Table 1 Growthofday10.5ratembryosculturedinthepresenceofall -*trans*-retinoicacid(RA)

	All-*trans*-retinoicacid(μM)			
	0(Control)	1	3	10
No.embryos	6	6	6	6
No.viableembryos	6(100%)	6(100%)	6(100%)	5(83.3%)
Crown-rumplength(mm)	4.28 ± 0.05	4.45 ± 0.07	4.29 ± 005	4.12 ± 0.22
Headlength(mm)	2.34 ± 0.04	2.40 ± 0.04	2.34 ± 0.06	2.19 ± 0.13
No.somitepairs	27.7 ± 0.21	27.5 ± 0.22	27.0 ± 0.37	25.6 ± 0.60**
No.embryoswithdeformedorgan	0	0	5(83.3%)**	5(100%)**
Branchialarch	0	0	0	4(80.0%)*
Neuraltube	0	0	5(83.3%)**	5(100%)**
Opticvesicle	0	0	5(83.3%)**	4(80.0%)*
Oticvesicle	0	0	5(83.3%)**	5(100%)**
Somite	0	0	2(33.3%)	3(60.0%)
Tail	0	0	2(33.3%)	1(20.0%)

Embryoswereculturedfor24hbytherollermethodinthemediumcomposedofpureratserum.Mean ± standarderrorofthemean(SEM) isshown.Asterisksindicatesignificantdifferencefromthecontrolvalue(* $P < 0.05$;** $P < 0.01$).

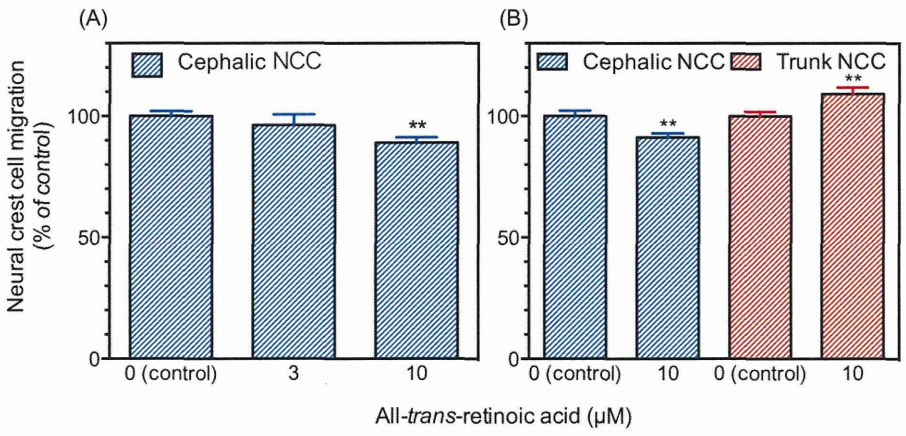

Fig. 5 Neuralcrestcell(NCC)migration during24hofcultureinthepresenceof all-*trans*-retinoicacid(RA).(A)Effects ofRAoncephalicNCCs.(B)Effectsof RAontrunkNCCs.NCCmigrationwas calculatedastheratiooftheradiusfrom thecircularspreadoftheNCCsandwas normalizedtothecontrol.Themeans ± standarderrorofthemean(SEM)of 9–11neuraltubesareshown.Asterisks indicatestatisticallysignificantdifferencesfromthecorrespondingcontrol (**$P < 0.01$).

approximately10%,indicatingtheRAhasoppositeeffectson cephalicandtrunkNCCs(Fig.5B).

DISCUSSION

Inthepresentstudy,weestablishedasimple *in vitro* NCCmigration assaythatenabledeasyassessmentoftheeffectsofchemicalson NCCmigrationindevelopmentaltoxicitystudies.However,the presentmethodisnotsuitableforscreeningbecausetheconcentrationsfoundtobeeffectivedonotprovideinformationonoverall embryotoxicity.Wholeembryocultureorgeneralcytotoxicity assaysincombinationwiththepresentNCCmigrationassayshould beusefulforexaminingthespecificeffectsofchemicalsonNCCs. OneadvantageofthepresentNCCmigrationassayisthatgeneral cellulartechniquesandtoxicogenomicanalysesfortoxicological mechanisticstudiesareeasilyapplicabletoNCCsisolatedbythe removalofneuraltubes,asdescribedfortheimmunocytochemistry methodology.

Byusingthismethod,wefoundthatRAhadoppositeeffectson themigrationofcephalicandtrunkNCCs.Thereductionin cephalicNCCmigrationinducedbyRAisconsistentwithprevious reportsthatdescribedtheinhibitoryeffectsofRAoncephalic NCCsasapathogenicmechanismunderlyingcraniofacialmalformation(Prattetal.1987;Menegolaetal.2004).Inthepresent study,weobservedhypoplasiaofthe3rdbranchialarch,theformationofwhichisdependentonmigratedcephalicNCCs,atthe sameRAconcentrationthatreducedcephalicNCCmigration. EnhancementoftrunkNCCmigrationisnotdirectlyrelatedtoany knowndevelopmentaltoxicityofRA.However,theseopposite effectsoncephalicandtrunkNCCswillmakeiteasytoinvestigate themechanismsunderlyingRAeffectsonNCCsbyallowingcomparativeanalysis.

Whenevaluatingtheresultsofthepresent NCCmigrationassay, itshouldbenotedthatalteredmigrationisnotnecessarilyadirect effectofthechemicalonthemotilityofNCCs.BecauseNCC migrationinthepresentassaywasdeterminedasthecircularspread ofthecells,thenumberofcellscaninfluencetheresult,thatis,a decreaseinthenumberofcellsinducedbycytotoxicitycanresultin areducedmigrationindex.Itisalsopossiblethatalteredncc migrationisduetotheeffectsofchemicalsontheneuraltube.This

isbecauseNCCmigrationinthepresentassayisdependentonthe presenceofaneuraltube,asevidencedbythereducedmigrationof NCCswhoseneuraltubedetachedfromtheculturesurface.

Inconclusion,weestablishedasimplemigrationassaythat enablesinvestigationoftheeffectsofchemicalsonratNCCs.By usingthisassaymethod,wefoundthatRAhasoppositeeffectson themigrationofcephalicandtrunkNCCs.

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CONFLICT OF INTEREST

Theauthorsdeclarethattherearenoconflictsofinterest.

REFERENCES

DouarinNL,KalcheimC.1999.Theneuralcrest,2ndedn,Vol.9.New York:CambridgeUniversityPress.

FullerLC,CorneliusSK,MurphyCW,WiensDJ.2002.Neuralcrestcell motilityinvalproicacid.ReprodToxicol16:825–839.

HallBK.2009.Neurocristopathies.In:Theneuralcrestandneuralcrest cellsinvertebratedevelopmentandevolution,2ndedn.NewYork: Springer.p269–293.

KawakamiM,UmedaM,NakagataN,TakeoT,YamamuraK-I.2011. NovelmigratingmouseneuralcrestcellassaysystemutilizingP0-Cre/ EGFPfluorescenttime-lapseimaging.BMCDevBiol11:68.

KimJ,LoL,DormandE,AndersonDJ.2003.SOX10maintains multipotencyandinhibitsneuronaldifferentiationofneuralcreststem cells.Neuron38:17–31.

KraftJ.1992.Pharmacokinetics,placentaltransfer,andteratogenicityof 13-*cis*-retinoicacid,itsisomer,andmetabolites.In:Morriss-KayGM, editor.Retinoidsinnormaldevelopmentandteratogenesis.NewYork: OxfordUniversityPress.p267–280.

MenegolaE,BrocciaML,DiRenzoF,MassaV,GiaviniE.2004.Rela- tionshipbetweenhindbrainsegmentation,neuralcrestcellmigrationand branchialarchabnormalitiesinratembryosexposedtofluconazoleand retinoicacid *in vitro*.ReprodToxicol18:121–130.

NagaseT,SanaiY,NakamuraS,AsatoH,HariiK,OsumiN.2003.Roles ofHNK-1carbohydrateepitopeanditssyntheticglucuronyltransferase genesonmigrationofratneuralcrestcells.JAnat203:77–88.

PrattRM,GouldingEH,AbbottBD.1987.Retinoicacidinhibitsmigration ofcranialneuralcrestcellsintheculturedmouseembryo.JCraniofac GenetDevBiol7:205–217.

UsamiM,MitsunagaK,NakazawaK,DoiO.2008.Proteomicanalysisof seleniumembryotoxicityinculturedpostimplantationratembryos.Birth DefectsResBDevReprodToxicol83:80–96.

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LETTER TO THE EDITOR

Various definitions of reproductive indices: A proposal for combined use of brief definitions

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Severalreproductiveindices,suchaslivebirthindex,arecalculated asendpointstobeevaluatedintoxicitytestsconcerningreproductiveeffectsofchemicals.Theseindicesareusefultocorrect forvariationsresultingfrominfertilityandmultiplepregnancy,for example,thevariednumbersofpups,amongtreatmentgroupsand dams,respectively.Inthetoxicitytestreports,thereproductive indicesareusedwiththeirdefinitions,usuallyexpressedascalculationformulae,todescribewhattheymean.

Despitetheirfrequentuse,however,thedefinitionsoftherepro-ductiveindiceshavenotbeenstandardized;thatis,theyaredifferentamonglaboratories,andareconfusing.Forexample,thelive birthindexis"numberoflivenewborns/numberofimplantation sites × 100"insomelaboratories,butis"numberoflivenewborns/ numberoftotalnewborns × 100"inothers,aslistedinTable1. Thesetwodefinitionsarequitedifferentfromeachotherinthatthe latterdoesnotinvolvepostimplantationloss,buttheformerdoes, thoughthelivebirthindexisoneofthemostimportantreproductive indices.Inmosttoxicitytestlaboratories,ontheotherhand,the definitionsofreproductiveindicescannotbechangedevenfor standardizationbecausetheyaredefinedasapartoflaboratory computersystems.

Inthedatabaseera,theconfusionofreproductiveindiceshas becomemoreseriousthanever,becausedatafromvariouslaboratoriesinthetoxicitydatabasesarefrequentlyconsultedatatimeas inmeta-analysesforbuildingquantitativestructure-activityrelationshipmodels.Inthemeta-analysisofreproductivetoxicitydata, reproductiveindicescannotbeusedastoxicologicalendpointstobe evaluatedunlesstheirdefinitions,usuallynotfoundintheabstract becauseoftheirlengthiness,areclearlyidentified.

Asasolutiontothisissue,wehereproposecombineduseofbrief definitionsthatdescribethemeaningofthereproductiveindices withsimplerwordsthanthecalculationformulae,forexample, "livenewborn/nidationrate"for"numberoflivenewborns/number ofimplantationsites × 100."Explanatorydescriptionsoftherepro-ductiveindiceswiththeirbriefdefinitions,forexample,"thelive birthindex(livenewborn/nidationrate)"attheirfirstappearancein theabstractandmaintextwouldbemosthelpful.

Inthisletter,weshowvariousdefinitionsofrepresentative reproductiveindicesandproposetheirbriefdefinitions.Wefound 14reproductiveindiceswith23definitionsbyabriefsurveyof toxicologicalreferencebooks(MansonandKang1989;Mizutani 1992;Saikikeiseinikansurudejitarukasagyogruupuiinkai1994; Econbichon1995;Parker2012)andcontractresearchorganizations'reportsinatoxicologicaldatabase(JapanExistingChemical DataBase,http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPage.jsp). Fromtheseindices,weshowsevenrepresentativeindicesand12 briefdefinitionsasexamples(Table1),butitisnotintendedthatthe briefdefinitionspresentedhereshouldbeusedastheyare.

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REFERENCES

EconbichonDJ.1995.Reproductivetoxicology.In:DerelankoMJ, HollingerMA,editors.CRChandbookoftoxicology.BocaRaton:CRC Press.p379–402.

MansonJM,KangYJ.1989.Testmethodsforassessingfemalereproductive anddevelopmentaltoxicology.In:HayesAW,editor.Principlesand methodsoftoxicology,2ndedn.NewYork:RavenPress,Ltd.p311–359.

MizutaniM.1992.Seisyokuhasseidokuseinojissai.In:TanimuraT, editor.Developmentaltoxicology.Tokyo:Chijinshokan.p143–167.(In Japanese.)

ParkerRM.2012.Reproductivetoxicitytesting-Methodology.In:Hood RD,editor.Developmentalandreproductivetoxicology:apractical approach,3rdedn.London:InformaHealthcare.p184–228.

Saikikeiseinikansurudejitarukasagyogruupuiinkai.1994.Saikikesei Yogo.In:NakadateM,editor.Toxicitytesting.Tokyo:NationalInstitute ofHealthSciences,BiologicalSafetyResearchCenter.p394–488. (InJapanese.)AvailableatURL:http://www.nihs.go.jp/center/yougo/ saiki.html.

Table 1 Representativereproductiveindicesandtheirdefinitionsappearedinreferencebooksandtoxicityreports

Reproductive index	Definition†								Exampleofbrief definition
	ReferenceBook				Contractresearchorganization'sreproductivetoxicitytestreport				
	Manson& Kang,1989Mizutani,1992Ecobichon,1995Saikikeisei,1994Parker,2012				LaboratoryA	LaboratoryB	LaboratoryC	LaboratoryD	
Implantation index				Implants/Corpora lutea	Implantationsites/ Corporalutea	Implantationsites/ Corporalutea	Implantationscars/ Corporalutea	Implantationsites/ Corporalutea	Nidation/luteum rate
		Implantations/Pregnant females							Nidation/pregnant rate
Gestation index		Femaleswithlive offspring/Pregnant females	Femaleswithlive offspring/Pregnant females	Femaleswithlive born/Femaleswith evidenceof pregnancy	Femaleswithlivepups/ Pregnantfemales				Livedelivered dam/pregnant rate
Delivery index						Femaleswhich deliveredliveborns/ Pregnantfemales	Damswithlive offspring/Pregnant dams	Pregnantfemaleswith livepupsatbirth/ Pregnantfemales	
		Pupsborn/Implantation sites			Pupsborn/Implantation sites		Offspringatbirth/ Implantationscars	Pupsborn/Implantation sites	Newborn/nidation rate
Livebirth index		(Viablepupsborn/ litter)/(Pupsborn/ litter)		Pupsbornalive/Total pupsborn	Livepupsonlactation day0/Pupsborn		Liveoffspringatbirth/ Offspringatbirth	Livepupsatbirth/Pups born	Live/totalnewborn rate
	Pupsaliveday 1/Pupsborn alive								Day1livepup/live newbornrate
		Liveborn/Implantation sites				Liveborn/Implantation sites			Livenewborn/ nidationrate
Birthindex			Offspringbornalive/ Implantations				Liveoffspringatbirth/ Implantationscars	Livepupsatbirth/ Implantationsites	
Viability index		Offspringaliveonday4 afterbirth/Liveborn	Offspringaliveonday4 afterbirth/Offspring bornalive		Livepupsonlactation day4/Livepupson lactationday0	Livepupsonpostnatal day4/Liveborn	Liveoffspringat4days afterbirth/Live offspringsatbirth	Livepupsonpostnatal day4/Livepupsat birth	Day4livepup/live newbornrate
	Pupsaliveday 7/Pupsalive day1								Daysx/ylive/live puprate
		Viablepupsborn/Dead pupsborn							Live/deadnewborn rate
Sexratio (atbirth)		Maleoffspring/Female offspring		Maleoffspring/Total offspring	Malepupsborn/Pups born		Maleoffspring/(Male offspring + female offspring)	Malesborn/Pups born	Male/totalpuprate
					Livemalepups/Live pups	Livebornmales/Live born			Livemale/live totalpuprate

†Commondescriptions,"numberof"and" × 100,"areomitted.

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Toxicomics Report

Proteomic analysis of ethanol-induced embryotoxicity in cultured post-implantation rat embryos

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ABSTRACT — Protein expression changes were examined in day 10.5 rat embryos cultured for 24 hr in the presence of ethanol by using two-dimensional electrophoresis and mass spectrometry. Exposure to ethanol resulted in quantitative changes in many embryonic protein spots (16 decreased and 28 increased) at *in vitro* embryotoxic concentrations (130 and 195 mM); most changes occurred in a concentration-dependent manner. For these protein spots, 17 proteins were identified, including protein disulfide isomerase A3, alpha-fetoprotein, phosphorylated cofilin-1, and serum albumin. From the gene ontology classification and pathway mapping of the identified proteins, it was found that ethanol affected several biological processes involving oxidative stress and retinoid metabolism.

Key words: Ethanol, Embryotoxicity, Proteomics, Rat

INTRODUCTION

Developmental toxicology is a rapidly growing area of proteomics; it is expected to provide mechanistic insights and protein biomarkers for the safety evaluation of chemicals (Usami and Mitsunaga, 2011). For example, expression changes in actin-binding proteins were considered to be involved in selenate embryotoxicity in the rat whole embryo culture (Usami *et al.*, 2008). Differences in strain sensitivity to cadmium-induced teratogenicity were related to unfolded protein response process and actin polymerization in the mouse limb-bud culture (Chen *et al.*, 2008). Furthermore, based on cluster analysis of proteins with expression changes in the embryonic stem cell test, chemicals were classified into highly embryotoxic and non- or weakly embryotoxic (Groebe *et al.*, 2010). It is thus important to accumulate proteomic analysis data in the field of developmental toxicology. In the present study, protein expression changes in day 10.5 rat embryos cultured for 24 hr in the presence of ethanol, a well-known developmental toxicant, were examined by two-dimensional electrophoresis (2-DE) and mass spectrometry (MS).

MATERIAL AND METHODS

Embryo culture and ethanol treatment

Day 10.5 embryos (plug day = day 0.5) of Wistar rats (Crlj: WI, Charles River Laboratories Japan, Inc., Kanagawa, Japan) were cultured for 24 hr (Usami *et al.*, 2008). Ethanol was diluted in Hank's balanced salt solution in two-fold and added to the culture medium composed of 100% rat serum at concentrations of 0, 65, 130, and 195 mM. Medium-sized cultured embryos (four embryos per treatment group) were selected for subsequent protein analyses. All animal experiments were carried out according to the guidelines for animal use of the National Institute of Health Sciences.

2-DE and MS analyses of embryonic protein

The analyses of 2-DE gels (one embryo per gel, four gels per treatment group) were carried out as previously reported (Usami *et al.*, 2009), except that the gels were stained with a fluorescent dye (Flamingo gel stain, Bio-Rad, Hercules, CA, USA) and scanned with a laser scanner (FLA-5100, GE Healthcare UK Ltd., Amersham Place, Little Chalfont, UK) at an excitation wavelength of

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473 nm. Quantitative differences in protein spots of more than 1.5-fold with statistical significance by the *t*-test at 5% probability level between the control and 195 mM ethanol groups, were regarded as ethanol-induced protein expression changes.

Classification and mapping of identified proteins

NCBInr GI numbers of the identified proteins were mapped to UniProtKB AC, and gene ontology (GO) terms were assigned using the UniProt web site (http://www.uniprot.org/) (Jain *et al.*, 2009; The UniProt Consortium, 2011). The occurrence of the GO terms (76 biological processes) of the proteins was counted with the CateGOrizer web tool in the "MGI_GO_slim2" ancestor terms using the multiple count method (http://www.animalgenome.org/tools/catego/) (Hu *et al.*, 2008). UniProtKB ACs of the proteins were queried against the KEGG PATHWAY for *Rattus norvegicus* with the KEGG Mapper on the GenomeNet web site (http://www.genome.jp/en/).

RESULTS

Effects of ethanol on the growth of cultured rat embryos

Ethanol inhibited the growth of cultured embryos at concentrations of 130 mM or higher in a concentration-dependent manner (Table 1). Deformed organs included branchial arch, heart, neural tube, optic vesicle, otic vesicle, somite, and tail (Fig. 1), which is in agreement with previous reports (Giavini *et al.*, 1992; Zhou *et al.*, 2011). Compared to blood ethanol levels found in humans, these embryotoxic ethanol concentrations are rather high; however, an ethanol concentration of 150 mM can be observed after acute alcohol intake in chronic alcoholics and 200 mM of ethanol has often been used in *in vitro* toxicological experiments (Li and Kim, 2003; Szabo *et al.*, 1994; Wentzel and Eriksson, 2008).

Effects of ethanol on embryonic protein expression

About 900 protein spots were matched through sixteen 2-DE gels (four gels per experimental group). Quality changes, i.e., appearance or disappearance, in the

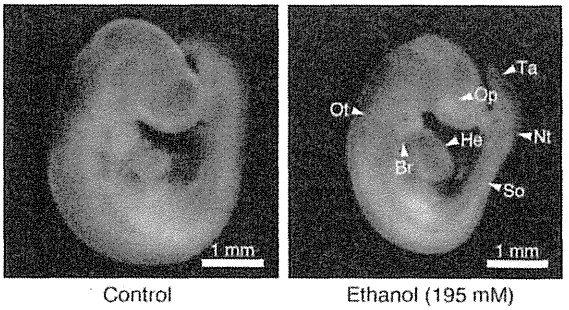

Fig. 1. Appearance of rat embryos cultured in the presence of ethanol. Rat embryos at the end of 24-hr culture are shown after removal of the embryonic membranes. Arrowheads indicate deformed organs. Br, branchial arch; He, heart; Nt, neural tube; Op, optic vesicle; Ot, otic vesicle; So, somite; Ta, tail.

Table 1. Growth of day 10.5 rat embryos cultured in the presence of ethanol

	Ethanol (mM)			
	0 (Control)	65	130	195
No. of embryos	6	5	6	5
No. of viable embryos	6 (100%)	5 (100%)	6 (100%)	5 (100%)
Crown-rump length (mm)	4.11 ± 0.15	3.99 ± 0.17	3.72 ± 0.29*	3.25 ± 0.28**
Head length (mm)	2.23 ± 0.11	2.16 ± 0.14	2.01 ± 0.22	1.74 ± 0.35**
No. of somite pairs	26.7 ± 0.52	26.4 ± 0.55	24.5 ± 2.81	21.2 ± 2.49**
No. of embryos with deformed organ	0	0	3 (50%)	5 (100%)**
Branchial arch	0	0	2 (33%)	4 (80%)*
Heart	0	0	1 (17%)	3 (60%)
Neural tube	0	0	2 (33%)	2 (40%)
Optic vesicle	0	0	2 (33%)	5 (100%)**
Otic vesicle	0	0	2 (33%)	5 (100%)**
Somite	0	0	3 (50%)	5 (100%)**
Tail	0	0	2 (33%)	4 (80%)*

Embryos were cultured for 24 hr by the roller method. Asterisks indicate statistically significant differences compared to the control group identified by Dunnett's multiple comparison test or Fisher's exact test (* $p < 0.05$; ** $p < 0.01$).

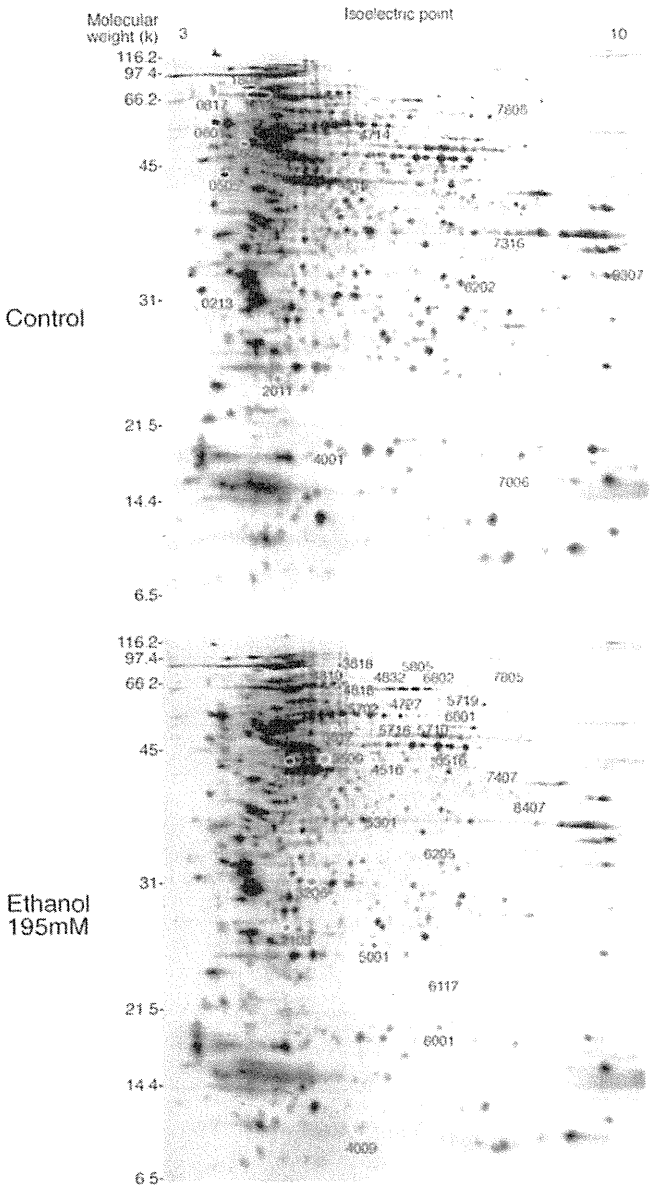

Fig. 2. Two-dimensional electrophoresis pattern of proteins from rat embryos cultured in the presence of ethanol. Representative gels are shown for the control and ethanol (195 mM) groups. Proteins with ethanol-induced expression changes are indicated by circles with standard spot numbers (SSPs); decreased proteins are indicated in the "control" gel (top) and increased ones in the "ethanol" gel (bottom).

protein spots were not observed. Ethanol-induced quantitative changes were noted in 44 spots, i.e., 16 spots were decreased and 28 spots were increased by 1.5-fold or more. The differences between the 195 mM ethanol group and the control group were significant and occurred for most proteins in a concentration-dependent manner (Figs. 2 and 3). Of these spots, 23 were analyzed by MS, resulting in the identification of 7 proteins that were decreased (Table 2) and 11 proteins that were increased (Table 3). Some proteins that were increased, e.g., alpha-fetopro-

Table 2. Proteins whose expression was decreased and their GO terms identified by two-dimensional electrophoresis analysis of rat embryos cultured in the presence of ethanol

SSP	Protein Name	UniProtKB AC	GO term for Biological Process
0505	Protein SET	Q63945	GO:0006334 nucleosome assembly
0601	Nucleosome assembly protein 1-like 1	Q9Z2G8	GO:0006334 nucleosome assembly
0817	Myristoylated alanine-rich C-kinase substrate	P30009	n.a.
1802	78 kDa glucose-regulated protein	P06761	GO:0006916 anti-apoptosis GO:0006983 ER overload response GO:0006987 activation of signaling protein activity involved in unfolded protein response GO:0021589 cerebellum structural organization GO:0021680 cerebellar Purkinje cell layer development GO:0030512 negative regulation of transforming growth factor beta receptor signaling pathway GO:0031398 positive regulation of protein ubiquitination GO:0042149 cellular response to glucose starvation GO:0043066 negative regulation of apoptotic process GO:0043154 negative regulation of cysteine-type endopeptidase activity involved in apoptotic process GO:0051603 proteolysis involved in cellular protein catabolic process
1813	Heat shock cognate 71 kDa protein	P63018	GO:0006351 transcription, DNA-dependent GO:0006355 regulation of transcription, DNA-dependent GO:0006950 response to stress GO:0045892 negative regulation of transcription, DNA-dependent GO:0051085 chaperone mediated protein folding requiring cofactor GO:0061077 chaperone-mediated protein folding
2011	Uncharacterized protein	D3ZRS6	n.a.
4714	Protein disulfide-isomerase A3	P11598	GO:0006662 glycerol ether metabolic process GO:0043065 positive regulation of apoptotic process GO:0045454 cell redox homeostasis

n.a., not available.

Table 3. Proteins whose expression was increased and their GO terms identified by two-dimensional electrophoresis analysis of rat embryos cultured in the presence of ethanol

SSP	Protein Name	UniProtKB AC	GO term for Biological Process
2103	Myosin light chain 3	P16409	GO:0002026 regulation of the force of heart contraction GO:0006936 muscle contraction GO:0006942 regulation of striated muscle contraction GO:0007519 skeletal muscle tissue development GO:0055010 ventricular cardiac muscle tissue morphogenesis GO:0060048 cardiac muscle contraction
2512	BWK4 AND Eukaryotic initiation factor 4A-II OR Eukaryotic translation initiation factor 4A1	Q5VLR5 AND Q5RKI1 OR Q6P3V8	GO:0006457 protein folding AND GO:0006950 response to stress GO:0006986 response to unfolded protein GO:0009100 glycoprotein metabolic process GO:0045454 cell redox homeostasis AND GO:0006413 translational initiation

3509	Eukaryotic translation initiation factor 4A1	Q6P3V8	GO:0006413 translational initiation
4009	Fatty acid-binding protein	P07483	GO:0006631 fatty acid metabolic process GO:0006635 fatty acid beta-oxidation GO:0006656 phosphatidylcholine biosynthetic process GO:0006810 transport GO:0015909 long-chain fatty acid transport GO:0032868 response to insulin stimulus GO:0042493 response to drug GO:0070542 response to fatty acid
5001	Adenine phosphoribosyltransferase	P36972	GO:0006166 purine ribonucleoside salvage GO:0006168 adenine salvage GO:0007595 lactation GO:0009116 nucleoside metabolic process GO:0032869 cellular response to insulin stimulus
6001	Cofilin-1	P45592	GO:0006606 protein import into nucleus GO:0007010 cytoskeleton organization GO:0022604 regulation of cell morphogenesis GO:0030030 cell projection organization GO:0045792 negative regulation of cell size
6516	Elongation factor 1-gamma	Q68FR6	GO:0006412 translation GO:0006414 translational elongation
4727 5702 5710 5716 6601	Protein disulfide-isomerase A3	P11598	GO:0006662 glycerol ether metabolic process GO:0043065 positive regulation of apoptotic process GO:0045454 cell redox homeostasis
4810 4818	Alpha-fetoprotein	P02773	GO:0001542 ovulation from ovarian follicle GO:0001889 liver development GO:0006810 transport GO:0010033 response to organic substance GO:0019953 sexual reproduction GO:0031016 pancreas development GO:0031100 organ regeneration GO:0042448 progesterone metabolic process GO:0060395 SMAD protein signal transduction
4832 5805 6802	Serum albumin	P02770	GO:0006810 transport GO:0006950 response to stress GO:0007584 response to nutrient GO:0009267 cellular response to starvation GO:0010033 response to organic substance GO:0019836 hemolysis by symbiont of host erythrocytes GO:0042311 vasodilation GO:0043066 negative regulation of apoptotic process GO:0046010 positive regulation of circadian sleep/wake cycle, non-REM sleep GO:0046689 response to mercury ion GO:0051659 maintenance of mitochondrion location GO:0070541 response to platinum ion

SSP 2512 contained two proteins.

Table 4. KEGG pathway mapping of proteins identified by two-dimensional electrophoresis analysis of rat embryos cultured in the presence of ethanol

Pathway ID	Pathway name	UniProtKB AC	Protein Name (Total number of mapped pathways)
rno04612	Antigen processing and presentation	P06761	78 kDa glucose-regulated protein (4)
		P11598	Protein disulfide-isomerase A3 (2)
		P63018	Heat shock cognate 71 kDa protein (9)
rno04141	Protein processing in endoplasmic reticulum	P06761	78 kDa glucose-regulated protein (4)
		P11598	Protein disulfide-isomerase A3 (2)
		P63018	Heat shock cognate 71 kDa protein (9)
rno05134	Legionellosis	P63018	Heat shock cognate 71 kDa protein (9)
		Q68FR6	Elongation factor 1-gamma (1)
rno03040	Spliceosome	P63018	Heat shock cognate 71 kDa protein (9)
rno04010	MAPK signaling pathway	P63018	Heat shock cognate 71 kDa protein (9)
rno04144	Endocytosis	P63018	Heat shock cognate 71 kDa protein (9)
rno05145	Toxoplasmosis	P63018	Heat shock cognate 71 kDa protein (9)
rno05162	Measles	P63018	Heat shock cognate 71 kDa protein (9)
rno05164	Influenza A	P63018	Heat shock cognate 71 kDa protein (9)
rno04360	Axon guidance	P45592	Cofilin-1 (4)
rno04666	Fc gamma R-mediated phagocytosis	P45592	Cofilin-1 (4)
rno04810	Regulation of actin cytoskeleton	P45592	Cofilin-1 (4)
rno05133	Pertussis	P45592	Cofilin-1 (4)
rno04260	Cardiac muscle contraction	P16409	Myosin light chain 3 (3)
rno05410	Hypertrophic cardiomyopathy (HCM)	P16409	Myosin light chain 3 (3)
rno05414	Dilated cardiomyopathy	P16409	Myosin light chain 3 (3)
rno00230	Purine metabolism	P36972	Adenine phosphoribosyltransferase (2)
rno01100	Metabolic pathways	P36972	Adenine phosphoribosyltransferase (2)
rno03060	Protein export	P06761	78 kDa glucose-regulated protein (4)
rno05020	Prion diseases	P06761	78 kDa glucose-regulated protein (4)
rno03013	RNA transport	Q5RKI1	Eukaryotic initiation factor 4A-II (1)
		OR	OR
		Q6P3V8	Eukaryotic translation initiation factor 4A1 (1)
rno03320	PPAR signaling pathway	P07483	Fatty acid-binding protein (1)

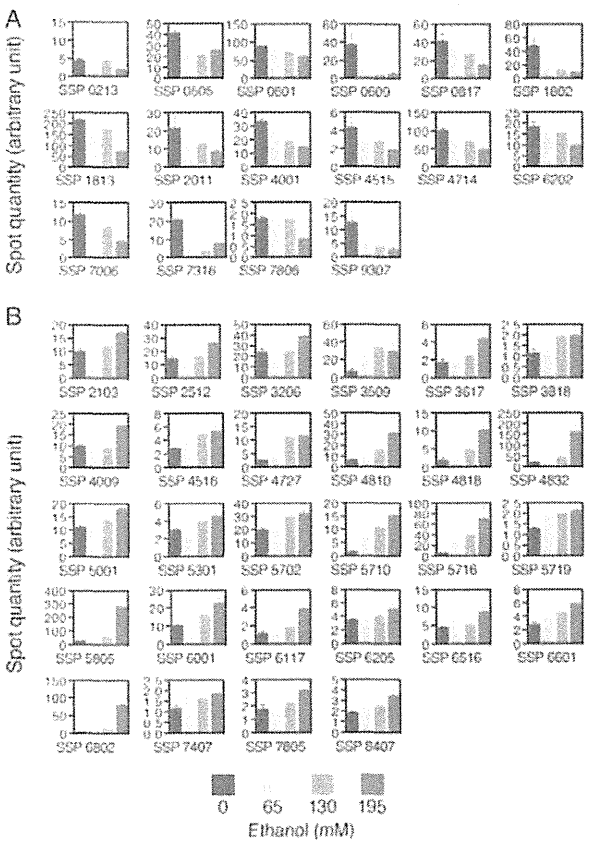

Fig. 3. Quantification of protein spots with expression changes in two-dimensional electrophoresis gels from rat embryos cultured in the presence of ethanol. Intensities of protein spots with ethanol-induced expression changes are shown. A. Protein spots with decreased intensity. B. Protein spots with increased intensity. Error bars indicate the standard error of the mean.

tein (standard spot numbers (SSPs) 4810, 4818), cofilin-1 (SSP 6001), and serum albumin (SSPs 4832, 5805, 6802), were the same as those identified as candidate proteins involved in embryotoxicity in our previous studies (Usami *et al.*, 2009, 2008); cofilin-1, which was increased, was found to be in its phosphorylated form.

Several protein spots were identified as charge variant forms of the same proteins, i.e., protein disulfide isomerase A3 (PDIA3; SSPs 4714, 4727, 5702, 5710, 5716, 6601), alpha-fetoprotein (SSPs 4810, 4818), and serum albumin (SSPs 4832, 5805, 6802). The quantities of spots that were identified as PDIA3 were increased (SSPs 4727, 5702, 5710, 5716, 6601), as well as decreased (SSP 4714) (Fig. 3). Because the PDIA3 spot with decreased quantity was the most acidic spot, it appeared that a basic pI shift of PDIA3 occurred in the groups exposed to ethanol.

Classification and mapping of proteins with ethanol-induced expression changes

According to their GO terms, the identified proteins were classified into various categories; the six major categories were "metabolism" (including 32% of the GO terms), "protein metabolism" (13%), "death" (9%), "developmental processes" (9%), "cell organization and biogenesis" (8%), and "stress response" (8%).

The identified proteins were mapped to 22 pathways using the KEGG pathway mapper (Table 4). Multiple proteins, i.e., PDIA3, 78-kDa glucose-regulated protein (SSP 1802), and heat shock cognate 71-kDa protein (SSP 1813), were mapped to the same two pathways, i.e., "protein processing in endoplasmic reticulum" (rno04141) and "antigen processing and presentation" (rno04612). Some proteins were mapped to multiple pathways, e.g., heat shock cognate 71-kDa protein (nine pathways), 78-kDa glucose-regulated protein (four pathways), and cofilin-1 (four pathways).

DISCUSSION

As mechanisms of ethanol-induced embryotoxicity, oxidative stress, and inhibited retinoid synthesis have been proposed (Goodlett *et al.*, 2005), which seems to be in accordance with the GO classification (32% metabolism and 8% stress response) of the proteins identified in the present study. In this context, expression changes in PDIA3 (also known as GRp58 and ERp57) are intriguing because it is an endoplasmic reticulum stress protein with oxidoreductase activity that regulates cellular redox homeostasis (Frickel *et al.*, 2004; Ni and Lee, 2007). PDIA3 is also involved in the nuclear translocation of retinoic acid receptor alpha (Zhu *et al.*, 2010) and its deficiency is embryonic lethal (Coe *et al.*, 2010). The identified proteins with GO terms classified into "death" may be involved in ethanol-induced apoptosis of neuronal cells, which has frequently been observed (Ahlgren *et al.*, 2002; Giles *et al.*, 2008). The present results also agreed with some biological networks that were perturbed by ethanol in cultured whole mouse embryos, involving cell death, reproductive system and antigen processing (Mason *et al.*, 2012). The pathways associated with multiple identified proteins may be more susceptible to ethanol, because these pathways could be affected at multiple steps simultaneously. On the other hand, the finding that

multiple pathways were associated with the same proteins might partially explain the complexity of ethanol-induced embryotoxicity.

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REFERENCES

Ahlgren, S.C., Thakur, V. and Bronner-Fraser, M. (2002): Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. Proc. Natl. Acad. Sci. USA, **99**, 10476-10481.

Chen, H., Boontheung, P., Loo, R.R., Xie, Y., Loo, J.A., Rao, J.Y. and Collins, M.D. (2008): Proteomic analysis to characterize differential mouse strain sensitivity to cadmium-induced forelimb teratogenesis. Birth Defects Res. A Clin. Mol. Teratol., **82**, 187-199.

Coe, H., Jung, J., Groenendyk, J., Prins, D. and Michalak, M. (2010): ERp57 modulates STAT3 signaling from the lumen of the endoplasmic reticulum. J. Biol. Chem., **285**, 6725-6738.

Frickel, E.-M., Frei, P., Bouvier, M., Stafford, W.F., Helenius, A., Glockshuber, R. and Ellgaard, L. (2004): ERp57 is a multifunctional thiol-disulfide oxidoreductase. J. Biol. Chem., **279**, 18277-18287.

Giavini, E., Broccia, M.L., Prati, M., Bellomo, D. and Menegola, E. (1992): Effects of ethanol and acetaldehyde on rat embryos developing *in vitro*. Vitr. Cell. Dev. Biol., **28A**, 205-210.

Giles, S., Boehm, P., Brogan, C. and Bannigan, J. (2008): The effects of ethanol on CNS development in the chick embryo. Reprod. Toxicol., **25**, 224-230.

Goodlett, C.R., Horn, K.H. and Zhou, F.C. (2005): Alcohol teratogenesis: mechanisms of damage and strategies for intervention. Exp. Biol. Med., **230**, 394-406.

Groebe, K., Hayess, K., Klemm-Manns, M., Schwall, G., Wozny, W., Steemans, M., Peters, A.K., Sastri, C., Jaeckel, P., Stegmann, W., Zengerling, H., Schopf, R., Poznanovic, S., Stummann, T.C., Seiler, A., Spielmann, H. and Schrattenholz, A. (2010): Protein biomarkers for *in vitro* testing of embryotoxicity. J. Proteome Res., **9**, 5727-5738.

Hu, Z.-L., Bao, J. and Reecy, J.M. (2008): CateGOrizer: A web-based program to batch analyze gene ontology classification categories. Online J. Bioinforma., **9**, 108-112.

Jain, E., Bairoch, A., Duvaud, S., Phan, I., Redaschi, N., Suzek, B.E., Martin, M.J., McGarvey, P. and Gasteiger, E. (2009): Infrastructure for the life sciences: design and implementation of the UniProt website. BMC Bioinformatics, **10**, 136.

Li, H. and Kim, K.H. (2003): Effects of ethanol on embryonic and neonatal rat testes in organ cultures. J. Androl., **24**, 653-660.

Mason, S., Anthony, B., Lai, X., Ringham, H.N., Wang, M., Witzmann, F.A., You, J.-S. and Zhou, F.C. (2012): Ethanol exposure alters protein expression in a mouse model of fetal alcohol spectrum disorders. Int. J. Proteomics, **2012**, 867141.

Ni, M. and Lee, A.S. (2007): ER chaperones in mammalian development and human diseases. FEBS Lett., **581**, 3641-3651.

Szabo, G., Puppolo, M., Verma, B. and Catalano, D. (1994): Regulatory potential of ethanol and retinoic acid on human monocyte functions. Alcohol. Clin. Exp. Res., **18**, 548-554.

UniProt Consortium (2011): Ongoing and future developments at the Universal Protein Resource. Nucleic Acids Res., **39**, D214-219.

Usami, M. and Mitsunaga, K. (2011): Proteomic analysis and *in vitro* developmental toxicity tests for mechanism-based safety evaluation of chemicals. Expert Rev. Proteomics, **8**, 153-155.

Usami, M., Mitsunaga, K., Nakazawa, K. and Doi, O. (2008): Proteomic analysis of selenium embryotoxicity in cultured postimplantation rat embryos. Birth Defects Res. B Dev. Reprod. Toxicol., **83**, 80-96.

Usami, M., Nakajima, M., Mitsunaga, K., Miyajima, A., Sunouchi, M. and Doi, O. (2009): Proteomic analysis of indium embryotoxicity in cultured postimplantation rat embryos. Reprod. Toxicol., **28**, 477-488.

Wentzel, P. and Eriksson, U.J. (2008): Genetic influence on dysmorphogenesis in embryos from different rat strains exposed to ethanol *in vivo* and *in vitro*. Alcohol. Clin. Exp. Res., **32**, 874-887.

Zhou, F.C., Zhao, Q., Liu, Y., Goodlett, C.R., Liang, T., McClintick, J.N., Edenberg, H.J. and Li, L. (2011): Alteration of gene expression by alcohol exposure at early neurulation. BMC Genomics, **12**, 124.

Zhu, L., Santos, N.C. and Kim, K.H. (2010): Disulfide isomerase glucose-regulated protein 58 is required for the nuclear localization and degradation of retinoic acid receptor alpha. Reproduction, **139**, 717-731.

Title

Effects of 13 developmentally toxic chemicals on the migration of rat cephalic neural crest cells in vitro

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ABSTRACT

The inhibition of neural crest cell (NCC) migration has been considered as a possible pathogenic mechanism underlying chemical developmental toxicity. In this study, we examined the effects of 13 developmentally toxic chemicals on the migration of rat cephalic NCCs (cNCCs) by using a simple in vitro assay. cNCCs were cultured for 48 h as emigrants from rhombencephalic neural tubes explanted from rat embryos at day 10.5 of gestation. The chemicals were added to the culture medium at 24h of culture. Migration of cNCCs was measured as the change in the radius (radius ratio) calculated from the circular spread of cNCCs between 24 and 48 h of culture. Of the chemicals examined, 13-*cis*-retinoic acid, ethanol, ibuprofen, lead acetate, salicylic acid, and selenate inhibited the migration of cNCCs at their embryotoxic concentrations; no effects were observed for acetaminophen, caffeine, indium, phenytoin, selenite, tributyltin, and valproic acid. In a cNCC proliferation assay, ethanol, ibuprofen, salicylic acid, selenate, and tributyltin inhibited cell proliferation, suggesting the contribution of the reduced cell number to the inhibited migration of cNCCs. It was determined that several developmentally toxic chemicals inhibited the migration of cNCCs, the effects of which were manifested as various craniofacial abnormalities.

Key words

Developmental toxicity; Embryo; Migration assay; Neural crest cell; Rat

INTRODUCTION

In vertebrate embryos, neural crest cells (NCCs) migrate to various tissues throughout the body and contribute to tissue organization; malfunction NCCs of can lead to dysmorphologies, tumors and syndromes called neurocristopathies (Hall 2009; Le Douarin & Kalcheim 1999). The inhibition of NCC migration has, therefore, been considered as a possible pathogenic mechanism underlying chemical developmental toxicity. It has been shown, for example, that all-*trans*-retinoic acid, a well-known teratogen, inhibits the migration of cephalic NCCs (cNCCs), causing branchial abnormalities in cultured mouse and rat embryos (Menegola et al. 2004).

The effects of chemicals on the migration of NCCs in mammals, however, have not been fully investigated, probably because no convenient experimental methods are available. The migration of NCCs has been examined by time-lapse video image analysis of fluorescence-labeled cells (Fuller et al. 2002; Kawakami et al. 2011), or by human neural crest stem cells with scratch assay (Zimmer et al. 2012). These methods are complicated and therefore not ideal for testing of chemicals in a common toxicity laboratory.

Recently, we established a simple in vitro assay that enabled examination of the effects of chemicals on the migration of cNCCs and trunk NCCs (tNCCs) (Usami et al. 2014b). In this method, NCCs are cultured as emigrants from isolated neural tubes of day 10.5 rat embryos. The cultured NCCs are exposed to test chemicals and their migration is determined as the radius ratio calculated from circular spread of the NCCs during the exposure period. Using this method we examined the effects of 13 developmentally toxic chemicals on the migration of cNCCs. We also examined the effects of chemicals on the proliferation of cNCCs, because this migration assay depends on the spread of cells and

can therefore be influenced by the cell number.

We selected developmentally toxic chemicals on the basis of our interest in our related study such as proteomics of embryos (Usami et al. 2014a; Usami et al. 2009; Usami et al. 2008) and metabolomics of hepatocytes (Kim et al. 2014) since there was little information about the effects of chemicals on the migration of cNCCs. However, we considered that the chemicals include both ones might affect cNCC migration, e.g., ethanol, and selenate, and ones might not, e.g., indium, and tributyltin, which was speculated from their potential to cause craniofacial abnormality.

MATERIALS AND METHODS

Animals

Wistar rats (Crj: WI, Charles River Japan Inc., Kanagawa, Japan) were used. Pregnant rats were obtained by mating female and male rats overnight, and the plug day was designated as day 0.5 of gestation. All the animal experiments were performed according to the guidelines for animal experiments of the National Institute of Health Sciences.

Chemicals

Acetaminophen (CAS 103-90-2), 13-*cis*-retinoic acid (CAS 4759-48-2), ibuprofen (CAS 31121-93-4), salicylic acid (CAS 54-21-7), selenate (CAS 13410-01-0), and selenite (CAS 10102-18-8) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Caffeine (CAS 58-08-2), ethanol (CAS 64-17-5), indium (CAS 22519-64-8), lead acetate (CAS 6080-56-4), phenytoin (CAS 57-41-0), and tributyltin (CAS 1461-22-9) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Valproic acid (CAS 1069-66-5) was purchased from Merck Co. (Darmstadt, Germany).

Culture of NCCs

Rat NCCs were cultured as emigrated cells from neural tubes of rat embryos at day 10.5 of gestation as previously described (Usami et al. 2014b), according to the culture schedule shown in Fig. 1. Neural tubes were excised from the rhombencephalic (for cNCCs) or trunk (for tNCCs) region of the embryos in Hanks' balanced salt solution with sharpened tungsten needles. The excised neural tubes were cultured in 35-mm culture dishes (BD Primaria; Becton, Dickinson and Company, Franklin Lakes, NJ) containing 2 ml of Dulbecco's Modified Eagle Medium with high glucose (DMEM; GIBCO, Life

Technologies Corp., Carlsbad, CA) and 10% (v/v) fetal bovine serum (GIBCO) at 37°C with 5% CO_2 for 48 h.

Phase-contrast images of cultured NCCs were recorded digitally at a magnification of ×10 with a microscope at 24 and 48 h of culture (BZ-9000; Keyence, Osaka, Japan). In the proliferation assay, the neural tube was removed at 18 h of culture, and the cell nuclei were stained with 4',6- diaminodino-2-phenylindole (DAPI, Invitrogen) and fluorescent images were photographed with the microscope at 48 h of culture. Representative photographs of the cNCCs are shown in Fig. 2.

Addition of chemicals

The chemicals were added at 24 h of culture by replacing the culture medium. For addition to the culture medium, caffeine, ethanol, salicylic acid, selenate, selenite, and valproic acid were directly dissolved in or diluted with the culture medium. Acetaminophen, 13-*cis*-retinoic acid, phenytoin, and tributyltin were dissolved in or diluted with dimethyl sulfoxide and 5 µl each of the solutions was added to 5 ml of the culture medium. Ibuprofen, indium, and lead acetate were dissolved in pure water and 100 µl each of the solutions was added to 4.9 ml of the culture medium.

The concentrations of the following chemicals in the culture medium were their embryotoxic concentrations obtained from the literature: acetaminophen (Weeks et al. 1990), caffeine (Robinson et al. 2010; Shreiner et al. 1986), 13-*cis*-retinoic acid (Lee et al. 1991), ethanol (Usami et al. 2014a), ibuprofen (Guest et al. 1994), indium (Usami et al. 2009), lead acetate (Zhao et al. 1997), phenytoin (Winn 2002), salicylic acid (Greenaway et al. 1985), selenate (Usami et al. 2008), selenite (Usami et al. 2008), tributyltin (Cooke et al. 2008; Adeeko et al. 2003), and valproic acid (Guest et al. 1994)

Migration assay of NCCs

The migration distance of NCCs was calculated as the increased radius of the circular spread of NCCs that emigrated from the neural tubes between 24 and 48 h of culture (Usami et al. 2014b). The outermost NCCs in each of the cultured neural tubes were connected with the polygon tool as if a rubber band were put around the cells, and its inner area was measured as a pixel count. Considering the polygon as a circle, its radius ratio was calculated: radius ratio = (radius at 48 h - radius at 24 h)/radius at 24 h. This ratio was then normalized as a percent of the simultaneous control to express the NCC migration for comparisons among experiments.

Proliferation assay for NCCs

NCC proliferation was evaluated as a ratio of the cell count at 48 h to that at 24 h of culture, and the effects of chemicals were examined. The cells were counted manually at 24 h on the phase-contrast image with the Cell Counter plugin of the ImageJ software (http://rsb.info.nih.gov/ij/, 1997–2009; Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA). The cell count at 48 h was estimated as the count of stained cell nuclei from the fluorescence image with the Hybrid Cell Count function of the BZ-X Analyzer software (Keyence). The average cell counts in an intact control group were 170.6 at 24 h and 272.1 at 48 h (n = 10). Two proliferation indices, the cell count ratio and the cell proliferation ratio, were calculated as follows: cell count ratio = cell count at 48 h/cell count at 24 h, and cell proliferation ratio = (cell count at 48 h - cell count at 24 h)/cell count at 24 h.

Although these indices are basically the same, the latter is more suitable for representing

the proliferation rate and the former is more useful for comparison with the migration index. These indices were normalized to the control to allow for comparisons among experiments.

Statistical analysis

Statistical significance of the difference between the experimental groups was examined by the Student *t* test at a probability level of 5%.