Trends in Improving the Embryonic Stem Cell Test (EST): an Overview

Roland Buesen, Anke Visan, Elke Genschow, Birgitta Slawik, Horst Spielmann and Andrea Seiler
Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) at the Federal Institute for Risk Assessment (BfR), D-Berlin

Summary
The embryonic stem cell test (EST) is an in vitro assay that has been developed to assess the teratogenic and embryotoxic potential of drugs and chemicals. It is based on the capacity of murine ES cells (cell line D3) to differentiate into contracting myocardial cells under specific cell culture conditions. The appearance of beating cardiomyocytes in embryoid body (EB) outgrowths is used as a toxicological endpoint to assess the embryotoxic potential of a test substance. Applying linear analysis of discriminance, a biostatistical prediction model (PM) was developed to assign test chemicals to three classes of embryotoxicity. In an international validation study the EST predicted the embryotoxic potential of chemicals and drugs with the same reliability as two other in vitro embryotoxicity tests, which employed embryonic cells and tissues from pregnant animals.
In a joint research project with German pharmaceutical companies we have successfully improved the EST by establishing molecular endpoints of differentiation in cultured ES cells. The quantification of cardiac-specific protein expression by intracellular flow cytometry has been studied in the presence of chemicals of different embryotoxic potential. The results obtained using molecular endpoints specific for differentiated cardiomyocytes employing FACS (fluorescence-activated cell sorting) analysis will be presented in comparison to the validated endpoint - the microscopic analysis of beating areas. FACS analysis provides a more objective endpoint for predicting the embryotoxic potential of chemicals than the validated method. Furthermore, flow cytometry promises to be suitable for high-throughput screening systems (HTS).
In addition, our partners from the joint project have improved the EST by developing protocols that stimulate differentiation of ES cells into neural and endothelial cells, chondrocytes and osteoblasts, because some substances might have embryotoxic effects on specific cell-types other than cardiomyocytes. These protocols have been successfully established at ZEBET and in the participating laboratories. Additionally, molecular endpoints have been established for the detection of specific differentiation pathways.
Furthermore, new prediction models (PMs) have been developed using single endpoints of the EST.

Zusammenfassung: Trends bei der Weiterentwicklung des Embryonalen Stammzelltests (EST): ein Überblick
Der Embryonale Stammzelltest (EST) ist ein in vitro-Test, der zur Abschätzung teratogener und embryotoxischer Eigenschaften chemischer Substanzen entwickelt wurde. Das Prinzip des EST beruht auf dem Vermögen embryonaler Stammzellen (ES-Zellen) der Zelllinie D3 kontrahierende Herzmuskelzellen auszubilden. Die Beurteilung einer Testsubstanz erfolgt über die Analyse der hemmenden Wirkung eines Stoffes auf die Entwicklung schlagender Herzmuskelzellen, die lichtmikroskopisch beobachtet werden kann. Mit Hilfe der linearen Diskriminanzanalyse konnte ein biostatistisches Prädiktionsmodell (PM) entwickelt werden, das eine Klassifizierung in nicht, schwach oder stark embryotoxische Substanzen erlaubt. In einer internationalen Validierungsstudie erwies sich der EST für die Beurteilung der embryotoxischen Eigenschaften chemischer Stoffe als ebenso zuverlässig wie zwei weitere in vitro-Tests, bei denen schwangere Tiere zur Gewinnung embryonaler Zellen und Gewebe verwendet werden.
In einem Verbundprojekt mit der deutschen pharmazeutischen Industrie konnte der EST erfolgreich durch die Etablierung molekularer Endpunkte für die Beurteilung der Differenzierung der ES-Zellen weiterentwickelt werden. Mit Hilfe der Durchflusszytometrie und der Bestimmung herzmuskelspezifischer Proteine wurden die differenzierungs-hemmenden Eigenschaften von Substanzen mit unterschiedlichem embryotoxischen Potenzial untersucht und die Ergebnisse anschliessend mit der validierten licht-mikroskopischen Analyse verglichen. Der Einsatz der FACS-Analyse (flourescence-activated cell sorting) im EST ermöglicht eine objektivere Bestimmung des embryotoxischen Potenzials einer Substanz als die validierte mikroskopische Methode und ebnet den Weg zur möglichen Automatisierung der Prüfung auf embryotoxische Eigenschaften im sog. „high-throughput screening (HTS)“.
Ein weiteres Ziel des Verbundprojektes war die Etablierung von Standardmethoden für die Differenzierung der ES-Zellen in weitere Zelltypen, um die embryotoxischen Wirkungen von Testsubstanzen auch auf andere Gewebe neben den Herzmuskelzellen im EST zu erfassen. So konnten in den teilnehmenden Laboren der Bayer AG und der Boehringer Ingelheim Pharma GmbH & Co. KG erfolgreich neue Methoden zur Differenzierung von ES-Zellen in neuronale, Endothel-, Knorpel- und Knochenzellen entwickelt werden. Ferner wurden für einige Gewebe differenzierungsspezifische Meßparameter etabliert.
Zusätzlich sind weitere Prädiktionsmodelle entwickelt worden, die auf der Verwendung einzelner Endpunkte des EST basieren.

Keywords: chemicals policy, REACH, German Chemicals Act, avoidance of duplication of testing, data sharing, non animal test methods, regulatory toxicity testing

This manuscript is an invited paper and contains the results presented in three posters at the Linz 2003 meeting. The authors were asked to discuss the trends in improving the EST further.
Received 16 January 2004; received in final form and accepted for publication 6 February 2004.

1 Introduction

In the field of reproductive and developmental toxicology, mandatory test guidelines have been developed to gain high-quality information about damaging health effects of chemicals and drugs. The Organization for Economic Co-operation and Development (OECD) passed several test guidelines (TGs) for the assessment of embryotoxic and teratogenic properties of chemical substances (OECD TG 414: the prenatal developmental toxicity study; OECD TG 415: the one-generation reproduction toxicity study; OECD TG 416: the two-generation reproduction toxicity study; OECD TG 421: the reproductive/developmental toxicity screening test; OECD TG 422: the repeated dose toxicity study combined with the reproductive/developmental toxicity screening test). The TGs call for different screening tests and multi-generation studies based on animal experiments. Their realisation often requires a large number of laboratory animals, e.g. mice, rats and rabbits. Thus, it is time-consuming and thereby expensive to get proper information on the embryotoxic or teratogenic potential of a test compound.

According to the European Commission's White Paper "Strategy for a Future Chemicals Policy" (Commission of the European Communities, 2001) and the recently released approach to a new chemical legislation (REACH system: Registration, Evaluation, Authorization of Chemicals; Commission of the European Communities, 2003) about 30,000 existing chemicals have to be toxicologically re-evaluated in the European Union by 2015. An implementation of current regulatory guidelines would cause a tremendous increase in the use of laboratory animals. Thus, economical as well as ethical aspects demand proper *in vitro* methods to examine the embryotoxic hazards of chemical substances.

Recently, an *in vitro* embryotoxicity test was developed at ZEBET: the Embryonic Stem Cell Test (EST; Spielmann et al., 1997). Using the capacity of embryonic stem cells (ES cells) to differentiate into several cell types, a reliable assay has been designed to assess embryotoxic/teratogenic properties of chemicals and drugs *in vitro*.

1.1 Current status of the EST

To perform the differentiation *in vitro*, murine ES cells of the permanent cell line D3 (Doetschmann et al., 1985) are maintained in an undifferentiated state in culture under conditions that inhibit differentiation by supplementing the culture medium with murine leukemia inhibitory factor (mLIF; Williams et al., 1988). Differentiation of ES cells is then induced by the withdrawal of mLIF. Using the "hanging drop" culture technique described by Rudnicki and McBurney (1987), ES cells form multicellular aggregates called embryoid bodies (EBs). Within the EBs the three germ layers endo-, meso- and ectoderm can develop and further differentiation into several cell types including contracting myocardial cells can take place (Doetschmann et al., 1985; Rudnicky and McBurney, 1987; Maltsev et al., 1994; Hescheler et al., 1997). The EST benefits from the fact that differentiation into contracting myocardial cells can be easily detected by microscopic inspection of EB outgrowths at day 10 of differentiation. In addition to the differentiation analysis, cytotoxic effects of the test substance on ES cells and 3T3 fibroblasts are analysed (Fig. 1). By using stem cells and differentiated (adult) fibroblasts, the assay takes embryonic as well as maternal toxicity into account. To assess the concentration of a substance which inhibits the development (inhibition of differentiation: ID_{50}) and proliferation (IC_{50}D3 and IC_{50}3T3) by 50% compared to the untreated control, dose-response profiles are performed.

Applying linear analysis of discriminance as the mathematical model to the results obtained in the EST, a biostatistical prediction model (PM) was developed to assign test compounds to three classes of embryotoxicity: *non-embryotoxic*, *weakly embryotoxic* and *strongly embryotoxic* (Genschow et al., 2002 and 2004).

In an international ECVAM validation study the EST was scientifically validated using a set of 20 reference compounds characterised by high-quality *in vivo* embryotoxicity data assessed in laboratory animals and humans. The EST predicted the embryotoxic potential of the 20 reference compounds with an accuracy of 78%. Remarkably, a predictivity of 100% was obtained for strong embryotoxic chemicals (Genschow et al., 2002 and 2004).

1.2 Improvements of the EST

Although the microscopic analysis of beating cardiomyocytes has been ap-

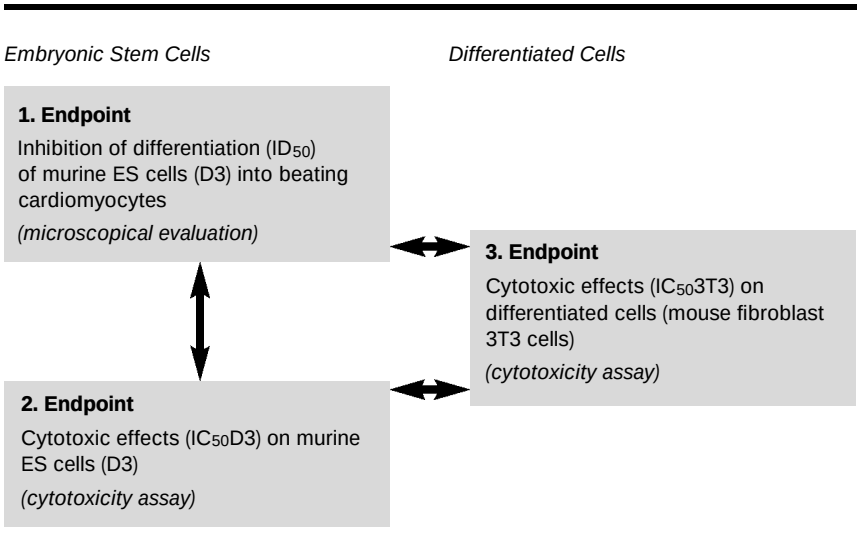


Fig. 1: The embryonic stem cell test (EST). Schematic overview illustrating the principle approach and the endpoints applied in the test to assess the embryotoxic potential of test compounds using two permanent mouse cell lines: 3T3 fibroblasts and D3 ES cells. Endpoints assessed from dose-response curves are: (1) inhibition of differentiation into contracting cardiomyocytes: ID_{50}, (2) cytotoxic effects on ES cells: IC_{50}D3, and (3) cytotoxic effects on 3T3 cells: IC_{50}3T3.

proved as a fast and reliable method, attempts have been made by our group and others to improve the EST protocol. Improvements were predominantly made for the following reasons:

1. Observing cellular areas of contracting cardiomyocytes is time-consuming and requires experience. Also, the differentiation into contracting myocardial cells takes 10 days. With regard to an automated application, e.g. high-throughput screening, it would be of great interest to simplify the detection and to shorten the duration of the assay.
2. The differentiation into myocardial cells only records effects on mesodermal development. This may lead to false negative results if the test compound affects cell types other than the myocardium.

In co-operation with German pharmaceutical companies (Bayer AG, Boehringer Ingelheim Pharma GmbH & Co. KG, Schering AG) ZEBET is improving the current EST protocol. Different approaches are being pursued:

1. The inhibition of differentiation of ES cells into cardiac muscle cells should be evaluated by more objective endpoints, e.g. quantification of cardiac-specific molecular markers. An analysis of differentiation-inhibitory effects of chemicals at the cellular level promises to be a simple but high-quality endpoint suitable for automated recording (high-throughput screening).
2. The establishment of additional endpoints of differentiation, e.g. neural cells, endothelial cells, chondrocytes and osteoblasts, and their detection via tissue-specific molecular markers would expand the set of toxicological endpoints of the EST.

In addition, the development of new prediction models (PMs) using single endpoints of the EST to assign test compounds to two classes of embryotoxicity (*non-/weakly embryotoxic* versus *strongly embryotoxic*) according to their *in vivo* data have been performed. For example in the field of drug development and for the new chemical legislation (REACH), the identification of strongly embryotoxic substances is most important. Therefore, a prediction model that distinguishes between two classes of embryotoxicity, e.g. *strongly embryotoxic* versus *non-/weakly embryotoxic*, is sufficient for these purposes and would simplify the test considerably.

The successful establishment of new molecular endpoints in the EST and the results of testing reference chemicals with the improved EST protocol were presented on three posters at the 11th Congress on Alternatives to Animal Testing in Linz, Austria (September 19th-21st, 2003).

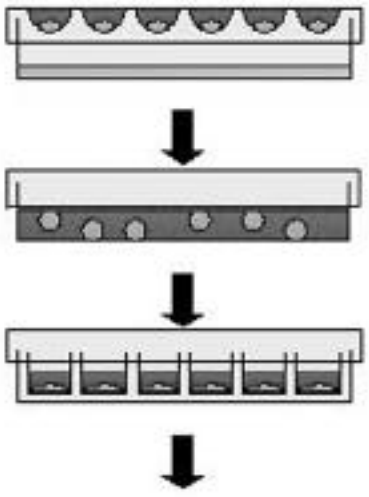
2 Improving the Embryonic Stem Cell Test (EST)

2.1 Implementing molecular endpoints of differentiation

The cell culture technique that allows murine ES cells to differentiate into contracting myocardial cells, the so-called "hanging-drop method", was used to detect adverse effects on differentiation of ES cells into cardiomyocytes (Spielmann et al., 1997; Fig. 2). ES cells in suspension were placed on the inner side of a Petri dish lid to enable the formation of "embryoid bodies" (EBs). After 3 days, EBs were transferred to bacterial Petri dishes. Bacterial Petri dishes were used to avoid adherence and outgrowth of the EBs during this stage of the culture. From day 5 to day 7 or day 10, EBs were cultivated in tissue culture-treated 24-well plates to allow adherence and further differentiation into cardiac muscle cells. For each step, culture medium was prepared containing the appropriate concentration of test chemical. At day 10 of differentiation, contracting cardiac muscle cells were evaluated by microscopic analysis of EB outgrowths. FACS analyses were performed at day 7 of differentiation.

For the establishment of molecular endpoints of differentiation, we were able to identify specific monoclonal antibodies (mABs) directed against sarcomeric α-actinin (Goncharova et al., 1992) and myosin heavy chain (MHC; Bader et al., 1982). Using these antibody preparations, a standard operating procedure (SOP) was established for reliable FACS analysis (Fig. 3). Time course experiments demonstrated that the strongest signals could be observed at day 7 of differentiation, indicating that a reduction in protein expression induced by embryotoxic compounds could best be monitored at day 7 of culture (Seiler et al., 2002 and 2004).

To prove the reproducibility and the reliability of FACS analysis, several experiments were performed to compare the newly established molecular endpoint (FACS analysis on day 7) to the validated microscopic evaluation at day 10 of differentiation. As shown in Figure 4, similar dose-response curves and



Day 1-3: Cultivation of ES cells in hanging drops, formation of "embryoid bodies (EBs)".

Day 3-5: Transfer of EBs to bacterial dishes.

From day 5: Cultivation of EBs in 24-well plates, Petri dishes or on glass cover slips, differentiation into different tissues.

Evaluation

Fig. 2: Differentiation assay of the EST.

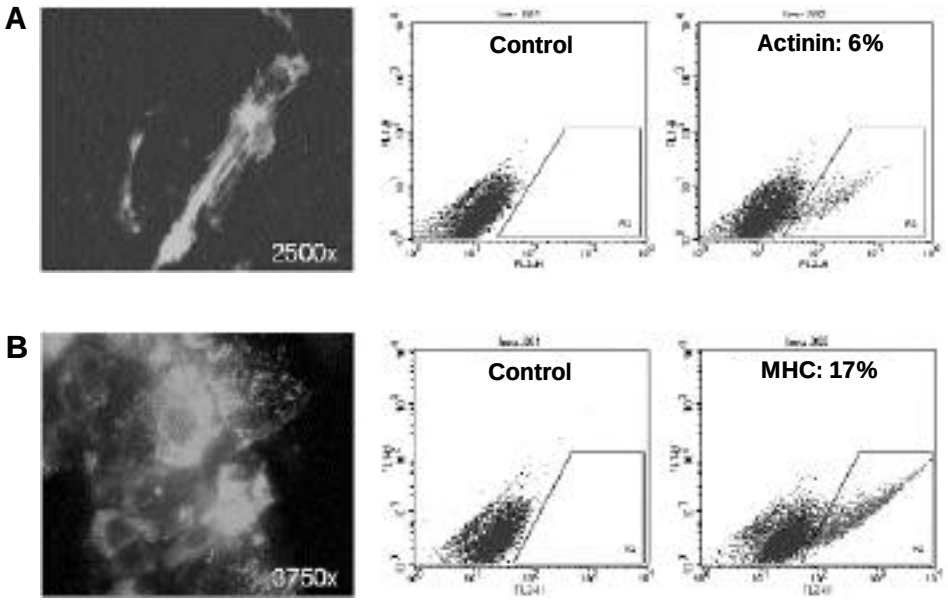


Fig. 3: Differentiation of cardiomyocytes from murine ES cells. Left panel: Microscopic images of an (A) *anti*-sarcomeric α-actinin (clone EA53) and (B) *anti*-sarcomeric myosin heavy chain (clone MF20) stained 10-day old embryoid body (EB). Right panel: Quantification of cardiac marker protein expression by intracellular flow cytometry. Representative dot blot analyses of *anti*-sarcomeric α-actinin and *anti*-sarcomeric myosin heavy chain stained 7-day old embryoid body. Cells stained without primary antibody were used as controls.

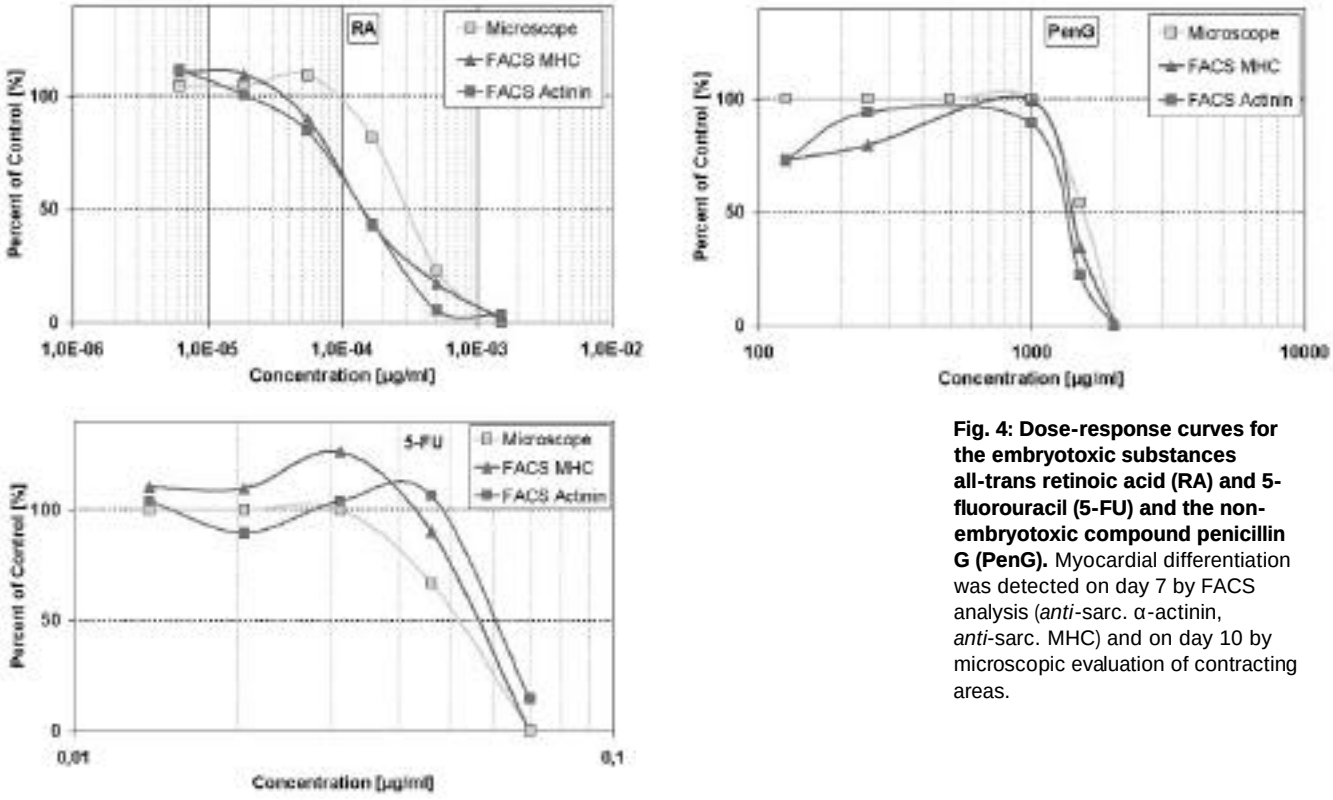


Fig. 4: Dose-response curves for the embryotoxic substances all-trans retinoic acid (RA) and 5-fluorouracil (5-FU) and the non-embryotoxic compound penicillin G (PenG). Myocardial differentiation was detected on day 7 by FACS analysis (*anti*-sarc. α-actinin, *anti*-sarc. MHC) and on day 10 by microscopic evaluation of contracting areas.

consequently almost identical ID_{50} values were obtained with both methods using positive (all-trans retinoic acid [RA] and 5-fluorouracil [5-FU]) and negative (Penicillin G [PenG]) reference chemicals.

2.2 Differentiation of mouse ES cells into multiple tissues and identification of molecular endpoints

A major aim of the joint BMBF project was the development of specific protocols that would stimulate differentiation of undifferentiated ES cells into other cell types such as neurons, endothelial cells, chondrocytes and osteoblasts. The laboratories of the Bayer AG and the Boehringer Ingelheim Pharma GmbH &

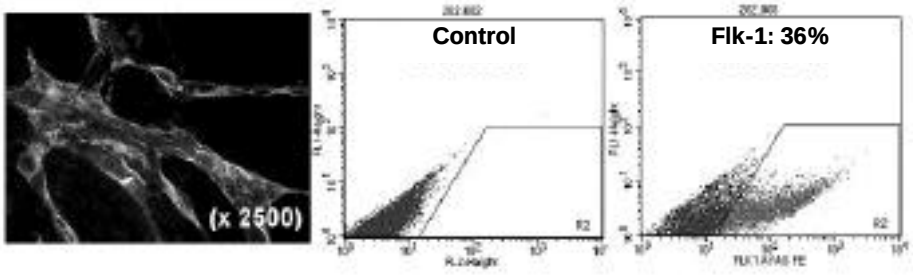


Fig. 5: Detection and quantification of endothelial cells differentiated from murine ES cells according to a protocol obtained from the Boehringer Ingelheim Pharma GmbH & Co. KG.
Left panel: Microscopic images of *anti*-Flk-1 (clone Avas 12α1) stained 11-day old embryoid bodies (EB). Right panel: Quantification of endothelial marker protein expression by intracellular flow cytometry. Representative dot blot analyses of *anti*-Flk-1. Cells stained without primary antibody were used as controls.

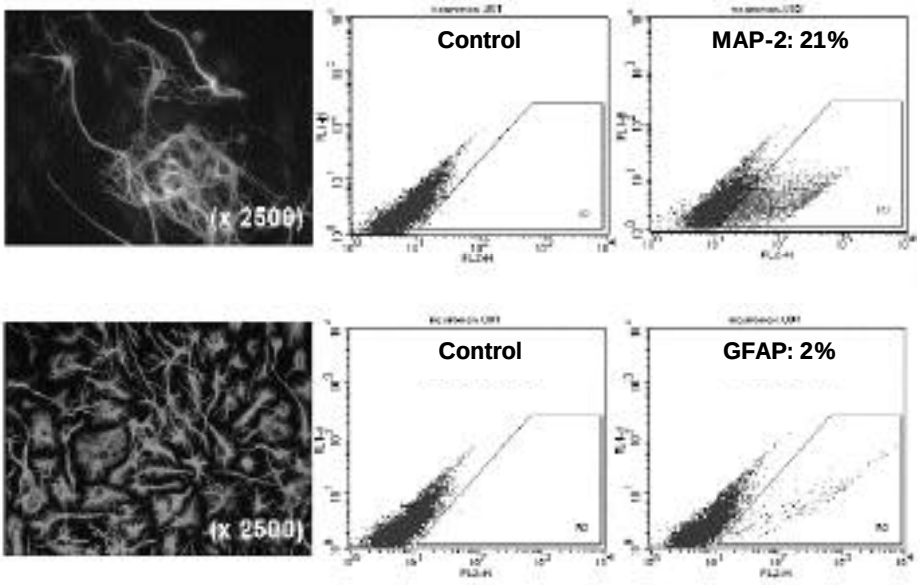


Fig. 6: Detection and quantification of neurons (A) and glia cells (B) differentiated from murine ES cells according to a protocol developed at the Bayer AG.
Left panel: Microscopic images of (A) *anti*-MAP-2 (clone AP-20) and (B) *anti*-GFAP (clone G-A-5) stained 27-day old embryoid bodies (EB). Right panel: Quantification of neural and glial marker protein expression by intracellular flow cytometry. Representative dot blot analyses of stained 27-day old embryoid body. Cells stained without primary antibody were used as controls.

Co. KG have developed culture protocols using defined media and growth factors to stimulate the differentiation into various cell types. These protocols were transferred to ZEBET for standardisation. After establishing the differentiation protocols at ZEBET, our goal was the detection of cell type-specific marker proteins that can be quantified by flow cytometry (see Fig. 5 and 6).

2.2.1 Endothelial cells

Endothelial cells were induced by supplementing the growth medium with vascular endothelial growth factor (VEGF) according to a protocol developed at the Boehringer Ingelheim Pharma GmbH & Co. KG (modified according to Vittet et al., 1996). As molecular marker of differentiation, VEGF receptor-2, also described as fetal liver kinase 1 (Flk-1; Hanahan, 1997), was chosen, because it is known to be expressed in embryonic endothelial cells (Millauer et al., 1993; Nishikawa et al., 1998). Differentiation from murine ES cells into endothelial cells could be detected and quantified on day 11 of development (Fig. 5).

2.2.2 Neural cells

Differentiation into mature neurons and glia cells was stimulated by serum-free culture conditions in combination with specific growth factors following the lineage selection protocol basically described by Okabe et al. (1996). A modified protocol was developed at the Bayer AG. Microtubule-associated protein 2 (MAP-2) was chosen for the detection of post-mitotic neurons (Tucker, 1990). It is a neuronal cytoskeletal protein that stabilises neuronal dendrites. The expression of MAP-2 is developmentally regulated. It is not found in neural precursor cells but is expressed in mature neurons. For detection of glial cells, e.g. astrocytes, the glial-fibrillary acidic protein (GFAP) was selected as molecular marker protein. GFAP is a cell-specific intermediate filament pro-

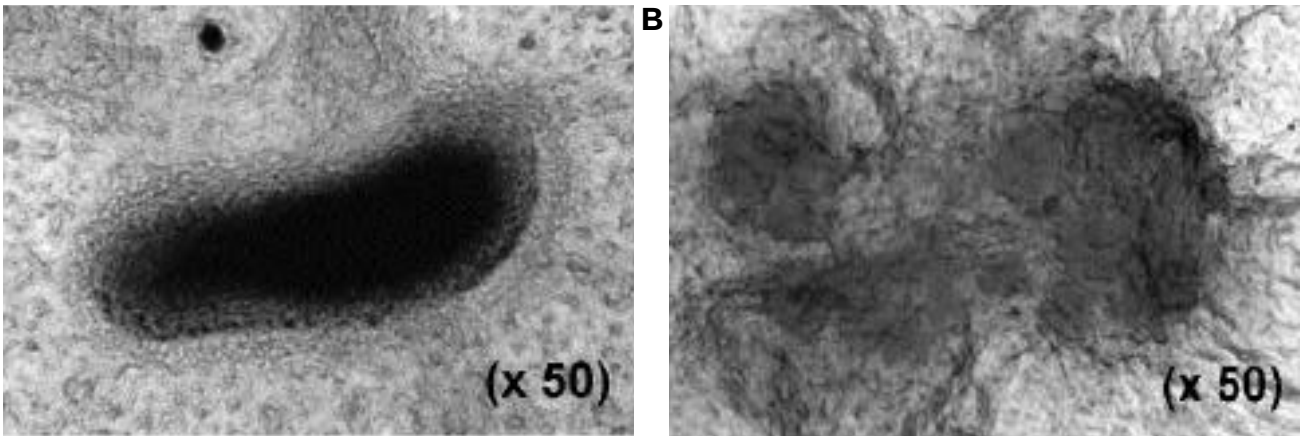


Fig. 7: Detection of chondrocytes and osteoblasts differentiated from murine ES cells according to protocols developed at the Bayer AG. Chondrocytes were stained with Alcian Green 2GX (A) and osteoblasts were stained with Alizarin Red S (B) on day 31 of differentiation.

tein in astrocytes (Debus et al., 1983). Specific staining of neurons and glial cells and their quantification by FACS analyses were performed on day 27 of differentiation (Fig. 6).

2.2.3 Chondrocytes and osteoblasts

To stimulate the differentiation into chondrocytes, culture medium was supplemented with bone morphogenic protein 2 (BMP-2), transforming growth factor β (TGF-β), insulin and ascorbic acid (modified according to Kramer et al., 2000), whereas osteoblasts were induced by the addition of ascorbic acid, β-glycerolphosphate and vitamin D3 (zur Nieden et al., 2003). Both protocols were developed at the Bayer AG. Histochemical staining for the detection of chondrocytes (Alcian Green 2GX) and osteoblasts (Alizarin Red S) were performed on day 31 of differentiation (Fig. 7).

3 Prediction of embryotoxic potential of drugs and chemicals

Applying the prediction model of the EST to the results of the ECVAM validation study, test chemicals were assigned to three classes of embryotoxicity according to their embryotoxic potential *in vivo*:
Class 1: *non-embryotoxic*,
Class 2: *weakly embryotoxic* and
Class 3: *strongly embryotoxic.*

However, in some cases assignment of chemicals and drugs to only two classes of embryotoxicity might be sufficient. For example, in the field of drug development, the identification of strongly embryotoxic compounds has the highest priority. Consequently, new prediction models allowing the assignment of test compounds to two classes of embryotoxicity (*non-/weakly embryotoxic* versus *strongly embryotoxic*) were developed recently (Genschow et al., manuscript in preparation).

The aim of these studies is to prove whether single endpoint values of the EST, e.g. ID_{50} (inhibition of differentiation of mouse ES cells), IC_{50}D3 (cytotoxic effects on mouse ES cells) or IC_{50}3T3 (cytotoxic effects on 3T3 cells), are applicable for the detection of embryotoxic properties of chemicals and drugs. Detailed *post hoc* analyses were performed using data from 27 chemicals obtained from experiments carried out for the EST during pre-validation and validation of the ECVAM trial (Genschow et al., 2002 and 2004).

Preliminary results indicate that single endpoints derived from mouse ES cells (differentiation or cytotoxicity) are highly predictive for the identification of strong embryotoxicants and that stem cell-derived endpoints are more predictive than cytotoxicity data of the mouse 3T3 fibroblast cell line (Genschow et al., manuscript in preparation). Single endpoint determinations would simplify the EST considerably and would fulfill the demand for new predictive screens for risk/hazard assessment according to the new chemical legislation, i.e. the REACH system, of the EU.

4 Outlook

In the field of consumer health protection an increasing interest is directed towards risk assessment. A broad range of compounds on the European market has to be re-evaluated, particularly with regard to their toxicological properties. The approach of a new chemical legislation in the European Union (REACH system) will force every producing company to perform toxicological data that allow a critical estimation.

An important objective for safety assessment of chemicals and drugs is to evaluate adverse effects on reproduction and embryonic development. The complexity of the reproductive system and the multiple targets for exogenic induction of malformations during embryonic development are the rationale for highly standardised animal experiments, e.g. screening tests or multigenerational studies, according to OECD test guidelines. Considerable efforts have been made in the past to reduce these animal experi-

ments by developing new alternatives for embryotoxicity testing *in vitro*.

The REACH system is an ambitious program to realise a toxicological re-evaluation of 30,000 chemicals that are currently on the market. But the estimation of the number of laboratory animals needed for a conventional implementation of existing OECD guidelines is dramatically high. Thus, testing these chemicals can hardly be accommodated without the use of predictive cell-based screening assays.

To date, the EST is the only scientifically validated mammalian *in vitro* system for developmental toxicology that does not require embryonic cells, tissues or organs from pregnant animals. However, this assay only considers embryotoxic effects of chemicals that have an impact on early embryogenesis. However, the identification of compounds that show adverse effects at this stage of embryonic development has high priority. According to the ECVAM Scientific Advisory Committee (ESAC) the EST is a scientifically validated test which is ready to be considered for regulatory purposes (Balls and Hellsten, 2002)

As presented at the 11th Congress on Alternatives to Animal Testing (A-Linz, 2003), the EST protocol has been improved considerably:

1. The quantification of tissue-specific proteins by FACS analysis provides a new, easily detectable and objective endpoint for embryotoxicity in the EST. FACS analysis can be automated, which is an important prerequisite to applying this method in high-throughput screening systems. Furthermore, by detecting molecular endpoints of differentiation, the assay duration could be reduced to 7 days in comparison to 10 days for the microscopic analysis.
2. The differentiation of ES cells into neural and endothelial cells, chondrocytes and osteoblasts, and the integration of these differentiation pathways into the EST protocol will expand the number of relevant endpoints considerably. This is especially important for test compounds that affect cell types other than cardiomyocytes. The implementation of new toxicological endpoints will lead to a more sensitive and reliable screening method to predict developmental toxicity *in vivo* from *in vitro* data.
3. New biostatistical analyses indicate that the EST may be simplified considerably by applying single stem cell-derived endpoints. Newly developed PMs based on single endpoint values will be of great interest with regard to a simple high-throughput screening method for candidates in drug development.

However, there are still some unresolved problems. For regulatory acceptance, the EST requires the enlargement of the database and the consolidation of the existing PM. Thus, more chemicals exhibiting different embryotoxic potential have to be tested and more data has to be collected. Furthermore, in its present form the EST is only applicable for compounds that do not need metabolic activation. However, the metabolic (in-)activation of xenobiotics can be an important prerequisite for proper determination of toxic actions of chemicals. The introduction of a metabolic system as an adjunct to the validated EST protocol is necessary. To improve the precision of the EST, particularly to prevent false negative classifications, more cell type-specific endpoints of differentiation have to be added to the validated EST protocol. A "Multiple Endpoint EST" designed in a hierarchical structure must be developed. In this procedure, differentiated myocardial cells would be the first cell type to be analysed followed by others, for example endothelial and neural cells. Given this, more PMs have to be developed for these complex test systems.

In conclusion, an improved and expanded EST protocol promises to be the first *in vitro* assay for developmental toxicology to be accepted by regulatory authorities. Furthermore, implementation of the EST in regulatory test guidelines would demonstrate the importance of *in vitro* assays as valuable components of the risk/hazard assessment process.

References

Bader, D., Masaki, T. and Fischman, D. A. (1982). Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol. 95*, 763-770.

Balls, M. and Hellsten, E. (2002). Statement of the scientific validity of the embryonic stem cell test (EST) – an in vitro test for embryotoxicity. – Statement of the scientific validity of the micromass test – an in vitro test for embryotoxicity. – Statement of the scientific validity of the post-implantation rat whole embryo culture assay – an in vitro test for embryotoxicity. *Altern. Lab. Anim. 30*, 265-273.

Commission of the European Communities, February 27, 2001. White Paper, Strategy for a Future Chemicals Policy, http://europa.eu.int/comm/environment/chemicals/01888_en.pdf.

Commission of the European Communities, May 7, 2003. Registration, Evaluation, Authorization of chemicals, http://europa.eu.int/comm/enterprise/chemicals/chempol/whitepaper/reach.htm.

Debus, E., Weber, K. and Osborn, M. (1983). Monoclonal antibodies specific for glial fibrillary acidic (GFA) protein and for each of the neurofilament triplet polypeptides. *Differentiation 25*, 193-203.

Doetschmann, T., Eistetter, H. R., Schmidt, W. and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol. 87*, 27-45.

Genschow, E., Spielmann, H., Scholz, G. et al. (2004). Validation of the embryonic stem cell test (EST) in the ECVAM international validation study on in vitro embryotoxicity. (*ATLA*, in press).

Genschow, E., Spielmann, H., Scholz, G. et al. (2002). The ECVAM international validation study on in vitro embryotoxicity tests. Results of the definitive phase and evaluation of prediction models. *ATLA 30*, 151-176.

Goncharova, E. J., Kam, Z. and Geiger, B. (1992). The involvement of adherens junction components in myofibrillo-genesis in cultured cardiac myocytes. *Development 114*, 173-183.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. *Science 277*, 48-50.

Hescheler, J., Fleischmann, B. K., Lentini, S. et al. (1997). Embryonic stem cells: A model to study structural and functional properties in cardiomyogenesis. *Cardiovasc. Res. 36*, 149-162.

Kramer, J., Hegert, C., Guan, K. et al. (2000). Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. *Mech. Dev. 92*, 193-205.

Maltsev, V. A., Wobus, A. M., Rohwedel, J. et al. (1994). Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ. Res. 75*, 233-244.

Millauer, B., Wizigmann-Voos, S., Schnurch, H. et al. (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell 72*, 835-846.

Nishikawa, S. I., Nishikawa, S., Kawamoto, H. et al. (1998). In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity 8*, 761-769.

OECD (1983). OECD Guidelines for the Testing of Chemicals. No. 415: One-generation Reproduction Toxicity Study, 8 pp. Paris, France: Organisation for Economic Cooperation and Development.

OECD (1995). OECD Guidelines for the Testing of Chemicals. No. 421: Reproduction/Development Toxicity Screening Test, 10 pp. Paris, France: Organisation for Economic Cooperation and Development.

OECD (1996). OECD Guidelines for the Testing of Chemicals. No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, 14 pp. Paris, France: Organisation for Economic Cooperation and Development.

OECD (2001). OECD Guidelines for the Testing of Chemicals. No. 414: Prenatal Developmental Toxicity Study, 6 pp. Paris, France: Organisation for Economic Cooperation and Development.

OECD (2001). OECD Guidelines for the Testing of Chemicals. No. 416: Two-generation Reproduction Toxicity Study, 8 pp. Paris, France: Organisation for Economic Cooperation and Development.

Okabe, S., Forsberg-Nilsson, K., Spiro, A. C. et al. (1996). Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech. Dev. 59*, 89-102.

Rudnicki, M. A. and Mc Burney, M. W. (1987). Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In E. J. Robertson (Ed). *Teratocarcinoma and Embryonic Stem Cells: A Practical Approach* (19-49). Washington D.C.: IRL Press.

Seiler, A., Visan, A., Buesen, R. et al. (2004). Improvement of an in vitro stem cell assay (EST) for developmental toxicity by establishing molecular endpoints of tissue-specific development. (*Reproductive Toxicology*, in press).

Seiler, A., Visan, A., Pohl, I. et al. (2002). Etablierung molekularer Endpunkte zur Weiterentwicklung des Embryonalen Stammzelltests (EST) mit embryonalen Stammzellen der Maus (Zelllinie D3). *ALTEX 19*, 3-11.

Spielmann, H., Pohl, I., Döring, B. et al. (1997). The embryonic stem cell test (EST), an in vitro embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem cells. *In vitro Toxicol. 10*, 119-127.

Tucker, R. P. (1990). The roles of microtubule-associated proteins in brain morphogenesis: A review. *Brain Res. Rev. 15*, 101-120.

Vittet, D., Prandini, M. H., Berthier, R. et al. (1996). Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood 88*, 3424-3431.

Williams, R. L., Hilton, D. J., Pease, S. et al. (1988). Myeloid leucemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature 336*, 684-687.

Zur Nieden, N. I., Kempka, G. and Ahr, H. J. (2003). In vitro differentiation of embryonic stem cells into mineralized osteoblasts. *Differentiation 71*, 18-27.

Acknowledgement

We gratefully acknowledge funding of this project by the Federal Ministry of Education and Research (BMBF; grant no.: 0312312), Bonn, Germany.

Correspondence to

Dr. Andrea Seiler
ZEBET im BfR
Diederdorfer Weg 1
D-12277 Berlin
Phone: +49-30-8412 2278
Fax: +49-30-8412 2958
e-mail: seiler.zebet@bfr.bund.de