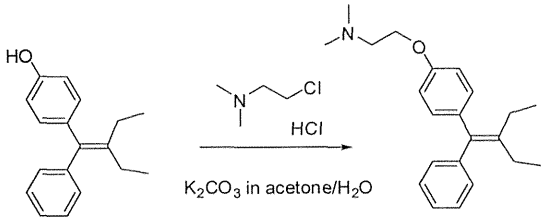

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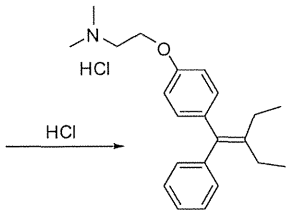

4 (YAK06)

Biology. All procedures using live animals in this study were conducted in accordance with the guidelines of the National Institute of Health Sciences, Japan.

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (CA, USA). Glutamate dehydrogenase (GLD) was purchased from Roche (Mannheim, Germany). β-Nicotinamide adenine dinucleotide (βNAD), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1-methoxy-5-methylphenazinium methyl sulfate (MPMS), lactate lithium salt and LY294002 were purchased from Sigma (MO, USA). DL-threo-β-benzyloxyaspartic acid (TBOA) and ICI182,780 were purchased from Tocris (MO, USA). U0126 was purchased from Promega (WI, USA). Assay kits for hormonal effects on HEK293/hERα and HEK293/hERβ reporter cells were purchased from Clontech (CA, USA).

Cell Culture. Primary cultures of astrocytes were prepared from the cerebral cortices of 3-day-old neonates of Wistar rats, as described previously.[31] Briefly, dissociated cortical cells were suspended in modified DMEM containing 30 mM glucose, 2 mM glutamine, 1 mM pyruvate and 10% FBS, and plated on uncoated 75 cm^2 flasks at the density of 600 000 cells/cm^2. A monolayer of type I astrocytes was obtained 12–14 days after plating. Nonastrocytes such as microglia were detached from the flasks by shaking and removed by changing the medium. Astrocytes in the flasks were dissociated by trypsinization, reseeded on uncoated 96-well microtiter plates at 20 000 cells/cm^2, and incubated until the cells became confluent (approximately 9–10 days after reseeding). In this culture, >98% of the cells were identified as type I astrocytes on the basis of positivity for GFAP and flattened, polygonal appearance.

Measurement of Extracellular L-Glu Concentration. Extracellular L-Glu concentration was measured by means of a colorimetric method according to Abe et al.[32] Briefly, 50 μL of culture supernatant was transferred to each well of a 96-well microtiter plate and mixed with 50 μL of substrate mixture consisting of 20 U/mL GLD, 2.5 mg/mL β-NAD, 0.25 mg/mL MTT, 100 μM MPMS and 0.1% (v/v) Triton X-100 in 0.2 M Tris-HCl buffer (pH 8.2). After 10 min incubation at 37 °C, the reaction was stopped by adding 100 μL of solution containing 50% (v/v) dimethylformamide and 20% (wt/vol) SDS (pH 4.7). In this reaction, MTT (yellow) is converted into MTT formazan (purple) in proportion to the L-Glu concentration. The amount of MTT formazan was determined by measuring the absorbance at 570 nm (test wavelength) and 655 nm (reference wavelength) with a microplate reader. The concentration of L-Glu was estimated from a standard curve, which was constructed in each assay using cell-free medium containing known concentrations of L-Glu. L-Glu clearance was shown as the amount of L-Glu taken up by astrocytes, which was calculated from the concentration difference in the medium.

Treatment with Test Compounds. L-Glu was dissolved at 1 mM in phosphate-buffered saline and diluted to 100 μM with the culture medium. Compounds **1**, **2**, **3**, and **4** were dissolved at 100, 100, 100, and 10 mM, respectively, in dimethyl sulfoxide (DMSO) and diluted to the required final concentrations with the culture medium. The concentration of DMSO in the medium was controlled to be below 0.1%, because we had already confirmed that 0.1% DMSO has no effect on L-Glu transport activity or cell viability (data not shown). Cells were incubated with test compounds for 24 h. TBOA (IC50 = 48 μM for GLAST, 7 μM for GLT1) was freshly dissolved at 1 mM in culture medium for each experiment. ICI182,780 (IC50 = 0.29 nM for ERs), U0126 (IC50 = 72 nM for MEK1, 58 nM for MEK2), and LY294002 (IC50 = 1 μM for class 1 PI3K, 19 μM for class 2 PI3K) were dissolved at 1, 5, and 5 mM, respectively, in DMSO, and the solutions were diluted with culture medium to yield the required final concentrations. These inhibitors were coapplied with 1 nM test compounds (**1**–**4**) for 24 h.

Assay Procedure for Hormonal Effects on HEK293/hERα and HEK293/hERβ Reporter Cells. Human embryo kidney 293 cells (HEK293) were grown in FBS (+) DMEM in 100 mm dishes. Cells were subcultured once or twice a week at about 80% confluence. A solution of 12.4 μL of 2 M calcium ion, 100 ng/well reporter or negative control vector (pERE-TA-SEAP or pTA-SEAP, Clontech), 50 ng/well expression vector (pcDNA3 ERα or pcDNA3 ERβ, generous gift from Dr. Shige-aki Kato, University of Tokyo, Japan), and 100 ng/well positive control vector (pSV-β-galactosidase, Promega) was diluted to a final volume of 10 μL/well. This mixture was carefully added dropwise to the same volume of HEPES solution with slow vortexing, and the mixture was incubated at rt for 20 min to obtain a precipitate. Cells from the exponential growth phase were seeded (3.0×10^4 cells/ml) into 96-well plates the day before transfection. The cells were incubated with fresh medium for 1 h, then 1/10 volume of precipitate was added to each well and incubation was continued for 24 h at 37 °C in an atmosphere of 5% CO_2 in air. The medium was replaced with fresh FBS (-) medium and incubation was continued for a further 24 h. Then the cells were incubated with test compounds for 24 h at 37 °C in an atmosphere of 5% CO_2 in air. SEAP activity (Great EscapeTM SEAP chemiluminescence kit 2.0, Clontech) and β-galactosidase activity (β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer, Promega) were measured with a Spectramax M5 microplate reader (Molecular Devices Japan, Tokyo, Japan). All transfections were performed in triplicate.

Statistical Analysis. Data were obtained from four independent experiments (averaged values of six wells for each) unless otherwise noted. Data are expressed as means ± SEM of these data. Tests of homogeneity of variance, normality, and distribution were performed to ensure that the assumptions required for standard parametric ANOVA were satisfied. Statistical analysis was performed by one-way repeated-measures ANOVA with post hoc Tukey's test for multiple pairwise comparisons.

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Author Contributions

†These two authors equally contributed to this Article. Individual author contributions: K.S. designed the biological experimental plan, performed biological experiments, data analysis, manuscript writing and preparation. J.K. and Y.S. performed experimental work. K.T. contributed to the data analysis. J.O., K.N. and Y.S. provided advice on the experimental direction. Y.O. carried out organic synthesis, data analysis and wrote portions of the manuscript. Y.S. carried out organic synthesis. T.O. designed and oversaw all organic chemistry studies, carried out organic synthesis and also performed data analysis and manuscript writing and preparation.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

βNAD; β-nicotinamide adenine dinucleotide; CNS; central nervous system; DMEM; Dulbecco's modified Eagle's medium; DMSO; dimethyl sulfoxide; E2; 17β-estradiol; ESI; electron spray ionization; FBS; fetal bovine serum; GLD; glutamate dehydrogenase; HEK-293; Human embryo kidney 293 cells; HRMS; high-resolution mass spectrometry; L-Glu; L-glutamate; MAPK; mitogen-activated protein kinase; MEK; mitogen-activated protein kinase/extracellular signal-regulated kinase; mERα; membrane-associated estrogen receptor α; mGluR5; metabotropic glutamate receptor 5; MPMS; 1-methoxy-5-methylphenazinium methyl sulfate; MTT; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; nERs; nuclear estrogen receptors; PI3K; phosphatidylinositol 3-kinase; Tam; tamoxifen; TBOA; DL-threo-β-benzyloxyaspartic acid; TLC; thin-layer chromatography; TOF; time-of-flight

■ REFERENCES

(1) Kumar, A., Singh, R. L., and Babu, G. N. (2010) Cell death mechanisms in the early stages of acute glutamate neurotoxicity. *Neurosci. Res. 66*, 271–278.

(2) Choi, D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron 1*, 623–634.

(3) Logan, W. J., and Snyder, S. H. (1971) Unique high affinity uptake systems for glycine, glutamic and aspartic acids in central nervous tissue of the rat. *Nature 234*, 297–299.

(4) Beart, P. M., and O'Shea, R. D. (2007) Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br. J. Pharmacol. 150*, 5–17.

(5) Sato, K., Matsuki, N., Ohno, Y., and Nakazawa, K. (2003) Estrogens inhibit L-glutamate uptake activity of astrocytes via membrane estrogen receptor alpha. *J. Neurochem. 86*, 1498–1505.

(6) Olivier, S., Close, P., Castermans, E., de Leval, L., Tabruyn, S., Chariot, A., Malaise, M., Merville, M. P., Bours, V., and Franchimont, N. (2006) Raloxifene-induced myeloma cell apoptosis: a study of nuclear factor-kappaB inhibition and gene expression signature. *Mol. Pharmacol. 69*, 1615–1623.

(7) Sato, K., Saito, Y., Oka, J., Ohwada, T., and Nakazawa, K. (2008) Effects of tamoxifen on L-glutamate transporters of astrocytes. *J. Pharmacol. Sci. 107*, 226–230.

(8) Bunch, L., Erichsen, M. N., and Jensen, A. A. (2009) Excitatory amino acid transporters as potential drug targets. *Expert Opin. Ther. Targets 13*, 719–731.

(9) Margueron, R., Duong, V., Bonnet, S., Escande, A., Vignon, F., Balaguer, P., and Cavailles, V. (2004) Histone deacetylase inhibition and estrogen receptor alpha levels modulate the transcriptional activity of partial antiestrogens. *J. Mol. Endocrinol. 32*, 583–594.

(10) Thompson, D. S., Spanier, C. A., and Vogel, V. G. (1999) The relationship between tamoxifen, estrogen, and depressive symptoms. *Breast J. 5*, 375–382.

(11) Grilli, S. (2006) Tamoxifen (TAM): the dispute goes on. *Ann. Ist. Super. Sanita 42*, 170–173.

(12) Sha, Y., Tashima, T., Mochizuki, Y., Toriumi, Y., Adachi-Akahane, S., Nonomura, T., Cheng, M., and Ohwada, T. (2005) Compounds structurally related to tamoxifen as openers of large-conductance calcium-activated K^+ channel. *Chem. Pharm. Bull. (Tokyo) 53*, 1372–1373.

(13) Robertson, D. W., Katzenellenbogen, J. A., Long, D. J., Rorke, E. A., and Katzenellenbogen, B. S. (1982) Tamoxifen antiestrogens. A comparison of the activity, pharmacokinetics, and metabolic activation of the cis and trans isomers of tamoxifen. *J. Steroid Biochem. 16*, 1–13.

(14) Weltje, L., vom Saal, F. S., and Oehlmann, J. (2005) Reproductive stimulation by low doses of xenoestrogens contrasts with the view of hormesis as an adaptive response. *Hum. Exp. Toxicol. 24* (9), 431–437.

(15) Perego, C., Vanoni, C., Bossi, M., Massari, S., Basudev, H., Longhi, R., and Pietrini, G. (2000) The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal co-cultures. *J. Neurochem. 75*, 1076–1084.

(16) Guillet, B., Lortet, S., Masmejean, F., Samuel, D., Nieoullon, A., and Pisano, P. (2002) Developmental expression and activity of high affinity glutamate transporters in rat cortical primary cultures. *Neurochem. Int. 40*, 661–671.

(17) Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol. Endocrinol. 13*, 307–319.

(18) Pappas, T. C., Gametchu, B., and Watson, C. S. (1995) Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. *FASEB J. 9*, 404–410.

(19) Grove-Strawser, D., Boulware, M. I., and Mermelstein, P. G. (2010) Membrane estrogen receptors activate the metabotropic glutamate receptors mGluR5 and mGluR3 to bidirectionally regulate CREB phosphorylation in female rat striatal neurons. *Neuroscience 170*, 1045–1055.

(20) Mannella, P., and Brinton, R. D. (2006) Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *J. Neurosci. 26*, 9439–9447.

(21) Szego, E. M., Barabas, K., Balog, J., Szilagyi, N., Korach, K. S., Juhasz, G., and Abraham, I. M. (2006) Estrogen induces estrogen receptor alpha-dependent cAMP response element-binding protein phosphorylation via mitogen activated protein kinase pathway in basal forebrain cholinergic neurons in vivo. *J. Neurosci. 26*, 4104–4110.

(22) Vasudevan, N., Kow, L. M., and Pfaff, D. (2005) Integration of steroid hormone initiated membrane action to genomic function in the brain. *Steroids 70*, 388–396.

(23) Alyea, R. A., Laurence, S. E., Kim, S. H., Katzenellenbogen, B. S., Katzenellenbogen, J. A., and Watson, C. S. (2008) The roles of membrane estrogen receptor subtypes in modulating dopamine transporters in PC-12 cells. *J. Neurochem. 106* (4), 1525–1533.

(24) Watson, C. S., Alyea, R. A., Hawkins, B. E., Thomas, M. L., Cunningham, K. A., and Jakubas, A. A. (2006) Estradiol effects on the dopamine transporter - protein levels, subcellular location, and function. *J. Mol. Signaling 1*, 5.

(25) Filardo, E. J., and Thomas, P. (2005) GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends. Endocrinol. Metab. 16* (8), 362–367.

(26) Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., and Prossnitz, E. R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science 11;307* (5715), 1625–1630.

(27) Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R. Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G

protein-coupled receptor homolog, GPR30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. *Mol. Endocrinol. 14* (10), 1649–1660.

(28) Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology 146* (2), 624–632.

(29) Kuo, J., Hamid, N., Bondar, G., Prossnitz, E. R., and Micevych, P. (2010) Membrane estrogen receptors stimulate intracellular calcium release and progesterone synthesis in hypothalamic astrocytes. *J. Neurosci. 30* (39), 12950–12957.

(30) Kisanga, E. R., Gjerde, J., Guerrieri-Gonzaga, A., Pigatto, F., Pesci-Feltri, A., Robertson, C., Serrano, D., Pelosi, G., Decensi, A., and Lien, E. A. (2004) Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. *Clin. Cancer Res. 10*, 2336–2343.

(31) Suzuki, K., Ikegaya, Y., Matsuura, S., Kanai, Y., Endou, H., and Matsuki, N. (2001) Transient upregulation of the glial L-glutamate transporter GLAST in response to fibroblast growth factor, insulin-like growth factor and epidermal growth factor in cultured astrocytes. *J. Cell Sci. 114*, 3717–3725.

(32) Abe, K., Abe, Y., and Saito, H. (2000) Evaluation of L-glutamate clearance capacity of cultured rat cortical astrocytes. *Biol. Pharm. Bull. 23*, 204–207.

Chapter 10

Efficient Hepatic Differentiation from Human iPS Cells by Gene Transfer

Kenji Kawabata, Mitsuru Inamura, and Hiroyuki Mizuguchi

Abstract

Establishment of protocols for the differentiation of hepatic cells from human embryonic stem (ES) and induced pluripotent stem (iPS) cells could contribute to regenerative cell therapies or drug discovery and development. However, the differentiation efficiency of endoderm-derived cells, such as hepatic cells, from human ES and iPS cells is poor because hepatic cells are differentiated via multiple lineages including endodermal cells, hepatic progenitor cells, and mature hepatocytes. We show here the protocols for efficient hepatic differentiation from human ES and iPS cells by adenovirus vector-mediated gene transfer.

Key words: ES cells, iPS cells, Hepatocytes, Adenovirus vector, Regenerative medicine, Drug development

1. Introduction

In vertebrate development, the liver is derived from the primitive gut tube, which is formed by a flat sheet of cells called the definitive endoderm (1, 2). Afterward, the definitive endoderm is separated into the liver buds and differentiated into hepatoblasts. The hepatoblasts can differentiate into both mature hepatocytes and cholangiocytes. Each step of cell growth and differentiation is tightly regulated by intra- and extracellular signaling (3). Activin A, fibroblast growth factors (FGFs), bone morphogenic protein (BMP), hepatocyte growth factor (HGF), and oncostatin M (OSM) are the most essential extracellular signaling molecules. At the intracellular level, the liver-enriched transcription factors, i.e., hepatocyte nuclear factors (HNFs), CCAAT enhancer binding protein (C/EBP) α and β, and hematopoietically expressed homeobox (HEX), are required for the hepatic differentiation (4, 5). Among these

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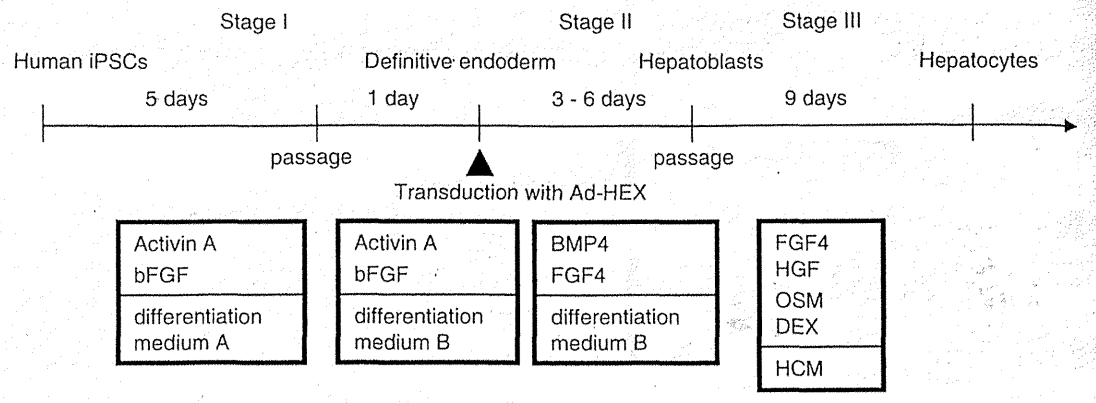

Fig. 1. A strategy for the differentiation of human iPS cells into hepatoblasts and hepatocytes. A schematic representation illustrating the procedure for differentiation of human iPS cells into hepatocytes is shown.

transcription factors, Hex is known to function at the earliest stage in hepatic differentiation (6). Targeted deletion of the HEX gene in the mouse results in embryonic lethality and a loss of the fetal liver parenchyma (7, 8). The hepatic genes, such as albumin, HNF4a, and prospero-related homeobox 1 (PROX1), are transiently expressed in the definitive endoderm of HEX-null embryos, and further morphogenesis of the hepatoblasts does not occur (9). Together, these findings underscore that HEX is essential for the definitive endoderm to adopt a hepatic cell fate.

Here, we show the protocol for the efficient differentiation of hepatoblasts from human ES and iPS cells. Our strategy is based on an imitation of in vivo liver development (Fig. 1). We have found that differentiation of hepatoblasts from the human ES and iPS cell-derived definitive endoderms, but not from undifferentiated human ES and iPS cells, could be facilitated by adenovirus (Ad) vector-mediated transient transduction of a HEX gene (10). Hepatoblasts derived from human iPS cells by HEX transduction were able to differentiate into functional hepatocytes in vitro. Furthermore, all the procedures for culture and differentiation were performed under serum/feeder cell-free chemically defined conditions. Our protocol based on Ad vector-mediated transient transduction under chemically defined conditions would provide a platform for drug screening as well as safe regenerative cell therapies.

2. Materials

2.1. Adenovirus Vectors

1. The human HEX cDNA (GenBank Accession No. BC014336) (Invitrogen, Carlsbad, CA).
2. Shuttle plasmid pHMEF5 (11).
3. Vector plasmid pAdHM41-K7 (12).

2.2. Cells

1. Human iPS cells (see Note 1).
2. Mitomycin C-inactivated mouse embryonic fibroblasts (MEF) (Hygro-Resistant Strain C57/BL6) (Millipore, Bedford, MA) (see Note 1).
3. HepG2 cells.

2.3. Medium and Growth Factors

1. Defined serum-free medium (hESF9): hESF-GRO medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with 10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, oleic acid conjugated with fatty acid-free bovine albumin, 10 ng/ml bFGF, and 100 ng/ml heparin (all from Sigma, St. Louis, MO).
2. Laminin from the Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma).
3. Twelve-well culture plate (Sumitomo Bakelite, Tokyo, Japan).
4. Laminin-coated tissue culture 12-well plate: Dilute laminin in PBS for a final dilution of 1:50. Add 1 ml of laminin solution to coat each well of a 12-well plate. Incubate the plates for 3–24 h at 37°C. Remove laminin solution and wash the well with PBS immediately before use.
5. Accutase (Invitrogen).
6. Differentiation medium A: hESF-GRO medium (Cell Science & Technology Institute) supplemented with 10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, and 0.5 mg/ml fatty acid-free bovine albumin (BSA) (Sigma).
7. bFGF (Sigma).
8. Activin A (R&D Systems, Minneapolis, MN).
9. Trypsin–EDTA: 0.0125% trypsin, 0.01325 mM EDTA (Invitrogen).
10. Trypsin inhibitor A: Differentiation medium A supplemented with 0.1% soybean trypsin inhibitor (Sigma).
11. Differentiation medium B: hESF-DIF (Cell Science & Technology Institute) medium supplemented with 10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, and 0.5 mg/ml fatty acid-free BSA.
12. FGF4 (R&D Systems).
13. BMP4 (R&D Systems).
14. Trypsin inhibitor B: Differentiation medium B supplemented with 0.1% soybean trypsin inhibitor (Sigma).
15. Hepatocyte culture medium (HCM) supplemented with SingleQuots (Lonza, Walkersville, MD).
16. HGF (R&D Systems).

Table 1
List of Taqman gene expression assays

Gene	Assay ID
AFP	Hs01040607_m1
ALB	Hs00910225_m1
CYP3A4	Hs00430021_m1
CYP7A1	Hs00167982_m1
CYP2D6	Hs02576168_g1

17. Oncostatin M (OSM) (R&D Systems).
18. Dexamethasone (Sigma).
19. Type I collagen (Nitta Gelatin, Osaka, Japan).
20. Type I collagen-coated 12-well plate (15 μg/cm²): Dilute type I collagen in PBS for a final dilution of 1:50. Add 1 ml of type I collagen solution to coat each well of a 12-well plate. Incubate the plates for 3–24 h at 37°C. Remove type I collagen solution immediately before use.

2.4. Analysis

1. Human fetal (22–40 weeks old) liver total RNA (Clontech Laboratories, Mountain View, CA).
2. Human adult (51 years old) liver total RNA (Clontech Laboratories).
3. RNeasy Plus Mini kit (Qiagen, Hilden, Germany).
4. Superscript VILO cDNA synthesis kit (Invitrogen).
5. Taqman gene expression assays (Applied Biosystems, Foster City, CA): The primer sequences are described in Table 1.
6. ABI PRISM 7700 Sequence Detector (Applied Biosystems).
7. P450-GloTM CYP3A4 Assay Kit (Promega, Madison, WI).
8. Rifampicin (Sigma).
9. Dimethyl sulfoxide (Sigma).
10. Luminometer (Berthold, Tokyo, Japan).

3. Methods

3.1. Adenovirus Vector Construction

1. Ad vectors were constructed by an improved in vitro ligation according to the method of Mizuguchi and Kay. (13, 14). The human HEX cDNA was inserted into pHMEF5, which contains the human elongation factor-1α (EF-1α) promoter, resulting in pHMEF-HEX.

2. The pHMEF-HEX was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7, resulting in pAd-HEX.
3. Ad-HEX, which contains the EF-1α promoter and a stretch of lysine residues (K7) peptides in the C-terminal region of the fiber knob, was generated and purified.
4. The vector particle (VP) titer was determined by using a spectrophotometric method (15).

3.2. In Vitro Definitive Endoderm Differentiation

1. Prepare human iPS cells, which were maintained on MEF on a gelatin-coated 25 cm^2 flask in human iPS cell culture medium (see Note 1).
2. Before the initiation of cellular differentiation, change the medium of human iPS cells for the defined serum-free medium hESF9.
3. Incubate the cells in a humidified atmosphere of 10% CO_2 and 90% air at 37°C overnight (see Note 2).
4. For induction of definitive endoderm, remove the hESF9 medium, add 1.0 ml Accutase per 25-cm^2 flask, incubate for 3 min at 37°C, and remove the Accutase (see Note 3).
5. Add 10 ml of cold hESF9 medium, resuspend the human iPS cells into a single cell suspension by pipetting, and centrifuge at 267 × *g* for 3 min at 4°C (see Note 4).
6. Aspirate the supernatant and resuspend the cells with 10 ml of cold differentiation medium A and centrifuge them at 267 × *g* for 3 min at 4°C.
7. Repeat step 6.
8. Aspirate the supernatant, and replace the medium with warm fresh differentiation medium A supplemented with 10 ng/ml bFGF and 50 ng/ml Activin A.
9. Transfer to a laminin-coated 12-well plate in a humidified atmosphere of 10% CO_2 and 90% air at 37°C (2.5×10^5 cells/well). The final volume of medium should be 1.0 ml per well (see Note 5).
10. Change the differentiation medium A supplemented with 10 ng/ml bFGF and 50 ng/ml Activin A every day.

3.3. In Vitro Hepatoblast Differentiation

1. After 5 days of culture, remove the medium, add 200 µl trypsin–EDTA per well, incubate the cells for 3 min at 37°C, and remove the trypsin–EDTA (see Note 6).
2. Resuspend the cell populations in 10 ml of cold trypsin inhibitor A and centrifuge them at 267 × *g* for 3 min at 4°C.
3. Aspirate the supernatant, resuspend the cells in 10 ml of cold differentiation medium B, and centrifuge at 267 × *g* for 3 min at 4°C.

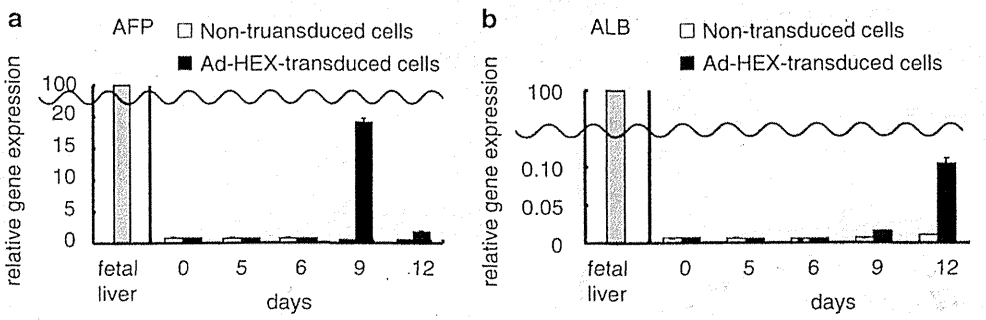

Fig. 2. Efficient hepatoblast differentiation from the human iPS cell-derived definitive endoderms by transduction of the HEX gene. Real-time RT-PCR analysis of the level of AFP (**a**) and ALB (**b**) expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPS cell-derived definitive endoderms (day 0, 5, 6, 9, and 12). The cells were transduced with Ad-HEX at day 6 as described in Fig. 1. The data at day 6 were obtained before the transduction with Ad-HEX. The *graphs* represent the relative gene expression levels when the level in the fetal liver was taken as 100.

4. Aspirate the supernatant and replace with warm fresh differentiation medium B supplemented with 10 ng/ml bFGF and 50 ng/ml Activin A.
5. Transfer the cells to a laminin-coated tissue culture 12-well plate (5.0×10^5 cells/well) and culture them in a humidified atmosphere of 10% CO_2 and 90% air at 37°C. The final volume of medium should be 1.0 ml per well (see Note 5).
6. After 24 h of culture, remove the medium, and add warm fresh differentiation medium B supplemented with Ad-HEX (3,000 VP/cell), 10 ng/ml FGF4, and 10 ng/ml BMP4 (R&D Systems) (see Note 7). The final volume of medium should be 500 µl per well.
7. Incubate the cells in a humidified atmosphere of 10% CO_2 and 90% air at 37°C for 1.5 h.
8. Remove the medium and replace with warm fresh differentiation medium B supplemented with 10 ng/ml FGF4 and 10 ng/ml BMP4, and incubate the cells in a humidified atmosphere of 10% CO_2 and 90% air at 37°C.
9. Change the medium every day (see Note 8).
10. After 3 and 6 days of culture in differentiation medium B, analyze the cells by RT-PCR (see Note 9) (Fig. 2).

3.4. In Vitro Hepatic Maturation

1. After 3 days of culture in differentiation medium B, add 200 µl trypsin–EDTA in each well, incubate the cells for 3 min at 37°C, and remove the trypsin–EDTA.
2. Resuspend the cell populations in 10 ml of cold trypsin inhibitor B and centrifuge them at $267 \times g$ for 3 min at 4°C (see Note 10).

3. Aspirate the supernatant, resuspend the cells in 10 ml of cold HCM and centrifuge them at 267 × *g* for 3 min at 4°C.
4. Aspirate the supernatant, and replace with warm fresh HCM supplemented with SingleQuots, 10 ng/ml FGF-4, 10 ng/ml HGF, 10 ng/ml, and 10^{-7} M dexamethasone.
5. Transfer into two wells of a type I collagen-coated tissue culture 12-well plate and incubate the cells in a humidified atmosphere of 10% CO_2 and 90% air at 37°C. The final volume of medium should be 1.0 ml per well (see Note 5).
6. Change the medium every 2 days.
7. After 9 days of culture in HCM, analyze the cells by RT-PCR and measure the cytochrome P450 activity (see Notes 9 and 11) (Fig. 3).

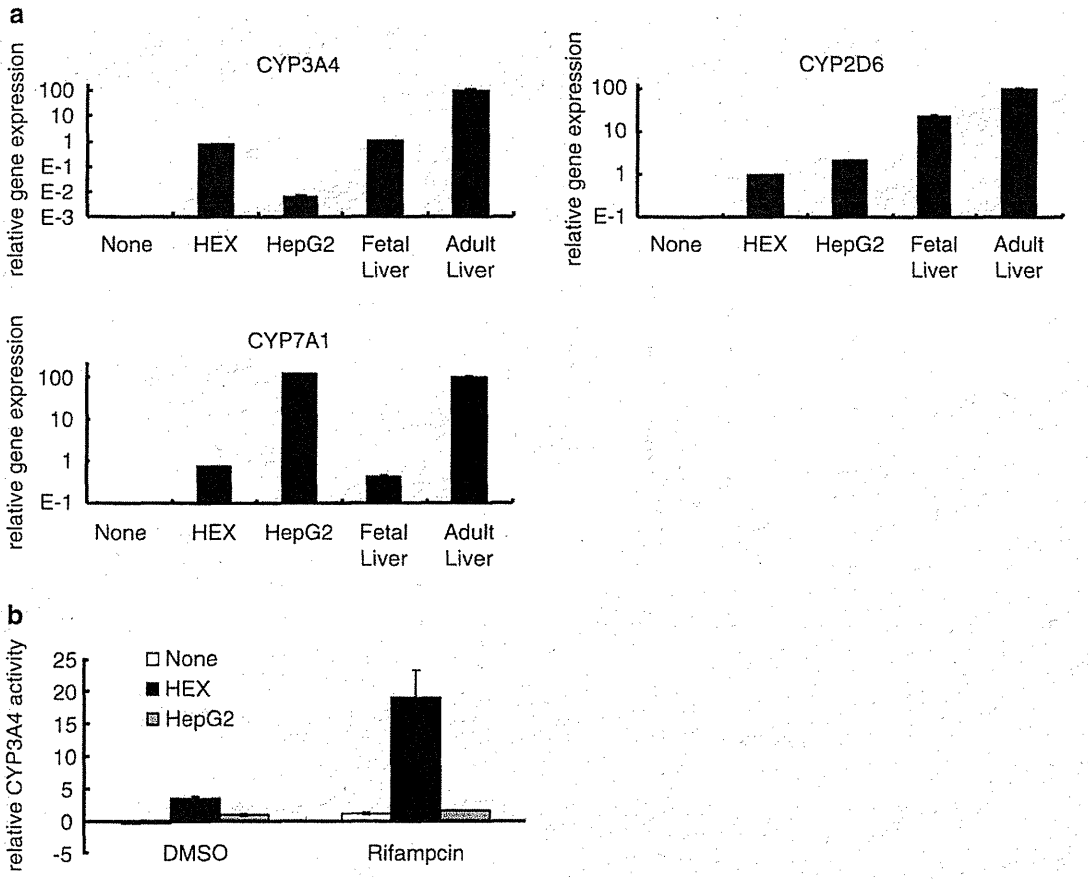

Fig. 3. Cytochrome P450 isozymes in human iPS cell-derived hepatocytes. (**a**) Real-time RT-PCR analysis of CYP3A4, CYP7A1, and CYP2D6 expression in human iPS cell-derived nontransduced cells (day 18), Ad-HEX-transduced cells (day 18), and fetal and adult liver tissues. (**b**) Induction of CYP3A4 by rifampicin in human iPS cell-derived nontransduced cells, Ad-HEX-transduced cells, and the HepG2 cell line. Data are presented as the mean ± SD from triplicate experiments. The *graphs* represent the relative gene expression level when the level in the adult liver is taken as 100. Abbreviations: *NONE* nontransduced cells, *LacZ* Ad-LacZ-transduced cells, *HEX* Ad-HEX-transduced cells, *DMSO* dimethyl sulfoxide.

4. Notes

1. Culture human iPS cells to maintain the undifferentiated states according to the original protocol (16, 17). Basically, human ES cells can be cultured, handled, and differentiated using the same protocol as human iPS cells described here.
2. Proceed to step 3 within 48 h. Attachment efficiency will be reduced if passage is performed after more than 48 h of culture in hESF9 medium.
3. By this operation, the feeder cells are removed and only the human iPS cells remain in the flask.
4. Determine the number of cells using a hemocytometer and adjust the concentration precisely. An excessive number of cells per well results in the presence of undifferentiated cells after 5 days of culture with differentiation medium. Also, strain the cell suspension with a cell strainer to obtain a uniform single cell suspension, since cell clusters will result in the appearance of undifferentiated cells after 5 days of culture with differentiation medium.
5. Be sure to gently shake the plate left to right and back to front to obtain evenly distributed cells.
6. A low concentration of trypsin–EDTA can reduce cell damage by passage and promote cell survival. Detach the cells by brushing the medium on the cells.
7. Vortex the 1.5-ml tube supplemented with Ad-HEX.
8. Proceed to Subheading 3.4. for induction of hepatocytes after 3 days of culture in differentiation medium B.
9. Total RNA was isolated from human iPS cells, their derivatives, and HepG2 cells using an RNeasy Plus Mini kit. cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit. Real-time PCR was performed with Taqman gene expression assays using an ABI PRISM 7700 Sequence Detector. Relative quantification was performed against a standard curve, and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used in these methods are described in Table 1.
10. Do not dissociate the cell clusters into single cells. Passage the cells as the cell clumps.
11. To measure cytochrome P450 3A4 activity, lytic assays was performed by using a P450-GloTM CYP3A4 Assay Kit. For the cytochrome P450 3A4 activity assay, Ad-HEX-transduced cells and nontransduced cells as well as HepG2 cells were

treated with rifampicin, which is the substrate for CYP3A4, at a final concentration of 25 μM or DMSO for 72 h. The fluorescence was measured with a luminometer according to the manufacturer's instructions. HepG2 cells were cultured as per the instructions.

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References

1. Lavon, N. and Benvenisty, N. (2005) Study of hepatocyte differentiation using embryonic stem cells. *J Cell Biochem* 96, 1193–1202.
2. McLin, V.A. and Zorn, A.M. (2006) Molecular control of liver development. *Clin Liver Dis* 10, 1–25.
3. Snykers, S., De Kock, J., Rogiers, V., and Vanhaecke, T. (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: State of the art. *Stem Cells* 27, 577–605.
4. Kyrmizi, I., Hatzis, P., Katrakili, N., Tronche, F., Gonzalez, F.J., and Talianidis, I. (2006) Plasticity and expanding complexity of the hepatic transcription factor network during liver development. *Genes Dev* 20, 2293–2305.
5. Hunter, M.P., Wilson, C.M., Jiang, X., Cong, R., Vasavada, H., Kaestner, K.H., and Bogue, C.W. (2007) The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. *Dev Biol* 308, 355–367.
6. Bogue, C.W., Ganea, G.R., Sturm, E., Ianucci, R., and Jacobs, H.C. (2000) Hex expression suggests a role in the development and function of organs derived from foregut endoderm. *Dev Dyn* 219, 84–89.
7. Martinez Barbera, J.P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D., and Beddington, R.S. (2000) The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* 127, 2433–2445.
8. Keng, V.W., Yagi, H., Ikawa, M., Nagano, T., Myint, Z., Yamada, K., Tanaka, T., Sato, A., Muramatsu, I., Okabe, M., Sato, M., and Noguchi, T. (2000) Homeobox gene Hex is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochem Biophys Res Commun* 276, 1155–1161.
9. Bort, R., Signore, M., Tremblay, K., Martinez Barbera, J.P., and Zaret, K.S. (2006) Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Dev Biol* 290, 44–56.
10. Inamura, M., Kawabata, K., Takayama, K., Tashiro, K., Sakurai, F., Katayama K., Toyoda, M., Akutsu, H., Miyagawa, Y., Okita, H., Kiyokawa, N., Umezawa, A., Hayakawa, T., Kusuda-Furue, M., and Mizuguchi, H. Efficient generation of hepatoblasts from human ES cells and iPS cells by transient overexpression of homeobox gene HEX. in press.
11. Kawabata, K., Sakurai, F., Yamaguchi, T., Hayakawa, T., and Mizuguchi, H. (2005) Efficient gene transfer into mouse embryonic stem cells with adenovirus vectors. *Mol Ther* 12, 547–554.
12. Koizumi N, Mizuguchi H, Utoguchi N, Watanabe Y, and Hayakawa T. (2003) Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. *J Gene Med* 5, 267–276
13. Mizuguchi H and Kay M.A. (1998) Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum Gene Ther* 9, 2577–2583.
14. Mizuguchi H and Kay M.A. (1999) A simple method for constructing E1- and E1/

E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* 10, 2013–2017.

15. Maizel JV, Jr., White DO, and Scharff M.D. (1968) The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36, 115–125.
16. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, and Yamanaka S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872.
17. Nagata S, Toyoda M, Yamaguchi S, Hirano K, Makino H, Nishino K, Miyagawa Y, Okita H, Kiyokawa N, Nakagawa M, Yamanaka S, Akutsu H, Umezawa A, and Tada T. (2009) Efficient Reprogramming of Human and Mouse Primary Extra-Embryonic Cells to Pluripotent Stem Cells. *Genes Cells* 14, 1395–404.

総　説

ヒト iPS 細胞から肝細胞への分化誘導の現状と創薬応用

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総　説

ヒトiPS細胞から肝細胞への分化誘導の現状と創薬応用

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Current Status of Hepatic Differentiation from Human iPS cells and Application for Drug Development

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1. はじめに

創薬のプロセスは，一般的に開発費に1000億円超，1つの医薬品が製品化されるまでに10～15年を要する．その過程で数万～100万件の候補化合物の中から薬効，毒性などの評価を経て，1つが医薬品として承認を受ける．この過程を迅速化させ，開発成功率を向上させるための新しい技術のひとつとして，iPS細胞（induced pluripotent stem cells）技術に注目が集まっている．

ヒトiPS細胞から分化させた細胞（特に，肝臓，心筋，神経細胞等）は，医薬品開発研究の最上流の疾患のメカニズム解明や創薬ターゲット分子の検索研究だけでなく，化合物スクリーニングや薬効評価試験・安全性薬理試験・毒性試験・薬物動態試験等の前臨床試験においても活用が期待されている．細胞を用いた*in vitro*アッセイ系は，薬理作用（有効性）の評価や毒性評価のためにこれまでも活用されてきたが，多くは株化細胞や（ヒト）初代培養細胞を用いたものである．株化細胞はスループット性に優れているが，生体の状態（病態）を必ずしも反映しておらず，一方で，ヒト初代培養細胞は入手が限られ，ロット差も大きいこと，単一ロットの細胞を大量に得ることが困難であるという課題がある．また，動物由来の初代培養細胞や動物実験では，『種差の壁』のために，ヒト固有の薬理・毒性作用を見落とす可能性がある．ヒトiPS細胞由来分化誘導細胞は，これらの問題点の克服が期待できることから，大きな注目を集めている．

本稿では，産業界からのニーズが特に高い肝細胞に焦点をあて，ヒトiPS細胞から肝細胞への分化誘導の現状と創薬応用（特に毒性評価）への可能性について，著者らの最新の知見を中心に概説する．

2. ヒトiPS細胞由来肝細胞を用いた創薬研究

肝臓（肝細胞）は生体内外の物質の代謝，解毒，排出等に関与する主要な臓器（細胞）であり，医薬品は主に肝細胞で薬物代謝酵素により代謝され，抱合系酵素により解毒を受け，トランスポーターにより排出される．肝毒性は医薬品候補化合物の開発中止原因の主要なものであり，正常肝細胞を用いて将来起こりえる高い潜在的毒

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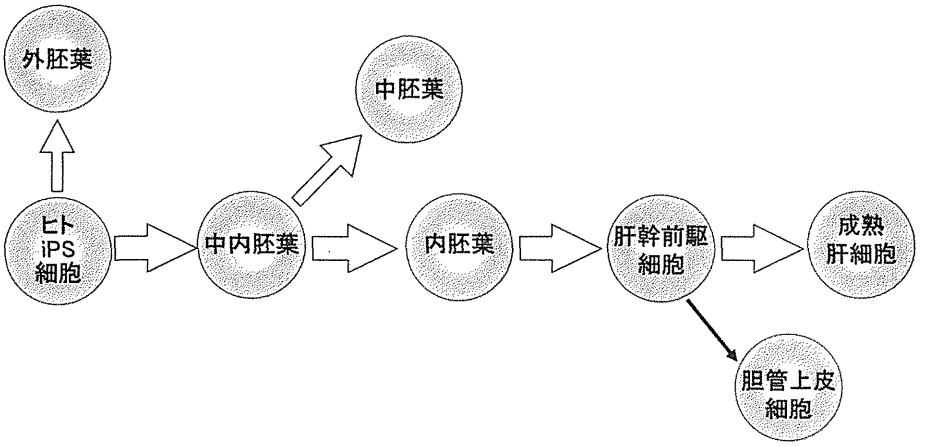

Fig. 1　ヒトiPS細胞から肝細胞への分化誘導
ヒトiPS細胞は中内胚葉，内胚葉，肝幹前駆細胞を経由して成熟した肝細胞へと分化する．

性発現を研究開発の初期段階に予測できれば，より安全性の高い医薬品を効率良く開発することにつながると考えられる．現在は，主にヒト初代培養（凍結）肝細胞（本稿では，ヒト凍結肝細胞も含めてヒト初代培養肝細胞と記載する）や肝ミクロソームを用いて，薬剤あるいは薬剤の代謝過程で生成する反応性代謝物による細胞傷害性等を試験する毒性試験や，薬物代謝酵素の誘導や阻害等の薬物動態評価試験が施行されている．しかしながら，ヒト初代培養肝細胞は高価であり，高機能なヒト肝細胞ロットの安定供給が難しいといった問題等から，ヒトiPS細胞由来分化誘導肝細胞を用いた毒性・薬物動態評価系の開発が期待されている．

また，薬物代謝酵素の活性は個人差が大きいことが知られているが（薬物代謝酵素の種類によるが，数十倍～千倍程度），将来的には，様々な個人由来のヒトiPS細胞由来分化誘導肝細胞を用いることで，個人差を反映した評価系が開発できる可能性もある．

3.　ヒトiPS細胞から肝細胞への分化誘導

3.1　ヒトiPS細胞から肝細胞への分化誘導の現状

ヒトiPS細胞から肝細胞への分化誘導は，先行して進められてきたヒトES細胞（embryonic stem cells）から肝細胞への分化誘導を応用して進められてきており，両者は共通の方法を用いて分化誘導できる．そこで本稿では，両者を区別することなく，紹介する．

ヒトiPS細胞は中内胚葉，内胚葉，肝幹前駆細胞を経由して成熟した肝細胞へと分化する（Fig. 1）．一般に，外胚葉由来の神経細胞や，中胚葉由来の心筋細胞への分化誘導に比べ，内胚葉に属する肝細胞や膵臓細胞への分化誘導は研究が遅れていた（Fig. 2）．しかしながら，

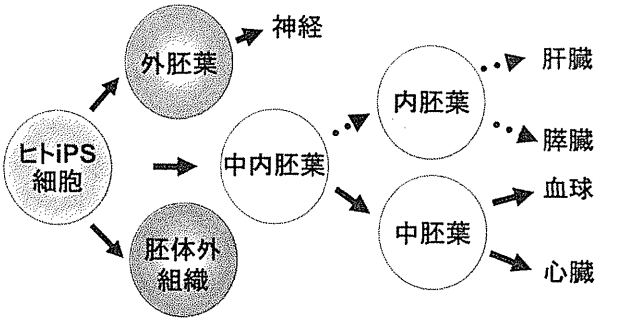

Fig. 2　ヒトiPS細胞から各胚葉への分化
神経細胞は外胚葉を，心筋細胞は中胚葉を，肝細胞や膵臓細胞は内胚葉を経由して分化する．

2005年にD'Amourらによって，アクチビンAが内胚葉を分化誘導できることが発見されて以来[1]，急速に研究が進展している．これまでに，ヒトiPS細胞から肝細胞への様々な分化誘導法が開発されているが（前述のように，ヒトES細胞から肝細胞への分化誘導法も含める），未分化ヒトiPS細胞から肝細胞への分化過程を，以下の3ステップあるいは4ステップに分けて分化誘導する方法が一般的である．即ち，(1) 未分化iPS細胞から内胚葉への分化ステップ（内胚葉分化），(2) 内胚葉から肝幹前駆細胞への分化ステップ（肝特異化），(3) 肝幹前駆細胞から肝細胞への分化ステップ（肝成熟化）〔あるいは肝幹前駆細胞から肝細胞への分化ステップを，(3) 肝（幹前駆）細胞の増幅と (4) 肝細胞の成熟化のステップに分ける〕に分け，個々の分化ステージで，発生段階を模倣したように，分化に必要な増殖因子やサイトカイン等を付加して分化させることが試みられている（詳細は代表的な総説[2, 3]を参照）．

(1)の内胚葉への分化ステップでは，アクチビンAの

付加がほぼ全てのプロトコールで用いられており，アクチビンAに加えFGF2（fibroblast growth factor 2）やWnt3aを付加して分化誘導する方法も知られている．

(2)の内胚葉から肝幹前駆細胞への分化ステップには，BMP（bone morphogenetic protein）シグナルとFGF（fibroblast growth factor）シグナルが必要なことが判明しており，BMP4やFGF4などを付加する方法が汎用されている．また，肝細胞への方向付けにおいてはDMSO（dimethyl sulfoxide）によるヒストンのアセチル化が有効であることも知られており，DMSOを用いた方法も報告されている[6]．

(3)の肝幹前駆細胞から肝細胞への分化には，HGF（hepatocyte growth factor）やオンコスタチンM（OsM），デキサメタゾン（DEX）などを用いて分化誘導する方法が一般的である．更に各分化ステップで，培地や細胞外マトリックス（Ⅰ型コラーゲンやマトリゲルが汎用される）の種類，血清やフィーダー細胞の有無等が各プロトコールで工夫されている．ヒトiPS細胞由来分化誘導肝細胞を再生医療に利用する場合には，血清やフィーダー細胞等の異種動物由来成分を排除し，かつ組成の明らかな培地（chemically defined mediumと呼ばれる）で分化誘導する必要があるが，同細胞を創薬研究に応用する場合にはそのような制限は必要ない．むしろ，創薬応用には可能な限り成熟度が高い肝細胞を分化誘導する必要があり，特に血清の付加は現時点では有用である（ただし，血清のロットチェックは必須である）．

以前は，胚様体（embryoid body: EB）形成法を用いて肝細胞への分化が試みられてきたが，最近では，EB形成を介さず，上述のように直接分化させる方法が一般的である．しかしながら，これらの増殖因子やサイトカインの添加だけからなる分化誘導法は，肝細胞への分化効率もまだまだ不十分なのが現状であり，更なる分化効率の向上が必要となっている．

3.2 分化ステージに応じた最適な転写因子の過剰発現を組み合わせたヒトiPS細胞から肝細胞への高効率分化誘導

著者らは，付加する増殖因子やサイトカインを単に最適化しただけの分化誘導法の改良では，劇的なヒトiPS細胞から肝細胞への分化効率の向上が期待できないのではないかと考え，個々の分化ステップの細胞に（肝細胞への分化に）適した転写因子を一過性に過剰発現させることで，効率よく肝細胞への分化を誘導する方法を開発した（Fig. 3）．すなわち，増殖因子やサイトカインを付加した従来の方法で細胞の外部環境を分化に適した状態にした上に，細胞内部から強制的に分化を生じさせるように適切な転写因子を発現させることで，分化効率を飛躍的に向上させる方法を考案した．

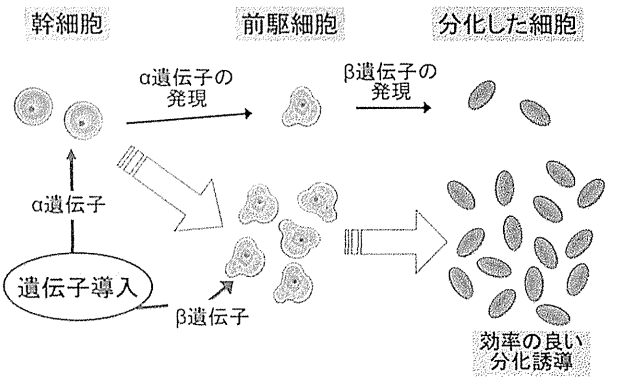

Fig. 3 機能遺伝子の導入による分化誘導効率の向上
適切な分化状態の細胞に効率よくかつ一過性に機能遺伝子を発現させることにより，目的の機能細胞を効率よく分化誘導することが期待できる．

当初は，未分化iPS細胞からアクチビンA処理で分化させた中内胚葉にSOX17（Sry-related HMG box 17）遺伝子を，内胚葉から肝幹前駆細胞への分化ステップではHEX（hematopoietically expressed homeobox）遺伝子を，肝幹前駆細胞から肝細胞への分化ステップではHNF4α（hepatocyte nuclear factor 4α）遺伝子を導入することで，高いアルブミン産生能や薬物代謝機能を有した肝細胞を効率よく分化誘導することに成功した[5,7]．更に最近では，ヒトES/iPS細胞から肝細胞への各分化ステップにおいて7種類の肝関連転写因子（FOXA2, SOX17, HEX, HNF1α, HNF1β, HNF4α, HNF6）を導入し，最も効率良く肝分化を促進できる転写因子を探索した結果，FOXA2（forkhead box protein A2）及びHNF1α（hepatocyte nuclear factor 1α）遺伝子を組み合わせて発現させることにより，更に効率良く成熟肝細胞を分化誘導することに成功した（Fig. 4）[8]．

このようにして作製した肝細胞は，80～90%以上の細胞がアルブミン，アシアロ糖タンパク質受容体，LDL（low density lipoprotein）取り込み能，インドシアニングリーン取り込み能，薬物代謝酵素（シトクロムP450 3A4, CYP7A1, CYP2D6等）陽性であり，ヒト初代培養肝細胞に匹敵する薬物代謝酵素の遺伝子発現レベルを示した．また，シトクロムP450酵素などで代謝される9種類の薬物の代謝プロファイルを調べたところ，ヒトiPS細胞由来分化誘導肝細胞の薬物代謝能はヒト初代培養肝細胞より低いものの（シトクロムP450酵素の種類により異なるが，ヒトiPS細胞由来分化誘導肝細胞はヒト初代培養肝細胞の1～40%程度の活性），いずれの薬物に対しても代謝能を有していることが確認された．各

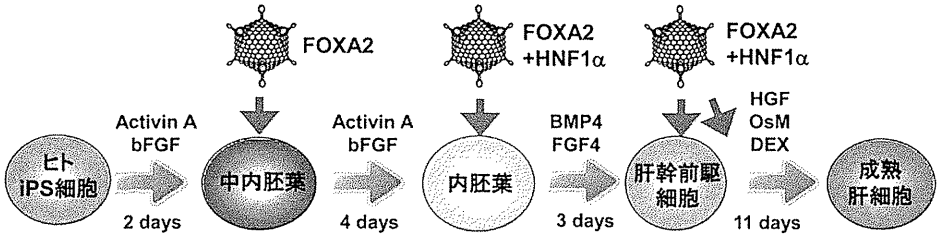

Fig. 4　液性因子と転写因子の導入を組み合わせることによるヒトiPS細胞から肝細胞への高効率分化誘導

ヒトiPS細胞をアクチビンAで培養することによって得られた培養3日目の中内胚葉に対して，FOXA2発現アデノウイルスベクターを作用させた．更に，アクチビンAで4日間培養した後，培養6日目の内胚葉に対してFOXA2及びHNF1α発現アデノウイルスベクターを作用させた．BMP4とFGF4を用いて3日間培養した後，培養9日目の肝幹前駆細胞に対してFOXA2及びHNF1α発現アデノウイルスベクターを作用させた．その後，肝幹前駆細胞をHGF，オンコスタチンM（OsM），デキサメタゾン（DEX）を用いて11日間培養することによって（培養12日目にFOXA2及びHNF1α発現アデノウイルスベクターを更に作用），高い薬剤代謝機能やアルブミン産生能等を有した肝細胞へ分化させることができる．

シトクロムP450酵素の遺伝子発現と代謝能との間に，ヒトiPS細胞由来分化誘導肝細胞とヒト初代培養肝細胞で乖離が認められたが，この原因としては，そもそもシトクロムP450酵素の活性は個人差が大きいことが知られており（数十倍～千倍程度の個人差），用いたヒトiPS細胞が低いシトクロムP450酵素活性の個人から樹立されていた可能性や，シトクロムP450酵素の活性発現に必要な補酵素群の発現が未だ分化誘導肝細胞では十分でないこと等が考えられた．今後，異なった個人から樹立したヒトiPS細胞由来分化誘導肝細胞を用いて，同様の検討する必要があるであろう．

一方，作製したヒトiPS細胞由来分化誘導肝細胞を用いて，薬剤に対する毒性評価についても検討した（論文投稿中）．肝毒性を生じることが知られている多種類の薬剤について，本分化誘導肝細胞を用いて細胞毒性評価試験を行ったところ，株化細胞であるHepG2細胞を用いた場合に比べ，より感度良く毒性（細胞傷害性）を示し，かつその毒性はシトクロムP450酵素の阻害剤を加えると部分的に消失した．したがって，シトクロムP450酵素で代謝された代謝物（反応性代謝物）によって生じた細胞傷害性を，分化誘導肝細胞が検出できることが明らかとなった．反応性代謝物は薬物性肝障害の主な原因と考えられており，ヒトiPS細胞由来分化誘導肝細胞で反応性代謝物による細胞傷害性を検出できたことは，極めて大きな意義をもつと考えられる．以上のことから，FOXA2及びHNF1α遺伝子を導入することにより，ヒトiPS細胞から薬物代謝能を有する肝細胞を効率良く分化誘導できるだけでなく，同細胞が薬物の毒性スクリーニングに使用可能であることが示唆された．

なお，細胞分化の各ステップでの転写因子（遺伝子）の導入には，機能性に優れ，独自開発した改良型アデノウイルスベクターを用いた．iPS細胞から肝細胞への分化のように，分化の各ステップが階層的に起こる場合には，各分化ステップでだけ導入遺伝子が機能するように（後の細胞分化に影響を与えないように），遺伝子発現期間は一過性であること，そして効率よく細胞集団を分化させるためには，100%の遺伝子発現効率で遺伝子発現させることが必須となるが，改良型アデノウイルスベクターはこのような目的に唯一叶うベクターである．本研究で用いた改良型アデノウイルスベクターは，細胞への感染に関与するウイルス表面タンパク質のファイバータンパク質のC末端領域にポリリジン配列（KKKKKKK；リジン（K）が7つ続くのでK7と略称）を遺伝子工学的に付与しており，細胞表面のヘパラン硫酸を認識して多くの細胞種に効率よく遺伝子導入が可能となる（Fig. 5）．本K7型アデノウイルスベクターは，未分化ヒトiPS細胞や，ヒトiPS細胞から分化した細胞に対しても，100%の効率で遺伝子導入が可能であった[5]．

著者らは，機能面で優れた様々なアデノウイルスベクターを開発しており，詳細は文献[9]を参照されたい．

3.3　3次元培養によるヒトiPS細胞由来分化誘導肝細胞の成熟化

ヒト初代培養肝細胞は，培養すると急速に肝細胞特異的な性質が失われていくことが知られている．例えば，アルブミンやシトクロムP450酵素の遺伝子発現は，最適化された培養条件で培養しても，48時間も培養すると，解凍（凍結肝細胞の場合）直後の遺伝子発現と比較すると10～100分の1程度にまで低下する．一方で，スフェロイド培養等の3次元培養や，繊維芽細胞や血管

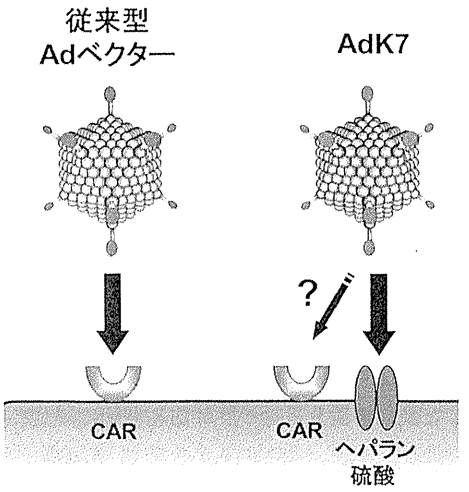

Fig.5　改良型アデノウイルスベクターの遺伝子導入特性
従来のアデノウイルス(Ad)ベクターはCAR(coxsackievirus adenovirus receptor)を認識して感染する．ポリリジン配列をファイバータンパク質のC末端領域に遺伝子工学的に付与したアデノウイルスベクター（AdK7）は，多くの細胞で発現しているヘパラン硫酸を認識して感染できるため，CAR陰性の細胞を含む多くの細胞への高効率な遺伝子導入が可能となる．

内皮細胞との共培養系でヒト初代培養肝細胞を培養すると，アルブミンやシトクロムP450酵素等の肝特異的な機能の減弱は，ある程度抑制されることが知られている．

そこで，細胞シート工学技術を用いることで，シート状に回収したSwiss3T3細胞とヒトiPS細胞から分化誘導した肝細胞とを積層3次元共培養し，肝機能の向上が可能か検討した（東京女子医大・先端生命医科学研究所大橋一夫先生，岡野光夫先生との共同研究）[10]．その結果，単層のヒトiPS細胞由来分化誘導肝細胞と比較し，肝細胞特異的な遺伝子発現量やアルブミン分泌量が有意に増加することが明らかとなった．また，ヒトiPS細胞由来分化誘導肝細胞の成熟化には，肝細胞とSwiss3T3細胞との物理的な接触が重要であることを見出した．更に，ヒトiPS細胞由来分化誘導肝細胞へ，1型コラーゲンゲルを重層することにより肝細胞成熟化が促進される一方で，コラーゲン合成阻害剤存在下においてはSwiss3T3細胞との積層3次元共培養時の成熟化が抑制されたことから，Swiss3T3細胞が産生する1型コラーゲンが肝細胞成熟化を担う主要な因子の1つであることが明らかとなった．最近では，簡便に3次元培養が可能な基材が各社から販売されており，培養法の改良によってもヒトiPS細胞由来分化誘導肝細胞の成熟化亢進が期待できる．

3.4　Direct-reprogrammingによる肝細胞への直接分化

近年，繊維芽細胞等の分化した細胞から，iPS細胞を介さずに，直接他の細胞に分化を誘導するDirect-reprogrammingに関する研究がトピックスとなっている．

古くは，膵臓細胞を肝細胞に分化誘導した研究（2000年）や，B細胞をマクロファージに分化誘導した研究（2004年）があるが，2008年以降，膵β細胞や神経細胞，心筋細胞，肝細胞等を，通常複数の転写因子を発現する遺伝子を導入して，繊維芽細胞から直接分化誘導した研究が相次いでいる．肝細胞についても，マウスの系であるが，繊維芽細胞からのDirect-reprogrammingの報告がある[11, 12]（ヒト細胞を用いた肝細胞へのDirect-reprogrammingについてはまだ報告例はない）．iPS細胞から分化誘導した細胞同様に，Direct-reprogrammingによって得られた細胞（肝細胞を含む）も，創薬研究に有用なツールとなる可能性はあるが，重要なのは最終的に得られる分化細胞の“分化度”と，分化細胞を大量供給できるか？という観点であり，この2点が満たされれば，iPS細胞から分化させたのか，あるいはDirect-reprogrammingであるのかは問題ではない．

分化細胞の大量供給という観点では，Direct-reprogrammingによって終末分化した細胞に直接分化させた場合には，通常，細胞は増殖能を失うことから大量供給は難しく，その前駆細胞を分化誘導する方が有用かもしれない．その場合，前駆細胞を成熟細胞に分化させる技術が必要になり，iPS細胞から目的細胞への分化誘導研究は，この過程での技術開発にも役立つことが期待される．

4.　おわりに

従来のヒトES/iPS細胞から分化誘導させた肝細胞は，機能面において初代培養肝細胞に比べて大きく劣っており，創薬研究への応用は困難であった．しかしながら，著者らが開発した分化誘導法により，創薬応用に向けて，ようやく最低限の解析が可能なレベルにまで分化した肝細胞を得ることが可能になった．

本稿では触れなかったが，著者らが分化誘導した肝細胞は，C型肝炎ウイルスに対する感染能も有しており[13]，肝炎研究のための有力な培養モデル系にもなる（同様な報告が最近，海外のグループからも報告された[14-16]）．一方で，ヒトiPS細胞由来分化誘導肝細胞を幅広く創薬研究に応用するためには，実験毎に3週間に及ぶ分化誘導を行うことは細胞供給の観点から効率が悪い．そこで現

在著者らは，分化途中の肝幹前駆細胞の段階で，分化細胞を大量に増幅できないかという課題にも取り組んでいる．今度，より一層高機能な（成熟度が高い）ヒトiPS細胞由来分化誘導肝細胞の作製法の開発（改良）を進めるとともに，本分化誘導肝細胞が創薬研究で広く活用されることを期待している．

なお，本稿で紹介した分化誘導法で作製されたヒトiPS細胞由来分化誘導肝細胞は，リプロセル社よりReproHepatoとして市販されている．

文　献

1) D'Amour K.A., Agulnick A.D., Eliazer S., Kelly O.G., Kroon E., Baetge E.E.: Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat. Biotechnol.*, 23, 1534-1541 (2005).
2) Snykers S., De Kock J., Rogiers V., Vanhaecke T.: In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem Cells*. 27, 577-605 (2009).
3) Baxter M.A., Rowe C., Alder J., Harrison S., Hanley K.P., Park B.K., Kitteringham N.R., Goldring C.E., Hanley N.A.: Generating hepatic cell lineages from pluripotent stem cells for drug toxicity screening. *Stem Cell Re.*, 5, 4-22 (2010).
4) Hay D.C., Zhao D., Fletcher J., Hewitt Z.A., McLean D., Urruticoechea-Uriguen A., Black J.R., Elcombe C., Ross J.A., Wolf R., Cui W.: Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. *Stem Cells*, 26, 894-902 (2008).
5) Inamura M., Kawabata K., Takayama K., Tashiro K., Sakurai F., Katayama K., Toyoda M., Akutsu H., Miyagawa Y.,Okita H., Kiyokawa N., Umezawa A., Hayakawa T., Furue MK., Mizuguchi H.: Efficient generation of hepatoblasts from human ES cells and iPS cells by transient overexpression of homeobox gene HEX. *Mol. Ther.*, 19, 400-407 (2011).
6) Takayama K., Inamura M., Kawabata K., Tashiro K., Katayama K., Hayakawa T., Furue MK., Mizuguchi H.: Efficient and selective generation of two distinct endoderm lineages from human ES and iPS cells by differentiation stage-specific SOX17 transduction. *PLoS One*, 6, e21780 (2011).
7) Takayama K., Inamura M., Kawabata K., Katayama K., Higuchi M., Tashiro K., Nonaka A., Sakurai F., Hayakawa T., Furue MK., Mizuguchi H.: Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4α transduction. *Mol. Ther.*, 20, 127-137 (2012).
8) Takayama K., Inamura M., Kawabata K., Sugawara M., Kikuchi K., Higuchi M., Nagamoto Y., Watanabe H., Tashiro K., Sakurai F., Hayakawa T., Furue MK., Mizuguchi H. : Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1α transduction. *J. Hepatol.*, 57, 628-636(2012).
9) 水口裕之：次世代アデノウイルスベクターの開発と生命科学研究への応用，*Drug Delivery System*, 25, 493-503 (2010).
10) Nagamoto Y., Tashiro K., Takayama K., Ohashi K., Kawabata K., Sakurai F., Tachibana M., Hayakawa T., Furue MK., Mizuguchi H.: Promotion of hepatic maturation of human pluripotent stem cells in 3D co-culture using type I collagen and Swiss 3T3 cell sheets. *Biomaterials*, 33, 4526-4534 (2012).
11) Huang P., He Z., Ji S., Sun H., Xiang D., Liu C., Hu Y., Wang X., Hui L.: Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature*, 475, 386-389 (2011).
12) Sekiya S., Suzuki A.: Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature*, 475, 390-339 (2011).
13) Yoshida T., Takayama K., Kondoh M., Sakurai F., Tani H., Sakamoto N., Matsuura Y., Mizuguchi H., Yagi K.: Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection. *Biochem. Biophys. Res. Commun.*, 416, 119-124 (2011).
14) Schwartz R.E., Trehan K., Andrus L., Sheahan T.P., Ploss A., Duncan S.A., Rice C.M., Bhatia S.N.: Modeling hepatitis C virus infection using human induced pluripotent stem cells. *Proc. Natl. Acad. Sci. USA.*, 109, 2544-2548 (2012).
15) Wu X., Robotham J.M., Lee E., Dalton S., Kneteman N.M., Gilbert D.M., Tang H.: Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. *PLoS Pathog.*, 8, e1002617 (2012).
16) Roelandt P., Obeid S., Paeshuyse J., Vanhove J., Lommel A.V., Nahmias Y., Nevens F., Neyts J., Verfaillie C.M.: Human pluripotent stem cell derived hepatocytes support complete replication of hepatitis C virus. *J. Hepatol.*, 57, 246-251 (2012).