Stem cells are of increasing scientific interest because of their potential use in a number of biomedical and research applications. Stem cells are inherently self-renewing and as a consequence can be cultured in an undifferentiated state or programmed to give rise to more specialised cells of representative of cell types found in specific human tissues or organs. These properties allow stem cell approaches to be used to investigate an array of diseases, and identify novel therapeutic targets for drug discovery programs. Equally, stem cell models and their differentiated progeny promise to significantly improve in vitro toxicity assessment. Indeed, an embryonic stem cell test for developmental toxicity has already been endorsed by ECVAM Scientific Advisory Committee. Other systems which rely on programmed stem cell culture to generate complex 3D tissue equivalents for the assessment of properties such as barrier function are also on the horizon. This presentation provides an overview of the use of stem cell technology to generate in vitro models of toxicity. Specific emphasis is placed the use of stem cell models as alternatives to testing in animals with regards their use in models of barrier function, hepatotoxicity and genotoxicity and in reproductive toxicology screens.
Poster

Establishing predictive molecular markers of differentiation as toxicological endpoints in the Embryonic Stem Cell Test (EST)

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In the field of reproductive toxicity mandatory test guidelines require in vivo experiments for the detection of the embryotoxic potential of chemicals and drugs. A promising alternative method for these purposes has been provided with the Embryonic Stem Cell Test (EST). This assay is based on the capacity of murine embryonic stem cells (ES cells) to differentiate in vitro into a variety of cell types. The EST is able to assess the embryotoxic potential of chemicals by the evaluation of inhibitory effects on differentiation of contracting myocardial cells which can be detected by microscopical analysis. Using a biostatistical prediction model (PM) the assay passed an international validation study and was able to predict the embryotoxic potential of test chemicals with an accuracy of 78%.

A joint project was carried out by ZEBET and German pharmaceutical companies to improve the EST by establishing molecular endpoints of differentiation. Cardiac-specific gene expression has been studied at protein and RNA levels by flow cytometry and real-time-PCR under the influence of 10 chemicals with different embryotoxic potentials. The results obtained using cardiac-specific molecular endpoints were comparable to the validated microscopic analysis of beating areas and led to the same predictive outcome. The data clearly demonstrated that the selected molecular markers provide objective endpoints of early embryonic differentiation and are able to predict developmental toxicity in vivo from in vitro data for reference compounds.

In conclusion, a modified EST holds promise to be a new predictive screening system for hazard assessment with regard to developmental toxicity.

The first two authors contribute equally.

Lecture

Human Umbilical Cord Blood Neural Stem Cell Line (HUCB-NSC) – implementation for studying developmental neurotoxicity

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Previously we have established non-immortalised, stable HUCB-NSC line with potential to differentiate into neuron, astrocyte and oligodendrocyte-like cells. In this report we are introducing HUCB-NSC as a model system for neurotoxicity testing. Standardised conditions for the growth and differentiation of HUCB-NSC line were established in multi-well plate format culture. The influence of selected growth factors and neuromorphogenes on cells differentiation and growth rate/survival has been tested in parallel.

Cells were incubated in the presence of twelve different combinations of growth factors and neuromorphogenes for 2 or 3 weeks at different plating densities. Their growth rate and survival was estimated by the MTT test and Live/Dead, Viability/Cytotoxicity Kit assays, as well as their differentiating potential by the quantified immunocytochemical expression of neural specific markers.

Under the standardised conditions the most potent for differentiation were: 1) CNTF and dBcAMP for neuron-like cells (~80% β-Tubulin III cells), 2) combination of PDGF-BB and RA for astrocyte-like cells (~60% S100-β² cells) and 3) PDGF-AA followed by T3 for oligodendrocyte-like cells (~10% of Gal-C⁺ cells). In addition, CNTF promoted, whereas dBcAMP and RA significantly depleted the HUCB-NSC number.

The availability of the HUCB-NSC line in culture promises a detailed examination of the compounds that influence the dynamics of neural somatic stem cells expansion and differentiation and opens possibilities for neurotoxicity testing.

**Poster**

**Expansion of the Embryonic Stem Cell Test: Differentiation into neural cells**

*Katrin Hayess, Roland Buesen, Katharina Schlechter, Birgitta Slawik, Horst Spielmann and Andrea Seiler*

Federal Institute for Risk Assessment (BfR), National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

The Embryonic Stem Cell Test (EST) is a validated *in vitro* assay that has been established to classify compounds with respect to their embryotoxic potential. The current experimental procedure involves differentiation of murine embryonic stem cells (D3) into contracting cardiomyocytes. However, potentially embryotoxic drugs may effect primarily other tissues than myocard. Consequently, this consideration prompted us to expand the EST to other major target tissues.

Here, we present a protocol for differentiation of murine embryonic stem cells into neurons designed with special regard to the testing of chemicals. This modified protocol is based on a monolayer differentiation procedure and offers the advantage of a reproducible development of neural cells in a comparatively short time. The differentiation of D3 cells into neural cells was characterised by analysis of neuron-specific marker gene expression using flow cytometry. In addition, the developing neurons were examined by immunofluorescence staining using neuron-specific antibodies. As a result, we were able to define neuron-specific molecular endpoints for the detection of chemical effects on embryonic development.

The expansion of the EST to more than one target tissue will considerably improve the accuracy of this predictive screen by preventing false negative results.

**Poster**

**In vitro approaches to developmental toxicity**

*Helena Hurst, Richard Clothier and Margaret Pratten*

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This study compared the effects of potential teratogens/embryotoxins *in vitro* on chick heart micromass (MM) model and the differentiation of cardiomyocytes with the D3 stem cell systems. White Leghorn 5 days old embryo hearts were dissociated to produce a cardiomyocyte suspension in Dulbecco’s Modified Eagle’s Media (DMEM). D3 murine embryonic stem cells (ESC) were induced to form Embryoid Bodies (EB’s) using the hanging drop technique upon removal of LIF. Cultures were incubated at 37°C in CO₂ (5% v/v in air) and observations made every 24 hours over 5 days. Culture viability was assessed using the resazurin reduction and total protein via the kenacid blue assays.

All-trans-retinoic acid (tRA) and sodium valproate (VPA) were used as controls. tRA significantly (P<0.05) reduces cell activity and beating whilst not affect total cell number. There is no cytotoxicity in the MM cardiomyocyte cultures when exposed to sodium valproate (VPA) up to 500 µM whilst all VPA concentrations (<500 µM) reduced contractile activity. The D3 studies with tRA and VPA were comparable with the MM results.

Blind studies have now been performed testing embryotoxic potential using structurally related compounds, one a known teratogen and the other non-teratogen (analogue). All studies were compared with D3 ESCs. This would provide an alternative battery of system/organs to the use of rat micromass, for embryotoxicity testing *in vitro* and for mechanistic studies of embryonic development perturbation.

This work was funded by a grant from the FRAME research council.
THEME 7, SESSION 7.1

Poster

In vitro embryotoxicity testing of some components of dental biomaterials by the Embryonic Stem Cell Test

Koichi Imai¹, Horst Spielmann², Tohru Hayakawa³ and Masaaki Nakamura¹

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The effects of some components of dental biomaterials on the differentiation of embryonic stem cells of the mouse cell line D3 (ES-D3 cells) were examined using the Embryonic Stem Cell Test (EST), which consists of three parameters: the differentiation rate into contracting myocard, 50% cell viability of ES-D3 cells and of Balb/c 3T3 cells, and clone A31 (3T3 cells), which were finally calculated by a designated formula. Of the 24 monomers tested, Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, Bis-GMA(6F), 6-HHMA, BPE-1300 and MTYA were classified as weak embryotoxic, and 2.0-EPDMA, 3.0-EPDMA, 4.0-EPDMA, 1.6-ADMA, 1.8-ADMA, 1.10-ADMA, MEPC, Phosmer M, BSNa, EDMABA, GAM, GMA, GMR, NPG, PTSNa and QTX as non embryotoxic. In the case of metal powders extracted in culture media, it was found that Ag was classified as weak embryotoxic, while Co, Cr, Ni and Pd were classified as non embryotoxic. On the other hand, among the standard chemicals used for atomic absorption spectrophotometry, it was found that Cr and Hg ions were classified as strong embryotoxic, and In, Sn, Sb and V ions were classified as weak embryotoxic, while Ag, Co, Cu, Ni, Pd and Zn ions were classified as non embryotoxic. Five plasticisers, dibutyl phthalate, n-butyl benzylphthalate, n-butyl phthalic anhydride, di-2-ethylhexyl phthalate, di-2-ethylhexyl adipate were classified as weak embryotoxic.

It is considered that further intensive study on the embryotoxicity of dental biomaterials is needed, in addition to conventional biological aspects, from the perspective of biological safety.

Poster

Proposal for modification of the Embryonic Stem Cell Test to expand its applicability

Koichi Imai¹, Horst Spielmann² and Masaaki Nakamura¹

¹Osaka Dental University, Department of Biomaterials, Osaka, Japan; ²BfR, ZEBET, Berlin, Germany

Developed by Spielmann et al. in 1997 and already validated in EU countries, the Embryonic Stem Cell Test (EST) protocol is a screening test method that easily and promptly analyses differentiation toxicity of chemical substances in vitro. It is not applicable to insolubles or very slight solubles, but to solubles. Modification is necessary to test dental biomaterials that have a variety of compositions and are either soluble or insoluble, or have a variety of usage. In the present study, we tried to modify the protocol for expanding EST applicability, by exchanging the treating vehicle from a liquid medium to a type I collagen gel matrix. Accordingly, we embedded the test samples in type I collagen gel matrix based on a three-dimensional cytotoxicity method. That is, a method was employed to estimate the in vitro embryotoxicity from the time of placement of the test substances to the differentiation of ES cells. We examined the influence of three kinds of dental restorative materials on the ES cells. The differentiation rate was the highest for glassionomer cement, followed by two kinds of light-cured composite resins, and was nil for dental amalgam. It seems possible to perform in vitro embryotoxicity testing of dental biomaterials using this modification. Although this modified protocol has not been subjected to a validation process, it can be positively assessed to some extent by an in vitro examination of provisional embryotoxicity levels of dental biomaterials with complex compositions.
Lecture

A perspective on embryonic and adult stem cells for in vitro and in vivo testing

Aernout Luttun
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Like recombinant DNA in the 1980’s and transgenic animals in the 1990’s, stem cell technology is well on its way to become a central research tool for this and the following decades. Stem cells are generally derived from two main sources: embryos or adults. They can be further subcategorised according to their differentiation potential. While embryonic stem cells are considered pluripotent given their ability to give rise to all somatic and germ line cell types, the differentiation repertoire of adult stem cells is more limited. On the other hand, unlike stem cells from adult sources, the use of embryonic stem cells has met with significant ethical concerns. Recently, adult stem cells were derived, termed “Multipotent Adult Progenitor Cells” (MAPCs) that have many features of embryonic stem cells, including the ability to differentiate into many cell types representing all three germ layers of the embryo. This broad differentiation potential along with the availability without ethical restrictions offers many possible applications. Here we give an overview on how stem cells in general, and MAPCs in particular, can be exploited in addressing diverse questions at different levels ranging from in vitro testing to in vivo therapy.

Lecture

Differentiation of hepatocyte-like cells from human embryonic stem cells and adult liver progenitors

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Primary cultures of differentiated human hepatocytes represent a powerful tool for basic and applied research, in particular for drug metabolism and hepatotoxicity testing, as well as for biotherapy of liver diseases. Hepatocytes are currently isolated from livers not used for transplantation or from lobectomies resected for medical purpose. However, these sources of tissue present various inconveniences including scarcity, limited availability and technical difficulties for cell isolation. Obtainment of differentiated human hepatocytes from stem cells is therefore indispensable and represents an exciting challenge. Several potential sources of stem cells have been considered in this respect, including Embryonic Stem Cells (ESC) and intrahepatic progenitors. Several groups have reported that, under appropriate culture conditions and stimulation by cytokines, growth factors or chemical reagents, ESC differentiate to hepatocyte-like cells expressing markers such as albumin and other plasma proteins, CK8/18, production of glycogen, expression and xenobiotic-mediated induction of cytochrome P450 genes. Similarly, intrahepatic progenitor cells have been shown to be able to generate hepatocyte-like cells expressing the above-mentioned markers. Another exciting and promising advantage of stem cells is the possibility to modify their genome by different approaches including gene transfer and siRNA-mediated gene down-regulation. The technical means allowing such modifications including homologous recombination and lentivirus vector-mediated transfection are now available. Evaluating the impact of up- or down-expression of candidate genes on the process of stem cell differentiation and/or on the phenotype of generated hepatocytes opens the way to basic and applied investigations on the process of human liver ontogenesis and liver diseases.
Lecture

Normal human neural progenitor cells: An in vitro model for testing developmental neurotoxicity

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The development of suitable test systems is important in order to protect foetuses and neonates against the toxic action of chemicals. Our goal is to establish a human in vitro model to test chemicals for their neurotoxic effects.

We have established the culture and propagation of Normal Human Neural Progenitor (NHNP) cells, which differentiate into different neuronal subtypes, astrocytes and oligodendrocytes.

To validate our cell model for testing developmental neurotoxicity, NHNP cells were exposed to different chemicals exhibiting neurotoxic effects. Cell differentiation, viability, apoptosis, migration and MAP kinase signaling were used as biological endpoints for testing the influence of these chemicals on these cells.

Exposure of undifferentiated NHNP cells to low concentration of HgCl₂ for one week lead to a significant decrease of differentiated neurons relative to astrocytes. Exposure of differentiating cells to HgCl₂ resulted in reduced cell migration. The cell viability was not affected.

Ethanol (EtOH) was reported to affect cellular signal transduction in the rodent brain. Exposure of undifferentiated NHNP cells to 200 mM EtOH for 30 and 60 minutes lead to a significant inhibition of ERK 1/2 phosphorylation. The cell viability was also not affected by EtOH treatment.

In summary, our preliminary data show that exposure of NHNP cells to neurotoxic compounds leads to alterations of endpoints, which were reported in in vivo studies.

Poster

Global in vitro predictive stem cell hemotoxicology for early drug screening and estimating pre-clinical and clinical trial dosing

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The HALO (Hemotoxicity Assays via Luminescence Output) Platform provides a standardised, validated in vitro proliferation test system with high-throughput and predictive capability for drug screening and all later stages of drug development. The newly developed HALO 7-population predictive paradigm can test compounds on 7 lympho-hematopoietic populations (2 stem cell, HPP-SP, CFC-GEMM; 3 progenitor, (BFU-E, GM-CFC, Mk-CFC); 2 lymphopoietic, T-CFC, B-CFC) derived from human, primate, dog, rat or mouse bone marrow target cells simultaneously. The response by these early cell populations, especially the stem cells, allows prediction of compound effects in all peripheral lineages. Since HALO has been validated against the Registry of Cytotoxicity Prediction Model, the IC₅₀, IC₇₅ and IC₉₀ values obtained from screening compounds on animal bone marrow cells can be used to calculate the estimated in vivo doses for pre-clinical studies. When primary human bone marrow target cells are used, the initial or clinically-relevant doses for human trials can also be predicted. If used during ADME/Tox compound screening, the HALO Platform provides a powerful decision-making tool that is an alternative surrogate assay for animal testing that can save time and money during drug development.
The Embryonic Stem Cell Test (EST) is a well-established and standardised in vitro method to predict the embryotoxic potential of chemicals and is based on the capacity of murine embryonic stem (ES) cells to differentiate under specific culture conditions into beating cardiomyocytes.

An efficient differentiation requires the use of cell culture medium supplemented with 15% foetal calf serum (FCS). Unfortunately, FCS has a great lot to lot variability with regard to its composition. Therefore selected batches of FCS have to be pre-tested if they support differentiation and proliferation of ES cells before the EST can be performed. In order to optimise the culture conditions in the EST our aim was to establish serum-free culture conditions. Chemically defined serum-free culture conditions would provide several advantages: (1) improved protocol transfer to other laboratories (2) improved reproducibility of the differentiation assay, (3) no interference of undefined serum component with the test substance (reproducible bioavailability) and (4) application of the EST in high/medium-throughput screening systems.

Chemically-defined serum-free media supplemented with several growth and differentiation factors known to be important for cardiogenesis in vivo have been tested. Our results demonstrate that under these conditions cardiac embryonic stem cell differentiation can be achieved in vitro in a highly standardised and reproducible manner. In addition, we were able to demonstrate the applicability of the serum-free EST to test chemicals with different embryotoxic potentials.

**Poster**

**The use of serum-free culture conditions in the Embryonic Stem Cell Test: Defined culture conditions for cardiac stem cell differentiation**

**Katharina Schlechter¹, Roland Buesen², Birgitta Slawik², Horst Spielmann² and Andrea Seiler²**

¹ University of Applied Sciences, Giessen, Germany; ² Federal Institute for Risk Assessment (BfR), National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

The Embryonic Stem Cell Test (EST) is a well established and standardised in vitro method to predict the embryotoxic potential of chemicals and is based on the capacity of murine embryonic stem (ES) cells to differentiate under specific culture conditions into beating cardiomyocytes.

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**Lecture**

**Current status of the Embryonic Stem Cell Test: The use of recent advances in the field of stem cell technology and gene expression analysis**

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All guidelines that are currently used for regulatory developmental toxicity testing of chemical and drugs are based on animal experiments. The most promising alternative is based on embryonic stem (ES) cells of the mouse. The ability to differentiate into numerous cell types has made ES cells a popular system to study gene function and developmental processes during differentiation in vitro. The Embryonic Stem Cell Test (EST) makes use of this capacity and is detecting developmental toxicants during stem cell differentiation into cardiomyocytes.

In the present study our investigations aimed at the further development of the validated EST protocol. Here we present improvements that focused primarily on (i) the quantitative assessment of drug effects at the cellular level, using a novel approach in which the expression of tissue-specific marker proteins under influence of the test chemical is quantified by intracellular flow cytometry in ES cells, (ii) the development of protocols for ES cell differentiation into various cell types other than cardiomyocytes and (iii) the standardisation and optimisation of ES cell culture and differentiation conditions in chemically defined serum-free medium.

An important strength of the serum-free, molecular approach is that in this way the ability of the test to monitor the cellular response to toxins could be expanded to proteins of many signal transduction pathways in a highly standardised form.
**Poster**

**Further evaluation of the optimised in vitro Embryonic Stem Cell Test (EST) by testing reference compounds and J&J compounds**

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The embryonic stem cell test was implemented at J&JPRD in order to help early drug research in the selection process of non-embryotoxic pharmaceutical compounds.

In order to gain a better yield of strongly contracting embryoid bodies, a few optimisations to the validated ECV AM protocol were executed. In a former study we already demonstrated that by using the optimised protocol and the prediction model of the validation study, the classification of 6 reference compounds reflected the *in vivo* classification.

The aim of this study was to test more compounds in the optimised EST in order to know better its prediction potential. In a first phase, 14 reference compounds used in the ECVAM pre-validation and validation study were tested. The results showed that the prediction by using the optimised protocol was comparable with the validation study. The compounds that caused misclassifications in the validation study also caused misclassifications with the optimised method.

In a second phase two analogous J&J compounds were tested of which the first one was teratogenic and the second one was not teratogenic *in vivo*. The results showed that the first compound caused an inhibition of differentiation at lower concentrations compared to the inhibition of cell growth, while the second compound caused inhibition of differentiation in the same dose range as cytotoxicity occurs. However the prediction model classified both compounds as moderate embryotoxic compounds.

More in house compounds with known *in vivo* data will be tested in order to optimise the prediction model.

**Lecture**

**Mimicking liver development by sequential exposure to hepatogenic cytokines: The key to differentiate rat bone marrow stem cells into hepatocyte-like cells**

*Sarah Snykers¹, Tamara Vanhaecke¹, Peggy Papeleu¹, Aernout Luttun², Catherine Verfaillie² and Vera Rogiers¹*

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Background/Aims: Differentiation of adult Bone Marrow Stem Cells (BMSC) into hepatocyte-like cells is commonly carried out by continuous exposure of the cells to a mixture of cytokines. In the present study, it was investigated whether the differentiation efficacy can be importantly enhanced by sequential exposure of the cells to liver-specific (LSP) cytokines [fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF), insulin-transferrin-sodium-selenite (ITS) and dexamethasone (Dex)] comparable to the secretion pattern occurring during *in vivo* embryonic liver development.

Methods: The differentiation process was characterised by means of quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis and immunofluorescence. ALB secretion was analysed using ELISA.

Results: Upon sequential exposure to LSP cytokines, BMSC-derived hepatocyte-like cells undergo different stages of hepatocyte differentiation, as seen during liver embryogenesis. Indeed, expression of the early hepatocyte markers alpha-foetoprotein (AFP) and hepatocyte nuclear factor (HNF)3β decreased as differentiation progressed, whereas levels of the late LSP markers albumin (ALB), cytokeratin (CK)18 and HNF1α were gradually upregulated, suggesting hepatocyte commitment and maturation. In contrast, simultaneous treatment with a mixture of all cytokines did not significantly alter the expression pattern of the LSP markers. Moreover, only upon sequential exposure to LSP cytokines, cells expressed phase I cytochrome P450 (CYP) proteins and showed significantly increased ALB secretions, pointing to a functional hepatic status of these cells.

Conclusion: Sequential induction of the differentiation process, analogous to *in vivo* liver embryogenesis, is crucial for *in vitro* differentiation of BMSC into mature hepatocyte-like cells.
Lecture

Human embryonic stem cells – The source of normal human cells for in vitro assays

Raimund Strehl¹, K. Emanuelsson¹, U. Karlsson¹, J. Lindqvist¹, A. Lindahl² and J. Hyllner¹
¹Cellartis AB, Göteborg, Sweden; ²RCEM1, Gothenburg University, Göteborg, Sweden

Background: The most commonly used models for toxicological studies today are animals or animal cells from rats, mice and other species followed by human cell lines. Animal experiments cause suffering while transferability of results from animal experiments and animal cells to humans is often low. Human cell lines are available, but usually have a low degree of functional differentiation. Human primary material is problematic with respect to quality and availability. Therefore there is a demand for available, normal and functional human cells for in vitro assays.

Procedure: Human Embryonic Stem Cells (hESC) offer unique possibilities and provide new opportunities to generate a virtually limitless supply of normal human cells for assays. Cellartis has established 30 hESC lines and generated extensive knowledge on methods for the culture, handling and characterisation as well as the differentiation of these cells. We are now directing our efforts towards the development of assays based on hESC and their differentiated derivates.

First generation assays based on undifferentiated hESC and derivates can be utilised to evaluate early human development in vitro and to detect embryotoxic and teratogenic effects in a human-relevant system. Once functionally differentiated somatic cells such as cardiomyocytes and hepatocytes can be created from undifferentiated hESC, these cell types can form the basis of an even more comprehensive generation of hESC based assays.

Poster

On the replacement of in vivo tests on germ cell mutagenicity and fertility impairment

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In contrast to drugs, industrial chemicals are not intended to be uptaken directly by humans. They mainly serve as intermediates for preparations and products. In case of proper use exposure to the general public seems, therefore, to be negligible. However, uncontrolled exposure may happen e.g. by accidents. Often little is known about the toxicological profile of a chemical substance. Consequently, a risk on human health can not be estimated. Realising this fatal situation, the EU enacted a Directive for the notification of new and existing chemicals. Impairment of fertility is one of the most important endpoints to be determined using in vivo generation studies, which are extremely time-, money-, and animal-consuming.

Besides the prediction of a carcinogenic risk, one of the most important issues of mutagenicity data is the detection of adverse effects on gametes and thus on fertility as well as on early embryonal development in mammals. For this purpose tests on mammalian germ cells need to be conducted. In practice, however, germ cell tests are rarely used because of the costs and the number of animals required. Therefore, we tried to develop a sensitive and predictive in vitro test system, which could serve as a model for mammalian germ cells: Murine female and male embryonic germ cell lines had been established in our laboratory and their sensitivity upon mutagen/non-mutagen treatment had been tested in comparison to adult cell lines. Applying linear discriminant analysis, all test chemicals used could be classified correctly!
Teratogenicity is one of the potentially critical side effects of new compounds. For chemicals used as drugs, embryotoxicity studies in two species have to be conducted to cover embryo-foetal development (ICH guidelines). These in vivo protocols are time-consuming, expensive and use a large number of animals. In developmental toxicology, many in vitro tests have been developed using a wide spectrum of cell and tissue cultures. The scope of this “internal validation” was to implement the Embryonic Stem Cell Test (EST) validated by the ECVAM in 1999 as a tool to detect the embryotoxic potential of new pharmaceutical compounds early in the drug development process. The “internal validation” with six well known compounds and 10 Roche compounds gave an overall accuracy for the embryonic stem cell test of 81%. The overall predictivity for this limited set of test compounds was very good (85% for non teratogenic compounds and 83% for strong teratogenic compounds). The relatively poor prediction (66%) for weakly teratogenic compounds was because only three weakly teratogenic compounds could be tested. Further testing of pharmaceutical compounds, ideally strong and weak teratogenic compounds, to improve the prediction is still necessary.