A chemical probe that labels human pluripotent stem cells.

Hirata, Nao; Nakagawa, Masato; Fujibayashi, Yuto; Yamauchi, Kaori; Murata, Asako; Minami, Itsunari; Tomioka, Maiko; Kondo, Takayuki; Kuo, Ting-Fang; Endo, Hiroshi; Inoue, Haruhisa; Sato, Shin-Ichi; Ando, Shin; Kawazoe, Yoshinori; Aiba, Kazuhiro; Nagata, Koh; Kawase, Eihachiro; Chang, Young-Tae; Suemori, Hirofumi; Eto, Koji; Nakauchi, Hiromitsu; Yamanaka, Shinya; Nakatsuji, Norio; Ueda, Kazumitsu; Uesugi, Motonari

Cell reports (2014), 6(6): 1165-1174

© 2014 The Authors. Published by Elsevier Inc.; This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/3.0/).
A Chemical Probe that Labels Human Pluripotent Stem Cells

Nao Hirata,1,2,9 Masato Nakagawa,3,9 Yuto Fujibayashi,4,9 Kaori Yamauchi,5,9 Asako Murata,1,2,9 Itsunari Minami,1 Mako Tomioka,1 Takayuki Kondo,3 Ting-Fang Kuo,1,2 Hiroshi Endo,2,8 Haruhisa Inoue,1 Shin-ichi Sato,1,2 Shinn Ando,1,2 Yoshinori Kawazoe,2 Kazuhiro Aiba,1 Koh Nagata,1 Eiichiro Kawase,2 Young-Tae Chang,6,7 Hirofumi Suemori,5 Koji Eto,3 Hiromitsu Nakauchi,8 Shinya Yamanaka,1 Norio Nakatsuji,1,5 Kazumitsu Ueda,1,4,9 and Motonari Uesugi1,2,*

1Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Kyoto 606-8501, Japan 2Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan 3Center for IPS Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan 4Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan 5Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan 6Department of Chemistry & MedChem Program of Life Sciences Institute, National University of Singapore, Singapore 117543, Singapore 7Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), Singapore 138667, Singapore 8Laboratory of Stem Cell Therapy, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan 9These authors contributed equally to this work

*Correspondence: nnakatsu@icems.kyoto-u.ac.jp (N.N.), uedak@kais.kyoto-u.ac.jp (K.U.), uesugi@scl.kyoto-u.ac.jp (M.U.) http://dx.doi.org/10.1016/j.celrep.2014.02.006

This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/3.0/).

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 (Shambrott 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.
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 stained (Figures 1G and 1J).
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 Piecuchszko, 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).
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 KB-3-1 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 KB-3-1 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 ABCB1 (MRP1), a transporter whose expression is unchanged upon cell differentiation (Chen et al., 2001; Nagata et al., 2000). Overexpression of ABCB1 did not result in export of KP-1, whereas calcein AM, a known substrate of ABCB1 (Ver-santvoort 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.

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.
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
ABC1 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 (Schnerrch 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 IC50 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 Bio-Rex.

**Characterization of KP-1**

hiPSCs (clone #201B7) were plated at a density of 2 x 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 hiPSCs 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 anti-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 DP2T camera. For flow cytometric analysis, the cells were washed twice with ice-cold PBS and dissociated with 0.025% trypsin-EDTA into a single cell suspension. Staining by KP-1 was quantified using a FACS Calibur flow cytometer (Becton Dickinson). For the experiments with transporter inhibitors, cyclosporin A or furmitremorin 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 (MRB90-1, MRB90-4, 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 (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-IRE5-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 mg/ml, BioAustralis). After 24 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 710; 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 x 10^5 cells per well, in DMEM containing 10% (v/v) FBS using a confocal microscope (LSM 710; 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 x 10^5 cells per well, in DMEM containing 10% (v/v) FBS and KP-1 (1 μM) with or without furmitremorin 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; Teco). The cells were also lysed with PBS containing 1% (v/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 triplicate.

**Selectivity Profiling of KP-1 with Hematopoietic Cells**

hESC clone Kyoto KheSC (KheESC)-3 was obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto), for 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. KheESC-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 (BioGF; 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 mg/ml sodium selenite, 2 mM L-glutamine, 0.45 mM s-monomethylglucamine, 50 μg/ml ascorbic acid, and 15% (v/v) highly filtered FBS (Celicell 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 CD34 (eBioscience), Pacific Blue anti-human CD34, allopococyanin (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 CaCl2 (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 day 12 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 KheESC-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 (FACS Aria 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 DP2T 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 FACS Calibur 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...
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 on the cells. The cells were treated with 2 mM 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.

ACKNOWLEDGMENTS

This work was supported in part by MEXT Leading Project, JSPS (LR018, 20228001, 23710254, 25221203, and 21591079), the Bio-Oriented Technology Research Advancement Institution of Japan (BRAIN), JST (CREST), and MEXT Leading Project, JSPS (LR018, 23710254, 25221203, and 21591079). The upgrade of the confocal microscope was supported by NEDO and Yokogawa Electric. S.Y. is a member without salary of the scientific advisory boards of iPierian, iPS Academia Japan, Megakaryon Corporation, and HEALIOS K.K. Japan. H.N. is a founder, shareholder, and scientific advisory board member of ReproCELL and Megakaryon. N.N. is a founder and shareholder of ReproCELL.


