Cell Pluripotency and Its Effect on Sensitivity to Chemical Exposure

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Abstract

Pluripotent stem cells (PSCs) possess unique characteristics that distinguish them from other cell types. Among them, human embryonic stem (ES) cells are gaining attention as a powerful tool for human toxicity assessment without the use of experimental animals, and the embryonic stem cell test (EST) was introduced for this purpose. However, some human PSCs, particularly induced pluripotent stem (iPS) cells, have not been thoroughly investigated and compared with other cell types or cell states in terms of chemical sensitivity. Aiming to close this gap, we assessed and compared several human PSC lines for their reaction to chemical exposure. We report that iPS cells are more sensitive to chemical exposure than other cell types, including ES cells. Specifically, RIKEN-2A human iPS cells were 1.5 times on average more sensitive to chemical exposure than KhES-3 human ES cells, and both were several times more sensitive to chemical exposure than non-pluripotent cell types. In addition, we showed that iPS cells subjected to naïve-state induction procedures exhibited a sharp increase in chemical sensitivity. However, upon passage of these naïve-like cells in conventional PSC culture medium, their sensitivity to chemical exposure decreased. In conclusion, we revealed differences in sensitivity to chemical exposure among different types and states of PSCs and that naïve-state induction could increase this sensitivity.

Introduction

Pluripotent stem cells (PSCs) possess unique characteristics that distinguish them from other cell types. They are able to self-renew, differentiate into all three germ layers, and produce a wide variety of somatic cell types(1,2). Recent works in the stem cell field, however, have

uncovered the existence of a spectrum of pluripotency states. Recent works in the stem cell field have uncovered the existence of a spectrum of pluripotency states. Initially, in 2007, it was discovered that human embryonic stem cells (ESCs) in vitro closely resemble mouse epiblast stem cells (EpiSCs), possessing primed pluripotency that is different from the naïve pluripotency of mouse ESCs, which are derived from pre-implantation blastocysts. Thus, the notion of different pluripotency states was first put forward when mouse EpiSCs were established and compared with human PSCs(3,4). Subsequently, it was established that the main features that make primed human ES cells in vitro similar to mouse EpiSCs are as follows: 1) their reliance on fibroblast growth factor (FGF) and not on leukemia inhibitory factor (LIF) for proliferation(5), as the naïve ESCs do(6), 2) a flattened morphology compared with the dome-like, well-defined colonies of naïve PSCs(7), 3) little or no expression of specific naïve-state markers, such as KLF2, KLF4, KLF17, SUSD2 and others(8,9), 4) cytoplasmic localization of TFE3 as opposed to nuclear localization in naïve state(10,11), 5) recovered DNA methylation compared to hypomethylation seen in naïve PSCs(12). The rapid appearance of different methods to induce naïve pluripotency in human PSCs(7,13–27) and developments in RNA sequencing technologies have uncovered that different methods produce different “states” of pluripotency, with several “intermediate” states existing on the naïve-primed spectrum(28,29), putting forward the idea of a pluripotency spectrum.

Human induced pluripotent stem cells (iPSCs), which are artificially derived from somatic cell lineages by introduction of the four Yamanaka factors, OCT3/4, SOX2, KLF4, and c-MYC(30), have for a long time existed in the gray area, with their status as ESC equivalents hotly debated(31–33). In particular, iPSC cultures appear more heterogeneous than ESC cultures(30,34) and may require the selection of “best-quality” cells. In 2009, an EOS selection system(34,35) was introduced as a tool for selecting such cells. This system takes

advantage of the fact that naïve PSCs express Oct3/4 from the distal enhancer (as opposed to the proximal enhancer in primed cells(3,4,36,37)), and enables the selection of high-quality naïve-like iPS or ES cells by GFP expression or antibiotic selection. The EOS system has been widely used for selecting high-quality cells and confirming their naïve-like state(22,38–40).

PSCs are also recently gaining attention as a tool for investigating toxicity without having to conduct live animal experiments, and the in vitro (mouse) embryonic stem cell test (EST) was developed specifically for this purpose(41,42). Shortly thereafter, the human EST (hEST) was developed, and recent reports showed its successful application to toxicity prediction(43,44). It has been suggested that ESCs show higher sensitivity to chemical exposure in viability assays than differentiated cells(45,46). However, the mechanisms of toxicity of different compounds to human ESCs have not been investigated in detail, and it is poorly understood how human ESCs compare with other cell types or cell states in terms of toxicological performance. Moreover, the use of human ESCs faces important ethical issues, and the replacement of human EST with an analogous iPS cell test is highly desired. Such human iPS cell test would not only allow screening for the general toxicity of many chemicals, but also enable the investigation of their effects on specific diseases, because iPSCs can be obtained from patients. To develop such tests, it is necessary to study iPSCs and their responses (sensitivity) to different chemicals. Nevertheless, reports directly comparing human ESCs and iPSCs of different qualities in terms of their reactions to toxicological tests are lacking.

In this work, we investigated the response of human PSCs to chemical exposure. The goals were to 1) compare the chemical sensitivity of ESCs with that of non-pluripotent cell types, 2) compare human ESCs and human iPSCs in terms of chemical sensitivity, and 3) determine

whether naïve-state induction methods, such as EOS selection and the application of naïve-state induction media, affect the chemical sensitivity of PSCs.

Results

Selection of chemicals and their initial analysis

As the first step of our analysis, we chose eight chemicals of known hepatotoxicity from the Drug Induced Liver Injury Rank (DILIrank) public database(47). This database is available at the US Food and Drug Administration (FDA) website (https://www.fda.gov/science-research/liver-toxicity-knowledge-base-ltkb/drug-induced-liver-injury-rank-dilirank-dataset) and contains 1,036 FDA-approved drugs. The drugs in the database are ranked according to their potential to cause drug-induced liver injury (DILI) based on the analysis of hepatotoxicity descriptions from FDA-approved drug labeling documents and available medical literature. All compounds in the database are divided into four classes: No-DILI-concern class (hereafter referred to as class I), vLess-DILI-concern (hereafter referred to as class II), vMost-DILI-concern (hereafter referred to as class III), and Ambiguous-DILI-concern (where the exact cause of liver injury could not be ascertained). In addition to vDILI-concern class, each chemical is assigned a DILI severity value ranging from 0 to 8.

We chose chemicals that represent classes I-III and various DILI severities for our experiments (Table 1). From class I, we chose chlorpheniramine, which has a DILI severity of 0 (no documented liver toxicity). From class II, we chose aspirin (DILI severity 0 but has some potential for liver damage, such as elevated alanine aminotransferase upon prolonged high-dose usage and signs of hepatic injury(48–53)), chlorpromazine (DILI severity 2 and “adverse reactions” label), clotrimazole (DILI severity 3 and “adverse reactions” label), and ibuprofen (DILI severity 3 and “warnings and precautions” label). From class III, we chose

126 atorvastatin (DILI severity 5 and "warnings and precautions" label), cyclosporin A (DILI
127 severity 7 and "warnings and precautions" label), and amiodarone (DILI severity 8 and "box
128 warning" label). No drugs from class IV (Ambiguous-DILI-concern) were used in this study.
129
130 Table 1. Eight chemicals with known hepatotoxicity effects ranked by their DILI score, DILI
131 severity, and their FDA labels.

	Compound name	Pubchem ID	vDILI-concern	DILI severity	Label section
1	Chlorpheniramine	2725	Class I	0	No match
2	Aspirin	8343	Class II	0	No match
3	Chlorpromazine	2726	Class II	2	Adverse reactions
4	Clotrimazole	2812	Class II	3	Adverse reactions
5	Ibuprofen	3672	Class II	3	Warnings and precautions
6	Atorvastatin	60823	Class III	5	Warnings and precautions
7	Cyclosporin A	5284373	Class III	7	Warnings and precautions
8	Amiodarone	2157	Class III	8	Box warning

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133 Next, to obtain information on cell responses to the eight chemicals *in vitro*, we searched the
134 literature for dose-response studies of the said chemicals in human or animal-derived cells
135 (Table 2). We chose studies that contained dose-response curves for the derivation of IC50
136 and IC10 values. In addition, to ensure comparability, we chose studies that evaluated the

effects of the chemicals specifically after 24 hours exposure (except aspirin and ibuprofen, for which we could not find dose-response studies with 24-hour exposure data). Then, we ranked the chemicals by their effect on cell viability according to the available data (IC50 and IC10 scores). This analysis was carried out to compare the effects on human ESCs in the next step.

Table 2. Eight chemicals ranked by their effect on cell viability according to literature, where the most effective chemical is ranked 8, and the least effective is ranked 1.

Rank in vitro	Chemical name	IC50, μM	IC10, μM	Cell line	Exposure time	Assay type	PMID
1	Aspirin	>10000	8000	HT-29 (colon cancer)	72h	MTT	10496355
2	Ibuprofen	5000	2500	HepaRG (human liver)	8h	visual	30264435
3	Chlorphenirамine	800	285	MDCK (dog kidney)	24h	CCK-8	30459739
4	Chlorpromazine	80	50	HepaRG (human liver)	24h	visual	30841456
5	Clotrimazole	80	20	MCF7 (breast cancer)	24h	BrdU	22347377
6	Amiodarone	70	15	HepG2 (liver cancer)	24h	ATP (CellTiterGlo)	23135547
7	Atorvastatin	60	10	SW620 (prostate	24h	MTT	25672364

				cancer)			
8	Cyclosporin A	30	1.2	fibroblasts, hepatocytes	24h	MTT	9209689, 25541063

Sensitivity of human ESCs to selected chemicals

Next, we applied the said chemicals to human ESC line KhES-3 and obtained IC10 values as described in Materials and Methods. Figure 1 shows the results of the cell viability analysis at different doses (the exact doses can be found in Sup. Table 1). Table 3 includes the obtained IC10 values in KhES-3 cell line, the ranks of the chemicals in KhES-3 cell line, the *in vitro* ranks obtained from previous analyses (Table 2), the DILI severity scores, and the vDILI-concern classes. The results reveal a general tendency for higher ranks of all categories to correspond, and the correlation coefficient for the *in vitro* results obtained by the literature search and our KhES-3 experiments was 0.9. The biggest difference was observed between *in vitro* and *in vivo* categories (*in vitro* ranks obtained in cell lines versus DILI severity scores and vDILI-concern classes, the data for which were obtained from findings in human organisms). Interestingly, for all eight chemicals we analyzed, the IC10 values were lower in ESCs than in differentiated cells, in many cases significantly lower. The biggest difference was observed for ibuprofen and chlorpheniramine, where ESCs were 7 and 8 times more sensitive to the chemicals than differentiated cells. There was no chemical that produced a less sensitive response in any of the cell types relative to the response in ESCs, and in all cases, ESCs were more sensitive.

Fig. 1. ATP assay results for KhES-3 cell line and eight chosen chemicals.

(1A) The results for amiodarone, aspirin, atorvastatin, and clotrimazole are shown. None of the four chemicals affected the viability of the cells relative to control at doses lower than Dose 3. For amiodarone, atorvastatin, and clotrimazole, the cell viability graph crosses the IC10 threshold (black) approximately at Dose 3, whereas for aspirin, it crosses the threshold at a point slightly higher than Dose 2. (1B) The results for chlorpheniramine, cyclosporin A, ibuprofen, and chlorpromazine are shown. None of the four chemicals affected the viability of the cells relative to control at doses lower than Dose 5. For chlorpheniramine and ibuprofen, the cell viability graph crosses the IC10 threshold (black) at Dose 4, whereas for chlorpromazine and cyclosporin A, it crosses the threshold between Doses 4 and 5. The slightly low value at Dose 9 is thought to be an experimental artefact, because it is present and the same for all four chemicals.

Table 3. Eight chemicals and their IC10 values in KhES-3 cell line as determined by the ATP assay, their ranks in KhES-3 cell line, their ranks in hepatocytes or other cell lines *in vitro*, their DILI severity scores, and their vDILI-concern classes. Correlation coefficients between ranks in KhES3 line and that from literature, and between KhES3 rank and DILI severity, are shown in the last row.

Chemical	IC 10 in KhES-3, μM	Rank in KhES-3	IC10 from literature, μM	Rank in vitro	DILI severity	vDILI concern
Aspirin	4000	1	8000	1	0	Class II
Ibuprofen	360	2	2500	2	3	Class II
Chlorpheniramine	36	3	285	3	0	Class I

Amiodarone	11	4	15	6	8	Class III
Clotrimazole	7	5	20	5	3	Class II
Chlorpromazine	3.5	6	50	4	2	Class II
Atorvastatin	1.8	7	10	7	5	Class III
Cyclosporin A	0.6	8	1.2	8	7	Class III
Correlation with rank in KhES-3				**0.9**	**0.6**	

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186 **Comparison of human ESCs and iPSCs by their sensitivity to chemical exposure**

187 Our next goal was to compare the toxicity response of human iPSCs and human ESCs. For
188 this purpose, we used the human iPS cell line RIKEN-2A and compared its response with
189 that of KhES-3 cell line. Figure 2 shows the ATP assay results for RIKEN-2A, and Table 4
190 shows the comparison of doses corresponding to IC10. We chose four chemicals as follows:
191 aspirin as the least potent chemical, atorvastatin as the most potent chemical (we did not
192 choose cyclosporin A because there are multiple reports that claim it generates an abnormal
193 dose- response curve due to the stimulation of cell proliferation at low doses), and two
194 chemicals in between (amiodarone and clotrimazole). The results demonstrate that the iPS
195 cell line was slightly more sensitive to chemical exposure than the ES cell line, as determined
196 by the ATP assay. The overall difference in doses that yielded IC10 did not exceed 2.5 times
197 (for the most potent chemical, atorvastatin), and the IC10 dose for amiodarone was the same.
198 There were no chemicals that yielded higher sensitivity in ES cells than in iPS cells, and in
199 all cases, the iPS cells were more sensitive.

200

Fig. 2. ATP assay results for RIKEN-2A human iPS cell line and four chosen chemicals. The results for amiodarone, aspirin, atorvastatin, and clotrimazole are shown. None of the four chemicals affected the viability of the cells relative to control at doses lower than Dose 3. For amiodarone, atorvastatin, and clotrimazole, the cell viability graph crosses the IC10 threshold (black) approximately at Dose 3, whereas for aspirin, it crosses the threshold at a point slightly higher than Dose 2.

Table 4. Four selected chemicals and their IC10 values in two human cell lines, ES cell line KhES-3 and iPS cell line RIKEN-2A, as determined by the ATP assay.

Chemical	IC10 in ES	IC10 in iPS	Difference
Aspirin	4000μM	2570 μM	1.5 times
Amiodarone	11μM	11 μM	0
Clotrimazole	7μM	3.5 μM	2 times
Atorvastatin	1.8μM	0.73 μM	2.5 times

Establishment of EOS stable cell line with "high-quality" iPS cells

Next, to manipulate the degree of pluripotency of the cells (i.e., the quality of iPS cells as described in (34,35)), we exploited the EOS system. To obtain a more homogeneous and more naïve-like population of cells, we introduced a *piggyBac* based EOS-C(3+)-GFP/puroR vector (PB-EOS) as previously described (22,35). The expression of this reporter is driven by mouse regulatory elements that are active in undifferentiated ESCs: a trimer of the CR4 element from the Oct3/4 (Pou5f1) distal enhancer and the early transposon (ETn) long terminal repeat promoter. The PB-EOS reporter was shown to be upregulated during naïve-state induction and to maintain visible expression in naïve-like cells (22). We established a

stable cell line, RIKEN-2A-EOS, that is resistant to puromycin (see Materials and Methods). Upon introduction of the vector, we found that the resultant line had different populations of GFP-expressing cells, namely, “bright” GFP cells and “dim” GFP cells. Because we observed this phenomenon in all four cell lines we tested, namely, KhES-1 human ES cells, KhES-3 human ES cells, RIKEN-1A human iPS cells, and RIKEN-2A human iPS cells, we concluded it to be an intrinsic property of EOS in PSC lines (Sup. Fig. 1). Because the expression of puromycin is coupled to GFP by the IRES insert in the EOS vector, all puromycin-resistant cells, by definition, express GFP with the Oct4 distal enhancer driving the expression. Therefore, GFP expression and puromycin resistance are indicators of the naïve-like state, and all puromycin-resistant cells should possess certain qualities of naïve cells. The heterogeneity of GFP expression and the differences between “bright” and “dim” populations need further investigation to clarify the exact differences between them, and are outside the scope of this study.

Application of naïve-state induction methods and effect on chemical sensitivity

To further advance the cells into the naïve state, we tried different methods of naïve-state induction (Sup. Fig 2 and Sup. Table 2). After trying different conditions, we found the highest percentage of round colonies with the highest increase in naïve-state markers upon applying a modified version of the YAP method. Round colonies obtained from RIKEN-2A-EOS-YAP cells (RIKEN-2A-EOS cells following YAP treatment) were positive for naïve-state markers SUSD2 and KLF17, and GFP expression was brighter in these colonies than in RIKEN-2A-EOS cells (Sup. Fig. 3). We next used the ATP assay to investigate differences in sensitivity to chemical exposure in RIKEN-2A-EOS-YAP cells and their progeny obtained after several passages in non-YAP conventional medium (AK02N). We performed the ATP assay on day 0 (RIKEN-2A-EOS-YAP immediately after establishment in YAP medium) and

passage days 2, 5, and 10 (RIKEN-2A-EOS-YAP-P2, -P5, and -P10, respectively) in AK02N medium. The results of the ATP assay indicate that the sensitivity of RIKEN-2A-EOS-YAP cells to chemical exposure drastically increased compared with that of control cells of the same line treated with the vehicle (DMSO), and IC10 could not be determined because all doses produced a sharp drop in cell viability relative to control cells. Interestingly, after several passages, the sensitivity gradually decreased (Fig. 3 and Table 5). We thus concluded that YAP treatment of EOS-selected cells produced a sharp increase in sensitivity of the cells to chemical exposure, which diminished upon returning the cells into the conventional AK02N medium and passaging them for several days.

Fig. 3. Changes in sensitivity to chemical exposure in RIKEN-2A-EOS-YAP cell line with passaging.

The following four chemicals were used to assess the viability changes: aspirin, amiodarone, atorvastatin, and clotrimazole. The black line indicates the IC10 threshold. The highest dose is Dose 1, and the lowest dose is Dose 8. Upper left: RIKEN-2A-EOS-YAP cells subjected to the EOS selection and subsequent modified YAP naïve-state induction method show significantly decreased viability relative to control for all four chemicals, and the viability values were below the IC10 threshold for all doses. After 2 passages (RIKEN-2A-EOS-YAP-P2), the viability was improved, with the chemicals not affecting the cells until Dose 5. After 5 passages (RIKEN-2A-EOS-YAP-P5), the viability values further improved, showing the resistance of the cells to the chemicals until Dose 3–4. After 10 passages (RIKEN-2A-EOS-YAP-P10), the cells became significantly less sensitive, showing resistance until Dose 3 (highest).

Table 5. Four selected chemicals and their IC10 values in human iPS cell line RIKEN-2A subjected to EOS selection, YAP treatment, and passaging in conventional AK02N medium, as determined by the ATP assay.

Chemical	RIKEN-2A-EOS-YAP	RIKEN-2A-EOS-YAP P2	RIKEN-2A-EOS-YAP P5	RIKEN-2A-EOS-YAP P10
Aspirin	<3μM	240 μM	730 μM	1100 μM
Amiodarone	<0.03μM	11 μM	3.6 μM	3.6 μM
Clotrimazole	<0.02μM	0.7 μM	5 μM	1.6 μM
Atorvastatin	<0.003μM	0.24 μM	0.36 μM	1.1 μM

Materials and methods

Chemicals

The following chemicals were used for toxicity assessment in this study (Table 1): amiodarone (TCI, A2530-1G), atorvastatin (TCI, A2476-1G), clotrimazole (Wako, 035-16021), aspirin (Wako, 015-10262), cyclosporin A (Wako, 031-24931), chlorpheniramine (Wako, 030-13271), chlorpromazine (Wako, 033-10581), and ibuprofen (Wako, 098-02641). All chemicals were diluted in DMSO to the desired concentrations.

Cell culture

ESCs and iPSCs were routinely cultured in StemFit AK02N (Ajinomoto) with the addition of 10 μM ROCK inhibitor Y-27632 (CultureSure, Wako 030-24023) and 1 μL/mL iMatrix-511 (Nippi, 892014). Routine splitting was performed by washing with PBS (room temperature), adding and subsequently removing TrypLE Express Enzyme (ThermoFisher, 12604013),

gathering the cells in AK02N medium through the addition of 10 μM Y-27632, centrifuging at 500 rpm for 5 min, aspirating the medium, resuspending the cells in new medium, and plating at the required density. All routine cultures were performed in 6-well plates (Greiner, 657160) at 37 °C and 5% CO2, and splitting was performed at 80% confluency.

ATP assay and IC10 assignment

The ATP assay was used to assess and compare the sensitivity of cell lines to chemical exposure. We define cell sensitivity as the decrease in viability of the cells relative to control samples (vehicle-treated cells in the same conditions). We used IC10 to compare the sensitivity, where IC10 is defined as the point at which the ATP-assay curve crosses the threshold set at 90% viability of the control sample. For the ATP assay, cells were seeded at a final concentration of 8,000 cells/well onto Falcon® 96-well black clear bottom flat-bottomed plates (353219) in total volume of 100 μL medium per well. The cells were then incubated for 24 hours at 37°C and 5% CO2. The next day, the medium was changed to new medium containing different doses of the chemicals under investigation. All chemicals used in this study were diluted with DMSO (vehicle), and the final dose of DMSO in each well was always 0.1% (including control sample). After exposure to the chemicals, the cells were cultured for 24 hours at 37°C and 5% CO2, and the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7571) was used for the ATP reading according to the manufacturer's instructions. The samples were subjected to plate reading using the Perkin Elmer EnVision 2104 plate reader.

EOS introduction for EOS-based cell selection

The (PB) EOS-C(3+)-GFP/puroR vector was a kind gift from Professor Akitsu Hotta (CiRA, Kyoto University). To introduce the vector, the cells were cultured to 80% confluency and

transfected using QIAGEN Effectene reagent (Cat. no. 301425) following the manufacturers instructions. The next day after the transfection, 0.5 μg/mL puromycin (Sigma, P9620-10ML) was added to the cells. After 5 hours, the medium was changed to 1 μg/mL puromycin medium, and dead cells were aspirated. The cells were left overnight, and the next day, the medium was changed again with aspiration of the dead cells. As a result, 2–3 colonies consisting of 5–7 cells remained, and these were subsequently expanded and their puromycin resistance was confirmed by applying 5 μg/mL puromycin to the culture medium.

Naïve state induction methods

We surveyed the literature for the most recent and well-established methods for naïve state induction, leading us to chose four: 5i/LAF (54), t2iLGoY (22), YAP (21), and XAV939-based method (23). We assembled the required media (red boxes in Sup. Fig. 2) and added the required additives (such as Y27632) in the quantities recommended in the original articles at designated times during the culture. The cells were cultured in 6-well plates at 37°C and 5% CO2, as instructed by the respective publications. However, we did not use feeder cells (contrary to the publications). The cells were assessed for shape change and stained for the presence of alkaline phosphatase (pluripotency marker) after a time period when the naïve-state induction should be complete according to the respective publications.

Modified YAP method

For the development of the modified YAP method, we first surveyed the literature to find out the effect of each of the additives. The t2iLGoY method was excluded, because it requires transfection and the overexpression of Nanog gene. The 5i/LAF method and the XAV939 method were excluded, because they require the presence of FGF, which is known to induce

differentiation in naïve mouse ESCs, leading them to transition from the naïve state to the primed state(55,56). Thus, we chose the YAP method as the basis for the culture method. We subsequently excluded LIF from the recipe because it affects the JAK/STAT signaling pathway, which is important for mouse ESCs when they are in diapause(57), and human embryos lack the diapause stage. CHIR99021 was excluded, because it drives the beta-catenin signaling pathway, which leads to the fibroblast phenotype in our cell lines (data not shown). Forskolin was excluded to avoid fluctuations in intracellular cyclic AMP. As a result, an original recipe was developed, which was based on YAP activator lysophosphatidic acid (LPA, Cayman Chemical, CAY-62215-1MG) and included standard ROCK inhibitor Y27632 and MEK inhibitor PD0325901 (Wako, 162-25291). This medium was applied to stable EOS cell lines to yield round colonies that were transferred and cultured in AK02N medium for further passaging.

Immunostaining

The immunofluorescence analysis was performed as follows. Round colonies of EOS-selected, YAP-treated cells were placed on 30-mm glass bottom dishes (MATSUNAMI, D11530H) and grown for 24 hours in YAP medium. On the day of immunostaining, the colonies were washed with PBS, fixed with 4% PFA (Wako, 163-20145) for 15 min at room temperature, and permeabilized with 0.5% Triton (Sigma-Aldrich, X100-5ML) in PBS with 10% FBS addition for 30 min. The following antibodies were applied: Primary anti-SUSD2 (Sigma, HPA004117-100UL) and anti-KLF17 (Sigma, HPA024629-100UL) in PBS with 10% FBS addition for 1 hour at room temperature. After 1 hour, the cells were washed with PBS twice and incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594) (Abcam, ab150080, 1/500 dilution) in PBS with 10% FBS addition for 1 hour at room temperature. Then the cells were washed 4 times with PBS, and 2 mL PBS per dish was added for

imaging. Imaging was performed on a KEYENCE BZ-9000 BIOREVO fluorescence microscope.

Alkaline phosphatase staining and imaging

For the alkaline phosphatase staining, the cells were briefly washed with PBS, fixed for 5 min with 4% PFA at room temperature, and stained with the Alkaline Phosphatase Staining Kit II (Stemgent, #00-0055) according to the manufacturer's protocol. Imaging was carried out using an Olympus CKX41 inverted microscope.

Discussion

Human PSCs are an invaluable tool for basic research, clinical applications, and toxicological studies. Nevertheless, the toxicological properties of undifferentiated human PSCs have not been properly investigated to date, and information on the chemical sensitivity of different cell lines in different states of pluripotency is lacking. In this work, we investigated the chemical sensitivity of human ESCs, iPSCs, and iPSCs cultured under EOS selection and naïve-state induction conditions. At the first stage of our work, we carefully selected eight chemicals having various degrees of toxicity in humans (specifically, the liver) and included all DILIrank categories of toxicity in our investigation. Then, we exposed the human ESC line KhES-3 to these chemicals and compared the response by rankings and, where possible, by known IC10 dose values. As expected, the response of ESCs to chemical exposure was more profound than that of other cell types. On average, ESCs were 5 times more sensitive to the exposure. Our *in vitro* results correlated well with the *in vitro* results obtained using other cell lines in the literature, with the ranks of chemicals correlating with a coefficient of 0.9. On

388 the other hand, the *in vivo* results did not produce a good correlation. Next, we compared
389 human iPSC line RIKEN-2A cultured in the same conditions as KhES-3, with KhES-3 cells.
390 The IC10 doses of three chemicals, atorvastatin, aspirin, and clotrimazole, were slightly
391 lower (1.5 times lower, on average) in iPSCs compared with ESCs, whereas the IC10 dose of
392 amiodarone was the same in the two cell lines. We thus concluded that RIKEN-2A is more
393 sensitive to chemical exposure than KhES-3.
394
395 We then exploited the EOS vector to select naïve-like cells, because EOS allows us to select
396 cells that express Oct3/4 from the distal enhancer, a feature of naïve cells. We established
397 four stable cell lines, confirmed their resistance to puromycin, and investigated the
398 expression of GFP in them. We found that all cell lines expressed GFP in different quantities.
399 Specifically, the population of each line was separated into two groups, “bright” cells and
400 “dim” cells, of which dim cells were the majority (80–90%).
401
402 One explanation for the presence of bright and dim cell populations in EOS cell lines is that
403 the bright cells are in a more naïve-like state, whereas the dim cells are in a less naïve state.
404 An alternative explanation is that the EOS vector was integrated in larger quantities into
405 some cells (20%) and in smaller quantities into the other 80%. However, we think that this
406 explanation is less likely because all four cell lines exhibited only two easily distinguishable
407 populations and did not have a spectrum of GFP expression, as would be expected if the
408 integration of the vector occurred in a stochastic manner. The fact that all four cell lines
409 exhibited this pattern (80% vs. 20%) also suggests that the EOS expression reflects the
410 strength of the Oct3/4 expression from the distal enhancer and not the quantity of the
411 integrated vector. Thus, we hypothesized that the reason why some cells possessed only low

412 levels of GFP was their incomplete naïveness. In response, we applied a modified YAP
413 naïve-state induction method that produced dome-shaped colonies and converted most of the
414 cells into the naïve state.
415
416 The dome-shaped colonies with clearly defined borders obtained from RIKEN-2A-EOS-YAP
417 cells were positive for the naïve-state markers SUSD2 and KLF17, and the GFP expression
418 was brighter in these colonies than in RIKEN-2A-EOS cells prior to YAP treatment (Sup. Fig.
419 3). Of note is that human ES cell line KhES-3 subjected to the same YAP treatment produced
420 similar round colonies and expressed the naïve-state markers KLF17, KLF2/4, PECAM1, and
421 SUSD2 as determined by immunofluorescence analysis (Sup. Fig. 4). We investigated
422 whether the resulting cells would exhibit increased sensitivity to chemicals and performed the
423 ATP assay with four chemicals (aspirin, amiodarone, atorvastatin, and clotrimazole) to assess
424 the chemical sensitivity of these cells. Indeed, we found that YAP treatment of the EOS-
425 selected cells significantly increased the sensitivity of the cells to chemical exposure, and this
426 sensitivity decreased upon returning the cells to conventional AK02N medium and passaging
427 them for several days. Of note is that the sharp increase in sensitivity in EOS-YAP cells was
428 also confirmed in KhES-3 cell line (Sup. Fig. 5) and thus should be regarded as a result of
429 EOS selection and subsequent YAP treatment rather than an artefact. It is tempting to
430 speculate that the sharp increase in sensitivity was due to differences in the cell media, as
431 RIKEN-2A-EOS-YAP cells (and KhES-3-EOS-YAP cells) were kept in a medium that
432 substantially differed from conventional AK02N medium. However, the results in Fig. 3
433 show a consistent gradual decrease in chemical sensitivity upon returning the cells to AK02N
434 medium. This gradual decrease cannot be explained by medium differences, because the cells
435 at P2, P5, and P10 were kept in the same AK02N medium and still retained the substantially
436 higher chemical sensitivity that decreased only with passaging. Thus, it can be concluded that

YAP treatment induced a change in the intrinsic properties of the cells themselves, such as changes in gene or protein expressions, leading to unknown mechanisms, including the increase of intake of chemicals by the higher expressions of channels, transporters, etc., or the increased activation of signaling reaction pathways.

Another important point to note is that our RIKEN-2A-EOS-YAP cells should not be equated with perfect naïve iPS cells, since we have not performed the whole gambit of experiments needed to confirm the naïve state, such as immunostaining with multiple antibodies (KLF2/4, PECAM1, TFE3(10,22)), RNA-seq analysis etc. Our immunostaining results for SUSD2 and KLF17 can be considered inconclusive. However, it should be noted that the goal of this study was not to produce perfect naïve cells but to investigate the influence of different pluripotent cell states on the chemical sensitivity of the cells. To this end, we have achieved this goal and revealed that RIKEN-2A iPS cells were slightly different from KhES-3 cells, and RIKEN-2A-EOS-YAP cells were significantly different (most sensitive), with the sensitivity gradually decreasing by passaging these cells in conventional AK02N medium.

It should be noted that RIKEN-2A-EOS-YAP cells obtained immediately after EOS transfection and selection by antibiotics appear to be in an unstable condition and thus are not suitable for toxicity assays. It also remains to be proven that an iPS-based system can predict liver toxicity in principle. Overall, our study provides important information about the chemical sensitivity of different human PSC lines, and should serve as an opening to the investigation of the chemical sensitivity of iPSCs.

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624 **Supporting Information Captions**

625

626 **Supplementary Figure legends**

627

628 **Supplementary Fig. 1 Four cell lines with EOS vector expressing different levels of GFP.**
629 Four cell lines, as indicated by the names in the figure, were subjected to introduction of (PB)
630 EOS-C(3+)-GFP/puroR vector (EOS) as previously described (Hotta et al., 2009). After
631 puromycin selection, stable cell lines resistant to puromycin were obtained. The cells of the
632 lines expressed GFP in different quantities, roughly dividing the cells of each line into two
633 populations: “bright cells” and “dim cells”. This phenomenon was present in all four cell
634 lines.

635

Supplementary Fig. 2. Four selected methods of naïve state induction.

Four methods (Theunissen et.al., 2014, Takashima et.al., 2014, Qin et.al., 2016, Zimmerlin et.al., 2016) were chosen to test naïve state induction (light blue, upper row). Since all four methods stated that they can be used with a conventional DMEM medium (number 1 through 4 in dark blue) and with TeSR-E8 (numbers 5 through 8 in dark blue), this yielded eight different conditions. Media bases for each corresponding condition are described in pink, and chemical additives, such as FGF or BSA, are described in green.

Supplementary Fig. 3. Immunofluorescence analysis of EOS line RIKEN-2A-EOS subjected to modified YAP naïve induction for the presence of naïve status markers (SUSD2, KLF17).

Analysis of RIKEN-2A-EOS line. EOS fluorescence is shown in green, the corresponding markers are shown in red.

Supplementary Fig. 4. Immunofluorescence analysis of EOS line KhES3-EOS subjected to modified YAP naïve induction for the presence of naïve status markers (SUSD2, KLF17, PECAM1).

Analysis of KhES-3-EOS cell line. EOS fluorescence is shown in green; the corresponding markers are shown in red. Note the increased green fluorescence of EOS vector, indicating increased expression of the EOS marker of naïve state.

Supplementary Fig. 5. KhES3-EOS-YAP cell line exposed to four chemicals. The sensitivity of the cells was significantly decreased compared to KhES3 line.

Supplementary Table legends

Supplementary table 1. Doses at which the respective chemicals were applied in the exposure experiments.

Supplementary table 2. Results of assessment of morphology and AP staining after the application of eight methods of naïve induction as outlined in Sup. Fig. 1 to KhES3 cells, with three different dish coatings.

Author contributions

YP designed the biological part of the study, performed all experiments and data analysis except the selection of the chemicals, and wrote the manuscript. JY helped with data analysis and manuscript revision. KK provided help with creating cell stock for new cell lines. HS performed selection of the chemicals and reviewed the manuscript. WF designed the study, reviewed the manuscript and supervised the project.

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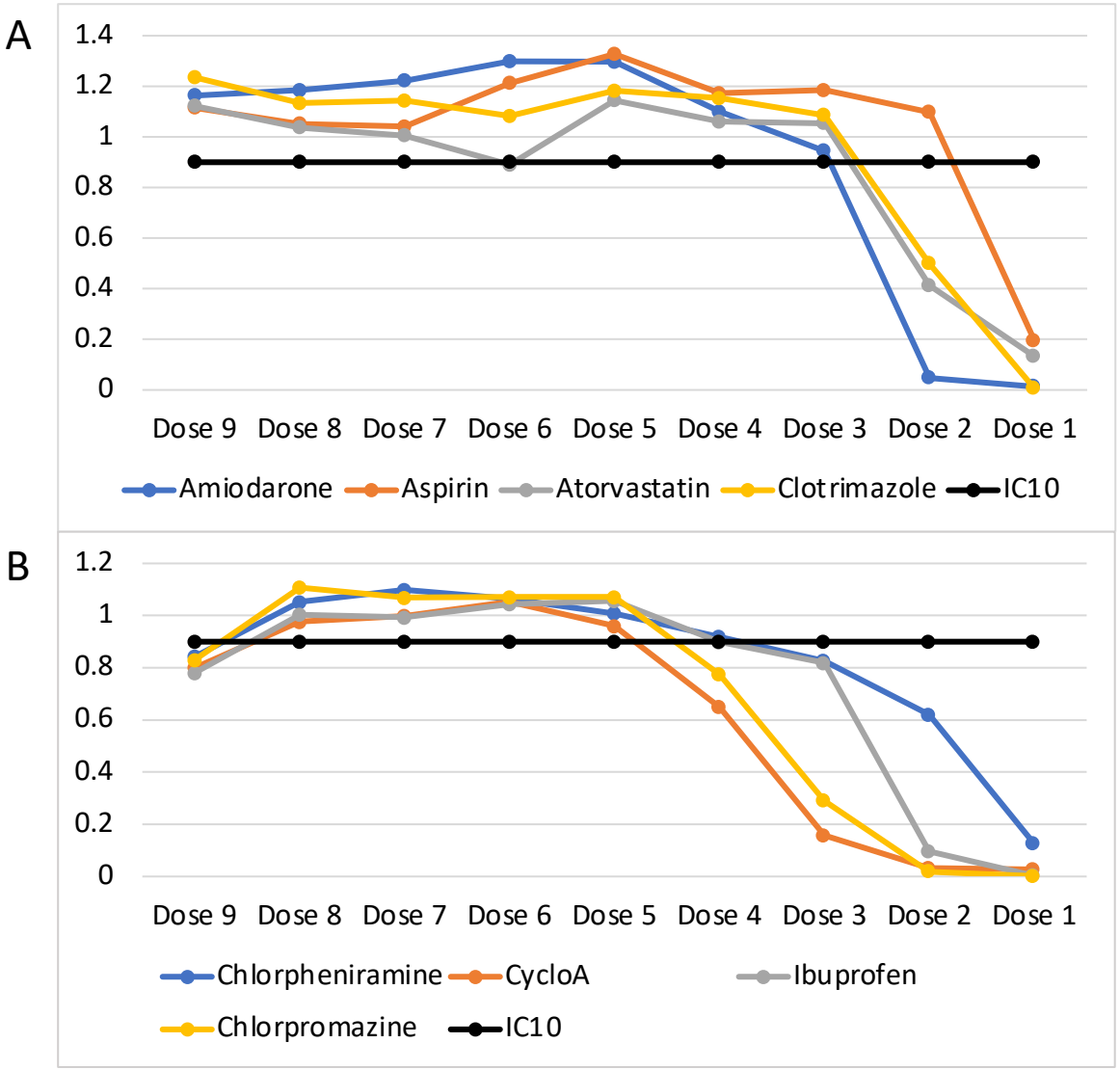
Fig. 1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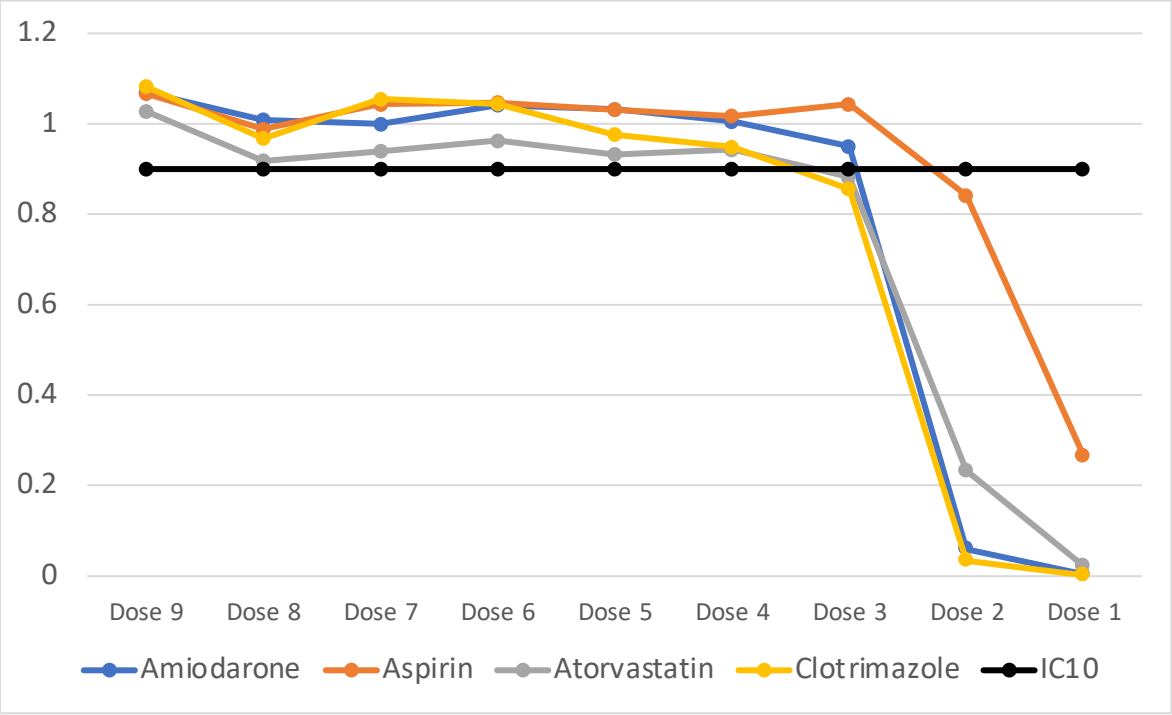
1.2
1
0.8
0.6
0.4
0.2
0
Dose 9 Dose 8 Dose 7 Dose 6 Dose 5 Dose 4 Dose 3 Dose 2 Dose 1
Amiodarone
Aspirin
Atorvastatin
Clotrimazole
IC10
B
1.2
1
0.8
0.6
0.4
0.2
0
Dose 9 Dose 8 Dose 7 Dose 6 Dose 5 Dose 4 Dose 3 Dose 2 Dose 1
Chlorpheniramine
CycloA
Ibuprofen
Chlorpromazine
IC10

Fig. 2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0.8
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0.2
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Dose 9
Dose 8
Dose 7
Dose 6
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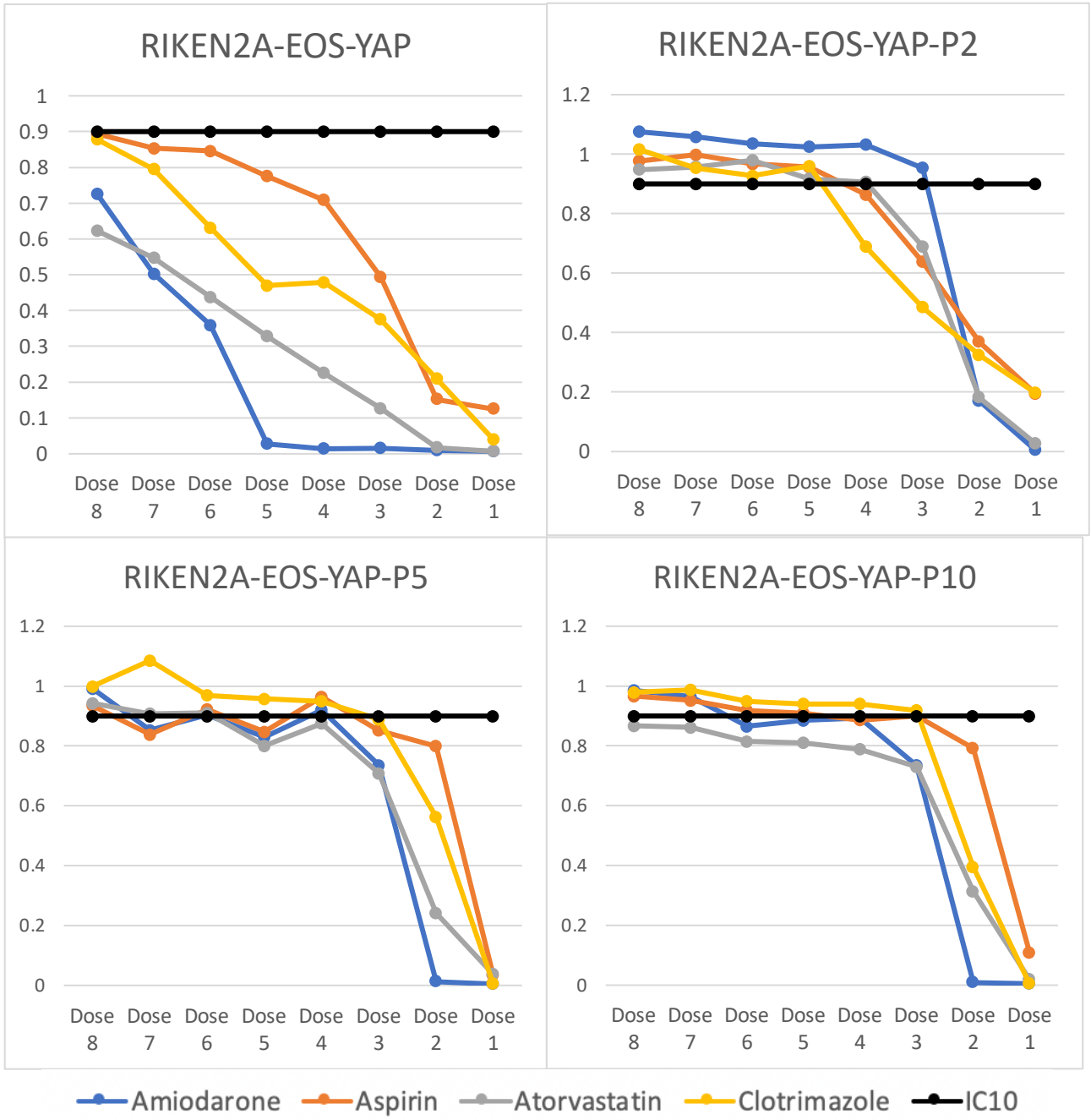
Dose 3
Dose 2
Dose 1
Amiodarone
Aspirin
Atorvastatin
Clotrimazole
IC10

Fig. 3


RIKEN2A-EOS-YAP
RIKEN2A-EOS-YAP-P2
RIKEN2A-EOS-YAP-P5
RIKEN2A-EOS-YAP-P10
Dose 8
Dose 7
Dose 6
Dose 5
Dose 4
Dose 3
Dose 2
Dose 1
Amiodarone
Aspirin
Atorvastatin
Clotrimazole
IC10