induced toxicity, identify toxicity pathways and potential biomarkers, and ultimately result in the development of a test model for this type of toxicity.

The *in vitro* models insufficiency of the *in vivo* resemblance is an impediment for the use of *in vitro* models for toxicity predictions. In that perspective, a future technology that seems promising is the organoid-on-a-chip, advanced culture devices that can recapitulate the 3D tissue structure and physiological conditions of normal tissues. In general, 3D systems exceed 2D cultures in regard to *in vivo* resemblance and the response to drugs and toxins[159]. The advances in tissue engineering, biomaterials, and microfluidic technologies enable the assembly of organoid-on-a-chip devices, multicellular 3D cultures approaching *in vivo* physiology. 3D organoids constructed of e.g. liver or cardiac cells show advantages in functionality compared to traditional cultures and maintain viability for 4 weeks or beyond[160]. These organoid-on-a-chip technologies might also offer the possibility to construct even more advanced multi-organ systems, so called

body-on-a-chip, which for example opens up for the appealing idea of allowing metabolism of a prodrug (or compound) in a liver organoid prior to the assessment of the active drug (or toxicant) in downstream tissues or target organoids[160].

As pointed out by Paul Jennings in 2015[161], there are two major issues that researchers and toxicologists in the future will have to address. First, the variety of choices when setting up an *in vitro* system is huge. The decisions of which cells to use, 2D vs 3D, static medium changes vs medium perfusion, etc. are not easy and will not be easier as we create more complex and sophisticated cell culture system. Secondly, how can we capture the large genetic and epigenetic diversity there is? A cell or cell type derived from one individual cannot recapitulate the population variability. The first issue is to some extent addressed in the different major international consortia such as the CiPA-initiative or the "Safety Evaluation Ultimately Replacing Animal Testing" (SEURAT-1), which aim towards evaluating available safety assessment models, and to develop and standardize innovative testing methods for future safety assessment. Studies, such as the ones conducted in this thesis, also aid in evaluating the field of new innovative *in vitro* models. Human iPSC have the potential of addressing the second issue, since they allow the possibility to generate target cells/tissues from any individual. However, increasing the complexity of an *in vitro* system, by for instance including a panel of cells derived from diverse genetic background, will unavoidably decrease the throughput of the system.

One appealing idea, supported by the results in this thesis, is that hiPSC, with the possibility to represent the population variability, can constitute the basic cell source for *in vitro* compound testing systems. These models can span from simple *in vitro* systems of lowly differentiated monocultures for high throughput toxicity screening of compounds, to complex *in vitro* systems of highly differentiated multi-cellular models for lower throughput in-depth mechanistic studies and biomarker identification.

Importantly, *in vitro* toxicology is a rapidly emerging field of science and it will most likely be crucial for future human chemical safety assessment. Using the new knowledge of hPSC biology together with omics technology in an integrated interdisciplinary manner will enable human *in vitro* systems to become these important toxicological tools, with the potential of replacing animal testing completely.

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