Original Article

Hand1-Luc Embryonic Stem Cell Test (Hand1-Luc EST): A novel rapid and highly reproducible *in vitro* test for embryotoxicity by measuring cytotoxicity and differentiation toxicity using engineered mouse ES cells

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ABSTRACT — The embryonic stem cell test (EST) is a promising alternative method for evaluating embryotoxicity of test chemicals by measuring cytotoxicity and differentiation toxicity using mouse ES cells. Differentiation toxicity is analyzed by microscopically counting the beating of embryonic bodies after 10 days of culture. However, improvements are necessary to reduce the laborious manipulations involved and the time required to obtain results. We have previously reported the successful stable transfection of ES cells (ES-D3) with the heart and neural crest derivatives expressed transcript 1 (Hand1) gene and the establishment of a 96-well multi-plate-based new EST with luciferase reporter assay 6 days after treatment with test chemicals. Now, we propose an even more rapid and easier EST, named Hand1-Luc EST. We established another cell line to monitor the Hand1 gene expression via a luciferase reporter gene. By mRNA analysis and luciferase assay, we examined in detail the luciferase activity during cell differentiation, which allowed us to reduce the time of measurement from day 6 to day 5 (120 hr). Furthermore, the protocol was improved, with, among others, the measurement of cytotoxicity and differentiation toxicity taking place in the same 96-well round bottom plate instead of two different plates. With the positive control, 5-fluorouracil (5-FU), and 9 test chemicals, data with high reproducibility and very low variation (CV < 50%) in the relevant endpoints were obtained. This study shows that the Hand1-Luc EST could provide an accurate and sensitive short-term test for prediction of embryotoxics by measuring cytotoxicity and differentiation toxicity from the same sample.

Key words: *In vitro*, Embryotoxicity, Differentiation, Reporter gene assay, Luciferase activity, Hand1 gene

INTRODUCTION

Decades ago, developmental toxicity testing, defined as the evaluation of the adverse effects of a substance from the conception period until the sexual maturation, could not avoid the use of animals because of a lack of other available scientific techniques. However, the isolation of embryonic stem (ES) cells (Evans and Kaufman, 1981), allowed researchers to understand the differentiation mechanism and a means to culture those newly discovered cells and thus create new protocols leading to alternative test methods evaluating embryotoxicity consist-

ing in all the adverse effects of a substance that can cross the placental barrier and harm directly the embryo leading possibly to teratogenicity or lethality. Among those, the embryonic stem cell test (EST) is the most promising protocol to date to test differentiation toxicity of chemicals. The EST (Spielmann *et al.*, 1997) was assessed by the European Center for Validation of Alternative Methods (ECVAM) (Genschow *et al.*, 2002) as an alternative testing method to evaluate the inhibition of cardiac differentiation from ES cells by chemicals within 10 days. In an ECVAM workshop in January 2003, this test was confirmed to be reliable and transferable to other labora-

tories (Bremer *et al.*, 2005; Spielmann *et al.*, 2006). Furthermore, the heart being the first organ to begin developing during organogenesis, the interaction of embryotoxic chemicals with the differentiation into cardiomyocytes is the most sensitive endpoint so far. However, this test failed to be incorporated in regulatory guidelines for the following reasons (Bremer and Hartung, 2004): The first weak point concerned a lack of clarity in discrimination between non- and weak/moderate embryotoxicants, and the prediction model needed to be improved. ECVAM also recommended applying the test to industrial chemicals, to demonstrate the reliability and relevance of the system. To enhance the reliability of the tests for wider applicability to a broader range of chemical substances, testing of embryotoxicants with several toxicological mechanisms was also required. Another important point was the lack of integration of the metabolic system. In addition to these regulatory acceptance considerations, the EST is still time consuming and requires high experimental expertise, for example in areas such as hanging drop culture, transfer of embryoid bodies, and visual assessment of cardiomyocyte differentiation.

In this context and in response to all of the current problems, our laboratory developed a novel high-throughput EST in 2011 (Suzuki *et al.*, 2011b). In that study, we used the same type of cells as used in the original EST (D3 and 3T3 cells). However, D3 cells were stably transfected with Hand1 or Cmya1 promoters upstream of luciferase reporter gene, which allowed us to see the influence of chemicals on both of the gene expressions translating differentiation toxicity. Hand1 and Cmya1 were chosen partly because of their important roles in the early stage of development of the heart (Reamon-Buettner *et al.*, 2009; Wang *et al.*, 2013) and therefore they are confirmed to be valid endpoints to measure cardiac differentiation toxicity. With this protocol, we were able to obtain data at day 6 after cell seeding, which was a noticeable improvement compared to the EST validated by ECVAM in 2002. The conclusion of the study was high predictability and accuracy with reduced test duration and manpower compared to the original EST. Furthermore, in 2012, we published another study (Suzuki *et al.*, 2012) in which we investigated Hand1- and Cmya1-ESTs to explore reproducibility by comparing a set of 6 well-known test chemicals, including hydroxyurea, at four different laboratories. We managed to obtain good correspondence among all four laboratories, proving the high potency of transferability, intra-laboratory variability, and inter-laboratory variability of the protocols. Since then, we have concentrated our efforts on the Hand1 gene expression, for two main reasons: First, more was known about this gene and there-

fore more data were available to prove the relevance of the test, and second, the luciferase signal obtained with Hand1 was much higher than that obtained with Cmya1 gene, and thus obtaining the data would be easier.

With this background, the aim was to develop a protocol which is fast, easy to perform and highly reproducible to respond to the needs demanded by the alternative methods organizations. At last but not least, this test was also created to accept a prediction model to be able to predict *in vivo* embryotoxicity of chemicals. We are now proposing a novel EST, the Hand1-Luc EST, based on the results we provided in 2011. This new test consists of testing chemicals with a cell line, transfected with the luciferase gene to detect the expression of Hand1. Compared to the present available alternative methods, we managed to reduce incubation time to 120 hr (5 days) without complex and delicate manipulations, allowing this test to be easily performed by researchers with average experience. Cytotoxicity and differentiation toxicity are measured on the same cells, avoiding more manipulations; and finally, in the results we obtained, no dispersion was observed for 3 runs of some reference chemicals tested (positive control and 9 test chemicals), showing statistical relevance of the test.

MATERIALS AND METHODS

Chemicals

Nine chemicals, 6-aminonicotinamide (6-AN), all-trans retinoic acid (RA), 5-bromo-2'-deoxyuridine (BrdU), dexamethasone (DEX), methoxyacetic acid (MAA), salicylic acid sodium salt (SA), ascorbic acid (AsA), acrylamide (AcA), D-(+)-camphor (CAM), and the positive control, 5-fluorouracil (5-FU), selected from the reference chemicals of the ECVAM international validation study (Brown, 2002; Genschow *et al.*, 2004) were purchased from Sigma-Aldrich (St Louis, MO, USA). 5-FU was chosen as the positive control as described in the article published for the prevalidation study of the original EST (Scholz *et al.*, 1999). The chemicals were dissolved in appropriate solvents, phosphate-buffered saline (PBS(-)) or dimethyl sulfoxide (DMSO). The purity of all chemicals tested was more than 98.0%.

Establishment of ES cell lines

Some E3.5 blastocysts of C57BL/6 mice were maintained individually on a feeder layer of mitomycin C-treated fibroblasts in Stem Medium (DS Pharma Biomedical, Osaka, Japan) supplemented with 1,000 units of leukemia inhibitory factor (LIF; ESGRO, Millipore, Tokyo, Japan) and 2-mercaptoethanol (2-ME) (Basic medium). Most

blastocysts hatched and attached to the feeders layers. The remaining inner cell masses were grown in the wells of a 96-well round-bottom plate. Seven days later, the colonies were transferred into new wells and dissociated mechanically by pipetting with trypsin. Cells named as KOB1-ES cells were cultured on a feeder layer of mitomycin C-treated fibroblasts in 60 mm cell culture dishes (BD Falcon, Tokyo, Japan) and passaged every 2-3 days.

Establishment of stable transgenic ES cells

Linearized Hand1-promoter-Luc plasmid (Suzuki *et al.*, 2011b) was transfected into KOB1-ES cells by a lipofection method (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The transfected cells were dissociated into single cells with trypsin/EDTA (ethylenediaminetetraacetic acid) (Nacalai Tesque, Kyoto, Japan) and transferred to 96-well tissue culture plates onto a feeder layer of mitomycin C-treated G418 resistant fibroblasts (Kitayama Labes, Nagano, Japan) in basic medium supplemented with 100 µg/mL of G418 (Nacalai Tesque). To isolate stable transgenic ES cells, the cells were cultured for 1 week and G418-resistant colonies were picked up, dissociated with trypsin/EDTA, and cultured. This selection process was repeated four to five times. The isolated clones were named as Hand1-ES (KOB1) cells. These cells were stored in a cell freezing solution (Cell Banker; Nippon Zenyaku, Fukushima, Japan) at -80°C.

Relationship between Hand1 mRNA expression and luciferase activity in Hand1-ES (KOB1) cells

To verify the relationship between Hand1 mRNA expression and luciferase activity, the Hand1-ES (KOB1) cells were seeded in wells of white, round-bottom, low-attachment 96-well plates (MS-9096W; Sumitomo Bakelite, Tokyo, Japan). The Hand1-ES (KOB1) cells were differentiated into cardiomyocytes, as follows: 750 cells were seeded in 100 µL of assay medium that consisted of high glucose (4.5 g glucose/L) Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 15% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM glutamine (Invitrogen), antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin; Nacalai Tesque), 1% non-essential amino acids (Invitrogen), 0.1 mM 2-ME, and 1,000 U/mL mLIF as described previously (Spielmann *et al.*, 1997). The cells were incubated for 0 to 240 hr at 95% humidity, 37°C, under 5% CO₂.

Expression levels of mRNAs of the Hand1 gene in the differentiated cells were analyzed by quantitative real-time PCR (Polymerase Chain Reaction) with a TaqMan probe (Mm00433931_m1) (Suzuki *et al.*, 2011b). Quan-

tification was done in a 7900H Fast Real-time PCR system (Applied Biosystems, Tokyo, Japan) with a two-step PCR procedure following the manufacturer's protocol. Hand1 mRNA expression levels were normalized against β-actin (ACTB) as an internal control. Characterization and expression pattern of Hand1 gene in the Hand1-ES (KOB1) cells were evaluated from 0 to 240 hr after cell seeding.

Luciferase activity induced by differentiation of Hand1-ES (KOB1) cells was measured periodically by adding 100 µL per well of Steady-Glo® Luciferase Assay System (Promega, Madison, WI, USA). Luminous intensity was measured in a micro-plate luminescence counter (TopCount NXT; Packard Bioscience Co., Meriden, CT, USA).

Analysis of luciferase activity profile during Hand1-ES (KOB1) cell differentiation into cardiomyocytes

To analyze the profiles of luciferase activities during differentiation of Hand1-ES (KOB1) cells into cardiomyocytes, 750 cells were seeded in 6 wells of the white, round-bottom, low-attachment 96-well plates with 3 replicates per plate (18 wells used). Until 192 hr after cell seeding, cell viability and luciferase activities were assessed every 3 to 6 hr. Cell viability of the Hand1-ES (KOB1) cells was measured periodically using CellTiter-Fluor™ Cell Viability Assay (Promega) in accordance with the manufacturer's instructions. Luciferase activity was quantified as previously described.

General overview of Hand1-Luc EST protocol

Cryopreserved cells were thawed in maintenance medium and cultured for 2 to 3 days in 60 mm plates coated with 0.1% gelatin solution (SIGMA). One day before the experiment, Hand1-ES (KOB1) cells were passaged once more and seeded at a concentration of 0.2 to 1.0 × 10⁶ cells/gelatin-coated 60 mm dish in 5 mL of the maintenance medium. For the cytotoxicity and differentiation toxicity assays, cells were seeded into a 96-well tissue culture plate (750 cells/well) in the white, round bottom, low-attachment 96-well plates. The design of the plate is shown in Fig. 1. The 36 peripheral wells were filled with 100 µL of PBS(-). The second column of the plate was set as the medium control (MC), in which 750 cells/well in 100 µL of the assay medium were added. The third column was the vehicle control (VC) containing 750 cells/well in 100 µL of the assay medium with a concentration of 1% PBS(-) or 0.1% DMSO. From the fourth to the tenth column, the wells were filled with 750 cells/well with an increasing concentration of chem-

Plate design:

MC: Medium control

VC: Vehicle control

BG: Background

C7-C1: concentration gradient of chemical

Fig. 1. Plate design. The medium control and the vehicle control are situated on the rows 2 and 3 of the plate respectively. The different concentrations of chemicals are shared from row 4 to 10 and the background is in row 11. The peripheral wells contain 100 μ L PBS (-).

ical beforehand dissolved either in PBS(-) or DMSO in a final volume of 100 μ L of the assay medium. The maximum concentration of chemical tested was the highest attainable concentration that could be dissolved in PBS(-) or DMSO. Finally, the eleventh column was used for the background, consisting of 100 μ L of the assay medium without cells.

Chemicals diluted in 50 μ L of assay medium were added into the plates containing cells between 2 and 4 hr after cell seeding (7 wells per column per concentration), followed by incubation for 120 hr at 37°C and 95% humidity under 5% CO₂. The viability of the Hand1-ES (KOB1) cells was measured by CellTiter-Fluor™ Cell Viability Assay. Cytotoxicity was expressed as the concentration of chemical that reduces the viability of cells to 50% of the control level against the vehicle control (ES-IC₅₀). Differentiation toxicity was analyzed in the same plate by detecting chemical-related changes in Hand1 promoter-derived luminescence during the differentiation of Hand1-ES (KOB1) cells into cardiomyocytes. Luciferase activity was measured. The inhibition of differentiation was expressed as the concentration of the test chemical that reduces the luminescence by 50% (ES-ID₅₀). A schematic overview of Hand1-Luc EST is shown in Fig. 2.

Curve fitting and estimation of the ES-IC₅₀ and the ES-ID₅₀

To estimate the ES-IC₅₀ and the ES-ID₅₀ based on the mathematical sigmoid curve for the concentration-response relationship of the surviving rate and relative luciferase activity, respectively, we adopted a 2-parameter logistic curve. For the curve fitting, we used the data of four values of concentration near to 50% of the surviving rate or the relative luciferase activity. When adopting the logit transformation for the values of the surviving rate or

the relative luciferase activity, the model for the 2-parameter logistic curve was as follows:

$$\text{logit}(p_i) = \log_e(p_i/(1-p_i)) = a + b x_i,$$

where p_i is the i th surviving rate or the i th relative luciferase activity and x_i is the i th log_r concentration with common ratio r ($i = 1, 2, 3, 4$), and a and b are parameters estimated using the ordinary method of least squares. ES-IC₅₀ or ES-ID₅₀ can be obtained as $r^{(-a/b)}$.

RESULTS

Hand1 expression and luciferase activity of Hand1-ES (KOB1) cells during differentiation into cardiomyocytes

The KOB1-ES cells established from blastocysts of C57BL/6 mice had a normal karyotype with the capability of differentiating into the three germ layers (endoderm, ectoderm, and mesoderm) (data not shown). By transfecting the Hand1-Luc plasmid, we obtained the Hand1-ES (KOB1) cells. To characterize the expression pattern of the Hand1 gene in the KOB1-ES cells, we used real-time PCR to investigate the expression of the gene from 0 to 240 hr. As shown in Fig. 3A, the Hand1/ACTB relative expression increased and reached a peak at 135 hr after cell seeding. It then decreased until 240 hr (10 days). In a complementary study to confirm the expression pattern of Hand1 through the luciferase transcription, the luciferase activity was also measured and the luminescence profile showed the same pattern as the Hand1/ACTB relative expression with a peak at 135 hr and a decrease of the activity until 240 hr (Fig. 3B).

In a separate experiment, we further analyzed the expression of the Hand1 gene with the luciferase activity

A novel rapid and highly reproducible *in vitro* test for embryotoxicity

Fig. 2. General Hand1-Luc EST protocol concept with the measurement of cytotoxicity and differentiation toxicity in the same plate. The two endpoints (IC_{50} and ID_{50}) are assessed at 120 hr after cell seeding.

Fig. 3. (A) Real-Time PCR showing the relative expression Hand1/ACTB. Each value is the result of a cell mixture of 10 wells/samples. Each data point represents the mean $\pm$ S.D. of 3 different experiments. (B) Luc activity to characterize Hand1 expression. Each value is the mean $\pm$ S.D. of 8 wells per samples.

every 3 to 6 hr, along with the cell proliferation (Fig. 4). The shorter interval time between measurements was used to investigate the peak of Hand1 gene expression more precisely. For each predetermined time, the cells were seeded in three different plates with three replicates per plate. The peak of luciferase activity, which was situated at 120 hr after the beginning of differentiation (cell seeding), and the expression decreased until 192 hr. Mean-

while, the cells were normally proliferating as shown in the viability assay (Fig. 4).

Reproducibility of cytotoxicity and differentiation toxicity of Hand1-Luc EST

Ten chemicals chosen from the ECVAM reference chemical list were analyzed by the Hand1-Luc EST, and two endpoints were assessed: IC_{50} (cytotoxicity) and ID_{50}

Fig. 4. Luciferase activity translating Hand1 gene expression with cell viability until 192 hr after cell seeding. Each point is the mean $\pm$ S.D. of 6 wells per samples repeated 3 runs. (■ Luciferase activity ● Cell viability).

Table 1. List of selected test chemicals in the Hand1-Luc EST.

Embryotoxic potential <i>in vivo</i>	Test chemicals	Abbreviation	CAS no.	Solvent*
Strong**	5-Fluorouracil	5-FU	51-21-8	PBS
	6-Aminonictinamide	6-AN	329-89-5	DMSO
	All-trans Retinoic acid	RA	302-79-4	DMSO
	5-Bromo-2'-deoxyuridine	BrdU	59-14-3	DMSO
Weak**	Dexamethasone	DEX	50-02-2	DMSO
	Methoxyacetic acid	MAA	625-45-6	PBS
	Salicylic acid Na salt	SA	54-21-7	PBS
Negative**	Ascorbic Acid	AsA	50-81-7	PBS
	Acrylamide	AcA	79-06-1	PBS
	D-(+)-Camphor	CAM	464-49-3	DMSO

*Solvents were chosen after laboratory evaluation before each first experiment.

**Strong embryotoxicants are defined as developmentally toxic in all species tested, inducing multiple developmental effects. Negative chemicals are defined as not developmentally toxic at maternally toxic exposures, but which may show some minor embryo/fetal toxicity, which cannot be separated from maternal toxicity. Weak embryotoxicants are chemicals of intermediate activity (Brown, 2002).

(differentiation toxicity). The positive control used was 5-FU, classified as a strong embryotoxicant. Three other strong embryotoxicants (6-AN, RA, and BrdU), three weak embryotoxicants (DEX, MAA, and SA) and three non-embryotoxic chemicals (AsA, AcA, and CAM) were chosen. The information on test chemicals is summarized in Table 1.

Cytotoxicity and differentiation toxicity are represented in Fig. 5 (mean of 3 runs). For the 6 positive chemicals (strong and weak), the ID_{50} was always lower than the IC_{50} , showing the relevance of the protocol, as dead cells cannot express luciferase. RA did not induce any cyto-

toxicity at concentrations at which differentiation toxicity was observed. A main trend was that the positive chemicals had a larger gap between IC_{50} and ID_{50} compared to the negative chemicals. As for the negative chemicals, ID_{50} was close to the IC_{50} , showing decreasing luciferase activity along with cell death. In the case of CAM, neither IC_{50} nor ID_{50} was observed, showing that this chemical had no effect on cytotoxicity or Hand1 expression.

Repeated for each experiment, numerous data ($n = 10$) were obtained for the positive control 5-FU. The mean, standard deviation, and coefficient of variation of all the IC_{50} and ID_{50} values were calculated, as shown in

A novel rapid and highly reproducible *in vitro* test for embryotoxicity

Fig. 5. Cytotoxicity and differentiation toxicity curves of the 9 chemicals chosen (□ differentiation toxicity curve, ● cytotoxicity curve). Those data show the mean $\pm$ S.D. of 3 runs.

Fig. 6. Variations of the data showed very low values (SD of 0.009 $\mu\text{g/mL}$ for the IC_{50} and 0.007 $\mu\text{g/mL}$ for the ID_{50} , and CV values of 25.27 for the IC_{50} and 25.09 for the ID_{50}). The data from the positive control were therefore shown to be reproducible with low variation (CV < 50%).

Concerning the test chemicals, all studies were performed under the same conditions as the positive control, and three runs were performed for statistical relevance. The results are presented in Table 2. For all the runs, the data did not statistically differ from each other, with CV values all remaining under 50%, varying from 5.43 to 33.74 for cytotoxicity and from 6.25 to 33.98 for differentiation toxicity. CAM did not trigger any IC_{50} or ID_{50} values. This chemical was soluble at a maximum concentration of 250 $\mu\text{g/mL}$ (in DMSO, laboratory investigation). The Hand1-Luc EST assay could therefore not be used to investigate the effect of this chemical at higher concentrations. The case of CAM revealed solubility as a limiting factor for the Hand1-Luc EST assay. Also,

Fig. 6. Variation of IC_{50} and ID_{50} values for 5-FU obtained from 10 runs.

Table 2. IC₅₀ and ID₅₀ values of the 9 chemicals tested in the Hand1-Luc EST.

<i>in vivo</i> class	Chemicals	IC ₅₀ (µg/mL)						ID ₅₀ (µg/mL)					
		1st	2nd	3rd	mean	SD	CV	1st	2nd	3rd	mean	SD	CV
Strong	6-AN	2.32	2.46	1.23	2.00	0.68	33.74	0.63	0.55	0.65	0.61	0.05	8.43
	RA	> 0.005*	> 0.005*	> 0.005*	> 0.005	-	-	0.00004	0.00004	0.00003	0.00004	0.00001	18.54
	BrdU	0.39	0.26	0.40	0.35	0.08	22.65	0.12	0.17	0.14	0.15	0.03	17.01
Weak	DEX	30.34	30.24	35.63	32.07	3.08	9.61	13.46	19.02	26.83	19.77	6.72	33.98
	MAA	679.34	636.88	610.24	642.16	34.85	5.43	481.70	389.88	439.96	437.18	45.97	10.52
	SA	560.03	589.09	522.46	557.19	33.40	6.00	316.35	393.92	357.65	355.97	38.81	10.90
Negative	AsA	41.12	32.87	28.87	34.29	6.25	18.22	44.60	44.48	38.75	42.61	3.34	7.84
	AcA	57.47	38.94	54.15	50.19	9.88	19.69	74.86	66.72	68.04	69.87	4.37	6.25
	CAM	> 250*	> 250*	> 250*	> 250	-	-	> 250*	> 250*	> 250*	> 250	-	-

* : The values could not be determined due to precipitation of the chemical at higher concentration.

IC₅₀ and ID₅₀ obtained values of 3 runs for each chemical. Mean, SD and CV were calculated.

RA did not trigger any IC₅₀ value. This substance highly affected the expression of Hand1, but did not kill cells at 0.005 µg/mL.

DISCUSSION

We developed a novel EST in response to the requirements of the ICATM (International Coordination on Alternative Test Methods) to avoid the use of animals for chemical testing when possible. As to meet regulatory acceptance and to be used worldwide, the aim of the present study was to create a simplified and not only time-, but also cost-reduced protocol. Hand1, being a key gene in heart and neural crest-derivative development (Firulli *et al.*, 1998; Riley *et al.*, 1998; Morikawa and Cserjesi, 2004; Barbosa *et al.*, 2007) was found to be a relevant and possibly reproducible endpoint to evaluate embryotoxicity (Suzuki *et al.*, 2011a). In the present study, we first created a new cell line transfected with the Hand1 luciferase gene Hand1-ES (KOB1) and we showed the relevance of transfection while comparing expression with D3 cells transfected with the same vector (data not shown). The peak of expression of the Hand1 gene was examined and 120 hr was found to be the best moment to measure the possible alteration of expression due to the chemical (Fig. 4). Furthermore, comparison between the cell viability and the luciferase activity showed that the variation of Hand1 expression was mainly dependent on DNA mechanisms and not on cell proliferation. This supports luciferase activity as a relevant and convenient method to measure Hand1 gene expression during differentiation. Those results were consistent with those we previously obtained in Hand1-ES cells derived from D3 cells (Suzuki *et al.*, 2011b).

Presently, there is no proposed EST protocol available

with final results obtainable in less than 5 days. It could be difficult to establish a protocol evaluating embryotoxicity of differentiated tissue in a shorter period, since cells require a certain time to differentiate into special tissues, while investigating toxicity earlier in the development would be investigating toxicity on tissues contained in the primary germ layers: ectoderm, mesoderm, or endoderm. Therefore, toxicity evaluation on specific differentiated tissues seems to be hardly possible at this time of differentiation. It would, however, be interesting to assess toxicity on a representative gene of each of those primary tissues. Furthermore, in the same way of thinking, embryotoxicity of some chemicals may intervene much later in the development and would not be seen in the short time period after the beginning of differentiation. This would then lead to false negatives (compared with *in vivo* results) in the final possible classification. Thus, investigation on those genes should not be avoided for a complete evaluation of embryotoxicity. Finally, it should be kept in mind that embryotoxicity is the result of chemical effect on many tissues, and the evaluation on a single type is not sufficient to obtain the same results as obtained in *in vivo* experiments. It would be considered as a great improvement to be able to gather experiments together to evaluate embryotoxicity. Other laboratories proposed new protocols considering the neuronal (Baek *et al.*, 2012; Hayess *et al.*, 2013) or the bone tissue (de Jong *et al.*, 2012; zur Nieden *et al.*, 2010). However, although showing promise and representing real advances in the domain, those protocols still require complicated manipulations that can hardly be automated with the current available technologies.

There have also been many other efforts made since the original EST developed by Dr. Spielmann and co-workers (1997) was validated by ECVAM. Many aspects of

the protocol have been described as difficult for instance, change of culture medium, transfer of embryoid bodies, microscopic observation of beating embryoid bodies, and handling of many plates containing another cell line (3T3). With the aim of establishing automated and rapid techniques, the FACS-EST provided the same sensitivity as the validated EST protocol, but had the advantage of reduced test duration (Buesen *et al.*, 2009; Seiler and Spielmann, 2011). Also, to confront the problems of time and subjective consideration in the evaluation of beating of embryoid bodies, a laboratory proposed an automated analysis of contractility using an automated system based on image recording of each well, resulting in an area (pixels) and frequency of contractility (Hz). This interesting approach allowed avoidance of observer bias and offered the same results as the original protocol (Peters *et al.*, 2008).

In the case of the Hand1-Luc EST, we use simple techniques that have advantages in terms of sensitivity, accuracy, and convenience. The cells are seeded in white, round-bottom, low-attached 96-well plates, which allow them to aggregate by gravity and then form embryoid bodies. This step effectively replaces the “hanging drop” culture. Also, 6 replicates of 7 concentrations of chemical are available per plate, and 3 runs are performed for each chemical, which translates to 18 replicates of 7 concentrations for each substance, fulfilling the requirements for statistical relevance. Moreover, in all of the currently available protocols, cytotoxicity and differentiation toxicity are measured separately. After examining the different possibilities, we were able to find specific reagents that allowed us to measure cytotoxicity and differentiation toxicity in the same plate. Thus, we reduced not only the time of manipulations, but also the cost. Measuring endpoints on the same samples also helped the results to be highly reproducible, with low variation between the different runs ($CV < 50\%$) (see Table 2 and Fig. 5).

The gap between the IC_{50} and ID_{50} value between the 3 runs of each chemical did not statistically vary, and the high reproducibility was again verified (Table 2). This makes Hand1-Luc EST a non-negligible experiment, in which a little variation in the endpoint values could be relevant and be used to classify chemicals more precisely. Luciferase gene reporter assay is a common and well-established technique (Brasier *et al.*, 1989) to evaluate gene expression, which gives further credit to the protocol in itself.

Regarding the analysis of the results, cytotoxicity and differentiation toxicity curves are obtained almost instantly by use of an Excel® calculation sheet (Microsoft Corp.).

A limitation of Hand1-Luc EST, and more generally, of *in vitro* experiments, is the solubility of chemicals in the vehicle, DMSO or PBS(-). The highest concentration aimed for in the protocol is 1,000 $\mu\text{g/mL}$. However, some chemicals cannot be dissolved at this concentration, and lower concentrations are then tested. There is no special issue in cases in which IC_{50} and ID_{50} values are triggered, even if the concentration of 1,000 $\mu\text{g/mL}$ cannot be tested. However, if lower concentrations fail to trigger any IC_{50} or ID_{50} values, then the results need to be analyzed with precaution, knowing that an IC_{50} or ID_{50} could possibly appear at higher concentrations. Despite the precipitation problem, Hand1-Luc EST can still be considered as relevant for embryotoxicity evaluation. Indeed, substances need to reach the fetus to be embryotoxic. This should basically induce the parameters of dissolution in blood stream and crossing of the placental barrier (Pacifici and Nottoli, 1995). A substance with low water solubility may therefore have fewer chances to exert its effect. Moreover, absorption, distribution, metabolism, and excretion (ADME) should be considered in the establishment of a possible prediction model (Verwei *et al.*, 2006). A substance hardly absorbed or distributed in the organism will have a low chance of reaching the fetus. Thus, the pH, clearance rate, and other parameters should be taken into account. Nowadays, there is also a particular focus on the evaluation of metabolite toxicity, a field that is not well established yet due to the difficulty of merging metabolism and original protocols. Unfortunately, Hand1-Luc EST is no exception to this problem. As already described in the literature, an approach with S9 or microsomes should be interesting, but the problem of the toxicity of those to the metabolic system needs to be overcome. The use of hepatocytes seems to be much more challenging, because of the incompatibility of medium between the two types of cells and the reduction of the metabolism power due to the toxicity of chemicals (Riebeling *et al.*, 2012).

In conclusion, Hand1-Luc EST is a promising protocol for the evaluation of embryotoxicity, with highly reproducible, time- and cost-reduced techniques to respond to the needs of the 3R (Replacement, Reduction, and Refinement). As was emphasized many times in this article, our new method for screening and evaluating embryotoxicity of chemicals is designed to be a protocol that could, in the future, become a guideline approved by the regulatory authorities. As missing in many new articles providing new ESTs, a way of classifying substances is the condition *sine qua non* to complete a protocol evaluating toxicity. The present study indeed does not provide any classification, but in view of the results (reproducibility and

low variation) we obtained, it should be highly acceptable to increase the number of chemicals tested and use those numerous data to build a prediction model.

Finally, given the fact that embryotoxicity is a complicated phenomenon involving many mechanisms from the early through the later stages of development, much work is needed to create improvements to reach the level of classification of currently available *in vivo* methods and to be able to replace the use of animals.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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A novel rapid and highly reproducible *in vitro* test for embryotoxicity

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