Original Article

Analysis of altered gene expression specific to embryotoxic chemical treatment during embryonic stem cell differentiation into myocardiac and neural cells

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ABSTRACT — Embryonic stem cells (ES cells), pluripotent cells derived from the inner cell mass of blastocysts, differentiate *in vitro* into a variety of cell types representing all three germ layers. They therefore constitute one of the most promising *in vitro* tools for developmental toxicology. To assess the developmental toxicity of chemicals using ES cells easily, identification of effective marker genes is a high priority. We report here altered gene expression during ES cell differentiation into myocardiac and neural cells on treatment with some embryotoxic and non-embryotoxic chemicals. Decreases in several undifferentiated markers such as Oct3/4 and Nanog, and elevated expression of genes associated with heart development or the central nervous system, respectively, were found on microarray analysis. Under differentiation of ES cells into myocardiac cells, 107 genes were substantially up-regulated. Decrease in the expression of 13 genes of these (Hand1, Pim2, Tbx20, Myl4, Myl7, Hbb-bh1, Hba-a1, Col1a2, Hba-x, Cmya1, Pitx2, Smyd1 and Adam19) was observed specifically by embryotoxic chemicals. Of the 107 genes up-regulated under differentiation into neurons, 22 genes (Map2, Cpe, Marcks, Ptbp2, Sox11, Tubb2b, Vim, Arx, Emx2, Pax6, Basp1, Ddr1, Ndn, Sfrp, Ttc3, Ubqln2, Six3, Dcx, L1cam, Reln, Wnt1 and Nnat) showed reduced expression specifically by embryotoxic chemicals. Almost all gene sets identified in this study are known to be indispensable for differentiation and development of heart and brain tissues, and thus may serve in early detection or prediction of embryotoxicity of chemicals *in vitro*.

Key words: Embryonic stem cells, Developmental toxicity, Embryotoxicity, Myocardiac cell, Neural cell

INTRODUCTION

Developmental toxicity is one of the most serious side effects of chemical compounds and evaluation of adverse effects on reproduction and embryonic development is therefore an important objective of toxicological safety assessment. Because with *in vivo* reproductive toxicity the influence of chemicals in processes of embryogenesis must be taken into consideration, it is difficult to predict using transformed cell lines. While usage of primary cells, cultures of dissociated cells from rat embryo limb buds or whole embryos from rat for alternative methods have been evaluated (Spielmann, 1998), it has been argued that these tests are unlikely to gain widespread acceptance (Bremer and Hartung, 2004). Moreover, it is generally acknowledged that the existing *in vivo* assays for

developmental toxicity are very time consuming, laborious, expensive and conflicting with the current need for rapid screening of potential drugs and chemicals. There is an increasing political and public demand for reduction in the use of laboratory animals.

Therefore, the development of alternative screening procedures is a high priority. To reduce the amount of animal experimentation, several *in vitro* systems have recently been developed using primary cells, cultures of dissociated cells from rat embryo limb buds (micromass test, MM test) (Flint and Orton, 1984) or whole embryos from *Xenopus*, the chicken or the rat (whole embryo culture, WEC) (Schmid *et al.*, 1985). However, these tests are unlikely to gain widespread acceptance and use (Bremer and Hartung, 2004).

Embryonic stem cells (ES cells) are pluripotent cells

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derived from inner cell mass of blastocysts which are known to differentiate *in vitro* into a wide variety of cell types representing all three germ layers (Evans and Kaufman, 1981). Under appropriate culture conditions some of them differentiates spontaneously into contracting myocardial cells (Scholz *et al.*, 1999). Moreover, various kinds of neurons such as the dopaminergic and the telencephalic precursor neurons can be induced efficiently by appropriate methods (Kawasaki *et al.*, 2000, Watanabe *et al.*, 2005). Many studies have focused on ES cell differentiation as a model for studying developmental biology while others have taken advantage of these remarkable cells as potential tools for drug or chemical embryotoxicity screening (Rohwedel *et al.*, 2001; Davila *et al.*, 2004). In the 1990s, several groups used ES cells to establish an *in vitro* developmental toxicity test, called the "Embryonic stem cell test (EST)" (Spielmann *et al.*, 1997). The EST was designed to predict embryotoxicity based on the inhibition of differentiation of ES cells into contracting myocardial cells in combination with cytotoxicity data for ES and 3T3 cells. This method has been validated by the European Centre for the Validation of Alternative Methods (ECVAM) as being reliable for the prediction of embryotoxicity *in vivo* (Genschow *et al.*, 2004). Using more than 20 reference compounds with different embryotoxic potencies (non-embryotoxic, weakly and strongly embryotoxic), the EST was shown to provide a correct judgment in 78% of all experiments. Remarkably, a predictivity of 100% was obtained for strong embryotoxicants. However, a major weakness of the EST is its reliance on a morphological endpoint (contracting myocardial cells) and the need for experienced personnel ensuring its reliable assessment. In addition, the assay time of 10

days is rather too long for screening purposes for drugs and chemicals. Myocardial cells were originally selected as target cells due to the very easy identification of contracting cells in bulk cultures of differentiating embryonic stem cells and due to the fact that the heart is the first organ which develops during organogenesis. However, additional major target tissues of teratogens such as the nerve system and skeletal system have to be included in order to obtain precise information about the teratogenic potential of chemicals (Bremer and Hartung, 2004). Therefore identification of critical endpoints for the early detection and prediction of embryotoxicity in major target tissues of teratogens is needed for development of convenient and accurate *in vitro* alternative tests for developmental toxicity.

The aim of the present study was to estimate alteration in the expression of genes related to developmental toxicity specific to embryotoxic chemicals during ES cell differentiation into myocardial and neural cells. DNA microarray analysis was applied to determine up- and down-regulated genes with 6 embryotoxic and 6 non-embryotoxic chemicals, several genes being identified as specifically altered by embryotoxic chemicals.

MATERIALS AND METHODS

Test chemicals

Twelve test chemicals (Table 1) used in the ECVAM international validation study (Brown, 2002; Genschow *et al.*, 2004) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemicals were dissolved in appropriate solvents, that is, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), or ethanol (EtOH) as indicated.

Table 1. List of selected test chemicals and the concentration for treatment

<i>In vivo</i> category	Chemicals	Myocardiac cell dose ($\mu\text{g/ml}$)	Neural cell dose ($\mu\text{g/ml}$)	Solvent
Strong-embryotoxic	5-Fluorouracil	0.05	0.04	PBS
	Hydroxyurea	4	4	PBS
	6-Amino nicotinamide	1	1.5	DMSO
	5-Bromo-2'-deoxyuridine	1.5	0.5	PBS
	Methotrexate	0.6	0.2	PBS
	all-trans-Retinoic acid	0.001	0.001	DMSO
Non-embryotoxic	Saccharin sodium salt hydrate	1000	1000	PBS
	Ascorbic acid	20	20	PBS
	Isoniazide	125	150	PBS
	(+)-Camphor	1000	1000	EtOH
	Acrylamide	25	25	DMSO
	PenicillinG	1000	1000	PBS

*Teratogenicity of test chemicals are summarized in previous reports (Smith *et al.*, 1983; Brown, 2002).

Cell lines and culture conditions

Mouse ES cells, line D3, purchased from American Type Culture Collection (ATCC) were cultured in high glucose (4.5 g glucose/l) Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 15% fetal calf serum (Hyclone, South Logan, UT, USA), 2 mM glutamine (Invitrogen), antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Nacalai tesque, Kyoto, Japan), 1% non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich) and 1,000 U/ml murine leukemia inhibitory factor (mLIF; Millipore, Japan), as described previously (Spielmann *et al.*, 1997). Cells maintained in 60-mm² cell culture dishes (BD Falcon, Tokyo, Japan) under 5% CO₂ and 95% humidity at 37°C were routinely passaged every 2-3 days.

Differentiation of ES cells into myocardial cells

Differentiation of mouse ES cells into myocardial cells was carried out under the EST protocol as described previously (Spielmann *et al.*, 1997; Seiler *et al.*, 2006). Briefly, 750 cells in 20 µl differentiation medium (as described under cell lines and culture conditions but without mLIF to allow differentiation) were placed on the lid of a petri dish filled with phosphate buffered saline (PBS, Invitrogen) and then incubated for 3 days at 37°C under 5% CO₂ and 95% humidity ("hanging drop" culture). During this period the cells formed aggregates referred to as embryoid bodies (EBs). After 3 days of "hanging drop" culture the EBs were transferred to bacterial petri dishes for another 2 days. On day 5, EBs were plated separately into wells of a 24-multiwell tissue culture plate (BD Falcon) to allow adherence and outgrowth and development of spontaneously contracting cardiac muscle cells. Differentiation was determined by contracting of myocardial cells under microscopic inspection at day 10.

Differentiation of ES cells into neural cells

Differentiation of mouse ES cells into neural cells was carried out under serum-free conditions as described previously (Watanabe *et al.*, 2005). Differentiation medium was prepared as follows: G-MEM supplemented with 5% Knockout serum replacement (KSR) (Invitrogen), 2 mM glutamine, 1 mM pyruvate (Invitrogen), 0.1 mM nonessential amino acids, and 0.1 mM 2-mercaptoethanol. ES cells were dissociated with 0.25% trypsin-EDTA to single cells and aliquots (5 X 10⁴ cells/ml differentiation medium) were seeded into 10 ml of differentiation medium in bacterial-grade dishes to generate floating cell aggregates spontaneously. Then, cell aggregates were replaced on differentiation medium in 24-multiwell tissue culture plates coated with poly-D-lysine, raminine and fibronectin,

at a density of 50 aggregates per well on day 5. Differentiation was determined by counting neurofilaments with microscopic inspection of differentiated cells labeled with Vybrant® DiI cell-labeling solution (LONZA, Tokyo, Japan) at day 10.

Determination of the genes involved in ES cell differentiation into myocardial and neural cells

In order to analyze global changes in gene expression during ES cell differentiation into myocardial and neural cells, gene expression was assessed by DNA microarray at 6 different time points (days 0, 2, 4, 6, 8 and 10). Three samples of mixtures of 40 EBs or 50 floating aggregates, respectively, were collected on days 0, 2, 4, 6, 8 and 10 of cardiac and neural cell differentiation. Total RNA was extracted using TRIzol solvent (Invitrogen) for gene expression analysis with the GeneChip system (Affymetrix, Japan) following the manufacturer's protocol. Briefly, double-strand DNA was synthesized from 3 µg of total RNA and the obtained cDNAs were used as templates for *in vitro* transcription. Fragmented *in vitro* transcripts were hybridized overnight onto Mouse Genome 430 2.0 microarrays (Affymetrix), stained, washed and scanned with an Affymetrix GeneArray scanner. The obtained image files were analyzed with the Affymetrix data suite system.

To determine the genes involved in myocardial and neural cell differentiation, significant alterations (more than 2-fold) in the obtained expression data were extracted by statistical analysis (ANOVA) of gene expression differences between day 0 and the other days. Then, genes which were substantially up-regulated during the differentiation processes were extracted on the basis of change in signal value of gene expression (a more than 1,000 signal value).

Treatment with embryotoxic and non-embryotoxic chemicals during ES cell differentiation into myocardial and neural cells

Differentiation of mouse ES cells into myocardial or neural cells with test chemicals was performed basically as described above. The cells were treated with test compounds on days 0, 3 and 5 with medium changes and differentiated cells were collected periodically from days 1 to 10. Total RNAs of all samples were extracted with TRIzol reagent. The dose levels of the test compounds were set on the basis of the maximum non-effect concentration in cytotoxicity assays or the minimum concentration for 100% inhibition in differentiation assays (Table 1). Cytotoxicity assays of test chemicals were performed as previously described (Scholz *et al.*, 1999) with minor modification. Briefly, 500 ES cells were seeded into a

96-multiwell tissue culture plate in differentiation medium with or without test chemicals. Medium was changed on days 3 and 5. Cytotoxicity was measured on day 10 using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Japan) based on the MTT assay, according to the manufacturer's instructions. Differentiation assays were performed basically as described above. The cells were treated with test compounds on days 0, 3 and 5 with medium changes and percentage inhibition of differentiation into myocardial or neural cells was calculated by counting the numbers of contracting EBs or neurofilaments, respectively.

Analysis of genes featuring altered expression on treatment with embryotoxic and non-embryotoxic chemicals during ES cell differentiation into myocardial and neural cells

To confirm the expression levels of the genes extracted in the DNA microarray analysis described above, quantitative PCR was used. Reverse transcription was performed with SuperScript III for 50 min at 42°C, and oligo (dT) as a primer, using 300 ng of total RNA. The mRNA was quantified with TaqMan gene expression assays (Applied Biosystems, Japan) on a 7900 Fast Real-Time PCR System, with a two-step PCR procedure according to manufacturer's protocol. The thermal cycling conditions were: 95°C for 20 sec for the first cycle, followed by 40 cycles of 95°C for 3 sec and 60°C for 20 sec. The TaqMan probes used in the present study are shown in Tables 2 to 5.

To estimate the genes involved in embryotoxicity, RNA samples from differentiated myocardial or neural cells treated with the embryotoxicants and non-embryotoxicants described above were analyzed by quantitative PCR at the time points with the highest expression of each gene.

RESULTS

Determination of genes involved in ES cell differentiation into myocardial cells

Global changes in the gene expression during ES cell differentiation into myocardial cells were analyzed by DNA microarray at 6 different time points (days 0, 2, 4, 6, 8 and 10). As shown in Table 2a, changes in gene expression of typical marker genes for an undifferentiated state (Oct3/4 and Nanog) decreased gradually during the cardiac differentiation. In addition, expression of 25 well-known mesoderm markers and several genes playing important roles in vertebrate heart formation (list in Table 2b) were analyzed. In the early differentiation stage

(days 0 to 4), expression of Bmp2, Cer1, Nodal and T (brachyury) was elevated, and increases in the expression of Gata4, Nkx2-5, Mef2a, Mef2c, Cdh2, Hand1, Hand2, Pitx2, Xin, Smyd1, Tbx20, Tbx5, Adam19, Tpm2, Myh6, Col1a2, Myl4, Myl7, Hbb-bh1, Hba-a1 and Hba-x were found in the middle and late differentiation stages (days 6 to 10) (Table 2b). These expression patterns demonstrated a rough concordance with those observed during differentiation in early embryogenesis *in vivo* (Guan *et al.*, 1999; Rohwedel *et al.*, 2001). Examples with more than 2-fold significant change were extracted by comparative analysis between day 0 and other days using the obtained expression data from microarray analysis. The genes substantially up-regulated more than 1,000 signal values were identified as candidate genes involved in ES cells differentiation into myocardial cells. Totals of 21, 39, 26, 65, and 10 were identified in days 2, 4, 6, 8 and 10, respectively, 82 genes being obtained without overlapping for further analysis (Table 3).

Determination of genes involved in ES cell differentiation into neural cells

As shown in Table 4a, the expression of Oct3/4 and Nanog decreased gradually during neural differentiation. In contrast, the expression levels of a neural progenitor marker, Nest (Nestin) (Kawaguchi *et al.*, 2001), was only increased in the middle differentiation stage (day 4 to 6). In the late differentiation stage, increases in 22 gene expressions of ectoderm markers, neurotransmitters, and several genes playing important roles in brain development were observed (Table 4b). These expression patterns roughly also reflected the gene expression patterns observed during the differentiation of early embryogenesis *in vivo* (Guan *et al.*, 1999; Rohwedel *et al.*, 2001). The genes substantially up-regulated more than 1,000 signal values in DNA microarray analysis were identified as candidate genes involved in ES cell differentiation into neural cells. Totals of 34, 44, 37, 33 and 53 were identified in days 2, 4, 6, 8 and 10, respectively, 85 genes being obtained without overlapping for further analysis (Table 5).

Analysis of genes with altered expression by treatment with embryotoxic and non-embryotoxic chemicals during ES cell differentiation into myocardial and neural cells

To estimate genes related to developmental toxicity, six embryotoxicants (5-fluorouracil, hydroxyurea, 6-aminonicotinamide, 5-bromo-2'-deoxyuridine, methotrexate and all-*trans* retinoic acid) and six non-embryotoxicants (penicillin G, saccharin sodium salt hydrate, ascorbic

Genes related to developmental toxicity

Table 2. Expression profiles of typical marker genes during ES cell differentiation into myocardial cell

Gene Symbol	Gene Title	Expression levels at each day						TaqMan probe ID
		0	2	4	6	8	10	
a) Marker genes of undifferentiation								
Oct3/4 (Pou5f1)	POU domain, class 5, transcription factor 1	2938.0 ± 213.3	3062.8 ± 182.9	2429.1 ± 64.9	313.5 ± 78.0	91.7 ± 9.2	81.2 ± 37.0	Mm00658129_gH
Nanog	Nanog homeobox	1507.5 ± 121.7	705.8 ± 78.8	1296.5 ± 101.4	236.2 ± 69.1	110.6 ± 22.4	106.7 ± 51.1	Mm01617761_gI
b) Genes that associated with heart development and mesoderm marker genes								
Bmp2	bone morphogenetic protein 2	17.2 ± 2.6	9.9 ± 2.0	43.2 ± 9.7	316.8 ± 47.8	297.0 ± 46.6	308.5 ± 90.4	Mm01340178_mI
Cer1	cerberus 1 homolog (Xenopus laevis)	2.3 ± 1.1	27.1 ± 5.4	58.0 ± 13.9	20.3 ± 7.2	11.1 ± 0.9	6.4 ± 3.9	Mm00515474_mI
Gata4	GATA binding protein 4	12.6 ± 8.4	6.8 ± 4.8	64.0 ± 8.1	292.9 ± 48.2	481.8 ± 68.2	351.4 ± 45.0	Mm00484689_mI
Nkx2-5	NK2 transcription factor related, locus 5 (Drosophila)	9.2 ± 3.7	10.3 ± 4.1	10.5 ± 3.0	104.5 ± 37.1	274.6 ± 70.6	185.1 ± 18.3	Mm00657783_mI
Mef2a	myocyte enhancer factor 2A	102.6 ± 9.2	91.8 ± 9.6	138.3 ± 25.4	308.6 ± 43.7	509.0 ± 82.4	570.0 ± 11.6	Mm00488969_mI
Mef2c	myocyte enhancer factor 2C	2.9 ± 1.9	2.7 ± 1.3	3.0 ± 2.2	160.4 ± 105.5	227.0 ± 31.5	165.9 ± 26.8	Mm00600423_mI
Cdh2	cadherin 2	70.9 ± 5.0	72.2 ± 24.3	275.9 ± 21.8	313.3 ± 33.0	386.2 ± 88.7	460.1 ± 121.9	Mm00483213_mI
Hand1	heart and neural crest derivatives expressed transcript 1	26.1 ± 7.1	12.7 ± 2.9	28.9 ± 5.6	1089.5 ± 122.1	724.3 ± 122.4	403.1 ± 84.9	Mm00433931_mI
Hand2	heart and neural crest derivatives expressed transcript 2	5.4 ± 3.0	4.4 ± 2.0	26.2 ± 11.0	1094.1 ± 157.9	1318.4 ± 117.1	1610.3 ± 176.1	Mm00439247_mI
Pitx2	paired-like homeodomain transcription factor 2	67.4 ± 10.4	81.4 ± 7.2	313.6 ± 46.2	229.9 ± 33.9	601.6 ± 62.0	945.9 ± 205.1	Mm00660192_gI
Xin (Cmyal)	cardiomyopathy associated 1	2.9 ± 4.6	1.4 ± 1.5	1.3 ± 1.7	6.8 ± 5.3	77.9 ± 26.6	70.3 ± 19.6	Mm00495998_mI
Smyd1	SET and MYND domain containing 1	7.7 ± 5.5	5.0 ± 1.5	1.6 ± 1.2	26.2 ± 11.7	127.9 ± 13.3	79.3 ± 10.4	Mm00477663_mI
Tbx20	T-box 20	8.4 ± 5.9	4.6 ± 3.4	17.8 ± 3.1	186.9 ± 60.7	259.8 ± 120.2	277.9 ± 21.5	Mm00451515_mI
Tbx5	T-box 5	0.8 ± 0.2	1.0 ± 0.5	1.8 ± 2.2	37.0 ± 4.9	66.9 ± 17.6	53.8 ± 1.9	Mm00803521_mI
Adam19	a disintegrin and metalloproteinase domain 19	241.4 ± 25.1	336.7 ± 65.7	249.8 ± 38.9	244.9 ± 34.8	397.4 ± 53.1	564.4 ± 76.3	Mm00803521_mI
Nodal	nodal	104.6 ± 10.1	101.8 ± 11.2	131.9 ± 22.8	21.4 ± 13.0	26.5 ± 6.1	16.2 ± 3.4	Mm00443041_mI
Tpm2	tropomyosin 2, beta	426.5 ± 50.1	278.9 ± 33.0	552.6 ± 80.0	650.1 ± 104.2	1168.5 ± 207.4	1414.0 ± 186.8	Mm00437172_gI
T	brachyury	252.3 ± 20.6	139.3 ± 11.5	1102.7 ± 258.6	224.4 ± 93.6	26.8 ± 8.2	22.4 ± 28.0	Mm01318252_mI
Myh6	myosin, heavy polypeptide 6, cardiac muscle, alpha	34.7 ± 6.7	39.2 ± 14.2	26.3 ± 7.1	104.2 ± 72.2	2254.6 ± 812.2	2258.4 ± 592.8	Mm00440354_mI
Col1a2	procollagen, type I, alpha 2	160.0 ± 16.4	15.3 ± 1.4	35.7 ± 6.1	302.0 ± 17.1	1717.2 ± 207.5	2979.0 ± 482.4	Mm01165187_mI
Myf4	myosin, light polypeptide 4	60.6 ± 10.5	20.0 ± 7.0	13.4 ± 1.7	351.2 ± 186.7	3182.0 ± 922.4	2974.6 ± 210.3	Mm00440378_mI
Myf7	myosin, light polypeptide 7, regulatory	60.7 ± 5.0	48.6 ± 9.1	71.7 ± 11.0	1020.7 ± 621.5	6749.0 ± 1400.3	5660.3 ± 533.3	Mm00491655_mI
Hbb-bh1	hemoglobin Z, beta-like embryonic chain	0.8 ± 0.9	0.6 ± 0.3	42.1 ± 2.9	4938.2 ± 2656.0	6285.8 ± 828.4	2521.9 ± 1062.3	Mm00433932_gI
Hba-a1	hemoglobin alpha, adult chain 1	12.6 ± 6.7	10.6 ± 2.1	37.2 ± 5.8	1836.8 ± 1357.2	3850.1 ± 1268.3	2409.0 ± 573.2	Mm00845395_sl
Hba-x	hemoglobin X, alpha-like embryonic chain in Hba complex	2.1 ± 2.1	3.0 ± 2.2	11.6 ± 1.0	1653.9 ± 1237.1	3329.4 ± 875.4	1114.2 ± 607.3	Mm00439255_sl

* Values represent means ± S.D. of data from three independent experiments.

Table 3. Expression profiles of the genes altered expression during ES cell differentiation into myocardial cells

Gene Symbol	Gene Title	Expression levels at each day						TaqMan probe ID	
		0	2	4	6	8	10		
I110038B12Rik	RIKEN cDNA 1110038B12 gene	638.1 ± 90.2	961.0 ± 114.5	1640.8 ± 141.6	1016.2 ± 119.9	510.0 ± 27.3	651.5 ± 41.6	Mm01167082_g1	
I500012F01Rik	RIKEN cDNA 1500012F01 gene	436.9 ± 19.5	456.7 ± 94.5	1389.2 ± 227.0	1145.3 ± 336.6	589.5 ± 198.9	825.1 ± 85.3	Mm01247987_m1	
I810014B01Rik	RIKEN cDNA 1810014B01 gene	415.8 ± 43.0	1001.3 ± 116.8	736.6 ± 65.1	434.7 ± 53.9	238.8 ± 14.5	258.3 ± 12.9	Mm01612218_m1	
I2310056P07Rik	RIKEN cDNA 2310056P07 gene	1406.7 ± 32.4	1222.5 ± 51.5	3369.0 ± 318.7	2835.2 ± 117.1	2087.8 ± 154.2	2151.3 ± 109.8	Mm00510335_m1	
I2410006H16Rik	RIKEN cDNA 2410006H16 gene	967.4 ± 62.1	1210.0 ± 119.1	2878.5 ± 284.3	2795.5 ± 147.0	1606.6 ± 75.8	2086.5 ± 364.0	Mm01331815_g1	
I3732413111Rik	RIKEN cDNA 3732413111 gene	945.5 ± 103.4	1736.1 ± 43.5	2160.1 ± 124.4	1961.3 ± 135.7	1728.2 ± 121.0	1671.1 ± 97.2	Mm01289003_m1	
Acta2	actin, alpha 2, smooth muscle, aorta	576.0 ± 59.9	32.3 ± 5.6	56.4 ± 7.2	1399.4 ± 736.4	7155.3 ± 846.8	7049.0 ± 976.5	Mm00725412_s1	
Actc1	actin, alpha, cardiac	37.2 ± 3.3	14.4 ± 1.5	79.0 ± 22.5	832.4 ± 66.1	7487.2 ± 1758.5	5836.5 ± 506.6	Mm01333821_m1	
Adk	adenosine kinase	1034.0 ± 99.6	2397.4 ± 442.4	1694.0 ± 289.7	948.4 ± 176.8	779.7 ± 160.4	688.9 ± 55.5	Mm00612772_m1	
Afp	alpha fetoprotein	0.9 ± 0.5	0.9 ± 0.2	10.0 ± 2.9	286.1 ± 23.3	5088.5 ± 775.6	5545.4 ± 1199.7	Mm00431715_m1	
Amot	angiomin	131.6 ± 3.8	262.9 ± 46.2	1521.8 ± 290.7	2310.7 ± 235.2	1761.1 ± 208.0	818.8 ± 92.0	Mm00462731_m1	
Apoa1	apolipoprotein A-I	1.5 ± 1.8	3.7 ± 3.2	13.0 ± 5.2	429.9 ± 126.5	2182.0 ± 230.5	2023.1 ± 808.8	Mm00437569_m1	
ApoE	apolipoprotein E	1467.6 ± 87.1	1036.7 ± 189.8	1497.7 ± 271.1	2997.8 ± 502.4	4148.6 ± 634.1	2688.4 ± 557.7	Mm00437573_m1	
Binp3	BCL2/adenovirus E1B interacting protein 1, NIP3	821.7 ± 42.7	554.9 ± 65.2	1923.9 ± 106.0	1559.3 ± 56.7	1536.8 ± 41.5	1720.2 ± 203.3	Mm00833810_g1	
Binp3l	BCL2/adenovirus E1B interacting protein 3-like	756.8 ± 86.3	684.5 ± 22.7	1645.2 ± 81.8	1651.5 ± 132.2	1476.8 ± 68.2	1437.7 ± 116.5	Mm00786306_s1	
Cachd1	cache domain containing 1	85.7 ± 8.4	330.2 ± 38.1	971.0 ± 121.1	551.8 ± 81.2	510.5 ± 87.1	445.6 ± 28.8	Mm01256098_m1	
Car4	carbonic anhydrase 4	53.6 ± 6.5	425.8 ± 29.0	830.5 ± 9.9	2083.2 ± 550.8	2194.5 ± 623.6	996.0 ± 304.2	Mm00483021_m1	
Cend2	cyclin D2	81.0 ± 4.0	73.7 ± 6.0	230.9 ± 40.9	753.8 ± 132.1	1515.9 ± 175.3	2168.1 ± 384.5	Mm00438071_m1	
Cem4l	CCR4 carbon catabolite repression 4-like	1496.8 ± 146.4	1382.0 ± 107.0	3131.3 ± 272.3	5440.7 ± 260.9	4502.1 ± 197.9	3219.7 ± 90.3	Mm00802276_m1	
Cd24a	CD24a antigen	887.0 ± 72.0	1166.0 ± 55.3	2242.3 ± 229.2	4953.8 ± 299.0	4795.9 ± 583.5	4372.3 ± 348.6	Mm00782538_sH	
Cldn7	claudin 7	354.0 ± 27.3	513.2 ± 72.9	1183.6 ± 99.5	1088.5 ± 109.4	1532.5 ± 185.8	1038.5 ± 260.5	Mm00516817_m1	
Clu	clusterin	428.4 ± 50.2	128.5 ± 12.7	282.1 ± 77.3	1174.2 ± 328.6	3055.5 ± 920.6	3171.1 ± 849.7	Mm00442773_m1	
Colla1	procollagen, type I, alpha 1	201.7 ± 13.0	12.4 ± 5.6	25.6 ± 2.3	594.3 ± 124.0	2125.2 ± 218.6	3398.3 ± 659.0	Mm00801666_g1	
Col3a1	procollagen, type III, alpha 1	29.5 ± 3.9	10.7 ± 2.9	7.4 ± 3.2	101.3 ± 60.9	2539.1 ± 805.8	5926.7 ± 656.4	Mm00802331_m1	
Col4a1	procollagen, type IV, alpha 1	300.2 ± 25.3	108.5 ± 15.2	442.5 ± 90.7	1639.5 ± 244.3	2808.8 ± 169.4	2852.4 ± 463.3	Mm00802372_m1	
Ctsl	cathepsin L	1713.9 ± 89.4	1716.9 ± 88.7	2069.2 ± 78.2	3272.1 ± 251.5	4223.0 ± 309.8	3258.7 ± 165.8	Mm00515597_m1	
Ddit4	DNA-damage-inducible transcript 4	301.5 ± 35.5	176.4 ± 26.9	1486.1 ± 194.5	999.6 ± 94.1	474.3 ± 38.9	385.2 ± 70.2	Mm00512503_g1	
Degs1	degenerative spermatocyte homolog 1 (Drosophila)	456.4 ± 38.1	996.4 ± 32.3	754.0 ± 88.7	708.9 ± 111.4	738.0 ± 149.0	636.6 ± 60.8	Mm00492146_m1	
Dnaj1	DnaJ (Hsp40) homolog, subfamily A, member 1	552.8 ± 21.0	1295.6 ± 92.6	882.5 ± 67.3	849.6 ± 95.1	826.2 ± 47.1	723.0 ± 40.6	Mm00787254_s1	
Dnm3a	DNA methyltransferase 3A	327.1 ± 28.7	1105.9 ± 138.9	329.9 ± 26.3	251.1 ± 61.3	231.3 ± 35.6	190.7 ± 25.5	Mm00432870_m1	
Dnm3b	DNA methyltransferase 3B	1140.9 ± 77.6	3658.2 ± 262.4	3519.9 ± 298.7	894.1 ± 189.8	287.1 ± 94.4	137.7 ± 67.0	Mm01240113_m1	
Emb	embigin	501.4 ± 73.4	1357.7 ± 208.6	2624.4 ± 508.2	3877.3 ± 394.1	3439.3 ± 358.8	1406.4 ± 281.9	Mm00515881_m1	
Eomes	omesodermin homolog (Xenopus laevis)	59.7 ± 8.7	132.0 ± 4.9	990.5 ± 153.6	162.4 ± 60.6	42.1 ± 11.5	25.9 ± 19.8	Mm01351985_m1	
Fn1	fibronectin 1	1633.2 ± 51.1	1105.1 ± 80.7	1296.0 ± 149.2	1990.2 ± 414.4	3599.1 ± 280.9	3668.2 ± 503.0	Mm01256734_m1	
Fst	folistatin	82.7 ± 23.0	174.7 ± 29.0	1221.7 ± 323.3	122.4 ± 21.0	67.3 ± 14.0	102.0 ± 34.9	Mm00514982_m1	
Fstl1	folistatin-like 1	330.9 ± 16.8	223.9 ± 9.5	542.7 ± 33.3	1488.5 ± 241.0	2014.2 ± 156.8	2646.8 ± 120.7	Mm00433371_m1	
Gja1	gap junction membrane channel protein alpha 1	779.2 ± 70.6	1266.2 ± 211.4	1588.8 ± 174.7	1062.1 ± 240.9	851.3 ± 223.2	736.0 ± 143.2	Mm00439105_m1	
Gnas	guanine nucleotide binding protein, alpha stimulating	1861.6 ± 196.2	1375.6 ± 33.6	3533.4 ± 116.4	5267.2 ± 424.1	5926.4 ± 748.6	5208.7 ± 474.9	Mm00456660_m1	
Gpx3	glutathione peroxidase 3	271.2 ± 29.3	214.7 ± 20.7	294.8 ± 17.7	1311.6 ± 244.1	2965.7 ± 366.8	2556.5 ± 367.1	Mm00492427_m1	
Gtl2	imprinted maternally expressed untranslated mRNA	947.8 ± 86.4	937.0 ± 95.0	1549.3 ± 229.6	2799.0 ± 294.0	4346.0 ± 666.7	4876.9 ± 767.5	Mm00522599_m1	
H19	H19 fetal liver mRNA	1729.6 ± 156.3	488.4 ± 83.5	1636.0 ± 153.3	7570.4 ± 514.6	8656.5 ± 676.3	7564.5 ± 429.0	Mm00469706_g1	

Genes related to developmental toxicity

Table 3. (Continued).

Gene Symbol	Gene Title	Expression levels at each day						TaqMan probe ID	
		0	2	4	6	8	10		
Hbb-y	hemoglobin Y, beta-like embryonic chain	0.2 ± 0.0	0.8 ± 0.7	4.2 ± 3.5	422.8 ± 312.9	2448.2 ± 719.3	1453.1 ± 776.3	Mm00433936_g1	
Igf2	insulin-like growth factor 2	156.7 ± 8.5	41.9 ± 7.3	103.7 ± 12.4	1626.2 ± 258.2	2528.6 ± 371.5	3081.9 ± 79.5	Mm00439565_g1	
Itm2a	integral membrane protein 2A	331.7 ± 28.9	624.6 ± 95.8	817.7 ± 84.6	707.4 ± 78.1	1351.5 ± 153.1	2946.5 ± 307.0	Mm00515208_m1	
Krt1-18	keratin complex 1, acidic, gene 18	1923.3 ± 52.7	477.3 ± 34.7	1562.2 ± 82.6	4878.3 ± 409.1	5592.3 ± 301.9	3931.5 ± 786.1	Mm01601706_g1	
Krt2-8	keratin complex 2, basic, gene 8	1845.8 ± 34.0	309.2 ± 21.0	1111.0 ± 62.1	3539.0 ± 289.3	4092.8 ± 263.2	2699.4 ± 323.4	Mm00835759_m1	
Limd2	LIM domain containing 2	610.5 ± 25.2	959.1 ± 134.7	1495.4 ± 134.3	1305.1 ± 102.1	1168.1 ± 104.2	1093.0 ± 53.2	Mm00549554_g1	
LOC434261	keratin complex 2, basic, gene 8	2045.6 ± 293.9	383.5 ± 29.4	1337.1 ± 140.8	4033.3 ± 270.9	4780.1 ± 215.8	3358.3 ± 457.3	Mm00835759_m1	
LOC671894	similar to myosin, heavy polypeptide 4,	17.5 ± 6.0	20.9 ± 2.7	8.8 ± 8.3	164.0 ± 49.6	2260.4 ± 626.5	2288.7 ± 722.7	Mm00600555_m1	
LOC672274	SRY-box containing gene 4	715.3 ± 39.7	1458.5 ± 108.8	1957.3 ± 108.3	2388.0 ± 186.3	2708.5 ± 301.4	2789.4 ± 84.4	Mm00486317_s1	
Mest	mesoderm specific transcript	989.2 ± 64.2	1663.4 ± 52.8	1131.0 ± 70.2	3243.0 ± 274.5	5048.6 ± 255.9	4319.9 ± 441.6	Mm00484993_m1	
Mkml1	makorin, ring finger protein, 1	609.5 ± 108.0	1573.4 ± 672.3	947.6 ± 412.6	238.0 ± 77.2	174.8 ± 72.0	154.9 ± 17.7	Mm00785672_s1	
Mtl	metallothionein 1	1760.1 ± 266.8	2054.5 ± 142.4	4943.3 ± 329.2	1375.4 ± 203.4	2397.6 ± 831.4	1687.2 ± 320.9	Mm00496660_g1	
Myh10	Myosin, heavy polypeptide 10, non-muscle	893.5 ± 60.0	712.8 ± 29.0	1266.9 ± 98.0	1689.3 ± 191.2	3063.2 ± 167.7	2720.1 ± 353.7	Mm00805131_m1	
Ncl	nucleolin	249.8 ± 26.3	763.7 ± 30.8	587.0 ± 82.6	296.3 ± 28.1	204.5 ± 24.1	154.8 ± 24.1	Mm00834059_g1	
Ox2	orthodenticle homolog 2 (Drosophila)	78.7 ± 13.8	1034.8 ± 73.3	910.1 ± 81.4	198.3 ± 64.5	95.8 ± 21.2	64.0 ± 32.8	Mm00446859_m1	
Peg3	paternally expressed 3	418.2 ± 28.1	394.6 ± 99.0	1023.0 ± 173.9	5128.3 ± 355.4	7072.1 ± 127.8	5923.2 ± 502.9	Mm00493299_s1	
Pfkfb1	phosphofructokinase, liver, B-type	987.2 ± 96.1	1192.0 ± 9.1	2584.2 ± 145.7	2237.6 ± 205.3	1501.7 ± 76.3	1466.1 ± 52.6	Mm00435587_m1	
Pgk1	phosphoglycerate kinase 1	857.4 ± 436.1	847.8 ± 234.0	4005.5 ± 337.6	3455.3 ± 563.1	2871.1 ± 524.0	2475.2 ± 536.0	Mm00435617_m1	
Pim2	provinial integration site 2	396.1 ± 19.1	1693.6 ± 110.7	2915.2 ± 94.6	345.1 ± 111.0	102.8 ± 5.3	74.0 ± 44.1	Mm00454579_m1	
Plagl1	pleiomorphic adenoma gene-like 1	482.7 ± 32.7	182.7 ± 27.8	278.1 ± 18.1	879.4 ± 424.9	5489.6 ± 846.0	6311.9 ± 713.6	Mm00494250_m1	
Pmp22	peripheral myelin protein	336.0 ± 27.3	53.3 ± 7.0	244.1 ± 31.1	1957.1 ± 320.3	3553.7 ± 242.6	3542.4 ± 731.0	Mm00476978_m1	
Podxl	podocalyxin-like	173.3 ± 18.7	294.8 ± 30.1	837.9 ± 73.4	3289.5 ± 318.0	3047.8 ± 404.3	1492.6 ± 307.8	Mm00449829_m1	
Postn	perostin, osteoblast specific factor	15.9 ± 7.7	2.6 ± 3.3	4.4 ± 2.5	75.7 ± 54.3	1591.2 ± 425.8	3267.2 ± 654.5	Mm00450111_m1	
Pou3f1	POU domain, class 3, transcription factor 1	110.3 ± 18.1	890.9 ± 289.1	447.7 ± 238.4	49.1 ± 27.4	26.0 ± 21.6	17.0 ± 10.5	Mm00843534_s1	
Prkcbp1	protein kinase C binding protein 1	311.4 ± 10.5	464.1 ± 28.3	1124.1 ± 165.7	829.3 ± 108.8	625.7 ± 55.4	506.0 ± 20.9	Mm00835499_m1	
Rbp4	retinol binding protein 4, plasma	58.6 ± 3.9	33.6 ± 4.8	63.5 ± 10.3	1506.8 ± 342.0	5612.4 ± 396.9	3873.9 ± 1197.7	Mm00803266_m1	
Rpl15	ribosomal protein L15	882.3 ± 80.2	1750.8 ± 90.3	1775.2 ± 45.3	2109.0 ± 123.2	1506.9 ± 148.5	1453.2 ± 163.0	Mm01727317_g1	
Rps3	ribosomal protein S3	197.5 ± 16.3	702.5 ± 22.3	815.0 ± 67.8	739.0 ± 105.3	461.4 ± 72.0	412.6 ± 13.9	Mm00656272_m1	
Serpinh1	serine (or cysteine) peptidase inhibitor, clade H, member 1	584.4 ± 17.5	213.2 ± 20.5	488.6 ± 42.2	1649.7 ± 54.5	2854.3 ± 72.8	3018.7 ± 246.8	Mm00438056_m1	
Slc16a1	solute carrier family 16, member 1	1311.3 ± 238.8	1942.0 ± 150.1	2526.9 ± 240.3	3397.3 ± 541.2	2849.7 ± 454.0	1379.0 ± 221.2	Mm00436566_m1	
Slc16a3	solute carrier family 16, member 3	514.4 ± 96.1	862.8 ± 162.4	1637.3 ± 500.7	2081.2 ± 239.8	1366.6 ± 138.5	1345.9 ± 95.0	Mm00446102_m1	
Slc7a7	solute carrier family 7, member 7	384.2 ± 17.5	996.3 ± 91.6	527.5 ± 17.7	197.9 ± 36.2	142.9 ± 25.4	92.3 ± 8.2	Mm00448764_m1	
Snrpn	small nuclear ribonucleoprotein N	2190.1 ± 234.6	4413.6 ± 83.6	4001.6 ± 270.3	2115.5 ± 88.3	1673.6 ± 98.3	1183.8 ± 118.1	Mm01310473_g1	
Sparc	secreted acidic cysteine rich glycoprotein	2530.3 ± 259.9	1107.8 ± 117.6	1639.1 ± 47.3	3864.6 ± 305.4	6630.8 ± 74.3	7314.5 ± 850.2	Mm00486332_m1	
Spink3	serine peptidase inhibitor, Kazal type 3	42.3 ± 10.4	28.4 ± 8.3	499.4 ± 84.6	2581.7 ± 719.0	2589.1 ± 578.2	1100.3 ± 320.8	Mm00436765_m1	
Tle4	transducin-like enhancer of split 4	658.7 ± 154.7	1609.5 ± 263.4	711.5 ± 219.5	277.6 ± 87.9	422.1 ± 92.4	411.7 ± 85.6	Mm00502265_s1	
Tpi1	triosephosphate isomerase 1	1350.3 ± 31.0	1288.1 ± 72.4	2836.0 ± 164.7	2174.1 ± 149.8	1730.0 ± 99.8	1952.4 ± 82.7	Mm00833691_g1	
Tpm1	tropomyosin 1, alpha	1606.3 ± 197.6	418.1 ± 60.9	775.2 ± 74.4	2000.7 ± 131.1	3876.3 ± 718.6	3216.8 ± 254.5	Mm00600378_m1	
Ttr	transferrin	4.6 ± 3.0	36.3 ± 7.1	157.6 ± 42.8	5897.2 ± 1256.8	9091.1 ± 253.9	6130.6 ± 1028.0	Mm00443267_m1	
Wbp5	WW domain binding protein 5	2767.4 ± 181.7	2530.3 ± 123.4	3218.9 ± 249.0	5288.1 ± 469.6	5871.2 ± 114.2	5501.9 ± 109.1	Mm00810180_s1	
Zfp3612	zinc finger protein 36, C3H type-like 2	364.6 ± 37.7	1205.4 ± 48.7	1640.3 ± 47.2	1037.6 ± 139.7	904.2 ± 99.4	854.2 ± 84.9	Mm00492049_s1	

* Values represent means ± S.D. of data from three independent experiments.

Table 4. Expression profiles of typical marker genes during ES cell differentiation into neural cells

Gene Symbol	Gene Title	Expression levels at each day					TaqMan probe ID	
		0	2	4	6	8	10	
a) Marker genes of undifferentiation								
Oct3/4 (Pou5f1)	POU domain, class 5, transcription factor 1	1879.4 ± 73.1	1742.6 ± 124.8	830.0 ± 78.4	858.8 ± 32.9	166.2 ± 10.8	89.2 ± 21.6	Mm00658129_gH
Nanog	Nanog homeobox	1745.5 ± 327.9	334.9 ± 20.9	849.1 ± 44.7	688.9 ± 35.4	162.5 ± 7.4	115.8 ± 20.5	Mm01617761_gI
b) Genes that associated with neural development and ectoderm marker genes								
Six3	sine oculis-related homeobox 3 homolog	19.0 ± 13.1	20.6 ± 2.3	26.5 ± 8.6	45.4 ± 4.4	73.4 ± 8.7	105.5 ± 21.5	Mm01237639_ml
Arx	aristaless related homeobox gene (Drosophila)	3.3 ± 2.7	0.7 ± 0.5	1.2 ± 0.7	23.1 ± 6.7	218.6 ± 12.8	453.4 ± 115.8	Mm00545903_ml
Dex	doublecortin	3.7 ± 3.8	1.5 ± 0.7	23.8 ± 6.1	138.1 ± 5.6	655.1 ± 42.5	1157.9 ± 124.3	Mm00438401_ml
L1cam	L1 cell adhesion molecule	26.9 ± 2.0	40.5 ± 8.3	33.7 ± 2.4	60.1 ± 9.2	198.0 ± 19.0	423.9 ± 51.6	Mm01193612_gI
Lhx1	LIM homeobox protein 1	0.7 ± 0.4	9.4 ± 6.0	12.8 ± 4.2	135.3 ± 12.6	275.4 ± 21.5	259.5 ± 52.0	Mm00521776_ml
Emx2	empty spiracles homolog 2 (Drosophila)	3.8 ± 2.8	2.1 ± 1.1	2.8 ± 1.8	29.2 ± 5.2	47.9 ± 5.5	67.2 ± 20.8	Mm00550241_ml
En1	engrailed 1	3.8 ± 2.9	2.9 ± 2.9	3.5 ± 0.8	10.0 ± 1.1	12.6 ± 3.8	14.2 ± 2.8	Mm00438709_ml
En2	engrailed 2	19.2 ± 3.2	13.9 ± 5.1	21.9 ± 4.6	88.9 ± 10.3	89.9 ± 9.7	97.4 ± 19.6	Mm00438710_ml
Wnt1	wingless-related MMTV integration site 7B	78.4 ± 11.6	51.5 ± 9.4	59.1 ± 5.8	208.1 ± 9.9	310.1 ± 42.5	246.6 ± 41.3	Mm01300555_gI
Pax6	paired box gene 6	23.9 ± 4.0	21.7 ± 5.9	93.7 ± 5.7	127.4 ± 6.9	285.0 ± 26.3	331.4 ± 23.7	Mm00443081_ml
Reln	reelin	10.7 ± 3.5	18.9 ± 4.4	39.5 ± 5.3	43.8 ± 5.7	124.0 ± 6.1	253.4 ± 27.2	Mm00465200_ml
Pou3f2	POU domain, class 3, transcription factor 2	6.3 ± 5.0	6.9 ± 3.4	14.8 ± 5.2	30.3 ± 1.7	105.3 ± 11.1	190.0 ± 47.8	Mm00843777_sl
Sox1	SRY-box containing gene 1	2.9 ± 3.4	8.4 ± 5.9	33.3 ± 2.5	38.3 ± 3.9	49.3 ± 2.1	45.4 ± 10.2	Mm00486299_sl
Nes	Nestin	85.3 ± 4.6	65.9 ± 10.2	81.7 ± 12.4	174.7 ± 19.5	138.6 ± 8.3	119.1 ± 13.3	Mm00450205_ml
Gad1	glutamic acid decarboxylase 1	39.1 ± 8.0	1.7 ± 1.3	3.5 ± 0.8	25.2 ± 4.5	361.3 ± 22.3	626.6 ± 137.3	Mm00725661_sl
Tph2	tryptophan hydroxylase 2	3.7 ± 3.4	3.8 ± 2.3	2.3 ± 2.4	3.2 ± 2.9	1.5 ± 1.5	12.1 ± 4.2	Mm00557717_ml
Foxg1 (Bf1)	forkhead box G1	4.9 ± 3.2	2.0 ± 2.9	51.8 ± 7.8	153.0 ± 18.3	549.0 ± 88.2	918.6 ± 126.0	Mm02059886_sl
Otx1	orthodenticle homeobox 1	1.8 ± 0.7	2.4 ± 2.0	2.7 ± 1.7	34.5 ± 10.5	41.4 ± 2.8	52.5 ± 10.5	Mm00550304_ml
Hoxb4	homeo box B4	4.9 ± 1.2	6.2 ± 5.3	9.3 ± 6.5	16.0 ± 1.4	14.2 ± 4.2	15.4 ± 2.5	Mm00657964_ml
Slc6a4	solute carrier family 6	2.7 ± 1.4	5.2 ± 4.0	3.1 ± 3.5	5.6 ± 4.3	3.1 ± 2.3	29.8 ± 12.1	Mm00439391_ml
Nkx2-1	NK2 homeobox 1	7.2 ± 5.3	6.6 ± 2.6	5.0 ± 4.1	5.9 ± 4.6	11.0 ± 3.0	18.2 ± 5.8	Mm00447558_ml
Map2	microtubule-associated protein 2	26.3 ± 2.3	17.1 ± 3.1	72.8 ± 8.3	222.2 ± 24.5	796.6 ± 33.8	1185.5 ± 196.1	Mm00485236_ml

* Values represent means ± S.D. of data from three independent experiments.

Genes related to developmental toxicity

Table 5. Expression profiles of the genes altered expression during ES cell differentiation into neural cells

Gene Symbol	Gene Title	Expression levels at each day						TaqMan probe ID	
		0	2	4	6	8	10		
C230070N19	0 day neonate cerebellum cDNA, RIKEN full-length enriched library	202.3 ± 19.3	153.2 ± 18.5	587.0 ± 49.2	1177.3 ± 112.5	2372.9 ± 87.9	3139.1 ± 671.2	Mm02524303_s1	
4933439C20Rik	RIKEN cDNA 4933439C20 gene	439.0 ± 98.7	509.0 ± 30.4	714.8 ± 153.8	722.1 ± 105.6	1693.1 ± 219.8	2640.3 ± 370.4	Mm02391143_m1	
Acat2	acetyl-Coenzyme A acetyltransferase 2	944.5 ± 49.0	1297.7 ± 73.6	1175.7 ± 66.5	1979.7 ± 140.7	1345.8 ± 57.2	1313.7 ± 111.4	Mm00782408_s1	
AK122525	cDNA sequence AK122525	394.2 ± 23.8	1003.4 ± 18.1	642.3 ± 37.2	500.2 ± 34.7	239.7 ± 11.6	247.0 ± 40.9	Mm03014377_m1	
Basp1	brain abundant, membrane attached signal protein 1	1062.9 ± 95.7	2025.0 ± 91.2	1768.8 ± 168.9	2052.1 ± 79.3	3798.7 ± 176.1	5018.5 ± 768.7	Mm02344032_s1	
Btg1	B-cell translocation gene 1, anti-proliferative	557.3 ± 34.2	452.1 ± 65.9	1057.4 ± 67.1	1262.6 ± 130.9	3731.2 ± 291.3	3861.7 ± 855.9	Mm02391761_m1	
Cachd1	cache domain containing 1	64.3 ± 8.8	408.2 ± 31.0	616.9 ± 33.8	640.6 ± 29.7	1091.8 ± 70.9	1106.7 ± 189.9	Mm01256122_m1	
Cend2	cyclin D2	55.9 ± 7.3	99.5 ± 16.5	428.0 ± 11.3	1350.3 ± 30.6	1459.5 ± 87.8	1986.1 ± 258.7	Mm00438070_m1	
Ccm4l	CCR4 carbon catabolite repression 4-like	1549.8 ± 77.4	1217.7 ± 41.4	2071.8 ± 122.8	2036.3 ± 41.1	4200.6 ± 96.2	4955.8 ± 519.5	Mm00802276_m1	
Cd24a	CD24a antigen	636.3 ± 20.0	752.7 ± 30.2	1705.2 ± 75.0	2211.9 ± 206.6	3043.2 ± 135.8	3570.9 ± 587.8	Mm00782538_sH	
Cited2	Cbp with Glu/Asp-rich carboxy-terminal domain, 2	521.6 ± 34.7	812.0 ± 14.5	1457.6 ± 75.2	1558.7 ± 46.2	2464.2 ± 98.5	2676.5 ± 447.0	Mm00516121_m1	
Col4a1	procollagen, type IV, alpha 1	144.8 ± 12.7	1163.7 ± 97.9	2500.3 ± 156.1	4715.7 ± 479.5	7829.7 ± 360.6	7921.8 ± 1484.1	Mm00802377_m1	
Col4a2	procollagen, type IV, alpha 2	60.7 ± 7.8	539.8 ± 40.5	1096.2 ± 65.3	2028.2 ± 149.2	4100.4 ± 196.3	4226.3 ± 702.9	Mm00802386_m1	
Cpe	carboxypeptidase E	120.6 ± 11.4	94.1 ± 10.2	329.1 ± 34.1	545.8 ± 65.8	1579.0 ± 207.3	2679.2 ± 442.3	Mm00516341_m1	
Crabp1	cellular retinoic acid binding protein 1	189.4 ± 12.1	55.2 ± 7.0	807.3 ± 88.4	727.0 ± 21.4	905.3 ± 16.2	603.0 ± 159.1	Mm00442776_m1	
Ctsl	cathepsin L	1798.7 ± 148.9	2470.1 ± 99.4	3356.4 ± 188.8	3947.0 ± 370.2	4090.7 ± 190.7	3682.4 ± 887.8	Mm00515597_m1	
Culn	cubilin (intrinsic factor-cobalamin receptor)	25.7 ± 4.7	285.3 ± 28.4	873.4 ± 94.4	1324.5 ± 224.3	497.8 ± 11.3	206.7 ± 38.2	Mm01325078_m1	
Dab2	disabled homolog 2 (Drosophila)	49.2 ± 8.1	235.8 ± 4.1	712.8 ± 14.9	918.9 ± 8.3	658.2 ± 4.4	510.0 ± 99.6	Mm00517751_m1	
Ddr1	discoidin domain receptor family, member 1	324.9 ± 104.8	142.0 ± 31.1	474.4 ± 26.1	668.2 ± 185.4	1705.7 ± 323.2	2340.3 ± 498.9	Mm00432251_m1	
Degs1	degenerative spermatocyte homolog 1 (Drosophila)	532.5 ± 23.0	1101.5 ± 58.9	940.0 ± 53.5	566.3 ± 26.2	512.8 ± 33.4	488.0 ± 90.7	Mm00492146_m1	
Dhcr24	24-dehydrocholesterol reductase	502.2 ± 70.3	1172.5 ± 64.2	792.3 ± 69.1	1032.4 ± 112.6	452.6 ± 40.2	243.1 ± 53.3	Mm00519071_m1	
Dnaj1	DnaJ (Hsp40) homolog, subfamily A, member 1	390.5 ± 5.3	939.8 ± 56.0	920.5 ± 35.7	913.9 ± 38.4	589.7 ± 16.4	871.0 ± 149.1	Mm01619878_g1	
Dnm3a	DNA methyltransferase 3A	280.4 ± 12.2	1169.2 ± 79.6	386.2 ± 8.7	298.0 ± 10.4	174.0 ± 13.6	155.2 ± 6.0	Mm00432881_m1	
Dnm3b	DNA methyltransferase 3B	1684.8 ± 57.3	4363.8 ± 375.4	3180.6 ± 129.7	1677.6 ± 58.9	793.5 ± 57.6	299.9 ± 66.1	Mm01240113_m1	
Dpysl2	dihydropyrimidinase-like 2	340.6 ± 29.9	200.8 ± 17.7	922.8 ± 115.1	1719.6 ± 117.4	2887.7 ± 132.4	3300.2 ± 413.6	Mm00515559_m1	
Fabp5	fatty acid binding protein 5, epidermal	1581.8 ± 79.4	1989.9 ± 153.9	2914.5 ± 109.1	4040.9 ± 216.9	3031.9 ± 125.8	2878.5 ± 448.2	Mm00783731_s1	
Fabp7	fatty acid binding protein 7, brain	6.1 ± 3.9	16.9 ± 1.9	289.9 ± 17.0	1306.1 ± 40.6	1535.4 ± 48.7	1680.2 ± 215.9	Mm00445225_m1	
Fads2	fatty acid desaturase 2	181.9 ± 4.1	720.4 ± 34.9	570.7 ± 34.6	665.9 ± 63.1	342.4 ± 19.5	252.6 ± 42.6	Mm00517221_m1	
Fasn	fatty acid synthase	1265.8 ± 72.0	2818.3 ± 157.4	1709.0 ± 122.0	1921.2 ± 199.7	883.9 ± 28.7	702.7 ± 123.3	Mm00662319_m1	
Fdps	farnesyl diphosphate synthetase	1429.3 ± 55.4	2967.0 ± 192.6	1919.2 ± 138.7	2085.1 ± 25.2	1018.8 ± 73.1	704.6 ± 121.9	Mm00836315_g1	
Fst	Follistatin	34.1 ± 8.0	157.7 ± 14.8	546.2 ± 26.2	532.5 ± 22.4	866.9 ± 16.9	1295.2 ± 251.5	Mm00514982_m1	
Fzd2	frizzled homolog 2 (Drosophila)	159.8 ± 20.7	476.4 ± 42.3	752.1 ± 133.5	732.4 ± 80.2	834.4 ± 87.1	636.8 ± 90.1	Mm02524776_s1	
Gnas	guanine nucleotide binding protein, alpha stimulating	940.0 ± 126.9	1169.4 ± 59.7	1931.0 ± 61.5	2594.7 ± 169.0	3230.9 ± 136.7	3446.9 ± 382.9	Mm00530548_m1	
Gtl2	imprinted maternally expressed untranslated mRNA	1841.7 ± 153.9	2476.2 ± 176.3	2727.0 ± 137.0	1698.1 ± 249.5	5475.8 ± 210.1	6949.2 ± 1353.9	Mm00522599_m1	
H19	H19 fetal liver mRNA	691.1 ± 31.4	385.5 ± 34.0	1490.0 ± 85.8	1954.7 ± 47.4	3867.0 ± 189.1	3818.3 ± 958.8	Mm00469706_g1	
H1f0	H1 histone family, member 0	530.4 ± 24.3	227.7 ± 23.3	1046.2 ± 48.1	1239.5 ± 31.4	3525.2 ± 257.2	3050.7 ± 575.8	Mm00515079_s1	
Hdbp	high density lipoprotein (HDL) binding protein	453.2 ± 11.9	961.9 ± 30.4	785.2 ± 35.1	710.3 ± 30.8	830.5 ± 15.0	815.1 ± 98.0	Mm00505507_m1	
Hmgb3	high mobility group box 3	274.9 ± 10.6	449.4 ± 24.5	920.8 ± 41.6	1109.6 ± 39.7	1164.9 ± 27.9	1263.2 ± 272.4	Mm01377544_gH	
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	960.7 ± 104.6	1072.4 ± 14.8	1437.6 ± 110.9	2062.5 ± 212.5	1641.4 ± 185.0	1688.7 ± 270.8	Mm00524111_m1	
Int6	integrator complex subunit 6	409.8 ± 12.6	585.9 ± 71.7	870.1 ± 95.5	1234.8 ± 24.2	2138.8 ± 121.7	2568.8 ± 311.7	Mm00493236_m1	
Iqgap1	IQ motif containing GTPase activating protein 1	662.4 ± 37.5	1515.9 ± 85.4	1043.1 ± 62.0	755.2 ± 36.8	485.0 ± 20.8	381.2 ± 57.2	Mm00443860_m1	
Lama1	laminin, alpha 1	251.0 ± 13.2	344.8 ± 23.8	838.0 ± 55.6	1436.9 ± 204.0	2907.6 ± 129.8	3253.8 ± 493.1	Mm00439445_m1	
Lamb1-l	laminin B1 subunit 1	524.4 ± 18.6	461.1 ± 37.6	1070.5 ± 98.2	1535.1 ± 143.6	3162.0 ± 294.1	3835.5 ± 822.0	Mm00801853_m1	
LOC672274	similar to Transcription factor SOX-4	603.4 ± 69.5	1021.7 ± 42.9	1730.9 ± 98.6	2283.8 ± 244.2	4493.9 ± 249.5	5724.9 ± 935.2	Mm00486320_s1	

Table 5. (Continued).

Gene Symbol	Gene Title	Expression levels at each day					TaqMan probe ID	
		0	2	4	6	8	10	
Lrpap1	low density lipoprotein receptor-related protein associated protein 1	640.3 ± 46.2	559.4 ± 17.8	1084.2 ± 70.5	1671.5 ± 84.1	1506.6 ± 43.9	1560.4 ± 305.3	Mm00660272_m1
Marcks	myristoylated alanine rich protein kinase C substrate	531.1 ± 65.8	712.6 ± 25.1	1418.4 ± 19.8	1856.1 ± 112.1	2982.9 ± 267.2	3554.8 ± 377.8	Mm02524303_s1
Mdk	midkine	920.6 ± 30.6	212.6 ± 30.4	2054.4 ± 119.3	2047.1 ± 106.9	1890.8 ± 114.2	1501.7 ± 172.4	Mm00440279_m1
Mest	mesoderm specific transcript	805.2 ± 49.1	1531.7 ± 67.0	3319.5 ± 278.4	4976.9 ± 510.6	4438.4 ± 93.8	2983.3 ± 280.2	Mm00484993_m1
Ndn	necdin	179.5 ± 12.4	193.1 ± 17.5	585.3 ± 60.3	762.9 ± 59.3	1880.7 ± 142.6	3531.1 ± 633.5	Mm02524479_s1
Nnat	neuronatin	146.4 ± 15.6	129.0 ± 23.7	895.2 ± 52.1	1531.2 ± 57.7	3623.0 ± 136.3	4695.9 ± 875.1	Mm00731416_s1
Nr6a1	nuclear receptor subfamily 6, group A, member 1	320.0 ± 22.6	681.0 ± 25.2	950.3 ± 19.3	556.9 ± 26.6	446.1 ± 19.6	305.8 ± 67.9	Mm00599848_m1
Nt5dc2	5'-nucleotidase domain containing 2	628.8 ± 39.8	1623.2 ± 43.5	1066.9 ± 107.1	1146.1 ± 78.3	719.8 ± 42.1	543.6 ± 115.8	Mm01211831_g1
Pa2g4	proliferation-associated 2G4	618.5 ± 24.7	1330.4 ± 36.2	822.0 ± 27.3	743.8 ± 37.3	489.4 ± 19.9	362.3 ± 43.7	Mm00650817_g1
Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	785.7 ± 64.3	1398.3 ± 108.1	1631.4 ± 279.2	882.2 ± 110.4	523.9 ± 85.8	298.6 ± 53.3	Mm00554306_m1
Pfkfb	phosphofructokinase, liver, B-type	1286.3 ± 117.3	2671.2 ± 115.6	2167.6 ± 237.8	2482.3 ± 182.2	1699.0 ± 134.2	1003.1 ± 168.0	Mm00435587_m1
Pfn2	profilin 2	143.6 ± 8.3	104.3 ± 3.8	368.3 ± 27.1	804.5 ± 29.9	1636.4 ± 42.9	2416.7 ± 383.1	Mm00450821_m1
Pik3cd	phosphatidylinositol 3-kinase catalytic delta polypeptide	330.0 ± 139.3	840.0 ± 198.9	357.8 ± 137.3	275.5 ± 131.0	193.1 ± 24.8	181.3 ± 65.7	Mm00435674_m1
Pim2	proviral integration site 2	317.2 ± 6.4	2606.6 ± 91.5	1647.6 ± 53.0	930.8 ± 49.8	193.1 ± 18.7	63.3 ± 13.9	Mm00454579_m1
Plagl1	pleiomorphic adenoma gene-like 1	209.7 ± 11.6	125.3 ± 11.2	600.5 ± 52.5	2462.7 ± 80.5	3740.1 ± 128.2	3571.7 ± 592.4	Mm00494250_m1
Plod2	procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	449.6 ± 26.7	544.0 ± 49.2	836.5 ± 79.1	1342.6 ± 134.5	2726.0 ± 143.5	2981.1 ± 568.2	Mm00478767_m1
Pou3f1	POU domain, class 3, transcription factor 1	122.4 ± 9.6	868.9 ± 119.2	682.6 ± 41.4	369.6 ± 28.5	181.2 ± 20.1	184.3 ± 52.5	Mm00843534_s1
Prdx4	peroxiredoxin 4	720.1 ± 71.4	1510.4 ± 28.4	1397.1 ± 47.9	1439.6 ± 56.8	1066.6 ± 60.5	768.5 ± 133.3	Mm00450261_m1
Prpl	proteoglycan 1, secretory granule	19.5 ± 3.3	239.6 ± 15.5	579.7 ± 37.8	991.2 ± 31.0	1922.0 ± 87.6	1992.8 ± 377.7	Mm01169070_m1
Ptbp2	polypyrimidine tract binding protein 2	229.5 ± 15.6	445.2 ± 40.6	741.7 ± 48.9	670.3 ± 28.3	1036.3 ± 96.0	1278.1 ± 215.6	Mm00497922_m1
Ptn	pleiotrophin	12.7 ± 3.2	22.9 ± 5.7	629.4 ± 31.7	336.7 ± 30.6	338.1 ± 55.3	185.9 ± 28.9	Mm00436062_m1
Rpl22	ribosomal protein L22	388.0 ± 33.4	591.4 ± 45.9	896.1 ± 121.1	641.2 ± 69.9	1290.3 ± 56.3	1543.4 ± 202.1	Mm00786206_s1
Serpinh1	serine (or cysteine) peptidase inhibitor, clade H, member 1	290.8 ± 18.7	350.6 ± 11.6	1044.3 ± 42.4	1629.3 ± 90.0	2105.1 ± 48.0	2469.9 ± 521.7	Mm00438056_m1
Sfrp2	secreted frizzled-related protein 2	66.0 ± 5.4	149.0 ± 7.9	534.8 ± 62.4	1257.4 ± 154.6	1073.0 ± 144.0	794.7 ± 99.2	Mm00485986_m1
Sfrs7	splicing factor, arginine/serine-rich 7	536.2 ± 34.2	840.4 ± 40.2	1253.2 ± 178.7	1108.0 ± 111.9	820.6 ± 59.5	1017.5 ± 147.5	Mm00524903_m1
Slc16a3	solute carrier family 16, member 3	523.6 ± 92.7	2239.6 ± 189.3	1088.1 ± 255.3	1355.5 ± 68.8	616.6 ± 110.5	273.4 ± 24.4	Mm00446102_m1
Slc7a7	solute carrier family 7, member 7	233.1 ± 8.2	831.7 ± 27.2	400.6 ± 30.0	334.4 ± 37.1	105.4 ± 13.4	67.7 ± 26.5	Mm00448764_m1
Slc9a3r1	solute carrier family 9, isoform 3 regulator 1	532.9 ± 36.9	1082.7 ± 72.2	922.5 ± 41.7	759.8 ± 53.6	314.2 ± 31.6	185.7 ± 32.4	Mm00488865_m1
Sox11	SRY-box containing gene 11	261.0 ± 22.2	342.7 ± 51.2	905.2 ± 168.0	1592.9 ± 378.6	3663.4 ± 316.3	4160.3 ± 1159.8	Mm01281943_s1
Spag9	sperm associated antigen 9	364.5 ± 13.3	309.4 ± 15.7	630.4 ± 69.3	819.5 ± 79.3	2073.9 ± 122.0	2544.9 ± 187.2	Mm00466201_m1
Sparc	secreted acidic cysteine rich glycoprotein	1203.1 ± 72.9	1902.2 ± 105.7	3489.8 ± 382.8	5369.8 ± 467.9	7701.8 ± 425.2	8203.7 ± 1365.2	Mm01295757_g1
Sqle	squalene epoxidase	718.1 ± 38.2	1449.7 ± 66.3	1020.6 ± 41.0	1215.6 ± 20.3	643.5 ± 45.6	533.3 ± 43.9	Mm00436772_m1
Stox2	storkhead box 2	170.6 ± 9.7	327.9 ± 16.2	822.4 ± 54.8	1361.4 ± 84.4	3099.1 ± 276.7	3993.4 ± 832.4	Mm00613305_m1
Tax1bp3	Tax1 binding protein 3	280.7 ± 20.2	310.6 ± 12.2	628.2 ± 50.7	904.2 ± 37.3	1993.8 ± 128.3	2705.2 ± 493.5	Mm01610965_m1
Tefl2	transcription factor 12	252.1 ± 15.3	234.7 ± 25.5	787.4 ± 62.9	962.8 ± 117.1	1628.4 ± 205.8	1868.0 ± 293.3	Mm00441699_m1
Tefl5	transcription factor 15	477.1 ± 131.7	1040.0 ± 19.3	436.7 ± 87.1	312.3 ± 89.7	73.5 ± 9.1	51.4 ± 12.6	Mm00493442_m1
Tle4	transducin-like enhancer of split 4, homolog of Drosophila E(spl)	668.1 ± 39.4	1582.2 ± 238.7	984.7 ± 41.2	757.3 ± 73.1	600.3 ± 41.0	660.8 ± 123.3	Mm00502265_s1
Ttc3	tetratricopeptide repeat domain 3	448.0 ± 27.7	482.8 ± 22.4	797.3 ± 35.9	1007.0 ± 81.3	1972.8 ± 35.6	2455.8 ± 431.5	Mm00493917_m1
Tubb2b	tubulin, beta 2b	627.8 ± 98.8	253.6 ± 13.8	491.9 ± 62.3	1022.4 ± 181.2	1958.5 ± 240.1	2658.1 ± 662.5	Mm00849948_g1
Ubqln2	ubiquilin 2	641.7 ± 65.1	710.1 ± 67.9	1406.7 ± 142.9	1716.9 ± 162.4	2360.3 ± 189.8	2847.2 ± 639.1	Mm00834570_s1
Vim	vimentin	1116.2 ± 56.5	879.6 ± 41.5	1147.9 ± 66.2	3617.5 ± 367.1	7209.4 ± 247.9	6546.1 ± 937.1	Mm00449201_m1

* Values represent means ± S.D. of data from three independent experiments.

Fig. 1. Confirmation of inhibition of neural differentiation treated with test chemicals. Differentiated cells were visualized by labeling with Vybrant® DiI cell-labeling solution. Each arrowhead represents neural filaments.

acid, isoniazide, (+)-camphor and acrylamide) used in the ECVAM international validation study (Brown, 2002; Genschow *et al.*, 2004) were added during ES cell differentiation into myocardial cells. Chemical dependent changes in expression of candidate genes (Tables 2b, 3, 4b and 5) were then compared between the cells treated with embryotoxics and non-embryotoxics.

In the present study, the doses of the test chemicals were set as maximum non-effect concentrations in cytotoxicity assays or minimum concentrations giving 100% inhibition in differentiation assays (Table 1). In the myocardial cell differentiation, more than 87% of EBs were contracted spontaneously with solvent control at day 10, however, no contracting myocardial cells were observed with embryotoxics at the concentrations described in Table 1 (data not shown). In the case of neuron differentiation, the percentage inhibition of differentiation was calculated by counting the neurofilaments. As shown in Fig. 1, treatment with all embryotoxics caused 100% inhibition of neuron differentiation at the doses in Table 1. However, no apparent effects on neuron differentiation were observed with any of the non-embryotoxic treatments, the doses of non-embryotoxic being set as the maximum non-effect concentrations in cytotoxicity assays. There were slight differences in the dose levels of part compounds between myocardial and neural cell differentiation, implying the sensitivity of the differentiated cells against test chemicals.

Comparison between gene expression with embryotoxics and non-embryotoxics during ES cell differentiation into myocardial cells are shown in Table 6. The expression of Hand1 gene in cells treated with embryotoxics was strongly reduced in comparison with the vehicle control at day 6. Significant differences between

gene expression with embryotoxics and non-embryotoxics were observed in the case of Hand1, Hbb-bh1, Hba-a1, Hba-x, Myl4, Myl7, Cmya1 and Smyd1. Weak but significant reduction of expression of Pim2, Tbx20, Col1a2, Pitx2 and Adam19 was also detected in cells treated with embryotoxics, but not the non-embryotoxics. In contrast, there were no apparent effects on expression of Bmp2, Cer1, Gata4, Nkx2-5, α -MHC(Myh6), Mef2a, Mef2c, Cdh2, Tbx5, Nodal, Tpm2 and T (brachyury) (data not shown). Altered expression of the 13 genes (Hand1, Pim2, Tbx20, Myl4, Myl7, Hbb-bh1, Hba-a1, Col1a2, Hba-x, Cmya1, Pitx2, Smyd1 and Adam1) specifically by embryotoxic chemicals were considered to be correlated with embryotoxicity.

In the case of neural differentiation, expression of 107 genes was examined using quantitative real-time PCR, data for several candidate genes being shown in Table 7. The expression of Map2 in the cells treated with embryotoxics was strongly reduced in comparison with vehicle control on day 10, significant differences between gene expression with embryotoxics and non-embryotoxics being observed. Similar results were found for Cpe, Marcks, Ptbp2, Sox11, Tubb2b, Vim, Arx, Emx2, Pax6, Basp1, Ddr1, Ndn, Sfrp, Ttc3, Ubqln2, Six3, Dcx, L1cam, Reln, Wnt1 and Nnat.

DISCUSSION

ES cells derived from the inner cell mass of blastocysts are a self-renewing population, and can be continuously cultured in an undifferentiated state (Evans and Kaufman, 1981; Thomson *et al.*, 1998). Their differentiation mimics the early processes involved in embryonic development, and in the present study we obtained data confirming this

Table 6. Comparison between marker gene expression with embryotoxics and non-embryotoxics during ES cell differentiation into myocardial cells

Gene	Day	Relative expression ^a	
		Embryotoxicant	Non-embryotoxicant
Pim2	4	0.20 ± 0.07	1.68 ± 0.61 **
Hand1	6	0.04 ± 0.03	1.11 ± 0.49 **
Hbb-bh1	7	0.02 ± 0.01	1.04 ± 0.74 *
Hba-a1	7	0.03 ± 0.01	1.29 ± 1.01 *
Hba-x	7	0.02 ± 0.03	0.89 ± 0.67 *
Myl4	8	0.03 ± 0.04	0.49 ± 0.45 *
Myl7	8	0.02 ± 0.02	0.32 ± 0.26 *
Cmya1	8	0.02 ± 0.04	0.57 ± 0.51 *
Smyd1	8	0.04 ± 0.04	0.55 ± 0.45 *
Tbx20	9	0.26 ± 0.15	1.65 ± 0.69 **
Pitx2	9	0.45 ± 0.20	1.76 ± 1.14 *
Adam19	10	0.48 ± 0.12	1.10 ± 0.16 **
Col1a2	10	0.16 ± 0.11	1.04 ± 0.65 *

^a: Values represent expression relative to control in the treatment group of six embryotoxants or non-embryotoxics (mean ± S.D.). **:p < 0.01. *:p < 0.05.

fact. Thus expression of typical marker genes (Oct3/4 and Nanog) for undifferentiated state decreased gradually during cardiac differentiation, and increase in expression of Myh6 (Ching *et al.*, 2005), typical marker gene for myocardial cell, was found (Tables 2a and b), suggesting that ES cells differentiated into myocardial cells. In addition, increase in expression of the well-known mesoderm markers and several genes playing important roles in vertebrate heart formation were evident (Table 2b). For example, increases in expressions of Nodal induced in period cardiac crescent formation (Lu and Robertson, 2004), the myocardial-specific transcription factors, Nkx2-5 (Hiroi *et al.*, 2001) and Mef2C (Lin *et al.*, 1997), and a heart looping mediating factor, Xin (Cmya1) (Wang *et al.*, 1999) were observed. These expression patterns reflected a rough concordance with the gene expression patterns observed during the differentiation of early embryogenesis *in vivo* (Guan *et al.*, 1999; Rohwedel *et al.*, 2001). This observation suggested that *in vitro* embryonic stem cell differentiation might replicate many processes that occur during *in vivo* embryogenesis, therefore might be an appropriate surrogate for the embryo with

Table 7. Comparison between marker gene expression with embryotoxics and non-embryotoxics during ES cell differentiation into neural cells

Gene	Day	Relative expression ^a	
		Embryotoxicant	Non-embryotoxicant
Sfrp	7	0.14 ± 0.17	0.79 ± 0.33 **
Wnt1	7	0.21 ± 0.36	2.55 ± 0.84 **
Vim	8	0.06 ± 0.07	0.62 ± 0.11 **
Baspl	9	0.06 ± 0.08	0.59 ± 0.10 **
Ddr1	9	0.06 ± 0.07	0.58 ± 0.10 **
Marcks	9	0.18 ± 0.21	1.44 ± 0.22 **
Ptbp2	9	0.18 ± 0.21	1.51 ± 0.31 **
Sox11	9	0.05 ± 0.06	0.90 ± 0.39 **
Ttc3	9	0.12 ± 0.14	0.90 ± 0.20 **
Tubb2b	9	0.10 ± 0.11	1.33 ± 0.32 **
Ubqln2	9	0.16 ± 0.18	0.96 ± 0.30 **
Six3	9	0.07 ± 0.09	0.48 ± 0.25 **
Arx	9	0.05 ± 0.07	0.65 ± 0.14 **
Dcx	9	0.10 ± 0.12	1.05 ± 0.54 **
Emx2	9	0.02 ± 0.04	0.74 ± 0.30 **
Reln	9	0.05 ± 0.06	0.79 ± 0.30 **
Map2	10	0.03 ± 0.04	0.71 ± 0.20 **
Cpe	10	0.06 ± 0.07	0.95 ± 0.28 **
Ndn	10	0.13 ± 0.16	1.47 ± 0.28 **
L1Cam	10	0.03 ± 0.03	0.75 ± 0.34 **
Pax6	10	0.03 ± 0.04	0.75 ± 0.22 **
Nnat	10	0.07 ± 0.08	0.75 ± 0.23 **

^a: Values represent expression relative to control in the treatment group of six embryotoxants or non-embryotoxics (mean ± S.D.). **:p < 0.01. *:p < 0.05.

respect to developmental toxicity.

The primary aim of the present study was to identify useful marker genes for determination of embryotoxics by transcriptome methods. When chemical dependent changes in expression of the selected genes (107 genes, Tables 2b and 3) were compared between ES cells treated with embryotoxic and non-embryotoxic chemicals by a quantitative PCR method, expression of 13 genes (Hand1, Pim2, Tbx20, Myl4, Myl7, Hbb-bh1, Hba-a1, Col1a2, Hba-x, Cmya1, Pitx2, Smyd1 and Adam19) was decreased specifically with the embryotoxic agents. A basic helix-loop-helix transcription factor, Hand1, is

known to display dynamic and spatially restricted expression patterns in the developing heart (Cross *et al.*, 1995). Mice that lack Hand1 die on embryonic day 8.5 from placental and extra-embryonic abnormalities (Firulli *et al.*, 1998; Riley *et al.*, 1998). In addition, mice harboring a conditional Hand1 knock out by cardiac-specific expression displayed defects in the left ventricle and endocardial cushions, and exhibited cardiac abnormalities (McFadden *et al.*, 2005). The intercalated disk protein Xin (Cmya1) was originally discovered in chicken striated muscle and implicated in cardiac morphogenesis (Wang *et al.*, 1999). Transcription factors Nkx2-5 and Mef2C activate the Xin gene, and mediate heart looping. The hearts of Xin α -null mice are hypertrophied and exhibited fibrosis, indicative of cardiomyopathy (Gustafson-Wagner *et al.*, 2007). Furthermore, mice lacking functional Adam19 exhibit severe defects in cardiac morphogenesis including ventricular septal abnormalities (Komatsu *et al.*, 2007). The Pitx2 transcription factor, activated solely in the left side of the lateral plate mesoderm, is also critical for proper heart looping, and it might regulate the expression of proteins such as the extracellular matrix protein to regulate the physical tension of the heart tissues on the different sides (Tsuda *et al.*, 1996). Moreover, the other genes were all known to be indispensable in heart development (Stennard *et al.*, 2003; Cohen-Haguenauer *et al.*, 1989; Maves *et al.*, 2009; Muscat *et al.*, 1988; Gottlieb *et al.*, 2002), implying that the 13 genes identified in the present study are intimately involved in embryotoxicity of chemicals. Changes in expression of key genes (Oct3/4, Brachury, Nkx2.5 and α -MHC) involved in cardiomyocyte development were reported during ES cell differentiation into cardiomyocytes with retinoic acid and LiCl (Pelizzer *et al.*, 2004), decreases in gene expression of Nkx2.5 and α -MHC being also found. In the present study, decreases in Nkx2.5 and α -MHC expression were clearly observed after treatment with retinoic acid (data not shown), but the changes in both genes were not specific to the 6 embryotoxicants used, so that they can not be selected as marker genes for embryotoxicity. Recently, gene expression during early stages of ES cell differentiation into cardiomyocytes were analyzed with and without exposure of embryotoxicants (monobutyl phthalate and 6-aminonicotinamide) using DNA microarrays, significant deviation of cultures of altered genes from the unexposed and embryotoxicant-exposed cells being reported. However no genes altered with the embryotoxicants were specified (van Dartel *et al.*, 2010a). Then a set of 26 genes altered in ES cells exposed with 5 embryotoxicants (all-*trans*-retinoic acid, methoxyacetic acid, valproic acid, monobutyl phthalate and 5-fluorouracil) was

reported by the same group (van Dartel *et al.*, 2010b). The report was the first identification of embryotoxicant-related genes by global expression analysis during early stages of ES cell differentiation into cardiomyocytes. In the present study we identified for the first time 13 genes with altered expression specific to treatment with 6 embryotoxicants (5-fluorouracil, hydroxyurea, 6-aminonicotinamide, 5-bromo-2'-deoxyuridine, methotrexate and all-*trans*-retinoic acid) on the basis of gene expression of ES cells differentiation into cardiomyocytes in comparison with 6 non-embryotoxicants (saccharin sodium salt hydrate, ascorbic acid, isoniazide, D-(+)-camphor, acrylamide and penicillin G). No genes identical with the 26 genes were found, suggesting that differences were due to the differentiation stage of ES cells or test chemicals used in the present study. The new identified genes are altered specifically by embryotoxicants, therefore, they are more informative and may help to improve *in vitro* reproductive toxicity methods.

Global changes in the gene expression during ES cell differentiation into neural cells were also analyzed by DNA microarray. Since the cerebral cortex sustains damage in many cases of brain malformation, a differentiation method inducing cerebral cortex neurons efficiently was selected in the present study (Watanabe *et al.*, 2005). Change in expression of typical undifferentiated marker genes (Oct3/4 and Nanog) decreased gradually during neural differentiation. In addition, increased expression of Map2 and Bf1, typical marker genes for neural cells, was found. When chemical dependent changes in expression of up-regulated genes (107 genes, Tables 4b and 5) were compared between ES cells treated with embryotoxic and non-embryotoxic agents by a quantitative PCR method, decreases in gene expression of 22 genes (Map2, Cpe, Marcks, Ptbp2, Sox11, Tubb2b, Vim, Arx, Emx2, Pax6, Basp1, Ddr1, Ndn, Sfrp, Ttc3, Ubqln2, Six3, Dcx, L1cam, Reln, Wnt1 and Nnat) were found specifically with embryotoxic chemicals. Map2 is a typical neural marker gene which is related to a microtubule associated complex essential for neurogenesis. Mutation of the reeler gene (Reln) is known to disrupt neuronal migration in several brain regions and give rise to functional deficits such as ataxic gait and trembling in the reeler mutant mouse (Yip *et al.*, 2000). Necdin (Ndn), a member of the MAGE (melanoma antigen) protein family, is expressed predominantly in terminally differentiated neurons. It is maternally imprinted and expressed only from the paternal allele, a deficiency of which was implicated in the pathogenesis of the neurodevelopment disorder Prader-Willi syndrome (Jay *et al.*, 1997). Moreover, the other genes were known to be indispensable in the formation of

the brain development (Joseph *et al.*, 1994; Pekny *et al.*, 1999; Wu *et al.*, 1999; Bhatt *et al.*, 2000; Frey *et al.*, 2000; Mallamaci *et al.*, 2000; Uwanogho *et al.*, 1995; Brault *et al.*, 2001; Grove, 2002; Kitamura *et al.*, 2002; Lagutin *et al.*, 2003; Duparc *et al.*, 2006; Kappeler *et al.*, 2006; Berto *et al.*, 2007; Resnick *et al.*, 2008; Woronowicz *et al.*, 2008; Jaglin *et al.*, 2009; Weimer *et al.*, 2009; Nakamura *et al.*, 2010). These results suggested that selected 22 genes were related to embryotoxicity, and might be useful for prediction purposes. Several studies have reported on gene expression of major neural-specific genes, such as Nestin, Synaptophysin, NFH, GFAP, Oligo2 and DM20 analyzed for estimation of the embryonic effects of carbamazepine and fluoxetine during ES cell differentiation into neurons (Murabe *et al.*, 2007; Kusakawa *et al.*, 2008). In the present study, although chemical dependent changes in expression of Nestin were compared between embryotoxicant and non-embryotoxicant-treated groups, no embryotoxicant-specific alterations in Nestin expression were observed (data not shown). Although the reasons are not yet clearly understood, it seems to Nestin may not be a useful marker gene for detection of embryotoxicity of chemicals.

The EST was developed in 1997, and was one of the most useful tests for developmental toxicity using mouse ES cells. However, various problems have been pointed out and it has not been generally accepted in test guidelines. One major weakness is its reliance on a morphological endpoint (contracting myocardial cells) and the need for experienced personnel ensuring reliable assessment. In order to solve this problem, various improvements have been introduced (Marx-Stoelting *et al.*, 2009). During the last decade the reported cases of usage for molecular markers instead of morphological endpoints have increased. The number of the selected marker genes is presently too limited to identify the best molecular endpoint for classification of embryotoxicity. However, identification of potential chemical effects on myocardic, osteogenic, chondrogenic and neural differentiation by quantitative PCR methods were reported using a myosin heavy chain (α -MHC/Myh6), Neurofilament, Osteocalcin and Aggrecan, respectively (zur Nieden *et al.*, 2004). Recently, new molecular endpoints of differentiation have been introduced with a flow cytometry method (FACS-EST) by cardiac myosin using antibodies against sarcomeric α -MHC (Seiler *et al.*, 2004; Buesen *et al.*, 2009). In the present study, although chemical dependent changes in gene expression of α -MHC were compared between embryotoxicant and non-embryotoxicant-treated ES cells by a quantitative PCR method, no embryotoxicant-specific changes in expression of α -MHC were found (data not

shown). In the present study, we identified myosin light chains such as Myl4 and Myl7 as the specific markers for embryotoxicants (Table 6). Cardiac muscle thick filaments are primarily composed of cardiac myosin which assembles into hexamers comprised of 2 heavy chains and 2 pairs of each light chain isoform (essential light chain and regulatory light chain) (Craig and Woodhead, 2006). These results imply that decreases in Myl4 or Myl7 gene expression caused reduction of cardiac myosin protein levels detected by antibodies against sarcomeric α -MHC. In the present study we could successfully identify several marker genes related to embryotoxicity by transcriptome methods. Although, further studies are required to confirm our identified genes, monitoring of the identified marker genes might be useful as endpoints in an ES cell based animal-free alternative test for embryotoxicity.

One other problem is that the assay period of 10 days of EST was rather long for screening purposes for drugs and chemicals. Reduction in the assay duration is advantageous in terms of simplicity, economy, and reproducibility. A recent study reported that quantitative FACS analyses of α -MHC marker proteins could be performed at day 7 and the assay period was thus reduced (Buesen *et al.*, 2009). The obtained marker genes in the present study were expressed at days 4 to 10 after induction of differentiation into myocardic or neural cells. Use of our identified genes can be considered of particular value for the endpoint because detectable expression levels were observed at days 4-6. Quantitative determination of their expression might be useful for improvement of EST. One of the most promising methods for detection of the marker genes is use of stable transgenic ES cells for detection with reporter genes. Employment of the GFP as a reporter gene connected to a tissue-specific promoter was reported for easy identification of myocardial cells derived from ES cells (Kolossoff *et al.*, 1998). However, stable transgenic ES cells for detection of marker gene expression by luciferase reporter genes would be useful for high-throughput screening, and we have establishing several examples for the propose of screening or prediction of embryotoxicity of chemicals in the future. In addition, humanization of *in vitro* developmental test methods is clearly required, as species differences in developmental toxicity are known for various chemicals such as thalidomide. To satisfy test requirements, use of human ES (Adler *et al.*, 2008) or iPS (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007) cells is very promising for the near future. The present findings provide an experimental model suitable for studies of embryotoxicity, although further investigations are now required to clarify the exact relationships between genes identified in the present study and embryotoxicity.

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