Neurotoxicity of Active Compounds – Establishment of hESC-Lines and Proteomics Technologies for Human Embryo- and Neurotoxicity Screening and Biomarker Identification

Martina Klemm and André Schrattenholz
ProteoSys AG, D-Mainz

Summary
Pharmaceutical and chemical industries are facing new challenges for hazard and risk assessment from regulatory agencies. Especially for potential embryotoxicity of active compounds, conclusions from animal testing remain problematic due to numerous species-specific effects. Developmental toxicity screening preferentially should be performed with human material.
Appropriate models are scarce or missing, and the development of a human in vitro model for the molecular characterisation of embryotoxic effects appears to be highly desirable. The outstanding advantages of a human embryonic stem cell (hESC) based in vitro screening model for embryonic neurotoxicity become clear from corresponding results from a murine ESC-screening system. This in vitro test system is based on neuronal differentiated murine embryonic stem cells and quantitative differential proteomic display techniques to identify biomarkers for neurotoxicity. Results are superior to those of conventional array technologies (nucleic acids), because the proteomic analysis covers posttranslational modifications.
Under the new strict guidelines for stem cell importation of the German Ministry of Health and a Central Ethics Commission for Stem Cell Research, it is now possible for the first time to exploit the outstanding features of human embryonic stem cells to establish an innovative screening method for embryo- and neurotoxicity and to identify toxicity biomarkers without using animal-based in vitro or in vivo systems.

Zusammenfassung: Neurotoxizität von Arzneimitteln – embryonale Stammzell-Modelle zur Identifikation diagnostischer Biomarker für humane Embryo- und Neurotoxizität
Im Rahmen der Arzneimittelsicherheit müssen pharmakologisch aktive Substanzen hinsichtlich ihres potenziellen embryotoxischen und teratogenen Gefahrenpotenzials überprüft werden. Da zahlreiche Chemikalien und Wirkstoffe gerade im Bereich der Embryotoxizität spezies-spezifisch wirken, erweist sich die Verwendung menschlicher Zellen zur Identifizierung humaner Entwicklungstoxizität als zwingend notwendig.
Die aussergewöhnlichen Vorzüge eines auf humanen embryonalen Stammzellen (ES) basierenden in vitro screening Modells zur embryonalen Neurotoxizität wird aus den dargelegten Resultaten eines murinen ES-Zell-Screening Systems ersichtlich. Dieses in vitro Testsystem zur Identifikation diagnostischer Neurotoxizitätsmarker basiert auf neuronal differenzierten murinen embryonalen Stammzellen und quantitativer differentieller Proteinmusteranalyse. Das proteomische Verfahren ist den rein nukleinsäurebasierten Testsystemen an Aussagekraft weit überlegen, da posttranslationale Modifikationen erfasst werden.
Durch die neuen gesetzlichen Regelungen (StZG) zu Import und Umgang mit humanen embryonalen Stammzellen (hES Zellen) ergibt sich jetzt auch in Deutschland erstmals die Möglichkeit diagnostische Toxizitätsbiomarker ohne in vitro und in vivo Tierversuche unter Einsatz einer innovativen Screening-Methode für Embryo- und Neurotoxizität zu identifizieren.

Keywords: human embryonic stem (ES) cells, embryotoxicity, neurotoxicity, quantitative differential proteomics, biomarker

1 Introduction

Medical research is dedicated to the continuous improvement of risk assessment for health protection. In the European Commission's regulatory framework of drug approval for treatment of patients, a certain set of standardised animal tests is performed according to the European Council directives (EC 1999 and 2001) and the guidelines of the International Conference on Harmonisation (ICH, 1993). The hierarchical decision-tree approaches defined by the European Commission (EC, 1989) and the Organisation for Economic Cooperation and Development (OECD, 1982 and 1996) for international standards of pharmacological and toxicological tests primarily address the adult organism. Currently comprehensive multigeneration studies are undertaken to provide information on all aspects of developmental toxicity. Developmental studies to address the specific risks of the developing embryo are not regulatory requirements and

Received 27 November 2003; received in final form and accepted for publication 12 February 2004

appropriate methods for the screening of toxic substance effects during embryonic development, especially on the part of developmental neurotoxicity, are essentially lacking.

The interpretation for human embryotoxicity remains problematic. There have been cases of toxic side effects with pathological consequences during development, which were only discovered after approval of the drug for therapeutic application in patients (dire case of Contergan). More than thirty compounds with highly toxic potential had to be withdrawn from the market by the FDA (Food and Drug Administration) between 1998 and 2001. In each case this toxicity was not detected in the required set of prior animal tests. One of the most recent examples is Lipobay (A. P. Li, Phase 1 Molecular Toxicology, Inc., Santa Fe, New Mexico). Among these 30, eight drugs had substantial side effects especially in women. Current animal tests are simply not sufficient to detect gender-specific predispositions in humans (Dr. K. Olden, Director of NIEHS). In the time after Contergan, only about 15% of those cases of drugs with highly toxic potential could be predicted safely by animal tests (Heywood, 1990 und Parke, 1994).

With the establishment of human embryonic stem cells, there now exists for the first time the chance to develop innovative *in vitro* analytical methods for early detection of human-specific embryotoxic risks of compounds and chemicals. The combination of hESC-based *in vitro* test models with cutting edge molecular analysis holds the promise to deliver with extreme sensitivity highly relevant functional and molecular marker end points for the early detection of embryotoxic effects of drugs or chemicals. In terms of relevance, proteomics is in principle far superior to RNA/DNA-based array technologies, because the relatively static and small human genome of approx. 30,000 genes is translated into a highly dynamic and complex proteome of several million protein molecules of posttranslationally modified isoforms. It is on this latter level where dynamic functional aspects of cellular events take place (Schrattenholz, 2001; Kitano, 2001; Lowe and Marth, 2003). Neuro- and embryotoxic substances bind to proteins, changing interactions in complex functional networks, sometimes on very fast time scales (e.g. phosphorylation), and the changes induced by this type of intervention are ideally analysed by proteomics technologies. Emerging molecular signatures are derived from functionally controlled hES cells differentiating towards mature neurons, key biomarker proteins are reliably identified and quantified, thus enabling very early understanding of side effects and innovative strategies for acceleration in drug development, validation and toxicity testing. After identification of developmental neurotoxicity markers on protein basis, affinity molecule-based protein chips for common readers can be developed for high throughput screening assays in industry.

2 Analysis of developmental neurotoxic substances – human embryonic or adult stem cells?

Stem cells have basically two characteristic properties: they can be maintained undifferentiated in culture for long periods of time, and they can be induced to specific organotypic differentiation. There exist embryonic and adult tissue-specific stem cells.

Embryonic stem cells are established from the inner cell mass (ICM) of 5-7 day old blastocysts (Evans and Kaufman, 1981; Martin, 1981) (Fig. 1).

For long-term cultivation of undifferentiated cells, embryonic stem cell cultures are maintained in co-culture with so-called "feeder" cells, i.e. mouse fibroblasts (Robertson, 1987) or in cell-free conditioned media (Smith and Hooper, 1987). The major inhibitory factor for differentiation in these cell culture systems is the cytokine LIF (Leukemia Inhibitory Factor) (Nichols et al., 1990). Embryonic stem cells display far-reaching pluripotent properties, i.e. they can differentiate into virtually every organ-specific human cell type, when cultured as embryoid bodies. Differentiation into diverse cell types of endodermal (e.g. pancreatic and hepatic cells), mesodermal (e.g. bones, muscle and blood cells, cardiomyocytes) or ectodermal origin (e.g. neurons and glial cells) is induced

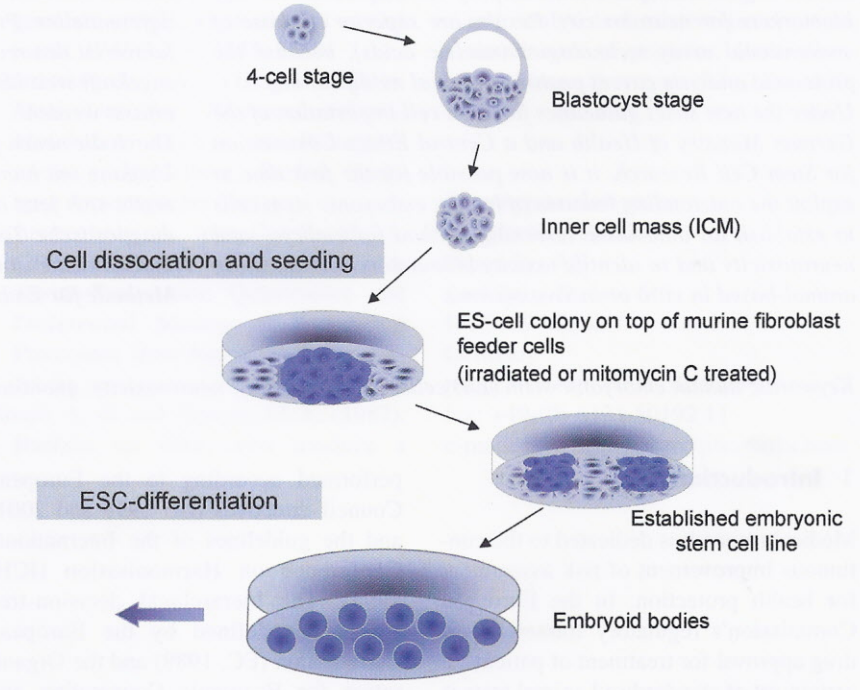


Fig. 1: Establishment of human and murine embryonic stem cells.

by LIF deprivation and the respective combination of growth factors.

Tissue- or organ-specific stem cells from adult tissues such as epidermis, hair, intestine, liver, haematopoietic system, brain or bone marrow have limited possibilities of proliferation and differentiation ("developmental restriction"). Due to their multipotent properties, these stem cells are mostly restricted to one tissue only. Plasticity of the adult stem cells, which means the ability to differentiate into cell types characteristic of another organ, was only demonstrated for haematopoietic stem cells, stromal cells of the bone marrow and multipotent adult precursors, which can be generated *in vitro* from certain cells of the bone marrow and certain neural cells (NIH stem cell report, 2001). These adult stem cells appear to have the capability to differentiate into tissues other than those from which they originated, namely

1) blood and bone marrow (unpurified haematopoietic) stem cells differentiate into the 3 major types of brain cells (neurons, oligodendrocytes, and astrocytes), skeletal muscle cells, cardiac muscle cells, and liver cells;

2) bone marrow (stromal) cells differentiate into cardiac muscle cells, skeletal muscle cells, fat, bone, and cartilage; and

3) brain stem cells differentiate into blood cells and skeletal muscle cells.

The establishment of a screening method for early developmental processes related to human embryonic neurotoxicity is feasible with embryonic but not with adult stem cells. Adult stem cells are derived from mature organs of adult individuals and a profiling for early embryonic differentiation and corresponding analysis of toxic effects is simply not feasible. The question, whether adult stem cells can be artificially re-differentiated is currently under intense investigation, but remains open for the time being. A further disadvantage of adult stem cells is their limited ability to differentiate and proliferate, which goes together with decreased lifetime *in vitro*. Moreover, only very small numbers of these cells are available (decreasing further with age), they are difficult to distinguish from surrounding cells and their derivation is difficult or impossible, depending on the organ. To date it remains unclear how adult stem cells function and how their potential could best be exploited (G. Q. Daley, chairman of the Whitehead Institute Task Force on Genetics and Public Policy).

In consequence, results with direct relevance for human developmental toxicity can only be obtained by human embryonic stem cell approaches. Only such models can provide information on the effects of compounds on all aspects of embryonic neuronal development.

3 Legal framework in Germany for import of human embryonic stem cells and strictly defined use

Import and use of human embryonic stem cells is generally prohibited in Germany under a so-called "stem cell law" (Stammzellgesetz, StZG, § 4, 1) (see Table 1, according to Commission staff working paper, Brüssel, 03.04.2003)

Permission can only be granted in exceptional cases and after a thorough review procedure by the responsible regulatory authority (Robert Koch Institut, RKI) in close agreement with a Central Ethics Commission. The following conditions have to be fulfilled: i) Stem cells must have been produced prior to the 1st of January 2002, according to the legal requirements of the country in which they originate; ii) embryos were generated for reproductive purposes and subsequently abandoned; iii) embryos were submitted to stem cell derivation without any financial connotations or financial advantage to the donors. Moreover, projects involving hESC are only permitted for basic research (§5) and under condition of no available scientific alternatives (§5).

On the 9th of September, 2003, ProteoSys AG, a biotechnology company in Mainz, obtained permission from RKI

	AT	BE	DK	DE	ES	FI	FR	GR	IE	IT	LU	NL	PT	SE	UK
Allowance: Acquisition of human embryonic stem cells from supernumerary embryos by law						x		x				x		x	x
Prohibition: Acquisition of human embryonic stem cells from supernumerary embryos **Allowance:** Importation of human embryonic stem cell lines (StZG)	x		x	x x											
Prohibition: Acquisition of human embryonic stem cells from supernumerary embryos					x		x		x						
No specific legislation regarding human embryo research		x								x	x		x		
Allowance: Derivation of human embryos for stem cell procurement by law															x
Prohibition: Derivation of human embryos for research purposes and for the acquisition of stem cells by law or by ratification of the convention of the council of Europe on human rights and biomedicine signed in Oviedo on 4 April 1997	x		x	x	x	x	x	x	x			x	x	x	

Tab. 1: Human embryonic stem cell research: regulations in EU member states (Commission staff working paper, Brüssel, 3. April 2003).

and the Central Ethics Commission to import hESC for a basic research project with the following topic: Development of an *in vitro* system for the analysis of neurotoxic effects of a selected set of compounds using differentiating human embryonic stem cells as a model.

4 Biomarker discovery in neuronal embryotoxicity: proteosys' approach

4.1 Objectives

A clear understanding of neurotoxic effects of medical drugs on human embryos during pregnancy would constitute a major contribution towards improved drug safety and preventive health care. The effects of neurotoxic substance application can be defined on the level of distinct molecular consequences in terms of immediate protein expression changes. Advanced proteomics technologies in correlation with synchronised functional/physiological measurements can provide a comprehensive, precise and quantitative molecular pattern analysis of the underlying mechanisms. The reduction of the enormous complexity of proteomic data, with exact quantification of differentially expressed proteins, helps to obtain meaningful interpretations of relevant events within complex and highly dynamic molecular interactions. Moreover the availability of human biomarkers for neuronal embryotoxicity could drastically improve early screening procedures for embryo- and neurotoxic substances.

Therefore, the main objective of ProteoSys' project is the identification of molecular mechanisms underlying human neurotoxicity of 20 selected substances. The corresponding events take place in early embryonic and specifically neural developmental stages and maturation processes, thus an in vitro test system based on hESC is ideally suited for functional and molecular analysis. Cells will be exposed to various concentrations of toxic compounds and analysed on the molecular level at various endpoints, representing precursors, early and mature stages of neurons. The differential proteomic analysis compares protein expression of hESC after substance application with untreated controls. The quantitative and reliably differential Proteomics technology applied by ProteoSys will generate molecular signatures of toxic conditions based on human biomarkers for embryonic neurotoxicity (specific post-translational modifications of proteins).

4.2 Proteomics technology platforms

4.2.1 Functional control

Proteins are multifunctional and highly dynamic modules. Because several million protein isoforms and modifications are generated from approx. 30,000 human genes, the necessity of rigorous pattern control of actual protein expression in a cellular system is evident. ProteoSys has established procedures for a correlational analysis of ionic currents, concentration changes of intra- and extracellular small molecules (like neurotransmitters), as well as physicochemical parameters (like temperature), which set the context and functionally define the meaning of protein changes.

4.2.2 Quantitative and differential protein detection

Proteins exist in a linear dynamic range over more than eight orders of magnitude. Quantitative display of protein patterns is achieved by integrated analytical platforms applying isotopic techniques at three levels of sensitivity. On the first level, which is fully automated, proteins are differentially detected, quantified and subsequently identified down to a level of approx. 1 femtomol (thousands of identifications per week). Detection of proteins down to a range of 50 to 100 atto moles is equally fast, but current limitations of mass spectrometry necessitate non-automated enrichment procedures for the identification. Very scarce proteins (approx. 1 to 50 atto moles) need special radioactive tracer methods, applying the proprietary MPD technology (Multi Photon Detection). This ultrasensitive method is based on protein labelling with so-called "electron capture"-isotopes (e.g. 125-I, 131-I), which on decay emit coincident events, which enable detection without background limitations.

The following chart gives an example from cancer research of quantitative differential protein pattern display as achieved by labelling samples with radioactive isotopes (Fig. 2):

4.2.3 Complexity reduction by fractionation

Affinity-based fractionations of phosphorylated, glycosylated and other functionally related posttranslational modifications have become important tools of system biology. These subproteomes constitute fractions (0.5 to 30%) of total protein content of samples and thus focus on crucial aspects of fast cellular signalling. Together with the mentioned display technologies and highly automated, reproducible procedures with reasonable throughput, we have established a flexible analytical basis for comprehensive molecular characterisation of mode of action and side effects.

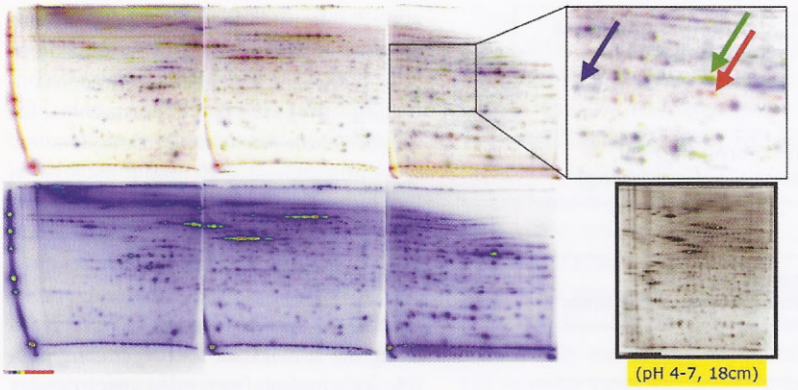
A further, even more focussed approach, called chemical proteomics, uses immobilised ligands for specific affinity capture of membrane receptors and interacting proteins from cellular compartments otherwise considered to be difficult in analytical terms.

The corresponding suite of proprietary protein analytical technologies and know-how enables ProteoSys AG to perform the comprehensive molecular description of the embryonic stem cell models, with the ultimate goal of obtaining signatures of biomarkers for developmental neurotoxicity.

5 "System biology and toxicoproteomics" using a human *in vitro* ES-cell screening system

In detail the research project granted is composed of two parts (Fig. 3).

In the first part of the project human embryonic stem cells will be cultivated in vitro and differentiated to functional neurons according to published procedures (Thomson et al., 1998; Itskovitz-Eldor et al., 2000; Pera et al., 2000; Richards et al., 2002). Undifferentiated hESC's will be characterised by surface antigens like SSEA-3, SSEA-4 (Solter and Damjanow, 1979), TRA-1-60, TRA-1-81, Oct-4 (Schöler et al., 1989) and GCTM-2 or by enzyme activities (alka-



S1	S2	S3
0.53 ±0.05	0.20 ±0.08	0.26 ±0.03
0.16 ±0.07	0.42 ±0.04	0.42 ±0.11
0.49 ±0.01	0.21 ±0.00	0.29 ±0.01
0.04 ±0.04	0.35 ±0.05	0.61 ±0.09
0.20 ±0.03	0.35 ±0.03	0.45 ±0.00
0.16 ±0.00	0.28 ±0.02	0.56 ±0.02
0.55 ±0.03	0.17 ±0.02	0.29 ±0.02
0.03 ±0.03	0.76 ±0.08	0.21 ±0.05
0.00 ±0.00	0.33 ±0.00	0.67 ±0.00
0.03 ±0.03	0.34 ±0.06	0.62 ±0.02
0.18 ±0.01	0.33 ±0.01	0.49 ±0.03
0.09 ±0.05	0.55 ±0.04	0.36 ±0.01
0.09 ±0.01	0.57 ±0.00	0.34 ±0.00
0.21 ±0.01	0.25 ±0.01	0.54 ±0.01
0.56 ±0.05	0.31 ±0.05	0.13 ±0.01
0.42 ±0.02	0.20 ±0.01	0.38 ±0.02
0.48 ±0.04	0.25 ±0.02	0.27 ±0.02
0.62 ±0.05	0.18 ±0.02	0.19 ±0.04
0.59 ±0.02	0.26 ±0.06	0.16 ±0.08
0.44 ±0.02	0.17 ±0.03	0.39 ±0.02

Fig. 2: Quantitative differential pattern control of neuronal differentiation with undifferentiated murine stem cells, early and late neurons. Small aliquots of three different biological samples were labelled with three different radioactive iodine isotopes (125-I, 123-I and 131-I), mixed together and jointly analysed in one 2D gel. The radioactive labelling enables a very reliable and exact quantification and differential detection of even subtle changes of protein expression patterns. The table shows differential expression levels for selected spots. Selection is based on statistical significance calculated using analysis of variance (ANOVA).

line phosphatase). Neurons differentiated from precursors are characterised by morphological criteria and immunohistochemistry (antibodies for neuron-specific proteins MAP-2 and synaptophysin). Subsequently and more importantly, a functional and physiological control of neurons based on their response to various neuron-specific stimuli (neurotransmitter, depolarisation, etc.) is performed, e.g. by calcium-imaging.

Together with specific agonists and antagonists these measurements allow fairly precise pharmacological characterisation of the major ionotropic and metabotropic receptors (for glutamate, GABA, acetylcholine, etc.) and voltage-dependent ion channels. This fine-tuning of physiological responses of the cellular system is a prerequisite for defining appropriate amplitudes of conditions, which later form the basis for the subsequent molecular analysis.

The exposition to 20 selected toxic test substances will be investigated during the stage of hESC differentiation into neuronal cells, as well as after reaching postmitotic mature status. The results from functional measurements will define the endpoints for generation of samples submitted to differential proteomics technologies. The tight functional control of the biological material is one of the most crucial steps of complexity reduction.

In the second part of the project, neuro-embryotoxic and neurotoxic effects of test substances will be analysed on the molecular level (Fig. 4).

Dose-response relationships of toxic effects induced by substances are essentially quantified by the influence of these compounds on normal functional signals measured by calcium imaging. Even subtle influences of toxic compounds on intracellular transient calcium concentration changes due to normal reactions caused by neurotransmitters or other physiological parameters will become apparent on a statistically significant level.

Based on the functional analysis (project part 1), protein pellets will be generated from treated and untreated neuronal differentiated human stem cells at appropriate end points, and will subsequently be submitted to a quantitative and differential protein pattern analysis (Fig. 5).

Small aliquots of up to three different biological samples will be labelled with three different radioactive iodine isotopes (125-I, 123-I and 131-I), mixed together and jointly analysed in one 2D gel. This part is for pattern control only; the bulk of the three samples will be kept for later tracer-controlled preparative 2D gels for protein identification. The radioactive labelling enables a very reliable and exact quantification and differential detection of even subtle changes of protein expression patterns (see also Fig. 4). The quantification moreover is essential for the understanding of the sequence of molecular events in the given time frame of experiments and thus for the biological interpretation (Schrattenholz and Cahill, 2003; Cahill et al., 2003; Vogt et al., 2003).

Identification of differential protein biomarkers is performed by mass spectrometry, for very scarce proteins after tracer-controlled enrichment procedures. Bioinformatics at ProteoSys, has established effective data-mining procedures, screening real molecular data of identified proteins and their respective modifications (e.g. phosphorylations) against complementary data from literature files or additional experiments (e.g. from nucleic acid arrays). Eventually, refined molecular signatures for human embryonic neurotoxicity are generated, providing the content for second generation high-throughput screening devices (e.g. antibody arrays against specific phosphorylated protein fragments). As shown in Figure 6, a rigorous design of correlated experimental levels enables the recognition of relevant contents of functional

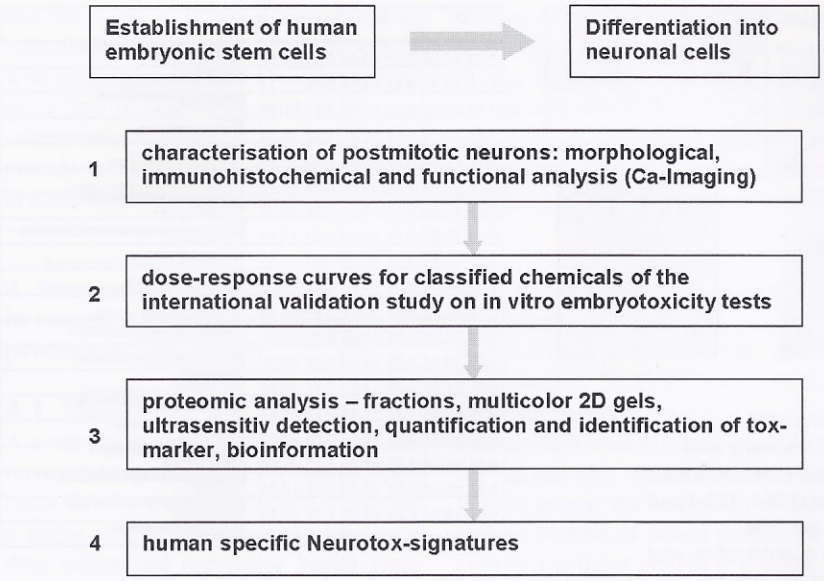


Fig. 3: System biology research approach. Project part 1 relates to *in vitro* culture and differentiation of hES-cells into mature neurons, and a corresponding correlational functional analysis; project part 2 covers the differential molecular analysis by proteomic techniques of dose-dependent consequences of exposure of cells to 20 selected toxic compounds in the human and murine *in vitro* ESC-based screening systems; final objective is the definition of human-specific embryonic neurotoxicity biomarkers.

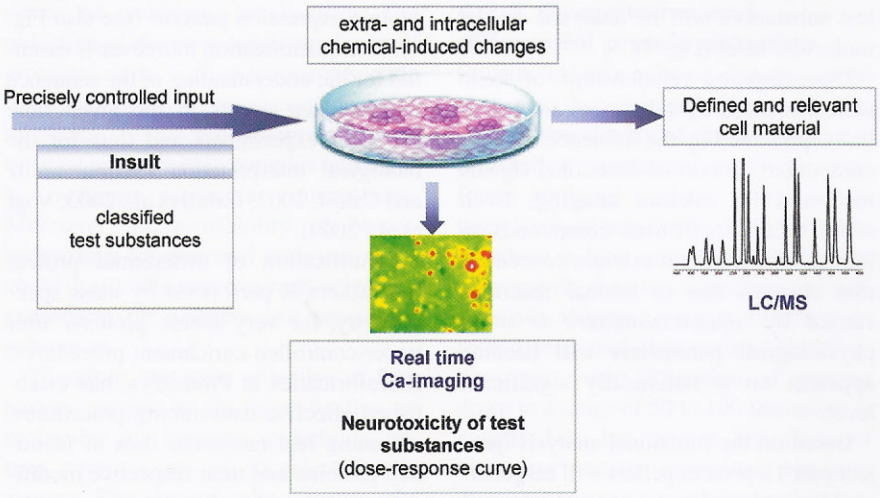


Fig. 4: Neurotoxicity of test substances. Functional correlational analysis, definition of physiological and/or pharmacological/toxic end points. The relevant cell status for effective neurotoxic concentrations of the test substances was functionally defined by the outcome of dose-response curves in calcium-signalling experiments.

proteinaceous information of innovative discovery and screening methods in the post-genome era, in the context of system biology.

The first step, the exact functional tuning of the respective model system, is also the most crucial step, because subsequent analytical methods are extremely powerful and do not show up previous mistakes. Well-controlled functional endpoints (and only they will allow, after molecular details become available, a correct interpretation of data) are submitted first to quantitative differential protein analysis, and then to tracer-controlled preparative experiments for identification or complete sequencing. Quantification of differential proteins is the basis for integrating related results from independent experiments and literature, generating focused hypotheses. In a second level of bioinformatics, iterative algorithms verify or falsify *in silico* leads using the actual data set (organising peptide information from mass spectrometry and positional, e.g. pI and molecular mass information form 2D gels).

6 Validation of the human *in vitro* ES-cell screening system for human-specific embryo- and neurotoxicity

In general, the selection of test substances is one of the critical points for validation studies. A substance can cause toxic effects in animals in high-dose, long-term studies, but this does not necessarily mean that it is a risk to human health. To reach our project goal, namely describing human-specific relevant signatures for a developmental neurotoxicity biomarker, we decided to select test chemicals from the ECVAM International Validation Study with good quality "segment II"- type in vivo data and/or human data as *in vivo* reference data and a murine based ESC-screening system for animal reference data. In this design, a distinction between human-specific embryotoxicity and general toxicity, and likewise discrimination between non-embryotoxic, weakly embryotoxic and strongly embryotoxic substances can be made. By means of this study critical issues concerning the interpretation of animal study data for human developmental toxicity (NIH stem cell report, 2001; Rosen, 2002), like specific ADME (Absorption, Distribution, Metabolism and Excretion of the substance) effects and combinatorial effects of biological, physical und chemical agents (lead, PCBs, pesticides, phthalates, endocrine disruptors) can be analysed. Comparative profiling of human and murine neuronal differentiated cell material, a key issue for human differential display toxicopro-

teomics and a better protection of health and safety of unborn human lives, can be accomplished.

We have already established a proprietary murine ESC-screening system based on neuronal differentiation and performed during *in vitro* cultivation, differentiation to functional neurons and functional-physiological tests. Based on the functional analysis of specific test substances, protein pellets are generated from treated and untreated neuronal differentiated murine stem cells at appropriate end point conditions, and the protein material is submitted to a quantitative and differential protein pattern analysis. The results clearly indicate the existence of murine molecular toxicity signatures (Sommer et al., 2003a and 2003b). Even with detection sensitivities of bulk methods for protein detection, neuro-specific proteins (stress-related proteins and protein isoforms) representing toxicity signatures were identified. To jointly analyse human and murine cell material in comparative proteomic analysis, the whole procedure must be developed and repeated with hESC-lines.

Now, for the first time, it seems possible to establish an innovative screening method for human-specific embryo- and neurotoxicity and to identify toxicity biomarkers without animal-based *in vitro* or *in vivo* systems by exploiting the outstanding features of human embryonic stem cells. Moreover, the various possible "organotypic" derivatives of embryonic stem cells (neural, cardiomyocyte, adipocyte, various muscle cell types) available today provide genetically homogeneous models for future, more comprehensive experiments, perfectly suited for innovative validation methods like RNAi.

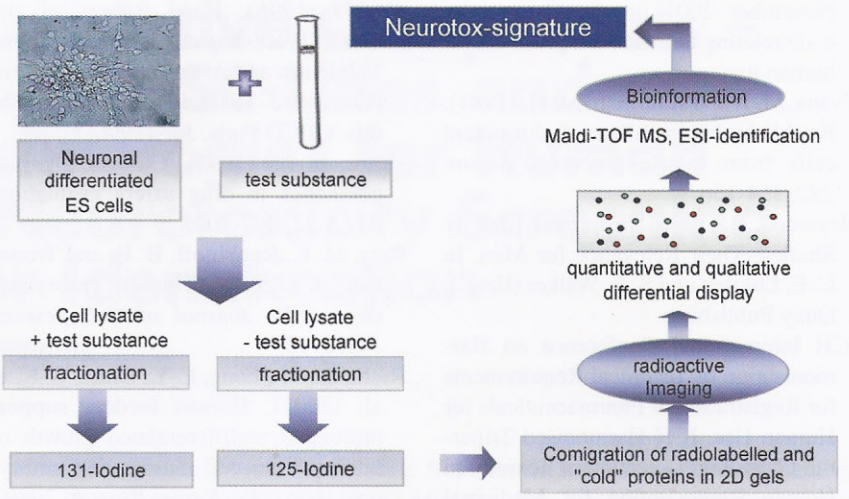


Fig. 5: Functional protein pattern comparison – "differential display". Samples from functionally verified stem cell cultures are prepared on a preparative scale. Subsequently they are differentially labelled by radioisotopic techniques, separated by 2-dimensional gel electrophoresis with immobilised pH gradients. Eventually they are fragmented and identified by automated peptide mass fingerprints via MALDI-TOF mass spectrometry.

Tab. 6: Sequence of steps in a system biology/proteomic study. ProteoSys has developed proprietary platforms for each of the stages (www.proteosys.com).

Sample	Defined state of differentiated hESC
Functional Control	Ca- or pH imaging, cytokine- or neurotransmitter release, morphological parameters
Sample preparation	Denaturation, inactivation of all protein activity, labelling with radioactive or stable isotope tracers
Pattern analysis	Two-dimensional gel electrophoresis, differential and quantitative protein detection
Protein identification	Mass spectrometry (MALDI-TOF, ESI-MS, MS/MS
Bioinformatics 1	Integration of external data from literature or independent experiments with actual quantitative protein expression; integrative project data base
Bioinformatics 2	Data Mining: hypothesis-driven structuring of project data base, focussed experimental verification

References

Cahill, M. A., Wozny, W., Schwall, G. et al. (2003). Analysis of relative isotopologue abundances for quantitative profiling of complex protein mixtures labelled with the acrylamide/D3-acrylamide alkylation tag system. *Rapid-Commun.- Mass-Spectrom. 17 (12)*, 1283-90.

Commission of European Communities, (2003). Commission staff working paper: Report on human embryonic stem cell research, Brussels.

EC Council Directive 89/341/EEC of 3 May 1989 amending Directives 65/65/EEC, 75/318/EEC and 75/319/EEC on the approximation of provisions laid down by law, regulation or administrative action relating to proprietary medicinal products.

EC Commission Directive 1999/83/EC of 8 September 1999 amending the Annex to Council Directive 75/318/EEC on the approximation of the laws of the Member States relating to analytical, pharmacotoxicological and clinical standards and protocols in respect of the testing of medicinal products.

EC Directive 2001/20/EC and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use.

EC Directive 2001/83/EC of the European parliament and of the council of 6

November 2001 on the Community code relating to medicinal products for human use.

Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotent cells from mouse embryos. *Nature 292*, 154-156.

Heywood, R. (1990). In Animal Toxicity Studies: Their Relevance for Man. In C. E. Lumley und S. R. Walker (Hrsg.), Quay Publishing.

ICH International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline: Detection of Toxicity to Human Reproduction for Medicinal Products. Recommended for Adoption at Step 4 of the ICH Process on 24 July 1993 by the ICH Steering Committee. ICH Secretariat, Geneva.

Itskovitz-Eldor, J. and Benvenisty, N. (2000). Differentiated human embryoid cells and a method for producing them. WO 00/70021.

Kitano, H. (Hrsg.) (2001). *Foundations of Systems Biology*. Boston: MIT Press.

Lowe, J. B. and Marth, J. D. (2003). A Genetic Approach to Mammalian Glycan Function. *Annu. Rev. Biochem. 72* (epub ahead of print).

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U S A. 78 (12)*, 7634-7638.

Nichols, J., Evans, E. P. and Smith, A. G. (1990). Establishment of germ-line competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development 110*, 1341-1348.

NIH stem cell report (2001). Internet: www.nih.gov/news/stemcell/scireport.htm

OECD (1982). OECD Guidelines for the testing of chemicals. OECD Paris.

OECD (1996). Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. OECD Paris, July 1996; 60 pp.

Parke, D. V. (1994). Clinical pharmacokinetics in drug safety evaluation. *ATLA 22*, 207-209.

Pera, M. F., Reubinoff, B. E. and Trounson, A. (2000). Human embryonic stem cells. *Journal of Cell Science 113*, 5-10.

Richards, M., Fong, C-Y., Chan, W-K. et al. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nature Biotech. 20 (9)*, 933-936.

Robertson, E. (Hrsg.) (1987). Embryo-derived stem cell lines. In E. Robertson (ed), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (71-112). Oxford: IRL Press.

Rosen, J. D. (2002). Acrylamide in food: Is it a real threat to public health? A position paper of the American council on Science and Health (acsh). http://www.acsh.org.

Schöler, H. R., Balling, R., Hatzopoulos, A. K. et al. (1989). A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. *EMBO J. 8*, 2543–2550.

Schrattenholz, A. (Hrsg.) (2001). *Methoden der Proteomforschung, Molekulare Analyse der Proteinexpression*. Heidelberg, Berlin: Spektrum Akademischer Verlag.

Schrattenholz, A. and Cahill, M. A. (2003). Potential of Comprehensive Toxico-Proteomics: Quantitative and Differential Mining of Functional Proteomes from Native Samples. *ATLA* (in press).

Smith, A. G. and Hooper, M. L. (1987). Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev. Biol. 121 (1)*,1-9.

Sommer, S., Klemm, M., Biefang-Arndt, K. et al. (2003a). Differential proteomics analysis of neuronal stress induced by homocysteic acid: phosphorylated and methylated isoforms of chaperones as molecular signature of homocysteic acid -induced excitotoxicity. ENS abstract, accepted for publication in European Journal of Neurology.

Sommer, S., Klemm, M., Biefang-Arndt, K. et al. (2003b). The molecular signature of homocysteic acid-induced neuronal stress. Submitted to publication.

Solter, D. and Damjanov, I. (1979). Teratocarcinoma and the expression of oncodevelopmental genes. *Methods in Cancer Research 18*, 277-332.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S. et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science 282 (5391)*, 1145-1147.

Vogt, J. A., Schroer, K., Hölzer, K. et al. (2003). Protein abundance quantification in embryonic stem cells using incomplete metabolic labelling with ^{15}N amino acids, MALDI-TOFMS, and analysis of relative isotopologue abundances of peptides. *Rapid-Commun.-Mass-Spectrom. 17 (12)*, 1273-82.

Correspondence to
Dr. Martina Klemm
ProteoSys AG
Carl-Zeiss-Strasse 51
D-55129 Mainz
Germany
phone: +49-(0)-6131 50192 34
fax: +49-(0)-6131 50192 11
e-mail: martina.klemm@proteosys.com