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Assessment of Embryotoxicity and Teratogenicity by the Embryonic Stem Cell Test

Kazuaki Nakamura, Shinji Kusakawa and Akito Tanoue
National Research Institute for Child Health and Development
Japan

1. Introduction

During pregnancy, a fetus can be affected by exposure to a variety of chemicals and pharmaceuticals. These effects can occur through exposure of the mother and subsequent placental transport. Thus, it is important to assess embryotoxicity (developmental toxicity without the observation of maternal toxicity) and teratogenicity (irreversible structure developmental effects without the observation of maternal adversities) prior to the marketing of compounds. The complexity of the reproductive system and the vast number of tissue targets for the exogenic induction of malformations during embryonic development are the rationale underlying the toxicity testing of chemicals in highly standardized animal experiments such as screening tests or multigenerational studies, according to specific Organization for Economic Co-operation and Development (OECD) test guidelines. All of these tests provide information on the biological effects of industrial chemicals or the pharmacological side effects. These guidelines generally specify time-consuming and expensive *in vivo* experiments, most of which are performed with mammalian species such as rats or rabbits. However, for both economical and ethical reasons, there is a great demand for alternatives to living mammals in the testing of chemical-induced adverse effects on reproduction and development. Over the past 30 years, various *in vitro* models have been developed to detect the teratogenic effects of chemicals. These test systems utilize either dissociated cells from the limb buds and brains of rat embryos (micromass test; (Flint & Orton 1984)) or whole embryos of rats (whole embryo culture test; (Freeman & Steele 1986)). Thus, these test systems must sacrifice living animals in order to obtain cells or embryos for each experiment. In recent years, stem cells have become important new tools for the development of *in vitro* model systems to test drugs and chemicals; they have also shown potential to predict or estimate toxicity. Among various stem cells, embryonic stem (ES) cells are the most valuable in developing *in vitro* model systems because they are able to self-renew and differentiate into every cell type of the mammalian organism; they therefore have higher plasticity than adult stem cells. The fact that stem cells are able to self-renew means that they can be continuously cultured in an undifferentiated stage, giving rise to more specialized cells such as heart, liver, bone marrow, blood vessels, pancreatic islets, or neuronal cells upon addition or removal of certain growth factors (Hoffman & Merrill 2007). In 1997, Spielmann *et al*. developed an *in vitro* model for the screening of embryotoxicity based on mouse (m) ES cells. This is termed the "embryonic stem cell test" (EST; (Spielmann et al. 1997)). The EST is based on the

assessment of three toxicological endpoints: (1) the morphological analysis of beating cardiomyocytes in embryoid body (EB) outgrowths compared to cytotoxic effects on (2) undifferentiated mES cells, and (3) differentiated NIH-3T3 fibroblasts. As an *in vitro* system, which mirrors both proliferation and differentiation, the EST was proved in an international European Centre for the Validation of Alternative Methods (ECVAM) validation study to be a reliable assay for the prediction of embryotoxicity *in vivo* (Genschow et al. 2004). Using a set of 20 reference compounds with different embryotoxic potencies (nonembryotoxic, weakly embryotoxic, and strongly embryotoxic), the EST was demonstrated to provide a correct judgment in 78% of all experiments. Remarkably, a predictive performance of 100% was obtained for strong embryotoxicants. As a consequence, the validated EST has been accepted and successfully introduced by many pharmaceutical companies as a tool for testing the developmental toxicity of lead compounds at an early stage in the research and development of new drug candidates (Whitlow et al. 2007). A major drawback of the classical EST is its reliance on a morphological endpoint (beating cardiomyocytes) and the need for experienced personnel to ensure reliable assessments of this endpoint (Buesen et al. 2009).

2. Technical details of EST

2.1 Classical EST

Murine ES cells are maintained in an undifferentiated state in culture under conditions that inhibit differentiation by supplementing the culture medium with murine leukemia inhibitory factor (mLIF; (Williams et al. 1988)). Differentiation of ES cells is then induced by the withdrawal of mLIF. Using the "hanging drop" culture technique described by Rudnicki and McBurney (Rudnicki & Mc Burney 1987), ES cells form multicellular aggregates called embryoid bodies (EBs; Fig. 1). Within the EBs, the three germ layers (endo-, meso-, and

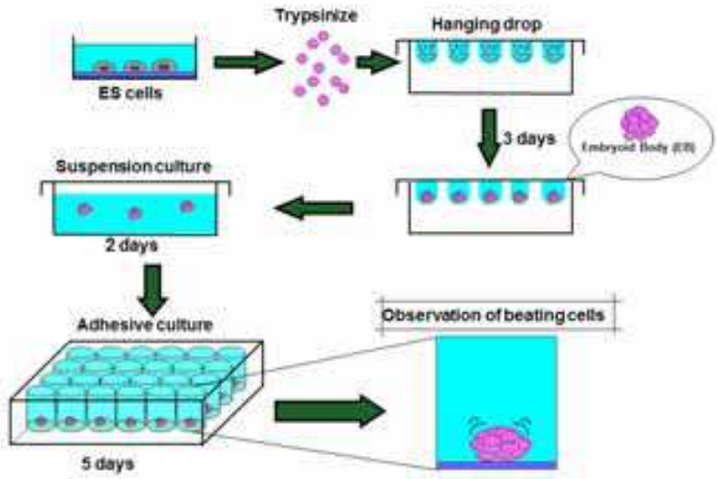

Fig. 1. Differentiation assay of the EST. ES cells are differentiated as EBs in hanging drops in differentiation medium. Differentiation medium containing 750 ES cells is placed on the lids of petri dishes filled with phosphate buffered saline (PBS) for 3 days. The EBs are transferred to suspension culture dishes and cultured for 2 days. They are plated onto 24-well tissue culture plates on day 5 and incubated for 5 additional days. To estimate the efficiency of differentiation from ES cells to cardiomyocytes, the distinctive beating movements of differentiated cardiomyocytes are analyzed under an inverted phase-contrast microscope.

ectoderm) can develop and the further differentiation into several cell types, including beating myocardial cells, can take place (Doetschman et al. 1985; Rudnicki & Mc Burney 1987; Maltsev et al. 1994; Hescheler et al. 1997). The EST benefits from the fact that differentiation into beating myocardial cells can be easily detected by microscopic inspection of EB outgrowths at day 10 of differentiation. In addition to the differentiation analysis, the cytotoxic effects of the test substance on ES cells and NIH-3T3 cells are analyzed. By using stem cells and differentiated fibroblasts, the assay takes embryonic as well as maternal toxicity into account. To assess the concentration of a substance which inhibits the development (inhibition of differentiation: ID_{50}) and proliferation (IC_{50}ES and IC_{50}3T3) by 50% compared to the untreated control, dose-response profiles are collected. A biostatistical prediction model was developed to assign test compounds to three classes of embryotoxicity: *non-embryotoxic*, *weakly embryotoxic*, and *strongly embryotoxic* (Genschow et al. 2002; Genschow et al. 2004).

2.2 Modification of EST

Arguments have been made that the classical, validated EST might be sufficient to assess the embryotoxic potential of chemicals (Marx-Stoelting et al. 2009). The differentiation of ES cells into cardiomyocytes is easy to achieve experimentally and is also highly reproducible. It has been reported that the spontaneous beating of cardiomyocytes represents a crucial first step during embryonic development. However, there is the possibility that the embryotoxic effects of chemicals might be overlooked if their underlying mechanisms of action consist of a very specific interaction with molecules that is expressed during the differentiation of ES cells into a cell type not well represented in the classical EST, such as a neuronal cell. It has also been argued that the EBs in the EST do not simply consist of beating cardiomyocytes, but also contain cells of other germ layers, including neuronal cells. Therefore, several suggestions have been made in regard to how to add further new endpoints (Buesen et al. 2004; Marx-Stoelting et al. 2009). These include additional ES cell differentiation endpoints, and the development of new molecular markers for the detection of toxic effects on embryonic development. One suggestion for the extension of endpoints was to differentiate ES cells into several different tissues, including nervous tissue, bone, cartilage, and epithelia. This approach would ensure that the effects on multilineage differentiation, and on tissue of endodermal, mesodermal, and ectodermal origin, would become detectable. It has been reported that the EC_{50} of thalidomide is ~30-fold lower when the effects were studied on the differentiation of ES cells triggered in the direction of bone tissue than in the classical EST with cardiac differentiation as the endpoint. Similarly, valproic acid (VPA) was more active (by a factor of ~10) in a regimen involving the induction of neuronal differentiation of ES cells (Marx-Stoelting et al. 2009). These results suggest that a multilineage analysis could increase the predictive performance of the EST. It is important to note that the observed differences in sensitivity correspond well with the two different developmental target tissues of thalidomide and VPA, which are primarily bone and the nervous system, respectively, but not the heart. Hence, the additional differentiation endpoints enabled the successful classification of the two agents, which was not possible with the classical EST (zur Nieden et al. 2004). This example not only demonstrates the relevance of additional differentiation endpoints, but also suggests that an increase in predictive value may be achievable using a combination of new differentiation endpoints with the introduction of new molecular markers (Marx-Stoelting et al. 2009). In

order to characterize the neural-tissue-specific toxicity of drugs, the stromal cell-derived inducing activity (SDIA) method, which promotes the differentiation of mES cells (Kawasaki et al. 2000; Kitajima et al. 2005), was introduced in the EST (Kusakawa et al. 2010) (Fig. 2). SDIA accumulates on the surface of PA6 stromal cells and induces efficient neuronal differentiation of cocultured ES cells in serum-free conditions without the use of either retinoic acid or EBs. A high proportion of tyrosine hydroxylase-positive neurons producing dopamine are obtained from SDIA-treated ES cells. (Kawasaki et al. 2000). This is one strategy for adding differentiation endpoints in the EST.

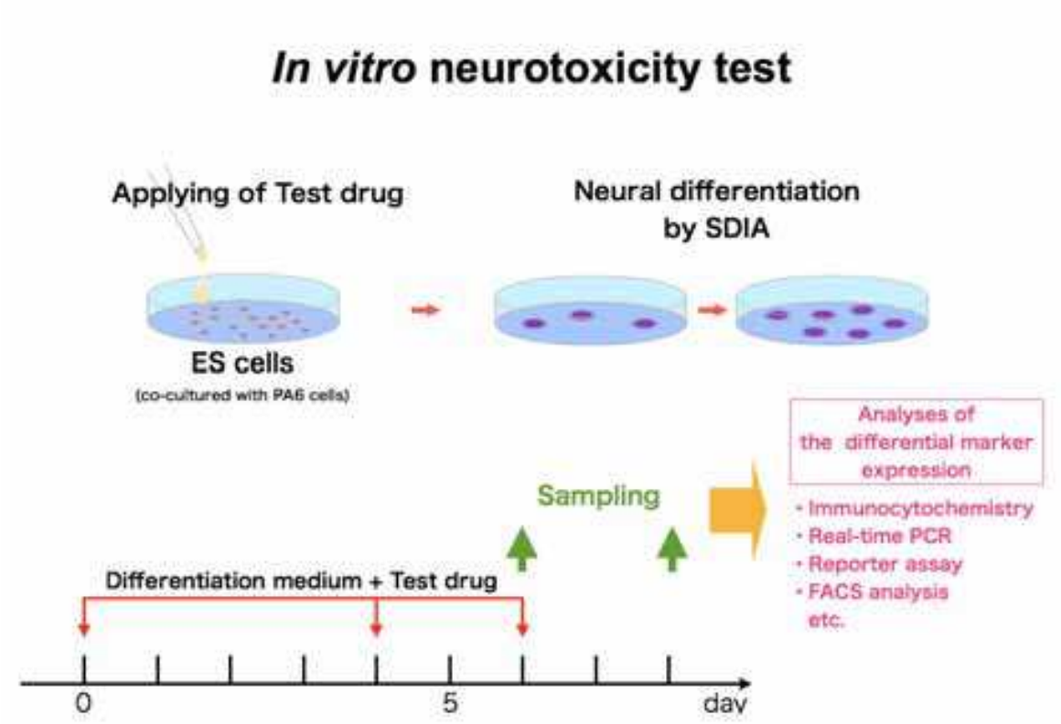

Fig. 2. An *in vitro* neurotoxicity test established using a neural differentiation system induced by the stromal cell-derived inducing activity (SDIA) method. By performing various analyses of neural marker expressions, the effects of test drugs on the developing neural system can be evaluated.

Several target genes and target proteins that could be used as molecular markers for the differentiation of specific embryonic tissues have been suggested and partially explored by methods employing quantitative real-time polymerase chain reaction (PCR) or fluorescence-activated cell sorting (FACS) analysis (Seiler et al. 2004; Seiler et al. 2006; Buesen et al. 2009). In addition to the analysis of endogenous molecular markers, reporter-based systems may represent an attractive alternative. For example, mES cells that have been stably transfected with a neural-tissue-specific promoter-driven green fluorescence protein (GFP) or luciferase reporter can be utilized as a detection system. With this system, the effects on neural cell differentiation can be detected in a high-throughput assay based on GFP expression or luciferase activity (Kusakawa et al. 2010) (Fig. 3). Thus, reporter-based systems are regarded as promising, and may offer potential new endpoints for the EST (Marx-Stoelting et al. 2009).

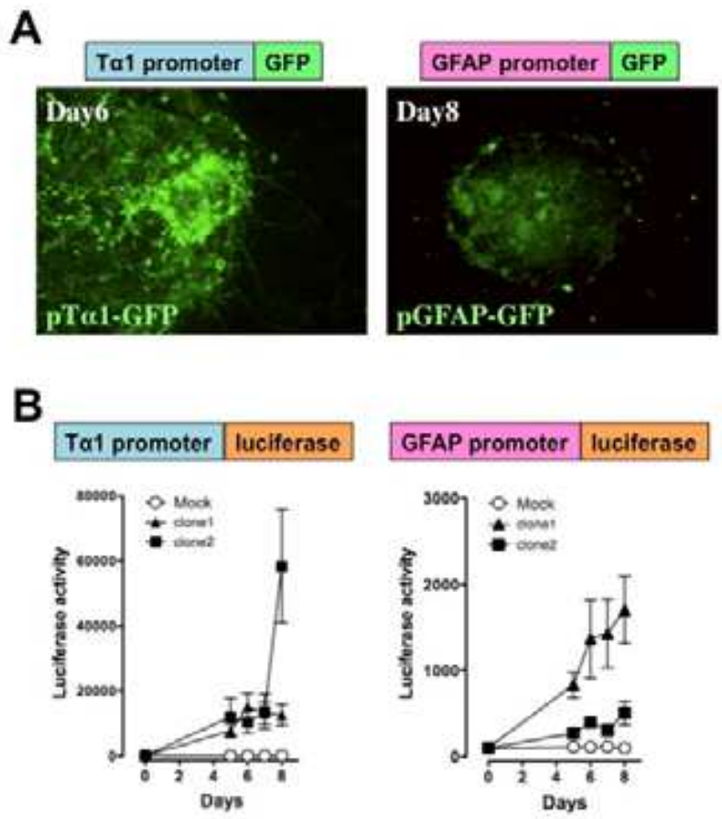

Fig. 3. Established ES cell lines that express reporter genes driven by each promoter of neuron marker gene, tubullin alpha 1 (Tα1), or glial cell marker gene, GFAP. In these stable cell lines, the expression of the reporter gene such as GFP (A) or luciferase (B) is observed, followed by neural differentiation.

3. Actual case using modified EST

3.1 Estimation of carbamazepine embryotoxicity using our modified EST

Carbamazepine (CBZ) is one of the most widely used antiepileptic drugs (AEDs), and is also used in the treatment of neuropathic pains and psychiatric disorders (Albani et al. 1995; Sindrup & Jensen 1999). CBZ is known to be a teratogen (Jones et al. 1989; Shepard et al. 2002). Pregnant women who undergo CBZ drug therapy have increased rates of congenital anomalies in the fetus, in particular, neural tube defects (NTDs), cardiovascular and urinary tract anomalies, and cleft palate. CBZ also induces a pattern of minor congenital anomalies and developmental retardation (Jones et al. 1989; Matalon et al. 2002). CBZ is structurally similar to tricyclic antidepressants, but shares remarkably similar clinical features to the structurally unrelated AED VPA, which has a short-chained fatty acid structure. CBZ is embryotoxic but is less teratogenic than VPA. It causes a spina bifida (an NTD) rate of approximately 0.5 to 1% and a cardiovascular anomaly rate of 1.5 to 2.0% (Ornoy 2006). VPA causes an NTD rate of approximately 2% and an increase of 4 to 8% in major congenital anomalies (Nau et al. 1991; Ornoy 2006). We attempted to characterize the tissue-specific embryotoxicity of CBZ using our modified EST (Murabe et al. 2007b).

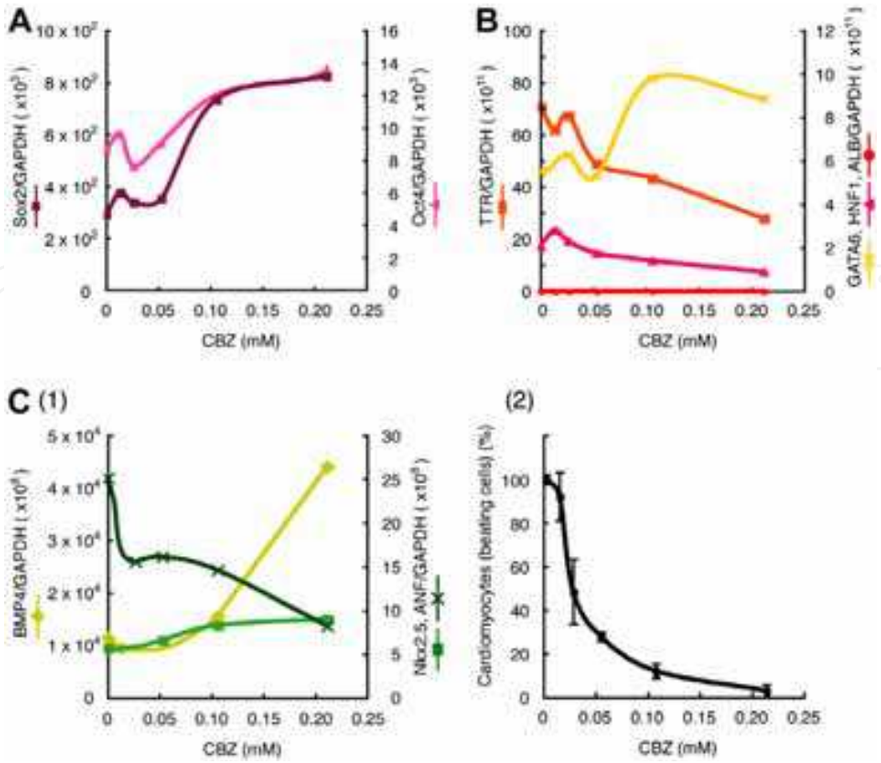

Fig. 4. Analysis of expression levels for mesodermal and endodermal differentiation with CBZ. The expression level of markers of the undifferentiated state, Sox2 and Oct4 (A), endodermal markers, GATA6, TTR, HNF1, and ALB (B), and mesodermal markers, BMP4, Nkx2.5, and ANF (C-1), were quantified at each concentration of CBZ with real-time RT-PCR. The frequencies of cardiomyocytes, identified by their distinctive beating movements, derived from ES cells were quantified at each concentration of CBZ (C-2) (Murabe et al., 2007b).

The tissue-specific effects of CBZ in the ES cell differentiation system were characterized at the molecular level. We used real-time reverse transcription (RT)-PCR on samples from day 5 of culture to determine the expression levels of tissue-specific genes in undifferentiated cells, and in cells differentiating into endodermal and mesodermal lineages (Fig. 4). The expression levels of Sox2 and Oct4 (undifferentiated markers) increased at high CBZ concentrations (Fig. 4A). In the differentiating endodermal lineage, only the expression level of the primitive marker, GATA6, increased (Fig. 4B); the expression levels of the markers of late differentiation stages, TTR and HNF1, decreased in a concentration-dependent manner. Albumin (ALB), a definitive endodermal hepatic marker, was not detected, suggesting that CBZ promoted initial endodermal differentiation but inhibited differentiation into mature endodermal lineages. In the mesodermal lineage, the expression level of the primitive marker, BMP4, increased in a dose-dependent manner (Fig. 4C-1). The expression level of an early cardiac marker, Nkx2.5, showed a slight increase. However, expression of a later stage cardiac marker, ANF, was reduced in a concentration-dependent manner. Under the same culture conditions but omitting CBZ, cardiomyocytes normally differentiate from EBs. We screened for cardiomyocyte differentiation at different concentrations of CBZ. We found that CBZ decreased the rate of undifferentiated ES cells differentiating to cardiomyocytes in a dose-dependent manner (Fig. 4C-2). Thus, based on the gene expression data and rates of

cardiomyocyte differentiation, it is evident that CBZ promoted the initial differentiation into mesodermal lineages, including primitive cardiomyocytes, but inhibited later differentiation into the mature mesodermal lineages. We also examined the expression levels of various neural markers: Nestin, which is a marker of early differentiation, and Synaptophysin (Syn) and Neurofilament H (NFH), which are later stage neuron-specific markers. The expression of all three markers increased in a dose-dependent manner in the presence of CBZ (Fig. 5A-1/2). The glial markers GFAP, an astrocyte-specific marker, and Oligo2 and DM20, oligodendrocyte-specific markers, were also elevated in a concentration-dependent manner (Fig. 5A-3). These results suggest that CBZ induces ES cells to differentiate into neurons and glial cells. This contrasts with our observation that VPA induces ES cells to differentiate into neurons but not glial cells (Murabe et al. 2007a). In order to compare the expression profiles of neuronal and glial markers between CBZ and VPA, we performed RT-PCR with samples on days 5 and 7. RT-PCR analysis revealed that the Nestin expression levels induced by CBZ were higher than those induced by VPA (Fig. 5B). Syn and NFH showed higher levels of expression after CBZ than VPA at day 5, but no expression of either marker could be detected at day 7 in the CBZ cultures. In contrast, the expression of Syn and NFH induced by VPA was increased in the day 7 culture. The DM20 expression levels in CBZ cultures were higher on days 5 and 7 than in the VPA cultures. The expression levels of other typical glial markers, such as GFAP, and Olig2, were very low (data not shown). Our immunocytochemical study with an antibody against β-III tubulin (a neuronal marker)

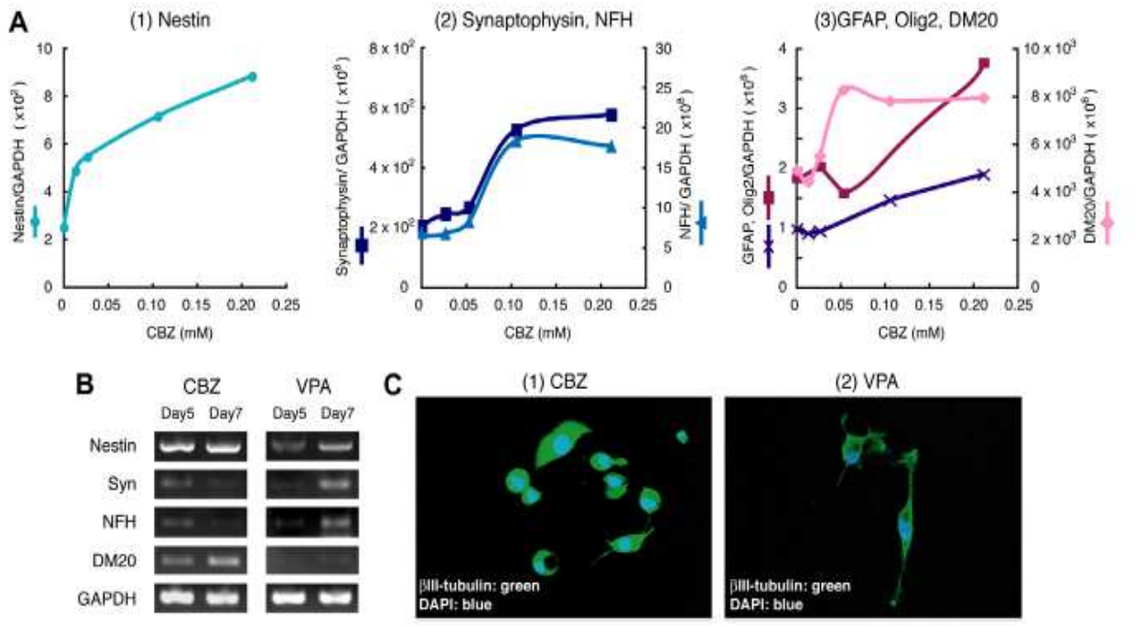

Fig. 5. Analysis of expression levels for ectodermal differentiation with CBZ. (A) gene expression levels of the ectodermal markers, Nestin (1), Synaptophysin, NFH (2), and GFAP, Olig2, DM20 (3), were quantified by real-timeRT-PCR. (B) The expression levels of ectodermal markers were quantified by RT-PCR in cultures treated with CBZ (0.11 mM) or VAP (1.50 mM). (C) Neural cells derived from ES cells cultured in the presence of CBZ (0.05 mM) or VPA (0.19 mM) were immunostained with an anti-βIII-tubulin antibody (Murabe et al., 2007b).

revealed that many positive cells were detected in samples on day 10 of cultures with either 0.05 mM CBZ (Fig. 5C, left) or 0.19 mM VPA (Fig. 5C, right). In the CBZ-administered group, the positive cells had an almost spherical shape and few had nerve processes. These cells had the appearance of immature neurons. In contrast, in the VPA-administered group, many of the positive cells had long nerve processes and had the appearance of mature neurons. These results suggest that: (1) CBZ induces neural lineage differentiation in ES cells but that the potential for neuronal differentiation is lower compared with VPA; and (2) CBZ induces differentiation of both neuronal and glial lineages, whereas VPA induces neuronal but not glial cells.

A cell viability assay was used to study the cytotoxic effect of CBZ on ES cells and NIH-3T3 fibroblasts. In both cell lines, CBZ inhibited the survival of cells in a dose-dependent manner (Fig. 6). There was no significant difference between the cytotoxic sensitivities of ES cells and NIH-3T3 fibroblasts to CBZ. The IC_{50} values were calculated as 0.24 and 0.31 mM for NIH-3T3 fibroblasts and ES cells, respectively. The therapeutic range of CBZ is 0.02 to 0.05 mM in serum. Thus, the IC_{50} values of NIH-3T3 fibroblasts and of ES cells were approximately 5- 15-fold larger than the therapeutic concentration. Neither cell line appears to show any significant response to CBZ within the therapeutic range. The cytotoxicity of CBZ for ES cells was much lower than that obtained for VPA in our system (Murabe et al. 2007a). To observe the cytotoxic and morphological effects of CBZ, ES cells and NIH-3T3 fibroblasts stained in MTT were observed on day 5 of the cytotoxicity assay (Fig. 6, bottom). In both cell types, cell densities were reduced in a concentration-dependent manner. In the high-dose CBZ group, NIH-3T3 fibroblasts showed strong indications of shrinkage or shape changes. In contrast, the ES cells contained many small, presumably undifferentiated cells, suggesting that CBZ strongly inhibited differentiation. This observation is almost the same as that found using VPA (Murabe et al. 2007a).

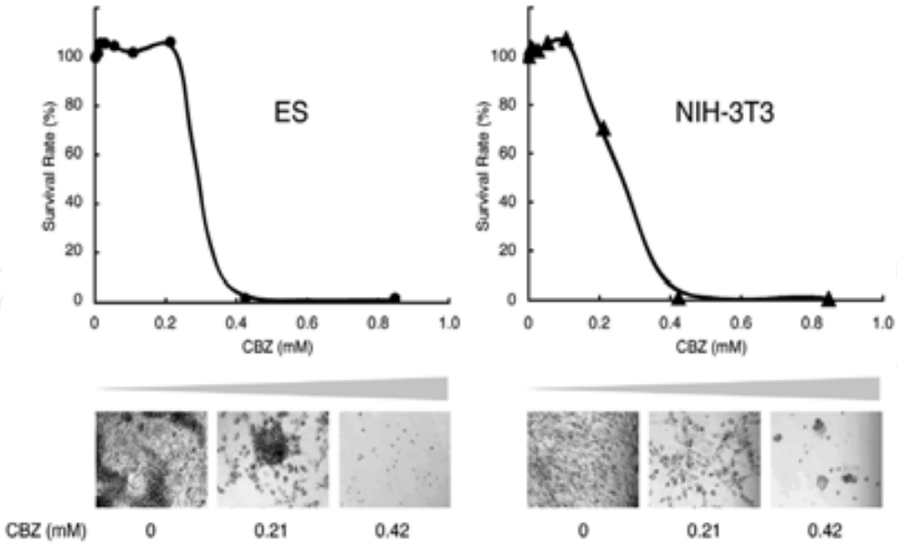

Fig. 6. Cytotoxicity assay on ES cells and NIH-3T3 fibroblasts with CBZ. Cells on day 10 of the assay were stained with MTT and solubilized. The activity of the mitochondrial enzyme of living cells was examined. The violet color of the MTT formazan, which is the enzyme product, was measured at an absorbance of 520 nm. On day 5, cells were stained with MTT (Murabe et al., 2007b).

3.2 Estimation of fluoxetine embryotoxicity using our modified EST

Twenty years have passed since fluoxetine, which is one of the selective serotonin-reuptake inhibitors (SSRIs), was introduced into clinical use; the class of antidepressants known as SSRIs is now used worldwide. Since their introduction, SSRIs have been recognized to be more effective and to have fewer side effects than older tricyclic antidepressants. For these reasons, treatment with SSRIs has become very popular, even among pregnant women, because approximately 10% of pregnant women exhibit symptoms of clinical depression and many are treated with antidepressants. Maternal use of SSRIs during pregnancy is of increasing public health concern, due to its wide prescriptive base for the treatment of depression and other disorders and its potential teratogenic effects on the developing fetus. Thus, we attempted to characterize the tissue-specific embryotoxicity of fluoxetine using our modified EST (Kusakawa et al. 2008).

A cell viability assay was used to study the cytotoxic effect of fluoxetine on ES cells and NIH-3T3 fibroblasts. In both cell lines, fluoxetine inhibited survival of cells in a dose-dependent manner (Fig. 7A and B), indicating that fluoxetine affected cell viability. The IC_{50} values were calculated at 1.79 μM for ES cells and 4.67 μM for NIH-3T3 fibroblasts. There was a significant difference between these two cell lines in their cytotoxic sensitivities to fluoxetine, indicating that ES cells were more sensitive to the toxicity of fluoxetine than NIH-3T3 fibroblasts were.

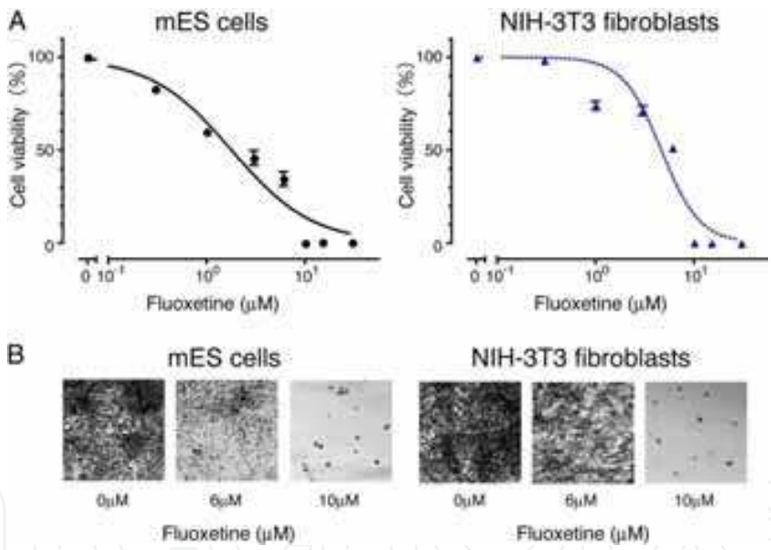

Fig. 7. Cytotoxicity assay on ES cells and NIH-3T3 fibroblasts with fluoxetine. (A) Cells on day 10 of the assay were stained with MTT and solubilized. The activity of the mitochondrial enzyme of living cells was examined. The violet color of the MTT formazan, which is the enzyme product, was measured at an absorbance of 520 nm. (B) On day 5, cells were stained with MTT (Kusakawa et al. 2008).

To characterize the tissue-specific effects of fluoxetine on the ES differentiation system at the molecular level, we examined the expression levels of typical tissue-specific genes by performing real-time RT-PCR analysis on our samples on days 5 and 10 of the differentiation assay (Figs. 8– 10). Under a control culture condition without the drug (0 μM fluoxetine), the undifferentiated markers Oct3/4 and Sox2 were highly expressed on day 5, and their expression levels decreased between day 5 and day 10. In fluoxetine-treated ES

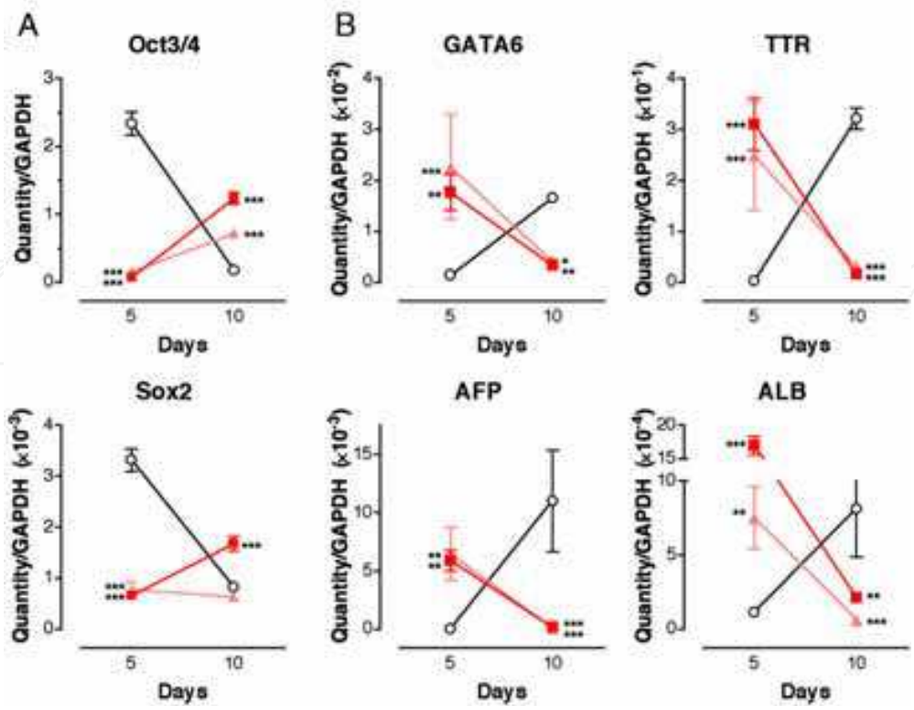

Fig. 8. Analysis of expression levels for the differentiated state and endodermal differentiation. The expression levels of the undifferentiated markers, sox2 and Oct3/4 (A), and endoderm markers, GATA6, TTR, AFP, and ALB (B), were quantified at each concentration of fluoxetine (0 µM: open circle; 1µM: closed triangle; 3µM: closed square) with real-time RT-PCR (Kusakawa et al. 2008).

cells, on the other hand, the expression levels of Oct3/4 and Sox2 were lower on day 5, but increased between day 5 and day 10 (Fig. 8A). There were significant interactions between fluoxetine treatment and the expression pattern of each undifferentiated marker. These results suggested that fluoxetine treatment affected the expression of the undifferentiated markers Oct3/4 and Sox2 in differentiating ES cells. Under the control culture condition, the expression levels of endodermal markers such as GATA6, TTR, AFP, and albumin (ALB) were low on day 5 and up-regulated from day 5 to day 10 (Fig. 8B). In fluoxetine-treated ES cells, in contrast, these endodermal markers were more strongly expressed on day 5, and down-regulated from day 5 to day 10. There were significant interactions between fluoxetine treatment and the expression pattern of each endodermal marker, suggesting that fluoxetine could also affect the differentiating endodermal lineage. The expression levels of BMP4 (a primitive marker), Nkx2.5 (an early cardiac marker), MLC-2v, and ANF (both later cardiac markers) were low on day 5 and increased between day 5 and day 10 under the control culture condition (Fig. 9A). In the fluoxetine-treated cells, on the other hand, these mesodermal markers were more highly expressed on day 5, and decreased between day 5 and day 10 (Fig. 9A). There were significant interactions between fluoxetine treatment and the expression pattern of each endodermal marker. Thus, fluoxetine treatment down-regulated the expressions of mesodermal markers as well as endodermal markers. In addition to studying gene expression levels in the mesodermal lineages, we examined how fluoxetine affected mesodermal cell differentiation. Ordinarily, in the absence of fluoxetine, cardiomyocyte can differentiate from EBs at different concentrations

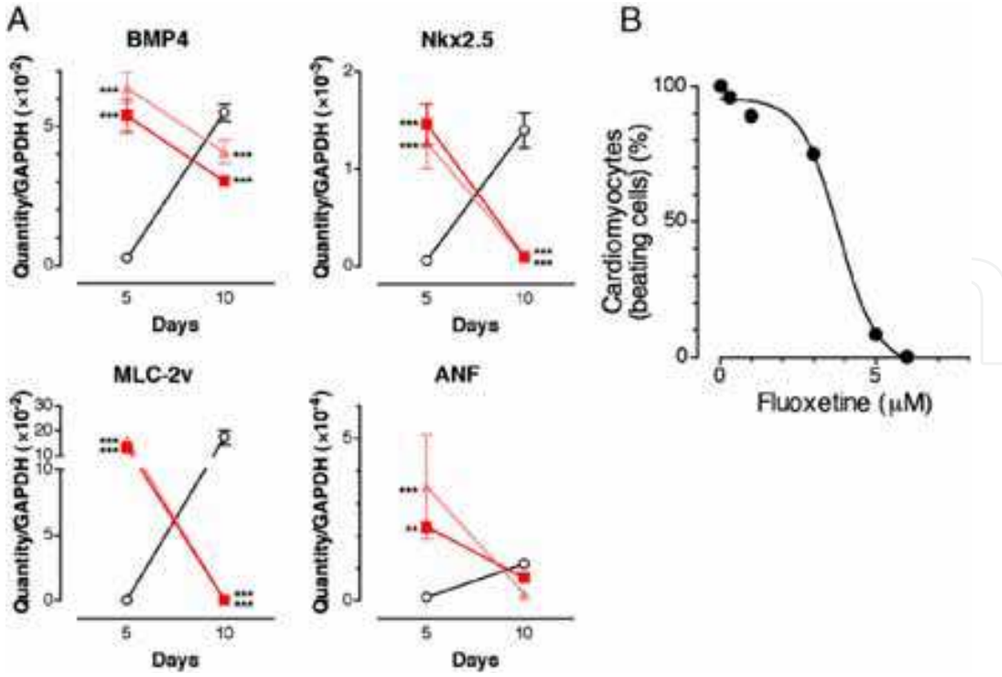

Fig. 9. Analysis of expression levels for mesodermal differentiation. The expression levels of the mesodermal markers, BMP4, Nkx2.5, MLC-2v, and ANF were quantified at each concentration of fluoxetine (0 μM: open circle; 1 μM: closed triangle; 3 μM: closed square) with real-time RT-PCR (A). The frequencies of cardiomyocytes derived from ES cells were identified by their distinctive beating movement and quantified at each concentration of fluoxetine (B) (Kusakawa et al. 2008).

of fluoxetine. We found that fluoxetine decreased the rate at which undifferentiated ES cells differentiated to cardiomyocytes in a concentration-dependent manner (Fig. 9B). The ID_{50} value was calculated at 3.79 μM. Thus, based on the gene expression data and the cardiomyocyte differentiation data, it was confirmed that fluoxetine could inhibit differentiation into mesodermal lineages.

In the absence of fluoxetine, the expression level of Nestin (a primitive neural stem cell marker) was low on day 5, and increased slightly on day 10. In fluoxetine-treated cells, in contrast, the expression level of Nestin was higher on day 5 and decreased between day 5 and day 10. Similarly, the expression levels of synaptophysin (a later neuron-specific marker), GFAP (an astrocyte-specific marker), and Olig2 (an oligodendrocyte-specific marker) in fluoxetine-treated cells were lower on day 5 and increased from day 5 to day 10 (Fig. 10A). There were significant interactions between fluoxetine treatment and the expression pattern of each ectodermal marker. The expression levels of GFAP and Olig2 increased in a concentration-dependent manner (GFAP: 3-fold increase at 1 μM and 18-fold increase at 3 μM; Olig2: 2-fold increase at 1 μM and 20-fold increase at 3 μM). We also performed an immunocytochemical study with antibodies against Neurofilament H (NFH) (a later neuron-specific marker) and GFAP (an astrocyte-specific marker). Among ES cells that had been treated with 3 μM fluoxetine, we detected many positive cells, both for NFH and for GFAP, on day 10 (Fig. 10B). In this fluoxetine-treated group, the positive cells had an almost spherical shape, and few had any nerve processes (Fig. 10B). These cells had the appearance of immature neurons. These results suggest that fluoxetine induces ectodermal

lineage differentiation in ES cells, but that the potential for neuronal differentiation is lower compared with that for glial lineages.

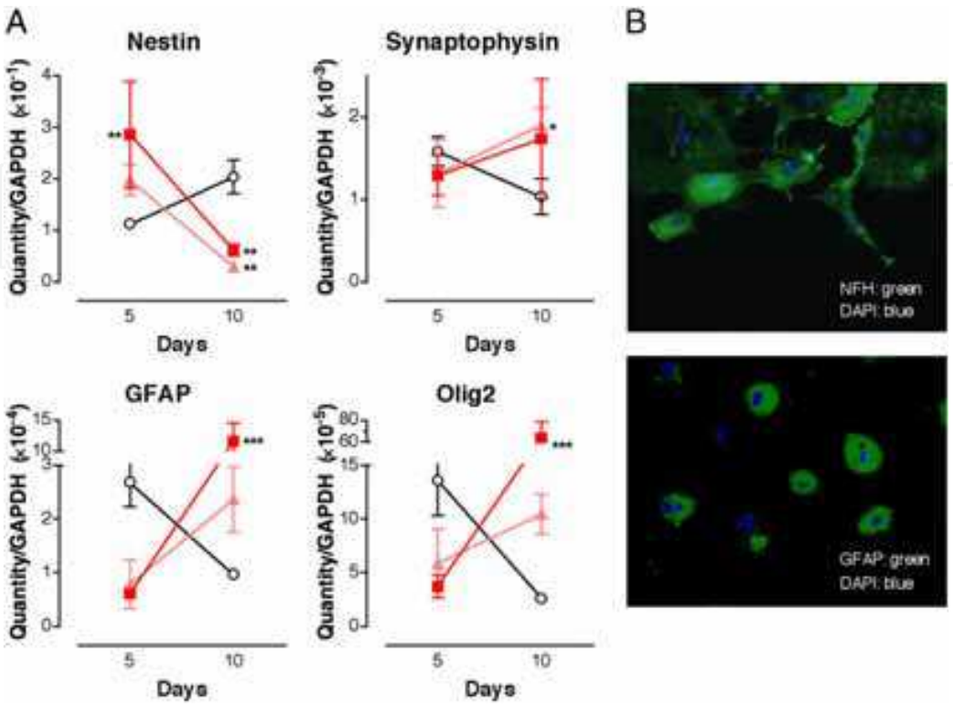

Fig. 10. Analysis of expression levels for ectodermal differentiation. The expression levels of the ectodermal markers, Nestin, Synaptophysin, GFAP, and Olig2 were quantified at each concentration of fluoxetine (0 µM: open circle; 1 µM: closed triangle; 3 µM: closed square) with real-time RT-PCR (A). Neural and glial cells derived from ES cells were cultured in the presence of fluoxetine (3 µM) and immunostained with NFH antibody and GFAP antibody (B) (Kusakawa et al. 2008).

4. Further improvement for EST

The issue of the necessity of adding a metabolic system to the EST to ensure the metabolic activation of potential embryotoxicants has been actively discussed (Marx-Stoelting et al. 2009). Potential experimental procedures are the use of cultured hepatocytes, especially human primary hepatocytes. Hepatocytes may be useful in systems when a preincubation step is applied to a chemical or a pharmaceutical and subsequently added to the ES cell culture with the active metabolite. Whether or not the development of a metabolic activation system for the EST is truly required has been the subject of much discussion, given the difficulties associated with it (Marx-Stoelting et al. 2009). Many hepatotoxicities arise from metabolites derived from drugs (Park et al. 2005). For this reason, it is important to use an assay system with cells that harbor drug-metabolizing enzymes to assess the drug toxicity accurately. In addition, maternal metabolism and fetoplacental interaction have to be taken into account, because some proteratogens require bioactivation to provide the active molecule (Brown et al. 1986). Therefore, the inclusion of a reliable metabolizing system would extend the usefulness of *in vitro* test procedures.

5. Conclusion

Among the various toxic tests, an EST can determine embryotoxicity and teratogenicity, and it does not require experimental animals. The EST has thus far been modified by being combined with real-time PCR, FACS analysis, GFP reporter analysis, or luciferase reporter analysis to detect molecular markers as new endpoints for the EST. In addition, some methods for ES cell differentiation into several cell types such as neural cells have been attempted to identify additional differentiation endpoints. However, further improvement of the EST is needed in order to investigate the harmful effects of chemicals and pharmaceuticals more exhaustively. For instance, a metabolic system designed to detect proteratogenic compounds has to be integrated in order to extend the applicability. Furthermore, a main advantage of embryotoxicity testing by the EST is the availability of human ES cells. Not all mammalian species are equally susceptible or sensitive to the toxic influences of a chemical. A compound that brings out defects in one species can have other or no effects on another species. Using two or more species for regulatory developmental toxicity testing currently covers the detection of genetic differences that influence the response to a chemical. A test system based on mES cells can now be adapted to human ES cells. The use of a humanized test system will have much greater predictive ability because some developmental pathways that could act as targets for chemicals are specific to human development. Thus, the EST can be significantly improved by combining tissue differentiation systems and/or metabolic systems, or by using human ES cells in the quick and accurate estimation of the *in vivo* embryotoxic effects of various medicines.

6. Acknowledgments

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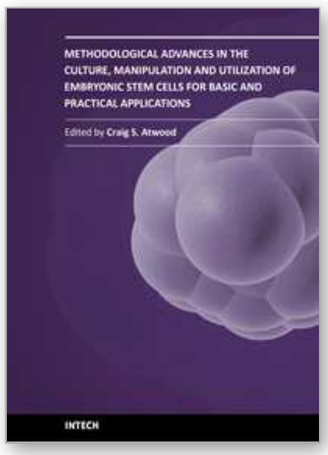

Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes methodological advances in the culture and manipulation of embryonic stem cells that will serve to bring this promise to practice.

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Slavka Krautzeka 83/A
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