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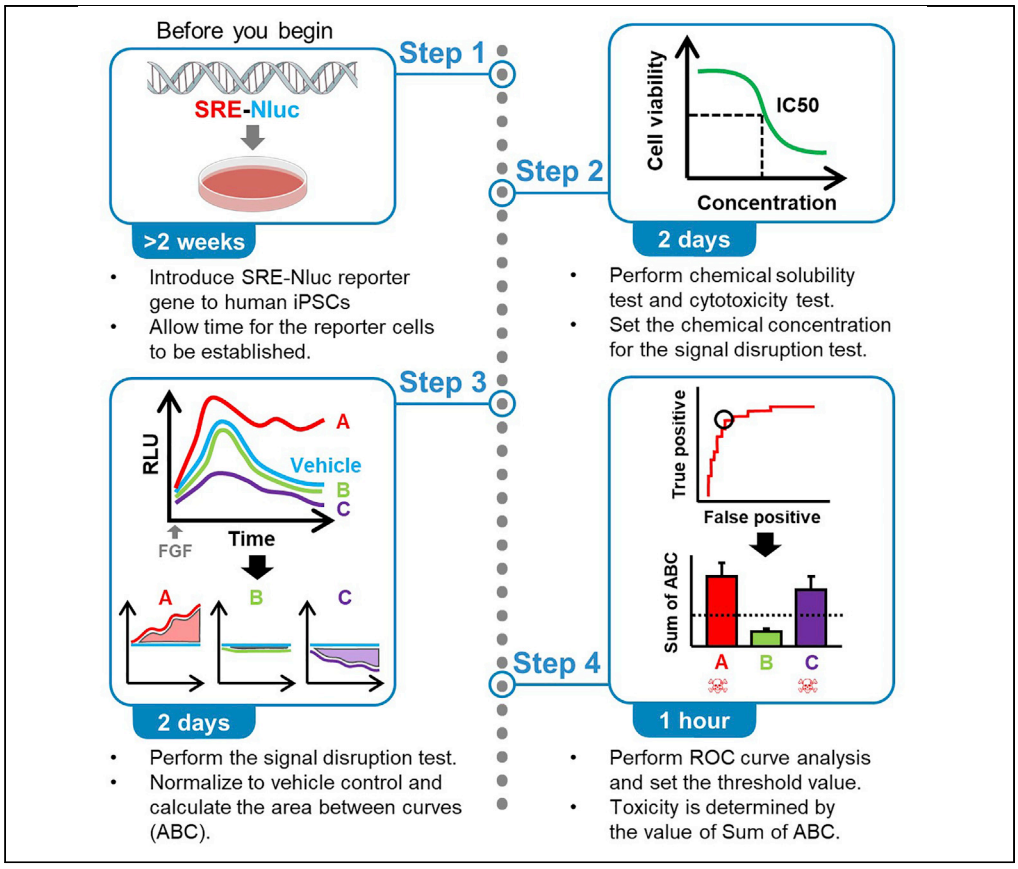
Protocol

Luciferase assay system to monitor fibroblast growth factor signal disruption in human iPSCs



We describe a protocol for a live-cell luciferase assay system for continuously monitoring fibroblast growth factor (FGF) signal disruption in human-induced pluripotent stem cells (iPSCs). Signal disrupting effects of chemicals are used as an indicator to evaluate toxicity. The assay is reliably predictive of the effects of limb malformation chemicals (AUC = 0.93). The current approach is limited to FGF signal disruption, and combinations with other types of signaling will be required to detect the effects of different toxicants.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A procedure for estimating developmental toxicants *in vitro* is described

The area between the curves is calculated as an indicator of the signal disruption

The assay is reliably predictive of the effects of limb malformation chemicals

Any signal disruption should be detectable by computing the area between the curves

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Luciferase assay system to monitor fibroblast growth factor signal disruption in human iPSCs

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SUMMARY

We describe a protocol for a live-cell luciferase assay system for continuously monitoring fibroblast growth factor (FGF) signal disruption in human-induced pluripotent stem cells (iPSCs). Signal disrupting effects of chemicals are used as an indicator to evaluate toxicity. The assay is reliably predictive of the effects of limb malformation chemicals (AUC = 0.93). The current approach is limited to FGF signal disruption, and combinations with other types of signaling will be required to detect the effects of different toxicants.

For complete details on the use and execution of this protocol, please refer to Kanno et al. (2022a).

BEFORE YOU BEGIN

Before conducting the FGF signal disruption assay, it is necessary to procure reagents for genome editing to generate a reporter cell line and characterize the established cells. The two key reagents required are a construct expressing the Nano-luciferase (*Nluc*) gene downstream of the serum response element (SRE) and a vector backbone with adeno-associated virus integration site 1 (*AAVS1*) homology arms of about 800 bp at both ends of the donor plasmid for efficient knock-in (Figure 1A). The following protocol has been used to successfully knock-in target DNA in the *AAVS1* region using CRISPR-Cas9 genome editing technology after introducing the SRE-Nluc plasmid into human iPS cells by electroporation (Kanno et al., 2022a, 2022b).

Plasmid preparation

Timing: 3 days

In this step, the DNA sequences encoding *Nluc* reporter gene downstream of the SRE are amplified from the pNL[NlucP/SRE/Hygro] vector and inserted into the bacterial plasmid pMK232 at approximately 800 bp *AAVS1* homology arms at both ends of the donor plasmid (Figures 1A and 1B; Table 1).

Day 1: In-Fusion cloning and E. coli transformation

1. Amplify DNA fragments from the pMK232 (CMV-OsTIR1-PURO) and pNL (NlucP/SRE/Hygro) plasmids by PCR.

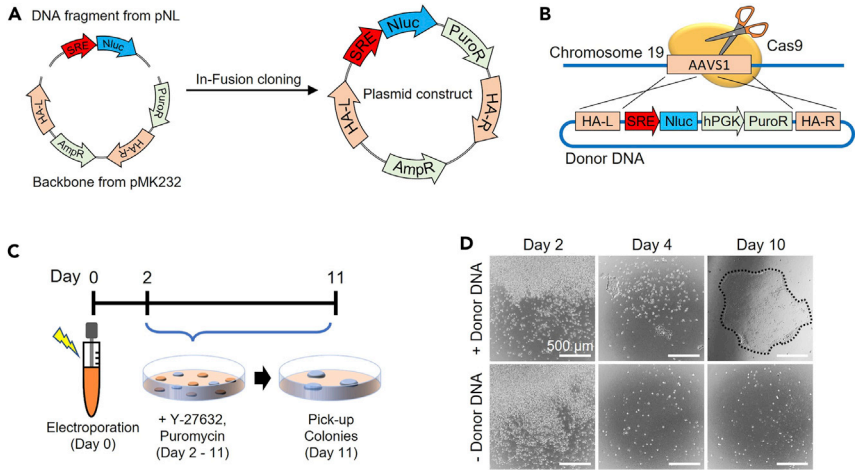

Figure 1. Generation of FGF signal reporter cells from human iPSCs

(A) Schematic illustration for plasmid vector construction. The donor plasmid used for genome editing is generated by inserting a DNA fragment containing the Nano-luciferase (*Nluc*) reporter gene downstream of the serum response element (SRE) into the backbone containing the adeno-associated virus integration site 1 (AAVS1) homology arms (HA-L and HA-R) using the In-Fusion reaction.

(B) Schematic diagram of targeted transgene insertion into the *AAVS1* locus in the human genome. The donor vector contains AAVS1 homology arms about 800 bp long at both ends of the donor plasmid for efficient knock-in. The plasmid construct contains the *Nluc* reporter gene under the control of SRE and the puromycin resistance gene (*PuroR*) under the control of the human phosphoglycerate kinase (hPGK) promoter.

(C) Schematic illustration of cell line establishment process. Human iPSCs are electroporated with gRNA and Cas9 protein with or without donor DNA (day 0) and then treated with puromycin from day 2 to day 11.

(D) Colony formation. Transfected cells grew and formed colonies, while non-transfected cells died in the presence of puromycin. Scale bar indicates 500 μm.

a. Set up PCR mixtures as follows:

Reagent	Final concentration	Amount
Plasmid (10 ng/μL)	1 ng/μL	1 μL
KOD ONE™ PCR Master Mix -Blue (2×)	1×	5 μL
Forward Primer (20 μM)	2 μM	1 μL
Reverse Primer (20 μM)	2 μM	1 μL
Nuclease-free water	n/a	2 μL
Total	n/a	10 μL

b. Perform PCR using a thermal cycler under the following temperature conditions:

PCR cycling conditions to amplify DNA fragments from the pNL (NlucP/SRE/Hygro) plasmid			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	120 s	1
Denaturation	98°C	10 s	30
Annealing & Extension	68°C	6 s	
Hold	4°C		

PCR cycling conditions to amplify backbone from the pMK232 (CMV-OsTIR1-PURO) plasmid			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	120 s	1
Denaturation	98°C	10 s	30
Annealing & Extension	68°C	70 s	
Hold	4°C		

Table 1. List of viable plasmids for gene editing

Plasmid	Characteristics	Antibiotic resistance	Source	Identifier
pMK232 (CMV-OsTIR1-PURO)	Contains *AAVS1* homology arms and *PuroR* gene	Amp	Natsume et al. (2016)	Addgene (Plasmid #72834)
pNL (NlucP/SRE/Hygro)	Contains the insert encoding *Nluc* reporter gene downstream of the SRE	Amp	Promega	Cat#CS177601

c. Using a subset of the PCR post-reaction mixture (approximately 10 μL), perform gel electrophoresis to confirm that the target DNA fragment has been amplified. For example, run a 1.5% agarose gel for 15 min at 100 V for DNA fragment from the pNL, while run 0.8% of agarose gel for 25 min at 100 V for backbone from pMK232. The expected lengths of the DNA fragments amplified from pNL and pMK232 are 1068 bp and 6975 bp, respectively.

Note: In this protocol, the extension time is designed to be short because of the high accuracy and amplification efficiency of DNA polymerase reagents (1–5 kb: 5 s/kb) used. In accordance with this manufacturer's protocol, a 2-step cycle PCR is utilized to prevent non-specific amplification (extra band or smear). If other PCR reagents are used, a typical 3-step cycle PCR can be performed (e.g., denaturation: 98°C, annealing: (Tm-5) °C, extension: 68°C).

2. The donor plasmid used for genome editing is generated using the In-Fusion® HD Cloning Kit (639633, Takara Bio, Shiga, Japan). The In-Fusion reaction can be performed without purification of the PCR products.
   a. Fragment the remaining circular plasmid template DNA by adding 1 μL of Dpn I to 10 μL of PCR product.
   b. Perform the Dpn I digestion reaction using a thermal cycler under the following temperature conditions:

Steps	Temperature	Time
Enzymatic reaction	37°C	60 min
Heat deactivation	70°C	5 min
Hold	4°C	

c. Set up In-Fusion cloning mixtures as follows:

Reagent	Amount
5× In-Fusion HD Enzyme Premix	1 μL
Backbone	1 μL
DNA fragment	1 μL
Cloning Enhancer	0.5 μL
Nuclease-free water	1.5 μL
Total	5 μL

d. Perform the In-Fusion cloning reaction using a thermal cycler under the following temperature condition:

Steps	Temperature	Time
In-Fusion reaction	37°C	15 min
	50°C	15 min
Hold	4°C	

3. Mix competent cells with In-Fusion cloning mixture and incubate on LB agar plates containing ampicillin to select for *E. coli* transformed with the plasmid constructed by In-Fusion cloning.
   a. Mix 30 μL of *E. coli* HST08 Premium competent cells with 1 μL of In-Fusion cloning mixture and place on ice for 30 min.
   b. Heat shock competent cells at 42°C for 30 s and then place on ice for 2 min.
   c. Add 300 μL of S.O.C. medium to the cells and shake at 37°C for 30 min.
   d. Spread competent cells on an LB agar plate containing 100 μg/mL of ampicillin and incubate at 37°C overnight (12–16 h).

Day 2–3: Colony PCR and plasmid propagation

4. Select well-formed colonies and perform colony PCR to amplify the target sequences in the plasmid vector to confirm whether the target vector has been introduced into *E. coli* cells.
   a. Set up PCR mixtures as follows:

Reagent	Final concentration	Amount
GoTaq® Green Master Mix, 2×	1×	5 μL
Forward Primer (20 μM)	2 μM	1 μL
Reverse Primer (20 μM)	2 μM	1 μL
Nuclease-free water	n/a	2 μL
Total	n/a	10 μL

   b. Select 6–10 individual colonies using a pipette tip and place a portion of each into an individual PCR mixture.
   c. Perform PCR using a thermal cycler under the following temperature conditions:

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	120 s	1
Denaturation	95°C	30 s	30 cycles
Annealing	65°C	30 s	
Extension	72°C	60 s	
Final Extension	72°C	300 s	1
Hold	4°C		

   d. Perform gel electrophoresis using 1.5% agarose gel at 100 V for around 15 min to confirm that the target sequence from the donor DNA has been amplified by colony PCR. When using the primer pair described in the key resources table, the expected length of the PCR product is 408 bp.
   e. Collect a portion of 3–5 of the colonies used for colony PCR and inoculate into Terrific Broth (TB) medium (+ 50 μg/mL ampicillin) in a 14 mL culture tube (352001, Corning). Allow to grow for 16–20 h at 37°C and 300 rpm in a shaking incubator.
   f. Purify plasmids propagated by *E. coli* using the Plasmid DNA Extraction Mini Kit (FAPDE001-1, FAVORGEN Biotech). Alternatively, PureLink™ Quick Plasmid Miniprep Kit (K210010, Thermo Fisher Scientific, Massachusetts, United States) and QIAGEN Plasmid Mini Kit (12123, QIAGEN, Hilden, Germany), and other kits for extracting plasmid DNA derived from *E. coli* can be used. Quantify the plasmid DNA concentration using a nanodrop spectrophotometer at an absorbance of 260 nm. Circular plasmids can be stored frozen at −20°C for at least 6 months.
   g. To confirm that the plasmid vector was constructed without DNA base substitutions, it is recommended to check the nucleotide sequence by Sanger sequencing.

Preparation of stable transgenic iPSCs

Timing: >11 days

In this step, the constructed plasmid vector is used as donor DNA to efficiently knock-in the *Nluc* gene downstream of the SRE and *PuroR* gene into the genomic *AAVS1* region of human iPS cells using the CRISPR/Cas9 system (Figures 1B–1D).

Day 1: DNA transfection by electroporation

5. Prepare the electroporation reagent by mixing guide RNA (gRNA) recognizing the *AAVS1* region, Cas9 protein, and the constructed plasmid.
   a. Prepare the gRNA complex as follows:

Reagent	Final concentration	Amount (for 10 times use)
Alt-R CRISPR-Cas9 crRNA (200 μM) (Purification grade: Desalting)	44 μM	2.2 μL
Alt-R CRISPR-Cas9 tracrRNA (200 μM)	44 μM	2.2 μL
1× TE Buffer	1×	up to 10 μL
Total	n/a	10 μL

   b. Heat at 95°C for 5 min to form a crRNA:tracrRNA complex (Alt-R guide RNA), and then return to room temperature (20°C–25°C). Alt-R guide RNA can be stored in a frozen condition at −20°C for 10 months.
   c. Prepare gRNA-Cas9 protein complex (RNP complex) as follows:

Reagent	Final concentration	Amount (for 10 times use)
Alt-R guide RNA (44 μM)	15 μM	3.4 μL
Alt-R Cas9 enzyme (62 μM)	12.4 μM	2.0 μL
Total	n/a	5.4 μL

   d. Incubate the mixture at room temperature (20°C–25°C) for 10–20 min. RNP complex can be stored in a frozen condition at −80°C for 5 months, avoiding more than two freeze-thaw cycles.
   e. Set up electroporation mixtures as follows:

Reagent	Final concentration	Amount (for 10 times use)
RNP Complex	≃15 μM	5.4 μL
Donor DNA	5 μg/60 μL	n/a
Resuspension Buffer R	n/a	up to 60 μL
Total	n/a	60 μL

6. Introduce the donor DNA and RNP complex into human iPS cells by electroporation.
   a. Detach human iPS cells from a culture plate and collect 1.0 × 10^6 cells in a 15 mL tube. Refer to "cell lines and culture conditions" section below.
   b. Centrifuge at 200 × g for 4 min, completely remove the supernatant, then resuspend the cells in 50 μL of Resuspension Buffer R.
   c. Mix 5 μL of the cell suspension (1.0 × 10^5 cells) with 6 μL of the electroporation mixture.
   d. Collect 10 μL of the cell-electroporation mixture in the Neon™ tip without air bubbles, and apply one electrical pulse at 1200 V for 30 msec.
   e. Immediately seed the cells into Geltrex-coated 24-well plates and culture in StemFlex medium (+ 10 μM Y-27632).

Note: The manufacturer has reported that the Cas9 Nuclease enzyme can be diluted to a working concentration and reused for later experiments with no loss in activity (data not shown). The manufacturer recommends storing CRISPR RNPs at 4°C for no more than 2 weeks because accidental contamination could result in bacterial or fungal growth.

Note: Electroporation may result in various levels of cell death. It is possible to change the medium a day after electroporation to remove dead cells and dead cell-derived undesirable factors in the medium. Because most cells are viable as single cells, it is recommended to replace the medium containing 10 μM of Y27632 to prevent cell death.

Note: Alternatives exist for iPS cell adhesion substrates and culture media (e.g., Matrigel® Matrix and mTeSR™-1 Media). However, their use is likely to alter the behavior and properties of the cells. It remains to be seen how the use of those alternatives will change the ability of this test method to detect developmental toxicity.

Note: Because electroporation requires specialized equipment, it may be possible to use a transfection reagent (e.g., lipofectamine 3000 reagent) as an alternative for delivering donor DNA or CRISPR RNPs into the cells.

Day 3–11: Puromycin selection

7. The donor DNA contains a puromycin-resistant gene, allowing for the selective growth of cells containing the knock-in on the puromycin-supplemented medium. See troubleshooting 1 and 2.
   a. On the second day after electroporation, replace the culture medium with StemFlex medium (+ 10 μM Y-27632, 0.5 μg/mL puromycin). Thereafter, change the medium once every 2–3 days.
   b. Culture the cells in StemFlex medium (+ 10 μM Y-27632, 0.5 μg/mL puromycin) for a total of 10 days until the cell colonies spread.

Note: In this protocol, the concentration of puromycin is based on the previous study (Steyer et al., 2018), but it is preferable to consider the minimum effective concentration because it varies based on the cell line. Since the previous study used a concentration of 0.2–0.5 μg/mL, it is recommended to consider this concentration range. In addition, a negative control group of iPS cells that do not knock-in donor DNA should be provided when considering the concentration. It is desirable to select the puromycin concentration at which the cells in the control group do not survive while the cells that have undergone electroporation survive and proliferate.

Note: It is expected that at least 5 colonies that expand to approximately 1 mm in diameter per well will be formed. In our experience, homozygous clones and heterozygous clones are obtained at a rate of one-third and two-thirds among the selected colonies, respectively.

8. Remove each colony individually to allow the cells to expand further.
   a. Discard the culture medium and wash the wells with phosphate-buffered saline (PBS), then place a piece of paper (2 mm square) soaked with TrypLE Select enzyme on cells and incubate at 37°C for 5 min.
   b. Transfer the filter paper with attached cells to wells pre-coated with Geltrex and filled with StemFlex medium (+ 10 μM Y-27632). Subsequently, disperse cells by gentle pipetting. Culture cells until they are fully expanded.

Note: It may be possible to use a gentler cell detachment reagent (e.g., Versene Solution) as an alternative to TrypLE Select, although this protocol does not include the same.

9. This step is performed to confirm the establishment of the signal reporter cell lines. To determine the zygosity of the gene knock-in in the established cell line, perform PCR with primers designed

against the regions flanking the knock-in. To confirm that the recombinant gene has been knocked in without insertion-deletion mutations, perform Sanger sequencing of the target region within the genome of the established cell line.

a. Wash approximately 60%–70% confluent cells in a 48-well or 24-well plate with PBS and lysate with 200 μL of TRIzol™ Reagent. Collect the lysate in a 1.5 mL tube.
b. Add 40 μL of chloroform (1/5 volume of TRIzol™ Reagent), shake vigorously for 15 s, and allow to stand for 2–3 min.
c. Centrifuge at 12,000 × *g* for 10 min at 4°C.
d. Remove the aqueous layer, add 60 μL of ethanol (300 μL per 1 mL of TRIzol™ Reagent), mix by inversion, and allow to stand for 2–3 min.
e. Centrifuge at 2,000 × *g* for 5 min at 4°C.
f. Discard the supernatant and add 200 μL of 0.1 M sodium citrate/10% ethanol (1 mL per 1 mL of TRIzol™ Reagent). Allow to stand for 30 min with occasional stirring.
g. Repeat the steps e and f.
h. Centrifuge at 2,000 × *g* for 5 min at 4°C.
i. Discard the supernatant and add 400 μL of 75% ethanol (1.5–2 mL per 1 mL of TRIzol™ Reagent). Allow to stand for 15 min with occasional stirring.
j. Centrifuge at 2,000 × *g* for 5 min at 4°C.
k. Discard the supernatant and centrifuge pellets air-dry for 5–15 min.
l. Add 60 μL of 8 mM NaOH to dissolve the DNA. Quantify the genome DNA concentration using a nanodrop spectrophotometer at an absorbance of 260. After further dilution with TE buffer, the genome DNA can be stored frozen at −20°C for at least 6 months.
m. Set up PCR mixtures as follows:

Reagent	Final concentration	Amount
DNA (40 ng/μL)	4 ng/μL	1 μL
KOD ONE™ PCR Master Mix -Blue (2×)	1×	5 μL
Forward Primer (20 μM)	2 μM	1 μL
Reverse Primer (20 μM)	2 μM	1 μL
Nuclease-free water	n/a	2 μL
Total	n/a	10 μL

n. Perform PCR using a thermal cycler under the following temperature conditions:

PCR cycling conditions to amplify DNA fragments from the pNL (NlucP/SRE/Hygro) plasmid			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	120 s	1
Denaturation	98°C	10 s	30
Annealing	61.5°C	5 s	
Extension	68°C	60 s	
Hold	4°C		

o. Using a subset of the PCR post-reaction mixture (approximately 10 μL), perform gel electrophoresis to determine the zygosity of the gene knock-in in the established cell line. For example, run at 1.5% agarose gel for 25 min at 100 V for DNA fragments. The expected lengths of the DNA fragments amplified are 4451 bp if knock-in is successful, but 2042 bp if knock-in is not successful.
p. Cut out the gel band of the amplified knock-in region, collect it in a 1.5-mL tube, and extract the DNA using GEL/PCR Purification Mini Kit (FAGCK 001, FAVORGEN Biotech Corp., Taiwan). Alternatively, Agarose Gel DNA Extraction Kit (11696505001, Merck, Darmstadt, Germany), QIAquick Gel Extraction Kit (28706X4, QIAGEN, Hilden, Germany), and other kits for extracting DNA from agarose gel can be used. Quantify the DNA concentration using

a nanodrop spectrophotometer at an absorbance of 260 nm. The DNA can be stored frozen at −20°C for at least 6 months.

q. To confirm that the recombinant gene has been knocked in without insertion-deletion mutations, it is recommended to check the nucleotide sequence by Sanger sequencing.

Note: When confirming the zygosity of genomic knock-in by PCR, genomic DNA derived from wild-type human iPS cells that have not undergone genome editing is prepared as a negative control group. If the band is seen only in the same position as its negative control (2042 bp), the target knock-in has failed. PCR bands at both the 4451 bp and 2042 bp positions indicate heterozygosity, while a PCR band at only the 4451 bp position indicates homozygosity. Heterozygous cell lines can also be used for the signal disruption test described below; however, the luminescence intensity is reduced to about half that of homozygous cell lines.

10. To evaluate the ligand responsiveness of the established reporter cell line, culture the cells in a medium containing a luminescent substrate (e.g., Enduradine or Vivazine). Subsequently, add the ligand (e.g., bFGF or EGF) and measure the luminescence intensity over time using a luminometer. StemFlex medium most likely contains various growth factors, including bFGF. To minimize the influence of the components of the medium and to reproducibly evaluate the signal activity, we recommend using a recombinant protein-based medium without animal products for the assay.
    a. Four days before adding the ligand, seed the established cell lines at 1.0×10^4 cells/well in 96-well white plates.
    b. The following day, change the medium to 200 μL/well of StemFlex medium.
    c. The day before the addition of the ligand, change the medium to 100 μL/well of APEL2 medium.
    d. Change the medium to 100 μL/well of APEL2 medium (+1% endurazine) at least 2 h before ligand addition, as it takes 2 h for the luminescent substrate to equilibrate in/out of the cell.
    e. Measure the luminescence intensity of each well immediately before adding the ligand. Add 11 μL/well of bFGF diluted to 20 ng/mL in APEL2 to a final concentration of 2 ng/mL.
    f. Measure the luminescence intensity of each well at regular intervals. The manufacturer's manual indicates that the luminescence can be stably detected for up to 72 h.

Prepare software and packages for analysis

Timing: 20 min

To assess the magnitude of signal disruption-based on temporal changes in FGF signal reporter activity, we developed the R software outlined in Kanno et al. (2022a). The steps below identify the prerequisites necessary for running the software.

11. Download and install R if it is not already installed. Instructions for this process can be found at https://cran.r-project.org/bin/windows/base/. We recommend using R v4.0.3 and higher. While older releases may work, the software has only been tested on recent versions of R.
12. Install the necessary packages (drc, mgcv).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli HST08 Premium Competent Cells	Takara Bio	Cat#SD1423
pMK232 (CMV-OsTIR1-PURO)	Natsume et al. (2016)	Addgene (Plasmid #72834)

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pNL (NlucP/SRE/Hygro)	Promega	Cat#CS177601
Chemicals, peptides, and recombinant proteins		
KOD One® PCR Master Mix -Blue- (Dye-containing 2×PCR Master Mix)	Toyobo	Cat#KMM-201
Dpn I	New England Biolabs	Cat#R0176
In-Fusion® HD Cloning Kit w/Cloning Enhancer	Takara Bio	Cat#639633
S.O.C. Medium	Thermo Fisher Scientific	Cat#15544034
Fast-Media® Amp Agar	InvivoGen	Cat#fas-am-s
GoTaq® Green Master Mix, 2×	Promega	Cat# M712B
Terrific Broth, modified	Merck	Cat#T0918
Glycerol	FUJIFILM Wako Pure Chemical	Cat#075-00616
Ampicillin Sodium	FUJIFILM Wako Pure Chemical	Cat#016-23301
Plasmid DNA Extraction Mini Kit	FAVORGEN Biotech	Cat#FAPDE001-1
Alt-R® CRISPR-Cas9 crRNA, 2 nmol (Purification grade: Desalting) (sequence for forward integration: ACAGTGGGGCCACTAGGGAC)	Integrated DNA Technologies (Richardson et al., 2016)	N/A
Alt-R® CRISPR-Cas9 tracrRNA 5 nmol	Integrated DNA Technologies	Cat#1072532
Tris-EDTA Buffer Solution (pH 8.0)	Nacalai Tesque	Cat#06890-54
Alt-R® S.p. HiFi Cas9 Nuclease V3, 100 µg	Integrated DNA Technologies	Cat#1081060
Neon™ Transfection System 10 µL Kit (components: Resuspension Buffer R, Neon Tips, Neon Tubes)	Thermo Fisher Scientific	Cat#MPK1025
Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Thermo Fisher Scientific	Cat#A1413202
StemFlex™ Medium	Thermo Fisher Scientific	Cat#A3349401
TrypLE™ Select Enzyme (1×), no phenol red	Thermo Fisher Scientific	Cat#12563011
PBS	Thermo Fisher Scientific	Cat#70013032
TRIzol™ Reagent	Thermo Fisher Scientific	Cat#15596018
Chloroform	FUJIFILM Wako Pure Chemical	Cat#038-02606
Ethanol (99.5)	FUJIFILM Wako Pure Chemical	Cat#057-00456
Trisodium Citrate Dihydrate	FUJIFILM Wako Pure Chemical	Cat#191-01785
6 mol/L (6 N)-Sodium hydroxide solution	Kishida Chemical	Cat#900-02205
GEL/PCR Purification Mini Kit	FAVORGEN Biotech	Cat#FAGCK 001
Dimethyl Sulfoxide	Merck	Cat#D4540; CAS: 67-68-5
CultureSure® Y-27632	FUJIFILM Wako Pure Chemical	Cat#030-24021; CAS: 331752-47-7
Puromycin Dihydrochloride	Thermo Fisher Scientific	Cat#A1113803
STEMdiff APEL2 medium	Veritas	Cat#ST-05275
Albumin, from Bovine Serum Cohn Fraction V, pH7.0	FUJIFILM Wako Pure Chemical	Cat#013-23291
Heat Stable Recombinant Human bFGF	Life Technologies	Cat#PHG0367V
Critical commercial assays		
Cell Counting Kit-8	Dojindo	Cat#CK04
Nano-Glo® Endurazine™ Substrate	Promega	Cat#N2571
Experimental models: Cell lines		
Human Induced Pluripotent Stem (iPS) Cells	RIKEN BRC Cell Bank	201B7 (RCB Cat# HPS0063, RRID: CVCL_A324)
Oligonucleotides		
Primer: pNL insert, Forward: TGGGGCAATTAATAGTAATCAT GAGCTCGCTAGC	This manuscript	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: pNL insert, Reverse: GGCGCAACCCCAACTAACTT GTTTATTGCAGC	This manuscript	N/A
Primer: pMK232 backbone, Forward: AGTTGGGGTTGCGCCTTTTCCA AGGCAGCC	This manuscript	N/A
Primer: pMK232 backbone, Reverse: TGATTACTATTAATTGCCCCAC TGTGGGGTGGAGGGGACAG	This manuscript	N/A
Primer: Colony PCR Forward: TGGGGCAATTAATAGTAATCATG AGCTCGCTAGC	This manuscript	N/A
Primer: Colony PCR Reverse: TCAGCCCATTTTCACCGCTCA GGACAATCC	This manuscript	N/A
Primer: For Sanger sequencing of plasmid constructs: CACTGCATTCTAGTTGTGGTT TGTCCA	This manuscript	N/A
Primer: For Sanger sequencing of plasmid constructs: TCAGCCCATTTTCACCGCTCAG GACAATCC	This manuscript	N/A
Primer: AAVS1 region PCR Forward: GGACCACTTTGAGCTC-TACTGGCTTCTGCG	This manuscript	N/A
Primer: AAVS1 region PCR Reverse: GCTGTCCTGGGCAAACAGCA-TAAGCTGGTCAC	This manuscript	N/A
Primer: For Sanger sequencing of stable transgenic iPSCs: GGCCGGTTAATGTGGCTCTGG TTCTGGGTAC	This manuscript	N/A
Primer: For Sanger sequencing of stable transgenic iPSCs: TCAGCCCATTTTCACCGCTCA GGACAATCC	This manuscript	N/A
Recombinant DNA		
Donor plasmid for gene editing	This manuscript	N/A
Software and algorithms		
R (version 4.0.3) (packages from CRAN)	R Core Team	https://cran.r-project.org/bin/windows/base/
All original code	This manuscript	Mendeley Data: https://doi.org/10.17632/79nmyyv99z.2

MATERIALS AND EQUIPMENT

Terrific Broth (TB) medium		
Reagent	Final concentration	Amount
Terrific Broth, modified	47.6 g/L	47.6 g
Glycerol	0.8%	8 mL
Distilled water	n/a	up to 1 L
Total	n/a	1 L
Autoclave at 121°C for 15 min. Stored at 4°C and warmed to 37°C. Typically used within one month once opened.		

1 M sodium citrate		
Reagent	Final concentration	Amount
Trisodium Citrate Dihydrate	1 M	29.4 g
Distilled water	n/a	up to 100 mL
Total	n/a	100 mL

Dissolve 29.4 g of trisodium citrate dihydrate in distilled water and volume up to 100 mL.
Sterilize in an autoclave at 121°C for 20 min. Stored at 20°C–25°C. Typically used within one month once opened.

0.1 M sodium citrate/10% ethanol		
Reagent	Final concentration	Amount
1 M sodium citrate	0.1 M	10 mL
Ethanol	0.1%	10 mL
Distilled water	n/a	up to 100 mL
Total	n/a	100 mL

Stored at 20°C–25°C. Typically used within one month once opened.

STEP-BY-STEP METHOD DETAILS

Reagent preparation and determination of the solubility of test chemicals

Timing: 1 day

Dissolve each test chemical in an appropriate solvent, either PBS or dimethyl sulfoxide (DMSO), according to the ECVAM study (Genschow et al., 2004). Because chemicals dissolved in DMSO are poorly water-soluble, check the concentration at which they can be dissolved in the culture medium.

1. Select appropriate stock solvents for each chemical based on the manufacturer's datasheet, and prepare the stock at a soluble concentration. Determine storage temperature and duration in accordance with the datasheet.
2. Dilute stocks of chemicals with low water solubility in the culture medium and check for precipitation.
   a. Dilute each chemical stock 100-fold in StemFlex medium.
   b. Centrifuge at 1,000 × *g* for 5 min and visually check for precipitation. If precipitation is observed rather than dissolution, dilute the stock solution with DMSO and then dilute 100-fold in the medium.

Cell lines and culture conditions

Timing: 2 weeks

Human iPSC line 201B7 (RIKEN Cell Bank, Japan) was routinely cultured in StemFlex medium (A3349401, Thermo Fisher Scientific, Waltham, MA, USA) on culture plates coated with Geltrex Matrix (A1413202, Thermo Fisher Scientific).

3. Geltrex coating of culture wells.
   a. Remove Geltrex from the −80°C freezer and thaw on ice.
   b. Mix ice-cold DMEM/F-12 and Geltrex at a ratio of 100:1 and add the Geltrex solution to culture wells in the following volumes:
      35 mm dish and 6-well plate: 1.5 mL/well.
      12-well plate: 600 μL/well.
      24-well plate: 300 μL/well.

48-well plate: 150 μL/well.
96-well plate: 60 μL/well.

c. Incubate the culture plate with 1% Geltrex solution at 37°C in a CO_2 incubator for at least 1 h for coating.

Note: To avoid repeated freezing and thawing, dispense into microtubes according to the volume used. Geltrex can be stored at 4°C for up to one week and at −80°C for up to six months. Make sure the Geltrex does not form bubbles.

4. Thaw the frozen stock of iPS cells.
   a. Pre-warm StemFlex medium (+ 10 μM Y-27632) to 37°C in a water bath.
   b. Remove the vial of cells from the storage container and quickly thaw in a 37°C water bath. Alternatively, add 1 mL of StemFlex medium (+ 10 μM Y-27632) to the vial if the stock volume is less than 200 μL, and thaw by pipetting gently.
   c. Collect the cell suspension in a 15 mL tube pre-filled with 4 mL of StemFlex medium and centrifuge at 200 × g for 4 min.
   d. Discard the supernatant and resuspend the cells in fresh StemFlex medium (+ 10 μM Y-27632).
   e. Discard the Geltrex solution from the pre-coated wells and seed the cells in culture plates at the following densities:
      35 mm dish and 6-well plate: 1.25×10^5 cells/well.
   f. The following day, change the medium to StemFlex medium if few single cells are present.

5. Clump passaging of iPS cells using Versene Solution. Following the recovery of the cells, start a culture in which the first passages are clumping passages to maintain cell-cell adhesion. Carry out passages when 60%–80% confluency is reached.
   a. Place the 6-well plate with 1% Geltrex solution in a CO_2 incubator in advance and incubate at 37°C for at least 1 h for coating.
   b. Pre-warm StemFlex medium (+ 10 μM Y-27632) to 37°C in a water bath, and equilibrate PBS and Versene Solution to room temperature (20°C–25°C).
   c. Discard the medium from the culture wells and wash the wells once with 2 mL of PBS.
   d. Add 1 mL of Versene Solution to the wells and let stand at room temperature for 5–8 min.
   e. Discard the Versene Solution and wash the wells 4–5 times with medium to remove the cells.
   f. Discard the Geltrex solution from the pre-coated wells and add a 1/5 volume of the cell suspension to the wells.

6. Single passage of iPS cells using TrypLE Select Solution. The second and subsequent passages are made by dispersion into single cells; passages are carried out when 60%–80% confluency is reached.
   a. Place the 6-well plate with 1% Geltrex solution in a CO_2 incubator in advance and incubate at 37°C for at least 1 h for coating.
   b. Pre-warm StemFlex medium (+ 10 μM Y-27632) to 37°C in a water bath, and equilibrate PBS and TrypLE Select Solution to room temperature (20°C–25°C).
   c. Discard the medium from the culture wells and wash once with 2 mL of PBS.
   d. Add 1 mL of TrypLE Select Solution to the wells and let stand at room temperature for 5 min.
   e. When cell adhesion is observed to weaken under a microscope, remove cells by gently pipetting 5–10 times with a 1000 μL pipette and collect the cells in a 15 mL tube pre-filled with 4 mL of StemFlex medium.
   f. Centrifuge at 200 × g for 4 min.
   g. Discard the supernatant and loosen the pellet by tapping 3–5 times.
   h. Suspend the cells in 2 mL of StemFlex medium (+ 10 μM Y-27632) and pipette 5–10 times.
   i. Take a subset of the cell suspension and mix it 1:1 with trypan blue to measure the number of viable cells.

j. Discard the Geltrex solution from the pre-coated wells and seed the cells in the wells at the following densities:
   35 mm dish and 6-well plate: 1.25 × 10^5 cells/well.
   12-well plate: 5.0 × 10^4 cells/well.
   24-well plate: 2.5 × 10^4 cells/well.
k. If the cells reach greater than 10% confluency the following day, change the medium to StemFlex medium.

Note: In an attempt to minimize the time spent on cell counting to prevent cell viability from declining and to reduce the variability of cell counts, it is recommended that replicate measurements be performed using a cell counter. If counting cells manually, dilution of the cell suspension is recommended to shorten the cell counting time.

Cytotoxicity test

Timing: 5–7 days

To distinguish whether a chemical has a specific adverse effect on the FGF signaling pathway, it is necessary to show that the effect occurs at concentrations lower than those that cause severe cell injury. Therefore, prior to evaluating the effects of chemicals on signaling pathways, the relationship between the chemical concentration and cell viability must be evaluated.

7. Seed the established cell lines in Geltrex-coated flat-bottomed 96-well culture plates at a viable cell density of 1.0 × 10^4 cells/well.

Note: Because evaporation of the culture medium may adversely affect the assay, fill the outermost wells of a 96-well plate with sterile water and only use the central 60 wells for the assay.

8. The following day, replace the medium with 100 μL/well of StemFlex medium.
9. When 40%–60% confluency is reached, perform the cytotoxicity test. Immediately prior to the addition of the chemical, replace the medium with 100 μL/well of StemFlex medium.
10. Add the chemical stock to the StemFlex medium so that the final concentration of the solvent, PBS or DMSO, is at an upper limit of 10% and 1%, respectively. This will result in a solvent concentration with an upper limit of 1% and 0.1% for PBS and DMSO, respectively, after addition to the wells.

Note: For chemicals that are not fully soluble in the medium due to low water solubility, mix the chemical stock with DMSO and then add to StemFlex medium at the final concentration determined by the solubility test. The final concentration of DMSO in the medium should also be 1% in this case.

Note: The maximum concentration of the chemical to be used in the toxicity study was set at 1000 μg/mL in a previous study (Genschow et al., 2004). Thus, 10000 μg/mL is the upper concentration limit of the chemical solution before addition. This will result in a final concentration of 1000 μg/mL after addition to the wells.

11. Further, dilute the test chemical solution from step 10 with StemFlex medium to make a 2-fold dilution series and add 11 μL to each well. For non-treated groups, provide a final concentration of 1% PBS or 0.1% DMSO, depending on the solvent used for the test chemical. (0 h).
12. Twenty hours after the addition of the test chemical, add 10 μL/well of CCK-8, followed by incubation at 37°C with 5% CO_2 for 4 h. For all tests, prepare a cell-free control as a blank with only CCK-8 reagent added to the medium. (20 h).

13. Measure the absorbance at 450 nm in each well 4 h after CCK-8 addition. (24 h).

Note: Similar to the CCK-8 assay, MTT assay and resazurin assay can also be used to evaluate cell growth inhibition caused by chemical exposure with the reduced activity of living cells as an indicator.

14. Subtract the absorbance of the blank from that of each sample well. Compare the absorbance readings obtained under the experimental conditions with those of the vehicle control group and calculate the cell viability as follows:

$$Cell\ viability = \frac{Abs_{sample} - Abs_{blank}}{Abs_{vehicle} - Abs_{blank}}$$

15. Perform a total of at least three biological replicates and calculate the relationship between drug concentration and cell viability. Then, fit a four-parameter Log-Logistic curve using the LL.4 model of the drc package, an R language program, to estimate the 50% inhibitory concentration of viability (IC_{50}).

Signal disruption assay

Timing: 6 days

16. Four days before adding the ligand, seed the established cell lines in 96-well white plates at 1.0×10^4 cells/well.

Note: Because evaporation of the culture medium may adversely affect the assay, fill the outermost wells of a 96-well plate with sterile water and use only the central 60 wells for the assay.

17. The following day, replace the medium with 200 μL/well of StemFlex medium.
18. The day before ligand addition, replace the medium with 100 μL/well of APEL2 medium.

Note: StemFlex medium contains a variety of growth factors, including bFGF. To minimize the influence of components of the medium and to reproducibly assess FGF signal disruption, a recombinant protein-based medium that does not contain animal products such as bFGF should be used for the assay.

19. Replace the medium with 100 μL/well of APEL2 medium (+1% endurazine) at least 2 h before adding the test chemical.

Note: It takes 2 h for the luminescent substrate to equilibrate in/out of the cell.

20. Measure the luminescence intensity of each well immediately before adding the chemical.
21. Add the chemical stock to APEL2 medium so that the final concentration of the solvent, PBS or DMSO, is at an upper limit of 10% and 1%, respectively. This will result in a solvent concentration with an upper limit of 1% and 0.1% for PBS and DMSO, respectively, after addition to the wells.

Note: For chemicals that do not completely dissolve in the medium due to low water solubility, the chemical stock should be mixed with DMSO and then added to the APEL2 medium at the final concentration determined by the solubility test described above. In this case, the final concentration of DMSO in the medium should also be 1%.

Note: The maximum concentration of chemicals to be used in the toxicity study was set to 1000 μg/mL in a previous study (Genschow et al., 2004). Thus, 10000 μg/mL is the upper concentration limit of the chemical solution before addition. This will result in a final concentration of 1000 μg/mL after addition to the wells.

Note: For chemicals with strong cytotoxicity, the half growth inhibitory concentration (IC_{50}) estimated from the cytotoxicity test should be used as the final concentration after addition to the wells.

22. Further, dilute the test chemical solution from step 21 with APEL2 medium to make a 2-fold dilution series with a total of 8 concentrations per chemical and add 11 μL to each well. For non-treated groups, administer a final concentration of 1% PBS or 0.1% DMSO, depending on the solvent of the test chemical. (-1 h).
23. Measure the luminescence intensity of each well immediately before ligand addition.
24. Add 12 μL/well of bFGF diluted to 20 ng/mL in APEL2 medium to a final concentration of 2 ng/mL. For non-treated groups, prepare a solution of PBS (+ 0.1% BSA) and the bFGF solvent diluted 1000-fold in APEL2 medium and add 12 μL/well. (0 h).
25. Measure the luminescence intensity of each well at repeated time intervals. The manufacturer's manual indicates that the luminescence can be stably detected for up to 72 h. See troubleshooting 3 and 4.

EXPECTED OUTCOMES

Signal disruption assay and calculation of area between the curves

After bFGF stimulation, the intracellular signaling activity should increase over 4–6 h, followed by slow decay. At this time, pre-exposure to signal-disrupting chemicals will result in signal kinetics that deviates from those of the control group. After the standardization process, the area between the curves (ABC) of the control group and the chemical-exposed group can be calculated to show the direction of the enhancement or diminution of the signal disrupting effect of the chemical and to quantify its strength (Figure 2A). An example of known developmental toxicants, all-*trans*-Retinoic acid (ATRA) and methylmercury chloride (MeHg), following exposure to signal reporter cells, is shown in Figures 2B and 2C. The effects should vary depending on the test chemical, such as negative impacts on the FGF signaling pathway in the case of ATRA or positive impacts in the case of MeHg. Furthermore, the dynamics of the disruption are expected to be quite different among chemicals. Exposure to ATRA tends to increase the extent of the signal disruption for up to 10 h and maintain the dysregulated level thereafter (Figure 2B). In contrast, exposure to MeHg causes maximal disruption early, at ~4 h, after which the extent of the disruption decreases (Figure 2C). By monitoring signal disruption and calculating ABC, we should be able to detect any type of signal disruption with high sensitivity (Figures 2B and 2C).

QUANTIFICATION AND STATISTICAL ANALYSIS

Calculation of area between the curves

1. Calculate the relative light unit (RLU) by dividing the luminescence intensity of each well at each time point by that of the non-treated group (solvent for the test chemical + solvent for bFGF).
2. Calculate the log ratio of the RLU of the chemical-exposed group to that of the untreated group (log [Fold change (FC)]) as follows:

$$\log FC = \log\left(\frac{Luc_{sample}}{Luc_{vehicle}}\right)$$

3. Fit the log FC time-series data to a smoothed spline curve using the gam function in the mgcv package of the R programming language.

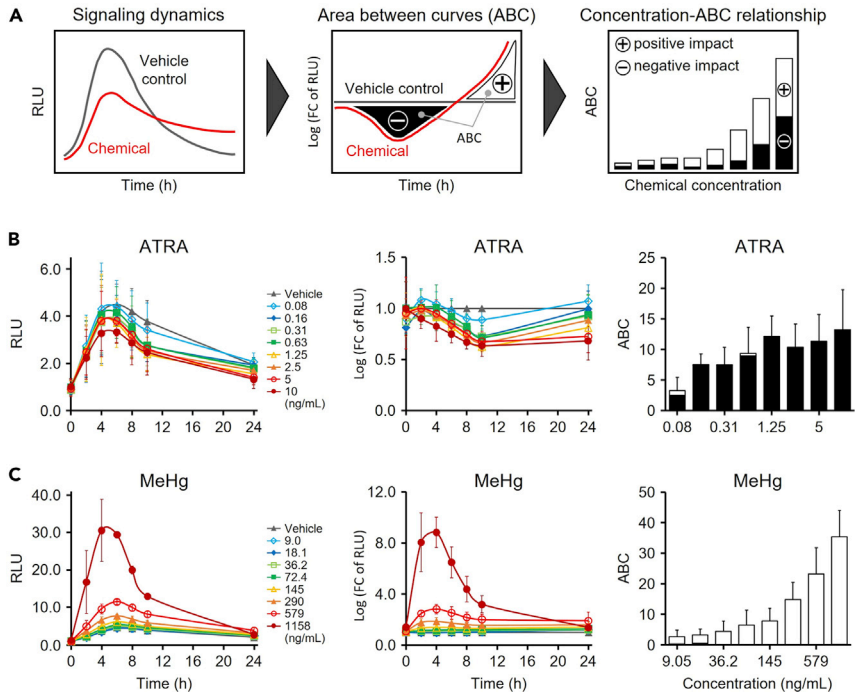

Figure 2. Quantification of signal disruption caused by a chemical

(A) Steps to calculate signal disruption. The iPSC reporters are stimulated with FGF, and time-course changes in luminescent intensity are measured in the presence of a potential developmental toxicant. Relative light unit (RLU) is calculated by dividing the resulting luminescent intensity by that without FGF stimulation at each time point. The log [fold change (FC)] is calculated by dividing the RLU value of the chemical exposure group by that of the vehicle control group and converting that to a binary logarithm. Smoothed spline curves are plotted to fit the time-course data of the log FC and used to estimate the area between curves (ABC).

(B and C) Example results of two developmental toxicants: all-*trans*-retinoic acid (ATRA) and methylmercury chloride (MeHg). The reporter cells were exposed to ATRA or MeHg at different concentrations or to the vehicle 1 h before (-1 h) treatment with bFGF (0 h). The live-cell luciferase assay was performed over 24 h (0–24 h). Time-course changes in the luminescent intensity were normalized and log-transformed to calculate ABC. Positive and negative impacts on signaling were observed with MeHg and ATRA, respectively. Data are presented as the mean ± S.D. (n = 3).

4. Based on the fitted function, calculate the distance between the two curves of the untreated group and the chemical-exposed group at the time point t_i as follows:

$$h_i = |a(t_i) - b(t_i)|$$

Note: At this point, the magnitudes of each effect of signal enhancement and attenuation can be calculated later by distinguishing whether the curve of the chemical exposure group exists in the upper or lower direction relative to the solvent control group.

5. The area of a small region of Δt is calculated by trapezoidal approximation, and ABC at each chemical concentration is calculated by integrating it as follows:

$$ABC = \sum_i \left[\frac{h_{i+1} + h_i}{2}\right] \Delta t_i$$

6. Furthermore, the sum of ABC is calculated using the same procedure (Figure 3A). Calculate the area of a small region of concentration Δc by trapezoidal approximation based on the relationship between chemical concentration and ABC, and then integrate it as follows:

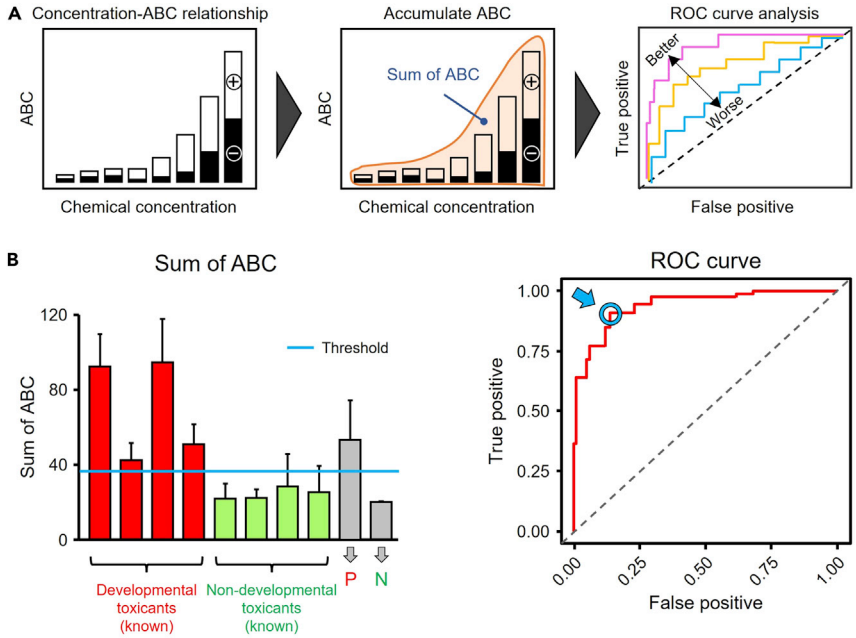

Figure 3. Threshold setting with receiver operating characteristic (ROC) curve analysis to determine teratogenic potential

(A) Steps for ROC curve analysis. The sum of ABC is calculated by accumulating ABC values at different concentrations, which is then used for plotting ROC curve (see (B)). The larger area under the ROC curve (AUC) represents the better classification performance of toxic and non-toxic chemicals.

(B) Drawing of ROC curves and determination of teratogenicity. Each sum of ABC for developmental and non-developmental toxicants is presented in a single graph. The detection accuracy (true positive and false positive) is calculated by changing the threshold value against the sum of ABC, which is plotted to draw the ROC curve. The optimal threshold of the ROC curve is the point closest to the upper left corner on the curve (arrow). The optimal threshold is used to determine the teratogenic potential of unknown chemicals.

$$Sum\ of\ ABC = \sum_i \left[\frac{h_{i+1} + h_i}{2}\right] \Delta C_i$$

Calculation of threshold and toxicological judgment

7. To identify the optimal threshold for prediction, perform a receiver operating characteristic (ROC) curve analysis based on a logistic regression model using the R package "pROC." Determine the threshold by calculating the closest point to the upper left corner of the ROC curve (the point at true positive 1.0 and false positive 0). See Figure 3B.
8. Determine the test chemicals whose sum of ABCs exceeds the threshold as developmental toxicants. See Figure 3B. See troubleshooting 5.

Note: In this statistical analysis, the effect size was measured using Hedges' g statistic, which indicates the degree of difference between two values independent of the sample size (Sullivan and Feinn, 2012). An effect size value of 0.2 or more indicates a small but important difference (Brydges, 2019). The signal disruption test is performed using chemicals with unknown developmental toxicity, and the test chemical is judged to be a positive substance if its sum of ABC exceeds the threshold with an effect size on Hedges' g > 0.2, and a negative substance if it does not.

LIMITATIONS

The signal disruption assay uses a luciferase reporter to evaluate the effect of a test chemical on intracellular FGF signaling activity. The luminescent response of this reporter may depend not only on the direct effect of the test chemicals on intracellular signal transduction but also on the stability of the test chemicals in an aqueous solution, cell membrane permeability, and the effect on the luciferase enzyme-substrate reaction. To confirm the membrane permeability of a test substance, estimation methods based on the physical property profile (Lipinski et al., 2001), Caco-2 model (Balimane et al., 2000), or cell-free permeation systems (Berben et al., 2018) have been reported. Chemicals that exhibit low cell membrane permeability under *in vitro* conditions are difficult to evaluate in signal disruption assays. The stability of the test chemical in an aqueous solution can be checked by measuring the temporal change in concentration using techniques such as high-performance liquid chromatography.

The effect of the test chemical on the luciferase enzyme-substrate reaction rather than intracellular signal transduction can be confirmed by using inhibition assays with purified luciferase (Thorne et al., 2012) or a stable cell line expressing luciferase downstream of a ubiquitous promoter such as CMV or CAG. If the test chemical affects the luciferase enzyme-substrate reaction, it is difficult to accurately evaluate it using the signal disruption assay.

The AAVS1 region, a safe harbor, is used as a knock-in target for the reporter gene. However, the AAVS1 locus contains the coding region for myosin-binding subunit 85 (MBS85). It has been reported that gene transfer to the AAVS1 region and subsequent downregulation of MBS85 alters myosin II-dependent cell contractility (Mizutani et al., 2015). It is necessary to account for the possible effect of this on the ability of this assay to detect developmental toxicity.

TROUBLESHOOTING

Problem 1

Adding puromycin to establish a cell line results in no cell survival and no cell colony formation.

Potential solution

In cases of poor knock-in due to degradation of gRNA or reduced enzymatic activity of Cas9, prepare a new reagent. Weakened cell activity due to electroporation or donor DNA cytotoxicity may make the cells more vulnerable to puromycin. Examine puromycin concentrations as described in step 7 of "preparation of stable transgenic iPSCs."

Problem 2

Too many cells survive after the addition of puromycin, colonies form in close proximity to each other, or colonies fuse with each other, making it impossible to pick up a single colony.

Potential solution

The puromycin concentration may be too low. A negative control group without donor DNA knock-in should be prepared, and the concentration at which the cells do not survive should be selected. The number of cells seeded in a well may be too large. A single colony can be picked up by decreasing the number of seeded cells so that the colonies are farther apart from each other.

Problem 3

Erratic measurement values are obtained on the same plate between wells that are undergoing the same treatment.

Potential solution

Variations may exist in the number of cells between wells. Before starting the assay, ensure that cells are growing evenly in each well of the same plate. Wells with extremely low or high cell numbers should not be used in the assay. Additionally, because luminescence is affected by exposure to

irradiation such as fluorescent light, cells should be shielded from light for several minutes before measurement with a luminometer by placing aluminum foil on the lid of the culture plate. Variations may also arise from pipetting errors that occur when adding reagents. Periodically calibrate pipettes and keep the pipette tilt constant when adding reagents and ensure that no bubbles are introduced. If bubbles are present in the medium, the luminescence intensity cannot be measured accurately by luminometers that measure luminescence from the top of the well.

Problem 4

Low luminous intensity is detected.

Potential solution

When measuring luminescence intensity from the top of the well, check for bubbles on the surface of the medium. If the luminescence intensity does not increase up to 3–4 fold after FGF treatment, the bioactivity of FGF may have been lost. Please prepare new reagents. Extending the detection time of luminescence to 5–10 s may help to detect a sufficient amount of luminescence intensity.

Problem 5

The signal disruption caused by chemical exposure cannot be detected by the signal disruption test.

Potential solution

If the FGF concentration is too high, the signal attenuation may be canceled out, and the effect of the chemical may be underestimated. To solve this problem, conduct preliminary tests with inhibitors (e.g., PD0325901, CCG-203971) or chemicals with known signal attenuating effects (ATRA) to determine the FGF concentration at which the effects can be detected.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Junji Fukuda (fukuda@ynu.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This study did not generate a new dataset. All data reported in this paper will be shared by the lead contact upon request.
- All original code has been deposited to Mendeley Data and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the reported data is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Software, S.K. and L.Y.; methodology, S.K., Y.O., and J.F.; writing – original draft, M.K., S.K., K.M., Y.O., T.K., and J.F.; writing – review & editing, M.K., S.K., K.M., Y.O., T.K., and J.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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