

1 **Prediction of broad chemical toxicities using**
2 **induced pluripotent stem cells and gene networks**
3 **by transfer learning from embryonic stem cell**
4 **data**

5

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1 **SUMMARY**

2 The assessment of toxic chemicals using animals has limited applicability to humans.
3 Moreover, from the perspective of animal protection, effective alternatives are also
4 desired. Previously, we developed a method that combines developmental toxicity
5 testing based on undifferentiated human embryonic stem (ES) cells (KhES-3) and gene
6 networks. We showed that $\geq 95\%$ accurate predictions could be achieved for
7 neurotoxins, genotoxic carcinogens, and non-genotoxic carcinogens. Here, we expanded
8 this method to predict broad toxicities and predicted the toxicity of 24 chemicals in six
9 categories (neurotoxins, cardiotoxins, hepatotoxins, nephrotoxins [glomerular
10 nephrotoxins/tubular nephrotoxins], and non-genotoxic carcinogens) and achieved high
11 prediction accuracy (AUC = 0.90–1.00) in all categories. Moreover, to develop a testing
12 system with fewer ethical issues, we screened for an induced pluripotent stem (iPS) cell
13 line on the basis of cytotoxic sensitivity and used this line to predict toxicity in the six
14 categories based on the gene networks of iPS cells using transfer learning from the ES
15 cell gene networks. We successfully predicted toxicities in four toxin categories
16 (neurotoxins, hepatotoxins, glomerular nephrotoxins, and non-genotoxic carcinogens) at
17 high accuracy (AUC = 0.82–0.99). These results demonstrate that the prediction of
18 chemical toxicity is possible even with iPS cells by transfer learning once a gene
19 expression database has been developed from an ES cell line. This method holds
20 promise for tailor-made safety evaluations using individual iPS cells.

1

2 **INTRODUCTION**

3 To date, chemical toxicity studies have been primarily conducted by *in vitro* testing in
4 cultured human cancer cell lines or in animals such as mouse, rat, and rabbit. However,
5 because these methods differ from testing in "normal" human cells, their applications
6 are limited¹. In addition, the use of animals has become a major issue from the
7 standpoint of animal welfare; in 2019, the U.S. Environmental Protection Agency
8 announced that research studies using mammals as well as funding for mammal studies
9 would be cut by 30% by 2025 and abolished by 2035².

10 The embryonic stem cell test (EST) reported by Scholz et al. was the first to
11 examine embryotoxicity *in vitro* using mouse fibroblasts, embryonic stem (ES) cells,
12 and cardiomyocytes differentiated from ES cells; such developmental toxicity testing
13 was previously performed only in animals^{3,4}. Later, this method was approved as a
14 scientifically valid alternative by the European Centre for the Validation of Alternative
15 Methods (ECVAM) (<https://tsar.jrc.ec.europa.eu/test-method/tm1999-01>). However, the
16 EST uses mouse cells, and species-specific differences must be clarified in order to use
17 this approach to evaluate toxicity in human. Subsequently, another research group
18 reported that in an EST based on a human cell system (hEST), changes in the
19 expression of homologous neurodevelopmental genes were similar to those observed in
20 the mouse system⁵.

1 In 2012, the U.S. Defense Advanced Research Projects Agency (DARPA) and
2 the NIH invested a huge sum on a national project to promote the development of
3 biomimetic systems, leading to rapid progress in the field⁶. These systems, which mimic
4 the (adult) human body and are constructed by filling tissues created in individual
5 compartments with culture fluid and connecting them together, are expected to be used
6 in human toxicity testing systems as an alternative to animals. Currently, however, very
7 little progress has been made in adapting these systems for application in developmental
8 toxicity testing, and no evaluation method has been established for determining how
9 accurately these systems mimic the function of the normal human body⁷. Realizing the
10 practical application of these systems as high-throughput toxicity screening tools is
11 likely to take several years.

12 For many years, the prediction of chemical toxicity has been carried out using
13 a method based on the physicochemical parameters of the chemicals, referred to as the
14 quantitative structure–activity relationship (QSAR)⁸. However, there is a limit to the
15 predictive ability of QSAR. One reason is that the mechanism that actually induces
16 toxic responses resides within the cell, so information about the chemical alone cannot
17 predict such responses. In this regard, it should be possible to detect toxicity more
18 accurately by obtaining information about the variation in gene expressions in cells, the
19 so-called 'hardware' that mediates responses to chemicals. In addition, as stem cells
20 differentiate, only the genes essential for that lineage is expressed and conserved

1 through DNA methylation⁹, whereas in pluripotent stem cells, a very large number of
2 genes are expressed, including transporters and transcription factors; consequently,
3 pluripotent cells are superior to differentiated cells as a tool for comprehensively
4 detecting toxic chemicals. In light of these considerations, we developed hEST-GN
5 (human embryonic stem cell test with gene networks), a prediction method that uses
6 information on feature gene networks based on massive gene expression datasets
7 obtained by exposing human ES cells to toxic chemicals as input data for machine
8 learning. Using this method, we achieved highly accurate predictions of developmental
9 toxicity categories¹⁰.

10 In this study, we expanded the hEST-GN and found that the prediction of 24
11 toxic chemicals in broad toxicity categories, including adult toxicity, can be achieved
12 with high accuracy. Furthermore, by selecting induced pluripotent stem (iPS) cells,
13 which can be used as an alternative to ES cells in toxicity testing, and using a gene
14 expression database created from ES cells, we were able to develop a method that can
15 predict chemical toxicity using iPS cell data via transfer learning; this approach
16 ameliorates the ethical issues related to ES cells. If this method could be further
17 improved, it is likely that it could contribute to the development of tailor-made,
18 individualized toxicity assessment/prevention using individual iPS cells, which would
19 have enormous clinical value.

20

1 **RESULTS**

2 **Development of an hEST-GN library for 24 chemicals and prediction using iPS**

3 A schematic of the chemical assay is shown in **Fig. 1a**. The human ES cell line KhES-3
4 was exposed to a total of 24 chemicals in six toxicity categories [neurotoxins,
5 hepatotoxins, cardiotoxins, two types of nephrotoxins (glomerular nephrotoxins and
6 tubular nephrotoxins), and non-genotoxic carcinogens] at six concentrations, including
7 vehicle (solvent) alone. The chemicals were carefully assigned to the toxicity categories
8 by referring to previous reports (**Table 1**). Gene expression data were obtained by
9 RNA-seq at two time points, 24 and 48 h, after exposure. At each time point, a principal
10 component analysis (PCA) was performed using transcription factor genes, and a total
11 of 20 genes from the top five PCs were extracted as feature genes. Using these genes,
12 gene network libraries were created for each of the 24 chemicals using the Graphical
13 Gaussian Model (GGM)¹¹. Similarly, the screened iPS cells were subjected to RT-qPCR
14 to obtain gene expression data for the same 20 genes and create gene network libraries.
15 Next, using ES cell library labels, a chemical toxicity prediction system trained by both
16 libraries from ES and iPS cells was developed via transfer learning¹² using support
17 vector machines (SVMs)¹³.

18

19 **Gene expression response database of ES cells for 24 chemicals**

1 To obtain the largest amount of data about the expression of genes that were perturbed
2 by the 24 chemicals, ES cells need to be exposed to chemicals at the maximum
3 concentration that does not cause an excessive degree of cell death. To this end, we first
4 performed ATP assays and then plotted regression curves to calculate inhibitory
5 concentrations (ICs) by carrying out serial dilutions of stock solutions containing the
6 maximum soluble concentrations of the 24 chemicals. ICs in the range of 0.1% to 50%,
7 at which cell death begins to be observed, were set as the maximum exposure
8 concentration (**Table S1, Fig. S1**). The estimated IC₅₀ and 95% confidence interval
9 (CI) for each chemical are shown in **Fig. 1b (Table S2)**. Serial dilutions were carried
10 out, with the maximum exposure concentration set as 1/1 to obtain 1/2, 1/4, 1/8, and
11 1/16 dilutions, and a six-step exposure including vehicle alone was performed and
12 repeated twice, yielding a total of $6 \times 2 = 12$ samples for each chemical. We collected
13 RNA 24 and 48 h after exposure to the 24 chemicals, performed transcriptome analysis,
14 and generated gene expression datasets for a total of $12 \times 24 \times 2 = 576$ samples.

15 To examine the characteristics of the 24 chemicals at the level of differentially
16 expressed genes (DEGs), we selected transcription factor–related genes (GO: 0006351)
17 from the 576 datasets (4,032 genes). After log-normalization, batch effect elimination,
18 and repeat merging, we generated DEG sets for which differences between each
19 exposure data and vehicle values were significant (FDR < 0.01 and $\log_2|\text{FC}| > 1$) and
20 presented them in a heatmap (**Fig. 1c, Fig. S2, S3**). According to this analysis, the

1 number of DEGs was higher at 48 h than at 24 h for all concentrations, and over time,
2 more genes were up- or down-regulated due to exposure to the chemicals. At both 24
3 and 48 h, valproic acid, a strong neurodevelopmental toxicant, elicited gene expression
4 patterns that were clearly distinct from those of the other chemicals. Similarly,
5 lithocholic acid, a mammalian bile acid and well-known carcinogen, yielded distinct
6 expression patterns.

7

8 **Construction of gene networks of the 24 chemicals by the Graphical Gaussian** 9 **Model (GGM)**

10 To obtain feature genes used in the prediction, we performed PCA on the basis of the
11 exposure data of each transcription factor gene, expressed as a log-fold-change (LFC)
12 in expression relative to vehicle after log-normalization and batch effect elimination.
13 There were 3,200 genes for the 24-h samples and 3,255 genes for the 48-h samples. For
14 both exposure times, it was difficult to clearly separate the chemicals by the toxicity
15 categories using two-dimensional PCA (**Fig. 1d**). Accordingly, we selected two genes
16 with maximum positive and negative loading values, which were considered to
17 contribute the most to the first to fifth PCs; at each time point, 20 genes were selected as
18 feature genes (**Table S3**). Among the 20 selected genes in each group, only *ACTR3*¹⁴,
19 which has been implicated in cell shape and motility, was common.

1 Using the 20 selected genes, we estimated sparse gene networks based on
2 GGMs using an L1 graphical lasso for each chemical at each time point (i.e., 24 and 48 h)
3 **(Fig. 2a, Fig. S4, Fig. S5)**. The figures illustrate the estimated 190 partial correlation
4 coefficients incorporated into the gene networks; edges with positive partial correlation
5 values between two genes are shown in green, and edges with negative values are shown
6 in red; the thickness, distance, and arrangement of the edges correspond to the degree of
7 correlation between the two genes. Because these genes were obtained from the top five
8 PCs that maximize the dispersion of the 24 chemicals using PCA, the estimated GGMs
9 that describe the networks of all 20 genes differed considerably among chemicals, and it
10 was difficult to classify the chemicals simply based on the network patterns as a whole.
11 Therefore, for actual predictions, we decomposed the networks into their constituent
12 edges rather than using them as a whole and used those with higher discriminative
13 potential for training data as features. In other words, the partial correlation coefficients
14 of the edges that are characteristic of the respective toxicity categories contributed to the
15 SVM discrimination.

16

17 **Prediction of six toxicity categories using KhES-3 cells and the GGM network**

18 Using the 190 partial correlation coefficients in the GGM as input data, we predicted the
19 toxicities of the 24 toxic chemicals in six categories using SVMs with
20 leave-one-out-cross-validation (LOOCV). For the predictions, we followed the

1 procedures described in a previous report on hEST-GN¹⁰ using four kernels (linear,
2 polynomial, RBF, and maximum entropy) and increased the number of top features
3 ranked by a t-test from 1 to 190. We also performed the prediction with the raw LFC
4 values of the 3,200 (24 h) and 3,255 (48 h) transcription factor genes at each of the five
5 concentrations relative to the vehicle-only expression. To compare the predictive
6 accuracy, the same number of input data used for the GGM (i.e., up to 190 genes) was
7 used as features. Predictions with the raw LFC values did not achieve a significantly
8 higher prediction performance than the mean predictions using 10 uniform random
9 numbers. On the other hand, in the prediction based on the GGM, the AUC values were \geq
10 0.90 for chemicals in all toxicity categories, and because the prediction accuracy or AUC
11 values were significantly high ($p < 0.05$), we concluded that prediction with high
12 performance is possible. Predictions were performed separately at 24 and 48 h, but
13 depending on the chemical, the time point at which a higher prediction accuracy could be
14 obtained differed; thus, neither time point was considered particularly superior in terms
15 of yielding a better prediction. Overall, these results demonstrated that hEST-GN based
16 on ES cell gene networks allows for the prediction of not only developmental toxicity but
17 also broad toxicity categories including adult toxicity.

18 In addition, to compare with predictions based on the QSAR theory, we
19 generated 5,666 molecular descriptors including 3D descriptors (**Table S4**) and
20 performed predictions using top 1 to 190 feature genes according to the aforementioned

1 method. None of the six categories, except for tubular nephrotoxin (accuracy of 91.7%),
2 gave a significantly high prediction result. The results of all predictions are presented
3 together in a table and as ROC curves (**Table 2, Fig. 2b, Fig. S6**). These results suggest
4 that chemical toxicity predictions that use the partial correlation coefficients of the GGM
5 as features can achieve significantly higher accuracy than predictions based on gene
6 expression values or QSAR. In the GGM-based prediction, the prediction accuracy for
7 each of the 24 chemicals was examined from the SVM results. This analysis revealed that
8 with respect to 16 chemicals (acetonylacetone, acrylamide, amitriptyline HCl,
9 atorvastatin, bucillamine, chlorpheniramine, chlorpromazine, digoxin, doxorubicin,
10 gentamicin, itraconazole, lithocholic acid, methapyrilene HCl, sunitinib, thioacetamide,
11 and valproic acid), the prediction accuracy was 100% for all six categories at 24 and 48 h.
12 On the other hand, for axitinib, cisplatin, and cyclosporin A, the prediction accuracy was
13 66.6%, suggesting that the prediction of these chemicals is difficult (**Table S5**).

14

15 **Pathway analysis of KhES-3 genes following exposure to 24 chemicals**

16 To determine the effects of exposure to chemicals on biological pathways, we performed
17 a Hallmark pathway analysis by Gene Set Enrichment Analysis (GSEA) using all genes.
18 When performing this analysis, we divided the samples into high-dose (1/1 and 1/2 doses)
19 and low-dose (1/8 and 1/16 doses). For hepatotoxins, cardiotoxins, and globular
20 nephrotoxins, differences were observed in the types of pathways that were induced or

1 suppressed in comparison with other toxic chemicals in the high-dose samples at 24 h
2 **(Fig. 2c, Fig. S7)**. In addition, the responses of ES cell genes to the toxic chemicals were
3 diverse and dependent on the type of chemical to which they were exposed and not
4 limited to specific pathways such as apoptosis. This observation suggests that it is
5 possible to predict toxicity categories on the basis of perturbed pathways that can be
6 detected by transcription factors. On the other hand, differences in concentrations or
7 among categories that may have been present at 48 h were not as pronounced as those at
8 24 h. However, analyses using available pathways based on human knowledge
9 accumulated in the past provide limited information. Instead, the computational
10 extraction of feature genes from the PCA of all genes without bias and predictions based
11 on their GGM networks are likely to be more effective.

12

13 **Selection of iPS cells as an alternative to human ES cells**

14 The results of the present and previous studies suggest that hEST-GN can predict not only
15 developmental toxicity but also broad toxicity categories with high performance.
16 However, there are still hurdles to overcome, including ethical issues, before this system
17 can be generally and widely accepted as a toxicity test. Accordingly, to make iPS cells a
18 possible alternative to hEST-GN, we performed pre-screening by comparing ATP assays
19 with ES cells exposed to 20 toxic chemicals across a wide range of categories. As
20 candidates, we used the top 20 cell lines selected from among Japanese male cell lines¹⁵

1 derived from healthy individuals, which had been examined and ranked in terms of their
2 differentiation potential into the three germ layers. For exposure concentration, we
3 adopted the IC50 that was determined using the KhES-3 cell line and examined the
4 toxicity response of human iPS cells. Among the candidate cell lines, we selected the top
5 three cell lines with well-correlated growth rates at IC50 (HPS4138, HPS4234, and
6 HPS4046) and confirmed the correlation coefficients of the growth rates at IC50 with
7 KhES-3 using 20 of the 24 toxic chemicals investigated in this study. HPS4138 had the
8 highest value of 0.94; accordingly, this cell line was used for the predictions as an
9 alternative to ES cells (**Table S6**).

10

11 **Prediction of chemicals in six toxicity categories using HPS4138 iPS cells**

12 For HPS4138, which was selected by screening, we performed ATP assays with the 24
13 chemicals (**Fig. S8, Table S7, Table S8**). As in the case of ES cells, the cells were
14 exposed to vehicle alone or five concentrations obtained by serial dilutions of stock
15 solutions containing the maximum exposure concentration (i.e., the maximum value
16 between IC0.1 and IC50 that did not cause an excessive degree of cell death); this
17 experiment was repeated twice. Gene expression data for 20 genes selected from KhES-3
18 cells at 24 and 48 h were obtained by qRT-PCR, and GGMs were created for each of the
19 24 chemicals based on LFC values relative to the vehicle. Partial correlation coefficients
20 were used for the prediction, as in the case of ES cells. In the prediction, data were created

1 by integrating iPS cell data with ES cell data as well as by transductive transfer learning,
2 in which toxicity category labels in the ES cell data were used for the learning to allow for
3 category prediction using iPS cells. Assessment was performed by LOOCV, similarly to
4 the predictions made using ES cells only. Chemicals in all categories except cardiotoxins
5 and tubular nephrotoxins yielded AUC values from 0.82 to 0.99, and the accuracy or
6 AUC was significantly higher than for results obtained with uniform random numbers.
7 Thus, although this approach was not perfect, the results of the prediction using HPS4138
8 were very accurate for most toxicity categories (**Table 3**). The summary of toxicity
9 category predictions for the 24 chemicals using HPS4138 are shown in **Fig. 3**. Prediction
10 was difficult for butylated HA, but satisfactory for the other chemicals (**Table S9**). These
11 results demonstrate that if a gene expression database for toxicity responses could be
12 created with ES cells, chemical toxicity prediction using iPS cells would also be possible
13 by means of transductive transfer learning. Our findings also raise the possibility of
14 achieving practical applications of toxicity testing using standardized or individualized
15 iPS cells in the future. A description of transductive transfer learning is available on our
16 web site at <https://hipst-gn.stemcellinformatics.org>.

17

18

1 **DISCUSSION**

2 Here we presented a proof-of-concept study that enables a toxicity hazard assessment
3 using human iPS cells and transfer learning based on the transcription factor gene
4 network libraries made from the gene expression data of human ES cells exposed to 24
5 chemicals for 6 categories.

6 In this paper, we clarified that i) human ES cells are sufficient to detect not only
7 developmental toxicities during embryogenesis but also broad toxicity categories such as
8 adult toxins (neurotoxin, cardiotoxin, hepatotoxin, and glomerular and tubular
9 nephrotoxins) and non-genotoxic carcinogens; ii) the chemical toxicity prediction using
10 transcription factor gene networks of human ES cells shows an AUC = 0.90–1.00, which
11 is significantly more accurate than predictions based on the QSAR theory or from raw
12 gene expression data; iii) there exist differences in the biological pathways affected by
13 the toxicity categories, suggesting the mechanisms that underlie the transcription factor
14 networks that control the pathways may be used to predict toxicity categories; and iv) the
15 gene network data from properly screened human iPS cells can successfully, although not
16 perfectly, predict the toxicity categories at significant accuracies once the toxicities are
17 learned by transfer learning using the models based on human ES cell data only.

18 Various alternative methods using pseudo-human systems, such as
19 differentiated human cell lines (HepG2, MCF-7, HeLa, etc.), have been reported¹⁶ since
20 animal protection has become a higher priority in research. However, these lines are often

1 derived from cancer or immortalized cells and thus have limited use¹⁷. On the other hand,
2 primary cells, which are assumed to resemble natural states in the human body, show
3 batch-to-batch variability¹⁷ and are difficult to collect at sufficient amounts. Furthermore,
4 it is difficult to extrapolate toxicity tests on some cell types to other target cell types due
5 to differences in cytotoxicity tolerance¹⁸. Performing a multi-target toxicity prediction
6 system based on stem cells, as we propose here, provides a more valid prediction of
7 toxicity to a larger range of cell types.

8 Toxicological assessment using the transcriptome is frequently used by the U.S.
9 EPA, Tox21 project and in Europe. Particularly, New Approach Methodologies (NAMs),
10 which are any technology, methodology, approach or combination thereof that can be
11 used to provide information on chemical hazards and risk assessments that avoids the use
12 of intact animals¹⁹, are often directional concepts using transcriptomics with other omics
13 or traditional toxicology methods. Our study indicated that transcription factor gene
14 networks exist in a master layer of biological pathways to activate molecular initiation
15 events (MIEs), in which the initial chemical trigger starts an adverse outcome pathway
16 (AOP) via DNA-binding, receptor activation, or a disturbance of cellular / organelle
17 systems²⁰, thus revealing toxicity reactions. Recent studies have endorsed the idea that
18 transcription factors of signal receptors might play a role in interfacing outside signals,
19 such as aryl hydrocarbon or androgen, to activate toxicological AOPs in HepaRGTM
20 cells²¹. In our system, we used 20 transcription factor genes from 5 PCs due to limited

1 resources and cost, but it should be possible to customize the set of genes and PCs to
2 reflect more accurately the specific AOPs in the endpoint organ. To pursue a full
3 coverage of endpoint organs and AOPs, RNA-seq analysis and a library of all 4,033
4 transcription factor genes for the test chemicals at low cost are needed.

5 Previous systems using QSAR theory depend on the information of chemicals
6 only and are thus inapplicable to mixtures such as food, Chinese or herbal medicines, and
7 other compounds to assess toxicity as a whole. Our hEST / hiPST-GN system detects the
8 cellular toxic events of these mixtures, providing the prediction of holistic cellular
9 reactions, making it a resource for industries that mix independent chemical ingredients,
10 including cosmetic, air-conditioner, and automotive companies who need to assess the
11 toxicity of mixtures in their final or intermediate products, media, emissions, detergents,
12 etc. In fact, more than 100 members from a wide variety of
13 industry-government-academia fields are involved in our non-profit consortium
14 (scChemRISC). By developing products from candidate substances that are predicted to
15 have little toxicity, our system will contribute to industry not only for efficiency but also
16 for human health.

17 Recently, toxicity reaction differences due to ethnicity, or genome haplotypes,
18 have been widely reported due to the globalization of foods and products among
19 countries. For example, catechin, which is contained in green tea and is widely consumed
20 in Asian countries, is reported to induce severe liver injury in the United States²². The

1 CiRA Foundation (Kyoto, Japan) has announced myiPS cells, a project in which
2 individuals can have their own iPS cells generated and banked. Our hiPST-GN system
3 could allow a tailor-made chemical toxicity assessment for these cells to detect individual
4 differences in toxic tolerance for different substances. Ideally, it also has the potential to
5 reduce medical accidents if myiPS cells could be used to diagnosis whether a medication
6 is toxic before receiving the treatment^{17,23}. In general, by performing a battery of toxicity
7 assessments using multiple iPS cell lines from individuals with various haplotypes, our
8 system may contribute to reducing toxicity accidents often caused by a small number of
9 test samples of limited genomic variances.

10 In conclusion, the largest advantage of our hEST / hiPST-GN is the ability to
11 perform toxicity hazard assessments for multiple endpoints with high accuracy in a short
12 amount of time and a low cost. We believe that our system will greatly benefit research
13 that will be affected by the lost funding for mammal studies designated to happen in 2035
14 by the U.S. EPA.

15

1 **FIGURE LEGENDS**

2 **Figure 1: Construction of a gene expression database for 24 chemicals**

3 (a) Schema of the chemical assay. hESC, human ES cells; hiPSC, human iPS cells. (b)
4 IC₅₀ for 24 chemicals. (c) Transcription factor genes differentially expressed following
5 exposure to 24 chemicals at 1/2 dose. (d) PCA of 24 chemicals in six toxicity categories at
6 two time points.

7

8 **Figure 2: Prediction of six toxicity categories using KhES-3 cells**

9 (a) Gene network representation of GGMs from KhES-3 cells. (b) ROC curves for the
10 prediction of chemicals in two toxicity categories (c) Pathway analysis for hepatotoxins
11 at 24 h and high-dose (1/1, 1/2 doses) samples.

12

13 **Figure 3: Summary of toxicity category prediction for 24 chemicals using HPS4138** 14 **cells**

15 Blue dots indicate predicted SVM values of iPS cell data, and filled and open markers
16 indicate true and false predictions, respectively. Black dots and bars indicate the means \pm
17 S.E.M. of SVM values for random data. In the tables, the label columns contain prior
18 knowledge regarding whether the chemical shows toxicity (P: positive) or not (N:
19 negative). The SVM column contains the SVM values of the iPS cell data. The expected
20 accuracy (Exp.Acc.) columns contain the prediction accuracy using random data. The

1 graphs below the tables show the probability distribution of the prediction accuracy using
2 random data. Black lines indicate the probability density estimated using the t distribution
3 (degrees of freedom = 9). Black shaded areas represent the upper 5%. Blue dashed lines
4 indicate the prediction accuracy using iPS cell data.
5

1 **EXPERIMENTAL PROCEDURES**

2 **Cell culture experiments**

3 The KhES-3 cell line was established at and provided by Kyoto University²⁴. The
4 protocol of this study was reviewed by the Ethics Committee of CiRA in accordance
5 with the "Guidelines for Derivation and Utilization of Human Embryonic Stem Cells"
6 by the Ministry of Education, Culture, Sports, Science and Technology, Japan. The iPS
7 cell lines were established from healthy Japanese donors at CiRA, Kyoto University,
8 and were approved for use by the Ethics Committee of Kyoto University.

9 Since it has been reported that the toxicity of antioxidants such as catechin is suppressed
10 in the presence of albumin²⁵, maintenance culture was carried out for all cell lines
11 including human ES cells using albumin-free Essential 8 Medium (Thermo Fisher
12 Scientific) in six-well feeder-free culture dishes coated with 5 µg/mL vitronectin
13 (VTN-N; Thermo Fisher Scientific). When seeding the cells, 10 µM CultureSure®
14 Y-27632 (FUJIFILM WAKO) was added, and medium exchange on day 1 and thereafter
15 was performed without Y-27632.

16

17 **Selection of toxic chemicals**

18 Twenty-four chemicals were selected and mainly included neurotoxins, hepatotoxins,
19 cardiotoxins, nephrotoxins, and non-genotoxic carcinogens (**Table 1**). The presence or
20 absence of toxicity was determined mainly on the basis of information regarding

1 toxicity and assorted disorders and diseases available at PubChem
2 (<https://pubchem.ncbi.nlm.nih.gov/>). With respect to neurotoxicity, among the
3 chemicals previously reported in the literature, those having only developmental
4 toxicity were classified as 'negative,' as the present study targeted adult toxicity. In
5 addition, when determining the presence or absence of hepatotoxicity, chemicals with
6 DILI rank ≥ 3 were considered hepatotoxic chemicals; with regard to others, those with
7 reliable reports of liver diseases were considered hepatotoxic. As for cardiotoxicity,
8 chemicals that have been reported to be associated with heart disease were considered
9 cardiotoxic. With regard to the kidney, due to its diverse and complex structure, the area
10 of damage was divided into two sites, namely, the glomeruli and renal tubules.
11 Chlorpheniramine and cyclopamine were the chemicals judged to be completely
12 'negative' and belonged to none of the toxicity categories examined in the present study.
13 On the other hand, 19 chemicals had multiple overlapping toxicities, whereas
14 acetonylacetone, bucillamine, and butylated HA had only one toxicity²⁶⁻⁷⁰.

15

16 **Chemical exposures and determination of IC logistic model equations**

17 DMSO or water was used as solvent (vehicle) for the 24 chemicals based on known
18 information (<https://pubchem.ncbi.nlm.nih.gov/>). For each of the 24 toxic chemicals, a
19 stock solution was prepared with the highest soluble concentration. First, in order to
20 perform the ATP assay to determine the exposure doses for testing, we performed 10

1 serial three-fold dilutions of the stock solution; the prepared exposure solution was
2 added to the cells (i.e., exposure) so that the concentration in the medium was 0.1% of
3 the exposure solution. The cells were cultured on 96-well black/clear flat bottom
4 TC-treated plates (Falcon), 8,000 cells were seeded, and the medium was exchanged on
5 day 1. The cells were exposed to chemicals on day 2. No medium exchange was
6 performed after exposure, and the ATP assay was performed 48 h after exposure. For
7 100 μ L of culture medium, 100 μ L of CellTiter-Glo[®] Luminescent Cell Viability Assay
8 (Promega Corporation) was added, and emitted light was measured with a 2104
9 EnVision Multilabel Plate Reader (PerkinElmer). From four luminescence
10 measurements for 10 concentrations and a blank as described above, regression analysis
11 was performed by fitting the three-parameter log-logistic model with the R-4.0.5 drc
12 package, and IC_{0.1} and IC₅₀ values were obtained (**Tables S1 and S2**). IC₅₀ values
13 were plotted using the ggplot2 package (**Fig. 1b**).

14

15 **RNA-seq analysis**

16 On day 2 after seeding, the cells were exposed to the chemicals. For each of the 24
17 chemicals, the exposure dose was set between IC_{0.1} and IC₅₀ depending on the degree
18 of cell death. Using this value as the maximum exposure dose, five serial two-fold
19 dilutions (1/1, 1/2, 1/4, 1/8, 1/16) were performed, and a total of six doses including a
20 solvent-only control (vehicle) were used. After exposure, no medium exchange was

1 performed, and samples were obtained at two time points (24 h and 48 h) with two
2 repeats, i.e., a total of $24 \times 6 \times 2 \times 2 = 576$ samples. After RNA purification using an
3 RNeasy Mini Kit (QIAGEN), sequence libraries were prepared for each sample using
4 TruSeq Stranded mRNA Library Prep/TruSeq RNA Single Indexes Set A & Set B
5 (Illumina, Inc.). For sequencing, high-throughput sequencing was performed using
6 HiSeq4000 (Illumina, Inc.). We used bowtie-2.2.5 with the option
7 "--very-sensitive-local" to map the obtained Illumina reads to Ensemble GRCh38r100
8 human cDNA and ncRNA sequences, added up the reads for each gene using MAPQ \geq
9 1 transcript, and obtained average counts of 28,652,809 and 28,291,682 reads for each
10 sample at 24 h and 48 h, respectively. From these, we selected only transcription
11 factor-related genes included in the Gene Ontology GO:0006351 using BioMart (4,032
12 genes). These genes were filtered using the statistical analysis language R-4.0.5 package
13 edgeR⁷¹ (<https://www.r-project.org/>) with the filterByExpr function min.count = 30,
14 min.total.count = 0, and then normalized to log₂ counts per million (logCPM) using the
15 voom function. Furthermore, the removeBatchEffect function was used to eliminate
16 batch effects.

17

18 **Differentially expressed gene analysis and principal component analysis**

19 At 24 h and 48 h, for a total of 122 groups including 120 conditions (five concentrations
20 each for 24 chemicals) and two solvent conditions (DMSO or water), we used a linear

1 model fitting with the lmFit function of the limma package⁷² in R and moderated
2 t-statistics with eBayes to analyze DEGs with respect to gene expression levels in terms
3 of the LFC between 24 chemicals and their corresponding solvent⁷³, and created a
4 heatmap of genes with an LFC > 1 and FDR (false discovery rate) < 0.01 using the
5 pheatmap package in R (**Fig. 1c, Fig. S2**). In addition, using the LFC values obtained
6 for 120 conditions, PCA was performed for each of the two time points (24 h, 48 h)
7 using the prcomp function in R (**Fig. 1d, Fig. S3**).

8

9 **Gene network construction by Graphical Gaussian Model (GGM)**

10 Based on results obtained in the aforementioned PCA, a total of 20 genes (two genes
11 each with the top positive and negative loading values in the first to fifth PCs) were
12 used to construct gene networks. To estimate the GGM for each of the 24 chemicals, we
13 used the aforementioned LFC values to calculate the sparse partial correlation
14 coefficient network with L1 graphical lasso using EBICglasso in the R package qgraph
15 (<https://cran.r-project.org/web/packages/qgraph/qgraph.pdf>)⁷⁴. For model fitting, regular
16 BIC with gamma = 0 was used, and regularization of sparsity was tried 1000 different
17 ways with nlambdas = 1000 for estimation. In the estimation, in order to avoid the
18 problem of covariance matrices failing to be positive definite, calculations were
19 performed with checkPD=FALSE (**Fig. 2a, Fig. S4, Fig. S5**).

20

1 **Prediction by support vector machine (SVM)**

2 The SVM program and protocol used in the present study were adopted according to the
3 report of Takahashi et al.⁷⁵ Four kernel functions were used: linear, polynomial, RBF,
4 and maximum entropy, and parameter types and combinations were calculated
5 according to the above report⁷⁵. In the calculation, 190 values of partial correlation
6 coefficients among 20 genes in the GGM for each of the 24 chemicals described above
7 were used as input data, and using LOOCV, the genes were ranked using the
8 two-sample t-test (two-sided) in each iteration, and the maximum accuracy and the
9 maximum AUC to achieve the maximum accuracy were recorded, varying the number
10 of values from 1 to 190. To statistically evaluate the maximum accuracy and AUC, 24 x
11 190 uniform random numbers were generated 10 times, and the maximum accuracy
12 with the maximum AUC were recorded in a similar fashion. One-sample t-test
13 (one-sided) was performed with the average values and standard deviations of
14 maximum GGM accuracies and AUCs obtained from these 10 attempts. For
15 comparison, LFC data for the transcription factor genes at five concentrations before
16 calculating the GGM (24 x 5 x 3,200 or 3,255 values in total) were used as input data,
17 and the maximum accuracy and the corresponding AUC value were recorded in a
18 similar manner. In addition, to compare with predictions based on QSAR, 5,666
19 molecular descriptors were created using alvaDesc (Affinity Science)
20 (<https://www.alvascience.com/alvades/>). We obtained information from PubChem DB

1 (<https://pubchem.ncbi.nlm.nih.gov/>) 3D Conformer data regarding 20 of the 24
2 chemicals; for the four other chemicals for which information could not be obtained
3 from PubChem DB (cisplatin, cyclosporin A, digoxin, and gentamicin), the SMILES
4 format was converted into 3D molecular descriptors using CORINA Classic
5 (https://www.mn-am.com/online_demos/corina_demo) and entered into alvaDesc.

6

7 **Gene set enrichment analysis**

8 We created LFC data for all genes by performing the same preprocess as for the
9 transcription factor genes (21,650 and 22,298 genes for 24 h and 48 h, respectively) and
10 divided them into high-dose (1/1/, 1/2) and low-dose (1/8, 1/16) groups to perform
11 GSEA for each group using the R package fgsea⁷⁶. As for the gene sets used, among the
12 MSigDB Collections provided by GSEA (<https://www.gsea-msigdb.org/gsea/index.jsp>),
13 we used 50 hallmark gene sets. The heatmap was generated with FDR-adjusted p values
14 obtained using the fgsea package.

15

16 **Selection of HPS4138 iPS cells**

17 The measurement of the differentiation potential to the three germ layers was performed
18 according to a previous report¹⁵, where we examined the expression ratio of two marker
19 genes for each layer (*PAX6*, *SOX2*, *BRA*, *NCAM*, *SOX17*, and *FOXA2*) by means of
20 fluorescence activated cell sorting. Among the ranked Japanese male cell lines derived

1 from healthy individuals, we used the top 20 cell lines according to their total ratios¹⁵.
2 These cell lines were kept in maintenance culture with StemFit AK02N medium
3 (Ajinomoto) and then cultured for two passages in maintenance culture using Essential
4 8 Medium (Thermo Fisher Scientific) as in the case of KhES-3. Among the 20 cell
5 lines, one underwent cell death, and the remaining 19 were subjected to pre-screening
6 using 20 chemicals with a wide range of toxicities (valproic acid, cyclopamine,
7 acrylamide, acetylacetone, chlorpromazine, chlorpheniramine, atorvastatin,
8 amiodarone, verapamil HCl, dimethoate, arsenic trioxide, quinidine, axitinib,
9 doxorubicin, gentamicin, ibuprofen, lithocholic acid, thioacetamide, butylated HA, and
10 methapyrilene HCl) by comparing the ATP assay results with those for ES cells. The
11 details of the ATP assay are described above. For exposure concentrations, the IC₅₀
12 determined with KhES-3 was used, and the growth rate of human iPS cells was
13 examined. Among the candidate cell lines, the top three cell lines (HPS4138, HPS4234,
14 and HPS4046) whose growth rates at IC₅₀ correlated well with that of KhES-3 were
15 selected, and again, the growth rate at IC₅₀ was confirmed by the ATP assay using 20 of
16 the 24 toxic chemicals examined in the present study (**Table S6**) to select the cell line
17 with the largest correlation coefficient, i.e., HPS4138.

18

19 **Gene expression data from HPS4138 by RT-qPCR**

1 The five serial exposure concentrations of the 24 chemicals for iPS cells were
2 determined by the ATP assay, as described above with respect to ES cells. For each of
3 the 20 genes at 24 h and 48 h used for the construction of the GGM for ES cells, the
4 primer sequence pair was designed using Primer 3 (version 0.4.0) based on the human
5 cDNA sequence data obtained from Ensembl GRCh38r100, and the obtained primer
6 sequence pair was synthesized (Hokkaido System Science). We confirmed whether the
7 target PCR product could be obtained from the primer sequence pair according to the
8 product size determined by electrophoresis. On day 2 after seeding, HPS4138 cells were
9 exposed to the 24 chemicals. With regard to exposure concentrations, time points, and
10 repeat experiments, we followed the experiments performed with ES cells. After
11 purification using an RNeasy Mini Kit, RNA was transcribed to cDNA using a
12 PrimeScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa), and the synthesized
13 primers for qRT-PCR and KAPA SYBR Fast qPCR Kit (KAPA BIOSYSTEMS) were
14 used to perform qRT-PCR with StepOnePlus (Applied Biosystems). ΔC_T values of the
15 resulting genes were obtained by subtracting from the C_T values the value of the internal
16 control (*GAPDH* gene). The average value of two repeated measurements at each
17 concentration was determined. In addition, for solvents (DMSO and water), ΔC_T values
18 were obtained by subtracting the value of *GAPDH* gene, and the average of all values
19 was determined. The difference, $\Delta\Delta C_T$, between the ΔC_T value at each concentration
20 and that of the solvent was determined and is referred to as LFC. Covariance matrices

1 among the 20 genes were calculated and used as input data in the construction of the
2 GGM.

3

4 **Transductive transfer learning by SVM**

5 A total of 380 edges in the GGM of ES cells and the GGM of iPS cells were used to
6 perform the prediction of chemicals in the six toxicity categories under conditions
7 similar to the SVM protocol described above. For learning, labels of the 24 chemicals in
8 ES cells were provided, whereas none of the labels of the 24 chemicals in iPS cells were
9 provided (i.e., zero); prediction was performed via transductive transfer learning. As in
10 the case of ES cells, prediction was also performed by replacing with uniform random
11 numbers 10 times only the values of the 24 x 190 input data for iPS cells, and similar to
12 ES cells, the one-sample t-test (one-sided) was used to assess the maximum accuracy
13 and the corresponding AUC value.

14

15 **DATA AVAILABILITY**

16 The source code of analysis program and the data supporting the findings of this study
17 are available from the corresponding author upon reasonable request. The RNA-seq
18 data reported in this paper have been deposited in NCBI's Gene Expression Omnibus
19 and are accessible through GEO series accession number GSE188203.

20

1 **SUPPLEMENTAL INFORMATION**

2 Supplemental Information includes eight extended data figures and nine supplemental
3 tables and can be found with this article online at:

4

5 **AUTHOR CONTRIBUTIONS**

6 WF conceptualized the research and led the project. JY drafted the manuscript. JY and
7 KK provided the stem cell data. JY, TW, WF, and JKY analyzed the data. HO, KI, MS,
8 TH, SS, HK, and HS selected the toxic chemicals. MO, and MKS provided the iPS cell
9 lines.

10

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20

1 **COMPETING INTERESTS**

2 None declared.

3

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Chemical Name	CAS RN	M.W	NT	HT	CT	GT	TT	NGC
Acetonylacetone								
Acrylamide	79-06-1	71.08						Group B2 https://www.epa.gov/iris
Amiodarone	1951-25-3	645.3						
AmitriptylineHCl	549-18-8	313.9						
Atorvastatin	134523-00-5	558.6						
Axitinib	319460-85-0	386.5						
Bucillamine	65002-17-7	223.3						
ButylatedHA	25013-16-5	180.24						Group 2B Precancerous Conditions [66]
Chlorpheniramine	132-22-9	274.79						
Chlorpromazine	50-53-3	318.9						
Cisplatin	14913-33-8	300						Group 2A http://ntp.niehs.nih.gov/npt/roc/e1eventh/profiles/s053cycl.pdf
Cyclopamine	4449-51-8	411.6						
CyclosporinA	59865-13-3	1202.6						Group 1 http://ntp.niehs.nih.gov/npt/roc/e1eventh/profiles/s053cycl.pdf Group 2B https://monographs.iarc.who.int/list-of-classifications
Digoxin	20830-75-5	780.9						Group 2A https://monographs.iarc.who.int/agents-classified-by-the-iarc/
Doxorubicin	23214-92-8	543.5						
Gentamicin	1403-66-3	477.6						
Ibuprofen	15687-27-1	206.28						
Itraconazole	84625-61-6	705.6						
LithocholicAcid	434-13-9	376.6						Precancerous Conditions [67] [68] Cystic fibrosis [69]
MethapyrileneHCl	135-23-9	297.8						Precancerous Conditions [70]
Sunitinib	557795-19-4	398.5						
Thioacetamide	62-55-5	75.14						Group 2B http://ntp.niehs.nih.gov/npt/roc/e1eventh/profiles/s172thio.pdf
ValproicAcid	99-66-1	144.21						
VerapamilHCl	152-11-4	491.1						

* Chemical names are given by referring to PubChem (2021.4.20 ver.).
Bold characters indicate positive toxicity.

NT, Neurotoxin; HT, Hepatotoxin; CT, Cardiotoxin; GT, Glomerular toxin (Renal toxin); TT, Tubular toxin (Renal toxin); NGC, Non-genotoxic carcinogen

Table 1. List of 24 chemicals

			NT (13)	HT (15)	CT (13)	GT (6)	TT (7)	NGC (9)
Random	24 h	Mean Accuracy (%)	85.8	83.7	83.3	87.5	87.0	87.0
		SD	8.13	6.92	6.81	6.21	6.65	5.38
		Mean AUC	0.86	0.82	0.85	0.88	0.86	0.87
		SD	0.09	0.11	0.09	0.07	0.11	0.06
	48 h	Mean Accuracy (%)	85.8	83.7	83.3	87.5	87.0	87.0
		SD	8.13	6.92	6.81	6.21	6.65	5.38
		Mean AUC	0.86	0.82	0.85	0.88	0.86	0.87
		SD	0.09	0.11	0.09	0.07	0.11	0.06

Molecular descriptors		Accuracy (%)	83.3	70.8	83.3	83.3	91.7	83.3
		AUC	0.83	0.76	0.90	0.82	0.86	0.60
RNA-seq log fold change	24 h	Accuracy (%)	75.0	75.0	79.2	79.2	75.0	66.7
		AUC	0.73	0.73	0.83	0.83	0.74	0.76
	48 h	Accuracy (%)	62.5	75.0	75.0	83.3	79.2	66.7
		AUC	0.71	0.73	0.78	0.66	0.61	0.79
GGM network coefficient	24 h	Accuracy (%)	83.3	91.7*	87.5	87.5	79.2	91.7
		AUC	0.73	0.99*	0.91	0.83	0.76	0.97*
	48 h	Accuracy (%)	87.5	83.3	91.7*	91.7	91.7	83.3
		AUC	0.93	0.79	0.9	1.00*	1.00*	0.80

p< 0.05

*p< 0.01

NT, Neurotoxin; HT, Hepatotoxin; CT, Cardiotoxin; GT, Glomerular toxin (Renal toxin); TT, Tubular toxin (Renal toxin); NGC, Non-genotoxic carcinogen

SD, Sample standard deviation; AUC, Area Under the ROC Curve; GGM, Graphical Gaussian Model

Table 2. Summary of prediction performance for KhES-3

			NT (13)	HT (15)	CT (13)	GT (6)	TT (7)	NGC (9)
Random	24 h	Mean Accuracy (%)	81.6	82.5	80.8	89.5	85.0	81.2
		SD	3.49	3.82	4.89	2.95	4.49	5.65
		Mean AUC	0.81	0.77	0.78	0.79	0.80	0.74
		SD	0.06	0.08	0.07	0.08	0.10	0.11
	48 h	Mean Accuracy (%)	81.6	82.0	80.4	88.7	86.2	81.2
		SD	5.27	4.82	5.21	2.03	4.43	4.04
		Mean AUC	0.77	0.81	0.8	0.84	0.77	0.77
		SD	0.09	0.05	0.07	0.10	0.13	0.08

GGM network coefficient	24 h	Accuracy (%)	87.5*	87.5*	79.2	87.5	79.2	91.7*
		AUC	0.90*	0.85*	0.81	0.84	0.71	0.82
	48 h	Accuracy (%)	91.7*	83.3	79.2	95.8*	83.3	83.3
		AUC	0.99*	0.85	0.75	0.94*	0.81	0.77

p< 0.05

*p< 0.01

NT, Neurotoxin; HT, Hepatotoxin; CT, Cardiotoxin; GT, Glomerular toxin (Renal toxin); TT, Tubular toxin (Renal toxin); NGC, Non-genotoxic carcinogen

SD, Sample standard deviation; AUC, Area Under the ROC Curve; GGM, Graphical Gaussian Model

Table 3. Prediction for HPS4138 cells

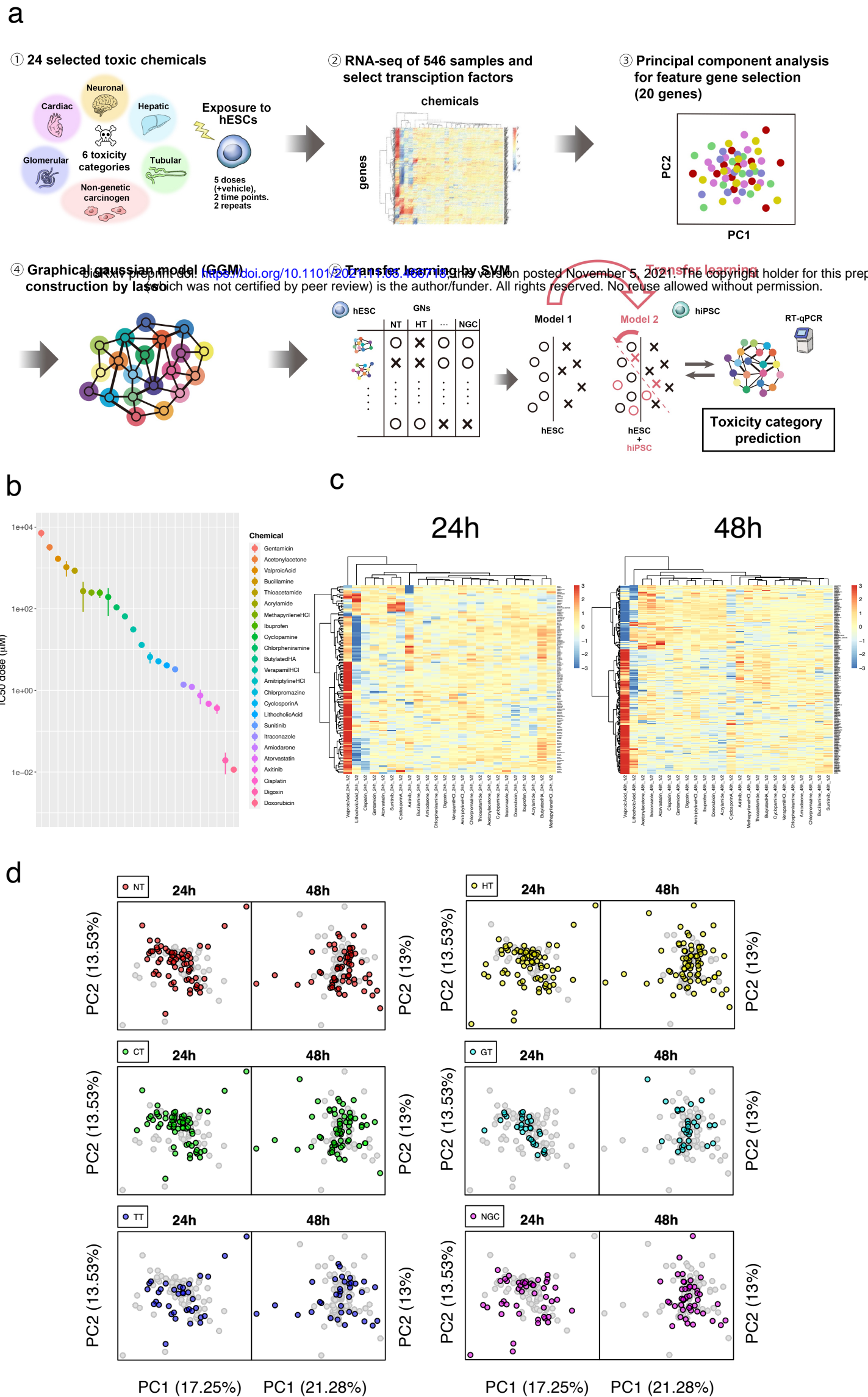
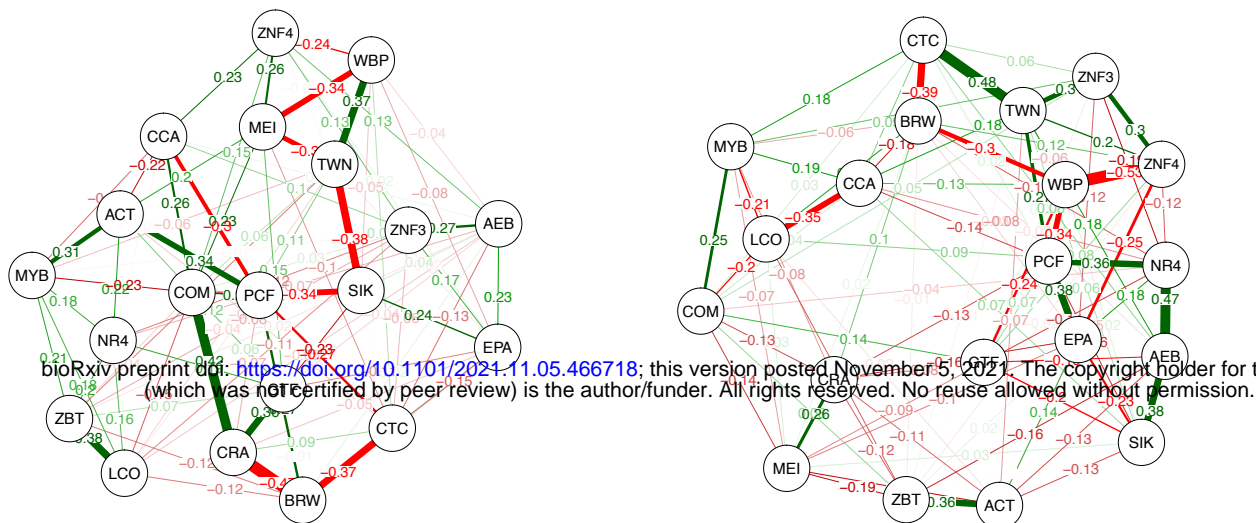


Fig. 1: Construction of a gene expression database for 24 chemicals

a 48h GGM

ValproicAcid

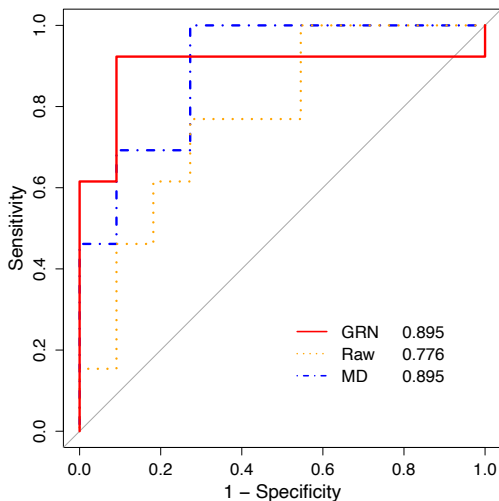
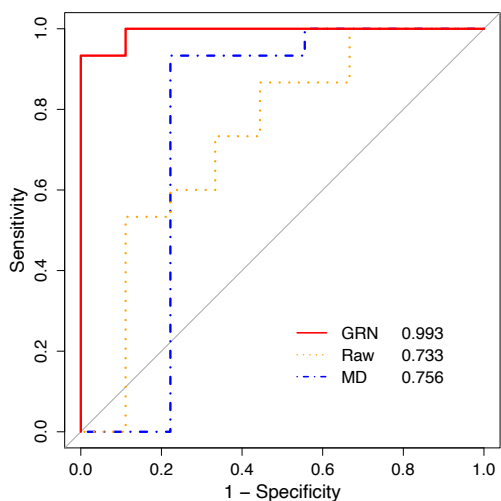
LithocholicAcid



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b HT 24h

CT 48h



c

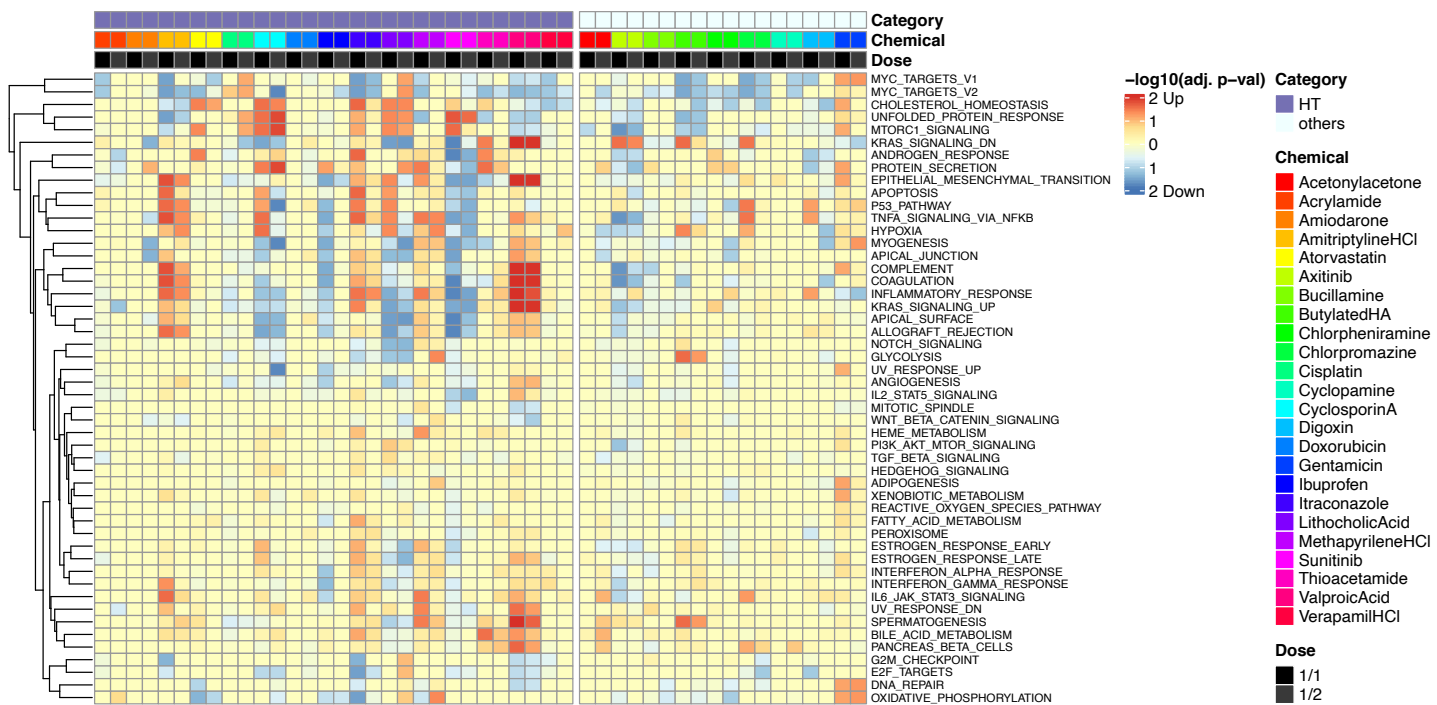


Fig. 2: Prediction of six toxicity categories using KhES-3 cells



Fig. 3: Summary of toxicity category prediction for 24 chemicals using HPS4138 cells