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

Summary of PhD thesis

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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 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. Possible developmental toxicants are mostly only suspected to be toxic in humans since the variations among species are high and the extrapolation to humans is difficult since the underlying mode of actions are often unknown.

It could lead for 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.

Most developmental toxicants produce abnormalities only during certain critical

periods of gestation. In line, some stages of development are more susceptible to toxicants than others. 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. It should be considered that some agents that are harmful to developmental 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.

1.3 Human embryonic stem cells

The first human embryonic stem cell (hESC) lines were isolated in 1998 by Thomson et al. They have been isolated either from the morula or from the inner cell mass of the blastocyst stage embryos between day 5/6 development. In human embryonic development the morula cleavage stage occurs 3 days after fertilization. At day 4/5 of gestation, the blastocyst consists of approximately 58 to 250 cells and pre-implantation is initiated. 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. Human ESCs isolated from pre-implantation blastocysts are derived from the inner cell mass cells.

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. 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.

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. 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. 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. 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.

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. Protocols have been established for the differentiation of hESCs into neural precursor cells as well as several differentiated cells such as motoneurons, glia cells, dopaminergic neurons, and cerebellar cells. 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. 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. Grafting of hESC derived dopaminergic cultures into 6-hydroxydopamine-lesioned parkinsonian rats can induce restitution of motor function, although a beneficial effect is not always observed. Thus, hESC derived neuronal cells seem to exhibit functionality *in vivo*.

1.5 ATP binding cassette transporters

ATP Binding Cassette (ABC) transporters form a special family of membrane proteins, characterized by homologous ATP-binding, and large, multispanning transmembrane domains. They can be found in every known organism, such as from microbes to humans. 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. 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. Several members of this family are primary active transporters, which significantly modulate the absorption, metabolism, cellular

affectivity and toxicity of pharmacological agents.

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

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 characterized by two identical halves, each with one NBD; exporting a wide range of diverse substrates.

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. 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.

It was already demonstrated that both ABCB1 and ABCG2 transporters are highly expressed in stem cells derived from tissues, such as brain, bone marrow, pancreas, and liver. In human neural progenitor cells the NESTIN positive precursors are also expressing the ABCB1 transporter. 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. 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.

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 characterization [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 characterization 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. Cells were expanded weekly by microdissection and further propagated on a feeder layer. The undifferentiated cell cultures were monitored daily by microscopical inspection, quantitative real-time PCR (qPCR), immunofluorescence and flow cytometry analysis.

The experiments presented were approved by two ethical panels, one from the European Commission and a second from Cremona, Italy.

3.2 Early embryonic neural differentiation

For the differentiation towards early neuroepithelial precursors a protocol which was described earlier 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. Cultures were kept for up to 10 days with medium changes every third day.

3.3 Chemicals

A training set of well established xenobiotics: 5-fluorouracil, 6-aminonicotinamide, all-trans retinoic acid, methotrexate, methylmercury chloride, valproic acid and warfarin was used to expose to the neural differentiating hESCs.

All chemicals were purchased from commercial sources. Chemical exposure begun 24 hours after plating of the embryoid bodies 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. The effects of the different chemicals on the neural differentiating cells were detected by resazurin assay and by qPCR analysis. Relative quantification was performed for each concentration of chemical by the ΔΔCt method ($2^{-\Delta\Delta Ct}$ formula) using the expression profile of the corresponding untreated control as reference.

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, and purified with Qiagen RNeasy mini kits (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The biological samples were taken for transcriptional profiling with the RNA quality indicator number higher than 8. 100 ng total RNA was taken as a starting material and amplified for 16 h with Genechip 3' IVT Express Kit as per the manufacturer's instructions. 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 normalization were done with Robust Multi-array analysis. The entire dataset was normalized with quantile normalization

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 for determining differentially expressed transcripts using cut off values of 5% error rate. 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. Besides the above mentioned conditions, fold change value with the threshold value ≥ ± 2.0 were used to filter the significantly expressed transcripts. 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.

3.11 Gene otology 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 AND DISCUSSION

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 cell-model;
ii) the definition of the time point for the optimal readout;

iii) challenging the cellular model with well known reference compounds.

4.1 The development of quality standards ensuring the robustness of the cell-model

The definition of the original pluripotent cellular material is one of the most relevant parameters for a robust test system. In order to define quality standards undifferentiated H9 cell cultures were monitored daily by microscopical inspection; the expression level of *OCT4* and *NANOG*, the frequently used markers pointing to the pluripotency status of stem cells, were analysed by qPCR and immunohistochemistry; and changes in the population's composition throughout the maintenance of the undifferentiated cell culture were detected by flow cytometry.

- Immunofluorescence analysis showed that undifferentiated H9 cells were expressing antigens for the pluripotency marker OCT4.
- Flow cytometry analysis demonstrating a high percentage of SSEA-4 expression and a low expression of SSEA-1 profile.
- High level of *OCT4* and *NANOG* expression was showed by qPCR analysis, while the lineage markers, such as *MAP2*, *MYH6*, *AFP* were absent.

According to our data a threshold was defined that has to be met in order to use cells for further neural differentiation.

4.2 The definition of the time point for the optimal readout

From the variety of differentiation protocols reporting on the successful differentiation into neuronal cell types, a protocol was selected 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 monitored by qPCR and immunofluorescence analysis.

- The neural differentiating hESCs expressed highly the neural β-TUBULIN III and NESTIN indicating their commitment into the neural differentiation.
- The pluripotency markers *NANOG* and *OCT4* showed a significant downregulation within 10 days of differentiation as compared to undifferentiated hESCs with qPCR analysis.
- Consistent expression of the neural related genes *PAX6*, *NESTIN*, *MAP2*, *OTX2*, *NCAM1*, and *NEFM* were determined by qPCR over time.
- Consistent expression of markers of the two tested ABC transporters: *ABCB1* and *ABCG2*.

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 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 toxicants known to interact with the embryonic development *in vivo*: 5-fluorouracil, 6-aminonicotinamide, methotrexate, retinoic acid, valproic acid and warfarin as well as the negative control substance D-mannitol.

- Cytotoxicity curves, in order to determine the effective chemical-dosage range, 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.
- In order to provide information on specific effects caused by the different toxcants on the neural precursors, selected relevant marker panel was analysed by qPCR.
- 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.

- 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, as it was expected from a negative control.

The presented data confirm our hypothesis that differentiating hESCs are able to detect specifically the hazard of xenobiotics even at non-cytotoxic concentrations, demonstrating the sensitivity of the cellular model.

4.4 Elucidation of the mode of action of methylmercury chloride

The environmental toxicant methylmercury chloride (MeHgCl), known to cause structural developmental abnormalities in the brain, was used as reference compound to develop a concept contributing to a mechanistic understanding of the toxicity of an investigated substance.

- 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 analysed by qPCR.
- Moreover, *ABCB1* was upregulated in a concentration-dependent manner, while *ABCG2* was downregulated.

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 order to enlighten the underlying mode of action, we performed to scan the expression changes in the complete human genome of MeHgCl-treated 10 days neural differentiated hESCs 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.
- The revealed pathways affected by MeHgCl treatment which remarkably corresponded to *in vivo* studies, were calcium signalling pathway, cell adhesion molecules, cell cycle, pathways in cancer, p53 and Wnt signalling pathway.
- Further validation of the 2 most upregulated and of the 2 most downregulated genes *RELN*, *NPPB*, *CDKN2B*, and *SAMD3* 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, 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* was 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. *RELN* was the most upregulated gene by MeHgCl treatment.
- 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* playing important role in the negative regulation of cell cycle as it encodes a cyclin-dependent kinase inhibitor, the p15lNK4B protein, was significantly downregulated by MeHgCl treatment.
- *SAMD3* one of the most sensitive genes upregulated by MeHgCl treatment, was belonging to the regulation of mitosis and cell cycle and also to the development of microtubule and cytoskeleton.

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. It caused cell cycle re-entry of the neuronal precursor cells and by that preventing their further maturation.

5. SUMMARY

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.

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II. Glavinas H, von Richter O, **Vojnits K**, Mehn D, Wilhelm I, Nagy T, Janossy J, Krizbai I, Couraud P, Krajcsi P.: Calcein assay: a high-throughput method to assess P-gp inhibition. Xenobiotica 41(8):712-9. (2011). IF: 2.707

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IV. **Vojnits K**, Ensenat-Waser R, Gaspar JA, Meganathan K, Jagtap S, Hescheler J, Sachinidis A and Bremer S: A transcriptomics study to elucidate the toxicological mechanism of methylmercury chloride in a human stem cell based in vitro test. Current Medicinal Chemistry. In press.

V. Klaric M, Winkler J, **Vojnits K**, Meganathan K, Jagtap S, Ensenat-Waser R, Hescheler J, Sachinidis A and Bremer-Hoffmann S: Current status of human pluripotent stem cell based *in vitro* toxicity. Frontiers of Bioscience Landmark. In press.

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XIV. **Vojnits K,** Ensenat-Waser R, Klaric M and Bremer-Hoffmann S: A human embryonic stem cell approach for toxicity assessment in human early neural development/neurulation. 8th World Congress on Alternatives & Animal Use in the Life Sciences (WC8), Montreal, Canada. (2011)