

Safety Evaluation Study Using Embryonic Stem (ES) Cells

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Embryonic stem (ES) cells are pluripotent stem cells that have the capacity for self-renewal and multilineage differentiation, and they have recently started to be used in the safety evaluation of chemicals. The novel *in vitro* embryotoxicity test (EST) that utilized the differentiation ability of mouse ES cells into cardiomyocytes was established in Germany. We could obtain results which were equal to the validation study performed in Europe and will apply the test system to a preliminary assessment of new chemicals. In addition, we have participated in one of the national projects to improve this test system to make it much simpler and more precise.

This paper is translated from R&D Report, "SUMITOMO KAGAKU", vol. 2007-I.

Embryonic Stem Cells (ES Cells)

1. What Are ES Cells?

Ontogenesis in mammals begins with a zygote where sperm has fertilized an egg. Embryonic stem cells (ES cells in the following) are a cell line established by collecting tissue (inner cell mass) that will become an embryo from the blastocyst formed from the division of a zygote in this ontogenesis and culturing it in a Petri dish (Fig. 1). This was first established with mice¹⁾ in 1981, and it was originally used mainly as a tool for producing genetically modified animals. However, since it was successfully established for humans²⁾ in 1998, there have been expectations for applications in regenerative medicine along with developments in techniques for inducing differentiation, and we have seen remarkable developments in the research.

ES cells have the two characteristics of "self-renewal," where they are capable of unlimited, undifferentiated proliferation, and "multilineage differentiation" where they can differentiate into all kinds of tissue cells that constitute the body (Fig. 2). At present, the applications in regenerative medicine are the field that is attracting the most attention, but they are used in a wide range of research fields, such as fundamental research in the field of developmental biology, pharmacology that uses differentiated cells, and develop-

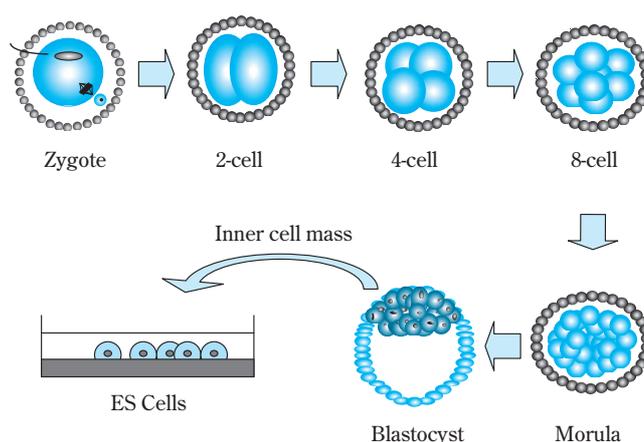


Fig. 1 Generation of the ES cell line

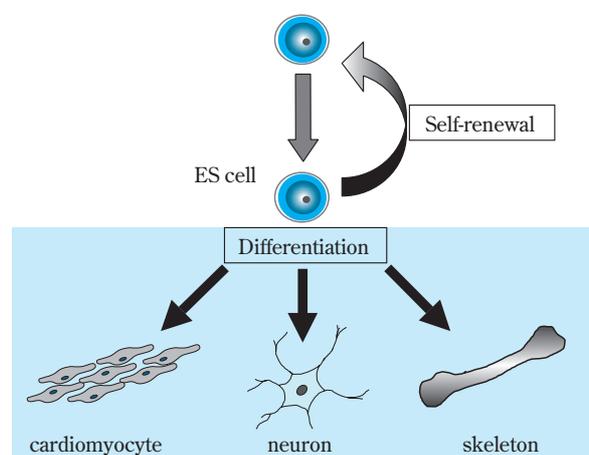


Fig. 2 Pluripotency of ES cells

mental toxicology that uses a differentiation process and which will be introduced in this paper.

2. Usefulness of Mouse ES Cells

Up to this point in time, there have been reports of the establishment of ES cell lines for many species of animals, but the most general-purpose ones are mouse ES cells. The reasons that can be cited for this are (1) that the cell culturing methods that are used have been established for a long time as a tool for fundamental research, (2) that cell lines have already been disseminated on a commercial basis and are easily acquired, and (3) that they are necessary for producing genetically modified mice. Since ES cells cannot return to their original undifferentiated state once they have been differentiated, the most care must be given to maintaining the undifferentiated state when they are cultured. The mechanism for keeping the mouse ES cells from differentiation has basically been explicated, and by adding a factor known as leukemia inhibitory factor (LIF) to the culture medium, it is possible to efficiently maintain the undifferentiated state. On the other hand, there are many points that are still unknown about that mechanism for ES cells from monkeys and humans, and at present, the undifferentiated state cannot be maintained just by adding an external factor; they are kept undifferentiated by coculturing with feeder cells.

Use in genetically modified mice can be cited as the technology that the creation of mouse ES cells has contributed the most to. For example, it is possible to create knockout mice by returning recombinant ES cells in which a gene with an unknown function has been knocked out to mouse embryos. Even though mice are small, a lot of information can be obtained from animals in terms of the physiological changes and their behavioral changes, and they can be thought of as an animal

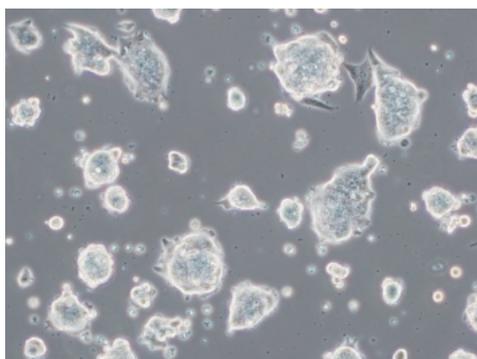


Fig. 3 Undifferentiated mouse ES cells

species that is suitable for the functional analysis of unknown genes.

Research using monkey or human cells is necessary for making ES cells practical in the field of regenerative medicine, but even at present the fundamental technology has been developed with mice because of the usefulness of the mouse ES cells, and the techniques where the knowledge that has been discovered is applied to primates, including humans, have become worldwide standards. (Fig. 3 : Undifferentiated mouse ES cells.)

Current State of *In Vitro* Toxicity Tests

1. Current State of *In Vitro* Testing in Safety Evaluations

Pharmaceutical agents, agricultural chemicals, cosmetics, industrial products and a wide variety of other chemical substances are present all around us, but to evaluate the safety of these for humans many toxicity tests that use laboratory animals are necessary at present. The items that are evaluated are diverse, and various effects, such as carcinogenicity, irritation, sensitization and reproduction, are actually investigated; those for which safe uses can be ascertained appear in the world as products. For these animal tests there are so-called *in vitro* test methods that use animal cells and microorganisms. It goes without saying that it is desirable to establish *in vitro* test methods as an alternative to animal tests, but test methods where evaluations can be made with small amounts of compounds in a short time make a large contribution to reducing development time and increasing efficiency. In addition, we can consider improvements in predictability in humans by using human cells and usefulness in clarifying toxicity mechanisms, since evaluations can be narrowed down to particular tissues or cells, as merits of *in vitro* test systems.

Research has been actively moved forward in Europe and the United States with the goal of developing alternative testing methods, and in terms of the public promotional organizations, the European Centre for the Validation of Alternative Methods (ECVAM) was established in 1991 in Europe, and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in the United States in 1993. They are developing alternative methods for various toxicity tests and carrying out validations and scientific evaluations. Up to this point in time

alternative methods using cells have been developed for three test systems (skin corrosion, phototoxicity and skin absorption) have been developed mainly targeting cosmetic materials, and they have been approved as Organization for Economic Cooperation and Development (OECD) test guidelines (Table 1). On the other hand, the Japanese Center for the Validation of Alternative Methods (JaCVAM) was established in 2005 in Japan as an organization for evaluating research into alternative methods.

Table 1 Evaluations of alternative (non-animal) test by OECD

Toxicity studies	OECD Test Guidelines (<i>In vitro</i>)
Skin Corrosion	No.430
	<i>In vitro</i> Transcutaneous Electrical Resistance
	No.431
	Human Skin Model Test
Phototoxicity	No.435
	<i>In vitro</i> Membrane Barrier Method for Skin Corrosion
Skin Absorption	No.428
	<i>In vitro</i> Method
Eye Irritation/Corrosion	— (unacceptable)
Acute Toxicity	— (unacceptable)
Repeated Dose Toxicity	— (unacceptable)
Carcinogenicity	— (unacceptable)
Teratology	— (unacceptable)
Reproductive Toxicity	— (unacceptable)

Table 2 *In vitro* embryonic toxicity studies

1. Mammals

	Study	Material	Index	Reference	
Cells	Cell Lines	MOT Assay	Ascitic mouse ovarian tumour cells	Inhibition of cell attachment to lectin-coated surfaces	Braun et al., 1982
		HEPM Assay	Human embryonic palatal mesenchyme cells	Cell proliferation	Pratt et al., 1985
		Gap junction	Chinese hamster ovary cells	Inhibition of gap junction	Trosko et al., 1982
		Embryonic Stem cell test	Mouse embryonic stem cells Mouse fibroblast cells	Cardiomyocyte differentiation Cell proliferation	Spielmann et al., 1997
Primary Cultures	Micromass culture	Rat embryo (limb bud)	Cartilage differentiation	Flint et al., 1984	
			Cell proliferation		
Organs	Palate culture Limb bud culture	Mouse embryo (palate) Mouse embryo (limb bud)	Fusion of secondary palate	Shiota et al., 1990	
			Cartilage differentiation Morphology	Freedman et al., 1982	
Whole embryo culture	Whole embryo culture	Rat embryo	Morphogenetic differentiation	Schmid et al., 1985	

2. Non-mammals (1970s – 1980s)

(1) Vertebrates

Fish, Avian or Frog embryo

(2) Invertebrates

Drosophila, Hydra, Sea urchin

2. Current State of *In Vitro* Testing in Reproductive Toxicity Testing

Among the safety evaluations, the items targeted by reproductive toxicity tests are wide ranging, going beyond the periods of formation of gametes (sperm and eggs) and reproductive function (conception, maintenance of pregnancy, delivery and nursing) as well as the succeeding periods of development and growth of pups. With the thalidomide disaster in 1961 that had phocomelia as its cardinal sign, the importance of reproductive toxicity tests when approving chemical compounds was confirmed by various countries around the world. In particular, teratogenicity tests, which detect morphological abnormalities in the next generation (fetus), are considered to be the most important among the reproductive toxicity tests. The fetus in the uterus is intimately connected to the maternal body through the placenta, and the development of the fetus is influenced by the metabolism and physiological changes of the mother, so there are limits to evaluations in simple *in vitro* systems. However, because of this importance as an item for evaluation, various methods had been proposed for a long time (Table 2) because *in vitro* test systems are extremely effective for preliminary assessment of new chemicals, if they can be used in screening in the initial stages of development.

Among the test systems that use mammalian cells and tissues, embryonic stem cell tests (EST) that use

the mouse ES cells, micromass cultures that use the limb buds of rat embryos and whole embryo cultures using early embryos of rats have been validated in Europe.³⁾ Examinations have also been done using non-mammals such as birds (chickens) and amphibians (frogs), and even further using much lower order animals such as insects (fruit flies) and flatworms (hydra), which are in invertebrates. The reason that lower order animals are used for predictions in higher order mammals is that many mechanisms that are common to all living creatures exist in the initial processes of ontogenesis, and with animal species that have a rapid alternation of generations, observations that cross generations are possible in a short period of time.

In Vitro Embryotoxicity Test (EST) Using Mouse ES Cells

1. EST Utility

In vitro embryotoxicity tests (EST in the following) using mouse ES cells have focused on the differentiation capabilities that ES cells have and were proposed by Dr. H. Spielmann et al.⁴⁾ of the Bundesinstitut für Riskobewertung (BfR), the Federal Institute for Risk Assessment in Germany. While conventional tests required animal embryos and fetal tissue for the test materials, EST is an alternative method that can be carried out only using cultured cells. Therefore, it has the merit of being able to thaw out the cells that have been frozen for storage and start the tests rapidly when necessary.

One other characteristic not found in conventional test systems is using two types of cells that have different properties. As was described earlier, since there is

an intimate relationship between the fetus and the mother, the supply of nutrients necessary for ontogenesis will become insufficient if the physiological state of the mother is made to deteriorate, so normal development will be impossible. Therefore, if both the effects on the mother and the effects on the fetus can be predicted in an *in vitro* system, the test system will have higher precision.

With EST, we assume the ES cells, which are immature cells, to be the “fetus” in animal experiments, and the fibroblast cells, which are differentiated cells, to be the “pregnant animal” in animal experiments. By comparing the effects on both types of cells in cultures, it is possible to ascertain the difference in sensitivity between the mother and the fetus.

2. Outline of Protocol

The two types of cell lines used in the tests are the mouse ES cell line (D3) and the mouse fibroblast cell line (Balb/c 3T3). The three endpoints in this test system are (1) the effects on differentiation of ES cells into contracting cardiomyocytes, (2) effects on the proliferation of ES cells and (3) effects on the proliferation of fibroblasts. An outline of the test procedure is given in the following.

(1) Differentiation into Cardiomyocytes (Fig. 4)

The hanging drop culture is performed as a first step. After isolating the undifferentiated ES cells into single cells by enzymatic digestion, drops of ES cell suspension in medium are placed on the inner side of the lid of the Petri dish (each drop containing approximately 750 cells). By this means the cells gradually aggregate in a single location due to gravity without coming into contact with the side sur-

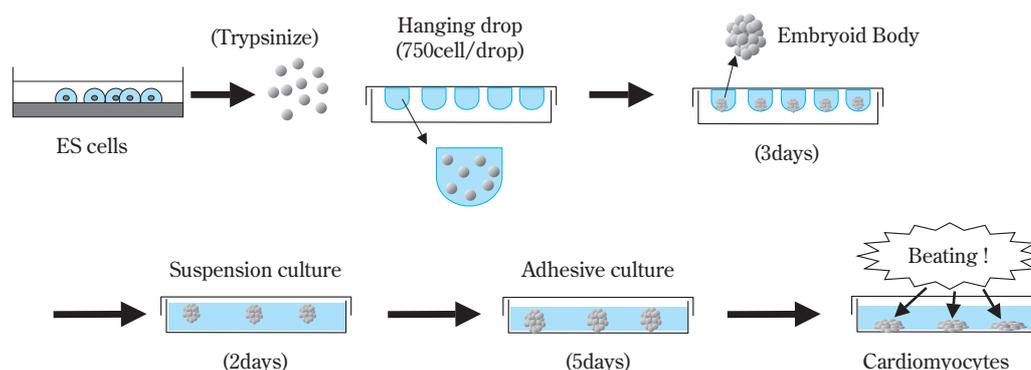


Fig. 4 Differentiation into cardiomyocyte

faces of the Petri dish, and aggregates called embryoid bodies are formed where there is differentiation into three embryonic germ layers (endoderm, mesoderm, endoderm) after three days. After another two days where this embryoid bodies are cultured in a suspension in a non-adhesive system in a Petri dish, the cell masses are transferred to a Petri dish with an adhesive system. After 10 days from the start of the hanging drop culture, the contracting cardiomyocytes are confirmed under a microscope.

(2) Cell Proliferation (Fig. 5)

After undifferentiated ES cells or fibroblasts are isolated into single cells by enzymatic digestion, approximately 500 cells are cultured in a small volume medium. After 10 days from the start of the culture, the number of cells in the medium is calculated using intracellular reductase on the final day after going through two medium exchanges.

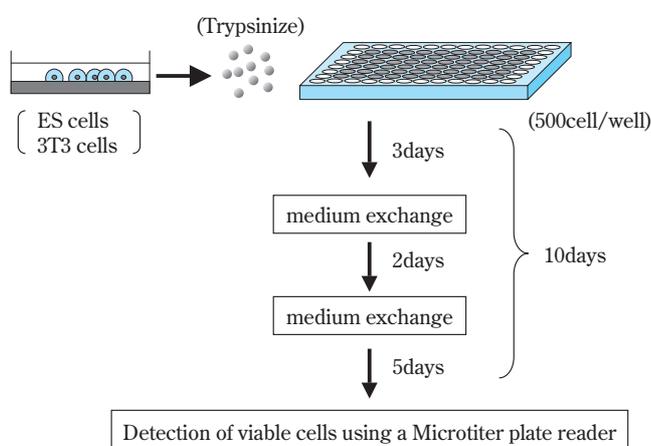


Fig. 5 Cytotoxicity Assay (ES and 3T3 cells)

(3) Experiments Using Chemical Compounds

The test chemical compound is added through the entire period from the beginning of the experiment to both the cardiomyocyte differentiation and the cell proliferation. Multiple concentrations are set for the chemical compounds, and the 50% inhibiting chemical compound concentrations, ID50 (cardiomyocyte differentiation) and IC50 (ES cell and fibroblast proliferation) are found in comparison with a control group in each of the experiments. Fifty percent inhibition is the

Function I	$5.92 \times \log(\text{IC50 } 3\text{T3}) + 3.50 \times \log(\text{IC50 } \text{D3}) - 5.31 \times \left(\frac{\text{IC50 } 3\text{T3} - \text{ID50}}{\text{IC50 } 3\text{T3}} \right) - 15.7$
Function II	$3.65 \times \log(\text{IC50 } 3\text{T3}) + 2.39 \times \log(\text{IC50 } \text{D3}) - 2.03 \times \left(\frac{\text{IC50 } 3\text{T3} - \text{ID50}}{\text{IC50 } 3\text{T3}} \right) - 6.85$
Function III	$-0.125 \times \log(\text{IC50 } 3\text{T3}) - 1.92 \times \log(\text{IC50 } \text{D3}) + 1.50 \times \left(\frac{\text{IC50 } 3\text{T3} - \text{ID50}}{\text{IC50 } 3\text{T3}} \right) - 2.67$

IC50 : 50% cytotoxic concentration, ID50 : 50% differentiation concentration
3T3 : mouse fibroblast cell line, D3 : mouse embryonic stem cell line

Non- embryotoxic	Function I > Function II, Function III
Weakly embryotoxic	Function II > Function I, Function III
Strong embryotoxic	Function III > Function I, Function II

Fig. 6 Prediction model of the EST

chemical compound concentration where the number of cell masses where cell contractions are found in the cardiomyocyte differentiations is half that of the control group and the chemical compound concentration with a number of viable cells is half that of the control group in cell proliferation. The three values that are obtained are substituted into the prediction model (Fig. 6), and the presence or absence of embryotoxicity is classified into three criteria (not embryotoxic and strong embryotoxic).

3. Accuracy of Tests

As described above, the validation study was conducted for confirming the accuracy of EST using ES cells, micromass cultures and whole embryo cultures. This validation study was carried out in 1996 -2000 as a single project with pharmaceutical companies, public institutions and the like in various European countries participating, with ECVAM as the overseeing facility. Six to seven chemical compounds were selected in each of the three classification criteria described above for the compounds used in validation study, and the study was carried out with a total of 20 chemical compounds. The results of the study were that in the aspect of detection sensitivity, it exhibited a detection sensitivity equal to that of conventional methods regardless of the fact that EST is a method that does not use animals at all. The concordance rate with *in vivo* animal tests obtained in the validation results were around 70 - 80%, and while, individually, the compounds which were classified as having a embryotoxicity of “weak” and “strong” were 84% and 83%, it was found that compounds not having embryotoxicity were at 68%, a tendency to be somewhat low (Fig. 7).

<i>in vivo</i> (animal study)		<i>in vitro</i> (EST)		
		Non-embryotoxic	Weakly embryotoxic	Strong embryotoxic
ECVAM	Non-embryotoxic	68%	32%	0%
	Weakly embryotoxic	16%	84%	0%
	Strong embryotoxic	10%	6%	83%
Sumitomo	Non-embryotoxic	50%	50%	0%
	Weakly embryotoxic	17%	83%	0%
	Strong embryotoxic	0%	0%	100%

in vivo = *in vitro*

Fig. 7 The validation results in ECVAM & Sumitomo-chemical

4. EST Evaluation

While, upon receiving the validation study results, the ECVAM Scientific Advisory Committee (ESAC), which is a consultative body of ECVAM, gave approval as an alternative test method, it has not gone to the point where the more global OECD test guidelines have been created for EST. In the ECVAM workshop that was held in 2003, some problems were pointed out.⁵⁾ In other words, the point that validation with industrial chemicals should be carried out for improving the predictive equations for increasing accuracy and because the chemical compounds used in the validation study was mostly pharmaceutical products; the point that most of the teratogenic substances used in the validation had strong cytotoxicity and validation should be carried out with teratogenic substances that have other mechanisms; the point that systems incorporating metabolism should be constructed, and the point that systems inducing differentiation into cells, such as nerve cells and bone, other than cardiomyocytes should be attempted were cited as problems.

5. Status of Work on EST at Sumitomo Chemical

After the introduction of the EST at Sumitomo Chemical, we carried out a validation study internally using commercial chemicals used in the European validation study.

In the European verification tests there were 20 chemical compounds, but we carried out the tests at Sumitomo Chemical using 17 among them that were on the market. In the results, the rate of agreement with *in vivo* animal tests, was 50% for substances not having embryotoxicity, and 83% and 100% for substances with weak embryotoxicity and strong embryotoxicity, and the results were comparable to those

obtained in the European validation study (Fig. 7). At present, we are carrying out additional tests using commercial products (pharmaceuticals, agricultural chemicals and industrial chemicals) for which teratogenicity data is known to confirm the detection sensitivity for chemical compounds having a wide range of actions, and we are currently confirming the detection sensitivity of EST and its characteristics.

Participation in National Project

In addition to the internal investigations into EST for *in vitro* detection systems for developmental toxicity, we are participating in a national project, and we are working on establishing a simple but high precision test system using a mouse ES cells.

1. Overview of National Project

The New Energy and Industrial Technology Development Organization (NEDO in the following) has started a project for “Development of methods for evaluating harmful effects using cultured cells,” planned for five years starting in 2006. In this project, three fields (teratogenicity, immunotoxicity and carcinogenicity) have been selected from among the toxicity tests that require high costs and long periods of time, and it aims to establish a test system that effectively detects toxicity in a short period of time using cultured cells.

2. Overview of Plan

The basic concepts of this project are “simple” and “high precision.” In this project, ES cells are differentiated into cardiomyocytes as with EST, but where the presence or absence of differentiation was confirmed in EST using a microscope, the gene for the light emitting enzyme in fireflies, which is called luciferase, is transfected into the ES cells in this project and detection is done using an automated system where in addition to “presence or absence” there is “degree” with the strength of the light emission as an indicator. Furthermore, using a multicolored luciferase reporter genes, which is technology developed by the National Institute of Advanced Industrial Science and Technology and Toyobo Co., Ltd., which are joint researchers, there is the goal of introducing technology for having multiple genes related to proliferation and differentiation emit light simultaneously and establishing an even more simple test system. In addition, in the selection of

differentiation marker genes, there is a possibility that a new marker with a sensitivity unknown up to this point can be discovered by comprehensive analysis of gene expressions that change in the differentiation process using a DNA chip. Furthermore, Sumitomo Chemical already has techniques for differentiating mouse ES cells into cardiomyocytes, nerve cells and osteoblasts, and through differentiations to various types of cells rather than cardiomyocytes only, we expect that we can construct a highly precise detection system.

Future Outlook

ES cells were first introduced in developmental toxicity tests among the various types of toxicity tests, and an *in vitro* test (EST) was established. Validation of EST was carried out in Europe, and it was possible to predict the results of *in vivo* animal tests with a probability of 70 - 80% with detection in the limited number of 20 chemical compounds. However, several problems have been brought out with the current method, and investigations are continuing within the ReProTect Project⁶⁾ that started in 2004 for this purpose. The *in vitro* test methods that are incorporated into the OECD guidelines currently only evaluate local effects *in vivo*, and no repeated dose toxicity or reproductive development toxicity for evaluations that include the systemic effects have been approved. However, the differentiation process for cells in the EST introduced in this paper has been reproduced in an *ex vivo* environment. Furthermore, the proliferation characteristics of the immature ES cells and mature cells are being compared, and *in vivo* evaluation systems that use animals are being modeled theoretically. The settings for use are limited, but it can be assumed that it will become a useful tool for chemical compound screening and elucidating toxicity mechanisms.

At Sumitomo Chemical, we are aiming at establishing highly accurate test methods that make use of ES cells while being active in the national project. After they are established, we can assume that they will be used internally in screening systems for chemical compounds, and that they will be useful in the early selec-

tion of candidate chemical compounds. With regard to the field other than developmental toxicity on the other hand, systems for preparing the specific differentiated cells on a large scale and detecting the effects on various types of cells can be established using the characteristics of ES cells.

We can think in terms of applications in large-scale assay systems by having a high throughput screening (HTS) and being able to use in the elucidation of toxicity mechanisms. By introducing primate cells in the future, we will work on bringing the construction of evaluation systems that improve the predictability for humans into our field of vision.

Acknowledgments

We would like to thank Dr. H. Spielmann of Bundesinstitut für Riskobewertung (BfR) as well as Dr. A. Seiler and Dr. K. Hayess of the Centre for Documentation and Evaluation of Alternatives to Animal Experiments (zebet) in BfR for their technical support at the time of the introduction of embryonic stem cell tests (EST) at Sumitomo Chemical.

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