at low density (2×10^3 cells/cm^2), and then cultured for 3 days. The numbers of hiPSCs passaged in PBS$^{ca/-}$ were higher than those passaged in PBS$^{-/-}$ (253G1: Fig. 2c, 201B7 & Tic: Supplementary Fig. 4c), suggesting that adding physiological concentration of Ca^{2+} to the dissociation solution increases cell survival rates by decreasing dissociation-induced apoptosis.

It is also known that enzymatic digestion damages hPSCs[5,8]. We first used dispase, an enzyme often used to passage hPSCs under serum- and feeder-free conditions (Table 1)[20]. Because we routinely use 0.025–0.6 U/ml dispase (0.05–300 mg/ml), depending on the enzyme activity and on storage conditions[14], excess dispase (1 U/ml) was used to evaluate its damaging effect with the expectation that dispase dissociation of cell-cell binding would decrease the size of cell clumps, resulting in apoptosis. However, addition of 1 U/ml dispase in PBS$^{-/ca}$ did not decrease hPSC clump size (253G1: Fig. 2d, 201B7: Supplementary Fig. 4d). Indeed, large clumps of annexin V-FITC-negative cells were also found when dispase was added to the PBS$^{ca/-}$ (253G1: Fig. 2e, 201B7: Supplementary Fig. 4e), and quantitative analysis by FCM revealed that the relative percentages of annexin V-FITC-positive apoptotic cells were not changed by addition of dispase (253G1: Fig. 2f, 201B7: Supplementary Fig. 4f). The results were the same when 0.25% trypsin was added to PBS$^{ca/-}$, despite trypsin having more potent protease activity than dispase (Supplementary Fig. 5a–c, e–g). These findings together suggested that adding proteolytic enzyme to the PBS$^{ca/-}$ dose not decrease the cell clump size and thus does not increase dissociation-induced apoptosis. Our results are consistent with a previous report that Ca^{2+} protects against trypsinization of cell-cell binding[10]. Because Ca^{2+} did not affect the cell-fibronectin binding during dissociation (Fig. 1bc, Supplementary Fig 1a), we next measured the re-attachment ability of hiPSCs to fibronectin-coated surfaces. To do this, hPSCs were incubated with dispase in PBS$^{ca/-}$, and replated in ESF9a medium with RI for counting the next day. The reattachment efficiency decreased with increasing concentrations of dispase, and the mean efficiency values at 1 U/ml dispase were smaller than those for PBS$^{ca/-}$ alone (253G1: Fig. 2g, 201B7: Supplementary Fig. 4g); a similar result was attained when trypsin was added to PBS$^{ca/-}$ (Supplementary Fig. 5dh). These results suggested that addition of enzyme damages cells by suppressing cell-fibronectin rebinding rather than by increasing apoptosis.

These results showed that enzyme-free solution containing a physiological concentration of Ca^{2+}, without Mg^{2+}, enables passaging of hPSCs with less cell damage than found using either divalent-free solution or Ca^{2+}-containing solution with enzyme (dispase or trypsin).

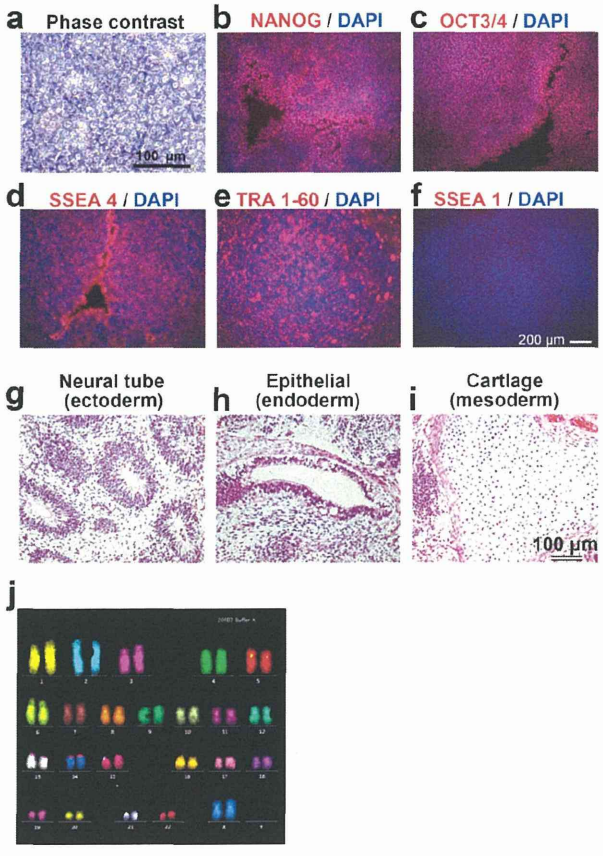

Figure 3 | Passage solution with Ca^{2+} and without Mg^{2+} supports long-term culture and pluripotency of hPSCs. The hiPSC 201B7 were cultured for 15 passages under the ESF-Fb-EzF condition. (a): Phsecontrast micrograph. (b–f): Immunocytochemistry showed that the cells expressed self-renewal markers, NANOG ((b): red), OCT3/4 ((c): red), SSEA4 ((c): red) and TRA 1–60 ((e): red), but not an early differentiation marker, SSEA1 ((f): red). The nuclei were stained with DAPI (blue). (g–h): Histological analysis with HE staining demonstrated that hiPSC-derived teratoma contained derivatives of all three germ layers: neural tube ((g): ectoderm), epitherial ((h): endoderm), and cartridge ((i): mesoderm). (j): FISH karyotype analysis showed a normal karyotype (46XX). Scale bars are 100 μm (a, g–i) or 200 μm (b–f).

Long-term culture of hPSCs with enzyme-free passaging. Next, we tried long-term culturing of hPSCs under enzyme-, serum-, and feeder-free culture conditions. Two hiPSC lines, 253G1 and 201B7, were successfully cultured for more than 10 passages in ESF9a medium on fibronectin-coated dishes by using the solution with Ca^{2+} and without Mg^{2+} (ESF-Fb-EzF condition), with both cell types expressing self-renewal markers (Supplementary Fig. 6a–c, j–l). Immunocytochemistry of embryoid bodies derived from the two cell lines indicated that pluripotency was maintained (Supplementary Fig. 6dm). Unexpectedly, karyotype abnormalities were found not only under the ESF-Fb-EzF condition (Supplementary Fig. 6ir) but also in the sister cultures under the other conditions (Supplementary Fig. 6hopq), suggesting that the abnormalities were induced before enzyme-, serum-, and feeder-free culture.

To confirm the karyotype normality, we newly performed long-term cultures using hiPSC 201B7 and Tic lines. The 201B7 cell line was pre-validated to ensure a normal karyotype, and then cultured under the ESE-Fb-EzF condition for more than 10 passages. The cells formed normal hiPSC colonies, which were tightly packed, and flat colonies consisting of cells with large nuclei and scant cytoplasm (Fig. 3a)[1–3] and expressed four self-renewal markers, NANOG, OCT3/4, SSEA4 and TRA 1–60 but not an early differentiation marker, SSEA1 (Fig. 3b–f). The cells differentiated into derivatives of all three primary germ layers *in vivo* using teratoma formation (Fig. 3g–i). The cells showed a normal karyotype (Fig. 3j). Karyotype after long-term culture was also normal in another hiPSC line, Tic (Supplementary Fig. 7), confirming that karyotype remained stable during the enzyme-, serum-, and feeder-free culture. These results suggested that enzyme-free culture is a useful method for routine culturing of hPSCs.

Cell sheet harvesting. Finally, we tried cell sheet harvesting using our enzyme-free solution. Cell sheet harvesting using special equipment such as a temperature-responsive surface and magnet followed by transplantation is one of the most promising approaches for applying hPSCs to regenerative medicine[21,22]. However, in this study, simple incubation in PBS with Ca^{2+} followed by gentle pipetting enabled us to harvest the cells as 2-mm-diameter sheets without cells splitting off (Fig. 4a–e) and without specialized equipment. Similar results were obtained for early-differentiated cells induced by bone

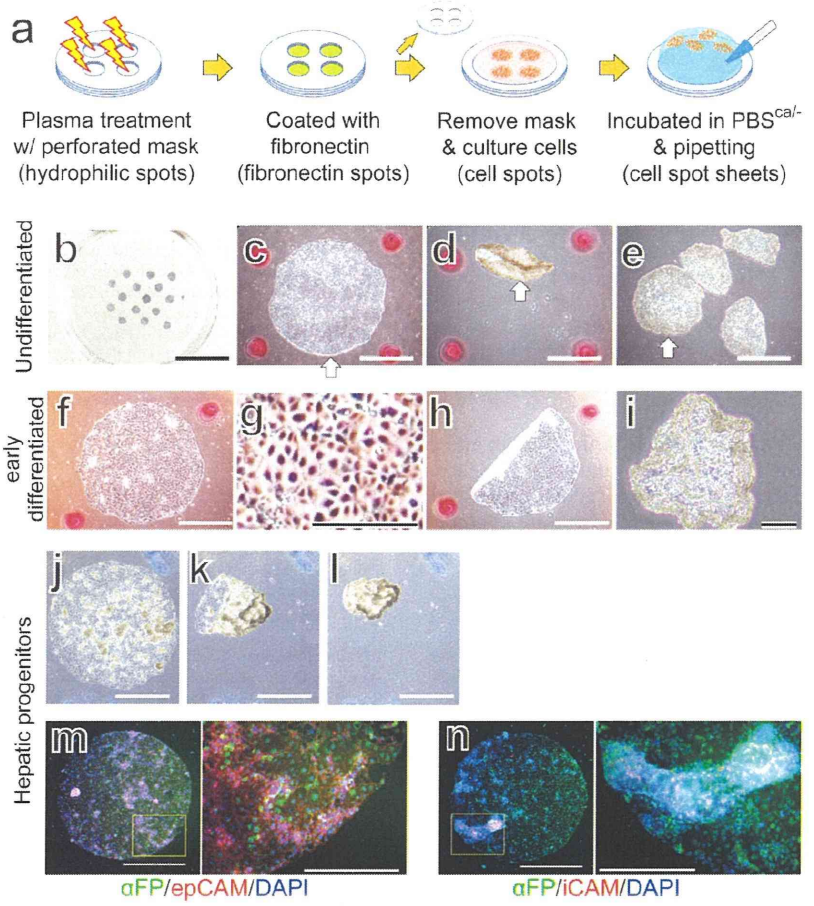

Figure 4 | Cell sheet harvesting. (a): Schematics of spot sheet formation. The hiPSCs (253G1) were cultured in ESF9a medium (undifferentiated: (b–e)), ESF6 medium supplemented with BMP4 for two days (early differentiated: (f–i)). The hiPSC (Tic)-derived hepatic progenitors (j–n). The illustrations were drawn by KO. (b): ALP staining of the hiPSCs plated on 2-mm-diameter fibronectin spots. Phase-contrast micrographs before (c, f, g, j), during (h, k), and after (d, e, i, l) 15 min in PBS$^{ca/-}$ followed by pipetting. (g) is a magnified image of part of (f). The white arrows indicate the same cell spot sheet (c, d, e). The red spots in (c–e, f, h, i) and blue irregular marks in (j–l) are position makers. (mn): Immunohistochemistry of hepatic progenitors using the early stages of liver development marker, α-fetoprotein (αFP: green), EpCAM (red), and iCAM (red). Nuclei were stained with DAPI (blue). The right panels are magnified images of the boxed areas in the left panels. Scale bars are 1 cm (b), 1 mm ((c–f, h, j–l), left of (mn)), and 400 μm ((g, i), right of (m,n)).

morphogenetic protein 4 (BMP4) (Fig. 4f–i) and for hiPSC-derived hepatic progenitors (Fig 4j–n), suggesting that the PBS with Ca^{2+} could be used to routinely and simply harvest cells as a large sheet without special equipment.

Discussion

The present study showed that cell-fibronectin and cell-cell binding are controlled separately by Mg^{2+} and Ca^{2+}, respectively, in hPSC cultures. Using enzyme-free solutions containing Ca^{2+} without Mg^{2+}, we successfully passaged hPSCs cultured under serum- and feeder-free conditions as large cell clumps that showed less damage than those passaged in divalent cation-free solution or with dispase or trypsin. The cells were also harvested as a cell sheet without the need for splitting off.

The cell clumps dissociated by PBS$^{ca/-}$ (1 mM Ca^{2+} and 0 mM Mg^{2+}) and represented in Fig 2d were smaller than those represented in Fig 1e. The decreased cell clump size might be caused by the DNase added in all enzyme-related experiments (Fig 2d–g, and Supplementary Fig 4d–g and 5) to reduce the abundance of free-floating DNA fragments derived from damaged cells. Such addition of DNase might reduce cell-cell attachments arising from the free DNA fragments, and thereby also reduce cell clump size. However, even in the presence of DNase, the cell clumps dissociated by PBS$^{Ca/-}$ without enzyme were significantly larger than those dissociated by PBS$^{-/-}$ without enzyme (Supplementary Fig 5ae).

Addition of enzyme increased the sizes of cell clumps in three of the four conditions in the presence of calcium (Fig 2d, Supplementary Fig 4d and 5ae). A possible reason for this size increase tendency is enzymatic digestion of some cell-fibronectin attachment that enabled cell colonies to detach more easily from the dish. Consequently, large colonies may be harvested intact with less splitting of cell-cell binding by pipetting.

Commonly, hPSCs are passaged with enzyme and in medium containing physiological concentrations of Mg^{2+} and Ca^{2+} (Fig. 5 upper right)[1,2]. Single-cell culture methods such as clonal isolation are achieved by dissociating cells in solutions containing low Mg^{2+} and Ca^{2+} concentrations (Fig. 5 lower left)[4,6,8]. In the present study we showed that hPSC cell-cell binding can be disrupted with less cell detachment from the dish surface in a solution containing high Mg^{2+}, but low Ca^{2+} concentrations (Fig. 5 upper left), and that large cell clumps and sheets can then be harvested by dissociating in low Mg^{2+} and high Ca^{2+} solution (Fig. 5 lower right).

The serum-, feeder-, and enzyme-free composition described herein could provide practical culture methods for controlling

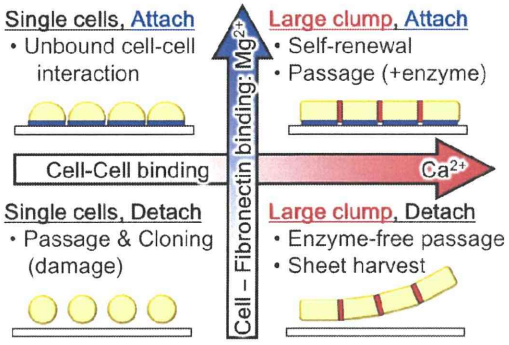

Figure 5 | Schematics of the effects of Mg^{2+} and Ca^{2+} on hPSCs culture.

hiPSCs physical interactions and thus enable further studies into the effects of such interactions and of endogenous and exogenous factors on cells, with the added benefit of eliminating instability caused by lot differences in enzyme. Moreover, such defined culture conditions could facilitate a stable and safe source of hiPSCs for potential clinical applications.

Methods

hPSCs culture. The hESC HUES8[19], H9 (WA09)[1], KhES1, and KhES3[23] lines were obtained from Harvard University (Cambridge, MA, USA), from WiCell Research Institute (Madison, WI, USA), or from Kyoto University (Kyoto, Japan). The hiPSC 201B7[2] and 253G1[18] lines were obtained from RIKEN BRC Cell Bank (Tsukuba, Ibaraki, Japan) through the National Bio-Resource Project for MEXT, Japan and the hiPSCs Tic[24] line (JCRB13331), which was derived from fetal lung fibroblasts (MRC-5), was obtained from JCRB Cell Bank (Osaka, Japan). hPSCs were maintained in a KSR-based medium on mouse embryonic fibroblast (MEF) feeder cells, and subcultured using CTK medium (KSR-MEF-CTK condition), as described in Supplementary Methods. In all experiments, hPSCs maintained in KSR-based medium on MEFs were transferred into serum-free medium, hESF9a on fibronectin-coated dishes, and passaged at least once before assaying (Supplementary Methods). The culture dishes were coated with 2 $\mu g/cm^2$ fibronectin from human plasma (063-05591; Wako) or from bovine plasma (F-1141; Sigma, St. Louis, MO, USA) in PBS for at least 30 min at 37°C, and then excess solution was removed. For subculturing, the cells were detached from the culture dish using 0.2–0.5 U/ml dispase (17105-041; Life Technologies, Grand island, NY, USA) in hESF9a medium and replated in hESF9a medium with 5 μM ROCK inhibitor (RI, Y-27632; Wako). The hESF9a medium (ESF-Fb-Dsp condition) was changed daily.

For long-term culture under enzyme-, serum- and feeder-free condition (ESF-Fb-EzF condition), the cells were passaged with enzyme-free passage solution containing divalent cation-free DMEM-F12 medium (Supplementary table 1) supplemented with the same factors found in ESF9a medium. For subculturing, the cells were rinsed twice with $PBS^{-/-}$ and once with the enzyme-free passage solution, before being incubated in the same solution for more than 15 min at 37°C, and then triturated with a 1-ml micropipette tip. The cells were finally harvested by gentle centrifugation (1 min at 10 G) or stood for a few minutes before replating in hESF9a medium with 5 μM RI. Medium was changed daily.

Embryoid bodies formation. *In vitro* differentiation was induced by the formation of embryoid bodies as described previously[14]. Undifferentiated hiPSCs were cultured by floating in DMEM-F12 medium supplemented with 20% KSR, 0.1 mM 2-mercaptoethanol, and MEM non-essential amino acids (Life Technologies). The floating embryoid bodies were then replated onto 1 mg/ml gelatin-coated dishes in DMEM with 10% FBS. The medium was changed every other day with the same floating culture solution.

Karyotype analysis. Metaphase spreads were prepared from cells treated with colcemid (KaryoMAX Colcemid, Gibco 15212-012, final concentration of 40 ng/ml, overnight treatment) or metaphase arresting solution (Genial Genetic Solutions Ltd., Cheshire, UK). We performed a standard G-banding or multicolour fluorescence in situ hybridization (FISH) karyotypic analysis on at least 20 metaphase spreads for each population. For FISH karyotype analysis, 24XCyte Human Multicolor FISH Probe Kit (MetaSystems GmbH Altlussheim, Germany) were used.

Cell detachment and dissociation assay. The dose response size and removal ratio of hPSCs cultured on divalent cations using 24-well plates were measured as follows. The cells cultured in ESF9a on fibronectin-coated dishes were detached and dissociated into cells clump using 0.2–0.5 U/ml dispase, and then were plated in ESF9a medium on 24-well plates coated with 2 $\mu g/cm^2$ fibronectin (Wako) at 37°C for more than 1 hour. At 4 or 5 days after plating, the attached cells were stained with 1 μM calcein-AM (Dojindo, Kamimashiki, Kumamoto, Japan), a fluorescent living cell dye, for 20 min at 37°C, and imaged as the control state. Then the cells were rinsed once with $PBS^{-/-}$, rinsed again with PBS containing various concentration of Ca^{2+} and Mg^{2+}, incubated in the same PBS for a further 15 min at 37°C, and then triturated 5 times with a 1-ml micropipette tip. For enzymatic digestion experiments, dispase or trypsin was added after 12 min incubation in PBS and left for 3 min. The cells were then triturated 5 times with a 1-ml micropipette tip in the presence of 1 mg/ml DNase I (Roche, Basel, Switzerland), 250 μg/ml trypsin inhibitor (Life Technologies), and 1 mg/ml BSA (sigma), and then 10X volumes of $PBS^{ca/-}$ were added before spinning down the cells. The detached cells were then transferred to another plate, and the remaining cells were imaged for green fluorescence to estimate detachment efficiency. The detached cell clumps were placed between cover slips and cell clump size was estimated based on fluorescent signal using Image J software (NIH, Bethesda, MD, USA). To estimate the cell clump sizes, randomly picked cell clumps for each condition in a test were analyzed with Excel software (Microsoft, Redmond, WA, USA). The cell clump size was converted from area (μm^2) into the number of cells by using the area of single cells, which was estimated to be 240 ± 86 μm^2 (mean ± SD, n = 107) in a separate experiment.

Teratoma formation. Teratomas were generated in severe combined immunodeficient (SCID) mice from 201B7 hiPSCs grown under ESF-Fb-EzF conditions for more than 10 passages. The cells harvested by dispase were resuspended in DMEM supplemented with RI (10 μM). The cells from a confluent single well in a 6-well plate were injected into the thigh muscle of a SCID (C.B-17/Icr-scid/scidJcl) mouse (CLEA Japan, Tokyo, Japan). Nine weeks after injection, tumors were dissected, weighed, and then fixed with 10% formaldehyde Neutral Buffer Solution (Nacalai Tesque, Kyoto, Japan). Paraffin-embedded tissue was sectioned and stained with hematoxylin and eosin (HE). All animal experiments were conducted in accordance with the guidelines for animal experiments of the National Institute of Biomedical Innovation, Osaka, Japan.

Alkaline phosphatase (ALP) staining, immunocytochemistry. The hPSCs were stained with an Alkaline Phosphatase Staining Kit II according to the manual (StemGen, Cambridge, MA, USA). Briefly, the cells were rinsed twice with $PBS^{+/+}$ and fixed with Fix Solution from the kit at room temperature for 4 minutes. The fixed cells were rinsed with PBS containing with 0.05% (v/v) Tween20 and incubated in AP Substrate Solution at room temperature for 20 to 30 minutes. Then the cells were rinsed with $PBS^{+/+}$ and photographed.

Immunocytochemistry was performed as described previously[14,17]. Briefly, hiPSCs were fixed in 4% formaldehyde with 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Then the cells were permeabilized and blocked with PBS containing 0.1–0.2% Triton X-100, 10 mg/ml BSA, 0.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$, and then reacted with primary antibodies in the solution. The primary antibody binding was visualized using secondary antibodies. Antibody information is listed in Supplementary Table 2. Nuclei were stained with 1 μM DAPI (Wako). Micrographs were taken using a BZ-8100 fluorescence microscope (Keyence, Osaka, Japan).

Flow cytometry (FCM). FCM analysis was performed as described previously[14,17]. All cells were removed from culture dishes using 0.02% (w/v) EDTA-4Na in $PBS^{-/-}$ and then fixed in 4% formaldehyde. The fixed cells were permeabilized and blocked with $PBS^{-/-}$ containing 0.1–0.2% Triton X-100 and 10 mg/ml BSA, and then reacted with primary antibodies in the solution. The primary antibody binding was visualized with secondary antibodies. Antibody information is listed in Supplementary Table 2. A cell sorter (JSAN, Bay Bioscience Co., Ltd., Hogo, Japan) was used for data acquisition.

Apoptosis. An annexin V-FITC apoptosis detection Kit was used to detect cell surface phosphatidylserine (BioVision, Milpitas, CA, USA). Cells were floating cultured for four hours in ESF9a solution following detachment and dissociation. For FCM analysis, the cells were re-dissociated by incubating in 0.02% EDTA solution followed by trituration using a 1-mL pipette tip. The living cells were then stained with FITC conjugate annexin V (1 : 100) and 50 μg/ml propidium iodide in binding buffer. The living cells nuclear were stained with 1 μg/ml Hoechst 33342 (Dojindo, Osaka, Japan).

Spot sheet formation. Silicone rubber masks made of polydimethylsiloxane (PDMS, Sylgard 184, 10 : 1 mix; Dow Corning) were perforated with 2-mm-diameter holes using a hole-punch. The bacterial culture dish (non-cell-attachment-treated dishes, Iwaki) with PDMS masking were treated for 60 seconds by air plasma to make hydrophilic spots (YHS-R, SAKIGAKE-Semiconductor Co., Ltd), then coated with 2 $\mu m/cm^2$ fibronectin for more than 1 hour at 37°C to make fibronectin spots. After rinsing twice with $PBS^{-/-}$, the PDMS mask was removed and the dish was sterilized under a UV lamp. hPSCs (253G1) cultured under the ESF-Fb-Dsp condition or hiPSC Tic-derived hepatic progenitors were dissociated in calcium- and magnesium-free solution, and then plated in ESF9a solution with 5 μg/ml RI or in CDM medium with 50 ng/ml FGF10 with RI.

Early differentiation was induced by 2 days cultivation in the ESF6 medium with 2 ng/ml recombinant human BMP4 (314-BP, R&D Systems, Inc, Minneapolis, MN, USA) as described previously[17]. Hepatic progenitors were differentiated based on the previously reported protocol[25]. Briefly, the hiPSC Tic line was passaged and grown for 2 days in CDM medium[26]. hiPSCs were then grown for 3 days in CDM/PVA medium[26] with 100 ng/ml activin, 20 ng/ml basic FGF, 10 ng/ml BMP4, and 10 μM LY294003 (9901, Cell Signaling Technology, Beverly, MA, USA), followed by 3 days

differentiation in CDM/PVA medium with 50 ng/ml recombinant human FGF10 (345-FG-025/CF, R&D Systems).

Data analysis. Image analyses were performed with Image J software (NIH). Statistical analyses were performed with R software (http://www.r-project.org).

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Acknowledgments

The authors would like to thank Reiko Terada at The University of Tokyo for the culturing of hPSCs, and Kumiko Higuchi at AIST for karyotype analysis. This work was supported in part by Grants-in-Aid for Scientific Research and the Project for Realization of Regenerative Medicine from the Ministry of Education, Science, Sports, Culture and Technology (MEXT) of Japan to KO and TM, by Promotion of Independent Research Environment for Young Researchers funding to KO, by an Adaptable & Seamless Technology Transfer Program through Target-driven R&D (A-Step) of the Japan Science and Technology agency (JST) to KO, and by grants from the Ministry of Health, Labour, and Welfare of Japan to KO and MKF. The funding bodies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

K.O. prepared all figures and supplementary figures. S.T. prepared figure 1, 2 and 4 and supplementary figure 1, 3, 4, 5 and 6. A.F., K.Y. and M.K.F. prepared Figure 3, 4 and Supplementary Figure 7. Y.I., Y.O. and M.A. prepared supplementary figure 6. Y.H., T.C., T.M. and M.A. prepared Supplementary Figure 2. K.O., Y.H. and M.K.F. wrote the manuscript text. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ohnuma, K. *et al.* Enzyme-free Passage of Human Pluripotent Stem Cells by Controlling Divalent Cations. *Sci. Rep.* **4**, 4646; DOI:10.1038/srep04646 (2014).

Supplement

Regenerative Medicine

Points to consider in the development of seed stocks of pluripotent stem cells for clinical applications: International Stem Cell Banking Initiative (ISCBI)

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1. Background and utility of this document

In 2009 the International Stem Cell Banking Initiative (ISCBI) contributors and the Ethics Working Party of the International Stem Cell Forum published a consensus on principles of best practice for the procurement, cell banking, testing and distribution of human embryonic stem cell (hESC) lines for research purposes [1], which was broadly also applicable to human induced pluripotent stem cell (hiPSC) lines. Here, we revisit this guidance to consider what the requirements would be for delivery of the early seed stocks of stem cell lines intended for clinical applications. The term 'seed stock' is used here to describe those cryopreserved stocks of cells established early in the passage history of a pluripotent stem cell line in the lab that derived the line or a stem cell bank, hereafter called the 'repository'. The seed stocks should provide cells with suitable documentation and provenance that would enable them to be taken forward for development in human therapeutic applications. WHO recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biologicals and for the characterization of cell banks were updated in 2010 and provide a number of definitions and guiding principles that may apply to stem cells. The term 'cell bank' is used to describe a stock of vials or other containers of cells with consistent composition aliquoted from a single pool of cells of the same culture history (for other specific definitions see PAS 84 [2] and WHO [3]).

Three important assumptions have been made in the preparation of this document. First, that seed stocks of hPSCs are used as starting materials to make cell banks for use in clinical trials. The cell banks made within a clinical trial would need to be established according to Good Manufacturing Practice (GMP) in a facility with a relevant product manufacturing license. These banks would need additional risk assessment focused on the new banking process/reagents and the specific intended clinical application. Second, it has been assumed that undifferentiated pluripotent stem cells would not be inoculated into patients. Third, where feeder cells are used to culture hPSC lines, their cellular nature and intimate contact with the therapeutic cells means that they should be subject to similar risk assessment and banking procedures as applied to the hPSC cells.

It is important to note that responsibility for establishing and updating national regulations for medicinal products relies on National Regulatory Authorities. Therefore, national requirements for cell therapy may vary considerably. Accordingly, it is not intended that this international consensus provides comprehensive guidance that will ensure compliance with requirements in any given jurisdiction. Rather, it is designed to aid the development of clinical grade materials by providing points to consider in the preparation of seed stocks of stem cell lines for use in cell therapy. It may arise that there are circumstances where it is not reasonably possible to meet specific procedures presented in this document. Where this is the case any alternative procedures should be justified and mitigate against any adverse consequences. Finally, this document could also serve as a useful reference to assist in the evaluation of potential sources of candidate cell lines for the development of cell-based medicines, and provide the links necessary to identify some of the key differences in regulatory requirements between countries.

2. Governance and ethics

2.1 General principles

Centers banking stem cell lines (hereafter called repositories) should adopt transparent and harmonized protocols for the collection, storage, access, and use of the cell lines that they curate. As part of a comprehensive governance structure, repositories should establish robust mechanisms for the authentication of *bone fide* users and should strive for equitable and transparent conditions of access and of material transfer (Appendices 1a, 1b and 2). Such protocols

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should be adopted according to internationally accepted principles for research ethics and in compliance with applicable legal, ethical and regulatory requirements (Appendix 3, 4 and 5). Furthermore, repositories should establish a system for documenting and monitoring performance with respect to such principles and requirements.

2.2 Key issues in determining provenance of pluripotent stem cell lines

Repositories should ascertain the provenance (source/origin) of the human biological specimens from which the pluripotent stem cell lines have been derived. International guidance exists for documenting the provenance of the cell lines [1,4–7].

Important issues to consider when evaluating provenance include:

- Evidence of free and voluntary informed consent, for the proposed research use, in conjunction with independent review and oversight, with particular attention given to disclosure of potential clinical and commercial applications.
- The extent to which reimbursement (e.g., expenses, financial incentives, monetary payments) were provided for donation of biological samples.
- The ability of the donor to withdraw original specimens, derived cell lines, data or otherwise to discontinue participation in research.
- The possibility that derived cell lines may be used for a wide range of research, possibly through a public repository.
- The establishment of robust systems for data security and traceability.
- The implementation of mechanisms for the protection of donor privacy and confidentiality. Particular attention should be given to the generation and use of genome sequence data.

Many national and more local jurisdictions have explicit policies governing the acquisition and use of human biospecimens for pluripotent stem cell derivation, particularly with regard to embryonic sources (Appendix 3 and 4). Prior to accepting a pluripotent stem cell line, a repository should determine its provenance by first documenting that the biospecimen was collected and the cell line derived in a manner broadly consistent with international standards for research ethics [4,5,6,7,20]; and second, to make a positive determination that the biospecimen was obtained in a manner consistent with applicable laws in the country of origin.

2.2.1 Provenance determination and international standards

Providers of cells should be able to demonstrate to the repository that they have met all applicable legal and ethical requirements associated with the procurement of a human biospecimen from which a pluripotent stem cell line was derived. Given the heterogeneity of national laws and regulations governing research and clinical applications, the depositor of a cell line should provide information that enables the repository governance structure to determine whether the conditions of derivation, use and distribution are broadly consistent with the repository's national regulation. Moreover, repositories should have in place a mechanism (e.g., 'horizon scanning', advisory board) to track changes in the legal and regulatory frameworks. In addition, repositories should verify and retain sufficient documentation to support a determination that each cell line has been obtained in accordance with international standards for research ethics.

Key principles include the following:

Independent review and oversight

The protocol for procurement of tissues, gametes or embryos for the purpose of generating a pluripotent stem cell line should be subject to independent scientific and ethical review. Review bodies include ethics committees, licensing bodies or committees responsible for oversight of research involving human subjects.

Voluntary informed consent

In addition to verifying appropriate informed consent, the repository should ascertain additional details regarding donor's disclosure when available (Appendix 1b). Numerous bodies and national policies recommend or require the disclosure of specific information to donors (particularly for hESC derivation). A number of jurisdictions have consent requirements that include, but are not limited to, disclosure of possible human transplantation, genetic modification, international sharing and commercial potential. Documentation of a robust informed consent process that addresses these requirements can serve to support wide distribution and utilization of the cell lines (Appendix 3 and 4). Informed consent requirements for stem cell derivation, use and banking have evolved over time and jurisdictional

variations may exist for different sources of biospecimens. In addition, standards for evaluating informed consent processes may need to be flexible and allow for context-specific considerations. For example, agreement to banking could include broad consent to future unspecified research (subject to appropriate security mechanisms and governance); whereas some protocols may be intended to develop a specific cell product. Donors should be notified of the possibility of future use in cellular therapies, commercialization of eventual products and of the international sharing of samples and of stem cell lines. Moreover, donors should also be informed of the limitations in privacy protection (see section 3.2 & 5.1) given the need to assure traceability for safety reasons (see section 6.9).

Gratuitous donation

Donors should not be paid to provide somatic cells, gametes or embryos for stem cell derivation, nor should they be reimbursed for any costs, such as tissue storage, prior to the decision to donate.

2.2.2 Compliance determination and access policies

Mechanisms should be in place to make a positive determination of compliance with both the ethical and legal requirements of the jurisdiction of biospecimen's origin, together with those of the jurisdiction where the cell line was derived, deposited, and will be used in research (Appendix 3 and 4). Furthermore, it is important to consider that there may be jurisdictional or funding agency restrictions on the types of cell lines eligible for research use as may be the case for hESC lines. To the extent feasible the repository should strive to compile complete provenance information for evaluation; however, it is ultimately incumbent on the end user of the cell line to determine that its provenance meets local ethics and legal requirements.

Repositories should also adopt transparent, flexible and equitable access policies. Given the importance of international collaboration, such policies should include procedures for deposit of cell lines of foreign origin, and for the distribution of cell lines to researchers in other jurisdictions. Among the policy criteria to be considered are the following:

- Mutual recognition via 'reciprocal policy agreements' allowing for transnational sharing of cell lines provided that the cell lines were derived by, or approved for use by, a licensing entity formally recognized as having adopted consistent ethical and legal requirements.
- 'Substantial equivalency' whereby criteria for cell line derivation, use, and banking in different jurisdictions involve ethical and legal requirements that are deemed to be 'broadly' or 'substantially' acceptable to the repository management and under applicable regulation.

3. Provenance and selection of donor tissue

3.1 Donor selection, screening and medical records

Eligibility criteria for embryo, cell or tissue donors intended for human transplantation are subject to national regulatory frameworks and institutional protocols in the jurisdiction of origin. As a general rule, donor eligibility determination requires screening for risk factors associated with infection and communicable disease. These are typically focused on serum human viral blood-born pathogens (e.g., HIV, hepatitis B virus, hepatitis C virus) and may also include other pathogens endemic to the donor's origin (e.g., human T-cell lymphotropic virus I&II, Chaga's disease, malaria). Donor testing for these agents may be required to be carried out under national licensed facilities.

For hESCs there are a number of considerations pertaining to donor screening protocols for assisted reproduction treatment (Appendix 4). For hiPSC evaluation, inclusion and exclusion criteria represent a starting point for risk-assessment or risk mitigation. In some cases, inclusion criteria may call for the collection of cells and tissues from patient groups with specific clinical (disease) indications. Any information regarding known disease indication should be associated with specific cell and tissue samples to support risk evaluation (see section 6.3). While cell lines derived from patients with inherited disease have been recognized as having potential scientific utility, they are unlikely to be suitable for development of general clinical applications.

Finally, regulatory authorities responsible for the evaluation of biological products consistently emphasise the value of a donor medical history. It is important to note that rules adopted in some jurisdictions may require a review of donors' relevant medical records and or a medical history screening; considerations for extended medical histories have been published by Murdoch *et al.* [8].

3.2 Allogeneic cell transplantation

The establishment of hiPSC repositories for human leukocyte antigen (HLA) haplotype representation to facilitate immune-genetic matching is a proposition already being pursued. Of particular interest are individuals who will be homozygous for common HLA haplotypes to maximise prospective histocompatibility matching, although it is important to note that rejection will also be mediated by other non-HLA associated molecules. In the establishment of these resources, health screening, medical history and life style documentation will be important sources of information the help assure the prospective patient safety as described below. However, defining what constitutes a a fully functional and 'safe' genetic state is more problematic and may not be resolved by development of autologous hiPSC lines as observed in mouse models. For hESCs derived from surplus IVF embryos, the risk of carrying genetic deficiencies has largely been presumed minimal. This is based on two presumptions: that the infertility of the donors is not in fact a congenital deficiency, and that the culture and manipulation of embryos *in vitro* does not result in genetic and epigenetic perturbations. For hPSC lines in general, it is not possible to screen for cell inheritable genetic or epigenetic conditions that are not known, and these risks are thus tolerated (Advisory Committee on the Safety of Blood, Tissues and Organs [SaBTO] [9]). In the case of some homozygous HLA haplotypes there are also disease associations (see section 4.4).

There is a reasonable prospect that in the near future there will be affordable access to personalized genomic sequence information. If genomic sequence information of banked hiPSC lines were also made openly available to research, then anonymized, or de-identified, donors could ultimately identify cell lines derived from them, or conversely be potentially identified by others [10]. Banking of hiPSC lines may, therefore, require greater attention to systems for preserving donor privacy [11].

3.3 Ongoing donor traceability and management of post-donation disease and adverse events in patient treatment

Ideally, there should be a mechanism that allows a link to be made between cell line and donors, but only in exceptional circumstances such as seeking reconsent or to facilitate reporting of serious post-donation disease e.g., hepatitis C virus, variant Creutzfelt–Jakob Disease (CJD). While this should be considered, a risk–benefit analysis should also be carried out taking into account the administrative costs, together with ethical and policy considerations that such a system could impose. Of course, the repository should ensure that there is an effective tracking system for the cellular materials, from reception of tissue to the point of release to users to support internal troubleshooting and to enable management of adverse events in clinical trials (section 6.9). To this end, the donor's informed consent should ideally allow for linkage to medical history and permission to re-contact. Linkage and re-contact will also raise, however, the possibility of donor(s) withdrawal (see section 2 and Appendices 3 & 4).

In cases where the institution that creates the seed stock is a separate entity from the procurement institution, the repository should retain sufficient records to allow traceability to the initial sample, while detailed information relating to procurement process and donor identity may remain with the organisation responsible for procurement (see section 6.8).

3.4 Advantageous capture of biological specimens

In certain jurisdictions it is required that donor blood samples be associated with embryos intended for assisted reproductive treatments. Consequently, there may be blood or other biological specimens associated with some banked embryos and similar arrangements may be in place for some hiPSC lines. While such samples could inform future investigations, they are unlikely to have been consented for this purpose and retention of blood samples from embryo donors may not be the best archive material to use for the purpose of microbial safety testing. In fact samples from the cell line seed stock may be more appropriate for this purpose as proposed by Murdoch *et al.* [8]. For discussion on the consent issues relating to the use of archive tissues for the generation of hiPSC lines see Lomax *et al.* [12].

3.5 Donor medical histories

In a number of jurisdictions a donor medical history may be required that identifies potential hazards in the past of the donor or their family and may also relate to aspects of the donor's lifestyle that may be associated with risk of infection. Repositories may wish to assure themselves that such information is accessible and even collate it in an anonymized, or de-identified, form (i.e., with donor name redacted); however, this may not be possible in some jurisdictions.

If medical histories are not collected at the time of donation, re-contacting donors may be difficult or impossible if, for instance, they have changed location or have become deceased. When establishing requirements for collecting donor medical histories, it is important to decide what information will be useful to collect [8].This will include risk factors such as sexual activity, drug abuse, cancer and family history of hereditary disease such as familial CJD. Finally, it is important to recognise that the management of donors may vary considerably in different jurisdictions, and in addition, the veracity of information provided by donors on certain risk factors may be difficult to determine. In conclusion, medical histories, in combination with donor virological testing, can be useful to screen out donated tissues carrying higher risk of transmitting certain infections or other disorders, and thereby mitigate against certain risk factors. However, these alone do not necessarily assure safety of cell lines selected for use in clinical products, which will require supplementary risk assessment and testing, as described in sections 4 and 5.

3.6 Disclosure of significant clinical information

In carrying out hPSC research, increasingly large genetic data sets are being generated. These will inevitably contain information on infectious disease and genetic inherited disorders that may be of relevance to the health of the donor and/or their relatives. The return of individual research results and incidental findings should be warranted and supported by informed donor consent, but also by protocols comprehensively detailing the nature of such findings, the mechanisms for disclosure and their management. Ideally, these procedures should be established prior to obtaining informed consent to donate. Moreover, such protocols should be transparent with regard to the conditions for such context-specific and qualified disclosure [5].

3.7 Withdrawal of bio-specimens and/or associated data

Obtaining medical information or other donor information on an ongoing basis constitutes human subjects research, and therefore, the participant has the right to discontinue participation (research withdraw). The extent to which a participant may withdraw will vary depending on the research protocol and applicable laws, but the withdrawal policy should be clearly described in the informed consent document. The following are common examples of withdrawal policies:

- Donors may request that donated embryos for hESC derivation, or somatic cells for hiPSC derivation, may be destroyed. However, it is generally accepted that derived hESC or hiPSC lines may continue to be used, and distributed materials cannot be recalled.
- Donors may request that all individually identifying information be removed from donated samples or resulting cell lines.
- Donors may request that further collection of medical information cease. Policies and legislation vary with regard to the status of medical information already associated with a cell line.
- Donors may request to withdraw consent up to the time their tissue is used to derive a cell line.
- Donors may request that they are no longer to be contacted by researchers.

Any or all of the above provisions may be applicable to a particular hESC or hiPSC line. Typically, donors are offered 'staged' withdrawal options where they may apply one or more of the options above, possibly at different time periods. It is important that the investigator or party responsible for interacting with the donor and the repository have clear procedures and protocols in place to act upon withdrawal requests in a timely and effective manner.

4. Safety assessment of hPSC seed stocks

Whilst microbiological contamination is the most immediately evident hazard from cells intended for human therapy, there are a number of additional factors that should be considered. These include the presence of transformed cells, expression of potentially damaging bioactive molecules and the appearance of novel surface molecules following *in vitro* isolation and culture. The presence of potentially tumorigenic cells is clearly undesirable in a cell culture intended for clinical application. However, the remaining non-microbiological factors are more difficult to evaluate in terms of safety and more experience in the use of hPSC lines will be needed to assess the exact nature of any risk to patients. This section considers the primary biological issues for hPSC lines that will have a critical impact on their safe use in cell-based medicines, and considers approaches to reduce the risk of these hazards employing a risk-based approach.

It is obviously desirable that each stem cell line established for clinical use should be available for use in a broad range of therapies. The specific clinical settings and therapies to be

developed from these seed stocks are unlikely to be known and it is therefore not possible to carry out a full risk analysis that would be needed to determine the testing regime for a cell line used for a wide range of therapies. The testing regime required for release of cell banks will, therefore, inevitably be based on the likely generic hazards associated with cell culture and the specific hazards associated with the origin and specific culture history of each cell line on a case by case basis (see sections 4 & 6). All testing used for release of clinical grade seed stocks should be performed by a qualified and accredited laboratory according to national and/or international regulation and guidance. Similar standards should be applied to any cell banks of partially differentiated or feeder cells.

It is recommended for a manufacturer using a cell line to produce a cell-derived biological product to focus testing and characterization on vials from the master cell bank (MCB) [3]. This practice can make testing regimes more efficient and ensures the MCB is fit, according to current best practice, for production of future working cell banks (WCBs). Additional testing of WCBs should be considered where justified based on science-based risk assessment, such as the risk of an expansion of a viral contaminant from culture reagents or a clonal expansion of karyologically abnormal cells. However, developing guidance [3,14] proposes that alternative strategies may be justified, such as exhaustive testing of each working cell bank as it is produced.

4.1 Microbiological hazards

4.1.1 General considerations on microbiological hazards

A very broad range of microorganisms could potentially contaminate hPSC lines and some may be able to grow in cell culture becoming a permanent and non-cytopathic component of the cell culture. In addition, some of these organisms may have the capacity to transform human cells and present a tumorigenic hazard for clinical use [9]. The primary risk of contamination arises from the donor tissue used to generate the cell line and the associated most likely contaminants will, to some degree, be different for hPSC lines derived from embryos, where contamination from the reproductive tract may need to be considered, compared to hiPSC lines isolated from blood or skin cells. In addition, donor history (section 2) and history of the cell line including storage conditions and detailed records of the reagents used (section), provide the key information to assess risk of contamination for each hPSC line. This risk assessment can then be used to establish the testing regime for the seed stocks of each cell line. Whilst virological testing of a donor is useful information in risk assessment, it does not guarantee freedom from viral contamination of a cell line derived from that donor's tissue. Thus, in addition to risk mitigation (see section 6.2 & 6.3), microbiological testing of a cell line will provide confidence in its safety for use in humans.

When cells are transferred from supplier to the manufacturer, a different set of conditions and reagents will apply and the appropriate testing regime for MCBs and WCBs established for generating the cell therapy product, will need to be reassessed. Moreover, regulators are likely to expect fully qualified cell banks for manufacturing purposes, as recommended for banks of cells used in other aspects of manufacturing [3,13]. With this in mind some stem cell line repositories may choose to perform testing on seed stock cell banks only for the most serious potential contaminants, whilst others may carry out a borader range of testing on their cells.

Highly sensitive molecular and cell culture based assays have been established and qualified for the evaluation of cells used in the manufacture of vaccines and biotherapeutics [3,13]. However, it is important to recognise that current qualified methods are not sufficiently broad ranging to provide an absolute guarantee of absence of microbial contamination. Deep sequencing technologies and microarray technologies [14–16] offer significant potential advances in the detection of virtually any agent in cell cultures, as has been demonstrated in cells used for vaccine manufacture [17,18]. However, they have yet to be proven and validated for use with cell banks for clinical use. Repositories should keep a 'watching brief' on emerging technologies and engage with their developer to assemble and analyze data that may be useful for clinical validation. Currently, such novel techniques lack appropriate validation for detection of different types of agents. It will be necessary to have widely available control materials and procedures to manage unqualified data as developed by WHO for sequencing [19], and by the Minimum Information About a Microarray Experiment (MAIME) workgroup [20]) to provide minimal datasets from microarrays for interpreting and assessing reproducibility of experiments.

4.1.2 Microbiological testing

The following sections discuss the typical microbiological tests that should be considered for seed stocks of hPSC lines intended for clinical use and an example of a possible core testing regime