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and astrocytes, as we performed here, from larger numbers of patients might result in the classification of sporadic AD.

To date, the clinical effectiveness of DHA treatment is still controversial (Freund-Levi et al., 2006; Quinn et al., 2010). It is of particular interest that one of two sporadic AD neurons accumulated intracellular Aβ oligomers and showed cellular phenotypes that could respond to DHA but the other did not, and this result may explain why DHA treatment was effective for some AD patients, those with the intracellular Aβ oligomer-associated type of AD, although the timing (that is, the stage of disease development) for starting the treatment would be another critical factor. These results may suggest that patient-specific iPSCs provide a chance to re-evaluate the effect of a drug that failed in AD clinical trials, depending on the selection of the patient type. In the present study, the amount of Aβ oligomers in our culture was not affected by DHA, although it would be effective for reducing cellular stresses, and reducing the oligomerization of Aβ was also presumed to be a candidate mechanism of DHA treatment (Cole and Frautschy, 2006). These results indicate that therapy with DHA would alleviate symptoms. Furthermore, the data showing that BSI treatment leads to a reduction in ROS formation at a relatively similar level (Figure 2G) in both AD and control cells might indicate an Aβ oligomer-independent effect, in addition to an Aβ oligomer-dependent effect, of BSI.

In any event, patient-specific iPSCs would provide disease pathogenesis, irrespective of the disease being in a familial or sporadic form, as well as enable the evaluation of drug and patient classification of AD.

EXPERIMENTAL PROCEDURES

Derivation of Patient-Specific Fibroblasts

Control and AD-derived human dermal fibroblasts (HDFs) were generated from explants of 3 mm dermal biopsies. After 1–2 weeks, fibroblast outgrowths from the explants were passaged.

iPSC Generation

Human complementary DNAs for reprogramming factors were transduced in HDFs with episomal vectors (*SOX2, KLF4, OCT4, L-MYC, LIN28*, and small hairpin RNA for p53). Several days after transduction, fibroblasts were harvested and replated on an SNL feeder cell layer. On the following day, the medium was changed to a primate embryonic stem cell medium (ReproCELL, Japan) supplemented with 4 ng/ml basic FGF (Wako Pure Chemicals Industries, Japan). The medium was changed every other day. iPSC colonies were picked up 30 days after transduction.

Statistical Analysis

All data are shown as mean ± SD. For comparisons of the mean between two groups, statistical analysis was performed by applying Student's t tests after confirming equality between the variances of the groups. When the variances were unequal, Mann-Whitney U tests were performed (SigmaPlot 11.2.0, Systat Software, USA). Comparisons of the mean among three groups or more were performed by one-way, two-way, or three-way analysis of variance followed by a post hoc test with the use of Student-Newman-Keuls Method (SigmaPlot 11.2.0). p values < 0.05 were considered significant.

ACCESSION NUMBERS

The Gene Expression Omnibus accession numbers for microarray data reported in this paper are GSE43326 (gene-expression comparison between control and AD clones), GSE43382 (gene-expression change along with the astroglial differentiation), and GSE43328 (gene-expression comparison of generated iPSCs).

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.01.009.

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(D) Quantitative data of (C) is shown. Each value indicated the ratio of the CellROX-stained area (an average of random 25 fields per sample) adjusted with DAPI counts. Data represent mean ± SD (n = 3 per clone). Two-way ANOVA showed significant main effects of DHA treatment ($F_{[1,32]} = 43.140$; $p < 0.001$) with a significant interaction between the APP mutation and DHA treatment ($F_{[3,32]} = 23.410$; $p < 0.001$). The DHA group in AD(APP-E693Δ) neural cells was significantly different from the other groups (**, $p < 0.005$).
(E) Real-time survival rate of control and AD neural cells with and without DHA showing cell viability. The numbers of control and AD(APP-E693Δ) neurons with Synapsin I-promoter-driven EGFP were sequentially imaged (average of 25 random fields per sample) and counted to assess the survival ratio (n = 3 per clone). Data represent mean ± SD (n = 3 per clone). In the cell-survival ratio, three-way ANOVA showed significant main effects of the APP mutation ($F_{[1,256]} = 377.611$; $p < 0.001$), DHA treatment ($F_{[1,256]} = 36.117$; $p < 0.001$), and time ($F_{[7,256]} = 65.272$; $p < 0.001$), with significant interactions between the APP mutation and DHA treatment ($F_{[1,256]} = 18.315$; $p < 0.001$), between the APP mutation and time ($F_{[7,256]} = 20.023$; $p < 0.001$), between DHA treatment and time ($F_{[7,256]} = 4.534$; $p < 0.001$), and among all three factors ($F_{[7,256]} = 5.277$; $p < 0.001$). Post hoc analysis revealed that, on day 14 and day 16, AD(APP-E693Δ) neural cells were more vulnerable in the long culture than control neural cells and that DHA treatment rescued the vulnerability (*, $p < 0.001$).
(F) Typical images of Synapsin::EGFP neurons used in real-time survival assay. The scale bar represents 50 μm.
(G) Cytotoxicity in neural culture derived from control and AD iPSCs after treatment with DHA (5 μM) for 16 days. Measured fluorescent lactate dehydrogenase (LDH) release served as a measure of cytotoxicity. Data represent mean ± SD (n = 3 per clone). Two-way ANOVA showed significant main effects of DHA treatment ($F_{[1,32]} = 16.710$; $p < 0.001$) with a significant interaction between APP-E693Δ mutation and DHA treatment ($F_{[3,32]} = 9.210$; $p < 0.005$). There was a significant difference in AD(APP-E693Δ) neural cells between the DMSO-control and DHA groups (*, $p < 0.05$).
(H) Aβ40 and Aβ42 secreted from iPSC-derived neurons into medium (extracellular Aβ) at day 16 of the long-term culture were measured at 48 hr after the last medium change. Data represent mean ± SD (n = 3 per clone).
See also Figure S4 and Table S2.

reagents, materials and analysis tools; Y.K., Y.O., Y.S., M.N., K.Y., S.Y., S.S., T.A., R.H., and S.U. recruited the patients; R.T., H.M., and S.Y. provided critical reading and scientific discussions; T.S., K.K., T.T., and K. Takahashi performed microarray analysis; T.A. performed karyotyping; A.W. performed bisulfite genomic sequencing; K.I. and D.W. performed electrophysiology; K. Tsukita, T.K., and H.H. produced the lentivirus; H.I., N.I., M.A., and T.K. wrote the paper. The experimental protocols dealing with human or animal subjects were approved by the institutional review board at each institute. S.Y. is a member without salary of the scientific advisory boards of iPierian, iPS Academia Japan, Megakaryon Corporation, and Retina Institute Japan.

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LETTER

STEM CELLS

Response to Comment on "Drug Screening for ALS Using Patient-Specific Induced Pluripotent Stem Cells"

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Our work and the study of Bilican *et al.* highlight the need for complementary assays to detect subtle phenotypic differences between control and mutant induced pluripotent stem cell lines.

We thank Bilican *et al.* for their thought-provoking comments and for providing new data about cell survival of motor neurons derived from control and mutant M337V induced pluripotent stem cell (iPSC) lines (*1*). In their new data presented in Fig. 1A of their Letter (*1*), Bilican *et al.* demonstrate that there is no difference in cytotoxicity determined by the lactate dehydrogenase (LDH) release assay between motor neurons derived from mutant M337V iPSC lines and control iPSC lines under basal conditions, which is compatible with our results reported in (*2*). In their original paper (*3*), Bilican *et al.* did not show the data for cytotoxicity of motor neurons derived from mutant M337V and control iPSCs under basal conditions measured by the LDH release assay. We agree with Bilican *et al.* that it is necessary to examine not only cell survival but also cytotoxicity using a variety of different assays including the LDH release assay, caspase-3 activity, and TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) staining under basal conditions and in the presence of stressors.

We understand that the LDH release assay in their original paper [Fig. 5C in (*3*)] was not measuring cytotoxicity under basal conditions but rather in the presence of stressful stimuli, which revealed the greater vulnerability of motor neurons derived from M337V mutant iPSCs versus control iPSCs. In our paper (*2*), we addressed the fact that our results from the LDH release assay measuring cytotoxicity [fig. S11C in (*2*)] confirmed our results from the cell survival assay [fig. S11, A and B, in (*2*)]. In the cell survival assay, we transfected motor neurons with HB9::GFP lentivirus, and we counted total green fluorescent protein (GFP)–positive motor neurons once every other day, focusing on overall cell survival at a fixed time point (day 10). We concluded that there was no difference in the cell viability of motor neurons derived from mutant M337V iPSCs versus control iPSCs.

Bilican *et al.* point out in their Letter (*1*), and we agree, that estimating an increased risk of death by real-time single-cell longitudinal survival analysis (single-cell assay) is an elegant approach. They used this approach in their paper (*3*) and determined a difference in survival of mutant iPSC–derived versus control iPSC–derived motor neurons over a 10-day time period under basal condition [Fig. 5B in (*3*)]. In their study, they used an HB9::GFP plasmid vector to label motor neurons. Regarding this point, Bilican *et al.* note that real-time single-cell longitudinal survival analysis may be a more sensitive technique for detecting dynamic phenotypes that may otherwise be masked by variations arising from static measurements. Their new results using the LDH release assay presented in their Letter do not show differences in cytotoxicity under basal conditions [Fig. 1A in (*1*)]. Other sensitive assays for detection of cytotoxicity under basal conditions such as caspase-3 cleavage could also be used.

In our study (*2*), we used the HB9::GFP lentivirus vector, which integrated into the genomic DNA of motor neurons with stable expression of the GFP fluorescence signal. We observed GFP expression by motor neurons for 10 days after repassage of the cells infected by HB9::GFP lentivirus 2 weeks previously. We suggest that labeling by HB9::GFP lentivirus may make it possible to continue analyzing GFP-positive motor neurons in long-term cultures for more than 10 days and to determine whether death of motor neurons under basal conditions can be recapitulated. We agree with Bilican *et al.* that clonal variation can make it difficult to identify potentially important survival phenotypes. It may take different techniques to interpret subtle cellular vulnerabilities of motor neurons labeled with the HB9::GFP plasmid vector (*3*) versus the HB9::GFP lentivirus vector (*2*). Discussion concerning labeling methods for specific cell types derived from iPSCs is extremely important for the accurate assessment of cellular vulnerabilities.

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We hope that Bilican *et al.*'s Letter and our Response will contribute to a clearer understanding of the subtle vulnerabilities of motor neurons derived from amyotrophic lateral sclerosis (ALS) patient iPSCs, which could become a useful platform for screening new drug candidates for treating this fatal disease.

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A Chemical Probe that Labels Human Pluripotent Stem Cells

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SUMMARY

A small-molecule fluorescent probe specific for human pluripotent stem cells would serve as a useful tool for basic cell biology research and stem cell therapy. Screening of fluorescent chemical libraries with human induced pluripotent stem cells (iPSCs) and subsequent evaluation of hit molecules identified a fluorescent compound (Kyoto probe 1 [KP-1]) that selectively labels human pluripotent stem cells. Our analyses indicated that the selectivity results primarily from a distinct expression pattern of ABC transporters in human pluripotent stem cells and from the transporter selectivity of KP-1. Expression of ABCB1 (MDR1) and ABCG2 (BCRP), both of which cause the efflux of KP-1, is repressed in human pluripotent stem cells. Although KP-1, like other pluripotent markers, is not absolutely specific for pluripotent stem cells, the identified chemical probe may be used in conjunction with other reagents.

INTRODUCTION

Human embryonic stem cells (hESCs) (Thomson et al., 1998) and induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007) have been serving as valuable tools for basic biological research and as promising resources for regeneration therapy. Despite advances, substantial challenges remain for the clinical application of stem cells. One safety concern has been posed by the appearance of teratomas in animal models transplanted with cell samples containing a small number of undifferentiated stem cells. Methods of detecting and ablating undifferentiated stem cells are required for safer stem cell therapy.

Antibodies against stage-specific embryonic antigens 4 and 5 (SSEA-4 and SSEA-5) have been used extensively to detect human pluripotent stem cells (Henderson et al., 2002; Tang et al., 2011; Thomson et al., 1998). SSEA-4 is a glycolipid that is expressed in early embryos and, for unknown reasons, is presented selectively on the surface of hESCs and embryonic carcinoma (EC) cells (Henderson et al., 2002). SSEA-5, which is classified as an H-type 1 glycan, is a recently identified antigen specifically expressed in human pluripotent stem cells (Tang et al., 2011). Other markers of human stem cells include Oct3/Oct4 and Nanog, which are transcription factors required for the maintenance of undifferentiated states of stem cells and are downregulated upon differentiation (Chambers et al., 2003; Mitsui et al., 2003; Niwa et al., 2000; Pesce and Schöler, 2001; Rosner et al., 1990). Although their antibodies are highly useful for detecting pluripotent cells, these unstable protein tools suffer from high cost and often require fixation and permeabilization of cells. Alkaline phosphatase is another routinely used marker of human stem cells (Shamblott et al., 1998; Thomson et al., 1995). Although the assay for its enzymatic activity provides a simple method for detecting stem cells, this housekeeping enzyme is expressed in a number of other cell types, and its specificity to pluripotent stem cells is a major concern. A small molecule fluorescent probe specific for human pluripotent stem cells would permit their rapid detection and separation. Furthermore, small molecule probes provide reversible detection that can be tuned by varying the dose. Stable, chemically defined, and cost-effective synthetic probes would offer significant advantages as tools for basic research and for lowering the risk of tumor formation in stem cell therapy.

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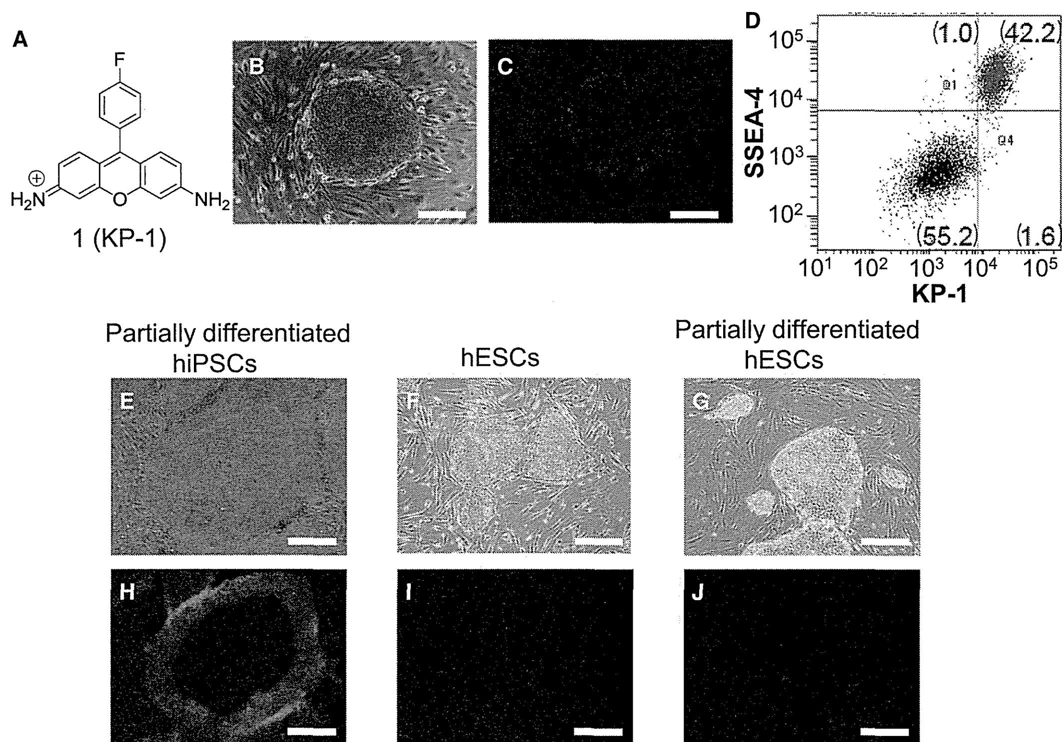

Figure 1. Discovery of KP-1
(A) Chemical structure of KP-1.
(B) Bright-field image of hiPSCs on mouse STO feeder cells. Scale bar represents 300 μm.
(C) Fluorescence image of hiPSCs on feeder cells incubated with KP-1 (2 μM) for 3 hr. Scale bar represents 300 μm.
(D) Flow cytometric analysis of a mixture of hiPSCs and feeder cells doubly stained with KP-1 and α-SSEA-4-Alexa 647. hiPSCs and feeder cells were dissociated with Accutase into single cells and stained with KP-1 (2 μM) for 3 hr and a fluorescence-labeled anti-SSEA-4 (α-SSEA-4-Alexa 647) for 30 min.
(E–J) Effects of cell differentiation on the staining pattern of KP-1. (E) Bright-field and (H) fluorescent images are shown of a partially differentiated hiPSC colony incubated with KP-1 (4 μM) for 4.5 hr. (F) Bright-field and (I) fluorescence images are shown of hESC colonies incubated with KP-1 (1 μM) for 2 hr. (G) Bright-field and (J) fluorescence images are shown of partially differentiated hESC colonies incubated with KP-1 (1 μM) for 2 hr. hESCs were treated with 500 nM retinoic acid for 4 days. Scale bars represent 450 μm.
See also Figures S1–S3.

RESULTS

Discovery of Kyoto Probe 1

To identify a fluorescent probe that is selective for human pluripotent stem cells, we screened 326 fluorescent compounds from chemical libraries (Ahn et al., 2007; Kawazoe et al., 2011). The image-based screening using human iPS cells (hiPSCs) isolated 21 molecules that stained hiPSCs more strongly than they stained feeder cells (mouse STO cells). We focused our subsequent efforts on a highly fluorescent rhodamine molecule (molecule 1, Kyoto probe 1 [KP-1]), which displayed the greatest selectivity (Figures 1A–1C). The excitation and emission spectra and fluorescent properties of KP-1 are shown in Figure S1A.

The selectivity of KP-1 for hiPSCs was confirmed by flow cytometry (Figures 1D, S1B, and S1C). Mixtures of hiPSCs and feeder cells were treated with KP-1 (Figure S1B), an Alexa Fluor 647-labeled anti-SSEA-4 (Figure S1C), or both (Figure 1D). When the cells were stained simultaneously with KP-1 and the anti-SSEA-4, KP-1 stained essentially all of the SSEA-4-positive cells, but not the SSEA-4-negative cells. Thus, KP-1 is capable of differentiating between hiPSCs and feeder cells. To examine the proportion of hiPSCs that is stained by KP-1, we carried out similar experiments using feeder-free culture conditions (Figures S1D–S1G). The results indicated that KP-1 stained 99.18% of hiPSCs, whereas an SSEA-4 antibody labeled 98.17% of hiPSCs.

An observation made during our evaluation of KP-1 further confirmed its specificity for pluripotent stem cells. When iPSCs are overgrown, central parts of the colonies tend to initiate differentiation, due to contact inhibition (Bortell et al., 1992; Green and Meuth, 1974). Treatment with KP-1 selectively stained the undifferentiated parts of such colonies, but not the central parts (Figures 1E and 1H). When similar experiments were conducted with colonies of hESCs (Suemori et al., 2006), the colonies were stained more strongly than the feeder cells (Figures 1F and 1I). When the colonies were partially differentiated by treatment with retinoic acid (Ben-Shushan et al., 1995), the differentiated parts of the colonies were less densely

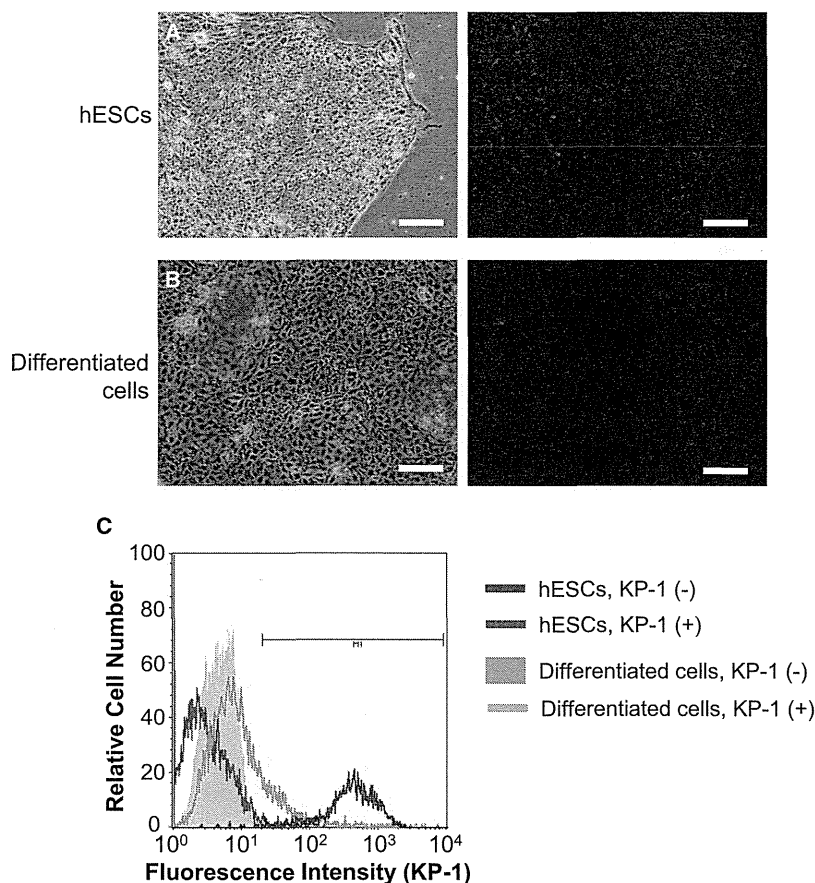

Figure 2. Selective Staining of hESCs by KP-1

(A and B) Fluorescence microscopic imaging of (A) hESCs and (B) ESC-derived differentiated cells in the presence of KP-1 (1 μM). Differentiated cells were derived from hESCs by treatment with retinoic acid (500 nM) for 4 days and treated with KP-1 (1 μM) for 1 hr. The left panels show bright-field images, and the right panels show fluorescence images. Scale bars represent 100 μm.

(C) Fluorescence histograms from flow cytometric analysis of hESCs and the ESC-derived differentiated cells.

See also Figure S2.

stained (Figures 1G and 1J). Flow cytometric analysis of hESCs and retinoid-treated differentiated cells revealed that ESCs were stained ~100-fold more strongly by KP-1 than the differentiated cells (Figure 2). These observations suggest that KP-1 is capable of distinguishing between pluripotent stem cells and differentiated cells.

Mitochondrial Localization of KP-1

What is the basis for the selectivity of KP-1? KP-1 appears to be cell permeable, and its subcellular localization overlaps with that of MitoTracker Red (MitoRed) (Minamikawa et al., 1999), a mitochondria-selective fluorescent marker (Figure S2). MitoRed labeled mitochondria both in hiPSCs and feeder cells; however, KP-1 stained mitochondria only in iPSCs (Figures S2A–S2E), indicating that KP-1 localizes in the mitochondria of human pluripotent stem cells. The staining pattern of KP-1 remained the same in the presence of CCCP, an uncoupling reagent that disrupts the mitochondrial membrane potential (Heytler, 1963; Kasianowicz et al., 1984), indicating that the staining properties of KP-1 are independent of the membrane potential (Figures S2F–S2I).

To isolate mitochondrial proteins that interact with KP-1, we synthesized a chloroacetyl derivative of KP-1 (Figure S3A). Although this highly reactive derivative is slightly less selective than KP-1, perhaps due to its rapid formation of covalent bonds to cellular proteins (Svensson et al., 2002), it still localized in mitochondria of hiPSCs (Figures S3B–S3I). We treated hiPSCs with the chloroacetyl derivative of KP-1 and used 2D SDS-PAGE to isolate mitochondrial proteins labeled with KP-1 (Figure S3J). Mass-sequencing analysis (Mann et al., 2001) of the fluorescently labeled bands revealed peptide sequences of aldehyde dehydrogenase 2 (ALDH2), a mitochondrial enzyme that has been reported to interact with a rhodamine derivative (Kim et al., 2011). Although binding to ALDH2 might account for the mitochondrial localization of KP-1, this abundant enzyme is expressed in numerous cell types (Greenfield and Pietruszko, 1977) and is not likely to be responsible for the selectivity of KP-1 for pluripotent stem cells.

KP-1 Selectivity and ABC Transporters

Concurrent with our study of KP-1, an independent project was investigating the expression levels of 44 ATP binding cassette (ABC) transporters in hESCs and iPSCs. ABC proteins transport hydrophobic small molecules and lipids across cell membranes in an ATP-dependent manner (Moitra and Dean, 2011; Ueda, 2011; Young and Holland, 1999) and are involved in protection against xenobiotics and cholesterol homeostasis (Ueda, 2011). The investigation with five lines of hESCs and three lines of hiPSCs showed intriguing expression patterns of four ABC proteins involved in xenobiotic efflux (Figure 3A). RT-PCR experiments revealed that both hESCs and iPSCs express ABCC1 (multidrug-resistance protein 1 [MRP1]) and ABCG2 (breast cancer-resistance protein [BCRP]) at detectable levels but have little, if any, expression of ABCB1 (MDR1) and ABCC2 (MRP2) transporters. Expression levels of ABCB1 and ABCG2 were markedly higher (29- and 24-fold, respectively) in differentiated cells prepared with retinoic acid, which express the differentiation marker, CDX2 (Bernardo et al., 2011; Niwa et al., 2005), than in human pluripotent stem cells (Figure 3B).

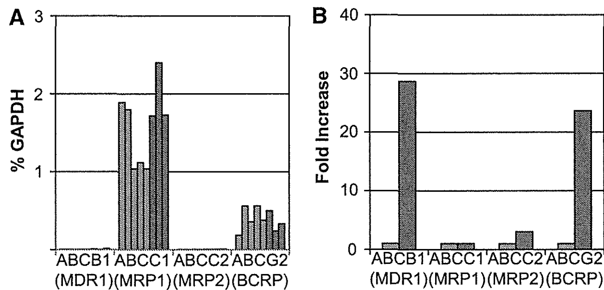

Figure 3. Expression Patterns of ABC Transporters in Human Pluripotent Stem Cells

(A) Comparison of mRNA expression levels of ABCB1 (MDR1), ABCC1 (MRP1), ABCC2 (MRP2), and ABCG2 (BCRP) transporters among hESCs (blue bar) and hiPSCs (red bar). Five hESC lines (KhESC-1, KhESC-2, KhESC-3, KhESC-4, and KhESC-5 in a left-to-right bar) and three hiPSC lines (201B7, IMR90-1, and IMR90-4, in a left-to-right bar) were examined.

(B) Fold increases of mRNA of ABCB1 (MDR1), ABCC1 (MRP1), ABCC2 (MRP2), and ABCG2 (BCRP) transporters between hESCs (blue bar) and differentiated cells (red bar). Each bar shows an averaged value of the five hESC lines (KhESC-1, KhESC-2, KhESC-3, KhESC-4, and KhESC-5).

See also Figure S4.

We hypothesized that the selective staining of pluripotent stem cells by KP-1 is due to increased expression of ABCB1 and ABCG2 in differentiated cells, resulting in the selective export of KP-1. To investigate the role of ABCB1 and ABCG2 in the selectivity of KP-1, we established cell lines that stably express ABCB1 or ABCG2 (KB/ABCB1 and KB/ABCG2, respectively) from the KB3-1 line of human epidermoid carcinoma cells, which have undetectable expression levels of those transporters (Taguchi et al., 1997; Ueda et al., 1987). We treated the cells with KP-1 for 2 hr, captured their images using a fluorescence microscope (Figures 4A and 4B), and quantified the signals (Figures 4C and 4D). Parental KB3-1 cells were strongly stained by KP-1, whereas fluorescent signals were significantly lower or undetectable in KB/ABCB1 and KB/ABCG2 cells. KP-1 staining of KB/ABCB1 or KB/ABCG2 cells was restored by treatment with cyclosporine A or fumitremorgin C (Figures 4A–4D), which are known inhibitors of ABCB1 (Tamai and Safa, 1990) or ABCG2 (Allen et al., 2002), respectively. Similar experiments were conducted with ABCC1 (MRP1), a transporter whose expression is unchanged upon cell differentiation (Chen et al., 2001; Nagata et al., 2000). Overexpression of ABCC1 did not result in export of KP-1, whereas calcein AM, a known substrate of ABCC1 (Versantvoort et al., 1995), was eliminated (Figures S4A and S4B). These results collectively suggest that KP-1 is a selective substrate for both ABCB1 and ABCG2.

We next examined the effects of transporter inhibitors on the selectivity of KP-1 for hESCs. When differentiated cells derived from ESCs were treated with cyclosporine A or fumitremorgin C, the differentiated cells were labeled by KP-1 approximately 10× more strongly than those untreated with the inhibitors (Figures 4E and 4F). These results indicate that the selectivity of KP-1 depends on its efflux via ABCB1 and ABCG2, whose expression is repressed in human pluripotent cells and induced upon differentiation.

Selectivity Profiling of KP-1 with Human Somatic Cells

If induction levels of ABCB1 and ABCG2 expression depend on the direction and degree of differentiation from pluripotent stem cells, the usefulness of KP-1 might be limited. Previous studies have reported that either ABCG2 or ABCB1 is induced in early hematopoiesis (Tang et al., 2010; Uchida et al., 2004; Zhou et al., 2001); therefore, we examined KP-1 staining of hematopoietic cells derived from hESCs (Takayama et al., 2008, 2010) (Figure 5A). Fluorescence-activated cell sorting (FACS) analysis showed that KP-1 distinguishes between SSEA-4-positive hESCs and human early hematopoietic cells expressing CD45, CD235, CD41a, or CD43, suggesting that KP-1 is useful for monitoring early hematopoiesis.

We next examined the ability of KP-1 to monitor other clinically important differentiation processes: cardiomyogenesis and neurogenesis. KP-1 was capable of distinguishing between hiPSCs and hiPSC-derived cardiomyocytes (Figures S5A and S5B), as confirmed by flow cytometric analysis (Figure 5B). We used RT-PCR to examine the expression patterns of 44 human ABC transporters in cardiomyocytes derived from hiPSCs (Figure S4C). Surprisingly, neither ABCG2 nor ABCB1 was induced during cardiomyogenesis. Instead, three ABC transporters, ABCA1, ABCC5, and ABCD3, were induced during differentiation (Figure S4C). The ability of two cell surface membrane ABC transporters, ABCA1 and ABCC5, to cause efflux of KP-1 was examined by overexpressing each transporter in HEK293 cells. However, no clear efflux of KP-1 was observed from these cells (data not shown). It is possible that cardiomyocytes have other ABC transporter-independent mechanisms for excluding KP-1.

In contrast to human cardiomyocytes, hiPSC-derived neuronal stem cells (Morizane et al., 2011) were as strongly stained by KP-1 as hiPSCs (Figures S5C and S5D). The inability of KP-1 to distinguish between hiPSCs and human neuronal stem cells prompted us to examine staining patterns of KP-1 in a range of human primary cells (Figures 6A–6I). FACS analysis showed that KP-1 stained human brain astrocytes as strongly as hiPSCs, consistent with our observation that KP-1 labels human neuronal stem cells. In contrast, KP-1 exhibited weaker staining patterns in human lung cells, human adrenal microvascular cells, human prostate epithelial cells, human hepatocytes, human bronchial epithelial cells, and human brain microvascular cells. These results are consistent with expression profiles of ABC transporters in human tissues (Langmann et al., 2003): human tissues associated with secretion (adrenal gland), metabolic activity (liver), barrier systems (lung, bronchia), and reproductive organs (prostate) tend to display strong expression of ABC transporters. Overall, the results suggest that KP-1 is useful for monitoring a wide range of differentiation processes from human pluripotent stem cells, with the exception of neurogenesis.

We also tested staining patterns of KP-1 with several human cancer cell lines (Figures 6J–6L). KP-1 exhibited weaker staining in HepG2 cells (hepatocellular carcinoma) and human EC (1156QE) cells than in hiPSCs, whereas HeLa cells, a cervical cancer cell line that displays low ABC transporter expression (Ahlin et al., 2009), were labeled by KP-1 as strongly as hiPSCs. Thus, KP-1 might find its use in classifying cancer cells.

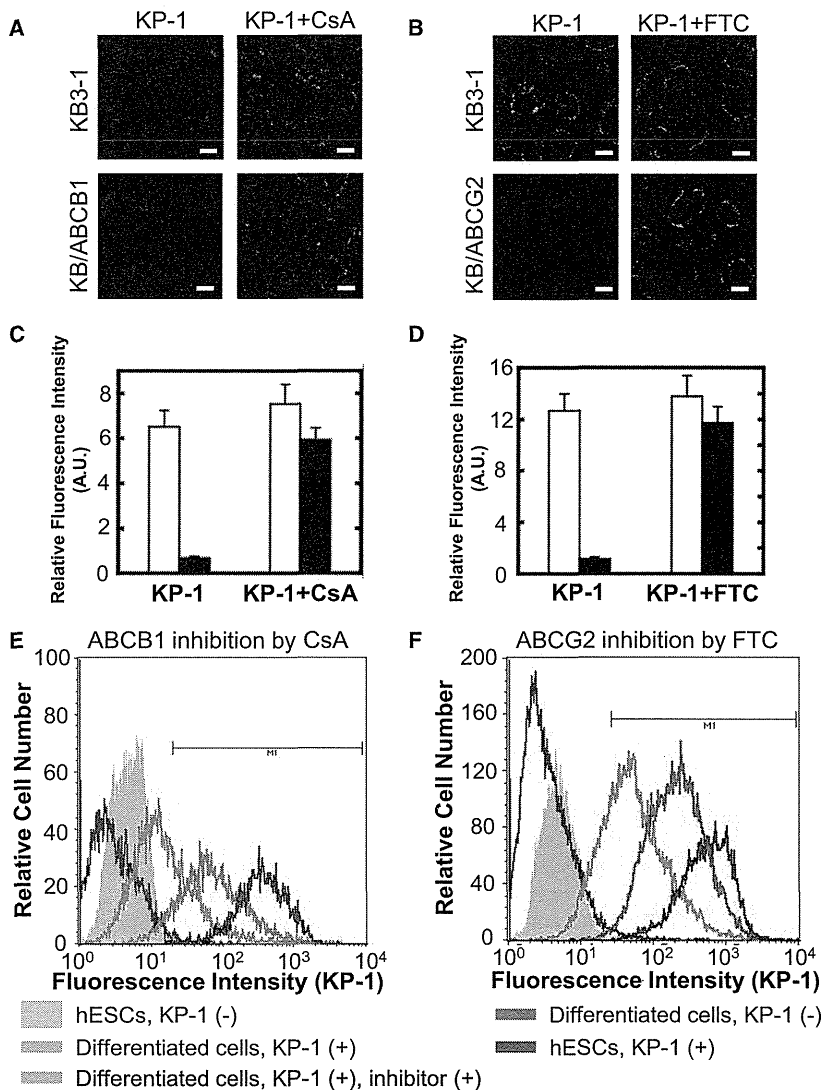

Figure 4. KP-1 Selectivity and ABC Transporters

(A) Fluorescence microscopic images of KB3-1 cells and KB/ABCB1 cells. The cells were treated with 1 μM KP-1 for 2 hr (left panel) or with 1 μM KP-1 for 2 hr in the presence of 5 μM cyclosporine A (CsA; right panel). Scale bars represent 2 μm.

(B) Fluorescence microscopic images of KB3-1 cells and KB/ABCG2 cells. The cells were treated with 1 μM KP-1 for 2 hr (left panel) or with 1 μM KP-1 for 2 hr in the presence of 10 μM fumitremorgin C (FTC; right panel). Scale bars represent 2 μm.

(C and D) Quantitative analysis of the fluorescence intensities of KP-1 in KB3-1 (open bar in C and D), KB/ABCB1 (solid bar in C), and KB/ABCG2 (solid bar in D) cells. It is evident that treatment with CsA or FTC restores the staining of KP-1.

(E and F) Fluorescence histograms from flow cytometric analysis of hESCs and ESC-derived differentiated cells in the presence of (E) 10 μM CsA or (F) 10 μM FTC. hESCs (red line) or differentiated cells (blue line) were incubated with 1 μM KP-1 for 1 hr or with 1 μM KP-1 and 10 μM inhibitor (pink line) for 1 hr. CsA and FTC were used as ABCB1 and ABCG2 transporter inhibitors, respectively. Shaded histograms and gray line represent hESCs and differentiated cells without KP-1, respectively. See also Figure S4.

KP-1 Staining during Reprogramming

One potential application of KP-1 might be its use in early detection of reprogrammed cells during reprogramming. To examine this potential application, the cells brightly stained by KP-1 at an early stage of reprogramming were isolated and cultured to determine whether they did in fact correspond to fully reprogrammed iPSCs at later stages. Four reprogramming factors (*Sox2*, *Oct3/Oct4*, *Klf4*, and *L-Myc*) were virally transfected into human adrenal microvascular cells, which have broad, strong expression of ABC transporters (Langmann et al., 2003). On day 7, the cells were treated with 2 μM KP-1 for 2 hr, then incubated overnight in fresh medium without KP-1. FACS analysis showed a significant increase in bright fluorescent cells among the transfected population (Figures S6A–S6D). The fluorescence intensity of the cells was greater than that of mock-transfected cells and as strong as in hiPSCs.

We isolated the 10% of cells most brightly stained by KP-1 and cultured them for another 3 weeks. Surprisingly, only a small portion (<0.1%) of the cells resulted in colonies of iPSCs after replating, and the reprogramming rates of the brightly stained cells were no higher than those of cells that were less strongly stained by KP-1.

During the course of the present study, the Yamanaka group independently obtained and published a similar result with an antibody against TRA-1-60, one of the most specific markers of human pluripotent cells (Tanabe et al., 2013). By day 7, ~20% of the transduced cells were positive for TRA-1-60. However, only a small portion (~1%) of the reseeded TRA-1-60-positive cells resulted in the colonies of iPSCs on day 28, and many of those cells turned back to be negative for TRA-1-60 during subsequent culture. Detailed analysis showed that reprogramming of cells treated with the four reprogramming factors is initiated much more frequently than was previously anticipated and that maturation, rather than initiation, is the limiting step of the reprogramming process. Together with our results with KP-1, these observations suggest that pluripotent markers do not necessarily allow early detection of pluripotent stem cells during reprogramming.

DISCUSSION

Results of the present study raise several questions for further investigation. First, what is the role of repressed expression of

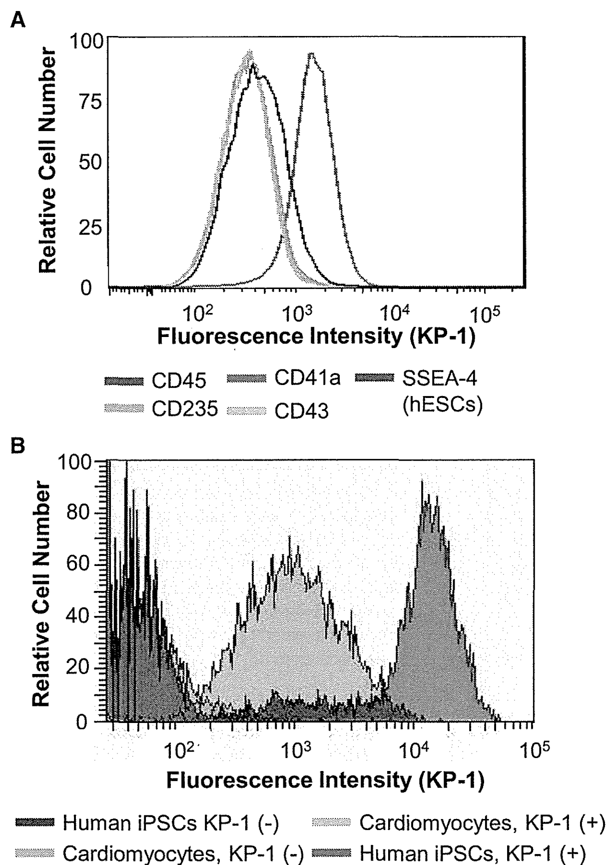

Figure 5. Selectivity Profiling of KP-1 with Hematopoietic Cells and Cardiomyocytes

(A) Flow cytometric analysis of KP-1 staining of hESCs and hematopoietic cells. hESCs and hematopoietic cells derived from hESCs were treated with 1 μM KP-1 for 2 hr. After the removal of KP-1, differentiated cells or undifferentiated cells were incubated for an additional 24 hr. Expression of cell surface molecules (CD45, CD235, CD41a, CD43, and SSEA-4) was analyzed by flow cytometry.

(B) Fluorescence histograms from flow cytometric analysis of hiPSCs and cardiomyocytes. hiPSCs and cardiomyocytes derived from hiPSCs (IMR90-1) were treated with 1 μM KP-1 for 1 hr. After the removal of KP-1, differentiated cells or undifferentiated cells were incubated for an additional 3 hr.

See also Figure S5.

ABCB1 and ABCG2 in pluripotent cells? Mixed results have previously been reported about the expression of ABCG2 in ESCs: Zeng et al. observed low expression levels of ABCG2 in human, but not mouse, ESCs (Zeng et al., 2009), and others detected high-level expression of ABCG2 in hESCs (Apáti et al., 2008). Our results support the model in which expression of ABCB1 and ABCG2 is repressed in hESCs and iPSCs. The finding that KP-1 stains neuronal lineages, as well as pluripotent stem cells, might provide insight. Both neuronal cells and pluripotent stem cells are usually protected by the blood-brain barrier or reproductive organs and, therefore, might not require extensive expression of ABC transporters. Reduced ABC transporter expression might make pluripotent stem cells and neuronal cells more sensitive to endogenous bioactive small molecules, permitting highly sensitive spatial and temporal responses to environmental signals. It is also possible that ABCB1 and ABCG2 inhibit undifferentiated states of hESCs. Studies to address these issues are in progress.

Second, how specific is the absence of ABCB1 and ABCG2 as a marker for human pluripotent cells? The expression pattern of ABC transporters might be one of many properties of human pluripotent stem cells and might not be conserved in other organisms. Previous screening of a similar chemical library with mouse ESCs identified a molecule that was not hit during the current screening (Im et al., 2010), consistent with recent findings of properties that differ between human and mouse pluripotent stem cells (Schnerch et al., 2010). Substrate specificities and expression patterns of ABC transporters might differ between pluripotent stem cells of humans and other species.

Finally, what are other potential applications of KP-1, in addition to detection of persistent undifferentiated stem cells in cell samples prior to transplantation? Unfortunately, our results demonstrated that KP-1 is not suited for early detection of pluripotent cells during reprogramming. However, another potential application might be monitoring of pluripotency during maintenance of hESCs or iPSCs. Visualization of hESCs or iPSCs using this stable synthetic molecule is reversible and amenable to fine-tuning via concentration and incubation time. No cytotoxicity of KP-1 was evident in hiPSCs under our standard staining conditions (1 or 2 μM for 2–7 hr), although cytotoxicity was detected after 48 hr of incubation at higher concentrations. The IC_{50} value at 48 hr was estimated to be 5.6 μM (Figure S6E). Thus, prolonged incubation should be avoided when KP-1 is used at high concentrations. The staining patterns of KP-1 are time dependent, due to the involvement of ABC transporters, and 4 hr of incubation usually provided clear results.

The use of KP-1 might lead to the discovery of compounds that increase the activity of ABC transporters, either by inducing differentiation of stem cells into ABC-expressing progeny or by increasing the activity of ABC transporters in pluripotent stem cells. KP-1 might also serve as a screening tool or starting point for the discovery and design of cytotoxic drugs that are selective for human pluripotent stem cells. In theory, cytotoxic compounds with ABC transporter selectivity similar to that of KP-1 should eliminate pluripotent stem cells from a mixture of differentiated cells and undifferentiated cells.

Despite the unanswered questions, the discovery of KP-1 and elucidation of its mechanism of action constitute significant steps toward the goals of understanding the unusual characteristics of and developing a highly specific probe for human pluripotent stem cells. We plan to distribute this chemical probe to the research community for further evaluation and for use in basic studies and clinical applications.

EXPERIMENTAL PROCEDURES

Chemical Library Screening

The chemical libraries of fluorescent compounds are combinations of fluorescent chemicals found or designed in multiple laboratories (Ahn et al., 2007; Kawazoe et al., 2011). hiPSCs (clone #201B2) were plated on SNL feeder cells in 24-well plates. Five days after plating, each fluorescent compound was added at the final concentration of 4 μM. After overnight

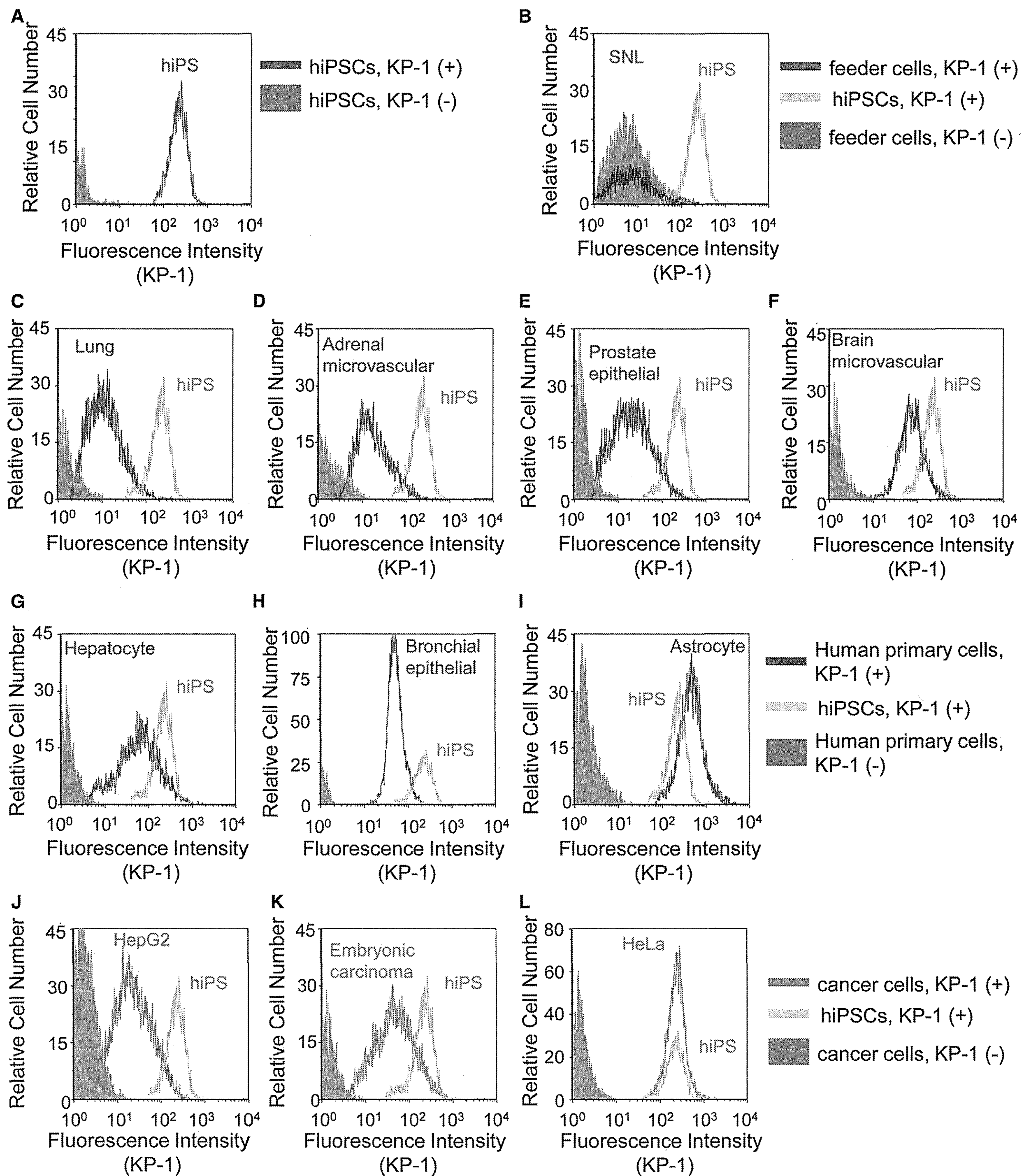

Figure 6. Staining Patterns of KP-1 with Human Somatic Cells

(A–L) Fluorescence histograms from flow cytometric analysis of (A) human stem cells, (B) feeder cells, (C–I) human primary cells, and (J–L) cancer cells. (A) hiPSCs, (B) mouse SNL cells, (C) human lung cells, (D) human adrenal microvascular endothelial cells, (E) human prostate epithelial cells, (F) human brain microvascular endothelial cells, (G) human hepatocyte cells, (H) human bronchial epithelial cells, and (I) human brain astrocyte cells are shown. (J) HepG2 cells, (K) human EC (1156QE) cells, and (L) HeLa cells are shown. The cells were treated with 2 μM KP-1 at 37°C for 2 hr. After the removal of KP-1, cells were incubated at 37°C for an additional 5–7 hr. See also Figure S6.

incubation, fluorescence microscopic images were captured using a Keyence Biorevo.

Characterization of KP-1

hiPSCs (clone #201B7) were plated at a density of 2×10^5 cells/well of a 6-well plate with mouse STO feeder cells. hiPSCs were also prepared under feeder-free conditions. Six days after plating, the cells were incubated with 2 μM KP-1 for 3 hr, and fluorescence microscopic images were then captured. The cells were dissociated into single cells with Accutase (Invitrogen) and stained with α-SSEA-4-Alexa 647 for 30 min at room temperature. After washing, flow cytometric analysis was performed using a MoFlo Astrios (Beckman Coulter Genomics).

Fluorescence Microscopic Imaging of hiPSC Colonies

hiPSCs (clone #201B7) were plated on SNL feeder cells in 24- or 96-well plates. Five days after plating, donut-shaped colonies of iPSCs were obtained. Differentiation of the central parts of the colonies was confirmed by immunostaining with an SSEA-1 antibody. The cells were incubated with 4 μM KP-1 for 4.5 hr at 37°C. Fluorescence microscopic images were taken using a Carl Zeiss Axioskop.

Fluorescence Microscopic Imaging and Flow Cytometric Analysis of hESCs and ESC-Derived Differentiated Cells

The hESC line, KhESC-1, was maintained as previously described (Suemori et al., 2006). To induce differentiation, hESCs were seeded onto a Matrigel-coated plate and cultured for 4 days with 500 nM all *trans*-retinoic acid (Sigma-Aldrich; R2625) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). After staining of hESCs or the differentiated cells with 1 μM KP-1 for 2 hr, the cells were rinsed with PBS and examined using an Olympus IX71 fluorescence microscope with a DP72 camera. For flow cytometric analysis, the cells were washed twice with ice-cold PBS and dissociated with 0.25% trypsin-EDTA into a single cell suspension. Staining by KP-1 was quantified using a FACSCalibur flow cytometer (Becton Dickinson). For the experiments with transporter inhibitors, cyclosporin A or fumitremorgin C was added at the concentration of 10 μM, during staining with KP-1.

Expression Profile of ABC Transporters

mRNA was extracted from five hESC lines (KhESC-1, KhESC-2, KhESC-3, KhESC-4, and KhESC-5) and three hiPSC lines (IMR90-1, IMR90-4, and 201B7). First-strand cDNAs were synthesized with reverse transcriptase (Applied Biosystems). Gene expression profiles were obtained by quantitative real-time RT-PCR, using TaqMan Array Gene Signature 96-well plates with 44 human ABC transporters (Applied Biosystems) and four housekeeping genes (GAPDH, 18S, HPRT1, and GUSB). Expression levels were normalized to GAPDH.

Experiments with KB3-1 Model Cells

The expression vector of myc-tagged ABCG2 in pCDH-EF1-MCS-IRES-Puro (System Biosciences) was introduced into 293T cells with psPAX2 and pMD2.G vector (Addgene). The lentivirus produced was used to infect KB3-1 cells, and a stable transformant, KB-ABCG2, was obtained by culturing the infected cells in medium containing puromycin (1 μg/ml). KB3-1, KB/ABCB1 (Taguchi et al., 1997), KB/ABCG2, and KB/ABCC1 (Nagata et al., 2000) cells were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified incubator (5% CO_2). The cells were subcultured for 24–48 hr in 35 mm glass base dishes (IWAKI), at a density of 4×10^5 cells per dish, in DMEM containing 10% (v/v) FBS. The cells were then incubated in DMEM containing 10% (v/v) FBS and KP-1 (1 μM) or calcein AM (2 μM) with or without an inhibitor (cyclosporine A: 5 μM, Wako Pure Chemicals Industries; fumitremorgin C: 10 μM, BioAustralis). After 2 hr incubation at 37°C, the treated cells were rinsed with PBS and observed in DMEM containing 10% (v/v) FBS using a confocal microscope (LSM 700; Carl Zeiss). For quantitative analysis, KB3-1 model cells were subcultured in poly-L-lysine-coated 96-well optical bottom plates (Nunc) for 24 hr, at a density of 2×10^4 cells per well, in DMEM containing 10% (v/v) FBS. The cells were then incubated for 1 hr in DMEM containing 10% (v/v) FBS and KP-1 (1 μM) with or without fumitremorgin C (10 μM). The treated cells were rinsed with the medium and further incubated without KP-1 for 1 hr at 37°C. After washing with PBS, fluorescence intensity was measured in PBS using a microplate reader (Infinite F200; Tecan). The cells were also lysed with PBS containing 1% (w/v) Triton X-100, and protein amounts were measured with BCA Protein Assay Reagent (Thermo Scientific). Fluorescence intensity was normalized to the protein amounts. Experiments were carried out in octuplicate.

Selectivity Profiling of KP-1 with Hematopoietic Cells

hESC clone Kyoto hESC (KhESC)-3 was obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto), after approval for hESC use was granted by the Minister of Education, Culture, Sports, Science, and Technology of Japan (MEXT), and the review boards for ethics at the University of Tokyo. KhESC-3 was cultured on irradiated mouse embryonic fibroblasts in a 1:1 mixture of DMEM and Ham F-12 medium, supplemented with 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 20% (v/v) knockout serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol, and 5 ng/ml basic fibroblast growth factor (bFGF; Upstate). The cells were passaged every 3 days to maintain them in an undifferentiated state. The mouse C3H10T1/2 cell line was purchased from the RIKEN BioResource Center and cultured in Basal Medium Eagle (Invitrogen), containing 10% (v/v) FBS and 2 mM L-glutamine. ESC differentiation medium was Iscove-modified DMEM, supplemented with a cocktail of 10 μg/ml human insulin, 5.5 μg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM L-glutamine, 0.45 mM α-monothioglycerol, 50 μg/ml ascorbic acid, and 15% (v/v) highly filtered FBS (Cellect Gold; ICN Biomedicals), in the absence or presence of the cytokines. Human vascular endothelial growth factor (VEGF) was purchased from R&D Systems. The following antibodies were used: phycoerythrin (PE) anti-human CD43 (eBioscience), Pacific Blue anti-human CD45, allophycocyanin (APC) anti-human CD41a, Pacific Blue anti-human CD235 (Glycophorin A) (BioLegend), and PE anti-human SSEA-4.

In order to differentiate hESCs into hematopoietic cells, small clumps of hESCs (<100 cells treated with PBS containing 0.25% trypsin [Invitrogen], 1 mM $CaCl_2$ [Sigma-Aldrich], and 20% (v/v) knockout serum replacement [Invitrogen]) were transferred onto mitomycin-treated or irradiated C3H10T1/2 cells, and cocultured in hematopoietic cell differentiation medium with VEGF (20 ng/ml), which was replaced every 3 days. On day12 after starting differentiation, KP-1 was added to the culture medium (final concentration, 1 μM). The cells were incubated for 2 hr, then washed twice with PBS, and changed to ESC differentiation medium. Undifferentiated KhESC-3 was used as positive control, and all cells were treated with KP-1. Differentiated cells or undifferentiated cells were collected after 0, 6, and 24 hr, using treatment with 0.25% trypsin-EDTA (Invitrogen). Expression of cell surface molecules and KP-1 fluorescence were analyzed by flow cytometry (FACSAria II; Becton Dickinson).

KP-1 Staining of Cardiomyocytes

Cardiac differentiation was carried out as previously described with modifications (Minami et al., 2012; Wang et al., 2011). In brief, hiPSCs were cultured on 3.5 cm culture dishes coated with human laminin 211 (BioLamina). To enhance generation of cardiac colonies, WNT signaling inhibitors were added for days 3–9 of cardiac differentiation. Cardiac colonies were harvested on day 15 and cultured for 7–10 days in floating culture. A majority of the prepared cells expressed the cardiac markers: cardiac troponin T, α-actinin, and NKX2.5. hiPSCs or iPSC-derived cardiomyocytes were treated with 1 μM KP-1 for 2 hr. The treated cells were rinsed with the medium and further incubated without KP-1 for 3 hr at 37°C. Fluorescent images were captured using an Olympus IX71 with a DP72 camera. For flow cytometric analysis, hiPSCs and iPSC-derived cardiomyocytes were dissociated into single cells by treatment with trypsin for 10 min. Flow cytometric analysis was performed with a FACSCalibur flow cytometer.

Flow Cytometric Analysis of Human Stem Cells, Feeder Cells, Human Primary Cells, and Cancer Cells

Human lung cells, human prostate epithelial cells, human brain microvascular endothelial cells, human hepatocyte cells, human bronchial epithelial cells, human brain astrocyte cells, and human EC (1156QE) cells were purchased from DS Pharma Biomedical. Human adrenal microvascular endothelial cells were

purchased from Primary Cell. Human lung cells were cultured in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine (Gibco) and 25 mM HEPES (Gibco). Human prostate epithelial cells, human brain microvascular endothelial cells, human hepatocyte cells, and human brain astrocyte cells were cultured on plates treated with attachment factor (Cell Systems) in CSC complete recombinant medium (Cell Systems) supplemented with 2% (v/v) human recombinant growth factor (Cell Systems). Human bronchial epithelial cells were cultured in BEBM medium (Lonza) supplemented with 0.4% (v/v) bovine pituitary extract (Lonza), 0.1% (v/v) human epithelial growth factor (Lonza), 0.1% (v/v) hydrocortisone (Lonza), 0.1% (v/v) epinephrine (Lonza), 0.1% (v/v) transferrin (Lonza), 0.1% (v/v) insulin (Lonza), 0.1% (v/v) retinoic acid (Lonza), 0.1% (v/v) triiodothyronine (Lonza), and 0.1% (v/v) GA-1000 (gentamicin, amphotericin-B; Lonza). Human EC (1156QE) cells were cultured in DMEM without sodium pyruvate (Nacalai) supplemented with 10% (v/v) FBS. Human adrenal microvascular cells were cultured in mesenchymal stem cell medium (ScienCell) supplemented with 5% (v/v) FBS (ScienCell), 1% (v/v) mesenchymal stem cell growth factor (ScienCell), and 1% (v/v) penicillin/streptomycin (ScienCell). The cells were subcultured in 6-well plates (Falcon) at a density of 1×10^5 to 5×10^5 cells per well. The cells were treated with 2 μM KP-1 at 37°C for 2 hr. After the removal of KP-1, cells were incubated in fresh medium at 37°C for an additional 5–7 hr. For flow cytometric analysis, the cells were washed with PBS and dissociated with 0.25% trypsin-EDTA into a single cell suspension. Staining by KP-1 was quantified using a FACSAria II flow cytometer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.006.

AUTHOR CONTRIBUTIONS

N.H., A.M., M.N., N.N., K.U., and M.U. conceived the project. N.H. and M.U. wrote the manuscript and analyzed the data with the support of M.N., N.N., and K.U. A.M. performed initial screening of a chemical library, and M.N. conducted the validation of KP-1 with hiPSCs and examined the effects of KP-1 during reprogramming. N.H. conducted chemical synthesis, mechanistic analysis of KP-1, validation with human somatic cells, and flow cytometric analyses. Y.F. performed profiling of ABC transporter expression and experiments with ABC transporter model cells. K.Y. carried out validation experiments with hESCs.

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Review

THE EMBO JOURNAL

iPS cells: a game changer for future medicine

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Abstract

The induced pluripotent stem cell (iPSC) technology is instrumental in advancing the fields of disease modeling and cell transplantation. We herein discuss the various issues regarding disease modeling and cell transplantation presented in previous reports, and also describe new iPSC-based medicine including iPSC clinical trials. In such trials, iPSCs from patients can be used to predict drug responders/non-responders by analyzing the efficacy of the drug on iPSC-derived cells. They could also be used to stratify patients after actual clinical trials, including those with sporadic diseases, based on the drug responsiveness of each patient in the clinical trials. iPSC-derived cells can be used for the identification of response markers, leading to increased success rates in such trials. Since iPSCs can be used in micromedicine for drug discovery, and in macromedicine for actual clinical trials, their use would tightly connect both micro- and macromedicine. The use of iPSCs in disease modeling, cell transplantation, and clinical trials could therefore lead to significant changes in the future of medicine.

Keywords cell transplantation; cohort study; disease modeling; future medicine; iPSC clinical trial; patient stratification
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See the Glossary for abbreviations used in this article.

Introduction

Like any other scientific advance, the iPSC technology (Fig 1) was established on the basis of numerous findings by past and current scientists in related fields (Yamanaka, 2012). Although the detailed mechanisms underlying the reprogramming process during iPSC generation are still being elucidated, the final products, which had previously been inaccessible, show promise for multiple purposes related to understanding disease mechanisms and strengthening the skills critical for patient treatment (Takahashi & Yamanaka, 2013). Although the iPSC technology still requires improvements and refinement, its contributions to disease modeling and cell transplantation studies are already well-recognized. New technologies, including direct cellular reprogramming and gene-editing, are optimizing the application of the iPSC technology for future medicine.

From this time onward, the progress in iPSCs and associated technologies is expected to engender novel criteria for patient stratification and for the regulation of clinical trials based on drug responsiveness, and the iPSC technology will contribute to more precise medicine in the future.

Disease modeling

The study of disease mechanisms and therapies is being enhanced by iPSC technology-based disease modeling. Following the first report of human iPSCs in 2007, the initiation of iPSC disease modeling was started by the generation of iPSCs using somatic cells from aged patients (Dimos *et al*, 2008) and patients with many types of diseases (Park *et al*, 2008), and the variety of diseases being modeled continues to grow (Supplementary Table S1). It is known that drugs used in animal models are not always effective for human beings (Inoue & Yamanaka, 2011). For example, a systematic study of inflammation showed that the gene expression changes in mice had little correlation with the changes seen in humans (Seok *et al*, 2013). Many genetic variants associated with human diseases are located in non-coding regions that show relatively little evolutionary conservation, which means that their introduction in animals is unlikely to result in phenotypes relevant to human diseases (Merkle & Eggan, 2013). Moreover, it may also be difficult to simultaneously recapitulate the gain and loss of function of the disease-causative proteins in human diseases (Winklhofer *et al*, 2008) by generating simple transgenic or knockout mice. In addition, one of the statin drugs, compactin, barely reached a human clinical trial level, since it was not effective for rats, in spite of being properly validated in humans (Tobert, 2003). Such discrepancies highlight the significance of using human cells for drug evaluation.

Of prime importance is the establishment of a *de facto* standard of disease modeling, including the quality control of iPSCs, as shown by previous reports summarized in Supplementary Table S1. However, iPSC disease modeling is faced with several obstacles. It has been revealed that heterogeneous cell populations exist after differentiation from iPSCs, and cells are not able to synchronize the developmental stages of cell populations (Kitaoka *et al*, 2011). These disparities in the differentiation efficiency and maturation among clones are considered to originate from incomplete reprogramming, genetic background variability (Soldner & Jaenisch,

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Glossary

ALS	amyotrophic lateral sclerosis
Aβ	amyloid β
BiP	immunoglobulin heavy-chain binding protein
ESC	embryonic stem cell
iPSC	induced pluripotent stem cell
GSK-3β	glycogen synthase kinase-3β
HLA	human leukocyte antigen
p-tau	phospho-tau (Thr231)
PRDX4	peroxiredoxin-4
t-tau	total tau

2012), epigenetic memory (Kim *et al*, 2010) or erosion of X-chromosome inactivation (Mekhoubad *et al*, 2012). There are several points that need to be addressed to overcome these obstacles facing iPSC disease modeling (Table 1), as follows:

Robust differentiation or purification/enrichment of target cells

Using a cell-specific promoter or cell-surface antigen, it is possible to isolate and obtain target cells with the same degree of maturation (Kitaoka *et al*, 2011; Egawa *et al*, 2012; Sandoe & Eggan, 2013; Yu *et al*, 2013), even though perfect purity is not yet possible.

One of the robust differentiation methods is to induce transcription factors for direct differentiation, *i.e.* direct reprogramming, which can be used to induce specific types of cells, including neurons (Vierbuchen, *et al*, 2010; Son *et al*, 2011; Qiang *et al*, 2013), cardiomyocytes (Ieda *et al*, 2010), blood cell progenitors (Szabo *et al*, 2010), hepatocyte-like cells (Huang *et al*, 2011; Sekiya & Suzuki, 2011) and cartilaginous tissue (Hiramatsu *et al*, 2011), as well as to determine the germ cell fate (Nakaki *et al*, 2013). Using this approach, disease modeling is possible (Qiang *et al*, 2011; Son *et al*, 2011; Rhinn *et al*, 2013). The major advantage of the direct cellular reprogramming/induced cell technology is that it works well in large cohorts of samples. On the other hand, there is a limit in the number of original somatic cells used as a resource, meaning that, while the induced cells are suitable for a large cohort analysis, they are not indicated for use in a large-scale analysis using a single line.

The direct cellular reprogramming/induced cell technology also has advantages in terms of the multi-sample analysis, cost and time, and cellular maturation; iPSCs are preferable in terms of gene-editing, the fact that they are an unlimited resource, and because they can differentiate into a great variety of cells. Although direct cellular

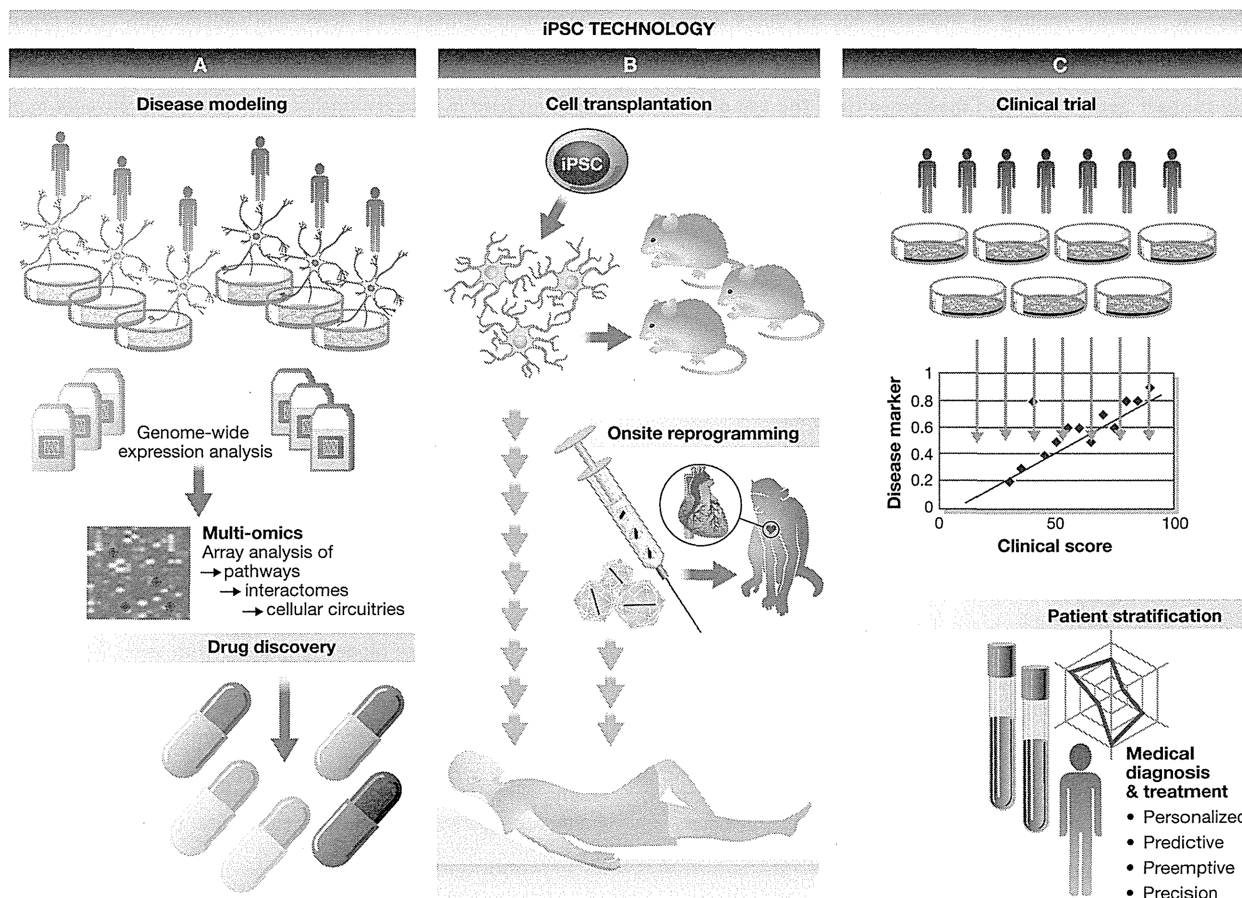

Figure 1. iPSC technology contributes to 'Disease modeling', 'Cell transplantation', and 'Clinical trial'.
(A) Disease modeling using patient iPSCs for 'Multi-omics' and 'Drug discovery'. (B) Instead of 'Cell transplantation', 'Onsite reprogramming' may be applied. (C) iPSCs from a large cohort of patients can be applied to 'Clinical trial' and 'Patient stratification'.

Table 1. Points in disease modeling

1. Robust differentiation or purification/enrichment of target cells
2. Mimicking of disease niche by additional conditions
3. A highly sensitive detection system
4. Optimal control setting
5. Validation with human sample and/or other disease models

programming was revealed to have the disadvantage of not being able to generate a renewable source of programmed cells, several labs have recently shown that programming can be achieved for a proliferating population of neural precursor cells that can then be propagated and subsequently differentiated into mature neurons and glia (Marchetto & Gage, 2012). In addition, the fusion of the direct cellular reprogramming technology with iPSCs would produce a hybrid technology that promotes the merits of both technologies (Imamura & Inoue, 2012), and this has already been reported for neurons (Hester *et al*, 2011; Zhang *et al*, 2013), hepatoblasts (Inamura *et al*, 2011) and myocytes (Tanaka *et al*, 2013). This hybrid technology will be even more useful after iPSCs can be generated more rapidly, easily and inexpensively.

Mimicking of disease niches by additional conditions

Genetic factors may not manifest functional defects in iPSC models under basal culture conditions, and might require the use of stressors to challenge the cell cultures (Kim *et al*, 2013). In addition, many neurodegenerative diseases are late-onset diseases, and their key phenotypes may not manifest themselves easily within a short period of time in culture. To mimic the aging process, cellular stress can be imposed, or trophic factors can be depleted.

Selective susceptibility of neuronal cell types in many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), can be induced by pathological changes in the neurons, as well as in their interacting partners (Xu & Zhong, 2013).

A highly sensitive detection system

The challenge of elucidating subtle but significant phenotypes in long-term cultures requires the application of multiple complementary readouts. A real-time single-cell longitudinal survival analysis using fluorescent reporter genes has enabled the determination of differences in cellular survival (Bilican *et al*, 2012). Single-cell expression profiling should clarify the levels of population heterogeneity within *in vitro* cultures, and advances in media culture platforms and automated cell processing should provide the accuracy and consistency that will be required (Citri *et al*, 2012; Yu *et al*, 2013). In addition, specific antibodies against intracellular pathogens have been tested (Kondo *et al*, 2013), and will continue to be developed.

Optimal control settings

Recent genome-wide association studies have demonstrated that every person possesses disease-relevant single-nucleotide polymorphisms, and it is therefore impossible to categorically define iPSCs that represent perfect non-disease control. Nonetheless, we think that deductive and inductive control (Inoue & Yamanaka, 2011) are valid for deriving iPSC-positive (disease) and negative (non-disease) controls. Deductive controls would include non-disease iPSC/embryonic stem cell (ESC) lines, together with gene-edited, isogenic iPSC lines (see Supplementary Table S1). Many methods have been used in the past for gene targeting in pluripotent stem cell lines (Sandoe & Eggan, 2013). Using the isogenic control, a labor reduction and noise cancellation from clonal variations would be possible. On the other hand, multiple clones must be analyzed so as to avoid off-target effects even in the isogenic control lines. If isogenic cell lines are compared, there is no clear answer at present regarding how many isogenic pairs should be analyzed (Merkle & Eggan, 2013).

In addition, when deductive clones are generated by introducing mutations into control human iPSC/ESC lines, protective alleles may intercept the expression of disease-phenotypes.

Validation with human samples and/or other disease models

Although iPSC technology provides novel resources, there is still room for improvement. It is still necessary to better validate the phenotypes with other systems, and to confirm that the phenotypes do not stem from the fragility of the technology by using human samples and other models. In this regard, there are some experimental conditions that only the iPSC technology can provide, such as the co-culture of disease cells and healthy control cells.

Cell transplantation

The iPSC technology is contributing to the study of cell transplantation. The advantages of iPSC are as follows: Autologous cells, which suppress the risks of rejection and infection, could be used; diseases caused by single gene defects could be addressed by made-to-order gene replacement in cells and allogenic cells from healthy people could be used.

A report of a mouse model of sickle-cell anemia, a genetic blood disorder caused by a defect in the β-globin gene, provided a proof-of-concept illustration of the therapeutic use of iPSCs (Hanna *et al*, 2007). In that study, a mutant iPSC line with gene correction by homologous recombination was used for transplantation into mutant mice to cure the disease. This exemplified the potential of regenerative medicine using iPSCs (Takahashi & Yamanaka, 2013). It was shown using a non-human primate PD model that autografts caused only a minimal immune response in the primate brain, and autografts have an advantage over allografts even at immunologically privileged sites (Morizane *et al*, 2013).

In contrast, the use of autologous iPSCs from every individual would necessarily result in high medical costs. Since it takes more than three months to generate iPSCs using the current methods, such a time line is hardly optimal for the effective treatment of certain disorders, such as spinal cord injury (Nakamura & Okano, 2013; Takahashi & Yamanaka, 2013). Furthermore, autografts from sporadic disease cases might harbor disease phenotypes. For these reasons, the importance of considering the use of allogeneic iPSC lines for transplantation therapy must be emphasized. Multiple iPSC clones could easily be generated from the diversity of donor candidates with validated health conditions and the types of human leukocyte antigen (HLA) needed for generating clinical-grade iPSC clones (Takahashi & Yamanaka, 2013). Matching the three major types of HLA loci between the recipient and donor is expected to result in less immune rejection after transplantation following bone marrow transplantation. One of the most feasible methods for iPSC therapy, therefore, will be based on the collection of iPSC stocks

derived from various HLA-homozygous donors under Good Manufacturing Practice (GMP) compliance (Nakajima *et al*, 2007; Okita *et al*, 2011; Takahashi & Yamanaka, 2013).

Some new technologies related to cell transplantation have been emerging. Dynamic patterning and structural self-formation of complex organ buds in 3D stem cell culture, including the generation of various neuroectodermal and endodermal tissues, have been discovered (Sasai, 2013). Another example of tissue generation was illustrated by the injection of wild-type rat pluripotent stem cells into the blastocysts of Pdx1-deficient mice, which are unable to grow a pancreas, and this resulted in the generation of normally functioning rat pancreatic tissue (Kobayashi *et al*, 2010). The self-organization of tissue development, a major advantage of pluripotency-mediated strategies, would be valuable not only for the next generation of organ transplantation, but also for disease modeling (Takahashi & Yamanaka, 2013). Furthermore, on-site reprogramming technology (Yu *et al*, 2013) has already been applied to the production of β-cells (Zhou *et al*, 2008) and cardiomyocytes (Qian *et al*, 2012; Song *et al*, 2012; Inagawa *et al*, 2012), and it will progress following the development of improved delivery methods. Immunological cells, including T cells (Nishimura *et al*, 2013; Vizcardo *et al*, 2013; Wakao *et al*, 2013), are also expected to be used for cell therapy.

New iPSC-based medicine

The iPSC technology has opened new possibilities for generating continuous supplies of progenitor cells for toxicity screening. A toxicity assay using iPSCs would be the first step in clinical trials (iPSC clinical trials). Proof-of-concept toxicity studies performed with human iPSC-derived differentiated cell types (Guo *et al*, 2011; Medine *et al*, 2013) support the concept of large-scale human cell-based toxicity screens. Drug-induced side effects in the liver, heart and brain have been thoroughly studied. It is both feasible and effective to use iPSC-derived cells between the drug discovery phase and development phase as clinical trial 'Phase 0.5'. However, there are several limitations to the sourcing of these cells, such as the achievement of fully mature phenotypes.

While stem cell-based hepatocyte toxicity assays are still at an early stage of development, proof-of-concept studies of known toxicants have been performed (Scott *et al*, 2013). It was also demonstrated that iPSC-derived cardiomyocytes could be treated using a subset of known arrhythmogenic drugs (Guo *et al*, 2011; Lahti *et al*, 2012). Applying electrophysiology methods to study the response of iPSC-derived cardiomyocytes to drug treatment provided prospective results, but such results are limited to the different experimental setups and the number of drugs evaluated in each study has been small (Deshmukh *et al*, 2012).

The results of these preliminary studies indicate that the toxic compounds that are already well known and have known mechanisms of action should be tested first with iPSC-derived cells, and the requirements, properties and differentiation protocols for the cells derived from standard iPSCs should be decided based on these findings.

In contrast to the drug-induced hepatotoxicity and cardiotoxicity, the mechanisms of which are relatively easy to discern, the reverse-translation of neuronal side effects into discrete cellular mechanisms and toxicity pathways for *in vitro* screening remains a challenge. However, proof-of-concept studies using the high-content analysis of different cell types are expected to be conducted by analyzing the features of neurodevelopment, including neurite outgrowth and synaptogenesis (Scott *et al*, 2013).

In an aging society, one of the unmet medical needs is that of drug development for Alzheimer's disease (AD). We previously analyzed the neural cells from AD patient iPSCs, and found that there are subgroups among AD cells. This indicates that clinical AD may need to be reclassified into different sub-types, and that the prediction of the drug responsiveness may be possible based on the different sub-types (Kondo *et al*, 2013). If we scale up the study, such as by performing an iPSC clinical trial of Phase 1.5, and generate AD and control iPSCs from a larger cohort of patients, it may become possible to select responders and non-responders to specific drugs, leading to a Phase II clinical trial only for responders. Or we could identify a responder marker, after actual clinical trials of a drug, using the iPSCs from responders and non-responders in the trials. This identified marker could then be used to enrich the responders in the next step, leading to higher success rates. Another report also showed that the neurons generated from iPSCs derived from four AD patients showed significantly higher levels of Aβ40 in the culture medium of the neurons generated from three of the four patients, supporting the concept of the heterogeneity of AD (Israel *et al*, 2012).

There have also been other reports showing patient stratification with the differential drug responsiveness (Table 2). For example, several clinical trials for spinal muscular atrophy (SMA) have been conducted. The completed clinical trials demonstrated that valproic acid (VPA) is only beneficial to a restricted subset of SMA patients, and that there are responders and non-responders (Garbes *et al*, 2013). The drug responsiveness of neuronal cells derived from responder iPSCs and non-responder iPSCs to VPA was compatible

Table 2. Responder selection in a disease with differential drug responsiveness

Disease	Drug	Marker	Total (*n*) control/disease/responder	Reference
Retinitis pigmentosa	α-Tocopherol	RP9 mutation	6 1/5/2	Jin *et al* (2011)
Alzheimer's disease	β-secretase inhibitor γ-secretase inhibitor	Aβ(1-40), GSK-3β, p-tau/t-tau	6 2/4/3	Israel *et al* (2012)
Spinal muscular atrophy	VPA	CD36	2 0/2/1	Garbes *et al* (2013)
Alzheimer's disease	DHA	Aβ oligomer, BiP, PRDX4	7 3/4/2	Kondo *et al* (2013)

with the results of the clinical trials (Garbes *et al*, 2013). Although large clinical trials have been conducted with α-tocopherol (vitamin E), no statistically significant change in visual function of retinitis pigmentosa (RP) patients was found (Jin *et al*, 2011). The underlying mutations causing the disease in the patients tested in the above clinical trials were not revealed, and the variability of individual responses to these drugs is unknown. However, a recent study showed that the rod cells derived from iPSCs of RP patients showed differential responsiveness to vitamin E, suggesting that RP may be divided into subgroups by the drug responsiveness (Jin *et al*, 2011). Therefore, the iPSC technology can contribute to micromedicine, including drug discovery based on cellular and molecular analyses, as well as to macromedicine, including patient stratification based on cellular and molecular analyses of participants in clinical trials or cohort studies (Fig 2).

iPSC clinical trials may make it possible to identify a drug-responsive subgroup of patients with a specific disease, and a more precise Phase II clinical trial could thus be performed (Fig 3). The iPSC clinical trial approach could be applied to a large cohort analysis with medical records and genome information. A genome analysis provides ample information, but it is hard to establish sporadic disease models on the basis of such findings. We found that the Aβ metabolisms differed according to the respective APP mutations (Kondo *et al*, 2013), and an APP mutation that protects against Alzheimer's disease was recently reported (Jonsson *et al*, 2012). These findings suggest that, besides the genomic analyses, iPSC-derived cells would be useful for precise analysis of the individual genes and proteins. In addition when a new mutation is found, an analysis of target cells derived from iPSCs would provide an answer to the question of whether the mutation is pathogenic or not (Egashira *et al*, 2013).

We believe that iPSCs can be game changer that will help to avoid the possibility that a candidate drug tested in a clinical trial might be irrationally dropped based on the old rules. The previous clinical diagnoses are now changing based on the results of the genome analysis and multi-omics analysis of patient samples, including iPSCs. In addition, patients can be stratified based on the drug responsiveness of their iPSC-derived cells, which, as a consequence, could lead to a new type of diagnosis and stratification. A genetic diagnosis of sporadic diseases is difficult, but a drug response-based diagnosis might be possible based on the effectiveness of drugs in clinical trials (Fig 4). The required conditions for iPSCs used *in vitro* are different from those used for cell transplantation. The development of technologies for generating budget-conscious personalized iPSCs rapidly, homogenously and easily will be required for such iPSC-based clinical trials. To make iPSC clinical trials a reality, the regulatory system would need to be changed.

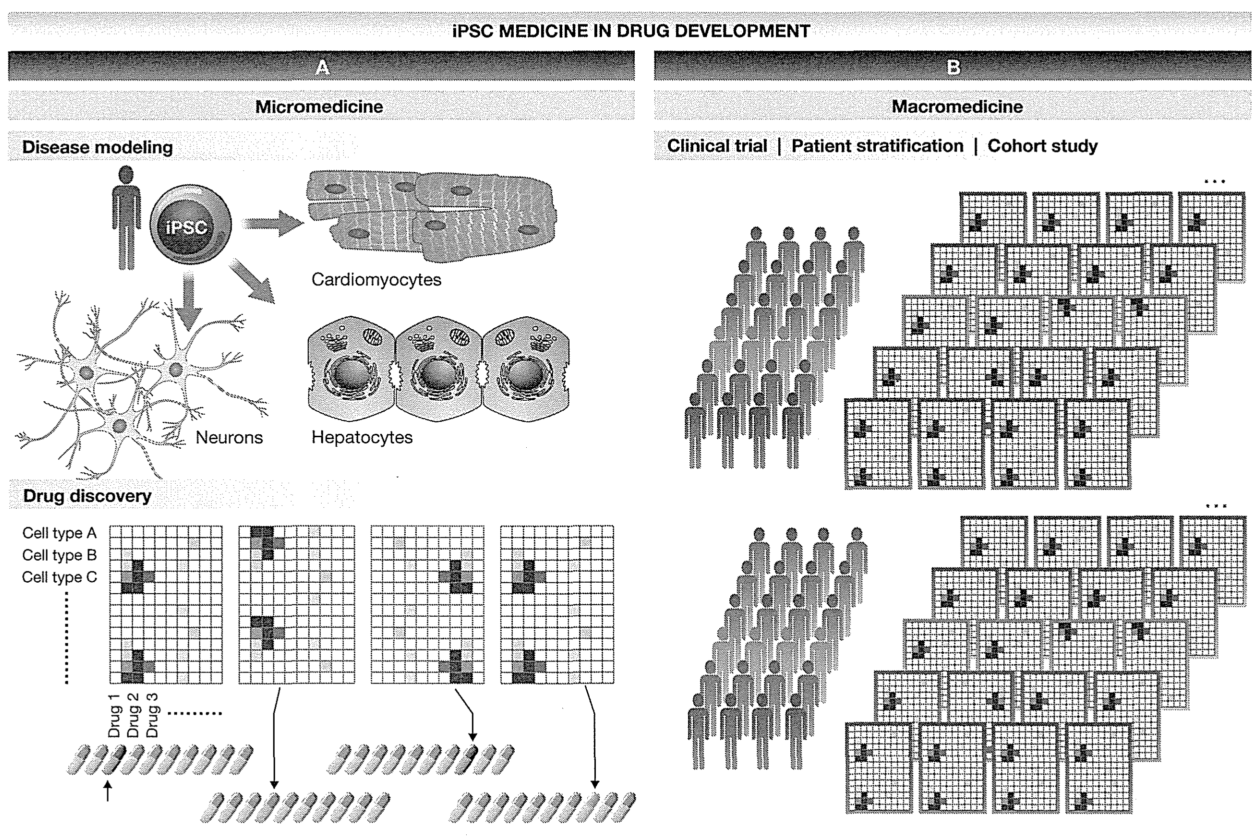

Figure 2. iPSC medicine in drug development ranges from 'Micromedicine' to 'Macromedicine'.

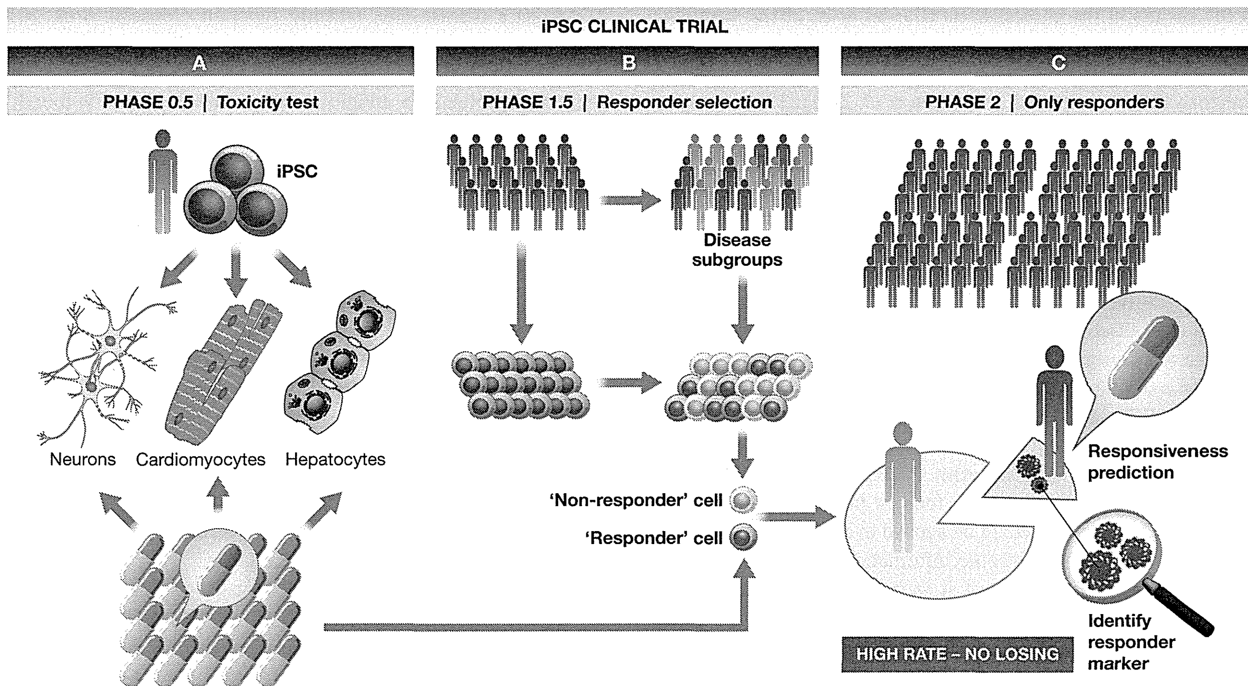

Figure 3. 'iPSC clinical trial' for phase 0.5, 1.5, and 2.

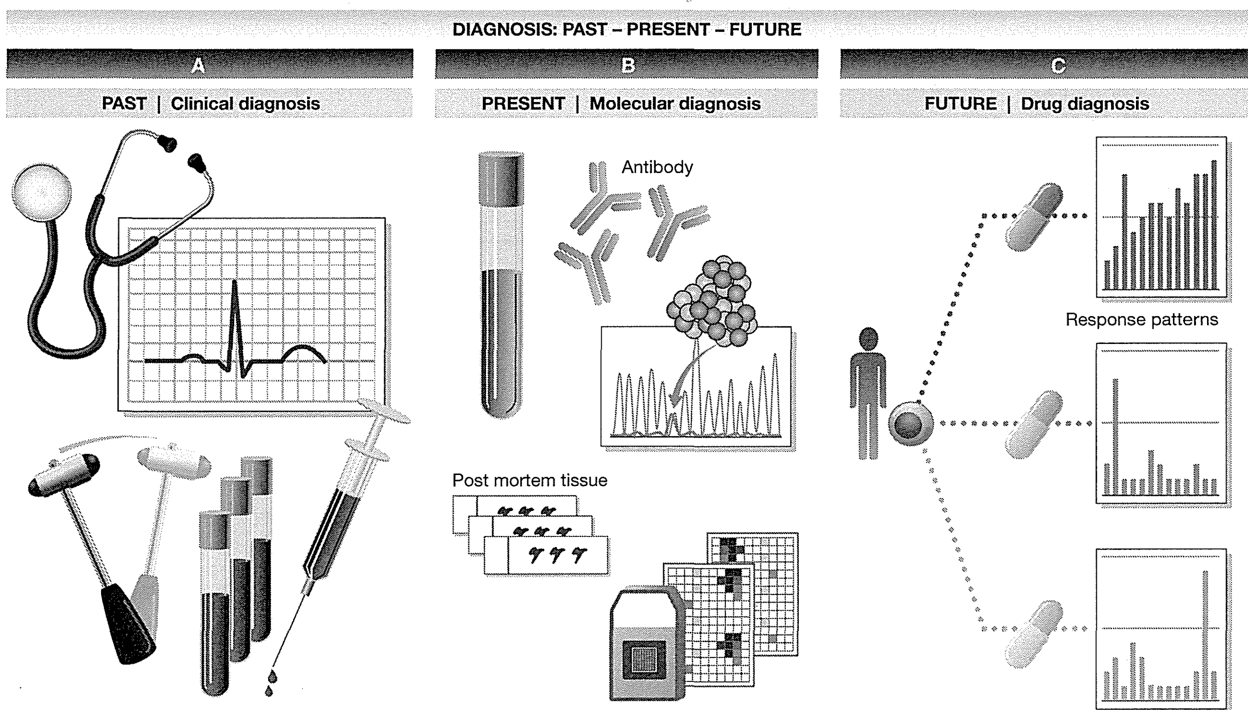

Figure 4. Patient diagnoses in the past, present, and future.