Letter

Assessment of technical protocols for novel embryonic stem cell tests with molecular markers (Hand1- and Cmya1-ESTs): a preliminary cross-laboratory performance analysis

Noriyuki Suzuki¹, Norihisa Yamashita¹, Naoteru Koseki², Toru Yamada², Yutaka Kimura³, Setsuya Aiba³, Tomoyasu Toyoizumi⁴, Mika Watanabe⁴, Ryo Ohta⁴, Noriho Tanaka⁴ and Koichi Saito¹

¹Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., 1-98, Kasugadenaka 3-chome, Konohana-ku, Osaka 554-8558, Japan

²Safety Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., 3-1-98 Kasugade-naka, Konohana-ku, Osaka 554-0022, Japan

³Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8574, Japan

⁴Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan

(Received March 2, 2012; Accepted June 4, 2012)

ABSTRACT — The Hand1- and Cmya1-ESTs are novel short-term tests for embryotoxic chemicals using genetically engineering mouse ES cells for luciferase reporter gene assays. These ESTs allow convenient determination of differentiation toxicity and cell viability in a short duration with high throughput 96-well microplates for prediction of embryotoxicity of chemicals. To assess the Hand1-EST technical protocol, we firstly compared reporter gene assay and cytotoxicity test data for a representative compound (hydroxyurea) from four different laboratories with tests carried out under the same experimental conditions. Extensive investigations of the Hand1- and Cmya1-ESTs were then performed to explore reproducibility by comparing a set of 6 well-known test chemicals, including hydroxyurea, across the laboratories. The results gave good correspondence in all four laboratories, indicating that transferability, intra-laboratory variability and inter-laboratory variability of the present technical protocols of the ESTs were sufficient to conduct further validation studies.

Key words: In vitro, Embryotoxicity, Embryonic stem cell test, Embryonic stem cells, Differentiation, Reporter gene assay

INTRODUCTION

Embryonic stem cells (ESCs) can be maintained undifferentiated in culture where they may differentiate into ectodermal, mesodermal and endodermal lineages. Since they thus follow the same developmental pathways as the developing embryo, this provides a theoretical basis for differentiating ESCs being used as effective tools to analyze embryotoxic compounds *in vitro*. The embryonic stem cell test (EST) was developed as an *in vitro* method for detection of embryotoxic hazards, based on three endpoints, i.e. cytotoxicity with ESCs and 3T3 fibroblasts in addition to ESC cardiac differentiation inhibition (Spielmann *et al.*, 1997). Recently, embryotoxicity of a variety of compounds like pharmaceuticals (Paquette *et*

al., 2008; Eckardt and Stahlmann, 2010), cosmetics (Chen *et al.*, 2010), industrial chemicals (de Jong *et al.*, 2009) and nanoparticles (di Guglielmo *et al.*, 2010), has been assessed with the EST. Although it is considered a promising *in vitro* test for developmental toxicity, there are also increasing demands for *in vitro* alternatives that can provide a less expensive and higher through-put means for testing. For screening purposes, the current experimental procedure of the EST with visual assessment of contracting cardiomyocytes requires extensive experimental expertise and is time-consuming with a rather long test duration of 10 days. Previously, we identified several marker genes as specifically altered by embryotoxic chemicals (Suzuki *et al.*, 2011a) and also reported the establishment of 96 well multi-plate based new ESTs with

Correspondence: Koichi Saito (E-mail: saitok5@sc.sumitomo-chem.co.jp)

luciferase reporter assays using stable transgenic ESCs for detecting molecular markers (Hand1: heart and neural crest derivatives expressed transcript 1 or Cmya1: cardiomyopathy associated 1) (Suzuki *et al.*, 2011b). The newly developed Hand1- and Cmya1- ESTs provide new rapid and sensitive *in vitro* methods for screening embryotoxics. In the present study, we performed a preliminary cross-laboratory performance analysis of Hand1- and Cmya1- ESTs. The objectives were to confirm transferability, intra-laboratory variability and inter-laboratory variability of their technical protocols in four independent laboratories to conduct further validation studies for development of an OECD test guideline with Hand1- or Cmya1-ESTs methods.

MATERIALS AND METHODS

Collaborating laboratories

Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd. (the lead laboratory), Safety Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Tohoku University Graduate School of Medicine and Hatano Research Institute, Food and Drug Safety Center, participated in the present study.

Chemicals

The test chemicals, hydroxyurea (HU), 6-aminonicotinamide (6-AN), boric acid (BA), valproic acid sodium salt (VAP), penicillin G sodium salt (PenG) and ascorbic acid (AsA), used in the ECVAM international validation study (Genschow *et al.*, 2004) were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in appropriate solvents, that is, PBS, dimethyl sulfoxide (DMSO). Purity of test chemicals was more than 98.0%. All reagents, chemicals and culture media used in the present study were purchased by the lead laboratory and distributed to the other three participants.

Hand1- and Cmya1-ESTs

Differentiation toxicity was analyzed by detecting chemical-related changes in Hand1 and Cmya1 promoter derived luminescence during the differentiation of Hand1- and Cmya1-ES cells into cardiomyocytes, as described in a previous report (Suzuki *et al.*, 2011b). Briefly, cells in 50 μ l of differentiation medium (750 cells/well) were seeded in white round bottom low attachment 96-well plates (Sumitomo Bakelite, Co. Ltd., Tokyo, Japan). Chemicals dissolved in 50 μ l of differentiation medium were added to the plates ($n = 12$), followed by incubation for 6 days at 37°C under conditions of 5% CO₂ and 95% humidity. Luciferase activity was

measured with differentiated cells lysed by adding 100 μ l/well of Steady-Glo® Luciferase Assay System (Promega, Madison, WI, USA) at room temperature for 30 min with light shaking. Luminous intensity was measured in individual microplate luminescence counters in four laboratories. Inhibition of differentiation was expressed as the concentration of test chemical reducing the luminescence by 50% (Hand1- or Cmya1-ES-ID₅₀, calculated from the concentration-response curve). Cytotoxicity in individual transgenic ES cells was determined using the following method as previously described (Scholz *et al.*, 1999) with minor changes. Cells were seeded into 96-well tissue culture plates (500 cells/well) in differentiation medium with or without test chemicals. Cytotoxicity in BALB/c 3T3 fibroblasts (ATCC) was assessed using the method detailed earlier (Scholz *et al.*, 1999). Viability of transgenic ES cells and BALB/c 3T3 cells was measured on day 6 using the CellTiter Glo® Non-Radioactive Cell Proliferation Assay (Promega), according to the manufacturer's instructions. Cytotoxicity was expressed as the chemical concentration reduces the viability of cells to 50% of the control level (Hand1- or Cmya1-ES-IC₅₀ and 3T3-IC₅₀; determined from concentration-response curves). To predict the embryotoxic potential of the test chemicals into the classes strong, weak and non-embryotoxic, a biostatistically based prediction model (PM) was applied (Genschow *et al.*, 2004). The three endpoints obtained in each experiment were used to calculate Function I, II and III as shown below:

$$\begin{aligned}\text{Function I} &: 5.92 \times \log(3T3-IC_{50}) + 3.50 \times \log(ES-IC_{50}) \\ &\quad - 5.31 \times (3T3-IC_{50} - ES-ID_{50})/3T3-IC_{50} - 15.7. \\ \text{Function II} &: 3.65 \times \log(3T3-IC_{50}) + 2.39 \times \log(ES-IC_{50}) \\ &\quad - 2.03 \times (3T3-IC_{50} - ES-ID_{50})/3T3-IC_{50} - 6.85. \\ \text{Function III} &: -0.125 \times \log(3T3-IC_{50}) - 1.92 \times \log(ES-IC_{50}) \\ &\quad + 1.50 \times (3T3-IC_{50} - ES-ID_{50})/3T3-IC_{50} - 2.67.\end{aligned}$$

The function providing the highest value was used to determine the classification of embryotoxicity as shown below:

Non-embryotoxic potency, if I > II and I > III

Weak-embryotoxic potency, if II > I and II > III

Strong-embryotoxic potency, if III > I and III > II

Strong-embryotoxic potency was defined as developmentally toxic in all species tested, and with a high A/D (adult/developmental toxicity) ratio. Weak-embryotoxic potency was defined as developmentally toxic in multiple (but not all) species, with a high A/D ratio. Finally, non-embryotoxic potency was defined as chemicals not developmentally toxic at maternally toxic exposures (Brown, 2002).

Statistics

Statistical analysis of the dose-response curves from differentiation toxicity and cytotoxicity assays was carried out using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA). Correlations between each endpoint of Hand1- and Cmya1- ESTs in individual laboratories were assessed based on pairwise scatter plots and determination coefficients (R^2) were determined for each endpoint.

RESULTS AND DISCUSSION

In the present studies to examine transferability, intra-laboratory variability and inter-laboratory variability of the technical protocols of Hand1- and Cmya1- ESTs among independent laboratories, differentiation toxicity and cytotoxicity of HU were first assessed. Data for the three endpoints from the four laboratories for Hand1-EST (Hand1-ES- ID₅₀, Hand1-ES-IC₅₀ and 3T3-IC₅₀) are given in Fig. 1. The average and standard deviations of Hand1-ES-ID₅₀, Hand1-ES-IC₅₀ and 3T3-IC₅₀ for combined data for the four laboratories were 3.1 ± 0.3 , 5.4 ± 1.1 and 3.9 ± 0.7 $\mu\text{g/ml}$, respectively. The respective %CVs were 10, 20 and 18%. Thus, the values for each endpoint from the four laboratories were very similar with low variability, indicating high transferability of the new technical protocol.

In the second experiment, the three endpoints for two strong-embryotoxic chemicals (HU and 6-AN), two weak-embryotoxic chemicals (BA and VAP) and two non-embryotoxic chemicals (PenG and AsA) were deter-

mined with both Hand1- and Cmya1-ESTs (Table 1). The obtained endpoint values were relatively similar among all four laboratories. Furthermore, when values for HU in the second experiment were compared to those in the first experiment (Fig. 2), they were found to be almost the same in the four laboratories, demonstrating low intra-laboratory variability of the Hand1-EST. In the BA and

Fig. 1. Inter-laboratory variability for the three endpoints with hydroxyurea using the Hand1-EST.

Fig. 2. Intra-laboratory variability for the three endpoints for hydroxyurea in each laboratory using the Hand1-EST.

Table 1. Endpoint values of test chemicals in each laboratory with the Hand1- and Cmya1-EST

Chemicals	Abbreviation	Labs	3T3 -IC ₅₀	Hand1-ES -ID ₅₀	Hand1-ES -IC ₅₀	Cmya1-ES -ID ₅₀	Cmya1-ES -IC ₅₀
Hydroxyurea	HU	A	4.08	2.90	5.30	4.26	4.79
		B	4.62	3.22	6.84	7.53	6.87
		C	4.07	1.75	4.05	7.03	6.87
		D	2.95	3.71	5.95	2.62	2.02
6-Aminonicotinamide	6-AN	A	2.26	0.50	1.39	0.90	1.48
		B	2.98	0.34	0.89	0.58	0.91
		C	1.65	0.75	0.69	1.18	0.83
		D	1.63	0.78	0.63	0.66	0.76
Boric acid	BA	A	350.00	27.98	134.39	15.46	234.96
		B	409.27	92.37	144.64	177.30	154.70
		C	437.60	148.40	226.51	152.03	198.10
		D	243.72	32.10	10.06	14.68	135.72
Valproic acid sodium salt	VPA	A	638.69	23.73	364.04	42.33	346.88
		B	577.36	28.43	371.07	115.39	348.03
		C	500.57	84.09	678.02	404.41	672.95
		D	382.99	40.34	108.93	18.82	122.31
Penicillin G sodium salt	PenG	A	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a
		B	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a
		C	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a
		D	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a	> 1000.00 ^a
Ascorbic acid	AsA	A	7.45	21.60	10.44	51.87	22.91
		B	5.98	34.34	20.54	28.28	45.68
		C	60.63	70.25	80.00	80.00	68.78
		D	5.52	1.12	53.39	1.41	43.14

Note. Endpoint values were calculated graphically from the concentration response curves from the mean values of 12-well samples per each concentration.

^a ID₅₀ and IC₅₀ concentrations above 1,000 µg/ml were not determined any more.

VAP exposure cases, tendencies for lower ES-IC₅₀ values were evident in laboratory D with both ESTs. No clear decreases in luciferase activities of Hand1- and Cmya1-ES were observed with PenG exposure up to the concentration of 1,000 µg/ml, so that Hand1- and Cmya1-ES-ID₅₀ values could not be determined. High values for the three endpoints were observed with AsA in laboratory C. To clarify reasons for the variation among the laboratories, correlations between each endpoint of Hand1- and Cmya1-ESTs in individual laboratories were calculated by pairwise scatter plots, determination coefficients (R^2) of individual endpoints then being analyzed (Fig. 3). The values of Hand1-ES-ID₅₀ were almost the same as those of Cmya1-ES-ID₅₀ in all four laboratories ($R^2 = 0.86$ to

1.00). Moreover, the IC₅₀ values were also similar between Hand1-EST and Cmya1-ESTs with the test chemicals ($R^2 = 0.98$ to 1.00). Differences in preparation of test chemicals might be one of the reasons of variation of ES-ID₅₀ and ES-IC₅₀ values among the laboratories.

The test chemicals were here classified by the PM reported previously (Genschow *et al.*, 2004) using three endpoints (Table 2). In our previous study (Suzuki *et al.*, 2011b), HU, a strong embryotoxic compound, was misclassified as “weak” with both ESTs. In the present study, HU was also classified as “weak” with both ESTs in three laboratories. The strong embryotoxic chemical, 6-AN, was predicted to be “strong” by the both ESTs in all laboratories. Two weak embryotoxic chemicals (BA and VPA)

Cross-laboratory performance analysis of novel ESTs

Fig. 3. Correlations between each endpoint of Hand1- and Cmya1-ESTs in individual laboratories, calculated based on pairwise scatter plots and determination coefficients (R^2).

were evaluated to be “Weak” with both ESTs in all laboratories. Of the two non-embryotoxic chemicals, PenG was classified as “Non-” with both ESTs in all laboratories, AsA being classified as “Weak” in two of the four. The same overall accuracy (79.2%) was calculated by comparison between the embryotoxic classification and *in vivo* embryotoxic potential of all the tested agents with Hand1- and Cmya1-ESTs (Table 2). These results indicate that the present technical protocols of the ESTs should present no problems regarding transferability, as well as intra-laboratory and inter-laboratory variability.

The Scientific Advisory Committee of the European Centre for the Validation of Alternative Methods (ECVAM) has validated the EST as an *in vitro* method for detection of embryotoxic hazard, the EST being shown to provide a correct judgment in 78% of all experiments and a predictivity of 100% of strong embryotoxicants using more than 20 reference compounds with different embryotoxic potencies (strong, weak and non-embryotoxic) (Genschow *et al.*, 2004). However, the Committee also requested a follow-up workshop, which led to the rec-

ommendation of further test development before use for regulatory purposes (Spielmann and Vogel, 2006). Optimization for successful regulatory implementation including improvement of the PMs, expansion of the database, adoption of a metabolic system and addition of major target tissue cells to be tested were proposed (Bremer and Hartung, 2004). Various modifications have been applied to analyze molecular markers in the EST, for example, using quantitative real-time PCR analysis (Festag *et al.*, 2007; Pellizzer *et al.*, 2004; zur Nieden *et al.*, 2004). Moreover, FACS-EST methods were developed (Buesen *et al.*, 2009). These modified ESTs still remain to be validated.

In the present study, transferability, intra- and inter-laboratory reproducibility of the ESTs was confirmed in individual four laboratories according to the new technical protocol with Hand1- and Cmya1-EST, although further investigations are now required to refine the PM for evaluation of embryotoxicity. The Hand1- and Cmya1-ESTs allow convenient determination of differentiation toxicity and cell viability in a short duration high-throughput

Table 2. Classification of test chemicals in each laboratory with the Hand1- and Cmya1-ESTs

	Chemicals	Embryotoxic potential <i>in vivo</i>	Laboratories				Concordance (%)	Overall Accuracy (%)
			A	B	C	D		
Hand1-EST	HU	strong	weak	weak	strong	weak	75	79.2
	6-AN	strong	strong	strong	strong	strong	100	
	BA	weak	weak	weak	weak	weak	100	
	VPA	weak	weak	weak	weak	weak	100	
	PenG	non	non	non	non	non	100	
	AsA	non	non	non	weak	weak	50	
Cmya1-EST	HU	strong	weak	weak	weak	strong	75	79.2
	6-AN	strong	strong	strong	strong	strong	100	
	BA	weak	weak	weak	weak	weak	100	
	VPA	weak	weak	weak	weak	weak	100	
	PenG	non	non	non	non	non	100	
	AsA	non	non	non	weak	weak	50	

96-well microplate format by chemiluminescent detection. Therefore it becomes much easier to make expanding database of abundant test chemicals, and modify the prediction method with adoption of a metabolic system using our proposal ESTs. Previously we could successfully identify several marker genes related to embryotoxicity during ES cell differentiation into neural cell (Suzuki *et al.*, 2011a). Additional stable transgenic ES cells for detection of marker gene expression during ES cell differentiation into neural cells by luciferase reporter genes would be useful to evaluate embryotoxicity of central nervous systems.

From the present study, we conclude that the Hand1- and Cmya1-ESTs are transferable across laboratories. Moreover, intra- and inter-laboratory reproducibility using the present technical protocols was confirmed in four individual laboratories. The present findings provide initial evidence that further validation studies are warranted to develop an OECD test guideline with Hand1- or Cmya1-ESTs methods.

ACKNOWLEDGMENT

This study was supported by NEDO (the New Energy and Industrial Technology Development Organization).

REFERENCES

- Bremer, S. and Hartung, T. (2004): The use of embryonic stem cells for regulatory developmental toxicity testing *in vitro* - the current status of test development. *Curr. Pharm. Des.*, **10**, 2733-2747.
- Brown, N.A. (2002): Selection of test chemicals for the ECVAM international validation study on *in vitro* embryotoxicity tests, *ATLA*, **30**, 177-198.
- Buesen, R., Genschow, E., Slawik, B., Visan, A., Spielmann, H., Luch, A. and Seiler, A. (2009): Embryonic stem cell test remastered: comparison between the validated EST and the new molecular FACS-EST for assessing developmental toxicity *in vitro*. *Toxicol. Sci.*, **108**, 389-400.
- Chen, R., Chen, J., Cheng, S., Qin, J., Li, W., Zhang, L., Jiao, H., Yu, X., Zhang, X., Lahn, B.T. and Xiang, A.P. (2010): Assessment of embryotoxicity of compounds in cosmetics by the embryonic stem cell test. *Toxicol. Mech. Methods*, **20**, 112-118.
- de Jong, E., Louisse, J., Verwei, M., Blaauboer, B.J., van de Sandt, J.J., Woutersen, R.A., Rietjens, I.M. and Piersma, A.H. (2009): Relative developmental toxicity of glycol ether alkoxy acid metabolites in the embryonic stem cell test as compared with the *in vivo* potency of their parent compounds. *Toxicol. Sci.*, **110**, 117-124.
- di Guglielmo, C., López, D.R., De Lapuente, J., Mallafre, J.M. and Suárez, M.B. (2010): Embryotoxicity of cobalt ferrite and gold nanoparticles: a first *in vitro* approach. *Reprod. Toxicol.*, **30**, 271-276.
- Eckardt, K. and Stahlmann, R. (2010): Use of two validated *in vitro* tests to assess the embryotoxic potential of mycophenolic acid. *Arch. Toxicol.*, **84**, 37-43.
- Festag, M., Viertel, B., Steinberg, P. and Sehner, C. (2007): An *in vitro* embryotoxicity assay based on the disturbance of the differ-

Cross-laboratory performance analysis of novel ESTs

- entiation of murine embryonic stem cells into endothelial cells. II. Testing of compounds. *Toxicol. In Vitro*, **21**, 1631-1640.
- Genschow, E., Spielmann, H., Scholz, G., Pohl, I., Seiler, A., Clemann, N., Bremer, S. and Becker, K. (2004): Validation of the embryonic stem cell test in the international ECVAM validation study on three *in vitro* embryotoxicity tests. *Altern. Lab. Anim.* **32**, 209-244.
- Paquette, J.A., Kumpf, S.W., Streck, R.D., Thomson, J.J., Chapin, R.E. and Stedman, D.B. (2008): Assessment of the Embryonic Stem Cell Test and application and use in the pharmaceutical industry. *Birth Defects Res. B Dev. Reprod. Toxicol.*, **83**, 104-111.
- Pellizzer, C., Adler, S., Corvi, R., Hartung, T. and Bremer, S. (2004): Monitoring of teratogenic effects *in vitro* by analysing a selected gene expression pattern. *Toxicol. In Vitro*, **18**, 325-335.
- Scholz, G., Pohl, I., Genschow, E., Klemm, M. and Spielmann, H. (1999): Embryotoxicity screening using embryonic stem cells *in vitro*: Correlation to *in vivo* teratogenicity. *Cells Tissues Organs*, **165**, 203-211.
- Spielmann, H., Pohl, I., Döring, B., Liebsch, M. and Moldenhauer, F. (1997): The embryonic stem cell test (EST), an *in vitro* embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem cells. *Toxicol. In Vitro*, **10**, 119-127.
- Spielmann, H. and Vogel, R. (2006): REACH testing requirements must not be driven by reproductive toxicity testing. *Altern. Lab. Anim.*, **34**, 365-366.
- Suzuki, N., Ando, S., Horie, N., Sumida, K. and Saito, K. (2011a): Analysis of altered gene expression specific to embryotoxic chemical treatment during embryonic stem cell differentiation into myocardiac and neural cells. *J. Toxicol. Sci.*, **36**, 569-585.
- Suzuki, N., Ando, S., Yamashita, N., Horie, N. and Saito, K. (2011b): Evaluation of novel high-throughput embryonic stem cell tests with new molecular markers for screening embryotoxic chemicals *in vitro*. *Toxicol. Sci.*, **124**, 460-471.
- zur Nieden, N.I., Kempka, G., and Ahr, H.J. (2004): Molecular multiple endpoint embryonic stem cell test - A possible approach to test for the teratogenic potential of compounds. *Toxicol. Appl. Pharmacol.*, **194**, 257-269.