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Generation of Human Induced Pluripotent Stem (iPS) Cells from Liver Progenitor Cells by Two Chemicals and the Clinical Application

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1. Introduction

The generation of human induced pluripotent stem human (iPS) cells from human cells by only chemicals is not reported. On the other hand, loss of microRNA-145 elevates Oct3/4, Sox2 and Klf4 (1). Furthermore, TGF beta ligand elevates Sox2 (2). Therefore, when we can find human cells with endogenous expressions of Oct 3/4 and Sox2, even if their expressions were slight, it would be possible for us to generate the human iPS cells from the cells by using micro RNA-145 inhibitor and/or TGF beta ligand as chemicals appropriately.

2. Materials and methods

2.1 Cell culture

2.1.1 Human liver biopsy specimens

Formalin-fixed and paraffin-embedded human postliving donor transplant liver biopsy specimens were obtained in our institutes. Liver biopsies from 10 living donor transplant recipients were collected at 1 week (two specimens), 6 weeks (five specimens), and 12 weeks (three specimens) posttransplant as part of a standardized protocol to rule out liver pathology following living donor transplantation. Zero specimens were collected to evaluate for suspected rejection. All human tissue procedures were approved by the Institutional Review Board. Oct3/4 positive cells were observed in specimens from all timepoints posttransplantation. In specimens from 1 week, Oct3/4 positive cells were present in a contiguous streaking manner from the central vein.

2.2 Induction of human iPS cells

For generation of human iPS cells, human liver progenitor cells with Oct 3/4 positive were derived from human liver biopsy specimens at first. Next, the cells were seeded at a density of 53104 cells per 6-well plate in human embryonic stem (hES) cells medium. The human liver progenitor cells with Oct3/4 positive were treated with 2'OMe-miR-145 as micro RNA-

145 inhibitor (100 nmol/L; 168 hours, after that, 50 nmol/L; 72 hours) and TGF-beta ligand (100 pM; 48 hours) in the hES cells medium.

Furthermore, the human iPS cell (hES cells-like) colonies were mechanically isolated and were subsequently re-plated and maintained on CF1 mouse feederlayers (Millipore) in hES cell medium.

2.3 Western blotting

The cells at semiconfluent state were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, and 0.1% SDS), supplemented with protease inhibitor cocktail (Roche). The cell lysate of MEL-1 hES cell line was purchased from Abcam. Cell lysates (20 mg) were separated by electrophoresis on 8% or 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The blot was blocked with TBST (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20) containing 1% skim milk and then incubated with primary antibody solution at 4 °C overnight. After washing with TBST, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr at room temperature. Signals were detected with Immobilon Western chemiluminescent HRP substrate (Millipore) and LAS3000 imaging system (FUJIFILM, Japan). Antibodies used for western blotting were anti-Oct3/4 (1:600, Santa Cruz), anti-Sox2 (1:2000, Chemicon), anti-Nanog (1:200, R&D Systems), anti-Klf4 (1:200, Santa Cruz), anti-c-Myc (1:200, Santa Cruz), anti-E-cadherin (1:1000, BD Biosciences), anti-β-catenin (1:200, BD Biosciences), anti-β-actin (1:5000, Sigma), anti-mouse IgG-HRP (1:3000, Cell Signaling), anti-rabbit IgG-HRP (1:2000, Cell Signaling), and anti-goat IgG-HRP (1:3000, Santa Cruz).

2.4 Immunocytochemistry

Cultured cells were fixed with 10% formaldehyde for 10 min and blocked with 0.1% gelatin/PBS at room temperature for 1 hr. The cells were incubated overnight at 4°C with primary antibodies against SSEA-4 (MC813-70; Chemicon), TRA-1-60 (ab16288; abcam), TRA-1-81 (ab16289; abcam), or Nanog (MAB1997; R&D Systems), AFP (Sigma), cTNT (NeoMarkers), DESMIN (Lab Vision), GFAP (DAKO), NKX2.5 (Santa Cruz Biotechnology), PDX1 (R&D systems), SMA (Sigma), SOX17 (R&D systems), TH (Chemicon), bIII-tubulin (Covance Research Products).

For Nanog staining, cells were permeabilized with 0.1 % Triton X-100/PBS before blocking. The cells were washed with PBS three times, then incubated with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) and Hoechst 33258 (Nacalai) at room temperature for 1 hr. After further washing, fluorescence was detected with an Axiovert 200M microscope (Carl Zeiss).

2.4.1 qRT-PCR

Total RNA was isolated from bulk cell culture samples or handpicked undifferentiated colonies using RNeasy columns (Qiagen) with on-column DNA digestion. cDNA was produced using oligo-dT15 priming and M-MLV reverse transcriptase (USB) according to the manufacturer's instructions at 42 °C for 1 h. About 50 ng of total RNA equivalent was typically used as template in 20 µl SYBR green PCR reactions (40 cycles of 15 s, 95 °C/60 s, 60 °C on Applied Biosystems 7300 instrumentation) that additionally contained 0.375 mM of each primer and 10 µl of SYBR green PCR mix (ABI). All primers used were confirmed to

amplify the predicted product at close-to-optimal efficiency without side products. Relative expression levels were calculated using the comparative Ct method, based on biological control samples and two housekeeping genes for normalization.

2.5 In vitro differentiation of human iPS cells

For immunocytochemistry, embryoid bodies (EBs) were generated from iPS cells with the hanging-drop method in MEF-conditioned medium. After 5 days, EBs were transferred to gelatin-coated plates and subsequently cultured for another 14 days in knockout DMEM (Invitrogen) supplemented with 20% FBS, 1mM L-glutamine, 1% non-essential amino acids, 0.1mM β-mercaptoethanol, and penicillin/streptomycin. For qRT-PCR, iPS colonies were mechanically isolated and re-plated on Matrigelcoated plates in MEF-conditioned medium. After 2 days, medium was replaced with medium for each of the three germ layers. Endodermal differentiation: RPMI1640 medium supplemented with 2% FBS, 100 ng ml⁻¹ activin A (R&D Systems), L-glutamine and penicillin/streptomycin for 3 weeks. For mesodermal differentiation: knockout DMEM supplemented with 100 mM ascorbic acid (Sigma), 20% FBS, 1mM L-glutamine, 1% non-essential amino acids, 0.1mM β-mercaptoethanol and penicillin/streptomycin for 3 weeks. For ectodermal differentiation: the cells were maintained in N2B27 medium for 7 days and the medium replaced with N2 medium supplemented with 10 ng ml⁻¹ bFGF2 (peprotech), 100 ng ml⁻¹ Sonic Hedgehog (R&D Systems), 10 ng ml⁻¹ PDGF (R&D Systems), L-glutamine and penicillin/streptomycin for 2 weeks. The medium was changed every other day. Furthermore, we could generate human normal hepatocytes from ChiPS cells in vitro, according to method of previous report (Ref. S1).

2.6 Bisulfite sequencing

Genomic DNA (1 mg) was treated with a CpGenome DNA modification kit (Chemicon) according to the manufacturer's recommendations. Treated DNA was purified with a QIA quick column (QIAGEN). The promoter regions of the human Oct3/4 and Nanog genes were amplified by PCR. The PCR products were subcloned into pCR2.1-TOPO. Ten clones of each sample were verified by sequencing with the M13 universal primer. Primer sequences used for PCR amplification were provided in a previous report (3).

2.7 Karyotyping and DNA fingerprinting analysis

Chromosomal G-band analyses and DNA fingerprinting analysis were performed in our laboratories.

2.8 Teratoma formation

Teratoma formation was performed as previously described (3).

2.9 Primers for RT-PCR gene primer sequence

Primers for RT-PCR gene primer sequence were performed as previously described (3). As for β-catenin, the primers for RT-PCR gene primer sequence was also performed as previously described (Ref. S1).

2.10 The evaluation of cancerous risk for the human iPS cell lines

The ChiPS cell lines as human iPS cell lines were inoculated intramuscularly into immunodeficient mice (Rag2^{-/-} Il2rg^{-/-}). Then, we compared microvessel density (MVD)

within teratomas in mice between both groups (p21 knockdown and control group). As for angiogenesis in teratomas from ChiPS cell lines, microvessel density (MVD) per high-powered field (h.p.f.) of teratomas was quantified by human-specific anti-CD31 immunofluorescence. $N = 3-6$; All values were mean \pm s.e.m.

3. Results

3.1 The generation of human induced pluripotent stem (iPS) cells

In 0 hour time point, though the expression of Oct3/4 was found and the each expressions of Sox2, Klf4 and Nanog was slight, Oct3/4, Sox2, Klf4 and Nanog were reactivated during the administration of chemicals (Fig. 1). However, by stopping of the administration, the reactivations for the expressions of each pluripotency-associated genes were silenced.

Around day 14 after the administration of chemicals, we observed human hES cell-like colonies (Fig. 2). The cells expressed hES cell-specific surface antigens, including SSEA-4, tumor-related antigen (TRA)-1-60, TRA-1-81 and Nanog protein (Fig. 3).

Furthermore, reverse transcription polymerase chain reaction (RT-PCR) showed that the cells expressed many undifferentiated ES cell-marker genes such as Oct3/4, Sox2, Nanog, etc, at levels equivalent to the hES cell line H9 (Fig. S1). Moreover, by Western blotting, protein levels of Oct3/4, Sox2, Nanog, etc were similar between the cells and hES cells (Fig. S2).

On the other hand, bisulfite genomic sequencing analyses evaluating the methylation status of cytosine guanine dinucleotides (CpG) in the promoter regions of pluripotent-associated genes, such as Oct3/4 and Nanog, revealed that they were highly unmethylated (Fig. 4). Thus, the cells were more similar to hES cells. We designated the human iPS cells as **Chemicals-human induced pluripotent stem (ChiPS)** cells.

Furthermore, in order to test pluripotency in vivo, we transplanted the ChiPS cell lines as human iPS cell lines subcutaneously into the dorsal flanks of immunodeficient (SCID) mice. Nine weeks after injection, we observed tumor formation. Histological examination showed that the tumor contained neural tissues (ectoderm), striated muscle (mesoderm), and gut-like epithelial tissues (endoderm) (Fig. 5), demonstrating that the cells were pluripotent. Moreover, we confirmed the expression of markers for all three germ layers by qRT-PCR analysis (Fig. 6).

On the other hand, chromosomal G-band analyses showed that human iPS cells had a normal karyotype of 46 XY (Fig. 7). In addition, DNA fingerprinting analysis confirmed that human iPS cells were liver progenitor cells origin (Data not shown). Thus, human iPS cells clones were derived from liver progenitor cells and were not a result of cross-contamination. Therefore we could generate ChiPS cells as human iPS cells from human cells by only chemicals.

3.2 The risk evaluation of malignant transformations for the human iPS cells lines

By using MVD within SCID mice that ChiPS cell lines as human iPS cell lines were transplanted, we performed the risk evaluation of malignant transformations for the cell lines (Fig. 8). The ChiPS cell lines between the p21 knockdown group and the control group were inoculated intramuscularly into immunodeficient mice (Rag2^{-/-} Il2rg^{-/-}). Then, we compared microvessel density (MVD) within teratomas in mice between the both groups. As a result, the MVD was significantly reduced within teratomas derived from the latter

compared to the former ($P<0.01$). Though cancer risk of patients with down's syndrome was less than healthy people and the MVD in the case of human iPS cells derived from patients with down's syndrome was also less than the MVD in the case of human iPS cells derived from healthy people (4), the MVD in control group of the present study was equal to the case (4) of patients with down's syndrome (Fig. 8 and Ref. 4). Therefore, our observation shows the increased risk for malignant transformations of ChiPS cells by the p21 knockdown (Fig.8), and the induction of p21 is necessary to avoid malignant transformations of ChiPS cells as human iPS cells.

4. Discussion

We could generate human iPS cells from human liver progenitor cells by only chemicals. The human iPS cells were similar to hES cells in morphology, proliferation, surface antigens, gene expression, and epigenetic status of pluripotent cell-specific genes. Furthermore, these cells could differentiate into cell types of the three germ layers in vitro and in teratomas. The reprogramming efficiency of generating ChiPS cells as human iPS cells ranged from 0.05%. Therefore, the efficiency was improved more than previous reports (3) (5) (6) (7). Moreover, though we previously found the induction of p21 was necessary to avoid malignant transformations of human iPS cells (8), the result was also true of the case of ChiPS cells in the present study. In conclusion, we could genetate ChiPS cells as human iPS cells from human cells by only two chemicals. Now, we try to generate human iPS cells from human normal somatic cells by chemicals used in this study. Perhaps, human iPS cells such as ChiPS cells would be useful as novel drug screening system in the near future (Fig. 9).

5. Figures and legends

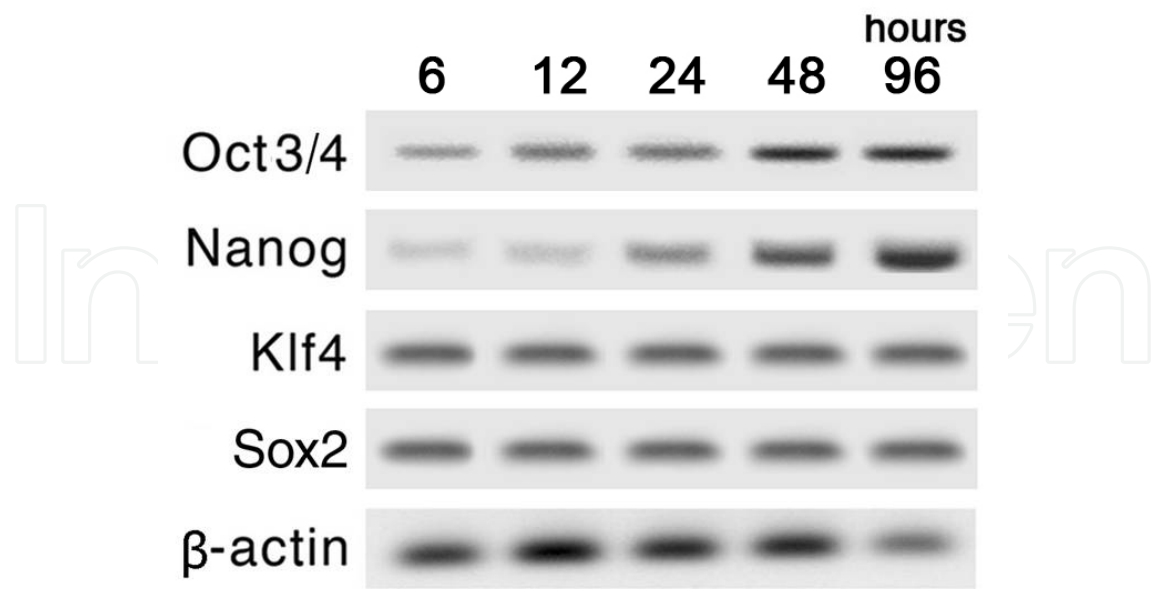


Fig. 1. Pluripotency-associated genes during treatment of human liver progenitor cells. Total RNA was prepared after various periods of time (6, 12, 24, 48 and 96 hr) and analyzed by reverse transcription-polymerase chain reaction for embryonic and pluripotency-associated genes (Oct3/4, Nanog, Klf4 and Sox2) and. Loading control, β -actin

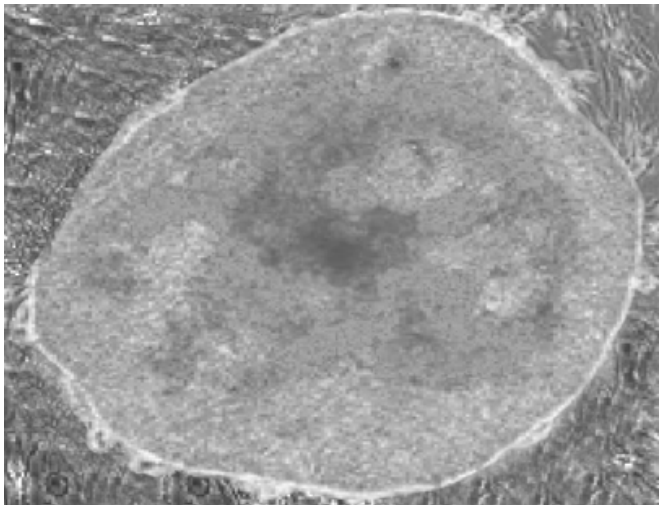


Fig. 2. Typical image of hES cell-like colony (ChiPS cells as human iPS cells)

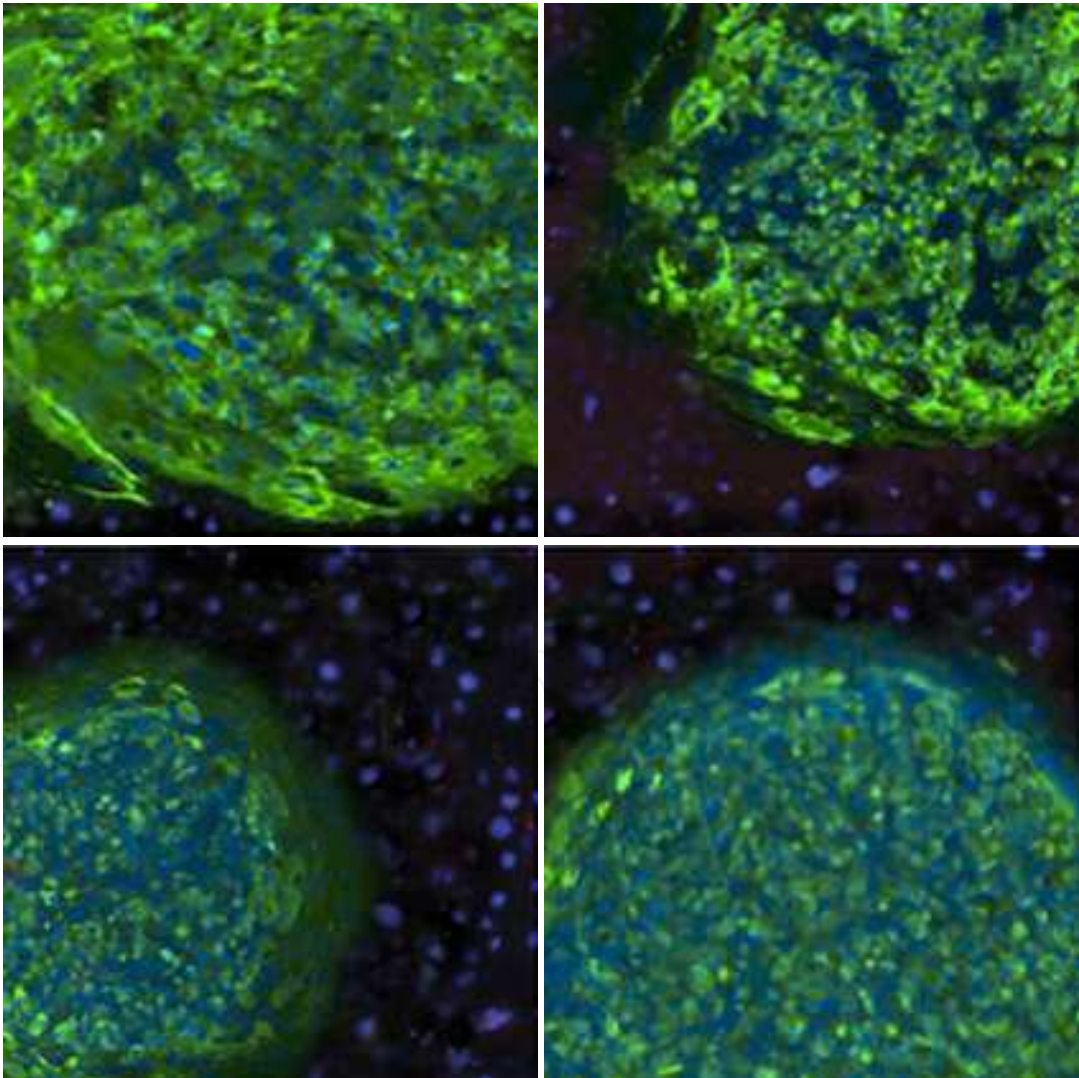


Fig. 3. Typical immunocytochemistry for SSEA-4, TRA-1-60, TRA-1-81, and Nanog (human iPS cells clone). Nuclei were stained with Hoechst 33342 (blue). Bars = 100 mm

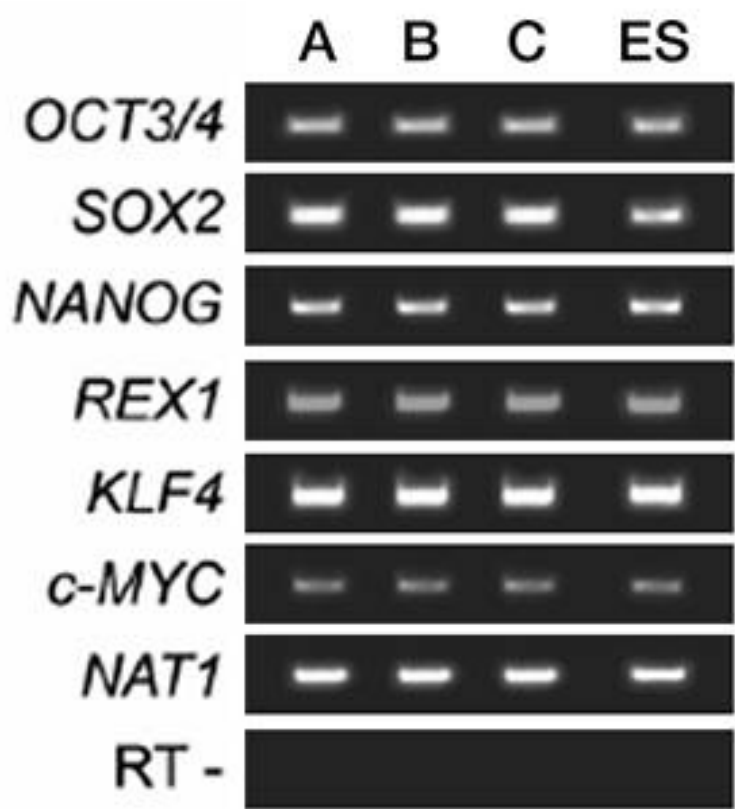


Fig. S1. RT-PCR analysis of ES cell-marker genes in human iPS cells

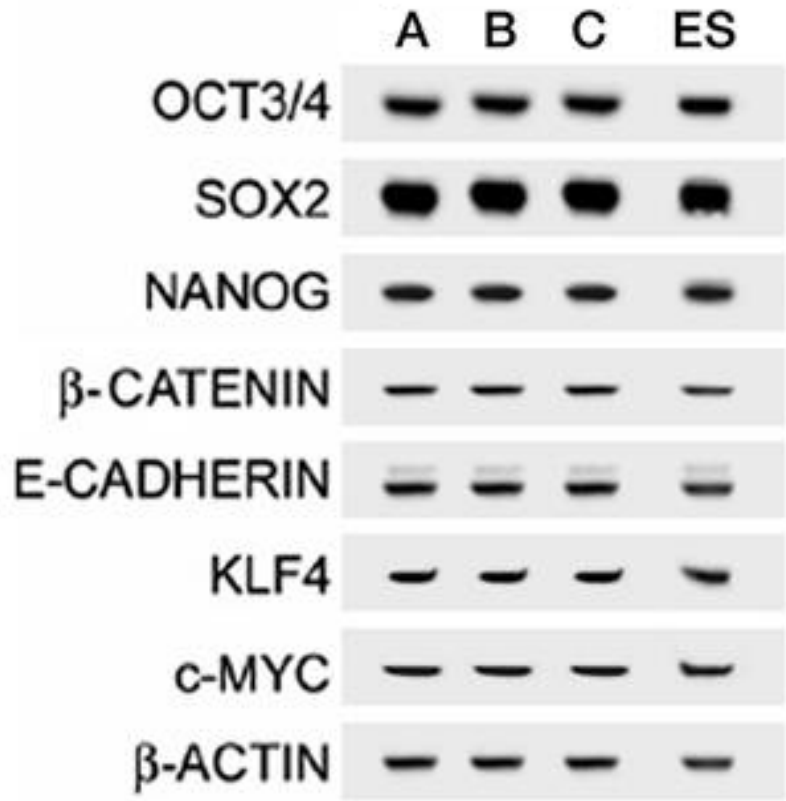
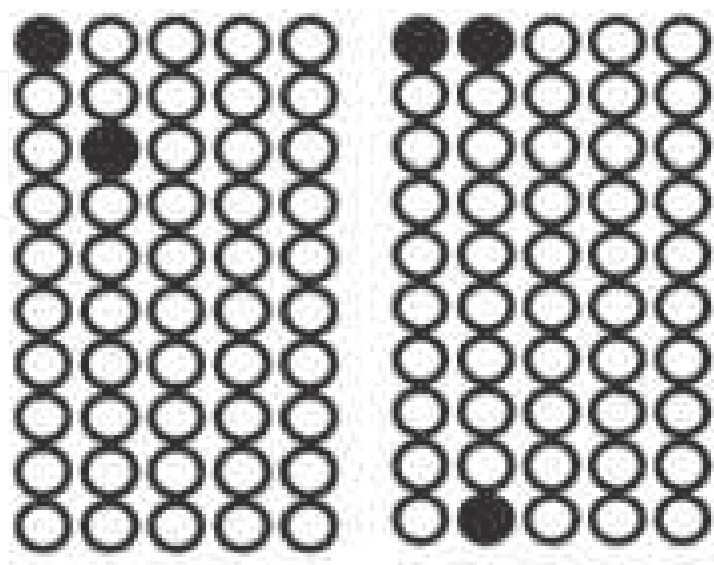
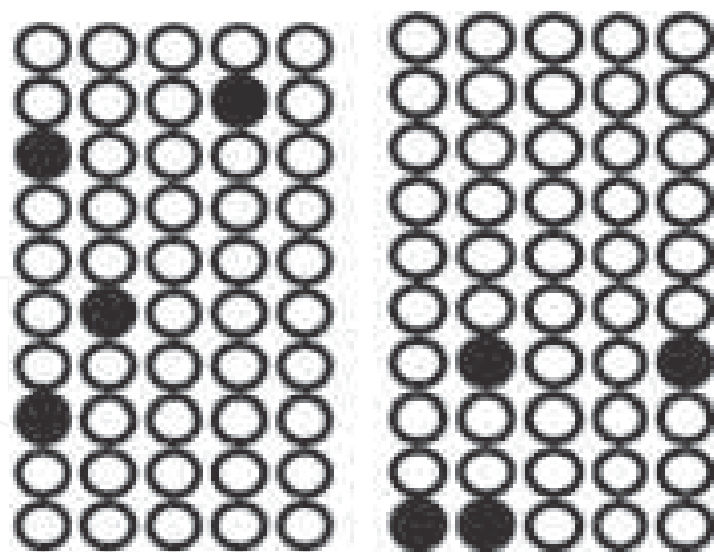


Fig. S2. Western blot analysis of ES cell-marker genes in human iPS cells

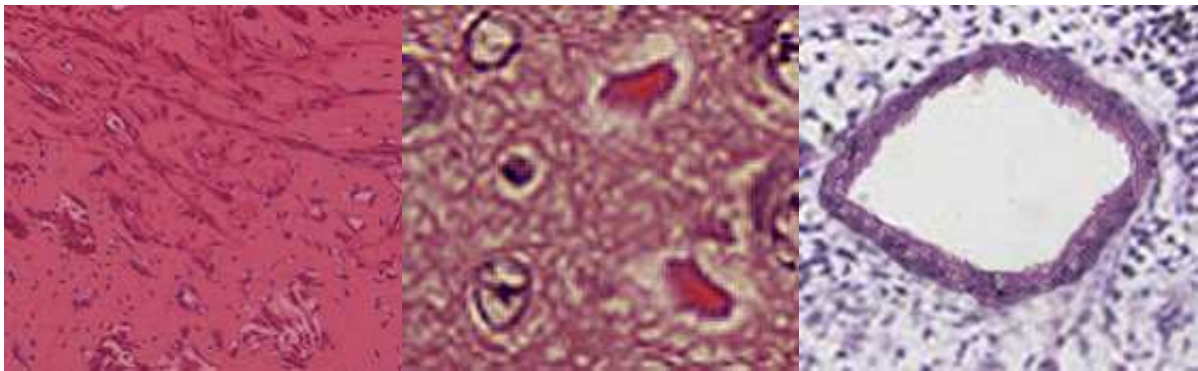


Left; Oct3/4 (human ES), Right; Oct3/4 (human iPS)



Left; Nanog (human ES), Right; Nanog (human iPS)

Fig. 4. Methylation analysis of Oct3/4 and Nanog promoter regions in human iPS **cells**. Bisulfite genomic sequencing of the promoter region of Oct3/4 and Nanog in iPS cells, human ES cells. The open circles indicate unmethylated CpG dinucleotides, while the closed circles indicate methylated CpG.s



Left (Muscle), Center (Neural tissue), Right (Gut-like epithelium)

Fig. 5. Teratoma formation in human iPS cells

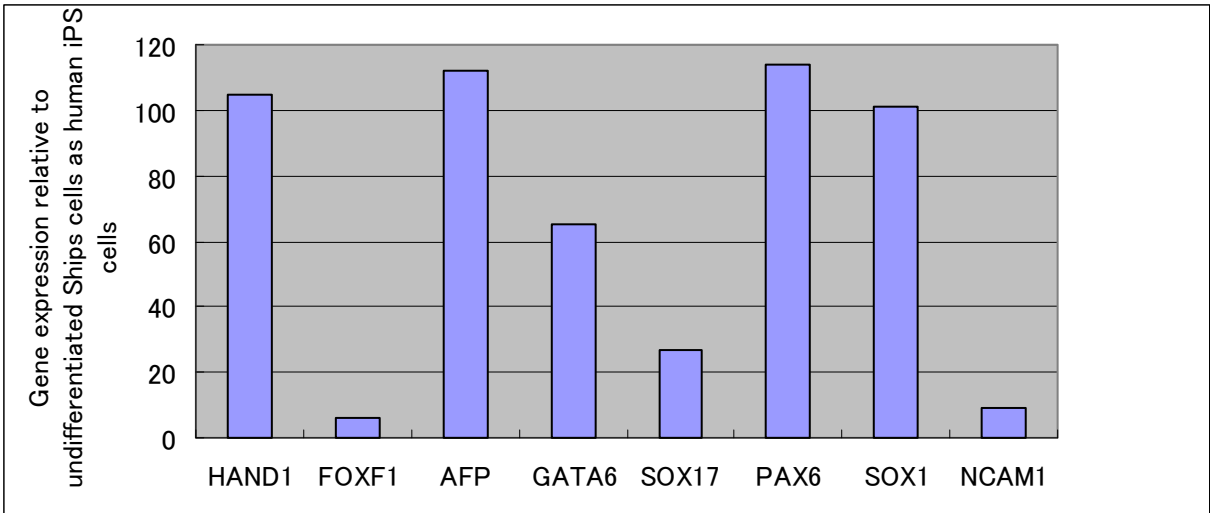


Fig. 6. In vitro differentiation of human iPS cells into all three germ layers. Quantitative PCR analyses of all three germ layer markers from differentiated human iPS cells after directed differentiation: mesoderm (HAND1, FOXF1), endoderm (AFP, GATA6, SOX17) and ectoderm (PAX6, SOX1, NCAM1). Data denote beta-actin-normalized fold changes relative to undifferentiated parental human iPS cells

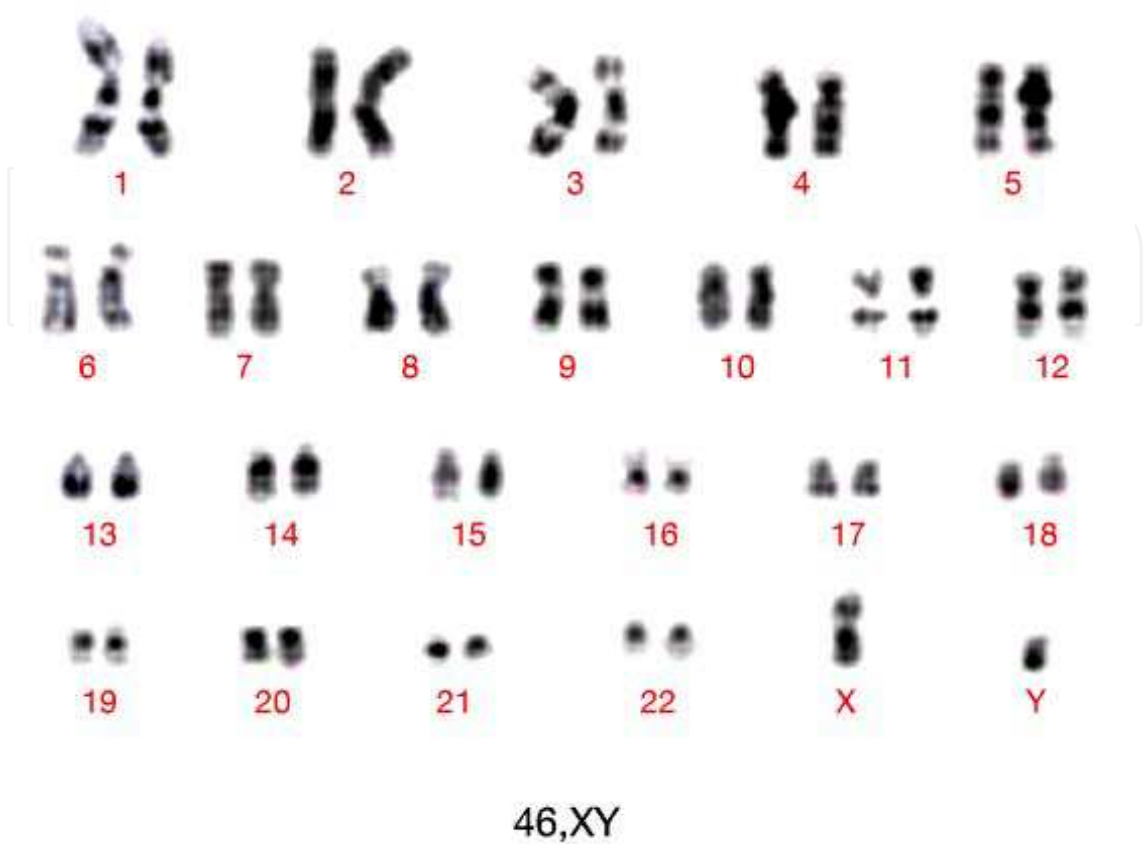


Fig. 7. Chromosomal G-band analysis in typical human iPS cells

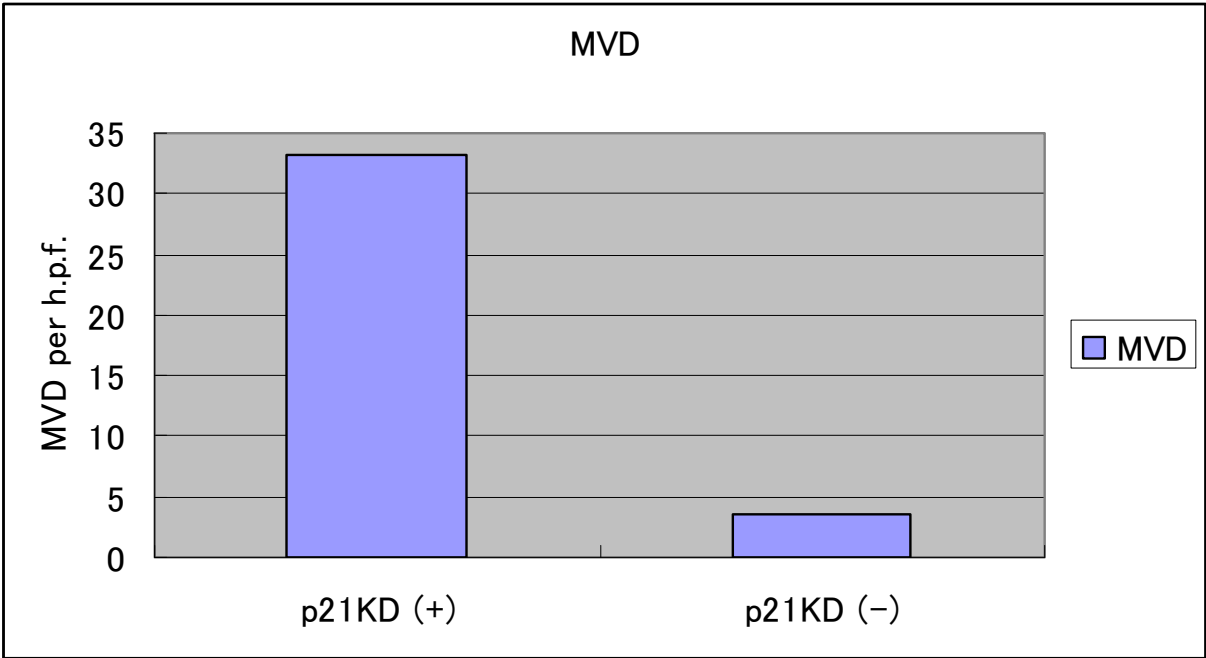


Fig. 8. The risk evaluation of malignant transformation for Chips cells as humsn iPS cell lines

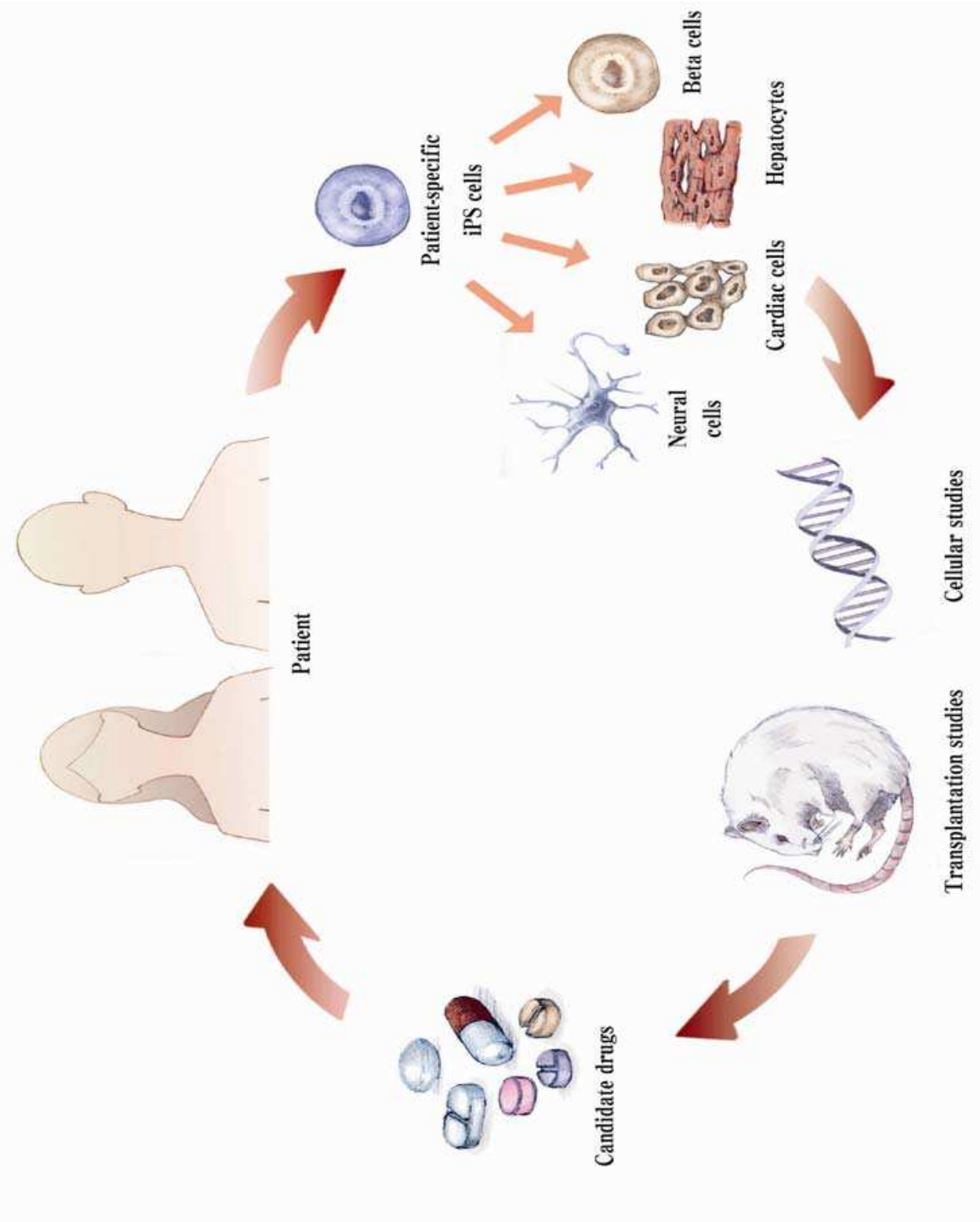


Fig. 9. A novel drug screening system by using Chips cells as human iPS cells

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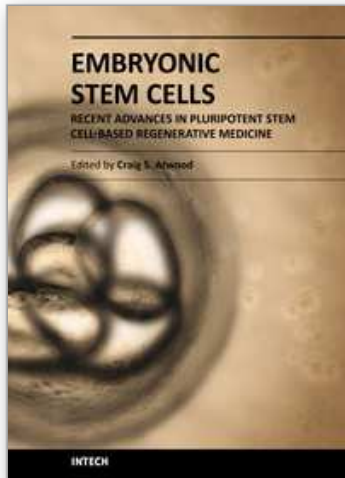
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7. Author contributions

H.M.: Conception and design, provision of study material, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; R.T.C: collection and/or assembly of data, data analysis and interpretation, manuscript assessment, final approval of manuscript; M.M.: manuscript assessment, final approval of manuscript.

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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