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

Florian Le Coz1, Noriyuki Suzuki1, Hirohisa Nagahori1, Takashi Omori2 and Koichi Saito1

1Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., 1-98, Kasugadenaka 3-chome, Konohana-ku, Osaka 554-8558, Japan
2Epidemiology and Biostatistics Laboratory, Faculty of Culture and Information Science, Doshisha University, 1-3 Tatara Miyakodani, Kyotanabe City, Kyoto 610-0394, Japan

(Received November 6, 2014; Accepted January 20, 2015)

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 embryotoxicants 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 consisting 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-
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 pro-
tocol which is fast, easy to perform and highly reproduc-
ible 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 test-
ing chemicals with a cell line, transfected with the luci-
ferase 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 com-
plex 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 con-
trol 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), dex-
amethasone (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 chemi-
cals 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 main-
tained individually on a feeder layer of mitomycin C-treat-
ed 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 6 wells of the plate is shown in Fig. 1. The 36 peripheral wells were filled with 100 μL of Steady-Glo® Luciferase Assay Medium (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-GloTM 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 × 10^6 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 was 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-
ical beforehand dissolved either in PBS(-) or DMSO in a final volume of 100 μL 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 μL of the assay medium without cells.

Chemicals diluted in 50 μL 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.

**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 μL PBS (-).

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\(\text{th}\) surviving rate or the i\(\text{th}\) relative luciferase activity and \(x_i\) is the i\(\text{th}\) log 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 the Hand1 gene using qRT-PCR, 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
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. Meanwhile, 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: IC50 (cytotoxicity) and ID50.
(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 cytotoxicity 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

---

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

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

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

![Graphs of various chemicals' activity](image)

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

**Fig. 6.** Variations of the data showed very low values (SD of 0.009 μg/mL for the IC$_{50}$ and 0.007 μg/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 μg/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,
RA did not trigger any IC<sub>50</sub> 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

---

**Table 2.** IC<sub>50</sub> and ID<sub>50</sub> values of the 9 chemicals tested in the Hand1-Luc EST.

<table>
<thead>
<tr>
<th>in vivo class</th>
<th>Chemicals</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>Strong</td>
<td>6-AN</td>
<td>2.32</td>
<td>2.46</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>&gt;0.005* &gt;0.005* &gt;0.005* &gt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BrdU</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>Weak</td>
<td>DEX</td>
<td>30.34</td>
<td>30.24</td>
</tr>
<tr>
<td></td>
<td>MAA</td>
<td>679.34</td>
<td>636.88</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>560.03</td>
<td>589.09</td>
</tr>
<tr>
<td>Negative</td>
<td>AsA</td>
<td>41.12</td>
<td>32.87</td>
</tr>
<tr>
<td></td>
<td>AcA</td>
<td>57.47</td>
<td>38.94</td>
</tr>
<tr>
<td></td>
<td>CAM</td>
<td>&gt;250* &gt;250* &gt;250* &gt;250</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

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

IC<sub>50</sub> and ID<sub>50</sub> obtained values of 3 runs for each chemical. Mean, SD and CV were calculated.
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 instantaneously 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 μg/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 μg/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.

ACKNOWLEDGMENTS

This study was supported by a research grant from the Ministry of Economy, Trade and Industry (METI) of Japan. We also would like to thank Mitsuhiko Nishida for his technical support.

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

REFERENCES


Suzuki, N., Ando, S., Yamashita, N., Horie, N. and Saito, K.
A novel rapid and highly reproducible in vitro test for embryotoxicity


