Chemical ablation of tumor-initiating human pluripotent stem cells

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The tumorigenicity of human pluripotent stem cells (hPSCs) is widely acknowledged as a major obstacle that withholds their application in regenerative medicine. This protocol describes two efficient and robust ways to chemically eliminate the tumor-initiating hPSCs from monolayer culture. The protocol details how to maintain and differentiate hPSCs, how to apply chemical inhibitors to cultures of hPSCs and their differentiated progeny, and how to assess the purity of the resultant cell cultures using *in vitro* and *in vivo* assays. It also describes how to rescue the cytotoxic effect. The elimination and the rescue assay can be completed within 3–5 d, the *in vitro* assessment requires another day, and the *in vivo* assessment requires up to 12 additional weeks.

INTRODUCTION

The unique ability of hPSCs to self-renew and to differentiate into the various cell types of the human body makes them exceptionally promising for regenerative medicine[1]. However, the same properties also make hPSCs tumorigenic, enabling them to generate teratomas after their *in vivo* injection[2]. One solution proposed to circumvent the tumorigenic properties of hPSCs when using them in regenerative medicine is to ensure that the cells transplanted into patients are fully differentiated cell types (e.g., mature cardiomyocytes or dopaminergic neurons). However, it has been demonstrated that even after prolonged *in vitro* differentiation protocols, residual undifferentiated cells remain in culture and retain their tumorigenicity[3,4], and that these residual pluripotent cells could be sufficient for teratoma formation[5,6]. Thus, it cannot be assumed that differentiation is 100% complete, and further safeguards need to be put in place to ensure that tumorigenic hPSCs are not transplanted into patients. Consequently, strategies for the removal of residual undifferentiated hPSCs from cultures of their differentiated progeny are essential for the safe implementation of hPSC-based cell therapies (reviewed in ref. 2).

In recent years, several methodologies have been suggested for the separation or elimination of hPSCs. These methods include the following:

- The use of immunologic targeting on the basis of pluripotent-specific antigens, for cell sorting of hPSCs[7–10] or for their elimination with cytotoxic antibodies[11,12].
- Genetically manipulating hPSCs to interfere with tumor-progression genes[13,14], to introduce 'suicide' genes[15,16], or to enable labeling and separation[17–19].
- Pharmacologically ablating the tumorigenic cells to enable efficient, robust and inexpensive hPSC targeting[7,20–23].

In this paper, we describe a protocol that we have developed to chemically ablate tumor-initiating hPSCs, by exploiting the unique protein expression and metabolic dependencies of the pluripotent state.

Chemical ablation of hPSCs

By performing an unbiased high-throughput screen of small molecules against hPSCs and various other cell types, we uncovered a unique vulnerability of hPSCs to perturbation in the biosynthesis of oleate. We showed that pharmacological inhibition of stearoyl-coA desaturase (SCD1), the key enzyme in oleate biosynthesis, efficiently removes undifferentiated cells from culture and prevents teratoma formation from these cells[20]. By implementing a complementary strategy, we performed a comprehensive gene expression analysis, and we identified the tight-junction protein claudin-6 as a specific cell surface marker of hPSCs. We then showed that clostridium perfringens enterotoxin (CPE), a toxin that binds several claudins including claudin-6, can efficiently kill undifferentiated hPSCs, thus eliminating the tumorigenic potential of hPSC-containing cultures[7]. Importantly, the protocol is equally relevant to human embryonic stem cells (hESCs) and to human induced PSCs (hiPSCs), regardless of their cellular origin and specific derivation technique. The protocol has been successfully applied to various differentiation protocols, including directed differentiation into neural, cardiac and endodermal cell types.

The simple chemical protocol presented here can be easily applied without specialist equipment for fast, robust and inexpensive elimination of undifferentiated hPSCs from culture. Specifically, this protocol could be of interest to investigators engaged in tumorigenicity studies of hPSCs, and to those involved in preclinical and clinical experiments with hPSC-derived cell types. Moreover, hPSC-free cultures could be valuable for investigators who are interested in pure cultures of differentiated cells for diverse purposes, such as disease modeling and drug screening. Therefore, this protocol could be useful for anyone interested in working with fully differentiated, hPSC-free cultures.

In principle, the methods described in this protocol should enable the elimination of PSCs of other species, including mouse, as inhibition of SCD1 has been shown to induce cell death in mouse PSCs[20], and claudin-6 has been shown to be a pluripotent marker of mouse PSCs as well[24]; however, the current protocol focuses on hPSCs, on which these methods have been extensively studied. Of note, although the current protocol could be combined with many differentiation protocols, the degree of purity that can be achieved, as well as the optimal exposure concentrations and durations, must be assessed for each particular cell type and medium conditions. In addition, it is important to validate

experimentally, as described in the protocol, that the chemical exposure does not jeopardize the viability and the functionality of the desired differentiated cells under the unique experimental conditions applied.

This protocol is intended for basic research and preclinical studies. Although it should be readily adjustable to clinical settings, several issues ought to be considered for such adaptation. First, clinical trials would require adaptation of the protocol to high-scale, clinically relevant differentiation processes. Second, further tests would be necessary to ensure that the differentiated cells remain fully functional (for example, electrophysiological assessments of neurons, insulin production in beta cells, etc.). Third, the teratoma formation assay in immunodeficient mice might not be sufficient for clinical applications, owing to the hostile xeno-microenvironment and lack of human inflammatory response, which might inhibit efficient teratoma formation in the mouse model; other animal models, such as 'humanized' mice, may thus be required as well.

Advantages and limitations in comparison with other methods

The methods for the removal of undifferentiated cells from culture can be divided into three categories: immunologic, genetic and chemical (**Fig. 1** and **Table 1**).

The immunologic methods can be further divided into three subcategories: separation by cell sorting by using antibodies against pluripotent-specific epitopes[7–10] or against specific epitopes of the differentiated cell type of interest[25,26]; elimination with primary cytotoxic antibodies against pluripotent-specific cell surface molecules[11,12]; elimination with secondary cytotoxic antibodies conjugated to primary antibodies against such pluripotent-specific molecules[7]; or elimination by photoablation by using nanogold particles targeted against antibodies that selectively mark pluripotent cells[27]. These methods are simple and rapid; however, their efficiency is currently limited, and multiple cycles of separation are normally necessary for effective elimination of undifferentiated cells from culture[9,28]. Moreover, these methods require single-cell dissociation, which is not compatible with some differentiation protocols. Cytotoxic antibodies may also lead to collateral damage in the nonpluripotent cell population.

The methods that are based on genetic manipulation can also be further divided into several groups: cell sorting on the basis of genetic labeling of pluripotent-specific genes[17] or labeling of genes that are specific for the desired differentiated cell type[18,19]; knocking out or knocking down genes that are required for teratoma development (but are dispensable for the differentiation protocol)[13]; overexpression of tumor suppressors[14]; and introduction of 'suicide' genes under constitutive[15] or pluripotent-specific[16] promoters. The main advantage of the genetic approach is that the manipulated cells are easily traceable. However, these methods are much more laborious and expensive, and they pose additional safety risks owing to potential off-target damages to the DNA. Cell sorting after genetic labeling also shares the caveats of antibody-based cell sorting (i.e., dissociation into single cells is required, and separation efficiency is currently restricted).

The chemical ablation of hPSCs can be achieved by supplementing the culture medium with small molecules that selectively kill undifferentiated hPSCs[20–29], by exposing the cells to a toxin that targets cell surface proteins selectively expressed on

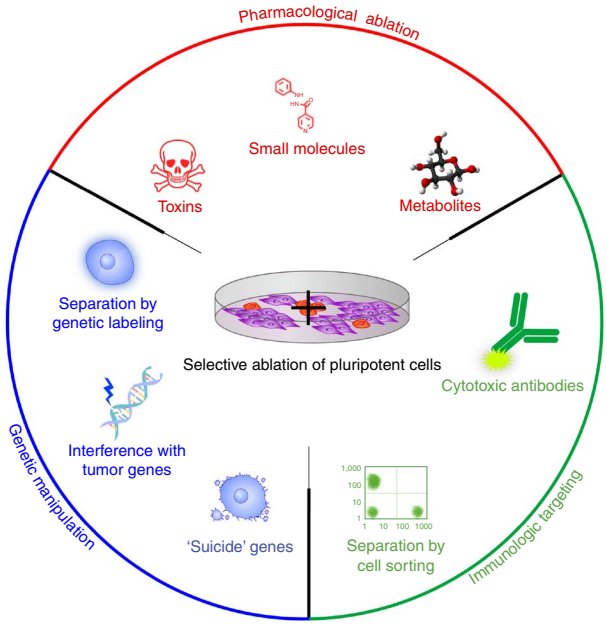

Figure 1 | Strategies for eliminating tumor-initiating human pluripotent stem cells. Residual undifferentiated hPSCs (depicted in red) are presented at the center of a sniper's scope, in an attempt to remove them from a differentiated cell population (depicted in purple). Three main approaches have been described to selectively eliminate hPSCs from culture: genetic manipulation (in blue), immunologic targeting (in green) and pharmacological ablation (in red). For each of these general approaches, the most prominent elimination strategies are presented. See the text and **Table 1** for a detailed comparison of these strategies.

hPSCs[7] or by exchanging the metabolites in the medium to create metabolic conditions that inhibit the survival of hPSCs[22,30]. The chemical ablation of hPSCs has the following advantages over the other techniques:

- It enables the proactive removal of undifferentiated tumorigenic cells, preventing the risk rather than dealing with its consequences.
- No genetic manipulation is necessary.
- Compared with cell sorting, this approach is more efficient, and it does not require single-cell dissociation, which may be advantageous in various clinical settings (for example, differentiation into complex structures).
- The chemical approach is cheaper than any other technique, it works faster than most techniques, and its application does not require any technical expertise.

In addition, although the methods based on cell sorting are only applicable *in vitro*, before cell transplantation, the pharmacological ablation (and some of the genetic manipulation approaches) may also be useful *in vivo*, for eliminating residual hPSCs after the injection of their derivatives, and even for eliminating hPSC-derived tumors after formation. Naturally, such potential *in vivo* applications warrant more research to enable their clinical translation.

The chemical approach also has its weaknesses: first, the chemical methods highly depend on culture conditions and medium compositions, so that the exact exposure durations and concentrations ought to be scaled for every cell type and differentiation

TABLE 1 | Strategies to eliminate tumor-initiating human pluripotent stem cells.

	Chemical ablation			Immunologic separation and elimination			Genetic manipulation			
Suggested approach	Small molecules	Toxins	Metabolites	Cell sorting with cell-surface markers	Primary cytotoxic antibodies	Secondary cytotoxic antibodies	Cell sorting with genetic labeling	KO/KD of teratoma genes	OE of tumor suppressors	Suicide gene introduction
Major examples	PluriSIns[20] Ceramide analogues[29] MitoBloCK-6 (ref. 21) Survivin inhibitors[23]	CPE[7]	Oleate depletion[20] Glucose depletion[22,30]	Claudin 6 (CLDN-6; ref. 7) TRA-1–60 (ref. 8) SSEA-3 (ref. 8) SSEA-5 (ref. 9) Lectins[10] Signal regulatory protein α (SIRPA)[25]	Podocalyxin-like protein 1 (PODXL)[11,12]	CLDN-6 (ref. 7) TRA-1–60 (ref. 27)	REX-1 (ref. 17) SOX-1 (ref. 18) MYL-2 (ref. 19)	Survivin[13]	p53 (ref. 14) Ink4/ARF[14]	HSV-tk: Constitutive promoter[15] NANOG promoter[16]
Main advantages	Proactive No genetic manipulation No single-cell dissociation Robust and efficient Fast, simple and inexpensive			Fast and simple			Easily traceable			
Main weaknesses	Potential nonspecific effects on differentiated cells Conditions need to be adapted for each differentiation protocol			—	Collateral damage		Requires single-cell dissociation Limited efficiency	—		
				Requires single-cell dissociation Limited efficiency (need for multiple cycles)			Risk for nonspecific genetic damages Laborious and expensive			

This table presents the main approaches currently available for removal of undifferentiated hPSCs from culture. Major examples from each category are mentioned, together with their key advantages and weaknesses. KO, knockout; KD, knockdown; OE, overexpression; HSV-tk, herpes simplex virus thymidine kinase type 1.

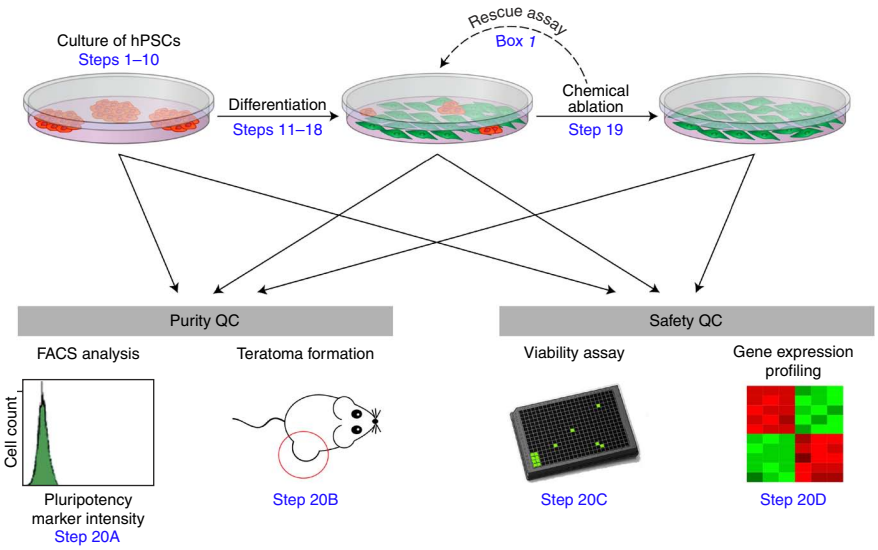

Figure 2 | A schematic overview of the chemical ablation methods described in the protocol. A schematic representation of the procedure: undifferentiated cells are cultured and maintained (Steps 1–10), differentiated into the desired cell type (Steps 11–18) and residual undifferentiated hPSCs are then chemically ablated by PluriSIn #1 or by CPE (Step 19). Next, cells are subjected to two types of quality controls (QC): purity QC, comprising an *in vitro* FACS analysis (Step 20A) and an *in vivo* teratoma formation assay (Step 20B); and safety QC, comprising a viability assay (Step 20C) and gene expression profiling (Step 20D). Untreated differentiated and undifferentiated cells are used as controls. Cytotoxicity rescue assay (**Box 1**) can be conducted to validate the cause of death.

protocol; second, to ensure that the differentiated cells remain intact and functional, the potentially deleterious effect of transient SCD1 inhibition or CPE exposure on differentiated cell cultures must also be evaluated separately for each cell type and differentiation protocol; third, this protocol is not suitable for separating undifferentiated hPSCs from their immediate derivatives, as a differentiation protocol of a few hours (or even a couple of days) may not be sufficient to completely abolish the cellular sensitivity to the chemicals. For clinical purposes, it will also be necessary to ensure that the chemical exposure does not hinder the engraftment of the cells. Despite these limitations, once they are scaled and validated for a particular differentiation protocol of interest, these chemical methods are probably the most cost-effective techniques currently available for the elimination of residual undifferentiated cells.

Experimental design

This protocol describes the chemical removal of hPSCs from culture, as schematically presented in **Figure 2**, and comprises three main parts: culture and differentiation of cells (Steps 1–18); chemical ablation of hPSCs within the culture (Step 19); and confirmation that the hPSCs have been successfully removed from the culture (Step 20).

Culture and differentiation of hESCs or hiPSCs. The protocol starts with the culture of hESCs or hiPSCs as undifferentiated cells (Steps 1–10), followed by their monolayer differentiation (Steps 11–18). In recent years, directed differentiation of hPSCs has advanced considerably (reviewed in ref. 31), resulting in many detailed differentiation protocols published[32–35]. As mentioned above, both of the methods described in this protocol can be adapted to various such differentiation protocols. For the sake of simplicity, however, the PROCEDURE below describes the elimination of residual undifferentiated hPSCs after spontaneous monolayer differentiation by fibroblast growth factor 2 (FGF2) withdrawal from the medium.

Chemical ablation of hPSCs. In the next part of the protocol, cells are exposed to the SCD1 inhibitor PluriSIn #1 (Step 19A) or to the toxin CPE (Step 19B). Note that these steps are mutually exclusive, so that cells are exposed either to PluriSIn #1 or to CPE, but not to both.

A direct comparison of SCD1 inhibition (Step 19A) and claudin-6 toxin-based targeting (Step 19B) brings up several important differences. Whereas the SCD1 inhibition-based elimination is relevant for differentiation protocols toward various cell types of all three embryonic germ layers[20], the CPE-based method is restricted to cell types that do not express any of the CPE-sensitive claudins[7], precluding its application to endodermal and other epithelial cell lineages. Other advantages of small molecules that inhibit SCD1, such as PluriSIn #1, over the CPE toxin, are the markedly lower costs of small molecules compared with proteins, and their easier penetration into 3D cellular structures. The advantage of CPE over SCD1 inhibition, in contrast, is its extremely fast and efficient mode of action, which allows obtaining fully purged cultures within 30–60 min[7]. The selection between these two options therefore depends on the particular desired application, and it should be guided by the aforementioned considerations.

The differentiated cell types present in culture, the culture conditions (such as the size of the culture plate) and the composition of the culture medium (and especially the oleate concentration in the medium) all influence the exposure duration and the concentration required for robust elimination of undifferentiated cells, and it should thus be determined in advance. The optimal experimental setting would be to expose the cells to the minimal compound/toxin concentration and for the minimal time duration that efficiently eliminate undifferentiated hPSCs without affecting their differentiated progeny. The PROCEDURE described below is based on culturing the cells in six-well culture plates with standard embryonic stem cell culture medium without FGF2, and on the 48–72-h exposure to PluriSIn #1 at 20 μM or 1-h exposure to CPE at 1 μg ml^{-1}. Other SCD1 inhibitors can be used instead of PluriSIn #1, but this could substantially change the necessary exposure duration and concentration.

Confirmation that hPSCs have been eliminated. The final part of the protocol is assessment of the success of hPSC elimination. This can be assessed *in vitro* by FACS analysis for pluripotency markers (Step 20A), and also *in vivo* by a teratoma formation assay in immune-compromised mice (Step 20B). In parallel to

Box 1 | Cytotoxicity rescue assay for SCD1 inhibition ● TIMING 1 d

If you have selected PROCEDURE Step 19A, an additional rescue experiment may be optionally performed. This assay is especially useful if one is interested in examining a new compound of interest that is suspected to kill undifferentiated hPSCs by inhibiting their SCD1 activity.

REAGENTS

- hPSCs (either hESCs or hiPSCs) ! CAUTION All relevant institutional and governmental regulations for the use of hPSCs must be followed.
- mTeSR medium (STEMCELL, cat. no. 05850) and spontaneous differentiation medium (Reagent Setup)
- Oleic acid (e.g., Sigma-Aldrich, cat. no. O1008)
- BSA (e.g., Sigma-Aldrich, cat. no. A7906) CellTiter-Glo luminescent cell viability assay (Promega, cat. no. G7570)

PROCEDURE

1. Perform Step 19A as described above, but when adding PluriSIn #1 to the culture medium also add oleate. Use five concentrations of oleate, ranging from 0.4 to 100 µM (with fourfold serial dilutions).

▲ CRITICAL STEP When using BSA-conjugated oleate, add the same concentrations of BSA to the control plates.

2. Perform Steps 20A and 20B to validate that supplementation of oleate can reverse the cytotoxic effect of PluriSIn #1.

performing these quality control analyses on the purged cultures, they should also be performed on differentiated hPSCs that are not subjected to selective chemical ablation (differentiated controls) and on undifferentiated hPSCs (undifferentiated controls). The safety of the protocol for the differentiated progeny can be assessed by measuring their viability (Step 20C) and by comparing the global gene expression profiles of exposed versus control cells (Step 20D). In some cases, further assays might help determine the lack of cytotoxicity to the cells of interest. Such assays include proliferation assays (for proliferating cells) or functional assays (for functional differentiated cells), but they are not detailed in the PROCEDURE.

Finally, for SCD1 inhibition only, a cytotoxicity rescue assay is described (**Box 1**). This assay is optional, and it does not need to be performed routinely. This assay is especially useful if one is interested in examining a compound of interest that is suspected to kill undifferentiated hPSCs by inhibiting their SCD1 activity.

MATERIALS

REAGENTS

- hPSCs (either hESCs or hiPSCs) ! CAUTION All relevant institutional and governmental regulations for the use of hPSCs must be followed.
- mTeSR medium (STEMCELL, cat. no. 05850) and spontaneous differentiation medium (Reagent Setup)
- Accutase (e.g., Sigma-Aldrich, cat. no. A6964)
- Matrigel (e.g., BD Biosciences, cat. no. 356234; see Reagent Setup)
- Gelatin solution, 0.1% (wt/vol) (e.g., Millipore, cat. no. ES-006-B)
- KnockOut DMEM (e.g., Invitrogen, cat. no. 10829-018)
- KnockOut serum replacement (KSR; e.g., Invitrogen, cat. no. 10828-028)
- L-Glutamine (e.g., Invitrogen, cat. no. 21051-016)
- Penicillin-streptomycin liquid (e.g., Invitrogen, cat. no. 15140122)
- MEM minimum non-essential amino acid solution (e.g., Invitrogen, cat. no. 11140-050)
- 2-Mercaptoethanol (e.g., Invitrogen, cat. no. 21985-023) ! CAUTION 2-Mercaptoethanol is toxic if inhaled, ingested or when in contact with skin. Wear gloves while working with it and handle it with care.
- PBS (e.g., Invitrogen, cat. no. 10010-015)
- DMSO (e.g., Sigma-Aldrich, cat. no. D2650)
- PluriSIn #1 if performing Step 19A (e.g., Sigma-Aldrich, cat. no. SML0682; see Reagent Setup) ! CAUTION Avoid contact and ingestion of PluriSIn #1. Wear gloves while working with it and handle it with care.
- Clostridium perfringens enterotoxin A protein (CPE), if performing Step 19B (Abcam, cat. no. ab63833; see Reagent Setup) ! CAUTION CPE is toxic if inhaled, ingested or when in contact with skin. Wear gloves working with it and handle it with care.
- Trypsin-EDTA, 0.25% (wt/vol) (e.g., Invitrogen, cat. no. 25200-056)
- Trypan blue solution (e.g., Sigma-Aldrich, cat. no. T8154)
- Conjugated anti-human TRA-1-60-PE antibody, if performing Step 20A (e.g., BD Biosciences, cat. no. 560884) ▲ CRITICAL Store it at 4 °C.
- BSA, if performing Step 20A (e.g., Sigma-Aldrich, cat. no. A7906)
- NOD.Cg-*Prkdc*^{scid}*il2rg*^{tm1Wjl}/SzJ (NSG) mice, if performing Step 20B (Jackson Laboratory, cat. no. 005557) ! CAUTION All experiments using mice must be performed in accordance with all institutional and governmental ethics and animal handling requirements.
- Ketamine, if performing Step 20B (Ketaset; Fort Dodge Animal Health, cat. no. 440761)
- Medetomidine, if performing Step 20B (Domitor; Pfizer Animal Health)
- Isoflurane, if performing Step 20B (e.g., Santa Cruz, cat. no. sc-363629Rx) ! CAUTION Isoflurane is toxic if inhaled or ingested; work with it inside a chemical fume hood and handle it with care.
- Tissue-Tek O.C.T. compound, if performing Step 20B (Sakura Finetek, cat. no. 4583)
- CellTiter-Glo luminescent cell viability assay, if performing Step 20C (Promega, cat. no. G7570)
- PerfectPure RNA cultured cell kit, if performing Step 20D (5 Prime, cat. no. 2302330)
- GeneChip human gene 1.0 ST array, if performing Step 20D (Affymetrix, cat. no. 901085)

EQUIPMENT

- Sterile biosafety tissue culture hood
- Chemical fume hood
- Sterile tissue culture incubator (37 °C, 5% CO_2 and 100% humidity)
- Cell culture centrifuge
- Plate-adapted inverted microscope
- Glass hemocytometer or automatic cell counter
- Refrigerator (4 °C) to store media

- Freezer (–20 °C) °C) to store Matrigel and PluriSIn #1 aliquots
- Freezer (–80 °C) to store CPE aliquots
- Sterile gloves, pipettes and filter tips
- Cell culture dishes (six-well plates) to grow, expand and purge hPSCs
- Conical centrifuge tubes (15 ml) to passage the cells
- Laboratory shaker
- Single-use filters (70 μm)
- Syringe, 1 ml, 26 gauge
- FACSAria III or similar flow cytometer
- FACS tubes (12 × 75 mm round-bottom test tubes)
- Eppendorf tubes
- Desiccator jar
- Surgical scissors
- Disposable plastic mold (e.g., Tissue-Tek Cryomold Intermediate, Sakura Finetek, cat. no. 4566)
- Luminescence plate reader
- PC with Windows operating system and internet connection
- Expander program (http://acgt.cs.tau.ac.il/expander/)

REAGENT SETUP

Spontaneous differentiation medium Mix KnockOut DMEM (410 ml), 15% (vol/vol) KSR (75 ml), 1% (vol/vol) L-glutamine (5 ml), 0.5% (vol/vol) penicillin-streptomycin (2.5 ml), 1% (vol/vol) 10 mM MEM minimum non-essential amino acid solution (5 ml), 0.01% (vol/vol) 1 M 2-mercaptoethanol (50 μl). ▲ **CRITICAL** This medium should be kept at 4 °C and used within 3 weeks.

Matrigel aliquot preparation Leave the bottle from the manufacturer to thaw overnight on ice at 4 °C. Divide the solution into aliquots and store them at −80 °C for up to 6 months. ▲ **CRITICAL** Each aliquot should be thawed overnight on ice at 4 °C when needed and kept on ice until it is dispensed in cell culture dishes. Matrigel freezes at −20 °C or 80 °C, stays in the liquid form if maintained on ice or at 4 °C and starts solidifying at room temperature (RT, ~25 °C) or in an incubator.

PluriSIn #1 stock solution Dissolve 1 mg of PluriSIn #1 in 469 μl of DMSO to prepare a stock concentration of 10 mM. ▲ **CRITICAL** Check that the molecule is well dissolved, divide the solution into aliquots and store them at −20 °C. This solution can be stored for a few months provided that repeated freeze-thaw cycles are avoided.

CPE aliquot preparation Prepare aliquots on arrival. ▲ **CRITICAL** Store the aliquots at −80 °C and avoid repeated freeze-thaw cycles. The aliquots can be stored for a few months under these conditions.

FACS buffer Dissolve 5 g of BSA (1% (wt/vol)) in 500 ml of PBS (1×). Store the solution at 4 °C for up to 1 month.

Ketamine/Domitor mixture Mix 0.83 ml of PBS, 0.25 ml of Domitor (1 mg ml^{-1}) and 0.165 ml of ketamine (100 mg ml^{-1}). ▲ **CRITICAL** Freshly prepare the mixture before use.

EQUIPMENT SETUP

Expander program Install the Expander program on your computer according to the instructions provided in ref. 36.

PROCEDURE

Culturing hPSCs on Matrigel-coated plates ● TIMING 1–2 weeks

1| Thaw Matrigel aliquots (Reagent Setup) overnight on ice at 4 °C. Dilute the Matrigel in DMEM at a ratio of 1:20 (vol/vol).

2| Coat six-well plates with 0.5 ml per well of thawed Matrigel. Prepare extra plates to be used to grow undifferentiated hPSCs for the FACS analysis and teratoma formation assays (plates to be used as controls in Steps 20A and 20B).
▲ **CRITICAL STEP** Matrigel aliquots should be kept on ice until they are added to the plates.

3| Transfer the plates to a shaker and leave them to rock at RT for 1–3 h. While the Matrigel is polymerizing, perform Steps 4–7.

4| Starting with hPSC plates at 75–80% confluence, aspirate the medium, and then wash the cells with Ca^{2+}/Mg^{2+}-free PBS. 1ml per well of PBS is sufficient for a six-well plate.

5| Add 0.5 ml per well of Accutase and put the plates back into the 37 °C incubator for 5 min.

6| As soon as hPSC colonies are detached from the surface of the plate, add 2 ml of DMEM, aspirate and dispense gently several times. Collect the cells into a 15-ml conical centrifuge tube.
▲ **CRITICAL STEP** hPSC colonies should become small cell clusters rather than single cells.

7| Centrifuge the hPSCs at 150*g* for 5 min at RT, aspirate the supernatant and resuspend the pellet in 6 ml of mTeSR1 medium.

8| Aspirate the Matrigel from the plates prepared in Steps 1–3, and then add 1 ml per well of mTeSR1 medium.

9| Plate the hPSCs from Step 7 at a ratio of 1:3 or 1:4 (one plate of hPSCs plated into three or four Matrigel-coated dishes). Prepare enough dishes for both the differentiation experiment and the control plates described in Step 2.

10| Incubate the cells at 37 °C and replace mTeSR1 medium every day until the cells are 75–80% confluent (usually, 3–4 d later).
▲ **CRITICAL STEP** Proceed to differentiation (Steps 11–18) only if the colonies look healthy and undifferentiated.

▲ CRITICAL STEP During the differentiation steps of the protocol (Steps 11–18), keep growing and passaging undifferentiated hPSCs, as they will be used as controls in Step 20.
? TROUBLESHOOTING

Spontaneous monolayer differentiation of hPSCs ● TIMING 5–7 d

11| Coat six-well plates with 1 ml per well of gelatin solution and leave it at RT for 15 min. Prepare extra plates to serve as controls for the FACS analysis and teratoma formation assay (plates to be used as controls in Step 20A and 20B). These controls will undergo differentiation (Steps 11–18) but not hPSC elimination (Step 19).

12| Aspirate the medium from the hPSC plates from Step 10, and then wash the cells with Ca^{2+}/Mg^{2+}-free PBS. 1 ml per well of PBS is sufficient to wash a six-well plate.

13| Add 0.5 ml per well of trypsin-EDTA for 1–2 min. As soon as hPSC colonies are detached from the surface of the plate, add 2 ml of serum-supplemented DMEM, aspirate it and dispense gently several times. Collect the cells into a 15-ml conical centrifuge tube.
▲ CRITICAL STEP When using trypsin-EDTA, hPSC colonies should dissociate into single cells.

14| Centrifuge the cells at 150*g* for 5 min at RT, aspirate the supernatant and resuspend the pellet in 6 ml of spontaneous differentiation medium.

15| Aspirate the gelatin from the plates prepared in Step 11 and add 1 ml per well of spontaneous differentiation medium (Reagent Setup).

16| Plate the hPSCs at a ratio of 1:8 (one plate of hPSCs plated into eight gelatin-coated dishes). Prepare enough dishes to include the control plates described in Step 11.
▲ CRITICAL STEP Spontaneous monolayer differentiation does not require optimization of the plating density—a ratio of 1:8 would usually range from 5×10^4 to 2×10^5 cells per well; however, most directed differentiation protocols are highly sensitive to plating density, and in these cases it is imperative to count the cells and plate the number determined in the differentiation protocol.

17| Incubate the cells at 37 °C and replace the spontaneous differentiation medium every day until the cells become 70% confluent (usually, 5–7 d later). If desired, differentiation can be followed by microscopy, as cells should start changing their typical undifferentiated morphology around day 3 of differentiation.
? TROUBLESHOOTING

18| Take a small sample of cells and evaluate the percentage of undifferentiated cells by FACS analysis for a pluripotency marker, as described in Step 20A. This evaluation is required so that the effect of the selective elimination of undifferentiated hPSCs (Step 19) can be determined. This percentage will greatly depend on the hESC or hiPSC lines used in the experiment. For most cell lines, it should range from 10 to 20% of the cells.

Selective elimination of undifferentiated hPSCs from culture.

19| Chemically ablate undifferentiated hPSCs with the SCD1 inhibitor PluriSIn #1 (option A) or with the toxin CPE (option B). See the INTRODUCTION for a comparison of these two options and for guiding considerations for selecting between them. If you select SCD1 inhibition (option A), consider also performing the cytotoxicity rescue assay described in **Box 1**.

(A) Chemical ablation of hPSCs with PluriSIn #1 ● TIMING 2–3 d for ablation, and 1–2 d for culture recovery

(i) Thaw PluriSIn #1 at RT.

(ii) Add PluriSIn #1 to fresh spontaneous differentiation medium at a ratio of 1:500 (vol/vol) to a final concentration of 20 μM. The total medium volume should be the standard culture volume (e.g., 2 ml per well in a six-well plate).
▲ CRITICAL STEP As PluriSIn #1 kills hPSCs by blocking oleate synthesis, this compound works most efficiently in oleate-free medium. If your medium contains high oleate concentrations (>20 μM) or high serum concentrations (>10%), it is advisable to change to low-oleate or low-serum medium for the duration of this assay.
▲ CRITICAL STEP The undifferentiated control plates from Step 10 should be treated the same way as the differentiated plates. The differentiated control plates from Step 17 should be handled similarly, but their medium should be supplemented with DMSO (0.2% (vol/vol)) instead of PluriSIn #1.

(iii) Aspirate the old spontaneous differentiation medium and replace it with the fresh PluriSIn #1-containing medium. Incubate the cells at 37 °C.

(iv) Repeat Step 19A(i–iii) after 24 h. If undifferentiated cells are still observed in culture, repeat Step 19A(i–iii) again after an additional 24 h.

(v) Wash the plates three times with PBS, and replace with fresh spontaneous differentiation medium. Culture the cells for 1–2 d before proceeding to the next steps, to allow differentiated cells to recover from potential transient effects and to allow residual undifferentiated cells (if any) to proliferate.

▲ **CRITICAL STEP** The ablation of undifferentiated hPSCs can often be observed by microscopy, as undifferentiated cells should no longer be visible in culture. Use undifferentiated hPSC culture plates as positive controls.

? TROUBLESHOOTING

(B) Chemical ablation of hPSCs with CPE ● TIMING 1.5 h for ablation, and 1–2 d for culture recovery

(i) Thaw CPE on ice.

▲ **CRITICAL STEP** CPE should be kept on ice at all times.

(ii) Add CPE to fresh spontaneous differentiation medium at a ratio of 1:2,000 (vol/vol) to a final concentration of 1 μg ml^{-1}. To spare the expensive reagent, the total medium volume can be lower than the standard culture volume (0.5 ml per well in a six-well plate).

▲ **CRITICAL STEP** The undifferentiated control plates from Step 10 should be treated the same way as the differentiated plates. The differentiated control plates from Step 17 should be handled similarly, but their medium should be supplemented with PBS instead of PluriSIn #1.

(iii) Transfer the plates to a shaker and leave them to rock at RT for 1 h.

(iv) Wash the plates three times with PBS, and replace with fresh spontaneous differentiation medium. Culture the cells for 1–2 d before proceeding to the next steps, to allow differentiated cells to recuperate from potential transient effects and to allow residual undifferentiated cells (if any) to proliferate.

▲ **CRITICAL STEP** The ablation of undifferentiated hPSCs can often be observed by microscopy, as undifferentiated cells should no longer be visible in culture. Use undifferentiated hPSC culture plates as positive controls.

? TROUBLESHOOTING

Quality control for the efficiency and safety of chemical ablation

20| Select and perform appropriate quality control assays for your investigation. The efficiency of chemical ablation can be assessed *in vitro* by FACS (option A) and *in vivo* by the teratoma formation assay (option B). The viability of the differentiated cells after the chemical ablation can be measured by a standard viability assay (option C). The gene expression profile of the differentiated cells can be examined by gene expression profiling (option D). Consider also including further assays that might help determine the lack of cytotoxicity to the differentiated cells, for example, proliferation assays (for proliferating cells) or functional assays (for functional differentiated cells, such as neurons, cardiomyocytes or hepatocytes).

(A) Assessing the purity of the purged culture by FACS ● TIMING 0.5 d

(i) Aspirate the medium from the plates prepared in Step 19, and from the undifferentiated control plates from Step 10 and the differentiated control plates from Step 17, and then dissociate the cells as described in Step 13. Filter the cells through a 70-μm filter.

▲ **CRITICAL STEP** Keep the cells on ice during FACS preparations.

(ii) Collect 10 μl of sample from each plate for cell counting by using trypan blue and a glass hemocytometer or an automatic cell counter.

(iii) Centrifuge the cells at 150*g* for 5 min at RT, aspirate the supernatant and resuspend each pellet in PBS-BSA solution at a density of 1.5 × 10^6 cells per ml. Distribute the cells in FACS tubes (200 μl per tube).

(iv) Add 5 μl of conjugated antibody to the cell-containing FACS tubes (a suitable antibody to detect undifferentiated hPSCs is TRA-1–60-PE). Vortex the mixture.

(v) Incubate the cells on ice in the dark for 30 min.

(vi) Add 300 μl of cold PBS-BSA and vortex the mixture.

(vii) Centrifuge the cells at 150*g* for 5 min at 4 °C, discard the supernatant and resuspend it in 500 μl of cold PBS-BSA. Repeat this step.

(viii) Place the tubes on ice in the dark for the duration of the FACS analysis.

(ix) Analyze the samples by using an FACS machine (e.g., FACSAria III and BD Biosciences).

(B) Assessing the purity of the purged culture by a teratoma formation assay ● TIMING 4–12 weeks

(i) Thaw Matrigel aliquots (Reagent Setup) overnight on ice at 4 °C.

(ii) Aspirate the medium from the plates prepared in Step 19, and from the undifferentiated control plates from Step 10 and the differentiated control plates from Step 17, and dissociate the cells as described in Step 13.
▲ **CRITICAL STEP** Keep the cells on ice during preparations for teratoma formation assay.
(iii) Collect a 10-µl sample from each plate for cell counting by using trypan blue and a glass hemocytometer or an automatic cell counter.
(iv) Centrifuge the cells at 150*g* for 5 min at RT, aspirate the supernatants and resuspend the pellets in cold culture medium at a density of 2×10^6 cells per 100 µl.
(v) Mix 100 µl of cell-containing medium with 100 µl of Matrigel in an Eppendorf tube.
(vi) Prepare fresh ketamine/Domitor mixture solution (Reagent Setup).
(vii) Weigh NSG mice, and anesthetize them by a s.c. injection of 0.12 ml per kg from the mixture prepared in the previous step. Mice should be asleep and areflexive after 10 min.
! **CAUTION** All experiments using mice must be performed in accordance with all institutional and governmental ethics and animal handling requirements. NSG mice must be housed under specific pathogen-free conditions.
(viii) Use a 26-gauge syringe to inject a 200-µl cell-containing mixture from Step 20B(v) s.c., above the hind leg of the mouse.
▲ **CRITICAL STEP** To allow statistical analysis of the results, use at least three mice for each experimental condition. Cells can be injected above both hind legs of each mouse.
(ix) Three weeks after cell injection, start following teratoma formation (at least once a week). Teratomas will appear as evident bulges at the location of cell injection. Once teratomas are detected, follow them daily and kill the mouse as described in the next step once the tumor reaches a volume of ~1 cm^2.
▲ **CRITICAL STEP** Do not allow teratomas to get larger, as they can cause unnecessary discomfort to the animals.
(x) Kill mice by exposing them to lethal doses of Isoflurane within a desiccator jar. Mice should be dead after <5 min.
(xi) By using surgical scissors, extract the teratomas and put them in a PBS-containing plate.
▲ **CRITICAL STEP** Do not allow the teratomas to dry.
(xii) Fill a cryomold intermediate plastic mold with O.C.T., put the teratoma inside, cover it with more O.C.T. and transfer it to liquid nitrogen for 5 min.
! **CAUTION** Liquid nitrogen is dangerous if it comes in contact with skin. Wear gloves and glasses while working with it, and handle it with care.
(xiii) Transfer frozen teratoma blocks to –80 °C for long-term storage. The cryopreserved teratomas can be sectioned, and the slides can then be subjected to histological analyses.

(C) Assessing the safety of the chemical exposure for the differentiated cells by a viability assay ● **TIMING 2 h**
(i) Measure the number of viable cells in the cell cultures from Step 19, using the CellTiter-Glo luminescence viability assay (Promega) according to the manufacturer's instructions.
(ii) Compare the cell viability in the cultures exposed to the chemicals and those exposed to control conditions. If the chemical exposure has not been detrimental for the cells, the viability difference should be similar to the percentage of residual undifferentiated hPSCs, which is known from Step 18.

(D) Assessing the safety of the chemical exposure for the differentiated cells by gene expression profiling. ● **TIMING 1 week**
(i) Purify RNA from the cell cultures obtained at Step 19 with an RNA purification kit, such as the PerfectPure RNA cultured cell kit (5 Prime), according to the manufacturer's instructions.
(ii) Subject your RNA samples to gene expression microarrays, such as the Affymetrix human gene 1.0 ST array, according to the manufacturer's protocol.
(iii) By using gene expression analysis software, such as Expander[37], derive a list of genes that are differentially expressed between the differentiated cells that have been exposed to the chemicals and those that have been exposed to control conditions.
(iv) By following the pipeline of the software, subject this list to a functional enrichment analysis in order to identify functional annotations that are significantly different between the two conditions.
? **TROUBLESHOOTING**

? **TROUBLESHOOTING**
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10	Extensive spontaneous differentiation in culture	Deterioration of culture conditions (e.g., because of insufficient medium replacement)	Manually transfer colonies that look good into new Matrigel-coated plates with fresh culture medium
17	The culture is already confluent, but differentiation is still ongoing	Plating density is too high	Plate again at a lower density or split the cells and continue differentiation
	There is substantial cell death in culture and few cells remain alive	Plating density is too low	Plate again at higher density
19A(v)	No cell death is observed in control hPSC cultures	The culture conditions decrease PluriSIn #1 potency	Try a higher concentration or a longer exposure duration Use low-oleate, low-serum medium
		PluriSIn #1 has not been properly dissolved or has been thawed and frozen too many times	Try another batch of the compound
	Massive cell death of differentiated cells is observed in the differentiated cultures	The culture conditions increase PluriSIn #1 potency	Try a lower concentration or a shorter exposure duration
		The differentiated cells are sensitive to SCD1 inhibition	Try Step 19B (CPE-based elimination of hPSCs)
19B(iv)	No cell death is observed in control hPSC cultures	The culture conditions decrease PluriSIn #1 potency	Try a higher concentration or a longer exposure duration
		CPE has not been properly prepared or has been thawed and frozen too many times	Try another batch of the toxin
	Massive cell death of differentiated cells is observed in the differentiated cultures	The culture conditions increase CPE potency	Try a lower concentration or a shorter exposure duration
		The differentiated cells are sensitive to CPE	Try Step 19A (PluriSIn #1–based elimination of hPSCs)
20D(iv)	The gene expression analysis shows that many genes have changed, but there is no consistency across replicates and no apparent biological meaning	Gene expression microarray is of low quality	Perform QC analysis to your microarrays as described in ref. 34 If necessary, repeat the microarray experiment (Step 20D)

● TIMING

Steps 1–10, culturing hPSCs on Matrigel-coated plates: 1–2 weeks; expansion of hPSCs can take from several days to several weeks, depending on the size of the experiment and the proliferation rate of the cells
Steps 11–18, spontaneous monolayer differentiation of hPSCs: 5–7 d
Step 19A, chemical ablation of hPSCs with PluriSIn #1: 2–3 d for ablation, and 1–2 d for culture recovery
Step 19B, chemical ablation of hPSCs with CPE: 1.5 h for ablation, and 1–2 d for culture recovery
Step 20A, assessing the purity of the purged culture by FACS: 0.5 d
Step 20B, assessing the purity of the purged culture by a teratoma formation assay: 4–12 weeks
Step 20C, assessing the safety of the chemical exposure for the differentiated cells by a viability assay: 2 h
Step 20D, assessing the safety of the chemical exposure for the differentiated cells by gene expression profiling: 1 week
Box 1, (Optional), cytotoxicity rescue assay for SCD1 inhibition: 1 d

ANTICIPATED RESULTS

The protocol presented here enables the removal of undifferentiated hPSCs from culture on the basis of unique vulnerabilities of these cells: their metabolic dependency on oleate biosynthesis, and their high claudin-6 expression that results in high CPE sensitivity. Depending on the differentiation protocol used and on the exact culture conditions, both of these methods

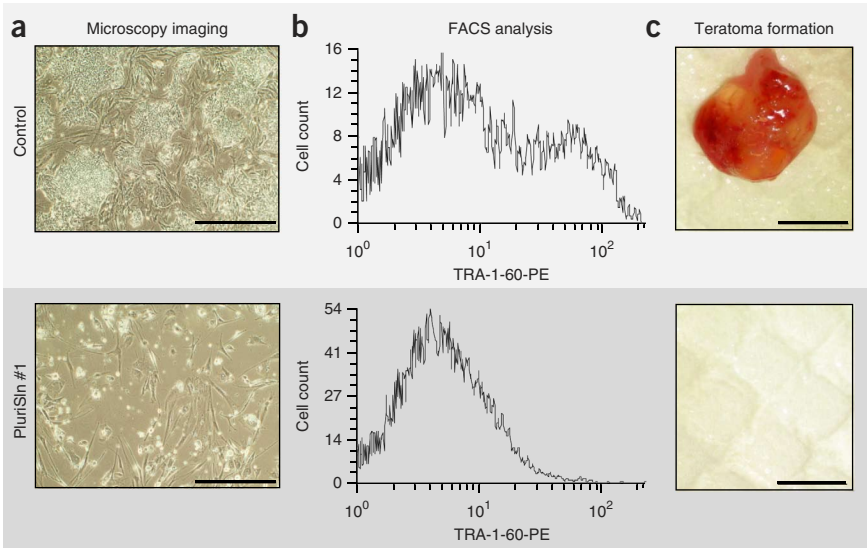

Figure 3 | Typical results of chemical ablation of hPSCs. (**a**) Bright-field microscopy images of a mixed culture of undifferentiated and differentiated hPSCs, after a 48-h exposure to control culture conditions (top) or to PluriSIn #1 (bottom). Scale bars, 100 µm. (**b**) FACS analysis of a mixed culture of undifferentiated and differentiated hPSCs, after a 48-h exposure to control culture conditions (top) or to PluriSIn #1 (bottom). (**c**) Representative image of a teratoma generated by injection of a mixed culture of undifferentiated and differentiated hPSCs into immunodeficient mice (top). Teratoma formation was evaluated 6 weeks after cell injection. A 48-h exposure to PluriSIn #1 prevented teratoma formation (bottom). Scale bars, 1 cm. Appropriate institutional regulatory board permission was obtained for the studies reported in **c**.

should result in over 99% depletion of the undifferentiated cells[7,20], significantly increasing the purity of the resultant culture and reducing its tumorigenicity. Following the spontaneous monolayer differentiation protocol described in Steps 11–18, the differentiated culture at day 7 will normally contain 10–20% residual hPSCs, whereas the undifferentiated control culture will comprise >90% hPSCs. After exposure to PluriSIn #1 or to CPE (Step 19), these cultures should contain <1% of the initial number of hPSCs. This can be roughly estimated by microscopy imaging (**Fig. 3a**), and it should be accurately reflected by the FACS analysis (Step 20A; **Fig. 3b**).

In the teratoma assay (Step 20B), injection of two million hPSCs according to the described procedure should result in teratoma formation in 100% of the cases, normally by 6 weeks' time. Therefore, the control mice that are injected with undifferentiated hPSCs should always develop teratomas. Mice that do not develop teratomas 12 weeks after injection will normally not develop them at all, and thus they can be declared 'teratoma-free'. Successful removal of undifferentiated hPSCs from culture should result in teratoma prevention (**Fig. 3c**).

The safety of the protocol for differentiated progeny can be appreciated by using a viability assay (Step 20C) or by gene expression profiling (Step 20D). In the viability assay, the expected difference between the number of viable cells in the treated culture and that in the control differentiated culture can be calculated based on the estimation of residual undifferentiated hPSCs obtained in Step 18; a much greater viability difference than expected would reveal that the chemical exposure has been detrimental for the differentiated cells. In the gene expression analysis, a decrease in the expression of pluripotency genes is expected if the culture contained many residual hPSCs before their chemical ablation. However, a safe exposure should not result in a marked decrease in genes that characterize the functional differentiated cell type, nor should it lead to significant increase in genes related to cellular stress or to cell death. When chemical ablation is performed with PluriSIn #1 (Step 19A), it is advisable to specifically examine genes related to endoplasmic reticulum (ER) stress; when chemical ablation is performed using CPE, one should examine genes related to the cell membrane integrity. In both cases, these genes should not change considerably.

Finally, the oleate rescue assay (**Box 1**) aims to test whether cell death has indeed occurred because of inhibition of SCD1 activity. High concentrations of oleate will completely reverse the phenotype, demonstrating a causal role for oleate depletion in the observed cell death.

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