移植法を開発し，角膜上皮疾患に対する臨床研究を実施してきた。その結果，一定の安全性および有効性が確認された[6,7]。しかし，術後視力が0.1以下にとどまる症例が多く，長期成績には限界があることが判明した。これは，移植した口腔粘膜が完全には角膜上皮化していないことに起因する。

一方，この数年iPS細胞を用いた角膜再生治療法（角膜上皮および実質の自家再生治療法，角膜内皮の他家再生治療法）の開発研究が行われてきたが，有望な結果が得られつつある。すなわち，iPS細胞から重層上皮前駆細胞，角膜上皮細胞への分化誘導[8]，純化したiPS由来上皮細胞シートの作製に成功したという。同様に，iPS細胞から角膜内皮の発生起源である神経堤細胞へ高効率に誘導する新規培養系の創出と，独自に開発した角膜内皮分化誘導法を用いて，角膜内皮様細胞への分化誘導にも成功したという。角膜内皮再生については，既に培養角膜内皮細胞を動物眼に移植することに成功している。さらに，慶應義塾大学の榛村重人博士らは角膜実質に存在する体性幹細胞の単離[9]と角膜内皮様細胞への分化誘導にも成功している。

このように，ヒトiPS細胞から角膜細胞を創出するための基本技術が整備されてきたことから，開発研究をいっそう加速させ，臨床研究（first-in-human）を7年以内に開始することを目指している。

3．ヒトiPS細胞由来ドパミン神経前駆細胞の移植によるパーキンソン病治療

パーキンソン病は厚生労働省の特定疾患に指定された進行性神経変性疾患で，ドパミン神経細胞の脱落により運動機能の低下を呈する神経難病である。わが国に約14万人の患者が存在する（2008年の統計）。

パーキンソン病に対しては主にL-ドパ（ドパミン前駆物質）を治療薬とする薬物治療が基本であるが，病気の進行とともにドパミンの合成にあたるドパミン神経細胞が枯渇し，薬の効果が十分発揮できなくなる。根本治療は十分な量のドパミン神経細胞の存在であるが，薬物療法においてもドパミン神経細胞の補充があれば相補的な効果が期待できる。

既に欧米では1980年代末から400例もの胎児中脳細胞移植が行われており，L-ドパに反応しないような重症例を除けば二重盲験試験[10]でもその有効性が示されている。すなわち，パーキンソン病に対する細胞移植治療というproof of conceptが確立している。

一方で，胎児を利用する方法には多くの問題点も指摘されている。倫理的な問題以外に，(1)1回の治療に5～10体の胎児が必要であるという量的問題，(2)術後に胎児中脳組織内に含まれるセロトニン神経細胞が関与していると考えられる不随意運動（ジスキネジア）がみられる症例があること，(3)他家移植であるために免疫抑制が必要ということなどである。

これらを解決するには，ヒトiPS細胞からドパミン神経細胞に相当する細胞を作製・純化して治療に供するという方策が考えられる。まずは患者由来自己細胞から，将来的にはヒト白血球型抗原適合正常人由来iPS細胞を用いた移植治療への移行が考えられる。

京都大学の高橋　淳博士らは，世界に先駆けて，カニクイザルES細胞から誘導したドパミン神経細胞移植によってカニクイザルパーキンソン病モデルの行動改善が得られることを報告した[11]。その後，ヒトES細胞からもドパミン神経細胞の誘導に成功し，カニクイザルモデルへの移植を行い，分化程度が進んだ移植細胞では腫瘍性増殖をきたすことなくサルモデルの行動改善が得られることを明らかにした。さらに，ヒトiPS細胞からもドパミン神経細胞を誘導し，サルモデル脳にドパミン神経細胞として生着していることを明らかにした。

術後の問題であったジスキネジアを回避するためには，目的細胞の純化が重要となるが，高橋らのグループは表面マーカーを用いたセルソーティングにより[12]世界で初めてヒトES，iPS細胞由来のドパミン神経前駆細胞を選別することに成功したとのことである。この選別されたドパミン神経前駆細胞は以下の性質を持つ：(1)高率にドパミン神経細胞を産生する，(2)増殖率ほぼゼロで，腫瘍性増殖の可能性が低い，(3)胎児細胞移植に用いられるヒト胎児中脳腹側組織と比べてドパミン神経マーカーの発現は同等であるが，セロトニン神経マーカーの発現も極めて低く，移植後のジスキネジアの発生を回避し得る。

以上のような研究基盤を背景に，わが国発の技術で世界のパーキンソン病治療に大きく貢献するための細胞移植治療が目指されている。その戦略を再度要約すると，(1)孤発性パーキンソン病患者の皮膚線維芽細胞からiPS細胞を樹立し，(2)浮遊培養系で神経誘導を行い，(3)ドパミン神経前駆細胞を選別し，(4)選別したドパミン神経前駆細胞の有効性と安全性をカニクイザル，ラットの疾患モデル，ならびに免疫不全マウスへの移植で検証し，(5)両側線条体に移植（局所麻酔下の定位的脳手術）するというもので，目的の達成を期待したい。本分野の詳細については本特集の森実飛鳥氏のレビューを参照されたい。

4．ヒトiPS細胞を用いた脊髄再生医療

わが国の脊髄損傷患者の数は50万人に近いという。抗痙剤，人工呼吸器，脊椎固定術などの医療の向上に伴い生命予後は改善されたが，脊髄損傷による麻痺とそれに伴う深刻な合併症は依然として患者を苦しめている。中枢神経系の再生を可能とし，脊髄損傷に対する革新的な治療法の開発が望まれる所以である。

米国では2010年10月にヒトES細胞を分化させた「オリゴデンドロサイト前駆細胞」を患者の損傷部位に注入して神経再生を促す治療法を利用する臨床試験がFDAの許可を得て開始された。わが国では，慶應義塾大学の岡野栄之教授らのグループを中心に，世界に先駆けて損傷脊髄に対する胎児由来神経幹細胞，ES細胞由来神経幹細胞移植の有効性が報告されている[13-15]。しかし，わが国における倫理的問題のため臨床応用は現在まで困難な状況である。最近，サルおよびヒトiPS細胞由来神経幹細胞についてマウスやサル脊髄損傷モデル動物を用いた安全性・有効性の検討が行われているが，ヒト臨床用に適切なヒトiPS細胞や細胞バンクの樹立，製品としての神経幹細胞への分化誘導，安全性の検討も含め，確認には時間が必要であるとのことである。なお，本分野の詳細については本特集海苔　聡氏のレビューを参照されたい。

5．iPS細胞を用いた再生心筋細胞移植による重症心不全治療法

従来の薬物療法やペースメーカ治療に不応性な難治性重症心不全は，補助人工心臓や心臓移植によらざるを得ない。しかし心臓移植は本邦はもとより世界中でドナー不足の状態にあり，ドナー心の恩恵を受けられる患者は極めて限られている。そこで，ヒト心筋細胞移植法の開発が待望されている。わが国では大阪大学の澤　芳樹教授らが重症心不全例への筋芽細胞シート移植による臨床研究を進めており一定の成果を収めている。

一方，慶應義塾大学の福田恵一教授らのグループは，(1)末梢血中の免疫細胞［T細胞[16]や樹状細胞］からiPS細胞を樹立，(2)iPS細胞をXeno　freeで培養するためiPS細胞自身を分化させ，フィーダー細胞とする方法の開発[17]，(3)iPS細胞から心筋細胞を分化誘導し，成熟させる方法の開発[18]，(4)心筋細胞を未分化幹細胞から分離し，純化する方法の開発[19]，(5)移植細胞の効率的な生着法や移植法の開発[20]などを基盤に，究極的には患者由来の安全性の高いiPS細胞を樹立し，これを大量培養した後に純化精製し，大量の心筋細胞（1×10^7個から1×10^8個）を心不全の患者の心臓に移植するべく研究を展開しようとしている。

Ⅱ．ヒトiPS由来細胞製品の臨床適用に関する規制環境の整備

わが国発の技術開発であるヒトiPS細胞を素材とした製品の再生医療における実用化を図るためには製品の品質や安全性の確保などとともに患者を用いた臨床試験が実施される必要がある。現在のところ，ヒトiPS細胞由来製品を用いた再生医療には2つのアプローチがある。

1つは薬事法下における製品の製造販売承認を目指したものである。すなわち，企業による研究開発，治験に入る前にヒトに適用する（first-in-human）に際して支障がないという評価，そして「治験」，「製造販売承認」，「臨床利用」という段階を踏むアプローチである。2つ目は，医師法下で行われる「ヒト幹細胞臨床研究」である。「ヒト幹細胞臨床研究」の実施の是非は，厚生労働省厚生科学審議会の議を経て，大臣から意見を聴くこととなっている。

ヒト幹細胞臨床研究を進めていく過程において，有効性および安全性の観点から公的保険制度における評価療養に該当すると評価された場合には「高度／先進医療」として公的医療給付の対象となる。このヒト幹細胞臨床研究が，さらにはシームレスに企業による製品開発につながることも期待されている。これに対し行政当局が開発早期から臨床使用に至るまでの必要な要件を示すことは，医学研究者や企業が研究・開発を合理的，効率的，効果的に進め，より迅速に実用化するために必須である。

また，規制側としても，近い将来に予想される製品の評価を円滑に進めるために審査上の留意点に関する共通の理解を深め，対応できる準備を早期に行う必要がある。さらに，国際競争面でも研究・技術開発のみならずガイドライン策定において先行することは，国際的優位性を保持するうえでも不可欠な要素である。

これを踏まえ，薬事法下ではいち早く平成12年（2000年）12月26日医薬発第1314号「ヒト又は動物由来成分を原料として製造される医薬品等の品質及び安全性確保について」において基本的考え方（別添1）や基本的技術要件（別添2）が示された。第1314号別添2については，その後の学問・技術の進歩，国際動向を踏まえて，平成18，19年（2006，2007年）度の厚生労働科学研究班（研究代表者：早川堯夫）により改訂作業が実施され，この成果をもとに，平成20年（2008年）2月に「ヒト（自己）由来細胞・組織加工医薬品等の品質及び安全性の確

保に関する指針(薬食発第0208003号)」および平成20年(2008年)9月に「ヒト(同種)由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針(薬食発第0912006号)」がそれぞれ通知された。

その後さらに,平成20～22年(2008～2010年)度の厚生労働科学研究班(研究代表者:早川堯夫)によりヒト体性幹細胞,ヒトES細胞,ヒトiPS細胞にそれぞれ由来する製品に特化した研究が行われ,その成果が5つの指針案として提示された[21]。そのうちの2つが「自己および同種由来のヒトiPS(様)細胞加工医薬品等の品質及び安全性の確保」に関するものである。これらの指針が目指すのは,患者益,国民益に資し,実用化の水先案内,牽引力,推進力としての役割である[22]。

臨床研究に関しては平成18年(2006年)7月3日付け厚生労働省告示　第425号「ヒト幹細胞を用いる臨床研究に関する指針」が臨床研究推進の一翼を担っていたが,最近その改訂版が平成22年(2010年)11月に出された(厚生労働省告示　第380号)。この科学的内容は,おおよそ医薬発第1314号および上記の薬事上の指針および指針(案)と同様のものとなっている。

Ⅲ. ヒトiPS細胞由来細胞を用いた創薬研究

創薬のプロセスは,一般的に開発費に1,000億円超,期間に10～15年を要する。その過程で約2万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るための新しい技術の1つとして,iPS細胞技術が注目されている。

医薬品開発段階における創薬研究としては,上流からさかのぼると,(1)疾患のメカニズム解明や創薬ターゲット分子の検索,(2)スクリーニング系の構築と化合物スクリーニング,(3)化合物の最適化や薬効評価試験・安全性薬理試験・毒性試験・薬物動態試験,(4)製造法の最適化(確立)や品質管理試験などのCMC(chemistry, manufacturing and control)試験,(5)臨床試験,と続く。iPS細胞を用いた創薬研究は,大きく分けて特定の疾患を反映した疾患由来のiPS細胞(疾患iPS細胞)を用いた研究と,健常人由来のiPS細胞を用いた研究に分けられるが,疾患iPS細胞は上記(1)(2)の研究段階に,健常人由来のiPS細胞は(1)(2)(3)の研究段階に利用可能と期待されている。

なお,iPS細胞自身がこれらの研究段階に利用されることは少なく,iPS細胞から特定の細胞に分化させた細胞が創薬研究には利用される。したがって,iPS細胞が創薬研究に利用できるか否か(あるいはどのような創薬研究に利用できるか)は,iPS細胞から分化させた細胞の"分化度"に大きく依存しており,未熟な分化細胞では実際のヒトにおける病態や状態を反映していないことが多く,利用できないことになる。

Ⅳ. 疾患iPS細胞を用いた創薬研究

患者生検組織から得た初代培養細胞を使用して作製された疾患iPS細胞は,疾患を引き起こす原因となる変異をゲノムに有しており,疾患標的組織細胞に分化させることで,疾患の表現型を再現できる可能性がある。この表現系や分化過程などを解析することで,その疾患の発症メカニズムの解明や,新たな創薬ターゲットを見出す研究への利用が期待されている。これまで,脊髄性筋萎縮症(spinal muscular atrophy:SMA)や家族性自律神経失調症,α1アンチトリプシン欠損症をはじめとする多くの疾患iPS細胞が作製されている(詳細は文献23を参照)。

疾患iPS細胞は,これまで解析が困難であった難治性疾患に対する新しいアプローチからの創薬研究を可能にした点で期待が大きい。課題としては,疾患iPS細胞を標的細胞に分化させても,必ずしも培養系では疾患(表現系)を再現できない場合があることが挙げられる。また,そもそも,iPS細胞の性質はクローンごとに比較的大きく異なっており,iPS細胞のクローンによる性質の違いや,クローンによる細胞の分化度の違いと,疾患によるiPS細胞(由来分化細胞)の表現系(疾患を反映した機能不全など)の違いを,区別して見極めることが必要である。

Ⅴ. 健常人由来のiPS細胞を用いた創薬研究

健常ヒトiPS細胞から分化させた細胞(特に,心筋,肝臓,神経細胞など)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・薬物動態試験など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,多くは株化細胞や初代培養(ヒト)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)をどの程度反映しているかに関して課題があり,一方で初代培養ヒト細胞は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(神経細胞では,そもそも

代培養ヒト細胞を得ることさえ困難である)。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。健常ヒトiPS細胞由来分化誘導細胞は，これらの問題点を克服できることが期待されることから，大きな注目を集めている。

以下，心筋，肝臓，神経細胞を例に，健常ヒトiPS細胞由来分化細胞を用いた創薬研究や技術開発研究の現状について解説する。また，これらの分化細胞を用いた研究は，各分化細胞への細胞の“分化度”が試験系の精度に大きく影響することから，効率のよい分化誘導法の開発が最も重要であり，分化誘導技術開発の現状についても述べる。なお，健常人由来のiPS細胞を用いた創薬研究は，ヒトES細胞を用いた同様の研究が先行しており，以下ではヒトES細胞とiPS細胞の両者を用いた研究について紹介する。

1．ヒトiPS細胞由来心筋細胞を用いた創薬研究

ヒトES細胞やiPS細胞から分化させた細胞の応用としては，研究・開発が最も進んでおり，特に薬物誘発性QT延長アッセイ系はリプロセル社（http://www.reprocell.com/），ChanTest社（http://www.chantest.com/），Cellular Dynamics International社（http://www.cellulardynamics.com/）により既に実用化されている。

薬物誘発性QT延長とは，心室筋の活動電位持続時間に相当する心電図のQT間隔が延長することを特徴とし，重篤な副作用を起こす原因となる。QT延長の主な原因としては，薬剤がK＋チャネルの形成サブユニットであるhERG（human *ether-a-go-go* related gene）チャネルの機能を阻害することであることが明らかとなっている。日米EU医薬品規制調和国際会議（International Conference on Harmonisation：ICH）において制定された安全性薬理試験ガイドラインにおいては，医薬品候補化合物の催不整脈作用，特にQT間隔延長作用の有無を検討することが求められており，hERG遺伝子を導入しhERG K＋チャネルを発現させたHEK293細胞やCHO細胞などを用いて，化合物のhERG K＋チャネルに対する機能抑制作用を調べる試験が安全性薬理試験として推奨されている。

リプロセル社が開発したQT延長試験（QTempo）は，ヒトiPS細胞由来の拍動心筋細胞を用いて心電図のQT間隔に相当する波形を無侵襲の電気生理学的な手法を用いて測定するcell-based QT延長試験法であり，QT延長だけでなく，拍動数の変化，K＋イオン以外の複数イオンチャネルへの影響も観察できることを特徴としている。hERG遺伝子を導入した細胞株を用いた試験と比較し，拍動心筋細胞を用いていることから，多種多様なイオンチャネルを発現していることなど，より正確な薬物誘発性QT延長試験が期待できる。

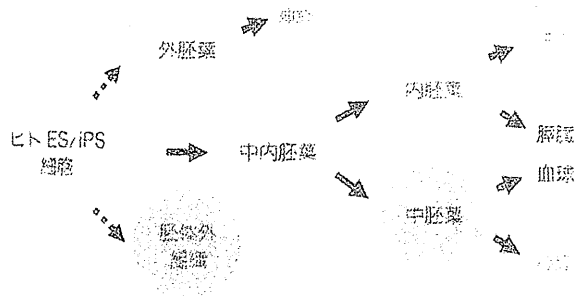

Fig. 3　ヒトES/iPS細胞から心臓，肝臓，神経細胞への分化誘導
ヒトES/iPS細胞から心臓（心筋細胞），肝臓（肝細胞），神経細胞への分化は，それぞれ中胚葉，内胚葉，外胚葉を通して起こる。

心筋細胞は，ヒトES/iPS細胞から中胚葉を経由して分化誘導される（Fig. 3）。ヒトES/iPS細胞から心筋細胞への分化誘導技術開発は，神経細胞と並んで比較的進んでいるが，ヒトES/iPS細胞をNogginとG-CSF（granulocyte-colony stimulating factor）を添加して培養すると心筋細胞へ効率よく分化することが知られている。ヒトES細胞由来心筋細胞はCellartis社（http://www.cellartis.com/）から，ヒトiPS細胞由来心筋細胞はiPSアカデミアジャパン社（http://www.ips-cell.net）から発売されており（Cellular Dynamics International社は製品化した細胞を販売），リプロセル社もヒトiPS細胞由来心筋細胞を発売している。

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，解毒などに関与する主要な臓器（細胞）であり，[illegible]に肝細胞で薬物代謝酵素により代謝され，[illegible]より解毒を受け，トランスポーターにより[illegible]肝毒性は医薬品候補化合物の開発中止[illegible]であり，正常肝細胞を用いて将来起こ[illegible]毒性発現を研究開発の初期段階に予[illegible]全性の高い医薬品を効率よく開発す[illegible]考えられる。

現在は，主に初代培養ヒト肝[illegible]用いて，薬剤あるいは反応性代[illegible]素により代謝された代謝[illegible]

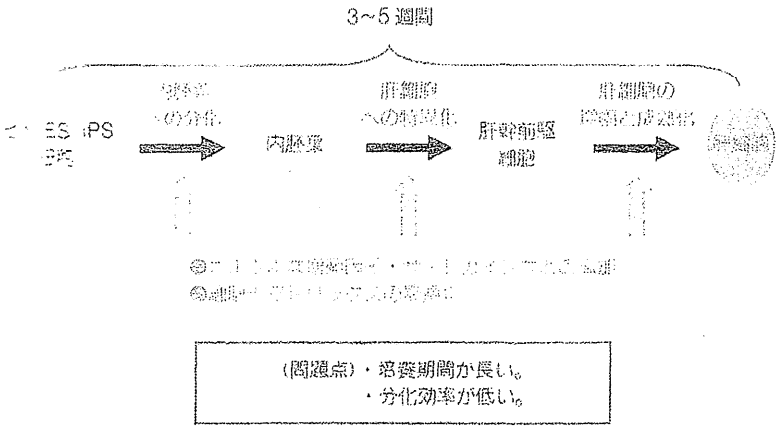

Fig. 4　ヒトES/iPS細胞から肝細胞への代表的な分化誘導法
ヒトES/iPS細胞から肝細胞への分化誘導は，内胚葉，肝幹前駆細胞を通して起こる。これらの各段階で，さまざまな増殖因子やサイトカインなどの添加，および細胞外マトリックスの最適化がなされる。しかしながら，培養期間が長いこと，分化効率が低いことが課題となっている。

験する毒性試験や，薬物代謝酵素の誘導や阻害などの薬物動態評価試験が施行されている。しかしながら，コストや高機能なヒト肝細胞ロットの安定供給の問題などから，ヒトES/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，将来的には，ヒトES/iPS細胞由来分化誘導肝細胞を用いた場合，個人差を反映した評価系が開発できる可能性もある。さらに，ヒトES/iPS細胞由来分化誘導肝細胞は肝炎ウイルス（B型肝炎やC型肝炎ウイルス）研究にも有用であり，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にも応用できる。

肝細胞は，ヒトES/iPS細胞から中内胚葉，内胚葉，肝幹前駆細胞などを経由して分化誘導される（Fig. 3，4）。ヒトES/iPS細胞から肝細胞への分化誘導法としては，さまざまな方法が開発されているが，ヒトES/iPS細胞から中内胚葉や内胚葉への分化にはアクチビンAなどを，内胚葉から肝幹前駆細胞への分化にはBMP4（bone morphogenetic protein 4）やFGF4（fibroblast growth factor 4）などを，肝細胞の成熟化にはHGF（hepatocyte growth factor）やオンコスタチンM（OSM）などを用いて分化誘導する方法が一般的である。しかしながら，これらの方法を用いて分化誘導された肝細胞の薬物代謝酵素活性は，初代培養ヒト肝細胞に比べると一般的には2～3オーダー以上低いことが多く，よりいっそうの分化誘導効率の改善が必要である。

筆者らは最近，細胞の外部環境（さまざまな液性因子の付加）を，発生段階を模倣したように変化させ分化誘導する上記の方法では限界があると考え，適切な外部環境の処方に加え，細胞内部からの分化指令が働くように細胞分化の適切な時期に適切な転写因子を発現させることで肝細胞への分化効率が飛躍的に亢進することを報告した。具体的には，中内胚葉から内胚葉への分化段階にはSox17（SRY-box containing gene 17）遺伝子を，内胚葉から肝幹前駆細胞への分化段階にはHEX（hematopoietically expressed homeobox）遺伝子を，肝幹前駆細胞から肝細胞へに成熟（分化）段階にはHNF4a（hepatocyte nuclear factor 4, alpha）遺伝子を発現させることで，80%以上の細胞がアルブミン陽性となり，各種薬物代謝酵素の遺伝子発現レベルも初代培養ヒト肝細胞に近いレベルにまで亢進した。なお，遺伝子導入にはウイルス表面蛋白質のファイバー領域を独自に改変し，未分化ヒトES/iPS細胞およびヒトES/iPS細胞から分化させた細胞へ100%の効率で一過性の遺伝子発現が可能な改良型アデノウイルスベクターを用いた。

今後，どのような創薬研究で本分化誘導肝細胞が使用できるのか，あるいは創薬研究に使用するためには，まだどのような改善が必要か，などを詳細に解析することで，さらなる分化効率の向上に必要な技術開発や応用研究へと進めていきたい。

本分化誘導系を用いた事業化に関しては，リプロセル社が進めていく予定である。ヒトES細胞由来分化誘導肝細胞に関しては，Cellartis社（http://www.cellartis.com/）が販売しているが，現状では高価であり，薬物代謝酵素活性のレベルも低いようである。

3．ヒトiPS細胞由来神経細胞を用いた創薬研究

これまでは正常ヒト神経細胞の入手は困難であったため，初代培養ヒト神経細胞を用いて神経細胞に対する薬効や毒性を試験することは不可能であった。ヒトiPS細胞由来分化誘導神経細胞を用いることで，新たな細胞評価系の構築が可能になる。例えば，ドパミン神経を誘導し，神経突起などの微細な神経細胞構造を指標として毒

Table　Direct-reprogramming による各種細胞の分化誘導例

雑誌名	年	号/頁	筆頭著者（責任著者）	種	元の細胞	誘導細胞	遺伝子
Nat Cell Biol	2000	2/879-887	Shen CN (Tosh D)	mouse	pancreas	liver	（被性因子）glucocorticoid, dexamethasone
Cell	2004	117/663-676	Xie H (Graf T)	mouse	B cell	macrophage	C/EBPα, η
Nature	2008	455/627-632	Shou Q (Melton DA)	mouse	pancreas	η-cell	Ngn3, Pdx1, Mafa
PNAS	2008	105/6057-6062	Funf R (Graf T)	mouse	fibroblast	macrophage	PU.1, C/EBPa
Nature	2010	463/1035-1041	Vierbuchen T (Wernig M)	mouse	fibroblast	neuron	Ascl1, Brn2, Myt1l
Cell	2010	142/375-386	Ieda M (Srivastava D)	mouse	fibroblast	cardiomyocyte	Gata4, Mef2c, Tbx5
Nature	2010	468/521-526	Szabo E (Bhatia M)	mouse	fibroblast	blood progenitor	Oct-4
J Clin Inv	2011	121/640-657	Hiramatsu K (Tsumaki N)	mouse	fibroblast	hyaline cartilaginous	c-Myc, Klf4, Sox9
Nat Cell Biol	2011	13/215-222	Efe JA (Ding S)	mouse	fibroblast	cardiomyocyte	Oct4, Sox2, Klf4, c-Myc, +small molecule
PNAS	2011	108/10343-10348	Pfisterer U (Parmar M)	human	fibroblast	dopamic neuron	LMx1a, Foxa2, Ascl1, Brn2, Myt1l
PNAS	2011	108/7838-7843	Kim J (Ding S)	mouse	fibroblast	neuron progenitor	Oct4, Sox2, Klf4, c-Myc
Nature	2011	476/220-223	Pang ZP (Wernig M)	mouse	fibroblast	neuron	NeuroD1, Brn2, Ascl1, Myt1l
Nature	2011	476/224-227	Caiazzo M (Dell'Anno MT)	mouse/human	fibroblast	neuron progenitor	Ascl1, Nurr1, Lmx1a
Nature	2011	476/380-383	Huang P (Hui L)	mouse	fibroblast	hepatocyte	Gata4, Hnf1a, Foxa3, inactivation of p19
Nature	2011	476/390-393	Sekiya S (Suzuki A)	mouse	fibroblast	hepatocyte	HNF4a, Foxa1 or 2 or 3
Nature	2011	476/228-231	Yoo AS (Crabtree GR)	human	fibroblast	neuron	miR-9/9*, miR-124 (a process facilitated by NEUROD2)
Plos One	2011	6/e21801	Lee ST (Roh JK)	human	fibroblast	Meuroshere	Neural stem cell line-derived cellular extract
Cell Stem Cell	2011	9/113-118	Ambasudhan R (Ding S)	human	fibroblast	neuron	miR-124, Brn2, Myt1l
Cell Stem Cell	2011	9/205-218	Son EY (Egan K)	mouse/human	fibroblast	neuron	Hb9, Isl1, Lhx3, Ascl1, Brn2, Myt1l
Cell Stem Cell	2011	9/71-9	Marro S (Wernig M)	mouse	hepatocyte	neuron	Brn2, Ascl1, Myt1l

三評価を行うことや，分化誘導ドパミン神経で構成された神経回路のカルシウムレスポンスに対する各種化合物の影響を評価することなどへの利用が考えられる。また，神経細胞だけでなく，グリア細胞を含む中枢神経系の構成細胞に対する細胞毒性などを評価することも可能となる。

ES/iPS 細胞から神経細胞への分化誘導は，これまでにさまざまな方法が開発されており，胚様体を形成させる方法，ストローマ細胞など（例：PA6 細胞）のフィーダー細胞上で分化させる方法（SDIA 法：stromal cell derived inducing activity）[27]，あるいはレチノイン酸などの誘導因子を用いて直接分化させる方法などが開発されている。

胚様体やフィーダー細胞を用いる方法はその後，ニューロスフェア（neurosphere；FGF2 などの増殖因子を含む無血清培地で浮遊培養した球状の神経幹/前駆細胞塊）を形成させて神経幹/前駆細胞を選択的に増幅させ，そこからニューロンやグリア細胞に分化させる方法と[28]，ニューロスフェアを介さず，直接ニューロンやグリア細胞に分化させる方法に分けられる。ヒト ES/iPS 細胞から胚様体を経てニューロスフェアが形成される効率は必ずしも高くなく，通常約 1 カ月程度の長時間を要するが，ニューロスフェアを形成した神経幹/前駆細胞は *in vitro* で増幅が可能であり，凍結保存も可能という長所も有する。

一方，ES/iPS 細胞を血清や増殖因子を除いた培養液で浮遊培養すると，胚様体様浮遊凝集塊を形成し，神経前駆細胞や神経細胞へ比較的効率よく分化することが知られている（SFEB 法：serum-free floating culture of embryoid bodies-like aggregates）[29]。最近の研究から，マウスでの検討ではあるが，血清や増殖因子を除いた培養液で浮遊培養された ES 細胞は，Zfp521 という核内蛋白質を発現させ，この蛋白質の働きで神経前駆細胞への分化が誘導されることが明らかになった[30]。

いずれの方法を用いても，今後は，個々の中枢神経系の構成細胞（ドパミン神経細胞，アストロサイトなど）への分化効率の上昇や，特定の構成細胞を純化する技術開発が必要となると考えられる。なお，リプロセル社はヒト iPS 細胞から分化誘導したドパミン神経細胞を発売している。

VI. Direct-reprogramming

近年，線維芽細胞などの末梢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，肝細胞などを，通常複数の転写因子を発現する遺伝子を導入して，線維芽細胞から直接分化誘導した研究が相次いでいる（Table)。

ES/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ほう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，ES/iPS細胞から目的細胞の分化誘導研究は，この過程での技術開発にも役立つことが期待される。

おわりに

本稿では，ヒトiPS細胞を素材として分化誘導して得られた細胞製品を重篤な疾病に適応しようとするいくつかの事例について触れたが，これ以外にも例えば本特集の別稿で述べられているように筋ジストロフィー患者からiPS細胞を作製し，それにジストロフィン遺伝子を導入することで，疾病の原因遺伝子を修復し，骨格筋前駆細胞に分化誘導した後，移植して体内での筋芽細胞への分化と骨格筋との融合によりジストロフィンの供給を期待するといったものから，血小板の作製，さらには臓器再生などさまざまな試みがある。また，ES/iPS細胞を用いた創薬研究としては，心筋細胞，肝細胞，神経細胞についての現状を述べたが，これらの細胞以外にも，例えば膵β細胞や免疫系細胞は，特に薬効評価系のための重要なターゲット細胞である。また，現在のところ，ほとんど分化誘導が困難な腎臓や小腸由来細胞も創薬研究には重要なターゲット細胞となり，今後のよりいっそうの基礎研究の発展が期待される。

[redacted]

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iPS細胞研究の道しるべ
遺伝子治療研究の貢献と教訓

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現在のiPS細胞研究に対する世間の大きな期待をみると，今から約15年前に遺伝子治療に対して向けられていた期待とオーバーラップするところがある．1990年代半ば～2000年にかけて，新世代の革新的な治療法として遺伝子治療が大きな期待と注目を集めていた．著者はこの時期，大学院博士後期課程の学生，そしてポスドク(米国留学)として，遺伝子治療のための基礎技術としてのベクター開発研究に従事し，まさにこの潮流の真っ直中にいた．当時我が国では，医学部を中心に多くの研究機関で遺伝子治療研究(臨床研究を含む)が進められており，その成果はマスコミでもたびたび大々的に報道されていた．しかしながら，2000年前後に欧米で起こった遺伝子治療臨床研究での有害事象(死亡事故や白血病の発症)や，多くの臨床研究(試験)で有効例が得られなかったことから，遺伝子治療は当初の期待を大きく裏切ることになった．臨床研究(試験)を通して分かったことであるが，当時は遺伝子治療を支える基礎技術(特にベクターをはじめとする遺伝子導入技術)がまだまだ未熟で臨床研究(試験)への移行を急ぎすぎたこと，過度な期待等がこの原因と考えられた．その後，もう一度基礎に戻って，ベクター技術をはじめとする遺伝子治療関連技術の改良研究が行われている．現在も，欧米を中心に地道な技術開発と臨床研究(試験)が行われており，一部の疾病に対しては明確な遺伝子治療の臨床効果も認められ，光明がかすかに見えつつある．研究開発当初に同じく期待を裏切った抗体医薬が，現在医療の現場で全盛を極めており，その成功に20～30年近い年月を要したことを考えると，遺伝子治療研究にもより一層の地道な基礎固めが必要とされているところである．

一方で，この遺伝子治療研究は大きな副産物も生み出している．遺伝子治療への応用を主な目的として開発されてきたレトロウイルスベクターやレンチウイルスベクター，アデノウイルスベクターは，今や生命科学研究を行う際の必須の基盤技術として，多くの研究室で日常的に使用されている．また，iPS細胞の誕生(発明)とその後のiPS細胞研究の進展は，遺伝子治療研究で開発された技術が基盤になっているものが多い．もちろん，iPS細胞の発明は，いわゆる山中4因子の同定が最重要であるが，レトロウイルスベクターやレンチウイルスベクターの普及がなければ発明は難しかったかもしれない．また，より安全なiPS細胞の作製を目指して，染色体を傷つけないインテグレーションフリーのiPS細胞を開発することの重要性は，レトロウイルスベクターを用いた造血幹細胞遺伝子治療で白血病が発症した事例が教訓となっている．さらに，インテグレーションフリーのiPS細胞を作製するために，現在使用されているセンダイウイルスベクターやプラスミドベクターも，遺伝子治療研究を通して開発・改良された技術である．iPS細胞を目的細胞に分化させる際に，著者らが細胞分化を制御するためのツールとして使用している改良型アデノウイルスベクターも，遺伝子治療研究の産物である．

現在，iPS細胞の創薬研究への応用が実用化に近づいてきており，今後，iPS細胞を用いた再生医療への応用にも期待が持たれる．iPS細胞の再生医療への応用には，まだまだ乗り越えなければならない技術・安全上のハードルがあるが，遺伝子治療での苦い経験を教訓とし，産官学が一体となって，一歩ずつ着実に前進していくことを期待したい．

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3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing

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ABSTRACT

Although it is expected that hepatocyte-like cells differentiated from human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells will be utilized in drug toxicity testing, the actual applicability of hepatocyte-like cells in this context has not been well examined so far. To generate mature hepatocyte-like cells that would be applicable for drug toxicity testing, we established a hepatocyte differentiation method that employs not only stage-specific transient overexpression of hepatocyte-related transcription factors but also a three-dimensional spheroid culture system using a Nanopillar Plate. We succeeded in establishing protocol that could generate more matured hepatocyte-like cells than our previous protocol. In addition, our hepatocyte-like cells could sensitively predict drug-induced hepatotoxicity, including reactive metabolite-mediated toxicity. In conclusion, our hepatocyte-like cells differentiated from human ES cells or iPS cells have potential to be applied in drug toxicity testing.

1. Introduction

Hepatocyte-like cells that are generated from human embryonic stem cells (hESCs) [1] or human induced pluripotent stem cells (hiPSCs) [2] are expected to be used in drug screening instead of primary (or cryopreserved) human hepatocytes (PHs). We recently demonstrated that stage-specific transient transduction of transcription factors, in addition to treatment with optimal growth factors and cytokines, is useful for promoting hepatic differentiation [3–6]. The hepatocyte-like cells, which have many hepatocyte characteristics (the abilities to uptake low-density lipoprotein and Indocyanine green, store glycogen, and synthesize urea) and drug metabolism capacity, were generated from hESCs/hiPSCs by combinational transduction of FOXA2 and HNF1α [6]. However, further maturation of the hepatocyte-like cells is required because their hepatic characteristics, such as drug metabolism capacity, are lower than those of PHs [6].

To promote further maturation of the hepatocyte-like cells, we subjected them to three-dimensional (3D) spheroid cultures. It is known that various 3D culture conditions (such as Algimatrix scaffolds [7], cell sheet technology [8], galactose-carrying substrata [9], and basement membrane substratum [10]) are useful for the maturation of the hepatocyte-like cells. Nanopillar Plate technology [11] used in the present study makes it easy to control the configuration of the spheroids. The Nanopillar Plate has an arrayed μm-scale hole structure at the bottom of each well, and nanopillars were aligned further at the bottom of the respective holes. The seeded cells evenly drop into the holes, then migrate and aggregate on top surface of the nanopillars, thus likely to form the uniform spheroids in each hole. Not only 3D spheroid cultures [12] but also Matrigel overlay cultures [13] are useful for maintaining the hepatocyte characteristics of PHs. Therefore, we employed both 3D

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spheroid culture and Matrigel overlay culture systems to promote hepatocyte maturation of the hepatocyte-like cells.

The hepatocyte-like cells generated from hESCs/hiPSCs are expected to be used in drug development. To the best of our knowledge, however, few studies have tried to predict widespread drug-induced cytotoxicity *in vitro* using the hepatocyte-like cells. To precisely determine the applicability of the hepatocyte-like cells to drug screening, it is necessary to investigate the responses of these hepatocyte-like cells to many kinds of hepatotoxic drugs.

In this study, 3D spheroid and Matrigel overlay cultures of the hepatocyte-like cells were performed to promote hepatocyte maturation. The gene expression analysis of cytochrome P450 (CYP) enzymes, conjugating enzymes, hepatic transporters, and hepatic nuclear receptors in the 3D spheroid-cultured hESC- or hiPSC-derived hepatocyte-like cells (3D ES-hepa or 3D iPS-hepa), were analyzed. In addition, CYP induction potency and drug metabolism capacity were estimated in the 3D ES/iPS-hepa. To determine the suitability of these cells for drug screening, we examined whether the drug-induced cytotoxicity is induced by treatment of various kinds of hepatotoxic drugs in 3D ES/iPS-hepa.

2. Materials and methods

2.1. hESCs and hiPSCs culture

A hESC line, H1 and H9 (WiCell Research Institute), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with Repro Stem medium (Repro CELL) supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (Sigma). Both H1 and H9 were used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan and furthermore, and the study was approved by Independent Ethics Committee.

Three human iPSC lines were provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331; Dotcom, JCRB Number: JCRB1327; Toe, JCRB Number: JCRB1338) [14,15]. These human iPSC lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with iPSellon (Cardio) supplemented with 10 ng/ml FGF2. Other three human iPSC lines, 201B6, 201B7 and 253G1 were kindly provided by Dr. S. Yamanaka (Kyoto University) [2]. These human iPSC lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with Repro Stem supplemented with 5 ng/ml FGF2.

2.2. In vitro differentiation

Before the initiation of cellular differentiation, the medium of hESCs was exchanged into a defined serum-free medium, hESF9, and cultured as previously reported [16]. The differentiation protocol for the induction of definitive endoderm cells, hepatoblasts, and hepatocytes was based on our previous reports with some modifications [3–5,17]. Briefly, in mesendoderm differentiation, hESCs were dissociated into single cells by using Accutase (Millipore) and cultured for 2 days on Matrigel (BD Biosciences) in differentiation hESF-DIF medium which contains 100 ng/ml Activin A (R&D Systems) and 10 ng/ml bFGF (hESF-DIF medium was purchased from Cell Science & Technology Institute; differentiation hESF-DIF medium was 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 bovine fatty acid free serum albumin [all from sigma]). To generate definitive endoderm cells, the mesendoderm cells were transduced with 3000 vector particle (VP)/cell of Ad-FOXA2 for 1.5 h on day 2 and cultured until day 6 on Matrigel in differentiation hESF-DIF medium supplemented with 100 ng/ml Activin A and 10 ng/ml bFGF. For induction of hepatoblasts, the DE cells were transduced with each 1500 VP/cell of Ad-FOXA2 and Ad-HNF1α for 1.5 h on day 6 and cultured for 3 days on Matrigel in hepatocyte culture medium (HCM) (Lonza) supplemented with 30 ng/ml bone morphogenetic protein 4 (BMP4) (R&D Systems) and 20 ng/ml FGF4 (R&D Systems). In hepatic expansion, the hepatoblasts were transduced with each 1500 VP/cell of Ad-FOXA2 and Ad-HNF1α for 1.5 h on day 9 and cultured for 3 days on Matrigel in HCM supplemented with 10 ng/ml hepatocyte growth factor (HGF), 10 ng/ml FGF1, 10 ng/ml FGF4, and 10 ng/ml FGF10 (all from R&D Systems). To perform hepatocyte maturation on Nanopillar Plate (a prototype multi-well culturing plate for spheroid culture developed and prepared by Hitachi High-Technologies Corporation) shown in Fig. 1B, the cells were seeded at 2.5×10^5 cells/cm^2 (Fig. S1) in hepatocyte culture medium (Fig. S2) supplemented with 10 ng/ml HGF, 10 ng/ml FGF1, 10 ng/ml FGF4, and 10 ng/ml FGF10 on day 11. In the first stage of hepatocyte maturation (from day 12 to day 25), the cells were cultured for 13 days on Matrigel in HCM supplemented with 20 ng/ml HGF, 20 ng/ml oncostatin M (OsM), 10 ng/ml FGF4, and 10^{-6} M dexamethasone (DEX). In the second stage of hepatocyte maturation (from day 25 to day 35), Matrigel was overlaid on the hepatocyte-like cells. Matrigel were diluted to a final concentration of 0.25 mg/ml with William's E medium (Invitrogen) containing 4 mM L-glutamine, 50 ug/ml gentamycin sulfate, 1 × ITS (BD Biosciences), 20 ng/ml OsM, and 10^{-6} M DEX. The culture medium was aspirated, and then the Matrigel solution (described above) was overlaid on the hepatocyte-like cells. The cells were incubated overnight, and the medium was replaced with HCM supplemented with 20 ng/ml OsM and 10^{-6} M DEX.

2.3. Adenovirus (Ad) vectors

Ad vectors were constructed by an improved *in vitro* ligation method [18,19]. The human EF-1α promoter-driven LacZ-, FOXA2-, or HNF1α-expressing Ad vectors (Ad-LacZ, Ad-FOXA2, or Ad-HNF1α, respectively) were constructed previously [3,4,20]. All of Ad vectors contain a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of hESCs, hiPSCs, and DE cells, in which transfection efficiency was almost 100%, and purified as described previously [3–5]. The vector particle (VP) titer was determined by using a spectrophotometric method [21].

2.4. Flow cytometry

Single-cell suspensions of hESC/hiPSC-derived cells were fixed with 2% paraformaldehyde (PFA) at 4°C for 20 min, and then incubated with the primary antibody (described in Table S1), followed by the secondary antibody (described in Table S1). Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences).

2.5. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from hESCs or hiPSCs 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 Taqman gene expression assays (Applied Biosystems) or SYBR Premix Ex Taq (TaKaRa) using an ABI PRISM 7000 Sequence Detector (Applied Biosystems). 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 S2.

2.6. Immunohistochemistry

The cells were fixed with 4% PFA. After incubation with 1% Triton X-100, blocking with Blocking One (Nakalai tesque), the cells were incubated with primary antibody (describe in Table S1) at 4°C for over night, followed by incubation with a secondary antibody (described in Table S1) at room temperature for 1 h.

2.7. ELISA

The hESCs or hiPSCs were differentiated into hepatocytes as described in Fig. 1A. The culture supernatants, which were incubated for 24 h after fresh medium was added, were collected and analyzed for the amount of ALB secretion by ELISA. ELISA kits for ALB were purchased from Bethyl. ELISA was performed according to the manufacturer's instructions. The amount of ALB secretion was calculated according to each standard followed by normalization to the protein content per well.

2.8. Urea secretion

The hESCs or hiPSCs were differentiated into hepatocytes as described in Fig. 1A. The culture supernatants, which were incubated for 24 h after fresh medium was added, were collected and analyzed for the amount of urea secretion. Urea measurement kits were purchased from BioAssay Systems. The experiment was performed according to the manufacturer's instructions. The amount of urea secretion was calculated according to each standard followed by normalization to the protein content per well.

2.9. Canalicular secretory assay

At cellular differentiation, the hepatocyte-like cell spheroids were treated with 5 mM choly-lysyl-fluorescein (CLF) (BD Biosciences) for 30 min. The cells were washed with culture medium, and then observed by fluorescence microscope. To inhibit the function of BSEP, the cells were pretreated with Cyclosporin A 24 h before of the CLF treatment.

2.10. Assay for CYP activity and CYP induction

To measure the cytochrome P450 2C9 and 3A4 activity of the cells, we performed lytic assays by using a P450-GloTM CYP2C9 (catalog number; V8791) and

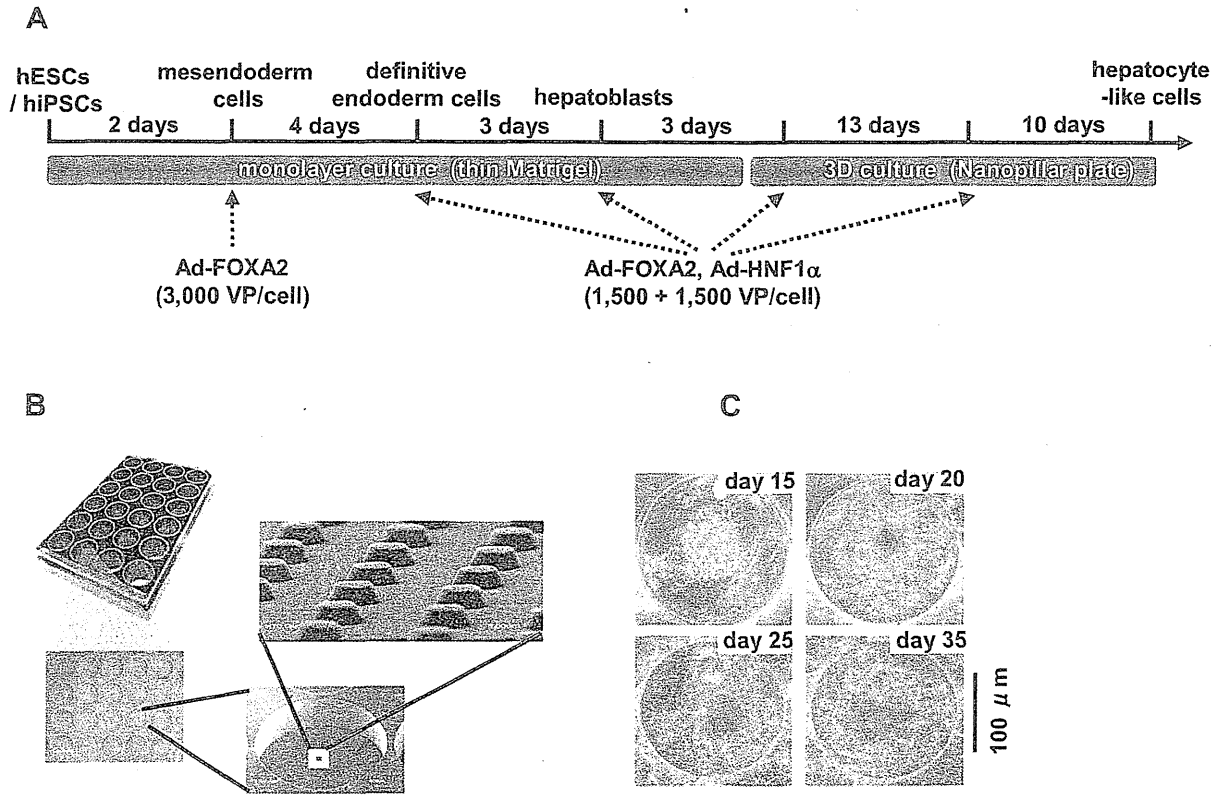


Fig. 1. Hepatocyte-like cells were differentiated from hESCs/hiPSCs by using Nanopillar Plate. (A) The procedure for differentiation of hESCs into 3D ES/iPS-hepa via mesendoderm cells, definitive endoderm cells, and hepatoblasts is presented schematically. In the differentiation, not only the addition of growth factors but also stage-specific transient transduction of both FOXA2- and HNF1α-expressing Ad vector (Ad-FOXA2 and Ad-HNF1α, respectively) was performed. The cellular differentiation procedure is described in detail in the materials and methods section. (B) Photograph display of a 24-well format Nanopillar Plate and its microstructural appearances of the hole and pillar structure. (C) Phase-contrast micrographs of the hESC-hepa spheroids on the Nanopillar Plate are shown. Scale bar represents 100 μm.

3A4 (catalog number; V9001) Assay Kit (Promega), respectively. We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold) according to the manufacturer's instructions. The CYP activity was normalized with the protein content per well.

To measure CYP2C9 and 3A4 induction potency, the CYP activity was measured by using a P450-Glo™ CYP2C9 and 3A4 Assay Kit, respectively. The cells were treated with rifampicin, which is known to induce both CYP2C9 and 3A4, at a final concentration of 10 μM for 48 h. The cells were also treated with Ketoconazole (Sigma) or Sulfaphenazole (Sigma), which are inhibitors for CYP3A4 or 2C9, at a final concentration of 1 μM or 2 μM, respectively, for 48 h. Controls were treated with DMSO (final concentration 0.1%). Inducer compounds were replaced daily.

2.11. Cell viability tests

Cell viability was assessed by the WST-8 assay kit (Dojindo) in Fig. 2D. After treatment with test compounds, such as Acetaminophen (Wako), Allopurinol (Wako), Amiodaron (Sigma), Benzbromarone (Sigma), Clozapine (Wako), Cyclizine (MP bio), Dantrolene (Wako), Desipramine (Wako), Disufliram (Wako), Erythromycin (Wako), Felbamate (Sigma), Flutamide (Wako), Isoniazid (Sigma), Labetalol (Sigma), Lefunomide (Sigma), Maprotiline (Sigma), Nefazodone (Sigma), Nitrofurantoin (Sigma), Sulindac (Wako), Tacrine (Sigma), Tebinafine (Wako), Tolcapone (TRC), Troglitazone (Wako), and Zafirlukast (Cayman) for 24 h, the cell viability was measured. The cell viability of the 3D iPSC-hepa were assessed by WST-8 assay after 24 h exposure to different concentrations of Aflatoxin B1 (Sigma) and Benzbromarone in the presence or absence of the CYP3A4 or 2C9 inhibitor, Ketoconazole (1 μM) or Sulfaphenazole (10 μM), respectively. The control refers to incubations in the absence of test compounds and was considered as 100% viability value. Controls were treated with DMSO (final concentration 0.1%). ATP assay (BioAssay Systems), Alamar Blue assay (Invitrogen), and Crystal Violet (Wako) staining assay were performed according to the manufacturer's instructions.

2.12. Primary human hepatocytes

Three lots of cryopreserved human hepatocytes (lot Hu8072 [CellzDirect], HC2-14, and HC10-101 [Xenotech]) were used. These three lots of crypreserved human hepatocytes were cultured according to our previous report [5].

2.13. Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student's *t*-test. All data are represented as means ± SD ($n = 3$).

3. Results

The 3D ES/iPS-hepa were generated from hESCs/hiPSCs as shown in Fig. 1A. Hepatocyte differentiation of hESCs/hiPSCs was efficiently promoted by stage-specific transient transduction of FOXA2 and HNF1α in addition to the treatment with appropriate soluble factors (growth factors and cytokines) [6]. On day 11, the hESC-derived cells were seeded at 2.5×10^5 cells/cm^2 (Fig. S1) on Nanopillar Plate (Fig. 1B), in hepatocyte culture medium (Fig. S2) to promote hepatocyte maturation. In addition, Matrigel was overlaid on the 3D ES-hepa to promote further hepatocyte maturation. The 3D ES-hepa with compact morphology that were adhesive to the substratum and had an optimal size (approximately 100 μm in diameter) were formed by using the Nanopillar Plate (Fig. 1C). The spheroids seem to be stable because they could be cultured for more than 20 days. We have confirmed that more than 90% of the cells that constitute the spheroids were alive, indicating that the necrotic centers are absent.

To investigate whether or not a 3D spheroid culture could promote hepatocyte maturation of the hepatocyte-like cells, various hepatocyte characteristics of the 3D ES/iPS-hepa were compared with those of the monolayer-cultured hESC- or hiPSC-derived hepatocyte-like cells (mono ES-hepa or mono iPS-hepa). The gene expression level of *ALB* peaked on day 20 in the mono ES-hepa, and then it was dramatically decreased after day 25 (Fig. 2A). In contrast, the gene expression level of *ALB* was

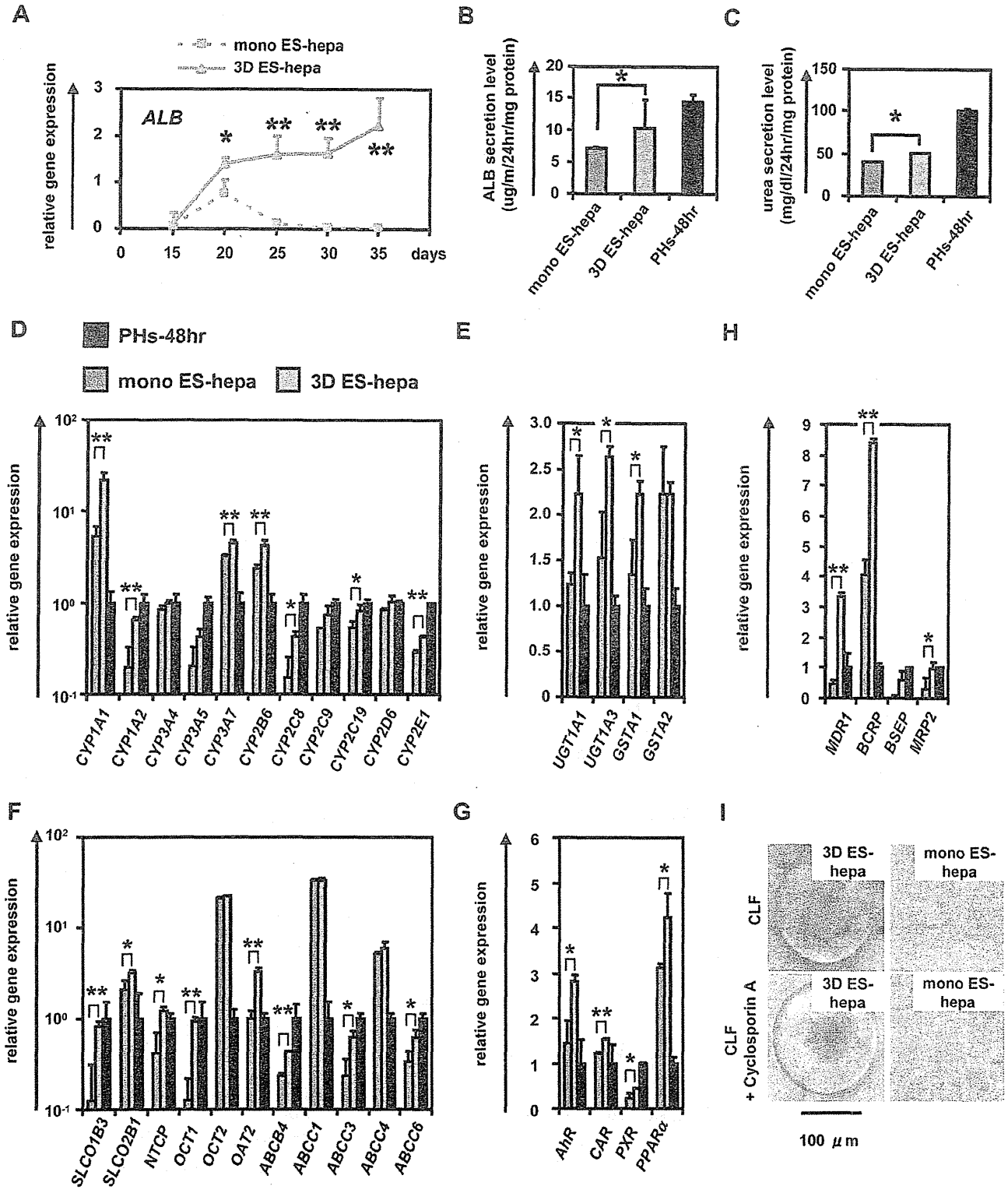


Fig. 2. *Hepatocyte functions in hESC-derived hepatocyte-like cells were enhanced by using Nanopillar Plate.* (A) The gene expression levels of *ALB* were measured by real-time RT-PCR on day 15, 20, 25, 30, and 35. On the *y* axis, the gene expression levels in PHs (three lots of PHs were used in all 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

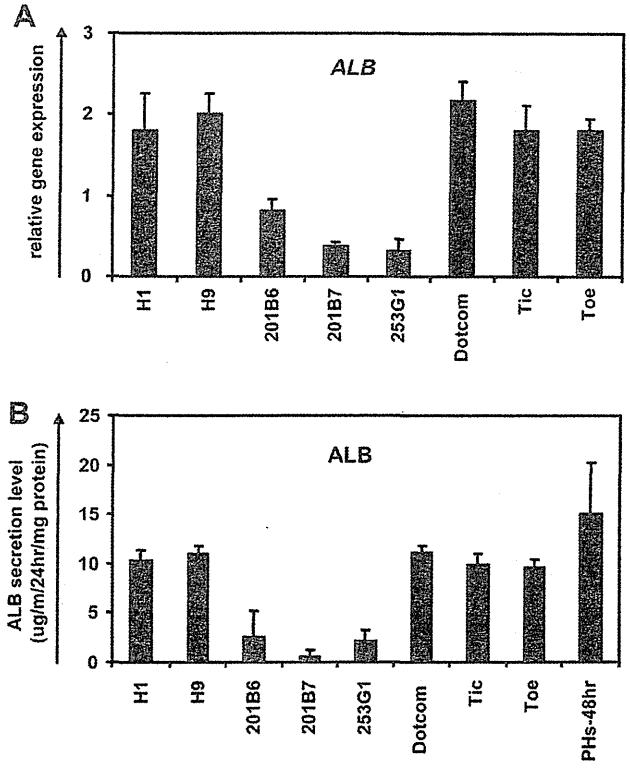


Fig. 3. Comparison of the hepatic differentiation capacities of various hESC and hiPSC lines hESCs (H1 and H9) and hiPSCs (201B6, 201B7, 253G1, Dotcom, Tic, and Toe) were differentiated into the 3D ES/iPS-hepa as described in Fig. 1A. (A) On day 20, the gene expression level of *ALB* was examined by 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.

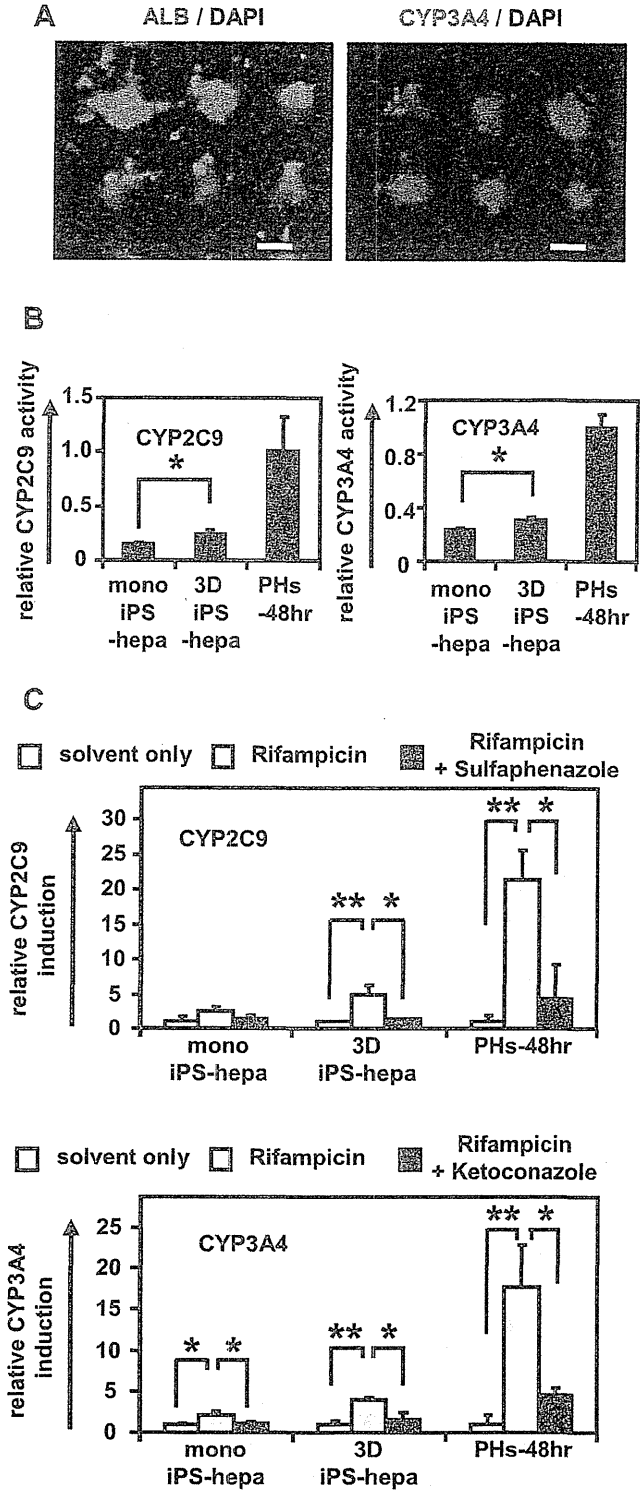
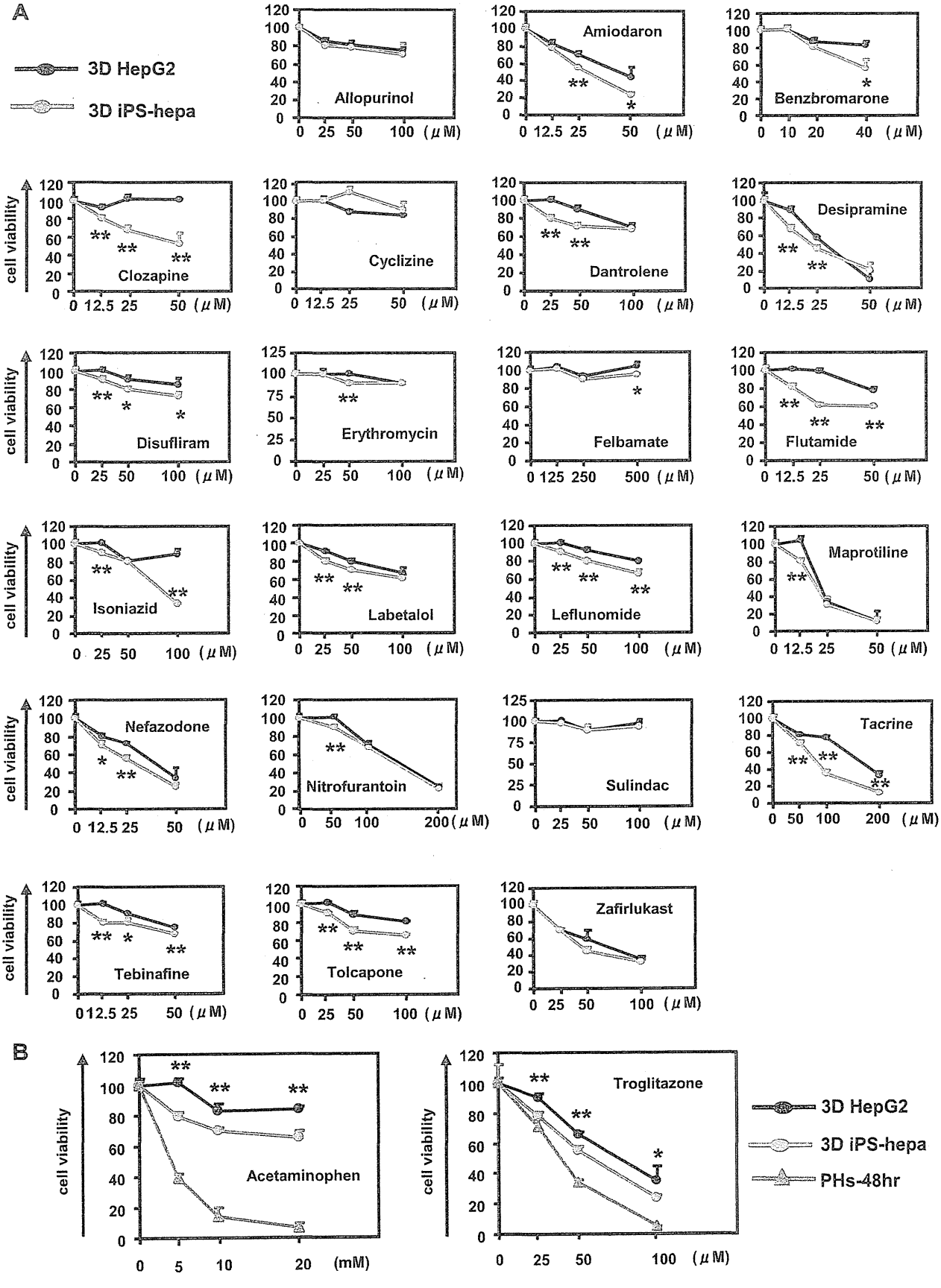


Fig. 4. Drug metabolism capacity and CYP induction potency were examined in the 3D iPS-hepa. (A) The 3D iPS-hepa (day 35) were subjected to immunostaining with anti-ALB (green) or CYP3A4 (red) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar represents 100 μm. (B) The CYP activity was 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
Acetaminophen
Troglitazone
(mM)
3D HepG2
3D iPS-hepa
PHs-48hr

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. $^{*}P < 0.05$; $^{**}P < 0.01$.

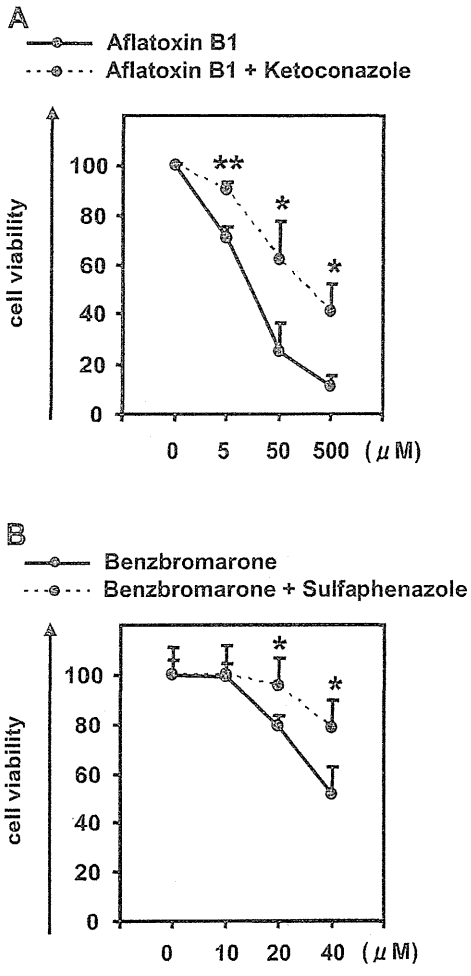


Fig. 6. Drug-induced cytotoxicity in the 3D iPS-hepa is mediated by cytochrome P450. (A, B) The cell viability of the 3D iPSC-hepa was assessed by WST-8 assay after 24 h exposure to different concentrations of (A) Aflatoxin B1 and (B) Benzbromarone in the presence or absence of the CYP3A4 or 2C9 inhibitor, 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.

Acknowledgments

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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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Commentary Open Access

Endodermal and Hepatic Differentiation from Human Embryonic Stem Cells and Human Induced Pluripotent Stem Cells

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Abstract

Induced hepatocytes differentiated from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) have a wide range of potential applications in biomedical research, drug discovery, and the treatment of liver disease. Differentiation of human ESCs and iPSCs into endodermal and hepatic cell types has been achieved by several methods, including addition of soluble factors into culture medium, transduction of differentiation-related genes, co-cultivation with other lineage cells, and a three-dimensional culture system. Each of these methods has an advantage from various points of view, such as the degree of maturation of differentiated hepatocytes, differentiation efficiency, clinical safety, and ease of handling. Currently, it is possible to select or combine the differentiation protocols to obtain ideal hepatocytes. The aim of this review is to describe the recent progress in endodermal and hepatic differentiation protocols from human ESCs and iPSCs in order to foster the suitable choice of induced hepatocytes on clinical and industrial applications.

Keywords: Embryonic stem cells; Induced pluripotent stem cells; Liver; Definitive endoderm; Differentiation

Introduction

The liver has many functions, including carbohydrate metabolism, glycogen storage, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins, and destruction of erythrocytes. The liver is composed of several types of cells, including epithelial, endothelial, and hematopoietic cells. Of these cells, hepatocytes play the most important role in major hepatic functions. Hepatocytes are thus useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly useful for drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models. For these applications, however, it is necessary to prepare a large number of the functional hepatocytes, which can no longer proliferate in *in vitro* culture. Isolated primary hepatocytes are the current standard *in vitro* model, because they express large amounts of drug-metabolizing enzymes and transporters [1]. However, isolated hepatocytes lose their differentiated properties, such as some cytochrome P450 activities that are induced by reference compounds, even under the optimized culture conditions [2,3]. Moreover, it can be difficult to set up long-term cultures with primary hepatocytes, because they can no longer proliferate in *in vitro* culture [4].

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body, and have the potential to provide an unlimited source of cells for a variety of applications [5-8]. Among the differentiated cells from ESCs and iPSCs, induced hepatocytes have a wide range of potential applications in biomedical research, drug discovery, and the treatment of liver disease. In this review, we provide an up-to-date overview of the wide variety of endodermal and hepatic differentiation protocols. These protocols were designed to reconstruct the *in vivo* environment in a variety of ways, including by addition of soluble factors into culture medium, transduction of differentiation-related genes, co-cultivation with other lineage cells, and use of a three-dimensional culture system.

Definitive Endoderm Differentiation from ESCs

Gastrulation of the vertebrate embryo starts with the formation of three germ layers: the ectoderm, mesoderm, and endoderm. The endoderm contributes to the digestive and respiratory tracts and their associated organs [9]. The endoderm differentiates into various organs, including the liver, pancreas, lungs, intestine, and stomach. To examine the molecular mechanisms of endoderm specification during early embryogenesis, endoderm differentiation from ESCs has been widely investigated as an *in vitro* model [10]. It has been reported that mouse ESCs have the ability to differentiate into definitive endoderm (DE) cells [11-13]. In recent studies, specific growth factors are used to generate DE cells from ESCs. In DE differentiation, it is well known that nodal signaling plays a crucial role and induces the expression of endoderm-related genes [14]. Activin A, a member of the nodal family, is a ligand of the type II activin receptor and can transmit a downstream signal by using Smad adaptor proteins [15-18]. Therefore, activin A is widely used to generate DE from ESCs. Although embryoid body (EB) formation is also used in the differentiation of ESCs, activin A could generate DE more efficiently than the EB formation [19]. In addition, using activin A with other factors such as fibroblast growth factor (FGF) 2 or Wnt3a proved to be more effective. Simultaneous addition of activin A and FGF2 could synergistically promote more efficient DE

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differentiation in comparison with using activin A alone [20,21]. It has also been found that DE differentiation was promoted by using activin A plus Wnt3a in comparison with activin A plus sodium butyrate [22].

Although DE differentiation methods using growth factors are useful strategies for generating DE with the ability to differentiate into hepatic or pancreatic lineages, they are not efficient enough for generation of homogenous DE populations [23,24]. To improve the DE differentiation efficacy, several groups have attempted a modulation of expression levels in endoderm-related transcription factors. It has been demonstrated that overexpression of SOX17, which is an integral transcription factor for DE formation, promotes DE differentiation, resulting in a DE differentiation efficacy of over 80% based on the estimation of c-kit/CXCR4 double-positive cells [24,25]. The FOXA2 transcription factor as well as SOX17 also functions as a crucial regulator of the initial intracellular signaling pathways in DE differentiation [26]. Overexpression of FOXA2 in ESCs enhances the efficacy of DE differentiation [27,28].

Hepatic Specification from ESC-derived DE cells

Hepatic differentiation is divided into two steps: hepatic specification and hepatic maturation. In hepatic specification, DE differentiates into hepatoblasts that express α-fetoprotein (AFP), transthyretin, and albumin (ALB) [29-31]. At this stage, repression of Wnt signaling and FGF 4 is necessary for hepatic specification [32,33]. Also, interaction of FGFs with bone morphogenetic protein (BMP) 2 and BMP 4 is important for the induction of hepatocyte-related genes [34-36]. The combination of FGF4 and BMP2 promotes hepatic specification from human ESC-derived DE cells [37]. Similar results were obtained by using the combinations of aFGF and BMP4, bFGF and BMP4, or FGF4 and BMP4 [37]. It has been reported that heterogeneous hepatoblast populations could be differentiated from DE cells by using the combination of BMP2/4 and FGF1/2/4 [20]. With respect to the generation of homogeneous hepatoblast populations, several studies have demonstrated that this can be accomplished by modulating the expression levels of hepatocyte-related transcription factors as well as DE differentiation stage. Overexpression of HEX, which is an integral transcription factor for hepatic specification, has been shown to promote hepatic specification, with the result that the expression levels of ALB and AFP are up-regulated in HEX-transduced cells [38-40]. Conditioned medium from human hepatocellular carcinoma cell line, HepG2, could also promote the hepatic differentiation from human ES cells [41].

Hepatic Maturation from ESC-derived Hepatoblasts

Hepatoblasts differentiate into two distinct lineages, hepatocytes and cholangiocytes. During the fetal hepatic maturation, the number of hepatoblasts decreases, and in turn, the number of mature hepatocytes increases [42]. In this process, AFP is highly expressed in the fetal liver, and then the number of AFP-positive cells decreases in a later maturation step and almost disappears in the adult liver [43,44]. Growth factors that are secreted by surrounding non-parenchymal liver cells, such as hepatocyte growth factor (HGF) and Oncostatin M (OsM), are essential for hepatic maturation [42]. HGF enhances hepatocyte proliferation but it inhibits biliary differentiation by blocking notch signaling [43]. OsM, which is expressed in hematopoietic cells in the fetal liver [45], promotes the hepatic differentiation from liver progenitor cells [42,43,46].

As mentioned above, growth factors that are necessary for *in vivo* hepatic development are utilized in hepatic differentiation from ESC-derived hepatoblasts. Measurement of urea synthesis [47], ALB production [47], glycogen storage [37], uptake low-density lipoprotein (LDL) [48], uptake and secrete Indocyanine Green [48], coagulation factor VII activity [49], have been used to verify if ESC-derived hepatocyte-like cells function adequately as hepatocytes. Measurement of the ability of human immunodeficiency virus (HIV)-hepatitis C virus (HCV) pseudotype viruses to enter into human ESC-derived hepatocyte-like cells, has also been used to estimate hepatic maturation [37]. Although HGF is widely used for inducing hepatic phenotypes (e.g., ALB and dipeptidyl peptidase IV expression) [50,51], this is not enough to induce functional maturation [51,52]. To generate functional hepatocytes, combinations of FGF, HGF, and a mixture of insulin-transferrin-sodium selenite (ITS), dexamethasone, and OsM are often used [53-55]. Combination of HGF, activin A, and Wnt3a promoted the differentiation of human iPSCs into mature hepatocyte-like cells [56]. Minor modifications to this strategy resulted in 70% to ~80% purity (based on estimating ALB-positive cells) of ESC-derived hepatocytes [57,58].

Because drug discovery is one of the most anticipated applications of ESC-derived hepatocyte-like cells, it is important to generate ESC-derived hepatocyte-like cells that have the same characteristics as primary human hepatocytes. Even when the various hepatic functions described above are observed in ESC-derived hepatocytes, expression level of hepatocyte-related genes in ESC-derived hepatocytes is often lower than that of human hepatocytes [59]. To generate functional hepatocytes which have characteristics similar to primary human hepatocytes, exogenous transduction of transcription factor genes that can control the expression of hepatocyte-related genes is suitable for efficient differentiation of hepatocyte-like cells from ESCs. Sequential transduction of the SOX17, HEX, and HNF4α genes, which are central regulators of liver development, in ESC-derived hepatoblasts has been shown to successfully induce mature hepatocyte-like cells that have the same features as primary human hepatocytes [60] (Figure 1). Furthermore, these hepatocyte-like cells could catalyze the toxication of several compounds, suggesting that the ESC-derived hepatocytes have potential for use in drug-screening applications. Overexpression of the Foxa2, Hnf4α, and c/EBPα genes into expandable liver-derived progenitor cells resulted in mature hepatocyte phenotypes [61]. Many other studies have shown the effect of the transduction of differentiation-related genes to promote hepatic differentiation from various origins (summarized in Table 1) [24,25,27,28,38,39,60,61,62-67], demonstrating that transduction of differentiation-related genes into ESCs would be a powerful strategy to generate mature hepatocyte-like cells.

Hepatic Differentiation from iPSCs

The iPSC technology raises the possibility of generating patient-specific cell types of all lineages [68,69]. Because drug metabolism capacity differs among individuals [70], it is difficult to make a precise

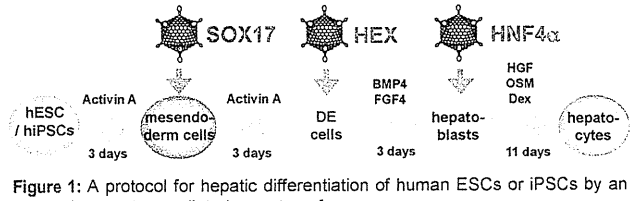

Figure 1: A protocol for hepatic differentiation of human ESCs or iPSCs by an adenovirus vector-mediated gene transfer.