Development of alternative methods to animal testing:
Human pluripotent stem cell lines as a tool for the detection of prenatal toxicity

PhD Thesis

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To “Mamu”, my beloved mother

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ABBREVIATIONS

ABC	ATP Binding Cassette
AFP	α fetoprotein
ANOVA	analysis of variance
ATP	adenosine triphosphate
BFGF	basic fibroblast growth factor
CDK	cyclin-dependent kinase
CDKN2B	cyclin-dependent kinase 4 inhibitor B
CDNA	complementary deoxyribonucleic acid
DAPI	4’,6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMSO	dimethyl sulfoxide
EASE	Expression Analysis Systematic Explorer
EB	embryoid body
EC	European Commission
EGE	European Group on Ethics in Science and New Technologies
ESC	embryonic stem cell
ESNATS	Embryonic Stem cell-based Novel Alternative Testing Strategies
EU	European Union
FACS	Facial Action Coding System
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GO	gene ontology
HESC	human embryonic stem cell
HPRT1	hypoxanthine phosphoribosyltransferase 1
ISSCR	International Society of Stem Cell Research
MAP2	microtubule-associated protein 2 in human
MEHG	methylmercury
MEHGCL	methylmecury chloride
MTAP2	microtubule-associated protein 2 in mouse

MYH6	myosin heavy chain 6
NBD	nucleotide binding domain
NCAM1	neural cell adhesion molecule 1
NEFM	neurofilament medium polypeptide 150 kDa
NEUROD1	neurogenic differentiation 1 transcription factor
NPPB	natriuretic peptide precursor B
OCT4	octamer-binding transcription factor 4
OECD	Organisation for Economic Co-operation and Development
OLIG2	oligodendrocyte transcription factor 2
OTX2	orthodenticle homeobox 2
PAX6	paired box gene 6
PC	principal component
PCA	principal component analysis
PBS	phosphatase buffered saline
PRC	poly-comb group protein
QPCR	quantitative real time polymerase chain reaction
REACH	Regulation for Registration, Evaluation, Authorisation and restriction of Chemicals
RELN	reelin
RNA	ribonucleic acid
SAMD3	sterile alpha motif domain containing 3
SSEA-1	stage-specific embryonic antigen-1
SSEA-3	stage-specific embryonic antigen-3
SSEA-4	stage-specific embryonic antigen-4
SOX1	neural commitment genes: sex determining region Y-box 1
TMD	transmembrane domain
TRA-1-60	tumor rejection antigen-1-60
TRA-1-81	tumor rejection antigen-1-81
TRA-2-49/6E	anti-alkaline phosphatase antigen-2-49/6E

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1. INTRODUCTION

The developing nervous system is often targeted by xenobiotics but the prediction of human prenatal toxicity is problematic as the concordance among the toxicological responses of different laboratory animal species can be low. Therefore, it is needed to conduct mechanistic tests on developmental toxicity which may reduce problems due to interspecies variations hence increase the safety assessment for human health point. *In vitro* tests, based on human pluripotent stem cells can elucidate toxicological mechanisms allowing to increase the safety assessment, and at the same time reducing the number of animal experiments which is a major objective in several European legislations. However, the usage of human embryonic stem cell lines in *in vitro* safety assessments is still not formally validated due to the different legislations in the Member States. It will support to set standards which mechanisms need to be detected by other test models also.

1.1 Alternatives to animal testing

Human society has undergone a chemical revolution in the last hundred years, reflected by the global production of chemicals that has dramatically increased from 1 million tonnes in 1930 to 400 million tonnes today [1]. That revolution brought man-made substances into all strands of life and most consumer articles, and can be found in everyday life products, such as computers, TV's, curtains, carpets, furniture, cosmetics and food. Human kind have developed a very high dependence on chemicals, however, the sufficient knowledge about their potential risks and long-term effects is still lacking.

Therefore, testing chemicals, consumer products or pharmaceuticals to figure out their potential harmful side effects is important for the protection of both the consumer and those involved in the manufacturing process. Laboratory animals have been widely used in the past for toxicity testing but recent European legislation actively promote the use of *in vitro* tests in order to increase human safety and to reduce the number of animals needed for safety assessments. For example the EC Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes requires the use of an alternative method to animal experiment if exists [2]. Moreover, it encourages the development, validation and

acceptance of methods which could **r**educe, **r**efine or **r**eplace (3 R's Principle) the use of laboratory animals. As a follow up of this request, stringent criteria have been developed which allow assessing the scientific validity of toxicological test methods. These criteria have been internationally accepted and summarized in the OECD Guidance Document 34 [3].

The 3 R's Principle is acknowledged and considered also by pharmaceutical regulatory authorities and by REACH (Regulation for Registration, Evaluation, Authorisation and restriction of Chemicals), the new European Union regulatory framework entered into force in June 2007. REACH includes the systematic examination of the chemicals produced in significant quantities within the European Union and accommodates non- validated methods as part of testing strategies [4].

Moreover, the fact that *in vivo* testing does not give precise predictions of human toxicity hazards, has spurred increasing interest from regulatory authorities and industry for *in vitro* tests. The US National Council's long-range vision for toxicity testing, prepared in response to a request by the US Environmental Protection Agency, reflects this opinion. The vision foresees a transformation of toxicity testing from a system based on whole-animal testing to *in vitro* methods evaluating changes in biological processes using cells and cell lines, preferably of human origin [5].

1.2 Developmental toxicity

Birth defects, defined as abnormal development of the foetus such as malformations, growth retardations and/or functional disorders, may lead to death or remaining impairment of humans through their entire life. Every year, approximately 6% of children are born with serious birth defects worldwide, although the origin of the birth defects is often unknown; the contribution of chemical exposure leading to adverse effects including pre-postnatal death is widely discussed [6]. Possible developmental toxicants are mostly only suspected to be toxic in humans since the variations among species are high [7-9] and the extrapolation to humans is difficult since the underlying mode of actions are often unknown.

This could lead to misinterpretations of the risk of chemicals as happened with Thalidomide which was perceived as a harmless sedative drug that was given to pregnant women in the late 1950s and early 1960s. However, the drug was strongly teratogenic, when

given in critical periods of embryo development, and caused more than 7000 children to be born with malformations [10].

Despite considerable amount of research conducted over the past 50 years, the cause of at least 50% of human congenital malformations remains unknown [11]. Roughly 18% of malformations can be attributed to genetic causes, such as chromosomal defects or mutation based Mendelian genetics, and 7% are caused by environmental factors. Of all malformations, 25% are multifactorial, as environmental factors acting on genetic susceptibility (Figure 1.1).

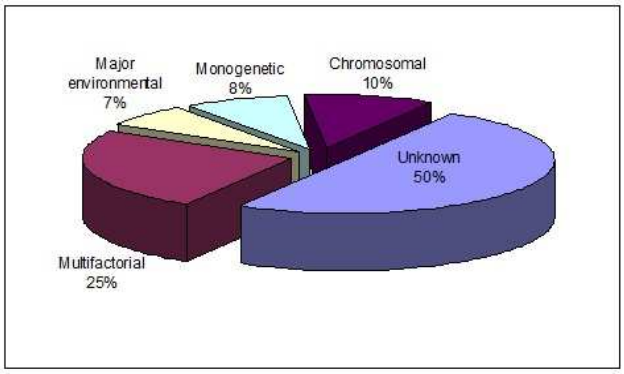

Figure 1.1: Major causes of congenital malformation. Modified from [11].

Most developmental toxicants produce abnormalities only during certain critical periods of gestation [9, 11]. In line, some stages of development are more susceptible to toxicants than others (Fig. 1.2). Exposure of the embryo to a toxicant during cleavage and the formation of the epiblast and hypoblast are unlikely to result in congenital abnormalities because the damaged or dead cells can be replaced since the cells are still multipotent so the embryo can recover. The period with maximal susceptibility to developmental harm is between the third and eighth weeks (the embryonic period), because most organogenesis occurs in this interval and interference with the processes may lead to gross malformations. Most organs are well established after the eighth week of gestation, making it unlikely that major malformations will be induced. Abnormalities arising during the third to the ninth month of gestation (the foetal period) tend to be functional or disturbances in the growth of established body parts. It should be considered that some agents that are harmful to development may cause their effects at the molecular level at an early stage, although the effects may be recognized only at later phase, perhaps postnatally, e.g. childhood cancer, DNT. Other agents may destruct already established structures. A recent literature analysis of

the standardised toxicological endpoints assessed in current *in vivo* developmental toxicity testing of chemicals revealed the frequency of endpoints with statistically significant findings for 202 developmental toxicants [12]. The data showed that the most common manifestations of developmental toxicity are post-implantation loss and death, abnormal offspring bodyweight, malformation of the skeleton, external limbs, digits, mouth, jaw and skull as well as malformations of the visceral (pertaining to the internal organs) neurological, urogenital and cardiovascular tissues.

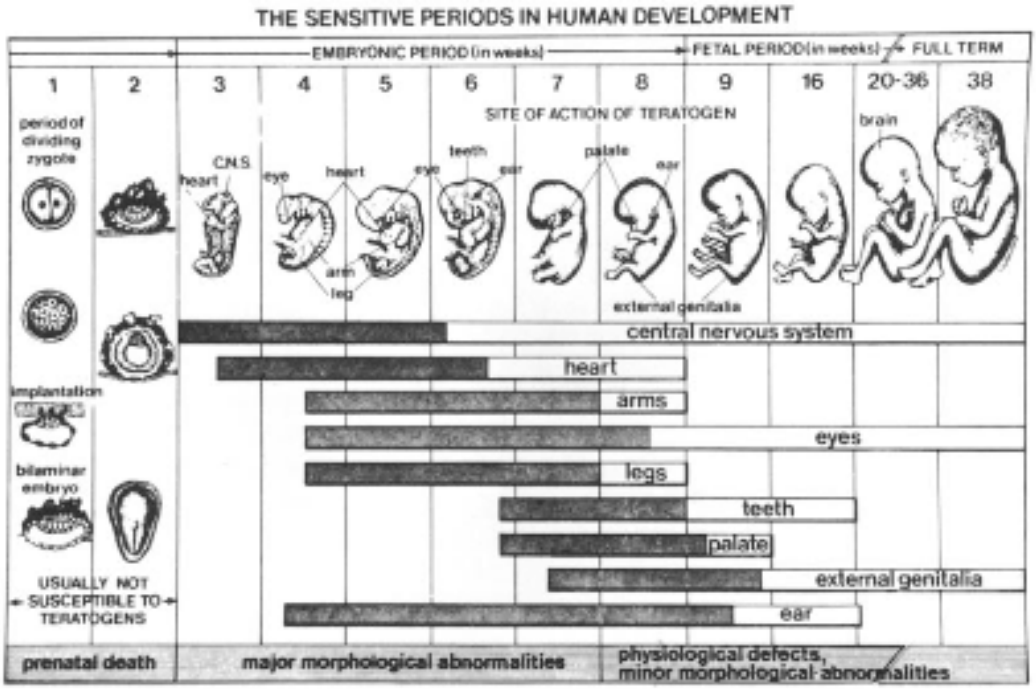

Figure 1.2: Periods and degrees of susceptibility of embryonic organs to teratogens. Black and white bars indicate respectively high and low susceptibility. [9].

To achieve a full understanding of the mechanisms of actions of developmental toxicants requires explanation of all events from exposure to the occurrence of the developmental defect [13]. This includes understanding of;

i) the toxicant's kinetics and means of absorption, distribution, metabolism and excretion within the mother and offspring;
ii) the toxicant's (or its metabolites) molecular interactions in the offspring or with maternal or extra-embryonic components supporting development;

iii) the consequences of the molecular interactions for the function of components in a cellular or developmental process;

iv) the consequences of the altered cellular functions or processes on the developmental outcome.

Normal mammalian development is extremely complex, and is still not understood in detail. Since it is a continuous development, the toxicants can interact at numerous points with an important molecular component, or even more, with several points further complicating the understanding of the mode of actions of developmental toxicants. Advances in research on signalling pathways (Table 1.1) and genetic regulatory circuits have identified key processes in embryo developmental [10, 13].

Table 1.1**:** Signalling pathways and their specific periods of activity during development. Modified from [13].

Period of development	**Signalling Pathway**
Early development (before organogenesis and cytodifferentiation) and later (during growth and tissue renewal)	Wingless-Wnt pathway
	Receptor serine/threonine kinase (TGF-β receptor) pahway
	Hedgehog pathway
	Receptor tyrosine kinase pathway
	Notch-Delta pathway
	Receptor-linked cytoplasmic tyrosine kinase (cytokine) pathway
Middle and late development (during organogenesis and cytodifferentiation) and late (during growth and tissue renewal)	Interleukin-1-Toll Nuclear Factor-Kappa B pathway
	Nuclear hormone receptor pathway
	Apoptosis pathway
	Receptor phosphotyrosine phosphatase pathway
Late and adult physiology (after cell types have differentiated)	Receptor guanylate cyclase pathway
	Nitric oxide receptor pathway
	G-protein coupled receptor pathway
	Integrin pathway
	Cadherin pathway
	Gap junction pathway
	Ligand-gated cation channel pathway

A report from the US National Research Council identified the most important signalling pathways for development in most animals [13]. Several of these pathways seem to be active only in specific periods of normal development, i.e. either before organogenesis and cytodifferentiation (development of specialised cell structures and functions) or after organogenesis and cytodifferentiation (Table 1.1). Studies of the alternation by developmental toxicants of these processes may enlighten the mode of actions of developmental toxicity. Present research effort has already lead to some understanding of molecular interactions and hence the mechanisms of some developmental toxicants (or their metabolites).

1.3 Human embryonic stem cells

The first human embryonic stem cell (hESC) lines were isolated in 1998 by Thomson et al [14]. They have been isolated either from the morula or from the inner cell mass of the blastocyst stage embryos between day 5/6 of development. In human embryonic development the morula cleavage stage occurs 3 days after fertilization [11]. At day 4/5 of gestation, the blastocyst consists of approximately 58 to 250 cells and pre-implantation is initiated [11, 15]. The blastocyst is a hollow sphere of cells containing an outer layer of trophoblast cells surrounding a small inner group of inner cell mass cells. The inner cell mass gives rise to the embryo proper plus several extra-embryonic structures, while the trophoblast gives rise only to extra-embryonic tissue, including the placenta [11]. Human ESCs isolated from pre-implantation blastocysts are derived from the inner cell mass cells [14, 15].

In contrast to most other cell lines, hESCs are genetically normal (diploid). Moreover, they are defined by two essential abilities: the capability of unlimited self-renewal whilst maintaining a stable genotype and phenotype, and the potential to differentiate into all the cell types of the human body, a characteristic that is defined as pluripotency [14, 16]. Human ESCs are;

i) round cells with a large nucleolus and scant cytoplasm;
ii) forming flat, tightly-packed and sharp-edged colonies;
iii) mitotically active, displaying active self-renewal;
iv) expressing cell surface markers, such as the stage specific antigens SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and the glycoproteins TRA-2-49/6E, and pluripotency markers as OCT4 and NANOG [15];

v) having high level of alkaline phosphatase activity;
vi) capable of differentiation into tissues, such as neural, cardiac, hepatic, mesenchimal, and many other cell types [17];
vii) forming embryoid bodies *in vitro* and teratomas when injected into immunodeficient mice [18].

Human ESCs cultures are challenging due to their growth in tight colonies and the still not fully understood conditions that ensure pluripotency or controlled differentiation. Currently optimal results can be obtained with feeder layers, special defined media, serum replacement, growth factors and a labor-intensive manual cutting technique.

Some of the striking potential benefits of stem cells compared to the other cell-based models used in *in vitro* toxicity testing are their human origin, normal genetic structure, relative stability, controlled genetic background, amenability to genetic modification, unlimited proliferation ability and their potential to generate a variety of cell types. They are thereby a more readily available source of human somatic cells and offer as such a very promising and innovative alternative for obtaining a large number of cells. Moreover, the stem cell technology provides a new tool for a better understanding of the mechanisms involved in drug-induced adverse reactions and to potentially predict toxic effects in humans [19].

1.4 Neuronal differentiation

The formation of the nervous system *in vivo* is an integrated series of complex developmental steps, which initiates during embryogenesis but continues during postnatal development. Embryonic neuronal development initiates by the formation of the neural plate from the ectodermal germ layer [11]. The neural plate folds to form the neural tube. The cells of the neural plate and early neural tube are highly mitotic neuroepithelial cells, which are organised in a pseudo-stratified single cell-layer neuroepithelium [11]. The central nervous system develops from these highly plastic neuroepithelium cells that proliferate, migrate from their places of origin to their final positions, differentiate, acquire regional identities and grow axons with a motile growth cone that is guided to form synapses with postsynaptic partners [11]. Through neural induction, the early central nervous system becomes organised into

regions, which develop increasing levels of complexity and finally gain the characteristics of the adult central nervous system. The first reflex movements and brain activities are seen in the sixth week of human embryo development [11].

The differentiation of hESCs towards the different germ layers of the embryo and their maturation into the different precursors and final tissue specific cells follows similar steps as those take place during human embryogenesis [20]. Protocols have been established for the differentiation of hESCs into neural precursor cells [21] as well as several differentiated cells such as motoneurons [22], glia cells [21], dopaminergic neurons [23, 24], and cerebellar cells [25]. The protocols based on hESCs usually yield a high percentage of neural precursors (more than 80%) and a significant number of target cells (up to 60%). However, the development of *in vitro* tests for neurotoxicity and developmental neurotoxicity has always proved to be challenging.

General RNA and protein markers for neural precursor cells are quite well established and include among others NESTIN, SOX1, PAX6, NEUROD1 [26]. However, the markers for specific lineages of stem cell derived-neuronal cells can vary between the different authors, what makes the results hard to compare. Moreover, hESC derived neural precursors transplanted into neonatal mouse brains incorporated into a variety of brain regions where they differentiated into astrocytes and neurons [27]. Grafting of hESC derived dopaminergic cultures into 6-hydroxydopamine-lesioned parkinsonian rats can induce restitution of motor function [28, 29], although a beneficial effect is not always observed [30, 31]. Thus, hESC derived neuronal cells seem to exhibit functionality *in vivo*.

The major drawbacks of currently available protocols are the low yield of some cell types [26] and the long culture periods for the differentiation (up to two months) [21]. The future challenge for test developers in the field of neurotoxicity is the establishment of test systems that could predict the effect of a chemical also on the functionality of the neuronal cells (e.g. electrophysiological properties, neurotransmitter activity, etc...). Further efforts are also needed in order to test the effects of unknown chemicals, to gather the data on intra/interlaboratory variability and define a prediction model that can categorize the test's outcome according to its stated purpose.

1.5 ATP binding cassette transporters

ATP Binding Cassette (ABC) transporters form a special family of membrane proteins, characterised by homologous ATP-binding, and large, multispanning transmembrane domains. They can be found in every known organisms, such as from microbes to humans [32]. To date, 48 members of the human family of ABC transporters have been identified which, based on their structural relatedness, are subdivided into seven families, designed ABC A-G [33, 34]. These evolutionary highly conserved multispan transmembrane molecules use the energy of ATP hydrolysis to translocate a broad spectrum of molecules across the cell membrane, participating in diverse cellular processes, including drug resistance and metabolism, transport of lipids and organic anions, and iron metabolism [35]. Several members of this family are primary active transporters, which significantly modulate the absorption, metabolism, cellular affectivity and toxicity of pharmacological agents [32].

The various ABC transporters in their two-dimensional structure are identical; they are built up from transmembrane domains (TMD) and nucleotide binding domains (NBD) **(**Figure 1.3).

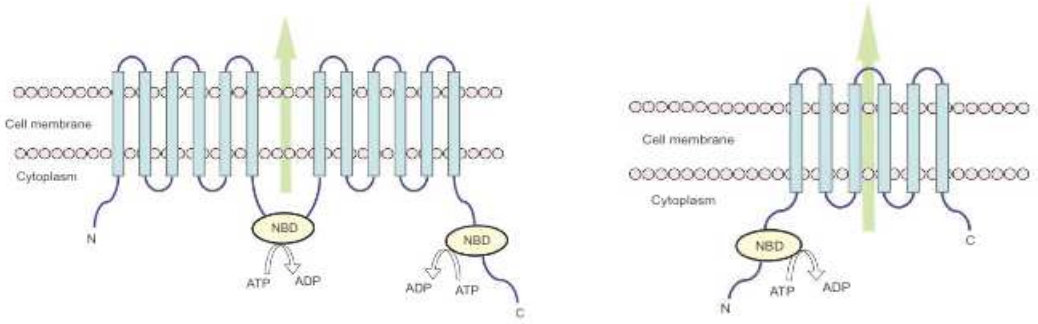

Figure 1.3**:** Structural features of full-length and half ABC transporters. [36].

As one of the best studied ABC transporters, the multidrug resistance P-glycoprotein (P-gp), also called as ABCB1, is a glycosylated membrane-associated full-length ABC transporter, comprising 1280 amino acids with a 170 kDa molecular weight. It is characterised by two identical halves, each with one NBD; exporting a wide range of diverse substrates [34, 35, 37].

Another member of the ABC transporter family that is intensively studied is ABCG2 (also known as MXR/BCRP1/ABCP), which is a membrane-associated half-transporter

containing 663 amino acids, and requires dimerization for its functional activity [38, 39]. Unlike other ABC half-transporters, which are localized to intracellular membranes at numerous compartments within the cell, ABCG2 is expressed exclusively in the plasma membrane. ABCG2 has been identified to confer resistance to anthracycline anticancer drugs, and is expressed in both malignant and normal tissues [40, 41].

It was already demonstrated by several research groups that both ABCB1 and ABCG2 transporters are highly expressed in stem cells derived from tissues, such as brain, bone marrow, pancreas, and liver [42-44]. However, the distribution and functional properties of these transporters were only recently studied in human neural progenitor cells, founding that the NESTIN positive precursors are also expressing the ABCB1 transporter [45]. Besides that the ABCB1 and ABCG2 transporters are useful as universal stem cell markers and are functionally expressed in human neural precursor cells protecting them from the possible effects of different xenobiotics, they may also play role in human neural precursors self-renewal by preventing differentiation [36]. Therefore, they can be used as a tool to study not only the regulation of neural stem cell differentiation and self-renewal but also their possible role of human neural developmental toxicity.

1.6 Legal situation of using human embryonic stem cells

The use of hESC is limited by ethical and legal considerations which have led to the establishment of guidelines and regulations concerning hESC research in Europe. The legal situation is complex and it is also mirrored in the rest of the world. In European Member States allowing hESC research, project proposals have to undergo strict scientific and ethical evaluation criteria, decided upon by competent national bodies [46]. The current situation of various positions worldwide is summarized in Table 1.2**.**

At the EU level, the Parliament has approved continued funding for hESC research within 7th Framework programme [47]. Human cloning for reproductive purposes, modification for the genetic heritage of human beings, which could lead to heritable changes and activities intended to create human embryos solely for research or therapeutic cloning are explicitly excluded from EU funding. In addition, the Commission will not fund research in

Member States where research is not permitted as laid down in Regulation (EC) No 1906/2006 [48].

To meet the ethical concerns, the International Society for Stem Cell Research (ISSCR) convened a task force of experts in science and medicine, ethics and law from 14 countries, which set up guidelines [49] for hESC research. These guidelines cover specify ethical standards for hESC research and seek to promote responsible, transparent and uniform practices worldwide.

Table 1.2: Global regulation of human embryonic stem cell research (update September 2008).

Policy	Countries
Prohibition of derivation of hESCs	Austria, Bulgaria, Germany, Ireland, Italy, Lithuania
Prohibition of the procurement of ESCs from human embryos but allowing by law for the importation of hESCs	Germany[a], Italy[b]
Prohibition of creation of human embryos for research including SCNT	Austria[c], Brazil, Bulgaria, Canada, Cyprus, Denmark, Estonia, France, Germany, Greece, Georgia, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania[c], Netherlands, Norway, Portugal, Romania, Slovakia[c], Slovenia, Switzerland, Taiwan
Allowing procurement of hESCs from supernumerary embryos by law	Australia, Belgium, Brazil, Canada, China, Czech Republic, Denmark, Estonia, Finland, France, Greece, Hungary, India, Israel, Japan, Mexico, Netherlands, Portugal, Russia, Singapore, Slovenia, South Africa, South Korea, Spain, Sweden, Switzerland, Taiwan, United Kingdom
Allowing creation of human embryos for research including SCNT	Australia, Belgium, China, India, Israel, Mexico, Russia, Singapore, South Africa, South Korea, Spain, Sweden, United Kingdom
No specific legislation regarding hESC research	Cyprus, Luxembourg, Malta[c], Norway, Poland[c], Portugal, Romania, Slovakia[c], Thailand, USA

[a] Germany has a law and hESC lines have to be derived before 01.01.02. [b] Italy has no law regarding the importation and IT scientists work on imported hESC lines. [c]Countries which voted against hESC research during Council Decision for FP7 [46].

1.7 Currently ongoing EU projects using hESCs for toxicology

Taking into account the ethical framework proposal by the European Group on Ethics of science and new technologies (EGE) projects have been funded so far in order to detect adverse effect specific to humans, as reported in the opinion n◦17 of EGE [50]. In total, 6 hESC projects related to the development of toxicity tests (Table 1.3) were financed under the 6th and 7th Framework Programme of the European Council.

Table 1.3: Examples of EU-funded projects that make different uses of hESCs.

Project acronym	Size	Target to organ/biological mechanism	Website
VITROCELLOMICS	9 partners ~ 3.000.000 €	Hepatocytes for drug testing	http://er-projects.gf.liu.se/~vitrocellomics
INVITROHEART	9 partners ~ 3.000.000 €	Cardiomyocyte for drug testing	http://er-projects.gf.liu.se/~invitroheart
CarcinoGENOMICS	21 partners ~ 10.500.000 €	Genomics-based *in vitro* testing for genotoxic and carcinogenic properties of chemicals	www.carcinogenomics.eu
ReProTect	21 partners ~ 10.500.000 €	hESCs for developmental toxicity	http://www.reprotect.eu
SCR&Tox	13 partners ~ 4.700.000 €	Development of human stem cell-based methods for toxicology testing	. http://www.scrtox.eu
ESNATS	31 partners ~ 12.000.000 €	Embryonic stem cell-based novel alternative testing strategies	http://www.esnats.eu

The focus hereby was on projects where animal experiments and other *in vitro* tests provide insufficient toxicological information due to interspecies variations. The projects concentrate on the detection of side effects of drugs, aiming to elucidate possible mode of actions of the chemicals. However, at the current stage of using hESC for toxicological applications, the identification of knowledge gaps in testing strategies is of high relevance in

order to select the cell type of interest as well as the most appropriate readout to identify relevant adverse effects of substances. Beside requirements on quality control issues (which is currently assessed in various projects), an agreement between various stakeholders could accommodate a directed test development:

i) which cell types have priority for the development of differentiation protocols. More guidance is needed which cell type and which mechanisms are known as biological target and should be addressed *in vitro*;
ii) which readouts are of interest. More “classical” readouts such as cytotoxicity or “functional” readouts, for example electric activity and the identification of biomarker by applying ‘omics’ technologies;
iii) the test chemical selection procedure normally faces the problem of understanding if the selected substances produce an adverse effect to the human since mainly animal data are available. In order to address the problem on the lack of a ‘golden standard’ for human cell based toxicity tests a peer reviewed list of reference compounds mimicking the most relevant mode of actions would be supportive.

2. AIMS OF THE STUDY

The ultimate goal of the thesis is to establish a human embryonic stem cell (hESC) based novel *in vitro* method for the assessment of toxicities affecting the human nervous system development. In order to achieve this general objective, the main aims of the present study are set as:

- Acquisition of expertise in stem cell technologies such as the maintenance of hESCs.
- Defining adequate culture conditions of hESCs based on their characterisation [pluripotency markers (*OCT4*, *NANOG*) for the measurement of culture stability (qPCR), morphology, protein and gene expression].
- Initial assessment of spontaneous hESC differentiation: embryoid bodies (EB) formation.
- Establishment of an initial protocol for neural differentiation (neuronal differentiation markers: *PAX6*, *NESTIN*, *NCAM1*).
- Quality control and detailed characterisation of undifferentiated hESCs.
- Development of a stable and reproducible neural differentiation culture/protocol.
- Characterisation of the differentiated model.
- Initial assessment of toxic response of neural tissues derived from hESCs exposed to known developmental toxicants (5-Fluorouracil, 6-Aminonicotinamide, Methotrexate, Methylmercury chloride, Retinoic acid, Valproic acid, Warfarin).
- Elucidation of the mode of action of Methylmercury chloride by transcriptomics analysis.

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3. MATERIALS AND METHODS

3.1 hESC line and maintenance culture condition

The NIH-registered H9 hESCs obtained from WiCell (USA) were cultured undifferentiated in 60 mm cell culture dishes (TPP, Switzerland) at 37 □C and 5% CO_2 on a layer of mitomycin C-inactivated primary mouse embryonic fibroblasts (pMEF, CF-1 strain Millipore USA), which were plated at a density of 15000 cells/cm^2, on gelatine coated dishes in the presence of the standard maintenance medium for undifferentiated hESCs [DMEM/F12 supplemented with 20% KO serum replacement, 1% non-essential amino acids, 2mM glutamine, 0.1mM β-mercaptoethanol and 4ng/ml human recombinant basic fibroblast growth factor (hr bFGF) (all from Invitrogen, USA)]. Cells were expanded weekly by microdissection and further propagated on a feeder layer.

3.2 Early embryonic neural differentiation

For the differentiation towards early neuroepithelial precursors a protocol which was described earlier [51] was followed with some modifications. Intact 6 days old H9 hESC colonies were detached by 1 mg/ml collagenase (Invitrogen, USA) treatment and left in suspension culture dishes for 3 days in hESC maintenance medium without bFGF to allow the generation of embryoid bodies (EBs). After this time, EBs were transferred onto single wells (one EB per well) of 96- wells plates coated with 10 μg/ml laminin [in water (Sigma, USA)] containing neural induction medium [DMEM/F12 supplemented with 1% non-essential amino acids, 1% N2 supplement, 2 μg/ml heparin (Sigma, USA) and 20 ng/ml bFGF (unless stated, all from Invitrogen, USA)]. Cultures were kept for up to 10 days with medium changes every third day (Figure 3.1).

3.3 Chemicals

A training set of well established xenobiotics (Table 3.1) was used to expose to the neural differentiating hESCs. Stock solutions at a concentration of 10 mM of each chemical

were generated in water (or in DMSO if solubility problems were found) and stored at -20 °C as reference stock solutions. Eight sequential working dilutions of 1:10 and 1:3 made in DMEM/F12 were generated freshly for each experiment, in such a way that the final solvent concentration (H_2O or DMSO) was 0.01% in each well. Even though initially the concentration range was between 10^{-3} M and 10^{-10} M with 10 units steps, this range had to be modified for Valproic acid (final range from 10^{-2} M to 10^{-10} M), due to the fact that the chemical had cytotoxic effect only at higher concentrations.

3.4 Chemicals exposure

Differentiating cultures were exposed to the desired final concentrations of the different chemicals by dilution of the working concentrations in neural induction medium taking a constant solvent concentration into account. Chemical exposure begun 24 hours after plating of the embryoid bodies (Figure 3.1) in the respective wells of the laminin coated 96-well plate (day 1 of differentiation), in order to allow the satisfactory attachment of the EB to the treated surface. Both the tested chemical and the neural induction medium were refreshed every third day during differentiation allowing a continuous exposure to the chemicals. Exposure lasted up to a total of 10 days and samples were taken for the different analysis on every third day and on day 10.

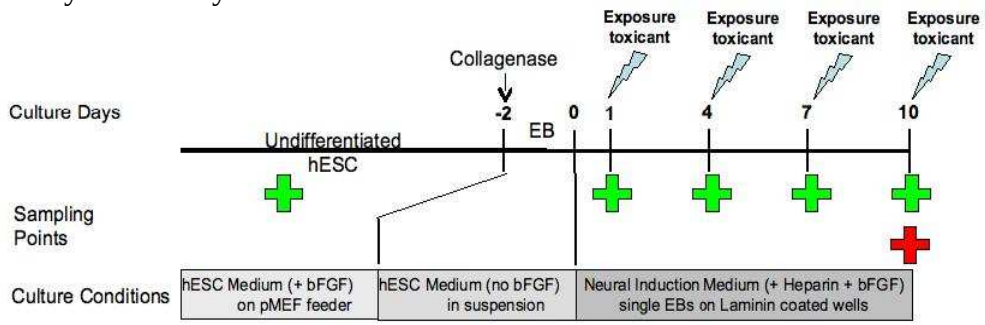

Figure 3.1: Scheme of the early embryonic neural differentiation protocol with exposure timing and sampling points. The exposure took place in a continuous manner with renewal of medium and toxicant every third day until the end of the test day on day 10, as indicated with red cross. Green crosses show the sampling points.

Table 3.1: Summary of the used compounds and their mode of action.

Chemical Name	Supplier	CAS Number	Mode of Action	Solvent used	References
5-Fluorouracil	Sigma	51-21-8	Induces cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA, inhibits the exoribonuclease complex	H_2O	[52-54]
6-Amino-nicotinamide	Fluka	329-89-5	Inhibits the NADP+-dependent enzyme	H_2O	[9]
All-trans retinoic acid	Fluka Sigma	302-79-4	Binds to heterodimers of the retinoic acid receptor, the retinoic X receptor and binds to retinoic acid response elements in the regulatory regions of direct target genes, activates gene transcription	DMSO	[55-58]
Methotrexate	Sigma	59-05-2	Inhibits the metabolism of folic acid	H_2O	[59-61]
Methyl-mercury chloride	Sigma	115-09-3	Potently affects the release of neurotransmitter from presynaptic nerve terminals, causing ultrastuctural changes, and accumulates within mitochondria	H_2O	[62, 63]
Valproic Acid sodium salt	Sigma	1069-66-5	Increases gamma-aminobutyric acid levels, alters the voltage dependent sodium channels, inhibits the histone deacetylase	H_2O	[64, 65]
Warfarin Pestanal	Fluka	81-81-2	Inhibits vitamin K-dependent coagulation factor	DMSO	[66-68]

3.5 Viability testing using Resazurin Assay

For each chemical one 96-well plate was used where each test concentration was performed in quadruplicates. In addition to that, on each plate, 4 wells containing EBs were selected to be grown in the absence of chemical as internal negative control. Additional 4 wells were used as solvent control with a 0.01% final concentration of DMSO or water. Resazurin assay was performed at least 3 times per each chemical to be analysed. The viability was assessed in three biological replicates on day 10 after plating of the EBs.

On day 10, the viability of the treated cultures were analysed by removing the used medium and the substitution of 100 µl of freshly prepared 10 µg/ml solution of resazurin (Sigma, USA) in neural induction medium. The cell cultures were incubated for 2 hours at 37 °C and 5 % CO_2 in the presence of resazurin. After the incubation period the cell-exposed

resazurin solution was transferred into an empty 96-well plate (the 100 µl into 2 independent wells with 50 µl each). Fluorescence signal at 590 nm was measured with a TECAN Infinite® 200 microplate reader (TECAN, Switzerland) after 544 nm excitation and blanking against the background wells containing only resazurin solution incubated for 2 hours at 37 °C and 5 % CO_2.

3.5.1 Calculation of IC_{50}, IC_{10} and Highest non cytotoxic concentration

The averaged fluorescence emission data (4 values per tested concentration) and the percentage of fluorescence change were plotted with the respective concentration of the chemical at day 10 of exposure. Cytotoxicity curves were created by using a spread-sheet program and the average of the independent replicates (at least n= 3) were generated using Prism 5.0 (GraphPad Software, USA) giving rise to the representative cytotoxicity curve of the analysed chemical. A regression formula was derived for the plotted curve using the aforementioned software and the concentration values for the IC_{50} and IC_{10} were extrapolated. Furthermore the highest non-cytotoxic concentration was determined as the last value before the decrease of the resazurin fluorescence, while the non toxic concentration was 1-fold lower compared to the highest non-cytotoxic concentration.

3.6 Isolation of RNA, cDNA synthesis and quantitative Real-Time PCR (qPCR)

RNA was isolated from stored samples as cell pellets (IC_{10}, highest non cytotoxic and non toxic concentration) using the RNeasy Microkit (Qiagen, Germany) following manufacturer's instructions and eluting in 12 µl elution buffer. As a rule 4 wells of cells exposed to the same concentration of the same chemical were pooled together to isolate sufficient RNA. cDNA was synthesised from 500 ng of RNA using M-MLV retrotranscriptase and random hexamers in a 1 hour reaction at 37°C in 25 µl of total volume. Gene expression was analysed by qPCR using the 7000 TaqMan System (Applied Biosystems, USA). Reactions were measured in duplicate using PCR Master Mix (Applied Biosystems, USA) following manufacturer's instructions. “Gene Expression Assays” primers and probe sets were purchased also from Applied Biosystem;

i) housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH, Hs99999905_m1*) and hypoxanthine phosphoribosyltransferase1 (*HPRT1, Hs01003267_m1)*;

ii) pluripotency markers: octamer-binding transcription factor 4 (*OCT4, Hs00999634_gH)* and *NANOG (Hs02387400_g1)*;

iii) neural commitment genes: sex determining region Y-box 1 (*SOX1, Hs01057642_s1),* paired box gene 6 *(PAX6, Hs00240871_m1), NESTIN (Hs00707120_s1),* orthodenticle homeobox 2 *(OTX2, Hs00222238_m1),* neural cell adhesion molecule 1 *(NCAM1, Hs00941821_m1),* microtubule-associated protein 2 *(MAP2, Hs00258900_m1)* and neurofilament medium polypeptide 150 kDa (*NEFM, Hs00193572_m1*);

iv) glial and oligodendrocyte lineage markers: glial fibrillary acidic protein (*GFAP, Hs00909238_g1*) and oligodendrocyte transcription factor 2 (*OLIG2, Hs00377820_m1)*;

v) endodermal lineage marker: α fetoprotein (*AFP, Hs00173490_m1*);

vi) the mesodermal lineage marker: myosin heavy chain 6 (*MYH6, Hs01101425_m1*);

vii) the ABC transporters: *ABCB1* (*Hs00184500_m1*), *ABCG2* (*Hs01053790_m1*).

Gene expression was scored by calculating the normalised ΔCt for each gene and for each sample by subtracting the Ct value determined for *GAPDH* from the Ct value obtained for the gene of interest. Genes were considered to be expressed in a sample when their normalised ΔCt is below 12 (implying that is 10000 times less expressed than the housekeeping genes), otherwise the gene would be addressed as not present. The resulting expression pattern was used to monitor the differentiation process and to judge the degree of differentiation. Only cultures with a certain expression pattern were used for further analysis. As such, the expression pattern served as quality criteria to judge the validity of the obtained toxicological data.

Relative quantification was performed for each concentration of chemical by the ΔΔCt method ($2^{-\Delta\Delta Ct}$ formula) [69] using the expression profile of the corresponding untreated control as reference.

3.7 Immunofluorescence analysis

Undifferentiated and differentiated H9 cells were fixed with 4% formaldehyde (Sigma) for 20 minutes at room temperature. Formaldehyde was removed by washing the cells 3 times with PBS buffer (Invitrogen). The cells were permeabilised for 15 minutes with 0.4% Triton X-100 (Sigma) and the reaction was stopped by using 2% of BSA (Sigma) in PBS, for 30 min at room temperature. Blocking buffer was removed by aspiration and replaced by primary antibodies against the pluripotent (OCT4, 1:500) and neural stem (NESTIN, 1:200) or neuronal (β-TUBULIN III, 1:500 and MAP2, 1:500) markers diluted in blocking buffer. The plates were incubated for 1 h at 37 °C. After the incubation, the plates were washed 3 times with PBS buffer, and the cells were incubated for 30 min at room temperature with the appropriate fluorescent-conjugated secondary antibodies (Alexa Fluor 546 goat anti-rabbit IgG (H+L) 1:1000 and Alexa Fluor 546 goat anti-mouse IgG (H+L) 1:1000) and DAPI dye. The cells were washed 3 times with PBS and imaged using Cellomics ArrayScan vTi platform (Thermo Scientific). For the image analysis, a 10x objective and two fluorescence channels with the filter set (XF 93) was used and 10 image/fields were collected per well. Quantitative image analysis was performed using the Cytotoxicity Bioapplication v.4 from Cellomics Scan Software.

3.8 Flow cytometry

For flow cytometry analysis, undifferentiated cell cultures were washed twice with 1x PBS and treated with Tryple Select (Invitrogen, USA) at 37 °C for 5 minutes in order to investigate the possible changes in the population’s composition throughout the maintenance of the undifferentiated cell culture. The reaction was stopped with growth medium and pipetted several times in order to generate a single cell suspension. Cell concentration was assessed by Trypan blue exclusion and the cells were transferred into a 2 ml Eppendorf tube after being passed through a 40 μM mesh to remove cell clumps. The cell suspension was centrifuged, washed once with 1x PBS and resuspended in 100 μl FACS buffer (1% FBS in 1x PBS). For staining 1.33 μl of each titrated antibody (generally 1:75 dilutions) was added to the cell suspension and incubated for 30 minutes at 4 °C. The antibodies used for the quality

control in the present study were CD29 (Alexafluor 488 conjugated), SSEA1 (CD15, pacific blue conjugated) and SSEA4 (Alexafluor 647 conjugated). All antibodies were purchased from Invitrogen (USA). After incubation, cells were diluted with 2 ml of FACS buffer and centrifuged at 200g for 5 minutes. Supernatant was removed and cells were washed twice more with 2 ml FACS buffer. For analysis, the washed cell pellet was resuspended in 1ml of FACS buffer and analysed using a FACS Aria, cell sorter (BD, USA). 50000 events were recorded and further analysed using FlowJo version 5 for PC (Tree Star Inc., USA). HESCs were selected based on the negativity for CD29 and population of SSEA4 and CD15 positive cells were measured using the respective negative controls.

3.9 Microarray analysis

For microarray analysis, the total RNA was isolated with Trizol (Invitrogen, Damstadt, Germany) from the 10 days neural differentiated hESCs treated with MeHgCl at highest non-cytotoxic, $IC_{0.5}$ and IC_{15} concentrations (0.025 µM, 0.09 µM, 0.27 µM, respectively), and purified with Qiagen RNeasy mini kits (Qiagen, Hilden, Germany). To avoid DNA contamination on column DNase digestion was performed as per the manufacturer's instructions. The RNA was quantified using NanoDrop N-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and the integrity of RNA was confirmed with a standard sense automated gel electrophoresis system (Experion, Bio-Rad, Hercules, CA, USA). The biological samples were taken for transcriptional profiling with the RNA quality indicator (RQI) number higher than 8. Total RNA (100 ng) was taken as a starting material and amplified for 16 h with Genechip 3' IVT Express Kit as per the manufacturer's instructions. 12.5 µg fragmented aRNA was hybridized on Affymetrix Human Genome U133 plus 2.0 arrays and the chips were placed in a GeneChip Hybridization Oven-645 for 16 h at 60 rpm and 45 ºC. For staining and washing, Affymetrix HWS kits were used on a Genechip Fluidics Station-450. For scanning, the Affymetrix Gene-Chip Scanner-3000-7G was used, and the image and quality control assessments were performed with Affymetrix GCOS software. All reagents and instruments were acquired from Affymetrix (Affymetrix, Santa Clara, CA, USA).

3.10 Microarray data analysis

Background correction, summarization and normalisation were done with Robust Multi-array analysis. The entire dataset was normalised with quantile normalisation method executable with R (Affy)-package carried out at probe feature level. Probe sets, having signal >= 6 (Log2 scale) in any one of the experimental conditions out of 12, were only chosen for statistical analysis and they were 26507 probe sets.

A linear model was implemented using R -LIMMA packages [70] for determining differentially expressed transcripts using cut off values of 5% error rate ($P<0.05$, determined by F-statistics with Benjamini and Hochberg Multiple Testing Correction). Array data sets from the untreated replicates as reference group were compared against highest non-cytotoxic, $IC_{0.5}$ and IC_{15} MeHgCl concentrations treated groups. Beside the above mentioned conditions, fold change value with the threshold value $\geq \pm 2.0$ were used to filter the significantly expressed transcripts. K-Mean cluster analysis was performed after transcript-wise normalisation of signal values to a mean of 0 and standard deviation of 1 using Elucidian distance measurement and k = 9, using Cluster 3.0 tool from Eisen lab [71]. Principal component analysis was performed using the Stats package in R with procomp function. The 'x' attribute of the procomp object was used to generate 2 dimensional scatterplots.

3.10.1 Gene ontology analysis

Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to investigate the differentially expressed genes for biological process, molecular function and cellular component from the individual transcripts. To define the biological impact and the biological response to experimental perturbation, dysregulation of significant genes were annotated based on the Expression Analysis Systematic Explorer (EASE) score enrichment P value ($P<0.01$) and clustering stringency at medium level as per the DAVID tool.

4. RESULTS

The use of stem cells for safety assessments requires a robust and reproducible differentiation protocol taking the unique characteristics of stem cells into account. The establishment of quality controls and acceptance criteria identifying valid experiments is therefore one prerequisite for the regulatory acceptance of stem cell based toxicity tests. In this study, a hESC model was established as basis for an *in vitro* test by focusing on key aspects such as

i) the development of quality standards ensuring the robustness of the cellular model;
ii) the definition of the time point for the optimal readout;
iii) challenging the cellular model with well known reference compounds.

These three major requirements need a close monitoring of the differentiation process and the definition on the amount of target cells as well as cells of other lineages. Practically this can be achieved by defining a panel of representative genes characterising the cell culture at the day of the readout.

4.1 The development of quality standards ensuring the robustness of the cellular model

The cellular model used in the study is based on the well defined H9 hESC line. Even if the cell line and their differentiation potential have been often described in the scientific literature, appropriate quality standards for toxicological application have not been developed so far.

Undifferentiated cell cultures are needed to start up a successful differentiation. The analysis of the starting culture is therefore a must. The daily microscopical inspection of undifferentiated H9 cultures gives already a first indication for major changes in the quality of the cell culture (Figure 4.1). Additionally, since the morphology assessment is a very subjective readout, the colonies were also routinely analysed to determine their pluripotency and surface marker expression and homogeneity by using immunohistochemistry and flow cytometry analysis. The presence of OCT4 and SSEA-4 and the absence of SSEA-1 and CD29 in the cell culture were used to detect changes in the population’s composition

throughout the maintenance of the undifferentiated cell culture. Nevertheless, since the determination of the undifferentiation status for toxicity testing should easily applicable for routine toxicity testing, as detailed as possible, qPCR approaches were used six days after passaging the cells. Undifferentiated colonies were examined for their expression profile of well-described markers associated with pluripotency or progressing differentiation.

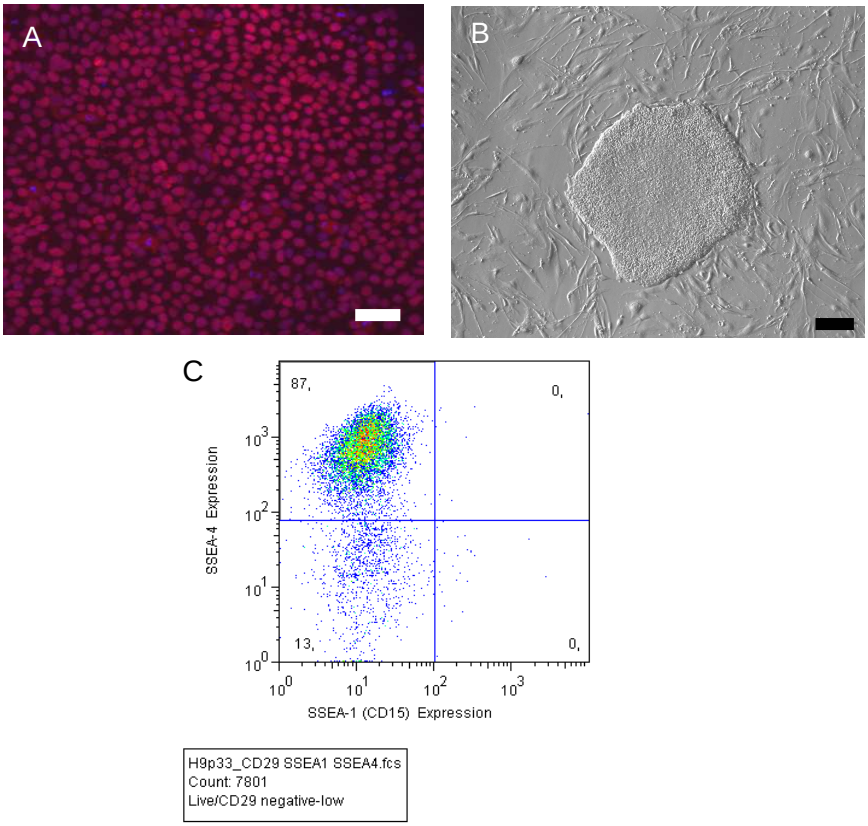

Figure 4.1: Characterisation of undifferentiated H9 colonies. (A) Immunofluorescence analysis showed that undifferentiated H9 cells are expressing antigens for the pluripotency marker OCT4 (red). Nuclei staining with DAPI are shown in blue in panel A. (B) Bright-field image of a 6 days old H9 hESC undifferentiated colony cultured on inactivated mouse feeder layer. Bars = 100 µm. (C) A representative FACS plot flow cytometry demonstrating a high percentage of SSEA-4 expression and a low expression of SSEA-1 profile.

A robust differentiation protocol is a precondition to assess the chemical effects on differentiating neural stem cell like cells. Rosette structures are a morphological marker indicating a successful neural differentiation that can be easily identified by experienced operators (Figure 4.2). The appearance of at least 3 neural rosette structures per well at day 8

of differentiation can be accepted as the first criteria for a successful differentiation. If more than 50% of the control wells contain at least 3 rosette structures, the more objective assessment criteria of immunostaining and qPCR were applied. The rosettes expressed highly β-TUBULIN III and NESTIN indicating their commitment into the neural differentiation.

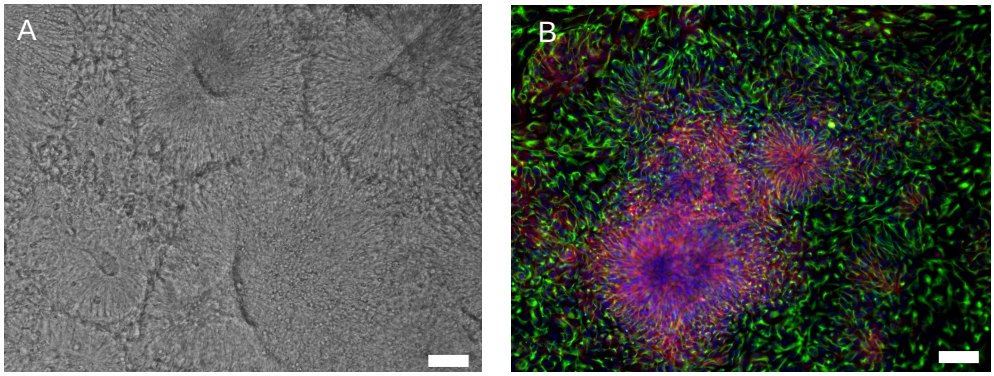

Figure 4.2: Characterisation of hESC-derived neural precursors *in vitro*. (A) Phase contrast image of the neural rosettes appearing already at day 3 after plating the embryoid bodies (EBs). (B) Immunostaining after 10 days of differentiation indicates that the majority of the cells are β-TUBULIN III (red) and NESTIN (green) positive. Nuclei staining with DAPI are shown in blue. Size bars represent 100 µm.

4.2 Monitoring of neural differentiation and determination of the optimal time point for read-out

Modifications of gene expression profiles (Figure 4.3) were analysed during differentiation of 10 days. The application of a neural differentiation protocol, as previously described, led to a downregulation of the *NANOG* gene and a significant *OCT4* downregulation within 10 days as compared to undifferentiated hESCs.

On the contrary, the transcription factor *PAX6* was significantly upregulated already at day 3 (26.5-fold higher than the undifferentiated hESCs), and reached a peak on day 7 with a 56.87-fold increase, then remained on a level of 34.5, 38.5 and 37-fold increase compared to undifferentiated control on days 8, 9 and 10.

NESTIN was continuously upregulated during the differentiation procedure with 2.3, 3.2, 3.9 and 4.9-fold on days 1, 2, 4 and 6, peaked on day 8 by 7.6-fold, and reached a plateau of 6.4-fold higher than control on day 10.

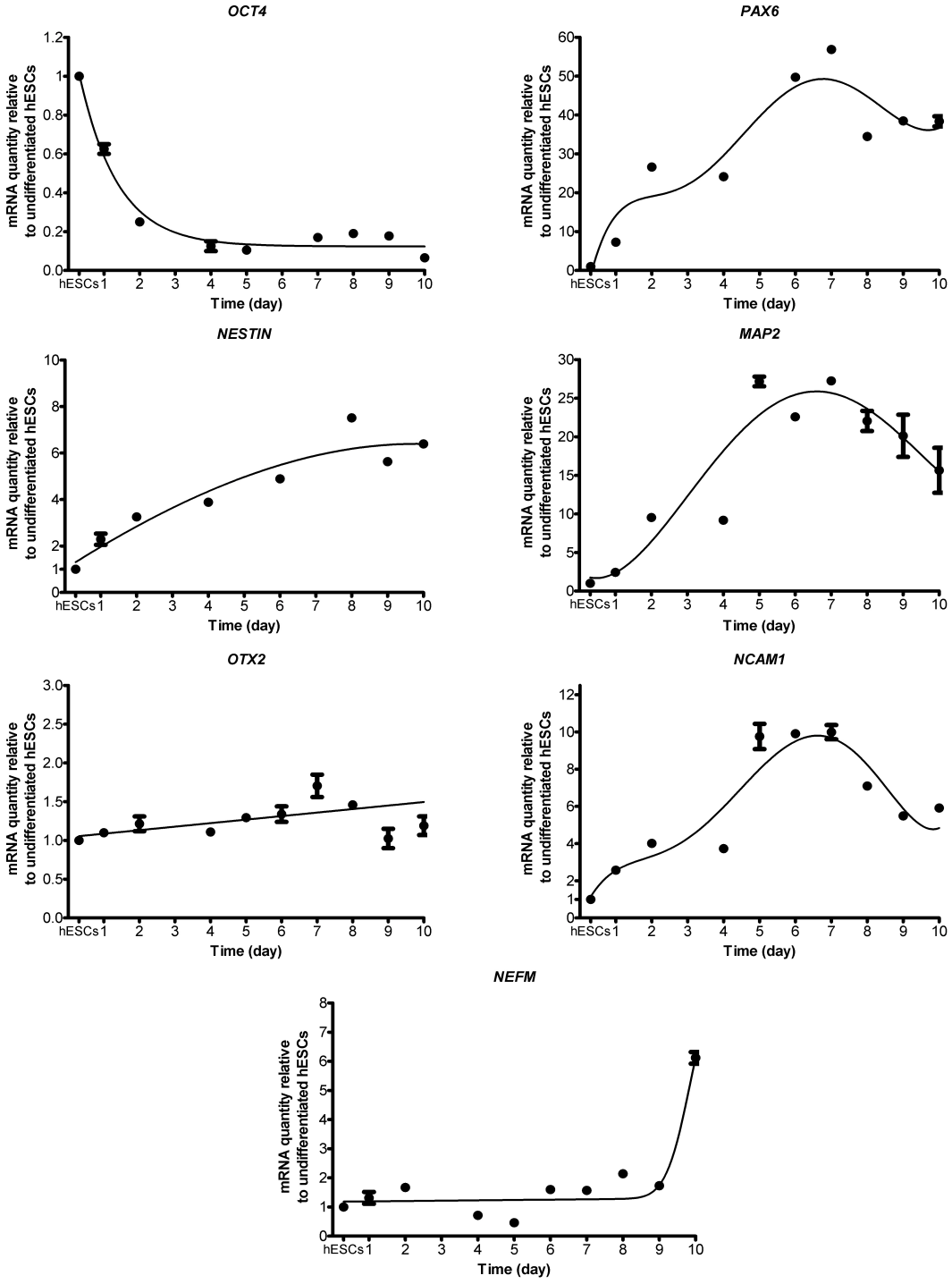

Figure 4.3: Relative quantification by qPCR of gene expression levels during differentiation of hESCs into neuronal precursors: *OCT4*, *PAX6*, *NESTIN*, *MAP2*, *OTX2*, *NCAM1*, and *NEFM*. Gene expression levels were normalised to the housekeeping gene *GAPDH* and the mean expression in the undifferentiated hESCs. N = 3.

Neural precursor differentiation caused significant *MAP2* upregulation with 9.5-fold at day 2, reached a peak on days 5 and 7 as 27.1 and 27.2-fold undifferentiated control levels. Hereafter the *MAP2* expression diminished to 22-fold on day 8.

The neural cell adhesion molecule 1, *NCAM1* expression reached a plateau on days 5, 6 and 7, showing 9.7, 9.9 and 10-fold higher than in undifferentiated hESCs, then decreased as 7-fold on day 8.

The expression of *NEFM* was slightly increased during the neuronal differentiation with 1.3, 1.6 and 2.1-fold on days 1, 2 and 8. On day 10 it showed a significant upregulation of 6.1-fold higher than control. In contrast, only a slight *OTX2* upregulation of 1.7-fold was evident on day 7.

According to the kinetic experiments performed, 10-day differentiation was defined as an optimal time point to assess chemical effects on differentiating neural stem cells.

4.3 Challenge of the cellular model with well known reference compounds

It is thought that the challenge of the cell model with well-known toxicants provides the first insights in the capacity of the cellular model to respond to chemical stress. In order to distinguish between unspecific toxicity and specific effects interfering with the differentiation process of neural precursors, the effective dosage range was determined by using a concentration-dependent inhibition of resazurin reduction. Resulting dose-response curves (Figure 4.4) were used to define IC_{50} as well as IC_{10} and highest non-cytotoxic levels as starting concentrations for the assessment of specific toxicity (Table 4.1). D-mannitol and methothrexate did not demonstrate a prominent cellular response at a high concentration and were therefore not considered for further evaluations.

Table 4.1: Average IC_{50}, IC_{10} and highest non cytotoxic values of each tested chemicals.

Chemical	IC_{50} (M)	IC_{10} (M)	Highest Non Cytotoxic (M)
5-Fluorouracil	$1.25x10^{-5}$	$1.00x10^{-6}$	$0.156x10^{-6}$
6-Aminonicotineamide	$36x10^{-6}$	$4.98x10^{-6}$	$0.3x10^{-6}$
D-Mannitol	N.P.	N.P.	N.P.
Methotrexate	N.P.	N.P.	N.P.
MeHgCl	$0.46x10^{-6}$	$0.23x10^{-6}$	$0.025x10^{-6}$
Retinoic Acid	$4.80x10^{-5}$	$3x10^{-6}$	$0.4x10^{-6}$
Valproic Acid	$6.60x10^{-3}$	$1.21x10^{-3}$	$44.3x10^{-6}$
Warfarin	$1.1x10^{-6}$	$44x10^{-9}$	$4x10^{-9}$

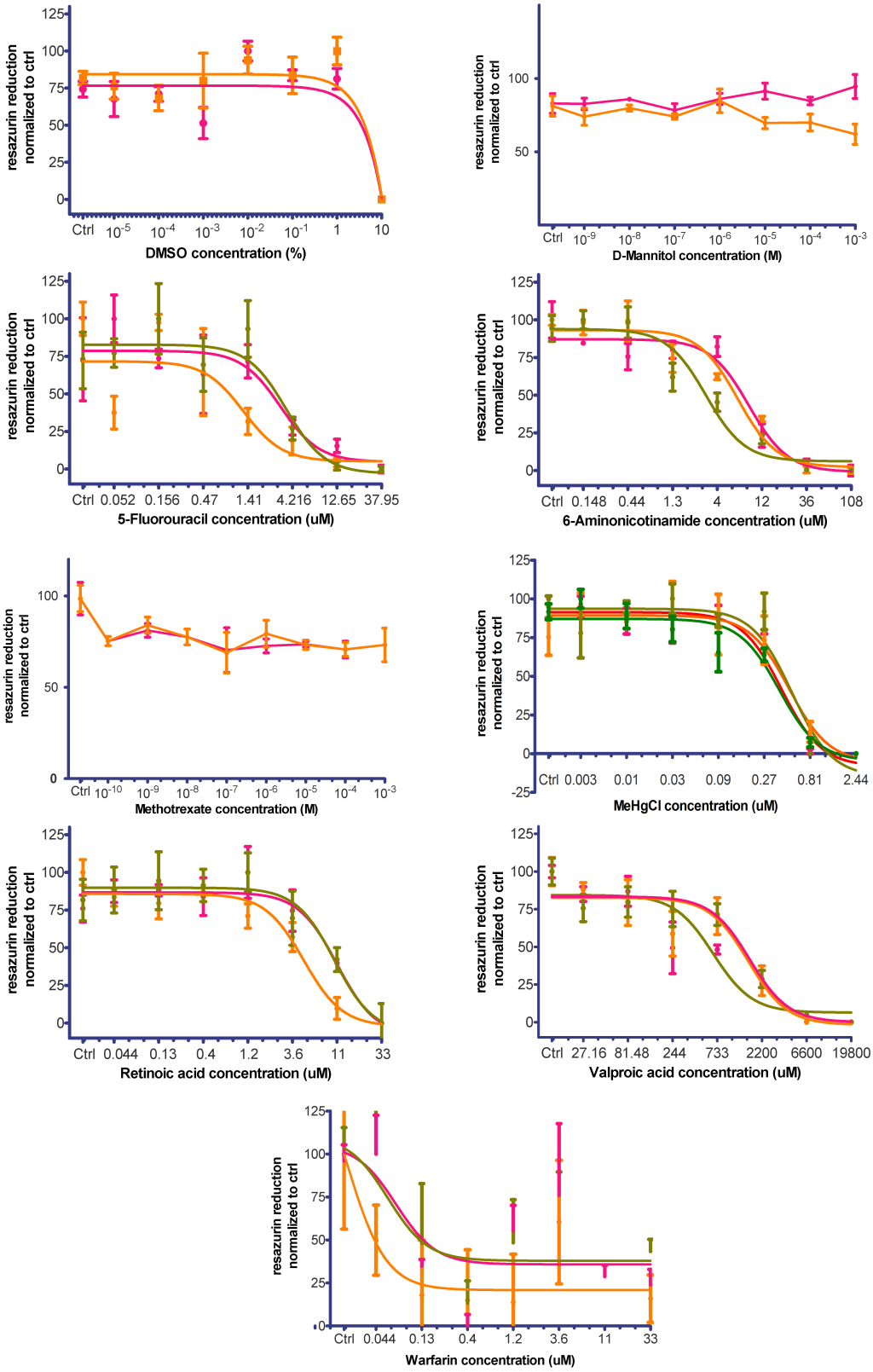

Figure 4.4: Resazurin based cytotoxicity. Data are given as mean ± S.E.M of 3 independent experimental runs.

4.4 Neuronal marker mRNA expression profile changes during toxicants exposure

Cytotoxicity can be accepted as an indicator for neurotoxicity; hence additional readouts pointing to specific effects on the differentiation into cells of the nervous system have to be developed. To accommodate this request, effects of toxicants on neural precursor differentiation were analysed by qPCR in treated and untreated control cell samples collected on day 10 of hESC differentiation. Changes in the expression of lineage specific marker genes such as *MAP2, NCAM1, NEFM, NESTIN, OCT4, OTX2* were used to detect chemical interference with the differentiating neural cells (Figure 4.5). The housekeeping genes *GAPDH* and *HPRT1* were used as internal controls.

MAP2 was significantly downregulated in a concentration-dependent manner. A 0.36-fold downregulation was measured at the highest concentration assessed with retinoic acid treatment, but already at the non-cytotoxic concentration its expression was lower, 0.55-fold, compared to the untreated control cells. While valproic acid and 5-fluorouracil caused upregulation, 1.3 and 2.1-fold at non-cytotoxic level and 1.5 and 6.1-fold changes at IC_{10}, respectively, no significant effect with the 6-aminonicotinamide and warfarin treatment were observed.

The expression level of *NCAM1* was significantly modified by valproic acid exposure of the differentiating cells with a concentration-dependent upregulation of 2.26, 2.68 and 2.75-fold from non-cytotoxic to IC_{10} concentration. *NCAM1* expression levels changed in a similar way with 5-fluorouracil treatment but with lower fold-changes, 1.38 and 1.75, while retinoic acid and 6-aminonicotinamide caused only slight upregulation, 1.33 and 1.17-fold at non-cytotoxic level which diminished at higher concentrations. Warfarin caused a decrease in *NCAM1* expression, 0.58-fold at non-cytotoxic concentration which increased to 0.78 at IC_{10}.

Retinoic acid, valproic acid and 5-fluorouracil significantly changed the *NEFM* expression profile with 2.6, 2.2, 3.87-fold increase at non-cytotoxic concentrations. Furthermore, this upregulation was concentration-dependent in the case of valproic acid treatment, reaching 5.17-fold increase at the IC_{10} concentration, whereas this fold increase decreased to 2.41 and 2.45-fold in the retinoic acid and 5-fluorouracil treated cells, respectively. 6-aminonicotinamide and warfarin did not affect the *NEFM* expression.

NESTIN expression was modified by retinoic acid and 5-fluorouracil causing slightly

concentration-dependent upregulation of this gene, reaching 1.59 and 1.31-fold at IC_{10} concentration, comparing to the untreated control cells. Valproic acid, 6-aminonicotinamide and warfarin caused its downregulation to 0.84, 0.92 and 0.61-fold at non-cytotoxic concentration; however at higher concentration levels this effect was reversed, the *NESTIN* expression level was similar to the control.

OCT4 was significantly upregulated already at non-cytotoxic concentration, reaching 2.1, 2.0 and 2.2-fold by the treatment of retinoic acid, valproic acid and 5-fluorouracil, respectively. But at higher concentrations, the effects were different: 5-fluorouracil caused a 3.44-fold upregulation at IC_{10} concentration, valproic acid only 1.11 and retinoic acid caused a 1.4-fold changes. In contrast, we observed a 0.72 and 0.61-fold downregulation at non-cytotoxic and IC_{10} concentrations caused by warfarin treatment. 6-aminonicotinamide caused a 0.33-fold downregulation at non-cytotoxic level, which increased to control level at IC_{10}.

OTX2 was upregulated by all of the chemicals at non-cytotoxic concentrations with 1.4, 2.96, 1.7 and 1.2-fold changes. On the other hand, warfarin caused no significant effect at this concentration (0.85-fold donwregulation). However, at higher concentrations, retinoic acid caused 0.66-fold downregulation, valproic acid 1.62-fold upregulation, 5-fluorouracil 2.32-fold upregulation, 6-aminonicotinamide 1.42-fold upregulation and warfarin 1.33-fold upregulation comparing to the untreated control cells.

In summary, retinoic acid treatment affected the neural differentiating cells causing significant downregulation of *MAP2* and *OTX2* and significant upregulation of *NCAM1, NEFM, NESTIN* and *OCT4*. All tested markers were upregulated by the valproic acid and 5-fluorouracil treatment. 6-aminonicotinamide caused *MAP2, NCAM1* and *OTX2* upregulation and *NEFM* downregulation, while it did not change *NESTIN* and *OCT4* expression. Methylmercury chloride treatment caused *MAP2*, *NCAM1* and *NESTIN* downregulations and *OCT4* upregulation, while it did not affect the expression of *NEFM* and *OTX2*. Warfarin treatment downregulated *NCAM1, NEFM* and *OCT4*, upregulated *OTX2* and did not affect *MAP2* and *NESTIN* expression. D-mannitol did not cause any significant changes in the gene expression profiles at low concentrations. The presented data confirm our hypothesis that differentiating hESCs are able to detect specifically the hazard of xenobiotics at non-cytotoxic concentrations (Table 4.2).

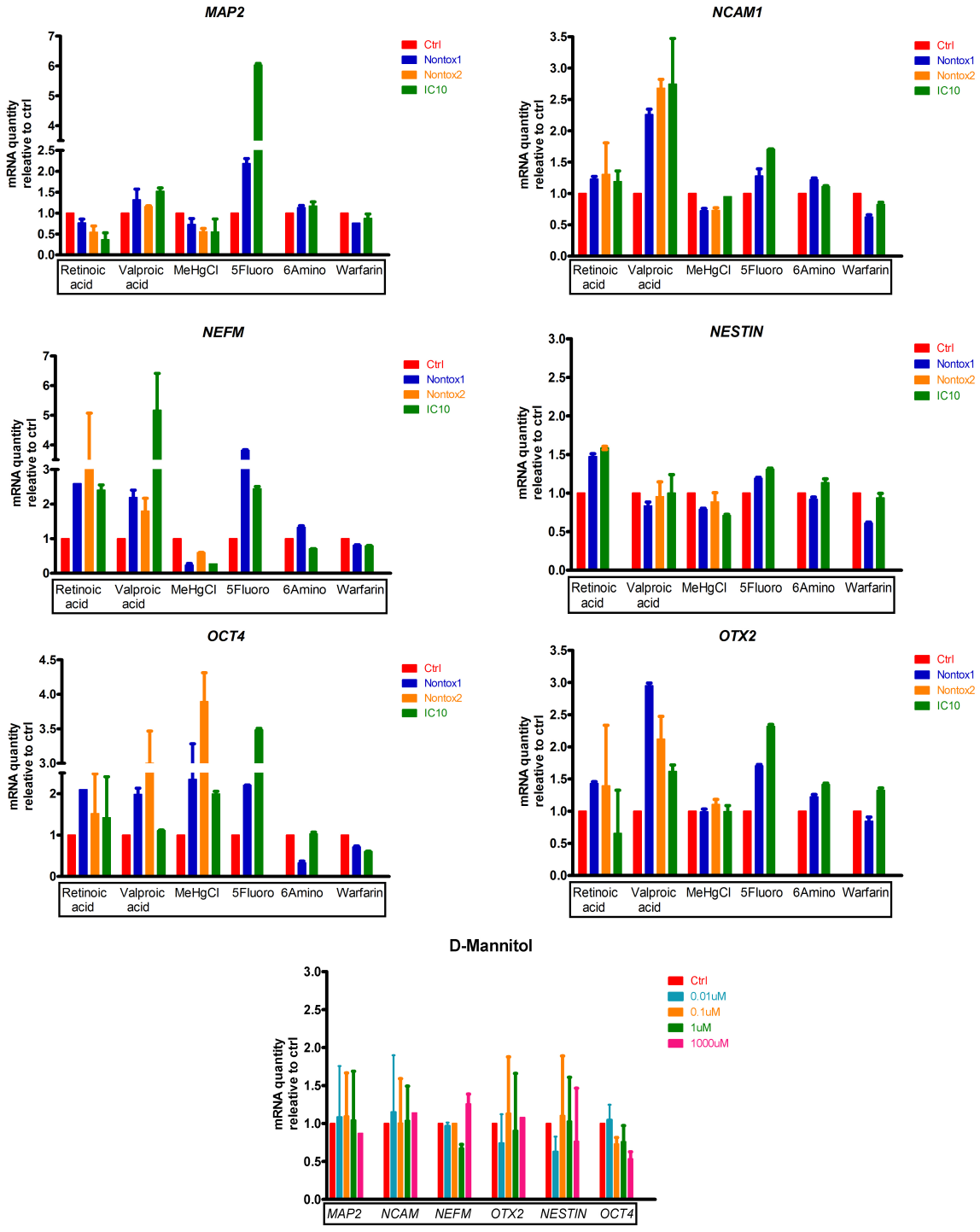

Figure 4.5: Relative quantification by qPCR determined effect of the tested toxicants. Gene expression levels were normalised to *GAPDH* and the mean expression in the untreated hESCs. Nontox2=the highest non-cytotoxic concentration, Nontox1=non toxic concentration. N=3.

Table 4.2: The table summarizes changes in the gene expression profile of cell cultures treated with different chemicals. ↑ means statistically significant upregulation, ↓ means statistically significant downregulation, while – indicates no significant changes at non toxic concentration.

Chemical	*MAP2*	*NCAM1*	*NEFM*	*NESTIN*	*OTX2*	*OCT4*
Retinoic acid	↓	↑	↑	↑	↓	↑
Valproic acid	↑	↑	↑	↑	↑	↑
5Fluorouracil	↑	↑	↑	↑	↑	↑
6Amino-nicotinamide	↑	↑	↓	-	↑	-
Methylmercury chloride	↓	↓	-	↓	-	↑
Warfarin	-	↓	↓	-	↑	↓
D-Mannitol	-	-	-	-	-	-

4.5 ABCB1 and ABCG2 mRNA expression of neural precursors

In order to evaluate the possible role and effect of the ABCB1 and ABCG2 transporters during chemical exposure on neural differentiating hESCs, their mRNA expression profile was analysed by qPCR during differentiation of 10 days. The *ABCB1* was highly expressed already on the 1st day of differentiation, 26-fold, reaching a plateau with 84-fold on day 6, compared to the undifferentiated hESCs. The *ABCG2* showed a continuous upregulation with 47, 379, 365-fold change on day 1, 7 and 10 (Figure 4.6).

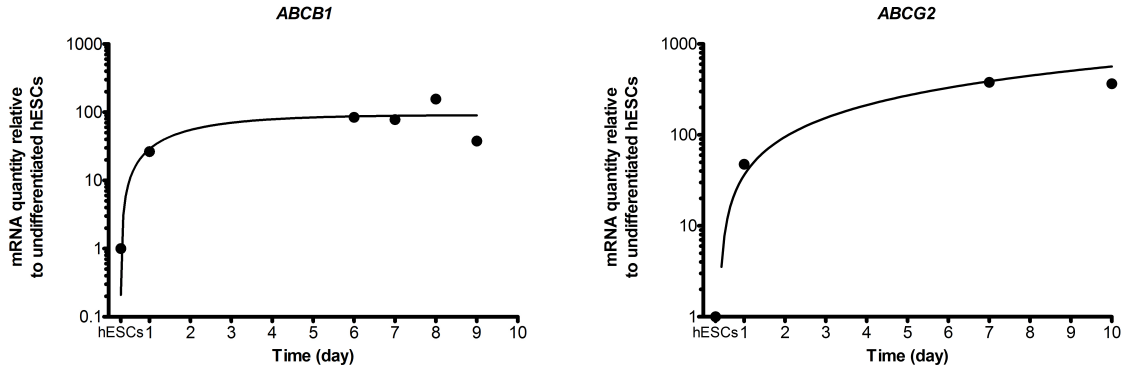

Figure 4.6: Relative quantification by qPCR of gene expression level of *ABCB1* and *ABCG2* during differentiation of hESCs into neuronal precursors. Gene expression levels were normalised to the housekeeping gene GAPDH and the mean expression in the undifferentiated hESCs**.**

The effects of MeHgCl on the two ABC transporters during neural precursor differentiation were analysed by qPCR in treated and untreated control cell samples collected on day 10 of hESC differentiation (Figure 4.7). The housekeeping genes *GAPDH* and *HPRT1* were used as internal controls. *ABCB1* was upregulated in a concentration-dependent manner reaching to 2-fold change at IC_{15} (0.27 µM). *ABCG2* was downregulated by 60±4% at IC_{15} concentration comparing to the control untreated cells.

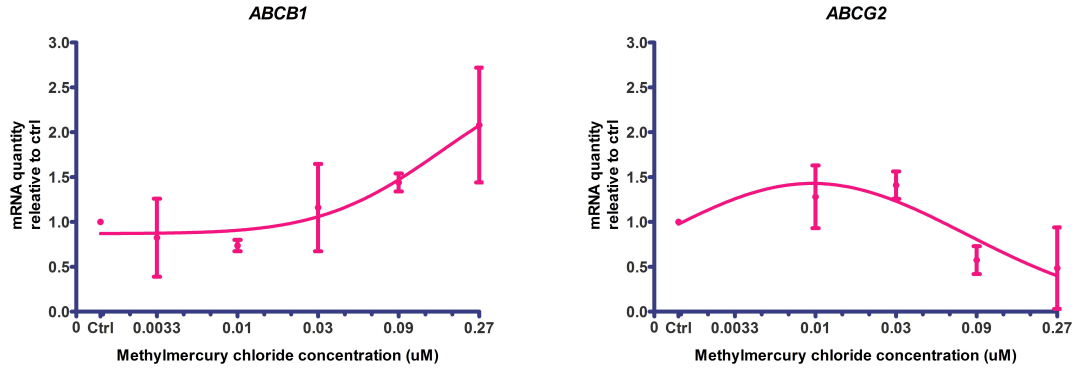

Figure 4.7: Relative quantification by qPCR determined effect of MeHgCl. Gene expression levels were normalised to *GAPDH* and the mean expression in the untreated hESCs. N=3.

4.6 Transcriptomics analysis

In order to elucidate the possible effects of MeHgCl on the developing human neural cells, MeHgCl treated (IC_{15}, IC_{05} and the highest non-cytotoxic concentrations) and untreated control cell samples collected on day 10 of differentiation were analysed using the Affymetrix HG-U133 Plus 2.0 arrays. After the robust multi-array analysis of the entire dataset (54000 probes) excluding internal controls and non- or low expressed transcripts, the remaining 26507 probe sets were investigated by principal component analysis to classify the variance in the data set (Figure 4.6). The first principal component (PC) axis accounts for 23.4% of the variance in the data set of variable transcripts showing a clear separation of the untreated cells from the MeHgCl treated cells. While the second and third principal component axes account for 21.9% and 11.5% of the variance respectively.

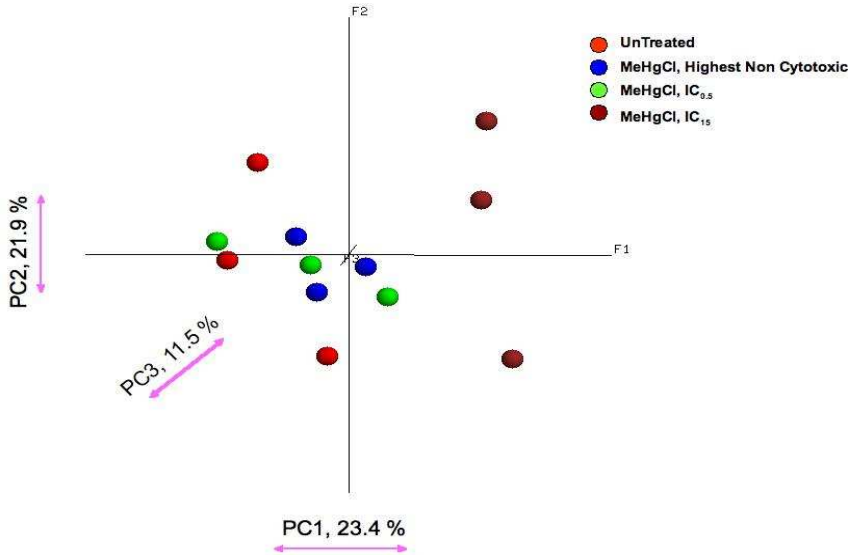

Figure 4.8: Principal component analysis (PCA) of untreated cells (red circles) and MeHgCl treated cells with the highest non cytotoxic (blue circles), $IC_{0.5}$ (green circles) and IC_{15} (brown circles) concentrations.

K-Mean clustering of the differently expressed probe sets obtained from ANOVA calculation resulted in 4 clusters as shown by the heatmap (Figure 4.7). Cluster X contains 203 genes that are highly expressed in the IC_{15} MeHgCl-treated cells. They are belonging to GOTERM_BP_5 associated with neurogenesis, neuron and central nervous system development, and cell migration and to the development of axon, dendrite, plasma membrane part, and cell junction in GOTERM_CC_5 resulted by the Gene Ontology Enrichment Analysis. In Cluster Ya and Yb the genes were downregulated in a concentration dependent manner by the toxicant treatment. Cluster Ya contains 279 genes and Yb contains 264 differently expressed genes. Gene Ontology and KEGG pathway analysis of Cluster Ya and Yb identified genes playing role in the development of mesoderm-derived organs, like vasculature, blood vessel, heart and kidney, but also genes associated with neural crest differentiation and development. Interestingly, genes from pathways in cancer were also identified. Cluster Z contains 61 genes which were highly upregulated in MeHgCl treated cells in a concentration dependent manner. These genes are belonging to the regulation of mitosis and cell cycle and also to the development of microtubule and cytoskeleton.

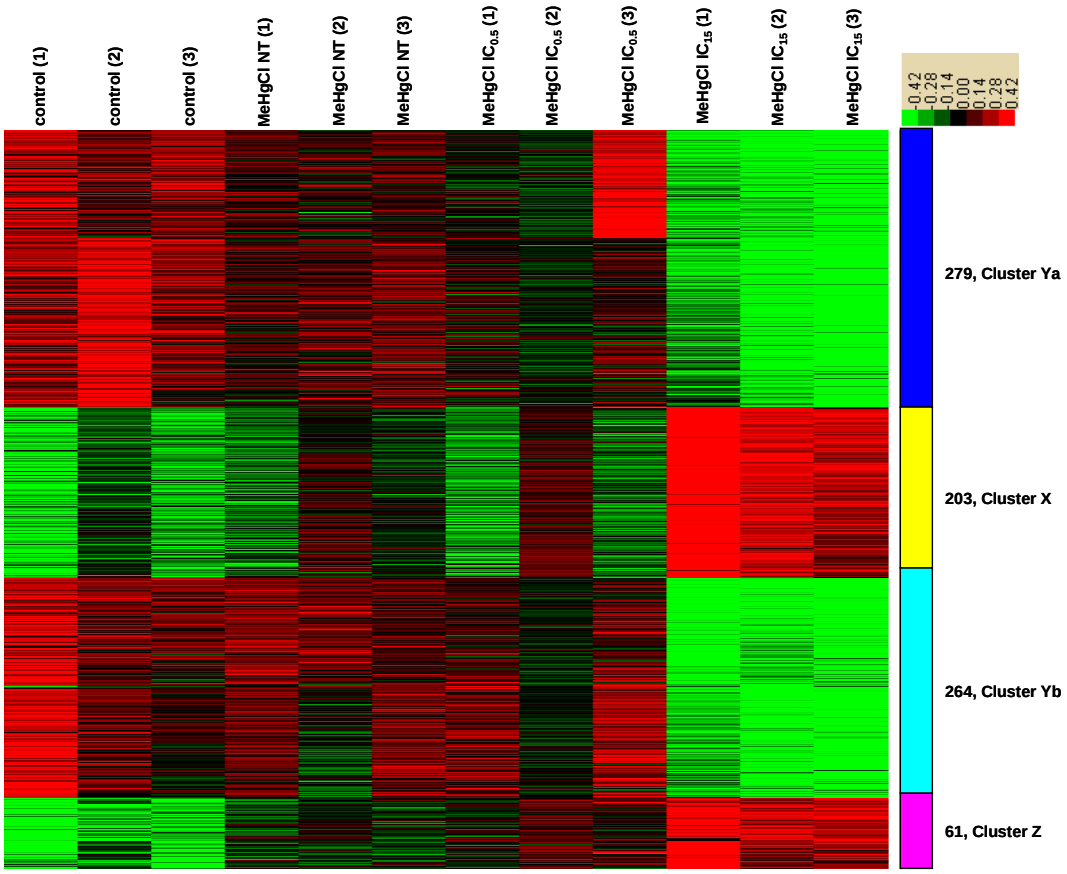

Figure 4.9: Heatmap of all differentially expressed genes by the MeHgCl treatment. Each row represents a single gene. Log2 transformed signal intensities are depicted in colour code. Red indicates high expression, dark grey intermediate expression and green low expression. Replicates are displayed in the vertical axis. Each cluster was defined based on comparable expression profiles during MeHgCl treatment. NT = highest non-cytotoxic concentration.

4.6 Biomarker analysis

For the validation of the microarray data, the top upregulated and downregulated genes from the different treated groups were combined and chosen for further analysis by qPRC the top 2 upregulated and downregulated genes from the significant overlapping genes. They were one-one representatives from the 4 clusters: X, Ya, Yb and Z, as *RELN*, *NPPB*, *CDKN2B*, and *SAMD3*, respectively. MeHgCl treated (0.27 μM – 3.3 nM) and untreated control cell samples were collected on day 10 of differentiation and the expression profile of *RELN*, *SAMD3*, *CDKN2B* and *NPPB* were detected against the housekeeping genes *GAPDH*

and *HPRT1*. The qPCR analysis (Figure 4.8) confirmed the results of the transcriptomics analysis as the chosen top genes were the most sensitive on the MeHgCl treatment.

RELN was significantly upregulated already at the lowest tested concentration (3.3 nM) arriving to a plateau of 3.3±0.3-fold change compared to the untreated control cells. *SAMD3* was upregulated in a concentration-dependent manner reaching to 5.9±1.6-fold change at IC_{15} (0.27 µM). *CDKN2B* and *NPPB* was significantly downregulated by 81±4% and 80±8% at IC_{15} concentration comparing to the control untreated cells, respectively.

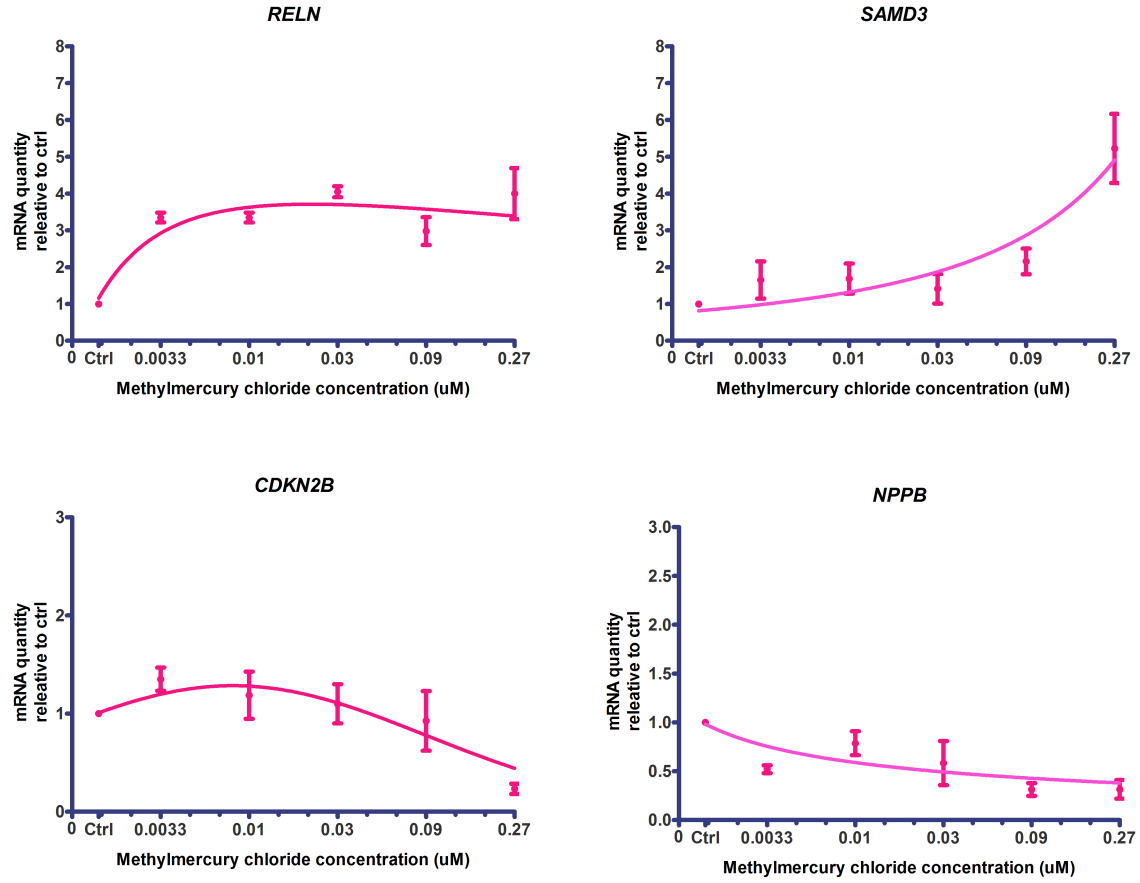

Figure 4.10: Possible biomarkers. Effects of MeHgCl on the mRNA expression of *RELN*, *SAMD3*, *CDKN2B*, and *NPPB* were determined by qPCR. Data are given as mean ± S.E.M of 5 independent experimental runs.

5. DISCUSSION

Embryonic stem cells have been proposed as cellular models for assessing developmental toxicity for several years [72]. Most of these models are using either differentiating pluripotent murine embryonic stem cells [54] or undifferentiated human embryonic stem cells [73]. In this study a human cell based test system is presented that aims to contribute to the detection of effects on the developing nervous systems such as neural tube defects which is one of the most common birth defects. The study describes the detailed development of a human stem cell based *in vitro* toxicity test aiming to identify chemicals interacting with the developing nervous system. The work focused particularly on the development of quality standards and acceptance criteria controlling the cellular model in order to accommodate the unique nature of stem cells that can differentiate theoretically into nay type of cells of the human body [74].

5.1 The development of quality standards ensuring the robustness of the cellular model

The definition of the original pluripotent cellular material is one of the most relevant parameters for a robust test system. However, the criteria to define pluripotency are multiple and are varying between stem cell based toxicity testing and other applications of stem cells such as regenerative therapy. Because of these discrepancies there is an urgent need to harmonise quality standard applicable for stem cell based toxicity testing [75]. In order to define quality standards, the most commonly used markers were evaluated for their suitability as quality controls for assessing routinely stem cell cultures in toxicological laboratories. *OCT4* and *NANOG* are the frequently used markers pointing to the pluripotency status of stem cells [76, 77]. In the presented study, a threshold that has to be met in order to use cells for further neural differentiation was defined. From the variety of differentiation protocols reporting on the successful differentiation into neuronal cell types [78], a protocol was selected [25, 71] and optimised to be stable over time and to provide sufficient number of toxicological target cells. The reproducibility of the differentiation protocol was judged with a panel of marker genes involving the three germ layers. The differentiation was considered as successful when showing a downregulation of the undifferentiated stem cell markers *OCT4*

and *NANOG* as indicators for pluripotent cells and a consistent expression of well-known neural related genes over time.

The above mentioned aspects refer to the assessment of the reliability of a test system. However, a test system has not only to demonstrate its reliability but also it needs to show relevance before it can be considered for safety assessment [79]. Therefore the scientific rationale of selecting the test system is of utmost importance. The described neural tube formation assay proved its biological relevance by comparing *in vivo* marker gene expressions with profiles occurring during neural cell differentiation *in vitro* [80]. Some of the described neural markers were converted into quality standards in order to monitor the successful differentiation of neural progenitor cells. The selected markers as described below reflect the developmental status after closure of the neural tube *in vivo*.

The intermediate filament *NESTIN* showed a continuous upregulation from day 2 on. This marker has been associated with neural progenitor cells appearing during early neurogenesis [81]. Other markers such as *PAX6*, *MAP2* and *NCAM1* were significantly upregulated later and reached the maximum expression at day 5. At this time point initial rosettes were detected in our culture plates. *PAX6* that is expressed in the developing nervous system appears in early neural cell cultures as well as in the maturation phase of neurons [82]. *MAP2* is generally considered as a postmitotic marker. The *MAP2a* isoform increases during maturation of neuronal processes while *MAP2b* and *MAP2c* are expressed during neurite outgrowth and their rearrangement in early foetal development [83]. The neural cell adhesion molecule 1, *NCAM1* is involved in cell-to-cell interactions as well as cell-matrix interactions during development of the nervous system [84]. *NEFM* showed a significant upregulation at day 10 which is comparable to the fact that is known to be expressed in late neural progenitor cells [85-87]. *OTX2* showed only a slight expression during our *in vitro* neural differentiation till day 10. Published *in vivo* data suggest that *OTX2* is required at a later stage of the neural differentiation process to regulate neuronal subtype identity and neurogenesis in the midbrain [88].

In contrast, expression of glial and oligodendrocyte lineage markers: *GFAP* and *OLIG2* were not detected as the selected differentiation protocol does not drive the cells towards the glial lineages [89, 90]. No expression of the endodermal lineage marker *AFP* and the mesodermal human cardiac differentiation marker *MYH6* was identified.

A high number of repetitions of the differentiation protocol allowed the definition of a narrow acceptance range of the gene expression levels (ΔCt values) allowing the comparison of data. Altogether, the cell model described in relevant literature was converted into a robust and reliable *in vitro* test following similar steps and time window as take place during human neurulation.

5.2 Challenging the cellular model with well known reference compounds

In order to understand if and how the cellular model responded to the chemical stress it was challenged with six known developmental toxicants: 5-fluorouracil, 6-aminonicotinamide, methotrexate, retinoic acid, valproic acid and warfarin as well as the negative control substance D-mannitol. Before analysing the specific effects on the neural cell differentiation, the effective dosage range was determined in order to avoid assessments at cytotoxic concentrations that might cover or influence the analysed gene expression profile. After 10 days of exposure of the neural differentiating culture to the selected chemicals, cytotoxicity curves were generated based on the resazurin reduction assay. Beside D-mannitol and methotrexate five chemicals demonstrated a concentration-dependent inhibition of resazurin reduction in the differentiating neural precursor cells. Since the applied cytotoxicity test is not providing information on specific effects on the neural precursors, the highest non-cytotoxic, IC_{10} and IC_{50} concentrations of the different chemicals were analysed by assessing the modulation of the selected marker panel relevant for neural precursor cells.

5-Fluorouracil is one of the oldest chemotherapy drug which has been in use for about 40 years [53]. It is a potent teratogen in all six tested species of laboratory animals: rat, mouse, rabbit, primate, hamster and guinea pig [52]. It induces multiple malformations in mice, rats and guinea pigs; cleft palate was produced in hamsters, and limb defects induced in rabbits [9]. In humans, it has been also associated with birth defects in one case [91]. 5-Fluorouracil must be considered as an unspecific control due to its general toxic effects based on its mode of action interacting with DNA integrity. In our experiments it was affecting the expression of all the neural related genes. Moreover, it significantly upregulated the gene expression of *OCT4*, indicating a stop of the neural differentiation process while keeping the cells in a pluripotency state.

6-Aminonicotinamide, a nicotinic acid antagonist, is a potent teratogen in multiple animal species. It caused cleft palate, skeletal defects, and hydrocephalus in mice which can derive from a spina bifida. In rats, cleft lip, cleft palate, digit and skeletal defects were primarily induced [9]. Eye defects were produced in rats. Eye, tail, visceral, and skeletal anomalies, and cleft palate were observed in rabbits' offspring. It also caused multiple effects in hamster and pig [9]. In our system the application of 6-aminonicotinamide during *in vitro* human neural precursor differentiation slightly affected the expression of all of the tested genes; *MAP2*, *NCAM1*, *NEFM*, *NESTIN*, *OCT4* and *OTX2*.

Retinoic acid is a metabolite of vitamin A that mediates the functions which are required for growth and development [92] via nuclear receptor binding. During early embryonic development it is one of the patterning signals in the developing nervous system and has a fundamental role in the specification of the anterior-posterior axis [93]. Retinoic acid exposure during the neurulation process can result in neural tube defects in rats [94]. Moreover, several cases have been reported that overdoses of retinoic acid during pregnancy caused human malformations; hydroureter, hydronephrosis and defects suggesting Goldenhar's syndrome [9]. In this study it was detected that the neural differentiating cells were sensitive to the retinoic acid treatment causing modifications of the mRNA expression profiles at non cytotoxic concentrations. Especially, the *NEFM* was significantly upregulated, while the postmitotic marker *MAP2* was significantly downregulated.

Valproic acid as an anticonvulsant and mood-stabilizing drug was first marketed in Europe in 1967, and later in the United States in 1978. Valproic acid is teratogenic in all of the six laboratory animal species tested. It induced malformations of multiple organs in mice, rats, and gerbils, renal and vertebral skeletal defects in rabbits, neural tube defects in hamsters, and craniofacial and appendicular skeletal defects in primates [9]. The first association between valproic acid and human malformation was discovered by Dalens and her colleges [95]. They reported on an infant who died 19 days after birth whose mother has taken 1000mg/day of valproic acid during gestation. The child suffered from retardation and had multiple malformations; peculiar facies, microcephaly, asymmetrical chest, hip dislocation, symphysis of toes and levocardia. In the period between 1976-1997, around 33900 malformed infants or foetuses were reported by the French Central-East Registry of Congenital Malformations. In 178 cases the mothers were treated with valproic acid in the first trimester

of pregnancy. The malformations consisted of 41 cases with spina bifida, 39 with congenital heart defects, 18 with orofacial clefts, 18 with hypospadias, 10 with preaxial limb defects and 66 with other malformations [9]. According to Schardein, the estimated risk of malformations in humans caused by valproic acid is 1-2% [9]. As previously described, valproic acid affected the expression profiles of all tested genes. Moreover, the detection of the concentration-dependent upregulation of *NCAM1* suggests that our system qualifies for mechanistic studies elucidating effects on physiological pathways leading to toxicities as observed for valproic acid.

Warfarin is an anticoagulant which was initially marketed as a pesticide against rats and mice. Later on it was used for preventing thrombosis and embolism in many disorders, and has remained popular ever since. It is the most widely prescribed oral anticoagulant drug in North America [96]. However, the exposure to warfarin during the pregnancy leads to a well-known embryopathy called foetal warfarin syndrome. It acts as an inhibitor of the synthesis of the vitamin K-dependent clotting factors and thereby it has been recently suggested that it can have also a direct teratogenic effect on the central nervous system development [68]. Our data suggest that warfarin would induce a change in the gene expression profile during neurulation, as the upregulation of *OTX2* and the systematic downregulation of *NEFM* and *OCT4*, that might explain the known physical abnormalities reported *in vivo*. A more detailed analysis of affected pathways might elucidate the toxicological mechanism of warfarin during neural development.

5.3 ABCB1 and ABCG2 transporters

ABCB1 and ABCG2 transporters can be used as universal stem cell markers as well as markers in neural precursor cells [45]. We were investigating whether the 10 days neural differentiating cells are expressing the ABCB1 and ABCG2 markers, and moreover, the MeHgCl treatment affects their mRNA expression profile or not. In our preliminary experiments it was found that both of the markers were highly expressed already at early stage of the neural differentiation. The expression of *ABCB1* reached a plateau on day 6 of differentiation, while the *ABCG2* showed a continuous increase.

The neural differentiating hESCs were exposed to MeHgCl from 0.27 μM to 3.3 nM

and the mRNA expression profile of *ABCB1* and *ABCG2* was detected by qPCR analyses. According to our results, both genes were affected by the treatment. *ABCB1* was upregulated in a concentration-dependent manner, while *ABCG2* was downregulated.

Since it is known that these transporters are playing important role in cell protection from different xenobiotics [36], a more detailed analysis might clarify their mechanism during neural development.

5.4 Elucidation of the mode of action of methylmercury chloride by transcriptomics analysis

It is already proven that methylmercury (MeHg) readily crosses the placenta and the effects of in utero exposure to MeHg are quite different from the effects associated with childhood or adult exposure due to the fact that the developing human brain is much more vulnerable to injuries caused by toxic agents than the brain of an adult [9]. Even pregnant women showing only mild or no symptoms of intoxication gave birth to infants with development disabilities [97]. Mental retardation, severe behavioural and sensory deficits including deafness and blindness, neuromuscular weakness and altered neurobehavioral development are among the toxic effects reported [9, 98]. MeHg exposure may also cause malformations manifested as skeletal variations, reduced ossification, abnormal brain structure, changes in neuronal migration and distribution patterns, and reduction in the number of brain cells [99-102].

It is reported in the literature that the lowest measured level of MeHg due to chronic low dose exposure related to neurobehavioral effects in the offspring is 10 ppm in maternal hair. This level can be accepted as a threshold indicator for the developmental effects of MeHg exposure [103]. Assuming a mercury hair-blood ratio of 250 [103], this level corresponds to 40 ppb = μg/L in maternal blood (0.16 μM). Although MeHg crosses the placenta without any problem and accumulates in the foetus, no significant difference has been reported on the measured levels of MeHg in maternal and chord blood [104]. Therefore, it can be assumed that the embryonic plasma concentration, 0.16 μM MeHg, is equal to maternal plasma concentration. Hence, the toxicological endpoints of our study with its response to even lower concentrations, such as 0.0625 μM and 0.025 μM can be accepted as

the proof of the sensitivity of our system.

Applying MeHgCl from 0.27 μM to 3.3 nM during 10 days neuronal precursor differentiation, the neural related genes *MAP2*, *NCAM1* and *NESTIN* showed significant downregulation in a concentration dependent manner, while the pluripotency marker *OCT4* was upregulated in qPCR analysis. These results indicate that MeHgCl interferes with the early neural differentiation by mechanisms different from general cytotoxic effects causing more specific effects than cell death on the neural differentiating cells. In comparison with previously published results using neural differentiated H1 hESCs exposed to 25 nM MeHgCl, *NCAM1* and *MAP2* was downregulated by the treatment at differentiation day 12, but *NESTIN* did not show any significant changes [51]. This difference can be explained by the slight time-shift of the different differentiation kinetics between the two hESC lines. In other studies using neural differentiated murine ESCs, the expression of the neuron specific cytoskeletal protein *Mtap2* gene was decreased [105], while in a similar approach with transcriptomics analysis the transcription- and development-related genes were downregulated and the neurulation and cell-motion related genes were upregulated by MeHgCl treatment overtime [106].

The molecular mechanisms of MeHg toxicity during human neurulation have currently not been identified, but *in vivo* and *in vitro* studies suggest mechanisms involving oxidative stress, cell cycle regulatory proteins, protein phosphorylation and intracellular calcium homeostasis [62, 63]. In order to enlighten the underlying mode of action, a scan of the expression changes in the complete human genome of MeHgCl-treated 10 days neural differentiated hESCs was performed by transcriptomics analysis. Among the genes differentially expressed by MeHgCl-treatment, the most upregulated transcripts were included in the GO categories related to neurogenesis and neuron development; cell morphogenesis involved in neuron differentiation; neuron and cell migration; central nervous system, brain, cerebral cortex, pallium, and forebrain development. The most downregulated genes were involved in blood vessel, vasculature, heart, skin, lung and kidney development; neural crest cell development and differentiation; and neural tube formation and closure. Moreover, the pathways affected by MeHgCl treatment which remarkably corresponded to *in vivo* studies, such as calcium signalling pathway, cell adhesion molecules, cell cycle, pathways in cancer, p53 and Wnt signalling pathway were revealed (Table 5.1).

Table 5.1: Similar pathways affected with MeHgCl treatment *in vitro* and *in vivo*.

Pathway	Pathway ID	Relationship inferred
Hemostasis	REACT:604	HIST1H3G; IGF1
Pancreatic secretion	KEGG:04972	TPCN2; TRPC1
Pathways in cancer	KEGG:05200	BIRC5; IGF1
Activation of TRPC channels by Netrin-1	REACT:22431	TRPC1
Aldosterone-regulated sodium reabsorption	KEGG:04960	IGF1
Allograft rejection	KEGG:05330	H2-T23
Amino sugar and nucleotide sugar metabolism	KEGG:00520	CYB5R3
Antigen processing and presentation	KEGG:04612	H2-T23
Autoimmune thyroid disease	KEGG:05320	H2-T23
Axon guidance	REACT:18266	TRPC1
Biological oxidations	REACT:13433	GSTM1
Calcium signaling pathway	KEGG:04020	TRPC1
Cell adhesion molecules (CAMs)	KEGG:04514	H2-T23
Cell Cycle, Mitotic	REACT:152	BIRC5
Colorectal cancer	KEGG:05210	BIRC5
Diabetes pathways	REACT:15380	IGF1
Dilated cardiomyopathy	KEGG:05414	IGF1
DNA Replication	REACT:383	BIRC5
Drug metabolism - cytochrome P450	KEGG:00982	GSTM1
Elongation arrest and recovery	REACT:1892	CCNT1
Endocytosis	KEGG:04144	H2-T23
Focal adhesion	KEGG:04510	IGF1
Gene Expression	REACT:71	CCNT1
Glioma	KEGG:05214	IGF1
Glutathione metabolism	KEGG:00480	GSTM1
Graft-versus-host disease	KEGG:05332	H2-T23
HIV Infection	REACT:6185	CCNT1
Hypertrophic cardiomyopathy (HCM)	KEGG:05410	IGF1
Intestinal immune network for IgA production	KEGG:04672	PIGR
Long-term depression	KEGG:04730	IGF1
Melanoma	KEGG:05218	IGF1
Metabolism of vitamins and cofactors	REACT:11193	CYB5R3
Metabolism of xenobiotics by cytochrome P450	KEGG:00980	GSTM1
mTOR signaling pathway	KEGG:04150	IGF1
Natural killer cell mediated cytotoxicity	KEGG:04650	H2-T23
Oocyte meiosis	KEGG:04114	IGF1
p53 signaling pathway	KEGG:04115	IGF1
Pausing and recovery of elongation	REACT:769	CCNT1
Phagosome	KEGG:04145	H2-T23
Progesterone-mediated oocyte maturation	KEGG:04914	IGF1
Prostate cancer	KEGG:05215	IGF1
Staphylococcus aureus infection	KEGG:05150	DSG1A
Systemic lupus erythematosus	KEGG:05322	HIST1H3G
Tight junction	KEGG:04530	TJP3
Transcription	REACT:1788	CCNT1
Transmembrane transport of small molecules	REACT:15518	SLC22A12
Type I diabetes mellitus	KEGG:04940	H2-T23
Viral myocarditis	KEGG:05416	H2-T23
Wnt signaling pathway	KEGG:04310	NELL2

Further validation of the 2 most upregulated and of the 2 most downregulated genes *RELN, NPPB, CDKN2B,* and *SAMD3* derived from each of the 4 clusters: X, Ya, Yb and Z confirmed the dose response relationship of the MeHgCl treated neural differentiated hESCs.

NPPB is a member of the natriuretic peptide family and encodes a secreted protein which functions as a cardiac hormone. The protein undergoes two cleavage events, one within the cell and a second after secretion into the blood. The protein's biological actions include natriuresis, diuresis, vasorelaxation, inhibition of renin and aldosterone secretion, and a key role in cardiovascular homeostasis. A high concentration of this protein in the bloodstream is indicative of heart failure. Mutations in this gene have been associated with postmenopausal osteoporosis and it is also accepted as clinical marker of the diseased heart. It was significantly downregulated during MeHgCl treatment and as by the K-Mean clustering and the GO analysis revealed its role in regulation of blood vessel size and vasodilation.

RELN is a large, with a relative molecular mass of 388 kDa, secreted extracellular matrix glycoprotein composed of 3461 amino acids that helps regulating processes of neuronal migration and positioning in the developing brain by controlling cell-cell interactions [107]. Neuronal positioning is critical for the formation of cytoarchitecturely distinct brain regions such as the cerebral cortex, hippocampus and cerebellum. In mammals the organisation of the cerebral cortex follows a stereotypic plan during development as neurons with similar morphologies and connections are positioned in the same layer. In the cerebral cortex of reeler mouse, *RELN* gene disrupted, the layer I is not discernible and the positioning of the cells comprising other layers is relatively inverted [108]. Moreover, recent studies have also suggested a role for *RELN* in axonal branching, synaptogenesis, and in neurodegeneration concluded from the findings that *RELN* was upregulated in the brain and in cerebrospinal fluid in Alzheimer’s disease patients [109].

In our cell system *RELN* was the most upregulated gene by MeHgCl treatment. It was belonging to the X cluster playing role in neuron generation; neuron, central nervous system, brain, cerebral cortex, pallium and forebrain development; cell morphogenesis and neurogenesis; and neuron, cerebral cortex, telencephalon and forebrain cell migration. According to our results, MeHgCl overactivated the reelin pathway by *RELN* upregulation. Reelin via binding to ApoER2/Vldlr complex and to integrins causes tyrosine phosphorylation triggering an intracellular signalling cascade which instructs neurons to occupy their proper

locations. Moreover, via activation of protein tyrosine kinases it stimulated the cyclin D1 causing crucial modifications in the cell cycle process of the neural differentiating hESCs.

CDKN2B plays an important role in the negative regulation of cell cycle as it encodes a cyclin-dependent kinase inhibitor (CDKI), the p15lNK4B protein. The cell cycle is controlled by molecular switches that regulate the activation and progression though the successive phases. The main switches include a group of Ser/Thr kinases called the cyclin-dependent kinases (CDK), their positive regulators cyclins, and their negative regulators CDKI, which include the INK4 or the Cip/Kip families. Each phase of the cell cycle starts and ends in response to increases or decreases in cyclin expression. Early G1 is initiated by increased levels of members of the cyclin D family resulting activation of the CDK4 and CDK6 complex. In order to assure the continuity of the cell cycle going into the later G1 phase and G1/S transition, the complex of p15INK4B/p16INK4A/p18INK4C/p19INK4D has to be activated to inhibit the CDK4/6.

MeHgCl at the IC_{15} concentration significantly downregulated the expression of *CDKN2B*. This gene was belonging to the Yb cluster, to biological processes regarding to positive regulation of cell communication and signal transduction, negative regulation of cell proliferation, cellular metabolic processes, phosphate and phosphorus metabolic processes, and, interestingly to response to nutrient. Our results suggest that the downregulation of *CDKN2B* can be crucial in the events caused by MeHgCl exposure during human neurulation, as activation of cell cycle checkpoint at any stage of the cell modifies the cell cycle process [110-112]. A breakdown in the regulation of this cycle leads to uncontrolled growth and contribute to tumour formation. Moreover, defects in many of the molecules that regulate the cell cycle also lead to tumour progression. Key among these are p53, and the cyclin-dependent kinase inhibitors, as p15lNK4B, p16lNK4A, p18INK4C, p19INK4D, p21, p27 and the retinoblastoma susceptibility protein. Furthermore, our findings correlate to previous *in vivo* studies proving that the re-entry in the cell cycle is a convergence point in many neurodegenerative diseases [113].

There is no functional information of *SAMD3*, even though in our cell system it was one of the most sensitive genes upregulated by MeHgCl treatment. It was between the genes grouped together in the Z cluster which were belonging to the regulation of mitosis and cell cycle and also to the development of microtubule and cytoskeleton.

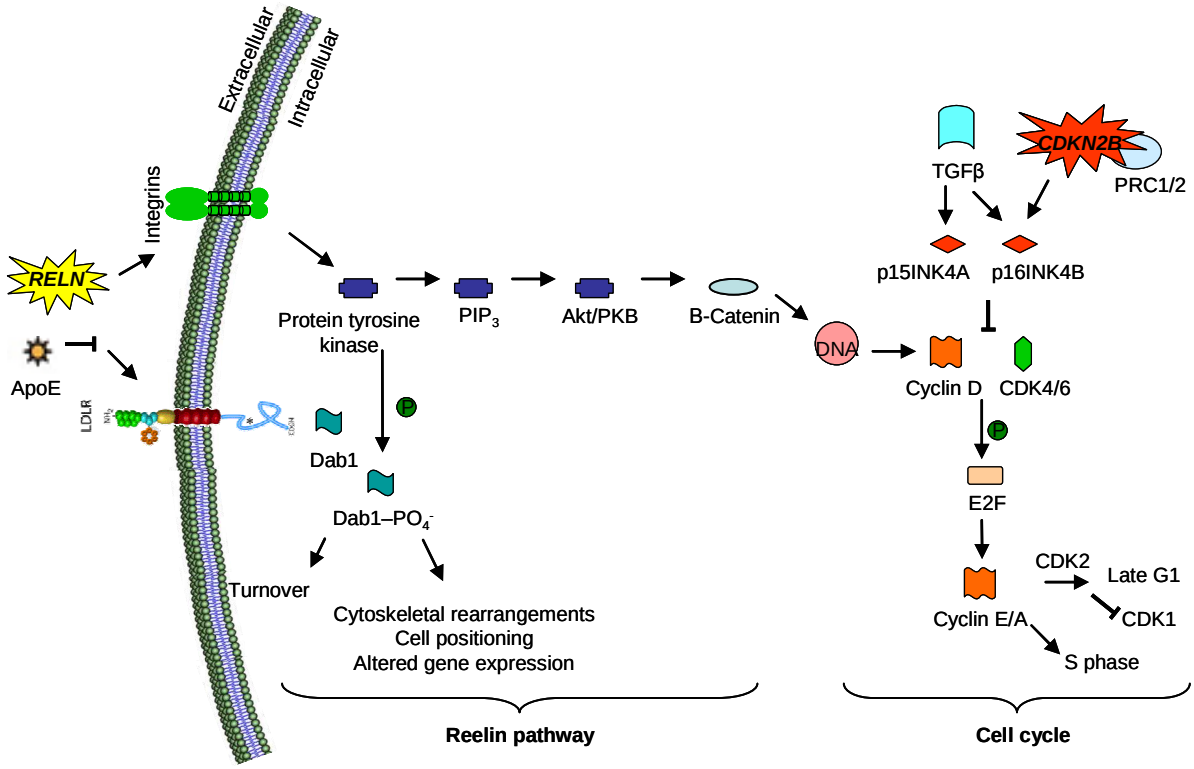

Figure 5.1: Possible pathway affected by MeHgCl treatment.

In summary, MeHgCl treatment of the neural differentiating hESCs modified the cell cycle by the stimulation of cyclin D1 via the high expression of *RELN*, and by the inhibition of the CDK4/6 inhibitor *CDKN2B*, through either the poly-comb group proteins PRC1/2 or the TGFβ pathway (Figure 5.1). It caused cell cycle re-entry of the neuronal precursor cells and by that preventing its further maturation.

Overall, our study demonstrates that well-described standard operating procedures and the introduction of stringent quality standards can support the overcoming of the currently anticipated challenges due to the instability of differentiation protocols. Within the context of our study, the development of a stem cell based cellular model which may serve as the basis of a predictive *in vitro* test that can be used to identify human developmental toxicants was described. The present study is the first which combined neural differentiating hESCs with sophisticated multiple endpoints readout in order to elucidate the mode of action of MeHgCl. After proving the sensitivity of the cell system with detecting mRNA changes by qPCR even at low MeHgCl concentration levels, it was classified for further transcriptomics analysis.

Furthermore, possible biomarkers were identified for detection of MeHgCl toxicity to neuronal precursor induction. It is the first time that molecular effects of MeHgCl toxicity on human neurulation linked to *RELN* upregulation and *CDKN2B* downregulation detected *in vitro* showed correlation to possible *in vivo* effects, such as defects of neural migration and cell cycle.

In the next steps, the transferability of the developed cellular model will be assessed as well as the suitability to elucidate critical pathways for developmental toxicity testing. The need for a more mechanistic oriented safety assessment in particular in the area of developmental toxicity testing has been demonstrated in several review studies that have evaluated the interspecies variations by the currently used *in vivo* tests [8, 9, 114]. Currently a lack of understanding on the mode of action of many developmental toxicants has led to a precautionary response of regulatory toxicology by safety testing in two species. However, a clear understanding of the mode of action for humans is a prerequisite to improve the hazard identification and risk management of chemicals as advocated by the National Research Council of the United States in its publication on “Toxicity Testing in the Twenty-first Century”.

6. SUMMARY OF NEW FINDINGS

- Detailed development of an *in vitro* toxicity test based on the directed neural differentiation of hESCs that includes quality and acceptance criteria as requested by competent authorities when toxicological test methods are used for regulatory safety assessments.
- Development of a robust differentiation protocol leading to neuronal precursor cells mimicking the embryonic neurulation process. The successful differentiation was confirmed by the consistent expression of well known neural related genes and a gene array involving marker genes of the three germ layers was defined in order to ensure lineage specificity of the toxicological target cells.
- The cellular model was converted into a predictive *in vitro* test for monitoring the effects of chemicals on differentiating hESCs and these results compared with the known *in vivo* effects. The test was challenged with six well known developmental toxicants that exhibit prenatal toxicity *in vivo*. The presented test system could detect little changes in the gene expression profile even at non-cytotoxic concentration which can lead to the identification of possible sensitive targets on the neurulation process, proving the sensitivity of the test system.
- The presented study is the first in the literature which combined neural differentiating hESCs with sophisticated multiple endpoints readout in order to elucidate the mode of action of MeHgCl.
- Possible biomarkers, such as *RELN*, *SAMD3*, *CDKN2B* and *NPPB* were identified for detection of MeHgCl toxicity to neuronal precursor induction. It is the first time that molecular effects of MeHgCl toxicity on human neurulation linked to *RELN* upregulation and *CDKN2B* downregulation detected *in vitro* showed correlation to *in vivo* effects, such as defects of neural migration and cell cycle.
- MeHgCl treatment of the neural differentiating hESCs modified the cell cycle by the stimulation of cyclin D1 via the high expression of *RELN*, and by the inhibition of the CDK4/6 inhibitor *CDKN2B*, through either the poly-comb group proteins PRC1/2 or the TGFβ pathway. It caused cell cycle re-entry of the neuronal precursor cells and by that preventing its further maturation.

7. ÖSSZEFOGLALÁS

Az embrionális fejlődés fázisában az idegrendszer gyakran válik xenobiotikumok, a szervezettől idegen kémiai anyagok célpontjává. Ugyanakkor az emberi születést megelőző toxicitás előrejelzése nem egyszerű feladat, mivel a toxikológiai válasz még a különböző laboratóriumi kísérleti állatfajok között is eltérő lehet. Ezért a fejlődés-toxikológia terén olyan tesztek kifejlesztésére van szükség, amelyek lehetővé teszik a fajok közötti különbségek okozta problémák kiküszöbölését, javítva az emberi egészség e pontjával kapcsolatos biztonságossági értékelés lehetőségét. Az emberi pluripotens őssejtek alkalmazásán alapuló *in vitro* tesztek amellett, hogy rávilágíthatnak toxikológiai mechanizmusokra - így elősegítve a xenobiotikumok veszélyességének megítélését, - egyben csökkentik a szükséges állatkísérletek számát is, ami a területtel kapcsolatos európai törvényhozás egyik fő céljaként fogalmazódott meg mostanában.

Emberi embrionális őssejt (hESC) vonalak izolálása vagy a szedercsírából (morula), vagy a hólyagcsíra (blastociszta) állapotú embrió belső sejttömegéből történik az egyedfejlődés 5.-6. napján. Ezek a sejtek - komoly etikai megfontolásokat követően - alkalmazhatóak gyógyszerfejlesztési célokra kihasználva két különleges képességüket: a készséget arra, hogy osztódás útján önmagukkal megegyező másolatokat állítsanak elő, vagyis az ön-megújítást, valamint azt a potenciált, hogy más sejt-formákká differenciálódjanak. A más, *in vitro* toxicitás tesztekben alkalmazott sejtvonalakhoz képest mutatott átütő előnyeik közé sorolható viszonylag magas stabilitásuk, kontrollált genetikai hátterük, irányítható genetikai módosíthatóságuk, korlátlan proliferációs készségük, és a változatos sejttípusokká való differenciálódási potenciáljuk. Ez utóbbi révén gyorsan rendelkezésre álló forrásai lehetnek emberi szomatikus sejteknek, ígéretes és gyors alternatívát nyújtva nagyszámú sejttömeg szüksége esetén. Mindezeken túl az őssejt technológia új eszközt kínál a gyógyszerek okozta nemkívánatos reakciókban szerepet játszó mechanizmusok megértéséhez is, lehetővè téve az emberi szervezetre gyakorolt esetleges toxikus hatások előrejelzését.

E dolgozat egy olyan hESC alkalmazáson alapuló tesztrendszert mutat be, amelynek célja, hogy hozzájáruljon a fejlődő idegrendszerre gyakorolt káros hatások detektálásához, mint amilyen például az idegcső hibás fejlődése, mely az egyik leggyakoribb születéskori

idegrendszeri rendellenesség. A disszertáció egy olyan emberi őssejt alapú *in vitro* teszt kifejlesztését írja le részletesen, amelynek célja a fejlődő idegrendszerrel kölcsönható vegyületek azonosítása. Munkánk során különös figyelmet fordítottunk sejtvonalra vonatkozó minőségi standardok kialakítására és a sejtes modellek elfogadási kritériumainak teljesítésére, melyeket az illetékes hatóságok a szabályozás kialakításakor a biztonságossági értékelésben alkalmazott toxikológiai tesztek esetében igényelnek.

Az irodalomban fellelhető számos protokoll közül, melyek neuronális sejttípussá történő sikeres differenciálódásról számolnak be, azt a módszert választottuk és optimizáltunk, amely alkalmasnak bizonyult időben stabil módon megfelelően nagyszámú toxikológiai célsejt előállítására. A differenciációs protokoll reprodukálhatóságát egy mindhárom csíralemezt reprezentáló markergén-készlet segítségével követtük kvantitatív PCR (qPCR), immunfluoreszcencia és flow citometriás analízis segítségével. A differenciációt sikeresnek tekintettük, amennyiben a pluripotens sejtek jellemzőjeként számon tartott *OCT4* és *NANOG* differenciálatlan sejt marker downregulációját tapasztaltuk, és emellett az idő előrehaladtával következetes módon megjelenő overexpressziót detektáltunk ismert idegrendszerre jellemző gének esetén, mint a *NESTIN*, *PAX6*, *MAP2*, *NCAM1*, *NEFM* és az *OTX2*. A differenciációs protokoll többszöri megismétlése lehetővé tette az adatok összehasonlítását megengedő szűk tartomány definiálását a génexpressziós szintek (ΔCt értékek) számára. Összefoglalva, a megbízható irodalmi forrásból származó modellt az emberi idegrendszer kialakulásának lépéseit követve és időablakát figyelembe véve robusztus és megbízható *in vitro* teszt módszerré alakítottuk.

A sejtes modell kémiai stresszhatásra való válaszának vizsgálata céljából a sejteket hat különböző toxikus anyag hatásának tettük ki, melyeket korábban az embrionális fejlődésre hatónak találtak *in vivo*. Választott molekuláink az 5-fluorouracil, 6-aminonikotinamid, methotrexate, retinolsav, valproinsav és a warfarin voltak. Negatív kontrollként D-mannitolt alkalmaztunk. Az idegsejt-differenciálódásra gyakorolt specifikus hatások elemzését megelőzően meghatároztuk a hatékony koncentráció tartományt abból a célból, hogy elkerüljük az értékelést a citotoxikus koncentráció tartományban, hiszen a citotoxikus hatás torzíthatja, vagy elfedheti a vizsgálni kívánt gén-expressziós profilt. A neurális differenciálódás 10 napját követően a kiválasztott anyagok jelenlétében az egyes anyagok citotoxicitását a resazurin redukciós módszerrel határoztuk meg. A D-mannitolon és

methotrexaton kívül öt anyag mutatott koncentráció függő resazurin redukció gátlást a differenciálódó neurális prekurzor sejtekben. Mivel az alkalmazott citotoxicitás teszt nem nyújt információt az idegrendszeri prekurzor sejtekre gyakorolt specifikus hatásokról, az egyes vegyületek okozta változást a kiválasztott, neurális prekurzor marker gén készlet qPCR analízisével is jellemeztük. Az mRNS expressziós szint profilokban bekövetkezett mennyiségi változások bizonyították sejtes modellünk alkalmasságát a fejlődő magzatot fenyegető veszélyes anyagok azonosításában.

Ezen felül, a toxikus anyagok hatásmechanizmusának megértéséhez hozzájárulva referencia vegyületként vizsgáltunk egy környezetszennyező toxikus anyagot, a metilhiganykloridot (MeHgCl), amely ismerten felelős agyszerkezeti fejlődéstani rendellenességek okozásáért. Annak ellenére, hogy jelentős mennyiségű adat áll rendelkezésre állatkísérletekből és Japánban illetve Irakban bekövetkezett mérgezéses esetekből, a MeHgCl hatásmechanizmusa az emberi egyedfejlődés során még mindig nem teljesen tisztázott és további elemzésre érdemes.

MeHgCl-t alkalmazva 10 napon keresztül a neuronális prekurzorsejtek differencációja során, 0,27 µM – 3,3 nM koncentráció-tartományban az idegrendszeri differenciációval összefüggő gének, mint a *MAP2*, *NCAM1* és *NESTIN* jelentős, koncentráció függő downregulációját tapasztaltuk, míg a pluripotencia marker *OCT4* upregulálódott a qPCR tanúsága szerint. Eredményeink arra utalnak, hogy a MeHgCl a közönséges citotoxicitástól eltérő mechanizmus révén zavarja a korai idegrendszeri differenciáciálódást, az egyszerű sejthalálnál specifikusabb hatást fejtve ki a fejlődő idegrendszeri sejtekre.

A jellemző hatásmód felderítése érdekében transzkriptomikai analízissel elemeztük az expressziós változásokat a MeHgCl jelenlétében 10 napos idegrendszeri differenciálódáson átesett hESC-k teljes genomján. A MeHgCl kezelés hatására megváltozott mértékben kifejezett gének közül a leginkább upregulált transzkriptek közé olyan gének tartoztak, amelyek a neurogenezisért, idegsejt fejlődésért, idegsejtté történő differenciálódásban résztvevő morfogenezisért, neuron- és más sejtvándorlásért, a központi idegrendszer, az agy, az agykéreg (pallium) és az előagy fejlődésért felelősek. A leginkább downregulálódott gének a vérerek, az érrendszer, szív, bőr, tüdő és vese fejlődéséért, a dúcléc sejtek fejlődésért és differenciálódásáért, valamint az idegcső keletkezésért és záródásáért felelősek. Ezenfelül olyan, - *in vivo* kísérleti eredményekkel figyelemre méltó összefüggést mutató - , MeHgCl

által érintett mechanizmusokat azonosítottunk, mint a kálcium jelátviteli útvonal, sejtadhéziós molekulák, sejtciklus, daganatos betegségekben érintett jelátvitel, p53 és Wnt signaling útvonalak. A két leginkább upregulált illetve downregulált gén, *RELN, NPPB, CDKN2B*, és *SAMD3* további vizsgálata megerősítette a MeHgCl koncentrációfüggő hatását a neurális differenciálódás alatt álló hESC sejteken.

Összegezve a MeHgCl kezelés módosította a neurális differenciálódás alatt álló hECS sejtek sejtciklusát a cyclin D1 stimulálásával a *RELN* magas expressziós szintje révén és a CDK4/6 inhibitor *CDKN2B* gátlása következtében, a poly-comb fehérjék közé tartozó PRC1/2 vagy a TGFβ útvonalon keresztül. Ez a sejtek állandó sejtciklusba való belépését okozta, megakadályozva ezzel a neurális prekurzor sejtek további érését es differenciálódását.

Általánosságban elmondhatjuk, hogy vizsgálataink igazolták, hogy jól leírt standard eljárások és szigorú minőségi szabványok bevezetése támogathat minket a differenciációs protokollok instabilitása révén előrelátható kihívások legyőzésében. E munkában egy olyan őssejtes modell kifejlesztését írtuk le, amely alapjául szolgálhat emberi fejlődési rendellenességeket okozó toxikus anyagok azonosítására alkalmas prediktív *in vitro* eljárásoknak. Tudomásunk szerint az itt közölt kísérletsorozat az első olyan munka, amely neurálisan differenciálódó hECS sejteket kombinált “omics” technikàval abból a célból, hogy fényt derítsen a MeHgCl hatásmechanizmusára. Miután sejtes rendszerünket megfelelően érzékenynek találtuk az mRNS szinteknek qPCR-rel még alacsony MeHgCl koncentrációknál is mérhető változásai alapján, a sejteket további transzkriptomikai elemzésnek vetettük alá. Azonosítottunk továbbá olyan lehetséges biomarkereket, amelyek a MeHgCl toxicitásnak a neuronális prekurzor indukcióval való összefüggésére utalnak. Elsőként találtunk összefüggést a MeHgCl emberi idegrendszer fejlődésére gyakorolt *in vitro* mérhető molekuláris hatásai, melyek az *RELN* upregulációjához és *CDKN2B* downregulációjához köthetők és az *in vivo* tapasztalt hatások között, mint a neurális migráció és sejt ciklus zavarai.

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