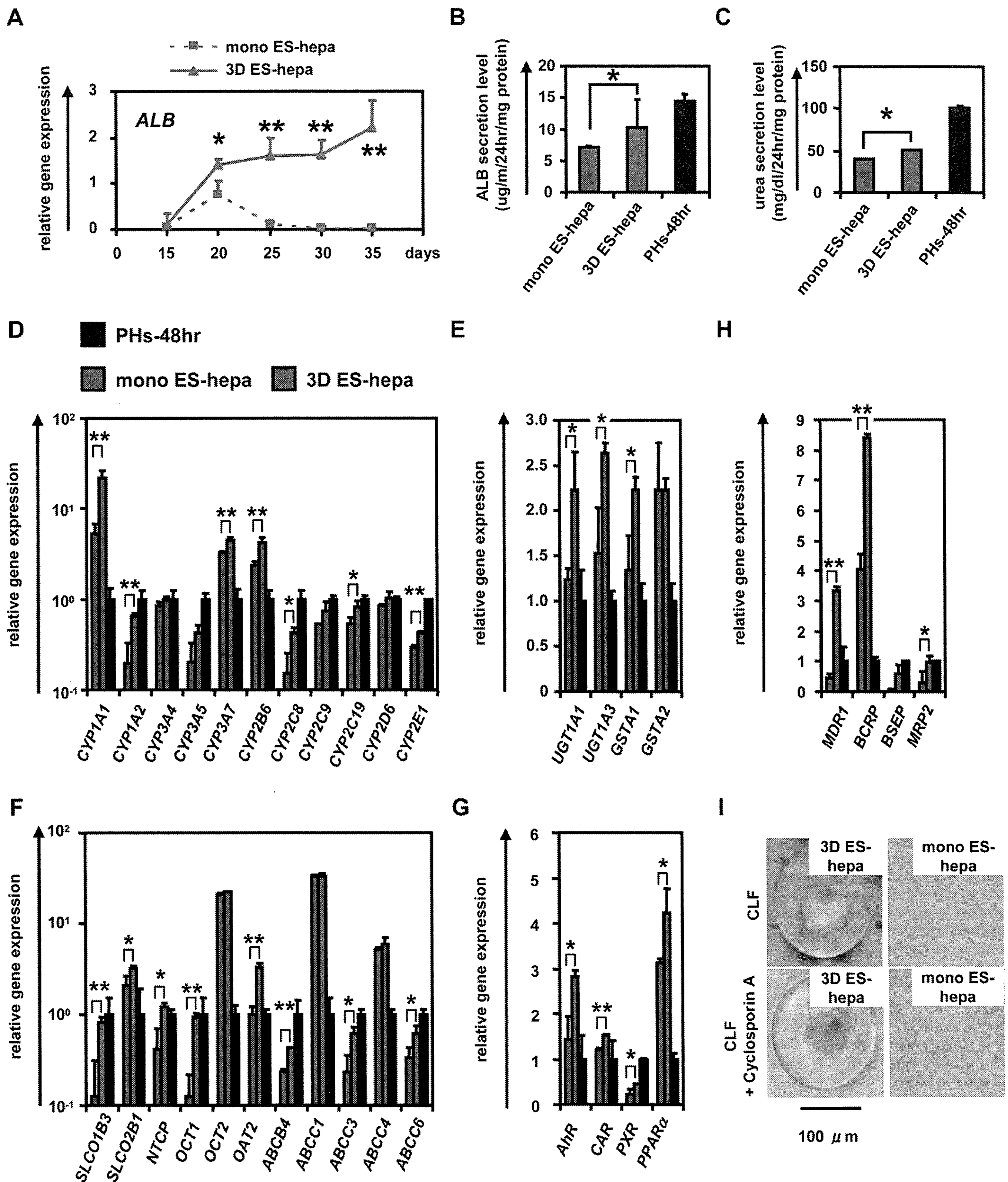


Fig. 2. Hepatocyte functions in hESC-derived hepatocyte-like cells were enhanced by using Nanopillar Plate. (A) The gene expression levels of *ALB* were measured by real-time RT-PCR on day 15, 20, 25, 30, and 35. On the *y* axis, the gene expression levels in PHs (three lots of PHs were used in all studies), which were cultured for 48 h after plating (PHs-48hr), were taken as 1.0. (B, C) The amount of ALB (B) and urea (C) secretion were examined in the mono ES-hepa (day 20), the 3D ES-hepa (day 35), and PHs-48hr. (D–H) The gene expression levels of CYP enzymes (D), conjugating enzymes (E), hepatic transporters (F), hepatic nuclear receptors (G), and bile canaliculi transporters (H) were examined by real-time RT-PCR in the mono ES-hepa, the 3D ES-hepa, and PHs-48hr. On the *y* axis, the expression levels in PHs-48hr were taken as 1.0. (I) The ability of bile acid uptake and efflux was examined in the mono ES-hepa and 3D ES-hepa. Choly-lysyl-fluorescein (CLF) (5 μM) was used for the observation of bile canaliculi uptake and efflux. To inhibit transportation by BSEP, the cells were pretreated with 1 μM Cyclosporin A. $^{*}P < 0.05$; $^{**}P < 0.01$.

moderately increased in the 3D ES-hepa until day 35 (Fig. 2A). These results suggest that the hepatocyte functions of the 3D ES-hepa are sustained for more than 2 weeks on the Nanopillar Plate, although those of the mono ES-hepa are rapidly devitalized (Fig. 2A and Fig. S4). Other hepatocyte characteristics, such as ability of ALB and urea secretion and gene expression levels of hepatocyte-related markers in the 3D ES-hepa were compared with those of the mono ES-hepa (Fig. 2B–H). Because the gene expression level of *ALB* in the 3D ES-hepa was the highest on day 35 and that in mono ES-hepa was the highest on day 20, various hepatocyte characteristics were compared on day 35 or day 20, respectively. The amount of ALB (Fig. 2B) and urea (Fig. 2C) secretion in the 3D ES-hepa was higher than those of the mono ES-hepa. The gene expression levels of CYP enzymes (Fig. 2D), conjugating enzymes (Fig. 2E), hepatic transporters (Fig. 2F), hepatic nuclear receptors (Fig. 2G), and hepatic transcription factors (Fig. S5) in the 3D ES-hepa were higher than those in the mono ES-hepa. The expression levels of most of the genes in the 3D ES-hepa were higher than those in the mono ES-hepa. Because the previous study [11] showed that hepatocyte spheroids expressed hepatocyte transporters similar to those of the bile canaliculi in native liver tissue, the gene expression levels of bile canaliculi transporters (Fig. 2H), as well as the ability of bile acid uptake and efflux, (Fig. 2I) were examined in the 3D ES-hepa. The gene expression levels of bile canaliculi transporters were increased in the 3D ES-hepa compared with those of mono ES-hepa and PHs (Fig. 2H). The bile canaliculi formation was visualized by BSEP fluorescent substrate: Cholyl-lysyl-fluorescein (CLF), which is inhibited by BSEP

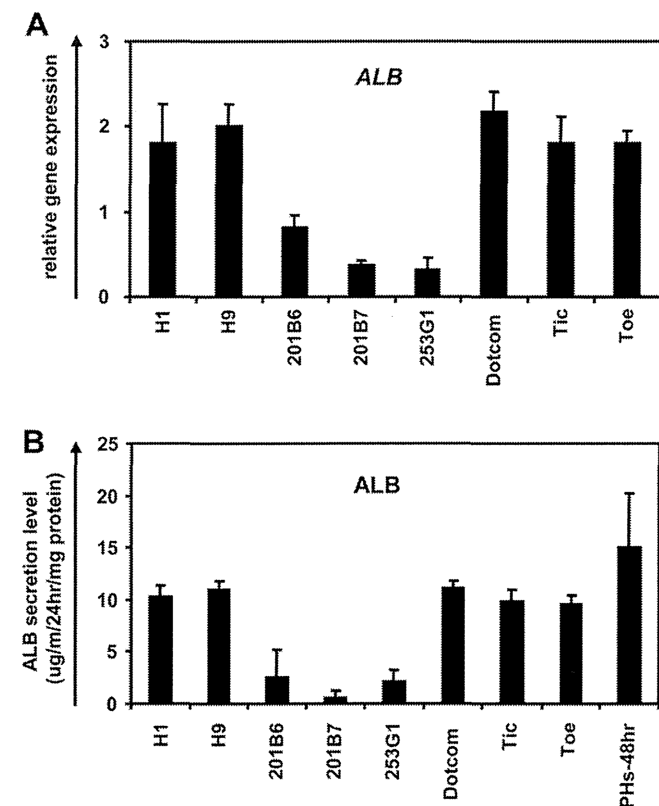


Fig. 3. Comparison of the hepatic differentiation capacities of various hESC and hiPSC lines hESCs (H1 and H9) and hiPSCs (201B6, 201B7, 253G1, Dotcom, Tic, and Toe) were differentiated into the 3D ES/iPS-hepa as described in Fig. 1A. (A) On day 20, the gene expression level of *ALB* was examined by real-time RT-PCR. On the y axis, the gene expression level of *ALB* in PHs-48hr was taken as 1.0. (B) On day 20, the amount of ALB secretion was examined by ELISA. The amount of ALB secretion was calculated according to each standard followed by normalization to the protein content per well.

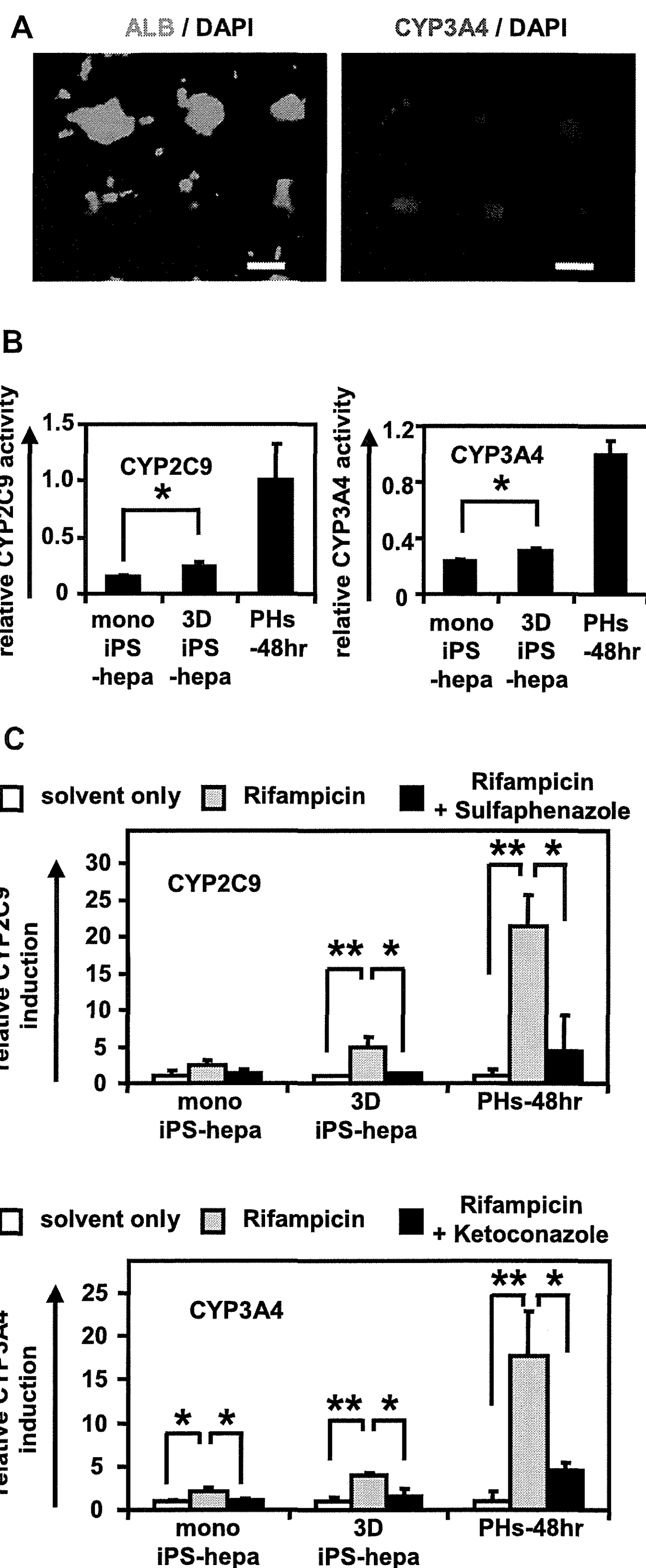


Fig. 4. Drug metabolism capacity and CYP induction potency were examined in the 3D iPS-hepa. (A) The 3D iPS-hepa (day 35) were subjected to immunostaining with anti-ALB (green) or CYP3A4 (red) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar represents 100 μm. (B) The CYP activity was measured in the mono iPS-hepa (day 20), the 3D iPS-hepa (day 35), and PHs-48hr. On the y axis, the CYP activity in PHs-48hr was taken as 1.0. (C) Induction of CYP2C9 (left) or CYP3A4 (right) by DMSO (solvent only; white bar), Rifampicin (gray bar), or rifampicin and CYP inhibitor (Sulfaphenazole or Ketoconazole, black bar) in the mono iPS-hepa, the 3D iPS-hepa, and PHs-48hr. On the y axis, the CYP activity of the cells that have been cultured in DMSO-containing medium was taken as 1.0. $^{*}P < 0.05$; $^{**}P < 0.01$.

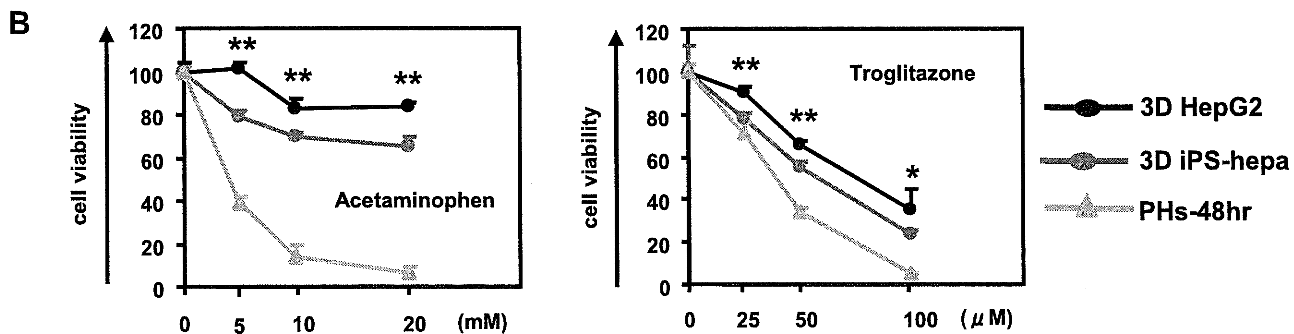
A

3D HepG2
3D iPS-hepa

Allopurinol
Amiodaron
Benzbromarone
Clozapine
Cyclizine
Dantrolene
Desipramine
Disufliram
Erythromycin
Felbamate
Flutamide
Isoniazid
Labetalol
Leflunomide
Maprotiline
Nefazodone
Nitrofurantoin
Sulindac
Tacrine
Tebinafine
Tolcapone
Zafirlukast

cell viability
(μM)

B



inhibitor Cyclosporin A [22,23]. More CLF was accumulated in the 3D ES-hepa than in the mono ES-hepa (Fig. 2I *upper panel*). Moreover, CLF accumulation was inhibited by Cyclosporin A treatment only in the 3D ES-hepa (Fig. 2I *lower panel*), demonstrating that the functionality of BSEP transporter in 3D ES-hepa was greater than that in mono ES-hepa. These results suggested that hepatocyte maturation was promoted by the culture on the Nanopillar Plate. It is likely that, compared to the monolayer culture condition, the 3D spheroid-culture condition is more similar to the *in vivo* condition.

It is important to select an hESC/hiPSC line that has a strong ability to differentiate into hepatocyte-like cells in the case of medical applications such as drug screening. In this study, two hESC lines and six hiPSC lines were differentiated into the hepatocyte-like cells, and then their gene expression levels of *ALB* (Fig. 3A) and ALB secretion levels (Fig. 3B) were compared. These results suggest that the iPSC line, Dotcom, was the suitable cell line for hepatocyte maturation. Therefore, the iPSC line, Dotcom, was used to examine the possibility of the 3D iPS-hepa for drug screening. The drug metabolism capacity and the CYP induction potency of the 3D iPS-hepa were compared with those of the mono iPS-hepa. We confirmed the expression of ALB and CYP3A4 protein in the 3D ES-hepa (Fig. 4A). The activity levels of CYP enzymes in the 3D iPS-hepa were measured according to the metabolism of the CYP2C9 or CYP3A4 substrates (Fig. 4B); the levels were higher than those of the mono iPS-hepa (Fig. 4B). We further tested the induction of CYP2C9 and CYP3A4 by chemical stimulation (rifampicin was used as a CYP2C9 or CYP3A4 inducer). Compared with mono iPS-hepa, the 3D iPS-hepa produced more metabolites in response to chemical stimulation (Fig. 4C). In addition, the CYP induction was inhibited by using CYP2C9 or CYP3A4 inhibitor (Sulfaphenazole or Ketoconazole, respectively). These results indicated that drug metabolism capacity and CYP induction potency in 3D iPS-hepa were higher than those in mono iPS-hepa.

Many researchers have tried to predict the drug-induced cytotoxicity *in vitro* using hepatocarcinoma-derived cells such as HepG2 cells [24,25]. HepG2 cells are less expensive than PHs and the reproducible experiments are easier to perform than they are with PHs, although 30% of the compounds were incorrectly classified as nontoxic [24,25]. To overcome these problems, hESC/hiPSC-derived hepatocyte-like cells are expected to be used to predict drug-induced cytotoxicity. To examine its applicability to drug screening, the 3D iPS-hepa were treated with various drugs, that cause hepatotoxicity. WST-8 assay was performed to evaluate cell viability (Fig. S6). The susceptibility of the 3D iPS-hepa to most of the hepatotoxic drugs was higher than that of the mono iPS-hepa (Fig. S7). Compared to the mono iPS-hepa, the 3D iPS-hepa were more suitable tools for drug screening. Next, the susceptibility of the 3D iPS-hepa to the hepatotoxic drugs was compared with that of the 3D spheroid cultured HepG2 cells (3D HepG2; the hepatocyte functions of 3D HepG2 cells are higher than those of monolayer cultured HepG2 cells [Fig. S8]). With most of the drugs, the cell viability of the 3D iPS-hepa was lower than that of the 3D HepG2 (Fig. 5A). These results indicated that the 3D iPS-hepa are more valuable tools for drug screening than the 3D HepG2. However, the susceptibility of the 3D iPS-hepa to Acetaminophen and Troglitazone was lower than that of the PHs which were cultured for 48 h after the cells were plated (Fig. 5B). These results might be due to the lower activity levels of CYPs in 3D iPS-hepa as compared as those in PHs. Taken together, 3D iPS-hepa are more valuable tools for drug screening than the 3D HepG2, although further maturation of 3D iPS-hepa is still required for 3D iPS-hepa to be an alternative cell source of PHs in the drug screening.

To examine whether drug-induced cytotoxicity is caused by CYP metabolites in 3D iPS-hepa, Aflatoxin B1 (mainly metabolized by CYP3A4 [26]) and Benzbromarone (mainly metabolized by CYP2C9 [27]) were treated in the presence or absence of a CYP3A4 and a 2C9 inhibitor, Ketoconazole and Sulfaphenazole, respectively (Fig. 6). The cell viability of 3D iPS-hepa was partially rescued by treatment with the CYP inhibitor. These results indicated that drug-induced cytotoxicity was caused by CYP metabolites of Aflatoxin B1 and Benzbromarone.

4. Discussion

Recently, it has been expected that human pluripotent stem cells and their derivatives, including hepatocyte-like cells, will be utilized in applications for the safety assessment of drugs. We have previously reported that combinational overexpression of SOX17, HEX, and HNF4α, or combinational overexpression of FOXA2 and HNF1α could promote hepatocyte differentiation [5,6]. However, the drug metabolism capacity of the hepatocyte-like cells generated by our previous protocol was still lower than that of primary human hepatocytes [6]. To generate more matured hepatocyte-like cells as compared with our previous protocol, we established a hepatocyte differentiation method employing not only stage-specific transient overexpression of hepatocyte-related transcription factors but also a 3D culture systems using a Nanopillar Plate, was established. Although the use of hepatocyte-like cells generated from hESCs/hiPSCs in application for drug toxicity testing has begun to be focused, to the best of our knowledge, there have been few studies that have investigated whether hepatocyte-like cells could predict many kinds of drug-induced toxicity.

3D culture spheroids were generated from hESCs/hiPSCs by using a Nanopillar Plate. The diameter of the spheroids was approximately 100 μm on day 35 of differentiation (Fig. 1C). Because it is known that the no-oxygen limitation would take place in spheroids up to 100 μm in diameter [28], the size of the spheroid might be important to generate spheroids with high viability. A Nanopillar Plate has a potential to regulate the spheroid diameter simply by culturing under optimized seeding condition, on its suitably designed pillar and hole structure [11]. Therefore, a Nanopillar Plate would be a suitable environment for the generation of 3D ES/iPS-hepa that show high viability and possess high level of hepatocellular functions.

The levels of many hepatocyte functions, such as ALB secretion ability (Fig. 2B), urea secretion ability (Fig. 2C), hepatocyte-related gene expressions (Fig. 2D–H), drug metabolism capacity (Fig. 4B), and CYP induction potency (Fig. 4C), of 3D ES/iPS-hepa were higher than those of mono ES/iPS-hepa. This might have been because the structural and functional polarity, which can be seen in the naïve environment of hepatocytes, of the hepatocyte-like cells was configured by a 3D culturing condition. Previous studies have shown that a 3D culture condition is suitable to maintain the hepatic characteristics of the isolated hepatocytes because this condition mimic *in vivo* environment [29,30]. These facts indicated that the 3D culture condition is a more suitable condition for the hepatocyte-like cells than the monolayer culture condition.

Two hES cell lines and six hiPS cell lines were differentiated into the hepatocyte-like cells in this study. The hiPS cell line, Dotcom, seemed to be a suitable cell line for hepatic differentiation (Fig. 3). Because the hepatic differentiation propensity differs among the

Fig. 5. The possibility of applying 3D iPS-hepa to drug testing was examined. (A) The cell viability of the 3D HepG2 (black) and 3D iPSC-hepa (red) were assessed by WST-8 assay after 24 h exposure to different concentrations of 22 test compounds. (B) The cell viability of the 3D HepG2 (black), 3D iPSC-hepa (red), and PHs-48hr (green) were assessed by WST-8 assay after 24 h exposure to different concentrations of Acetaminophen and Troglitazone. Cell viability is expressed as a percentage of cells treated with solvent only. $^{*}P < 0.05$; $^{**}P < 0.01$.

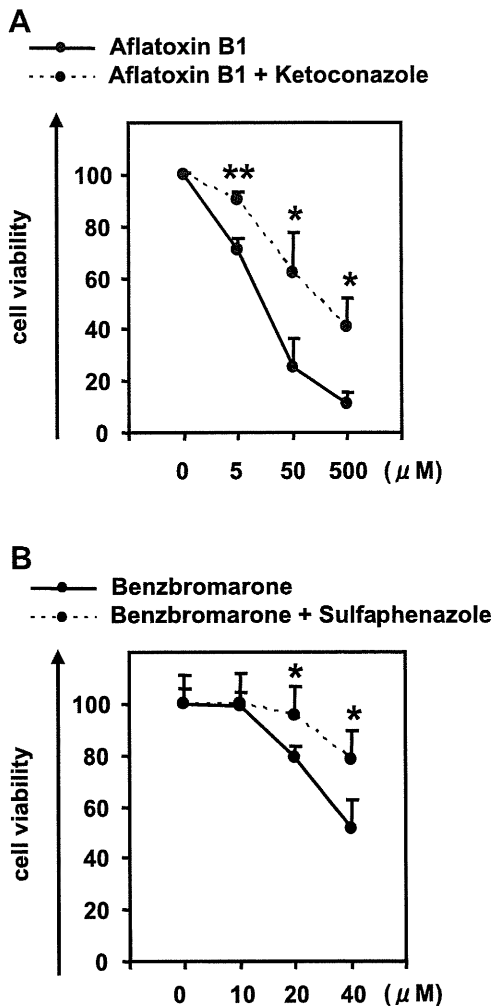


Fig. 6. Drug-induced cytotoxicity in the 3D iPS-hepa is mediated by cytochrome P450. (A, B) The cell viability of the 3D iPSC-hepa was assessed by WST-8 assay after 24 h exposure to different concentrations of (A) Aflatoxin B1 and (B) Benzbromarone in the presence or absence of the CYP3A4 or 2C9 inhibitor, Ketoconazole or Sulfaphenazole, respectively. Cell viability was expressed as the percentage of cells treated with solvent only. $^{*}P < 0.05$; $^{**}P < 0.01$.

hES/hiPS cell lines, it would be important to select an appropriate cell line for medical applications such as drug screening. However, the dominant reason for this hepatic differentiation propensity is not been well known. It would be interesting study to elucidate the mechanism of this propensity.

Although the drug metabolism capacity and CYP induction potency of 3D iPS-hepa were higher than those of mono iPS-hepa (Fig. 4B and C), they were still lower than those of primary human hepatocytes. The hepatic nuclear factors are known to be key molecules in the CYP induction of hepatocytes [30]. Therefore, overexpression of hepatic nuclear factors, which are not abundantly expressed in the hepatocyte-like cells (such as *PXR*), might upregulate the CYP induction potency of the hepatocyte-like cells.

3D iPS-hepa were more sensitive for detection of the drug-induced cytotoxicity than HepG2 cells that are widely used to predict hepatotoxicity [31,32] (Fig. 5). In addition, the decrease of cell viability, which was caused by hepatotoxic drugs, of 3D iPS-hepa was partially rescued by treatment with a CYP inhibitor (Fig. 6). These data suggest that the hepatocyte-like cells could detect the toxicity of the reactive metabolites that were generated by drug metabolizing enzymes such as CYP enzymes. Because in many cases, drug-induced hepatotoxicity is caused by the reactive metabolites produced by drug metabolizing enzymes [33], our finding that the hepatocyte-like cells could detect the toxicity of reactive metabolites should be of great potential for toxicological screening. Moreover, it might be possible to predict idiosyncratic liver toxicity by using hepatocyte-like cells generated from hiPSCs that were established from a patient with a rare CYP polymorphism. However, some compounds did not show any cytotoxicity (such as Cyclizine, Felbamate, and Sulindac) (Fig. 5). To apply the hepatocyte-like cells for wide-spread drug screening, generation of the hepatocyte-like cells are required to detect hepatotoxity in more sensitive manner. Previous studies showed that the depletion of conjugating enzymes [32] or knockdown of Nrf2 [34] expression are useful to upregulate the sensitivity to hepatotoxic drugs. Therefore, these approaches would be useful to generate more sensitive hepatocytes to toxic drugs.

5. Conclusions

In this study, we established the efficient hepatocyte differentiation method which employs not only stage-specific transient overexpression of hepatocyte-related transcription factors but also 3D spheroid culture systems by using Nanopillar Plate. To the best of our knowledge, this is the first study in which the hepatocyte-like cells, having enough hepatocyte functions, mediate drug-induced cytotoxicity against many compounds. Our hepatocyte-like cells differentiated from hESCs or hiPSCs have potential to be applied in drug toxicity testing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.11.029.

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ヒトiPS細胞研究の海外動向

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古江-楠田 美保

2006年に山中ら[1]によりマウス人工多能性幹細胞(iPS)細胞が発表され、瞬く間にその技術は世界中に広まった。ヒト胚性幹(ES)細胞は1998年にThomsonら[2]により樹立されており、日本国内でも2003年に京都大学再生医科学研究所においてヒトES細胞が樹立され[3]、ヒトES細胞の研究は開始されていた。しかし、倫理的な問題も含めた規制の厳しさによりそれほど多くの研究者がヒトES細胞を使っていたわけではなかった。しかし、2007年[4]にヒトiPS細胞作成が発表された後は、日本の幹細胞研究環境をがらりと変えた。国家戦略の一つとしてiPS細胞研究が10年間のロードマップに掲げられ、精力的に研究が進められるようになった。それは日本だけでなく、米国やEUにおいても戦略的に進められている。その進み方はあまりに早く、ここで記載する内容がもう数か月後には古くなっている可能性があるぐらい熾烈な国際競争となっている。

1—iPS細胞標準化

ヒトES/iPS細胞はこれまで研究ツールとして使用されてきたがん細胞株や不死化された線維芽細胞などの細胞とは異なる点が多い[5-7]。培養技術の差により研究室間のみならず、研究者間による結果の差も大きい。また、株間の差も大きい。さらに、長期継代を行っているうちに形質が変化する。ヒトES/iPS細胞の培養技術を短期間の実習で習得するのは難しい。ヒトES細胞研究を早くから行っていた米英においても、経験のある研究室に数ヶ月滞在したり、必要なノウハウを研究者どうしのコミュニティー内で情報交換している。ヒトES細胞の場合と異なり、ヒトiPS細胞は誰もが研究を手がけることが可能であるが、ヒトES細胞研究に関する基礎知識が培われていない国内においては培養維持に苦労している研究者も多い。

海外ではヒトES細胞を利用して研究を推進していくために、ヒトES細胞の標準化が必要であることが早くから認識され、2003年に国際幹細胞フォーラムが開催され、2005年より英国シェフィールド大学Andrews教授がリーダー(日本を含めた世界の研究者らが共同で推進[8])として推進しているInternational Human Stem Cell Initiatives (ISCI) プロジェクトが開始されている。2007年以降はヒトiPS細胞も加えられて標準化が進められている。ISCIでは、まず59株のヒトES細胞を集めて、フローサイトメトリーを用いた表面抗原の発現プロフィール、PCR-アレイを用いた未分化マーカー遺伝子発現、胚様体作成法により分化させた際の遺伝子発現、インプリンティング遺伝子、X染色体不活性化について解析が行われた[9]。

また、その研究結果を受けて各国の幹細胞バンクが参画するInternational Stem Cell Banking Initiative (ISCBI) が研究用幹細胞のバンキングの国際ガイドラインを発表し[10]、その日本語訳は京都大学再生医科学研究所・高田らにより日本再生医療学会雑誌「再生医療」に掲載されている[11]。2011年に、ISCIはヒトES細胞125株とヒトiPS細胞11株の樹立早期と長期継代後のサンプルを集め、ゲノム安定性の比較分析を実施し、ゲノムの変化などについて報告されている。しかし、ヒトES/iPS細胞の特性で最も重要である分化能についての評価が難しいことが問題となっている。マウスの場合にはgermline transmissionにより全能性が証明されるが、ヒトの場合はこれを検証することができない。そのためSCIDマウス等の免疫不全マウスに細胞を移植し、テラトーマを形成され三胚葉に分化することを確認することによって多能性が検証されている。しかし、テラトーマ形成法は、実験動物の個体差、細胞移植の技術的問題、形成されたテラトーマの組織の診断が難しい、などの問題があり、標準化が難しい。トリプシンEDTAにより分散させ継代すると分化能が失われるという事例が海外の研究者の間では語り継が

れているが、長期継代の間に形質が変化して分化能が変わる可能性も指摘されていることから、分化能については複数回の検査が求められる。欧州では動物実験の規制が厳しく、*in vitro*での再現性の高い評価法開発が望まれている[12]。これまでもヒトES細胞株のゲノム不安定については報告があり[13]、ヒトiPS細胞株についてもゲノム不安定性やそのメカニズムなどが報告されている[14]。倍加速度が早い異常クローンが出現した場合、5継代でほとんどの細胞集団が入れ替わると予測されている[15]。ヒトES/iPS細胞の品質検査として利用される未分化/分化マーカーの発現プロフィールは、培地やフィーダー細胞のロット、継代や培地交換のタイミングによっても簡単に変化する。国内では標準株を設定してほしいという要望を聞かれるが、たとえある株を標準株と設定したとしても、その形質を維持できるわけではない。仮に、細胞バンクや樹立機関から品質検査されたものを受け取ったとしても、各自が細胞の品質管理を行う必要がある。国際的には、品質評価の方法や技術の標準化が求められている[16]。

2—培地の開発状況

ヒトES/iPS細胞は、一般的に不活性化したマウス胎児組織由来線維芽細胞をフィーダー細胞（MEF）として使用し、牛血清あるいは代替血清knockout-serum replacement（KSR）と線維芽細胞増殖因子-2（FGF-2）[17]を添加した培地を用いて培養されている。フィーダー細胞とKSRを用いた培養法は多くのヒトES/iPS細胞株において安定した培養が可能であるが、動物由来成分を含むため培地間にロット差がある。さらに、培養した細胞に動物細胞表面に存在するシアル酸、Neu5Gc（N-グリコリルノイラミン酸）が確認される[18]。動物由来成分の代わりにヒト由来生物材料を用いる条件も開発されているが、創薬研究や細胞治療へ応用をめざして、病原体をできるだけ排除し、再現性ある結果や安定した品質を得るために、未知の成分を含まず、生産段階から流通経路が記録された既知の成分からなる無血清培地の使用が望まれている。国内では理解されていない場合もあるが、無血清培養とは単に血清を除いた基礎培地のみによる培養ではなく、既知の成分よりなる培地を用いたchemically defined serum-free culture[19]である。1975年にGordon H. Sato博士[20,21]が血清の役割とはそれに含まれるホルモン、増殖因子、接着因子などが細胞の増殖を促進することであり、これらの因子を基礎培地に加えることにより血清を代替できることを提言した。1979年に神経細胞培養用としてN2サプリメント[22]が開発された。その後、5因子あるいは6因子（+オレイン酸）に改良された[23,24]。その結果、神経細胞だけでなく様々な細胞の無血清培養が可能となった[25-30]。一方、1993年にPriceらによってインスリンを含む20因子から構成されているB27サプリメント[31]が開発されたが濃度が非公開となっている。現在、ヒトES/iPS細胞を培養するための培地は様々開発されており、海外では各自その研究目的により様々な条件を使用している。Thomsonらのグループにより2006年に発表されたmTeSR™1[32]とマトリジェルを使用している研究者は多い。また、N2サプリメントとB27サプリメントを合わせて使用する条件も報告されている[33]。しかし、これらの条件は動物由来成分を含むため、動物由来成分不含に改良されたTeSR™2、さらに新たに2011年に開発されたE8培地[34]の使用が試みられ始めているようである。また、StemPro®を用いている研究者も多い。著者らが開発したhESF9培地[35,36]や動物由来成分不含培地に改良したhESF-FXは必要最低限の組成からなるため、添加因子の影響が高感度に解析できる[37]。これら既知の組成からなる無血清培地は、未分化状態を維持するだけでなく、再現性高い分化誘導にも使用されている。Vallierらはアクチビン Aを添加した無血清培地CDMを未分化維持から分化過程に使用して高効率な肝細胞への分化誘導を行っている[38]。また、中辻らは無血清培地と低分子化合物によりサイトカインを使用せず心筋への分化誘導に成功している[39]。血清添加という万能に効く条件の代わりに、既知の成分を使用することにより再現性は確保されてきたが、培地は細分化してきている。それぞれの目的にあった培地を調整するカスタムサービスを行う企業も増えてきているようである。一つに決めるのではなく、その目的にあった培地が求められているのかもしれない。

3—企業の取り組み

これまでヒトES細胞の樹立は大学や研究機関で樹立されることがほとんどであった。しかし、欧米では早くから企業も取り組んでおり、欧米企業は2008年頃にはすでに創薬にiPS細胞を適用するために様々な対応を行ってきている。また、昨年末にはオクスフォード大学とファイザー、ロシュなどの製薬会社10社と23の大学が結束し、1,500株のiPS細胞株をバンキングし、今後の難病の研究と治療の開発に活用すると発表した。また、ウィスコンシン大学よりスピンアウトしたCellular Dynamics International（CDI）社もカリフォルニア再生医療機構（CIRM）から研究費を取得し、Buck Instituteからの施設をリースし、ヒトiPS細胞バンクを整備すると発表した。

4—おわりに

欧米では大学院生の研究テーマが企業との共同研究であることは珍しくなく、大学のシーズを吸い上げる機構がある。また、研究者間の交流も多いため情報の

入手も早い。iPS細胞作成という素晴らしい日本における発明が世界に先駆けて産業応用されるために、横並びで国内企業が互いに牽制しあうようなムードは撤廃し、各企業のオリジナリティーをいかしてアカデミアと連携して幹細胞実用化に向けて進むことを願ってやまない。

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生命と医療の倫理学　第2版

現代社会の倫理を考える〈第2巻〉

監修／加藤尚武、立花隆
著者／伊藤道哉
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要旨　初版刊行から10年が経ち、医療倫理を巡る情勢の激変に伴い全面改訂。とりわけ「終末期医療の決定プロセスに関するガイドライン」等、終末期医療ガイドラインの整備や、ヒトゲノム・遺伝子解析研究に関する三省指針改正、臓器の移植に関する法律の改正等、最新の状況を反映した内容に改めた。また未曾有の大震災の経験から、危機管理に関する内容を加え、医療現場スタッフの便も図った。

目次　第1章 患者の権利、医師の裁量、臨床倫理の原則、チーム医療、医療安全；第2章 告知、インフォームドコンセント；第3章 医療情報開示、個人情報保護；第4章 臨床試験、GCP、利益相反；第5章 遺伝子診断、遺伝子治療、遺伝カウンセリング、分子標的治療；第6章 クローン技術、幹細胞研究、再生医療、生殖補助医療、エンハンスメント；第7章 脳死、臓器移植；第8章 緩和ケア、QOL；第9章 終末期医療、安楽死、尊厳死、自殺幇助、生命維持治療の不開始・中止；第10章 平時と大災害の医療

著作権者：紀伊國屋書店/トーハン/日本出版販売/日外アソシエーツ

命を守る材料－人工血管から再生医療の最先端へ　東京理科大学坊っちゃん科学シリーズ〈3〉

編者／東京理科大学出版センター
著者／菊池明彦、曽我公平、牧野公子、柴建次、大塚英典
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要旨　人工血管、人工心臓、人工関節、金ナノ粒子による診断と治療、ドラッグデリバリーシステム、そして、最先端の再生医療を解説。

目次　第1章 血液に触れて使われる材料（人工血管;人工心臓 ほか）；第2章 骨などの硬い組織に用いられる材料（人工関節-金属・無機・高分子からなる複合材料；人工骨材料-セラミックスの多孔性材料）；第3章 診断に用いられる材料（ナノ粒子による蛍光バイオイメージング；金ナノ粒子による診断と治療 ほか）；第4章 ドラッグデリバリーシステム；第5章 再生医工学（組織工学の手法を用いた再生医療；生分解性材料を用いる再生医療 ほか）

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Induction of intermediate mesoderm by retinoic acid receptor signaling from differentiating mouse embryonic stem cells

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ABSTRACT Renal lineages including kidney are derived from intermediate mesoderm, which are differentiated from a subset of caudal undifferentiated mesoderm. The inductive mechanisms of mammalian intermediate mesoderm and renal lineages are still poorly understood. Mouse embryonic stem cells (mESCs) can be a good *in vitro* model to reconstitute the developmental pathway of renal lineages and to analyze the mechanisms of the sequential differentiation. We examined the effects of Activin A and retinoic acid (RA) on the induction of intermediate mesoderm from mESCs under defined, serum-free, adherent, monolayer culture conditions. We measured the expression level of intermediate mesodermal marker genes and examined the developmental potential of the differentiated cells into kidney using an *ex vivo* transplantation assay. Adding Activin A followed by RA to mESC cultures induced the expression of marker genes and proteins for intermediate mesoderm, odd-skipped related 1 (Osr1) and Wilm's Tumor 1 (Wt1). These differentiated cells integrated into laminin-positive tubular cells and Pax2-positive renal cells in cultured embryonic kidney explants. We demonstrated that intermediate mesodermal marker expression was also induced by RA receptor (RAR) agonist, but not by retinoid X receptor (RXR) agonists. Furthermore, the expression of these markers was decreased by RAR antagonists. We directed the differentiation of mESCs into intermediate mesoderm using Activin A and RA and revealed the role of RAR signaling in this differentiation. These methods and findings will improve our understanding of renal lineage development and could contribute to the regenerative medicine of kidney.

KEY WORDS: *kidney, Activin A, odd-skipped related 1, Pax 2, Wilm's tumor 1*

Introduction

Mammalian pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) (Evans and Kaufman, 1981; Martin, 1981) and induced pluripotent stem cells (iPSCs) (Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006) can differentiate into three germ layers. Number of studies demonstrated to differentiate into specific cell lineages from PSCs by cytokines and chemicals and provided useful insights into the molecular mechanisms of mammalian development *in vitro* and for future regenerative medicine. As to renal lineages, although several independent studies have been demonstrated to differentiate into these lineages from mouse ESCs (mESCs) (Bruce *et al.*, 2007; Vigneau *et al.*, 2007), directed differentiation methods into renal lineages have never been reported (Nishinakamura, 2008). Renal lineages are derived from intermediate mesoderm, which are differentiated from a subset of caudal primitive mesoderm (Dressler, 2009; Reidy and Rosenblum, 2009). To achieve directed differentiation into renal lineages, step-wise differentiation system through intermediate mesoderm is required.

We previously reported that treatment with Activin A and retinoic acid (RA) induces renal lineages from amphibian undifferentiated PSCs (Moriya *et al.*, 1993; Osafune *et al.*, 2002; Uochi and Asashima, 1996). As for the role of Activin A, it induces broad mesoendodermal derivatives from amphibian undifferentiated PSCs *in vitro* (Asashima *et al.*, 1990; Asashima *et al.*, 1991; Kaneko *et al.*,

Abbreviations used in this paper: iPSC, induced pluripotent stem cell; mESC, mouse embryonic stem cell; PSC, pluripotent stem cell; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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2008; Okabayashi and Asashima, 2003). As for the role of RA, it does not display any inducing activity in the amphibian differentiation assays, but it can modify the action of Activin A on mesoderm pattern formation in concentration- and time- dependent manners (Ariizumi and Asashima, 2001; Fukui and Asashima, 1994). In mammalian development, number of studies demonstrated that Activin-related genes have important functions in mesoderm formation and differentiation using mice deficient in these genes (Goumans and Mummery, 2000; Schier and Shen, 2000). Mice deficient in RA-related genes impair various tissue development including renal lineages (Duester, 2008). From these findings, we hypothesized that the sequential addition of Activin A and RA could induce renal lineages from mammalian PSCs.

In this study, we aimed to differentiate mESCs into renal lineages using defined culture conditions and dissect the inductive signal pathways for intermediate mesoderm differentiation. We previously developed chemically-defined, serum-free culture system for mESCs (Furue *et al.*, 2005). This culture system enables us to analyze the direct effects of various cytokines including extracellular matrix (ECM) proteins (Hayashi *et al.*, 2007), Wnt proteins (Nakanishi *et al.*, 2009), bone morphogenetic proteins (BMP) (Hayashi *et al.*, 2010b), and fibroblast growth factor (FGF) (Aihara *et al.*, 2010). In this culture system, we used Activin A and RA as the candidates of inducer to differentiate mESCs into renal lineage and examined the expression levels of the intermediate mesodermal marker genes in these cells. We characterized that these differentiated cells can integrated into developing kidney explant cultures. Furthermore, we used RAR agonists and antagonists to examine the involvement of RAR signaling in intermediate mesodermal differentiation from mESCs.

Results

Induction of intermediate mesodermal gene expression by RA combined with Activin A

We first asked whether RA together with Activin A induced intermediate mesoderm from mESCs. Undifferentiated mESCs were seeded in the defined medium supplemented with 10 ng/ml of Activin A on laminin-coated dishes (2μg/cm^2) at 0th day. From 0th to 5th day, 100 nM of RA was added in the culture medium; i.e., RA and Activin A was treated for 6, 5, 4, 3, 2, 1 days. No addition of RA was used as a control condition. Culture medium was changed every day. The differentiated cells were collected on day 6 (Fig. 1A) and assayed by quantitative RT-PCR for the gene expression of intermediate mesoderm markers, odd-skipped related 1 (Osr1) and Wilm's tumor 1 (Wt1). Osr1 is expressed in intermediate mesoderm in developing mouse embryos (Wang *et al.*, 2005). Wt1 is also specifically expressed in intermediate mesoderm mouse embryos at embryonic day (E)9.5 (Armstrong *et al.*, 1993) and has an essential role in kidney development (Kreidberg *et al.*, 1993). We found that the expression of both Osr1 and Wt1 was up-regulated in the differentiated cells treated with RA under most conditions (except for Osr1 expression with RA treatment from 5th day) compared with undifferentiated mESCs and differentiated cells without RA (Fig. 1B, C). Osr1 expression was highest in the cells treated with RA from 3rd day. Wt1 expression were comparable in the cells treated with RA from 2nd and 3rd day (Fig. 1B). Thus, we treated the cells with RA from 3rd day in the following experiments. Next, we examined the expression level of Osr1 and Wt1 in the

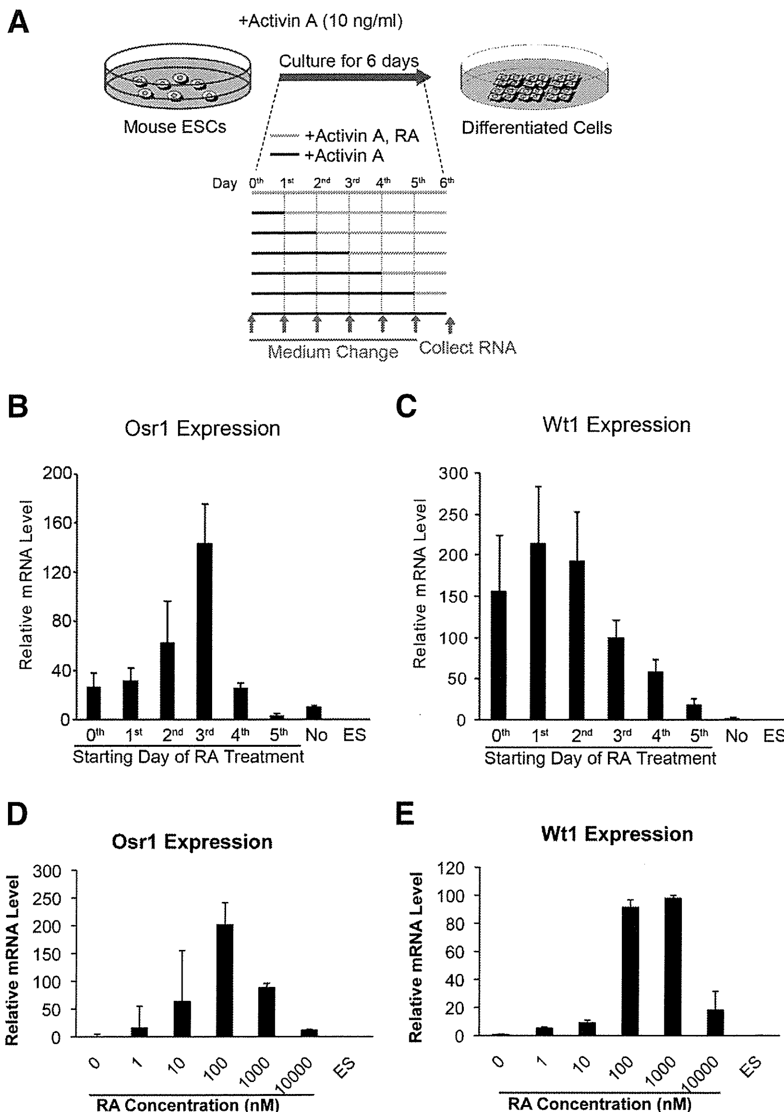

Fig. 1. Time- and concentration-dependent effects of retinoic acid (RA) on the induction of intermediate mesodermal marker gene expression in differentiated mouse embryonic stem cells (ESCs). (A) *Schematic representation of the experimental conditions.* **(B,C)** *Quantitative RT-PCR analysis of Osr1 and Wt1 mRNA levels in Activin A- and RA-induced mESCs cultured with 10ng/ml of Activin A (from 0th day) and 0.1 μM of RA (from each day as indicated in the graph) in ESF5 medium for 6 days.* **(D,E)** *Quantitative RT-PCR analysis of Osr1 and Wt1 mRNA levels in Activin A- and RA-induced mESCs cultured with 10ng/ml of Activin A (from 0th day) and various concentrations of RA (from 3rd day; concentration as indicated) in ESF5 medium for 6 days. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The relative amount of the undifferentiated mESCs (ES) is indicated as 1.0. Values are mean m SEM (n = 4).*

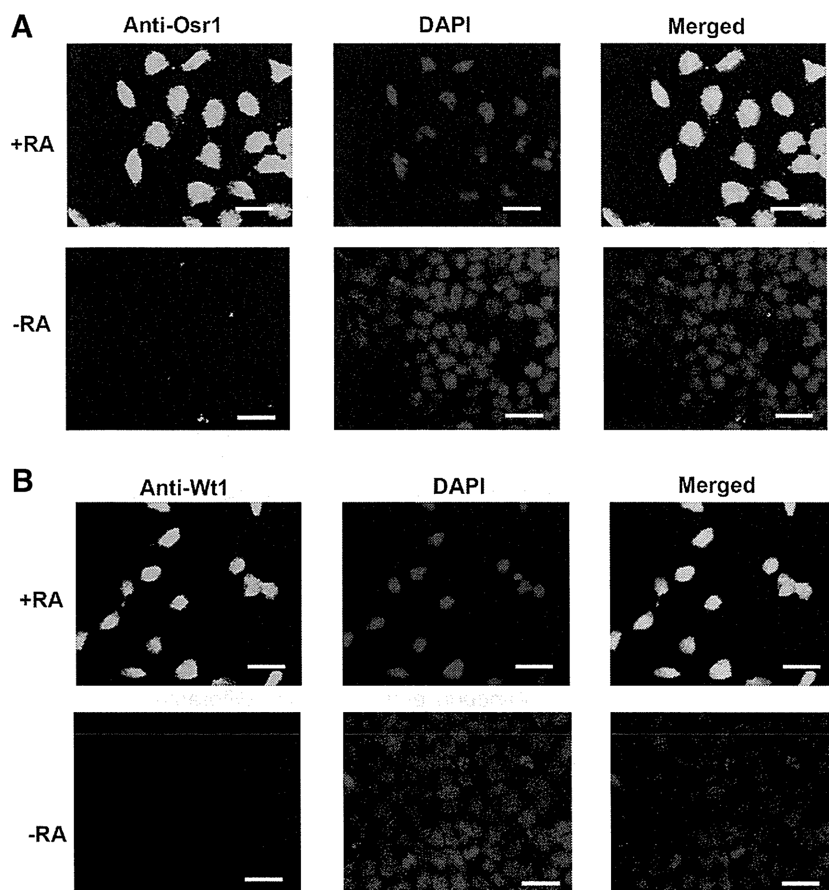

Fig. 2. Osr1 and Wt1 protein expression in differentiated mESCs treated with Activin A and retinoic acid (RA) by immunocytochemistry. *Immunostaining for Osr1 and Wt1 in cells cultured with or without 0.1 μM of RA (from 3rd day) for 6 days. Immunoreactivity of Osr1 and Wt1 was visualized with Alexafluor 488-conjugated second antibodies (green). Nuclei were stained with DAPI (blue). Scale bars indicate 40 μm.*

conditions with different RA concentrations by quantitative RT-PCR. The mRNA level of Osr1 was up-regulated dose-dependently in the RA-treated cells and was highest with 100 nM of RA treatment (Fig. 1D). The mRNA level of Wt1 was also up-regulated dose-dependently in the RA-treated cells and was highest with 100 or 1,000 nM of RA treatment (Fig. 1E). Thus, we used 100 nM of RA for all subsequent experiments. These results indicate that RA treatment of the appropriate timing and concentration increased Osr1 and Wt1 expression following Activin A treatment in differentiating mESCs.

Next, we examined the protein expression of Osr1 and Wt1 in the differentiated cells cultured for 6 days with or without RA treatment in these culture conditions by immunocytochemistry. Many RA-treated cells were positively stained with Osr1- and Wt1-specific antibodies while untreated cells were negatively stained with these antibodies (Fig. 2A, B). These results indicated that RA treatment induced the protein expression of intermediate mesoderm in the differentiating mESCs.

Next, we examined the time course of the marker gene expression of undifferentiated mESCs, epiblast, and undifferentiated mesoderm during the induction into intermediate mesoderm. We determined the expression level of Oct4 as an undifferentiated mESC marker (Okamoto *et al.*, 1990; Scholer *et al.*, 1990), Fgf5 as an epiblast marker (Rathjen *et al.*, 1999), and Brachyury (T) as an undifferentiated mesodermal marker (Wilkinson *et al.*, 1990) by quantitative RT-PCR. Oct4 expression was down-regulated from 3rd day onward, suggesting that differentiation from pluripotent state occurred in these culture conditions (Fig. 3A). Fgf5 expression was transiently up-regulated from 2nd to 4th day of induction, suggesting that the cells differentiated via epiblast state (Fig. 3B). Brachyury expression was up-regulated throughout the induction with a peak at 3rd day of induction, suggesting that the cells also differentiated via primitive mesodermal state (Fig. 3C). These results confirmed that the addition of RA following Activin A in these culture conditions induces the step-wise mesodermal gene expression program from undifferentiated mESCs.

Differentiation potential of RA-induced cells using an ex vivo transplantation system

We asked whether the differentiated cells induced by RA following Activin A had the differentiation potential for kidney cells, we transplanted the cells into developing kidney *ex vivo.* RA-induced differentiated cells transduced with green fluorescent protein (GFP) by retroviral infection were injected into developing whole kidney isolated from mouse E12.5 and cultured for 5 days. Cryosections of the transplanted kidney were then immunostained for laminin and Pax2 protein expression. Laminin is expressed in the basement membrane of tubule structures in the developing kidney (Ekblom *et al.*, 1980). Pax2 is expressed in collecting duct, metanephric mesenchyme and tubules derived from metanephric mesenchyme (Torres *et al.*, 1995). Some of the GFP-expressing transplanted cells co-expressed laminin (Fig. 4A) or Pax2 (Fig. 4B) in the cryosections of cultured kidney explants. In addition, there were no teratoma-like structures observed in the explants (data not shown), suggesting mESCs were fully differentiated from the pluripotent stage. These results indicated that the transplanted cells were successfully integrated into the tubule structures and mesenchyme of a developing kidney and that the differentiated cells treated with RA following Activin A had the differentiation potential for kidney cells.

Induction of intermediate mesoderm from mESCs by retinoic derivatives

We asked how RA induced intermediate mesoderm from differentiating mESCs. RA generally regulates cell signaling and gene expression through binding to the RA receptors (RAR) or retinoid X receptors (RXR) (Altucci *et al.*, 2007). Since RA can act as a ligand for both RAR and RXR, it is difficult to distinguish between the receptor signaling pathways. To examine which receptor signaling pathway is involved in the induction of intermediate mesoderm from mESCs, we used a pan RAR agonist, TTNPB (Afonja *et al.*, 2002; Lu *et al.*, 2008), and a pan RXR agonist, PA024 (Honda *et al.*, 2005; Ishida *et al.*, 2003; Takahashi *et al.*, 2002), to mimic the effect of RA in our culture conditions. We detected the

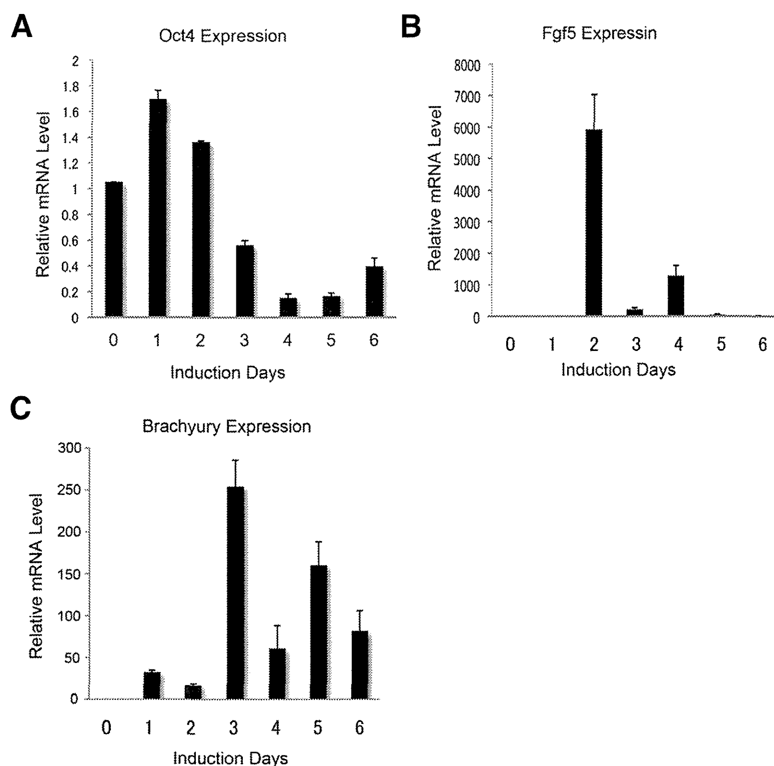

Fig. 3. The time course of differentiation marker expression in the differentiating mESCs treated with Activin A and retinoic acid (RA). *Quantitative RT-PCR analyses of mRNA levels of Oct4* **(A)***, Fgf5* **(B)***, and Brachyury* **(C)** *in the differentiated mESCs treated with Activin A and RA. Cells cultured with 10 ng/ml of Activin A (from 0th day) and 0.1 μM of RA (from 3rd day) were collected at 0th – 6th day. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The amount of the undifferentiated mESCs (0th day) is indicated as 1.0. Values are mean SEM (n = 4).*

expression level of intermediate mesodermal markers, Osr1 and Wt1, in the differentiated mESCs treated with different concentrations of TTNPB or PA024 by quantitative RT-PCR. While PA024 at any concentration had no effect on the expression of either Osr1 or Wt1, TTNPB up-regulated both of their expression level dose-dependently (Fig. 5A, B). These results suggested that RAR signaling pathway was involved in the induction of intermediate mesoderm from mESCs.

To confirm the involvement of RAR signaling pathway in the induction of intermediate mesoderm from mESCs, we used an RAR antagonist, LE135 (Umemiya *et al.*, 1997), together with RA. We detected the expression level of intermediate mesodermal markers, Osr1 and Wt1 in the differentiated mESCs treated with or without LE135. LE135 decreased their expression level induced by RA dose-dependently (Fig. 6A, B). These results indicated that RAR signaling was crucial for the RA-induced differentiation of mESCs into intermediate mesoderm.

Discussion

In this study, we demonstrated that treating undifferentiated mESCs with RA following Activin A induced intermediate mesoderm. Under these defined culture conditions, both mRNA and proteins expressions of Osr1 and Wt1 were induced by RA addition. We also showed that the differentiated cells transplanted into developing kidney *ex vivo* were integrated into tubule structures marked by Laminin and renal epithelium marked by Pax2. Thus, these results suggested that the differentiated mESCs induced by RA and Activin A can differentiate into renal lineages. Previous studies using embryoid bodies, which were formed from mESC aggregates, showed that mESCs could differentiate into renal lineages (Bruce *et al.*, 2007; Vigneau *et al.*, 2007); however, directed differentiation into renal lineage has been proven difficult to achieve due to the methods used. Although embryoid body differentiation methods are useful and convenient for differentiation into many cell types (Desbaillets *et al.*, 2000; Keller, 1995), this method allows ESCs to differentiate in a random manner influenced by cell-cell contact and cell-autonomous secreted signals in the embryoid bodies. Moreover, these culture methods usually use serum or serum replacement, which include many unknown cytokines to affect cell differentiation. These influences are minimized in our culture method using serum- and feeder-free defined monolayer culture conditions, whereby known chemicals and cytokines are used to control cell fate decisions more precisely.

We have identified that RA is crucial for the induction of intermediate mesoderm using mESCs differentiation models. Generally, RA has pleiotropic effects in mESC differentiation and mammalian development (Clagett-Dame and DeLuca, 2002; Mark *et al.*, 2006). In undifferentiated mESCs, RA inhibits their self-renewal and promotes their neurogenesis (Glaser and Brustle, 2005; Rohwedel *et al.*, 1999). On the other hand, under our culture conditions, Activin A was used to induce mesendoderm from mESCs before RA is applied (Johansson and Wiles, 1995; Kubo *et al.*, 2004), confirmed by the up-regulation of early mesodermal marker, Brachyury. Furthermore, treatment with Activin A prior to RA was needed to up-regulated the expression of Osr1 and Wt1. Thus, our results

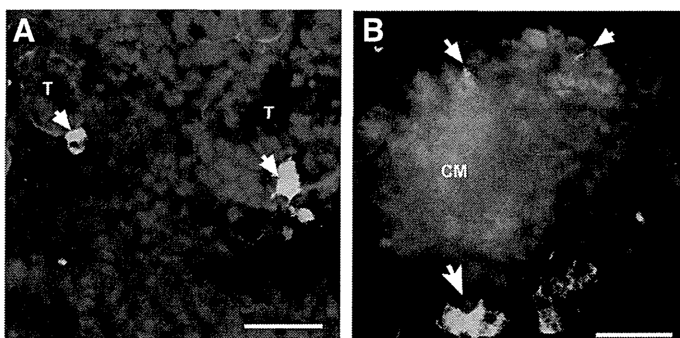

Fig. 4. The contribution of differentiated mESCS treated with Activin A and Retinoic acid (RA) to developing kidney *ex vivo*. *Immunohistchemistry for GFP, Pax2, and laminin expression in cryosections of cultured whole kidney containing GFP-positive transplanted cells. Differentiated, GFP-transduced mESCs cells cultured in ESF5 medium with 10 ng/ml Activin A (from 0th day) and 0.1 μM RA (from 3rd day) for 6 days were transplanted into cultured whole kidney collected from mouse embryos at E12.5. Immunoreactivity of GFP was visualized with Alexafluor 488-conjugated secondary antibodies (green). Immunoreactivity of laminin* **(A)** *and Pax2* **(B)** *was visualized with Alexafluor 594-conjugated second antibodies (red). Nuclei were stained with DAPI (blue). Arrows indicate cells double positive for GFP and differentiation markers. T, tubules; CM, condensed mesenchyme. Scale bars, 50 μm.*

suggested that RA directed intermediate mesoderm from primitive mesoderm. This step-wise, directed differentiation approach allows us to dissect the role of RA in mESC differentiation program and mammalian development.

We have also identified that RAR signaling pathway was involved in the differentiation into intermediate mesoderm. An RAR agonist specifically up-regulated the expression of intermediate mesodermal markers. Conversely, an RAR antagonist inhibited the RA-induced marker genes expression. These findings are consistent with the expression pattern of RAR and the phenotype of the RAR-deficient mice. RAR γ begins to express in the posterior region of the embryo at E8.0 (Ruberte *et al.*, 1990). Mice deficient in RARα and RARβ2 genes or RARγ impair renal development (Lohnes *et al.*, 1993; Mendelsohn *et al.*, 1999). Thus, our findings established an *in vitro* model to demonstrate the role of RAR signaling pathway in intermediate mesodermal induction using a mESC differentiation system. Our previous study show that RXR is involved in heart development using mESC differentiation system (Honda *et al.*, 2005), suggesting that downstream signaling pathways of RA may differ among the mesodermal tissue formation.

In conclusion, this study demonstrated that sequential addition of Activin A and RA induced intermediate mesoderm from mESCs. Together with our previous studies using amphibian pluripotent (animal cap) cells (Moriya *et al.*, 1993; Osafune *et al.*, 2002; Uochi and Asashima, 1996), our findings imply that the induction of renal lineages by Activin A and RA is conserved among vertebrates. Therefore, we also expect that these findings will be applied to human pluripotent stem cells (Takahashi *et al.*, 2007; Thomson *et al.*, 1998; Yu *et al.*, 2007). Since stem cells in the mature human mature kidney remain elusive (Yokoo *et al.*, 2008), the differentiation into renal lineages from human PSCs could be useful for the realization of regenerative medicine of the kidney and successful drug development for renal diseases.

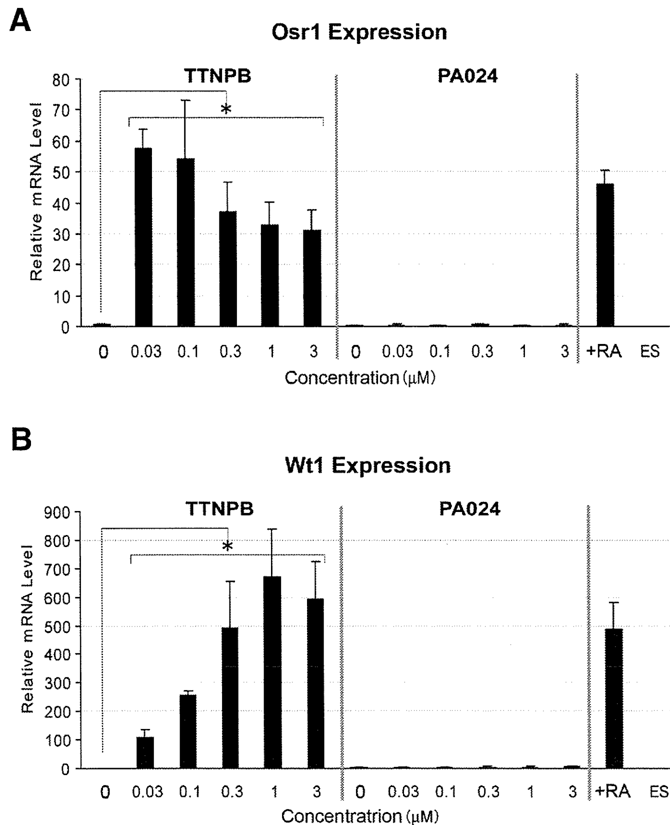

Fig. 5. The effect of retinoic acid receptor (RAR) and retinoid X receptor (RXR) agonists on intermediate mesodermal marker expression. *Quantitative RT-PCR analyses of* Osr1 **(A)** *and* Wt1 **(B)** *mRNA levels in the differentiated mESCs, treated with Activin A and retinoic derivatives. The cells were cultured with 10 ng/m Activin A (from 0th day) and various concentrations of an RAR agonist, TTNPB, or an RXR agonist, PA024 (from 3rd day) for 6 days. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The amount of differentiated mESCs without RA is indicated as 1.0. The values are mean SEM (*n *= 4).*

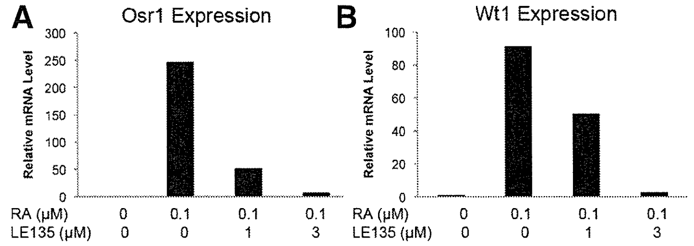

Fig. 6. The effect of a retinoic acid receptor (RAR) antagonist on intermediate mesodermal marker expression. *Quantitative RT-PCR analyses of* Osr1 **(A)** *and* Wt1 **(B)** *mRNA levels in the differentiated mESCs, treated with Activin A and RA with or without an RAR antagonist, LE135. The cells cultured with 10 ng/ml of Activin A (from 0th day) and various concentrations of LE135 with 0.1 μM of RA (from 3rd day) for 6 days. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The amount of differentiated mESCs without RA is indicated as 1.0. Values are mean.*

Materials and Methods

Cell culture

The mESC D3 (ATCC, CRL1934) line was routinely cultured as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2007). Briefly, the mESCs were cultured in 75-cm^2 plastic flasks (Corning), coated with 15 μg/ml of type I collagen (Nitta gelatin), in a humidified atmosphere of 5% CO_2 at 37°C in a defined ESF7 medium. The ESF7 consisted of ESF basal medium (Cell Science & Technology Institute) supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-ethanolamine, 20 nM sodium selenite, 9.4 μg/ml oleic acid conjugated with 2 mg/ml fatty acid-free bovine serum albumin (FAF-BSA; Sigma), and 10 ng/ml of leukemia inhibitory factor (LIF; Chemicon). For the differentiation experiments, mESCs were seeded at a density of 1×10^4 cells/cm^2 in ESF5 medium, which comprised ESF basal medium supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-ethanolamine, 20 nM sodium selenite added with 0.5 mg/ml FAF-BSA, and 10 ng/ml human recombinant Activin A (Ajinomoto pharmaceuticals), on 2 μg/cm^2 laminin-coated (Sigma) dishes. The medium was changed every day. After the indicated days of culture under differentiating conditions, retinoid derivatives, or 0.02% dimethyl sulfoxide (DMSO, Sigma) as a solvent control were added in the medium. The preparation of these chemical solutions was described previously (Honda *et al.*, 2005). Briefly, the RA (Sigma), RAR agonist (TTNPB; Sigma), RXR agonist (PA024, gifted from Dr. Kagechika (Takahashi *et al.*, 2002)), and RAR antagonist (LE135, gifted from Dr. Kagechika (Takahashi *et al.*, 2002)) were dissolved in DMSO to a stock concentration of 1 mM. The reagents were then filtered through a 0.22-μm membrane filter (Millex-LG, Millipore), and aliquots were stored at −20 °C. The concentrations of retinoid derivatives in each experiment are indicated in the Results section. Stock retinoid derivatives solution was diluted with DMSO (Sigma).

RT-PCR

RT-PCR was performed as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2007). Briefly, total RNA was extracted from the cultured cells using the total RNA extraction kit (Agilent) and reverse transcribed using Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed with SYBR Green PCR Master Mix according to the supplier's directions (Applied Biosystems) using an ABI Step One Plus sequence detector (Applied Biosystems). Relative mRNA expression of each sample was normalized against Gapdh expression. The primer sequences are as follows: Gapdh (FW: 5'-ACCCAGAAGACTGTGGATGG-3', RV: 5'- CACATTGGGGG-TAGGAACAC-3'), Brachyury (FW: 5'-TACCCCAGCCCCTATGCTCA-3', RV: 5'-GGCACTCCGAGGCTAGACCA-3'), Fgf5 (FW: 5'-GCTGTGTCT-CAGGGGATTGT-3', RV: 5'-CACTCTCGGCCTGTCTTTTC-3'), Oct4 (FW: 5'-TTCTGCGGAGGGATGGCATA-3', RV: 5'-TTTCCACTCGT-GCTCCTGCC-3'), Osr1 (FW: 5'- GACCGCGGCGGAACAAGATA-3', RV: 5'- CACTGTGGGCAGGCCATTCA-3'), Wt1 (FW: 5'-GCAACCACGGCA-CAGGGTAT-3', RV: 5'- GGGGCCACTCCAGATACACG-3'). All the results are expressed as the mean values with standard error ($n = 4$).

Immunostaining

Immunocytochemistry was performed as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2010a; Hayashi *et al.*, 2007). Briefly, mESCs were fixed in 4% (w/v) paraformaldehyde before being permealized with 0.1% Triton X-100, and then reacted with primary antibodies. The bound primary antibodies were visualized with AlexaFluor 488-conjugated anti-rabbit, anti-mouse, or anti-goat IgG or AlexaFluor 594-conjugated donkey anti-mouse, anti-rabbit, or anti-goat IgG (Invitrogen). The following primary antibodies were used: anti-Osr1 antibody (sc-68392 from Santa Cruz Biotechnology; 1:100), anti-Wt1 antibody (sc-192 from Santa Cruz Biotechnology; 1:100). anti-Pax2 antibody (covance; 10 μg/ml), anti-laminin antibody (Sigma; 8 μg/ml), and anti-GFP antibody (Abcam; 1:200).

***Transplantation of differentiated mESCs into developing kidney* ex vivo**

The developing kidneys of E12.5 embryos were collected from pregnant mice (C57BL/6JJcl, CLEA Japan) and cultured in DMEM + 10% FCS on the culture filter. Differentiating mESCs were infected with retrovirus (concentrated) carrying GFP on 5th day of induction. Retrovirus was concentrated from the culture medium of Plat-E cells (Cell Biolab) transfected with pMXs-GFP for 48 hours. At 6th day of induction, GFP-transduced, differentiated mESCs (10,000 cells / explant) were injected into cultured whole kidney by microinjection using mouth pipettes. After 5 days of *ex vivo* culture, the cultured kidney were fixed with 4% PFA in 4°C for 30 min. Fixed samples were cryopreserved in OCT compound using the sucrose substitution method. Cryopreserved sections were cut at 13 μm using a cryostat-microtome and used for immunostaining.

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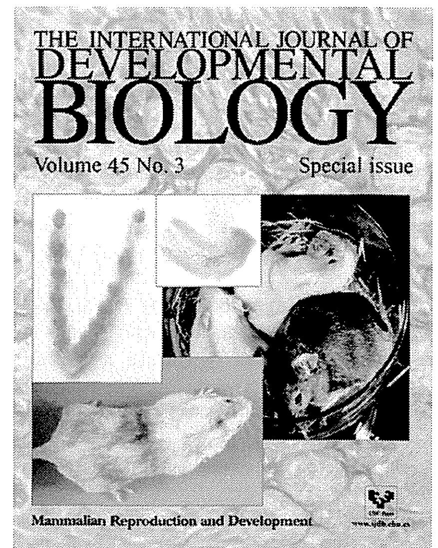

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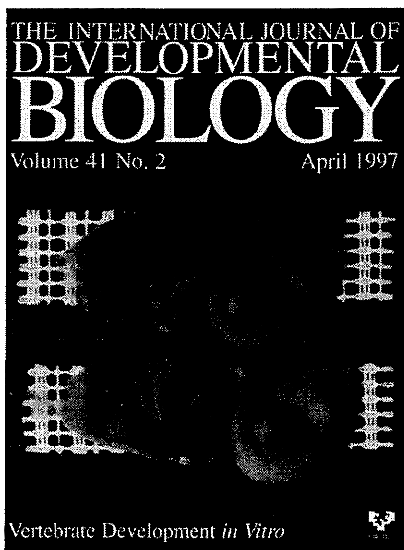

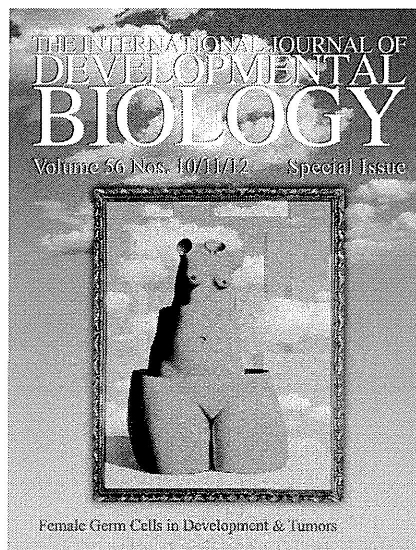

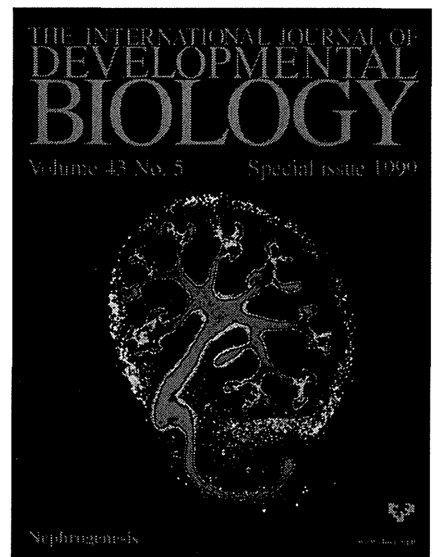

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HHEX Promotes Hepatic-Lineage Specification through the Negative Regulation of Eomesodermin

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Abstract

Human embryonic stem cells (hESCs) could provide a major window into human developmental biology, because the differentiation methods from hESCs mimic human embryogenesis. We previously reported that the overexpression of hematopoietically expressed homeobox (HHEX) in the hESC-derived definitive endoderm (DE) cells markedly promotes hepatic specification. However, it remains unclear how HHEX functions in this process. To reveal the molecular mechanisms of hepatic specification by HHEX, we tried to identify the genes directly targeted by HHEX. We found that HHEX knockdown considerably enhanced the expression level of eomesodermin (EOMES). In addition, HHEX bound to the HHEX response element located in the first intron of EOMES. Loss-of-function assays of EOMES showed that the gene expression levels of hepatoblast markers were significantly upregulated, suggesting that EOMES has a negative role in hepatic specification from the DE cells. Furthermore, EOMES exerts its effects downstream of HHEX in hepatic specification from the DE cells. In conclusion, the present results suggest that HHEX promotes hepatic specification by repressing EOMES expression.

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Introduction

The molecular mechanisms of liver development have been clarified by using model organisms such as chicks, *Xenopus*, *zebrafish*, and mice [1–2]. Although these models have many advantages, the molecular mechanisms of human liver development might be different from those of model organisms. The use of differentiation models from human embryonic stem cells (hESCs) for studying human development might resolve these problems, because these differentiation methods mimic human embryogenesis [3]. Previous reports have demonstrated that the definitive endoderm (DE) cells could be efficiently generated from hESCs in the presence of Activin A [4], and that the hESC-derived DE cells have the potential to differentiate into various DE-derived lineages, such as hepatocytes, pancreatic beta-cells, and small intestinal enterocytes[5–7]. In hepatic differentiation, Agarwal et al. reported that the typical gene expression profiles observed in the differentiation model from hESCs are similar to those observed in fetal liver development [8]. In addition, we previously reported that CCAAT/enhancer binding protein-mediated regulation of TGF beta receptor 2 expression determines the hepatoblast fate decision by using a differentiation model from hESCs [9]. The use of differentiation models from hESCs, rather than the usual model organisms, would provide great opportunities to expand our understanding of the molecular mechanisms.

A transcription factor, *hematopoietically expressed homeobox* (HHEX), is initially expressed in DE, and then its expression is restricted to the future hepatoblasts, which could segregate into both hepatocytes and cholangiocytes [10]. In the *HHEX*-null embryo, some hepatic gene expression levels are reduced and further hepatic development is prevented [11–12]. These studies indicate that the transcription factor HHEX plays an essential role in hepatic specification from DE. Recently, we reported that overexpression of HHEX by using adenovirus (Ad) vectors in the hESC-derived DE cells markedly promotes the hepatic specification [13]. Moreover, Kubo et al. demonstrated that HHEX promotes this process by synergistically working with bone morphogenetic protein 4 (BMP4), and they expected that HHEX might function with *HNF1 homeobox A* (HNF1α) [14], which is known to be its co-activator [15]. However, the functions of HHEX in this process

are not well understood, and the target genes of HHEX have not been investigated in detail. Therefore, we attempted to identify the target genes of HHEX in the hepatic specification by using a differentiation model from hESCs.

In the present study, to elucidate the functions of HHEX in hepatic specification from DE, we attempted to identify the target genes of HHEX by using the hepatic differentiation model from hESCs. To this end, the candidate target gene of HHEX were verified by performing ChIP-qPCR and luciferase reporter assays, and then loss-of-function assays were performed to clarify the functions of the candidate target gene in the hepatic specification. These results confirmed that *eomesodermin* (EOMES), which is known to regulate DE differentiation, is one of the crucial target genes of HHEX in human hepatic specification from the DE. Our report thus shows for the first time that HHEX promotes hepatic specification through the repression of EOMES expression.

Materials and Methods

hESCs Culture

A hESC line, H9 (WA09, WISC Bank, WiCell Research Institute), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (MEF) (Millipore) with ReproStem medium (ReproCELL) supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (KATAYAMA CHEMICAL INDUSTRIES). hESCs were dissociated with 0.1 mg/ml dispase (Roche) into small clumps and then were subcultured every 4 or 5 days. H9 was used following the Guidelines for Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan after approval by the institutional ethical review board at National Institute of Biomedical Innovation.

In vitro Differentiation

The differentiation protocol for the induction of DE cells and hepatoblasts was based on our previous report with some modifications [13–16–21]. Briefly, hESCs were dissociated by using dispase and suspended in MEF-conditioned ReproStem medium supplemented with 10 ng/ml FGF2, and then plated onto a growth factor reduced Matrigel (BD Biosciences)-coated dish. When hESCs reached approximately 80% confluence, the MEF-conditioned ReproStem medium was replaced with the differentiation RPMI-1640 medium (Sigma) containing 100 ng/ml Activin A (R&D systems) (the differentiation RPMI-1640 medium is consisted with RPMI-1640 medium (Sigma) supplemented with B27 supplement (Invitrogen) and 4 mM L-glutamine), and then cultured for 4 days. For induction of the hepatoblasts, the DE cells were cultured for 5 days in the differentiation RPMI-1640 medium supplemented with 20 ng/ml BMP4 (R&D Systems) and 20 ng/ml FGF4 (R&D Systems).

RNA Isolation and Reverse Transcription-PCR

Total RNA was isolated from hESCs and their derivatives using ISOGENE (Nippon Gene). cDNA was synthesized using 500 ng of total RNA with a SuperScript VILO cDNA Synthesis Kit (Invitrogen). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using an Applied Biosystems StemOnePlus real-time PCR systems. 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 this study are described in **Table S1 in File S2**.

Flow Cytometry

Single-cell suspensions of the hESC derivatives were fixed with 2% paraformaldehyde (PFA) at 4°C for 20 minutes and then incubated with the primary antibody, followed by the secondary antibody. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences). All the antibodies are listed in **Table S2 in File S2**.

ChIP-qPCR

ChIP assays were performed by using a Chromatin Immunoprecipitation Assay Kit (Millipore) according to the manufacturer's instructions. The hESC-derived cells (approximately 1.0×10^6 cells) were cross-linked with 1% formaldehyde at room temperature for 10 minutes. The cells were washed once with PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 1 mg/ml pepstatin A) and then harvested using a cell scraper. The cross-linked cells were centrifuged and resuspended with sodium dodecyl sulfate (SDS) lysis buffer with the protease inhibitors described above, and then incubated on ice for 10 minutes. The cells were sonicated to solubilize and shear cross-linked DNA. The resulting whole cells were centrifuged, and the supernatants were diluted in ChIP Dilution Buffer containing the protease inhibitors described above, then added to Protein A magnetic beads and rotated at 4°C for 30 minutes. Next, the supernatants of these cells were immunoprecipitated with anti-human HHEX antibody (Santa Cruz Biotechnology, sc-15129) or anti-goat IgG antibody at 4°C overnight with rotation. On the following day, the resulting supernatants were added to Protein A magnetic beads and rotated at 4°C for 60 minutes, then washed five times with Low Salt Immune Complex Wash Buffer (one time), High Salt Immune Complex Wash Buffer (one time), LiCl Immune Complex Wash Buffer (one time), and TE Buffer (two times) for 5 minutes per wash with rotation. Bound complexes were added to elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature for 15 minutes with rotation, and then the supernatants were added to 5 M NaCl and were eluted at 65°C for 4 hours. Immunoprecipitated DNA was purified by treatment with 0.5 M EDTA, 1 M Tris-HCl, and 10 mg/ml proteinase K at 45°C for 60 minutes and recovered by phenol/chloroform alcohol extraction and ethanol precipitation. Purified DNA was used as a template for qPCR according to the protocol described in the *RNA isolation and reverse transcription-PCR* section above. All the antibodies are listed in **Table S2 in File S2**. The primer sequences used in this study are described in **Table S1 in File S2**.

Plasmid Constructions

The promoter region of EOMES was cloned. To generate the 5′ untranslated region (UTR) of the EOMES-firefly luciferase reporter construct (pGL3-EOM-5UTR1000), a 1,000 bp 5′ UTR of the human EOMES was amplified by using the following primers: 5′-AGCGGTACCTTCCTCTCTACAAACCTTTCC-CACTGGG-3′ and 5′-TAACCATGGGCTTTGCAAAGCG-CAGACGGCAGCTGGCTGC-3′ (−1,000/−1 5′ UTR of EOMES; KpnI and NcoI restriction sites incorporated into sense and antisense primers, respectively, are underlined) and to generate the long 5′ UTR of the EOMES-firefly luciferase reporter construct (pGL3-EOM-5UTR4000), a 4,000 bp 5′ UTR of the human EOMES was amplified by using the following primers: 5′-CAGGGTACCGATAACACGTTTT-TAGTGGGGGTG-3′ and 5′-TAACCATGGGCTTTG-CAAAGCGCAGACGGCAGCTGGCTGC-3′ (−4,000/−1−5′ UTR of EOMES; KpnI and NcoI restriction sites incorporated into sense and antisense primers, respectively, are underlined).