Origin	Species	hepatic transcription factor genes	ref
ESCs	mouse	FOXA2	[27]
ESCs	mouse	FOXA2	[28]
ESCs	mouse	E-cadherin	[62]
ESCs	mouse	HEX	[38]
ESCs	human	SOX17	[24]
ESCs/iPSCs	human	SOX17	[25]
ESCs/iPSCs	human	HNF4α	[60]
ESCs/iPSCs	human	HEX	[39]
hepatic progenitor cells isolated from E14 fetal mouse	mouse	HNF4α	[63]
lineage-depleted OsM receptor β expressing bone marrow cells	mouse	HNF4α	[64]
human unbilical cord mesenchymal stem cells	human	hTERT	[64]
human mesenchymal stem cells	human	HNF4α	[65]
adult liver derived progenitor cells	mouse	FOXA2, HNF4α, c/EBPα	[61]
fibroblasts	mouse	HNF4α, FOXA1-3	[66]
fibroblasts	mouse	GATA4, HNF1α, FOXA3 (+ inactivation of p19Arf)	[67]

Table 1: Strategies for in vitro hepatic differentiation by using hepatic transcription factor genes.

prediction of drug toxicity by using primary human hepatocytes isolated from a single donor. A hepatotoxicity screening utilizing iPSC-derived hepatocyte-like cells would allow the investigation of individual drug metabolism capacity [71-77]. A study has shown the generation of hepatocyte-like cells from patient-specific human iPSCs [78-80]. In the same study, it was demonstrated that patient-specific iPSC-derived hepatocytes are a potential source for modeling diseases whose phenotypes are caused by protein dysregulation within adult cells. A novel drug discovery that reflects the individual genetic information would be possible by using an iPSC library representing different ethnic groups, sexes, and disease phenotypes.

Hepatic Differentiation by Co-culture and Three-dimensional Culture

In order to facilitate maturation of the ESC- or iPSC-induced hepatocyte-like cells and to enhance the differentiation efficiency of those cells, development of a differentiation system that more closely mimics progenitor development *in vivo* will be needed. Such culture system is also relevant to the culture of primary hepatocytes. Normal culture condition of hepatocytes *in vitro* differs substantially from the environment *in vivo*. Thus, it is difficult to maintain the physiological function of the hepatocytes. To overcome this difficulty, development of a culture system for highly functional hepatocytes is required. So far, co-culture methods with other lineage cells and three-dimensional culture methods have been used to support these challenges.

Co-culture methods have been attempted with primary hepatocytes and other kinds of cells [81-85], because cell-cell interactions are important in embryogenesis and organogenesis. In particular, heterotypic cell-cell interactions in the liver, such as interactions of parenchymal cells with non-parenchymal cells, play a fundamental role in liver function [86]. It has been reported that small hepatocytes could be induced to differentiate into mature hepatocytes by co-culturing with non-parenchymal cells *in vitro* [87]. Cell-cell interactions between embryonic cardiac mesoderm and definitive endoderm have been shown to be essential for liver development [88]. Transcription factors that are critical for hepatic development have been identified from these cell-cell interactions [88]. ES cells co-cultured with cardiac mesoderm showed spontaneous differentiation into hepatocytes [89]. These results suggest that the combined differentiation methods, such as addition of soluble factors into culture medium, transduction of differentiation-related genes or co-cultivation with other lineage cells, may further enhance the differentiation and maturation efficiency of hepatocytes.

Recently, numerous three-dimensional (3D) culture methods have been reported. Among these, the spheroid culture methods, which include the hanging-drop method and the float-culture method using culture dishes coated with non-adherent polymer, have been widely used to culture primary hepatocytes *in vitro*. As various micro-patterning technologies have been developed, various micro-patterned substrates, employing both surface engineering and synthetic polymer chemistry for utilizing spheroid culture, have been reported [90,91]. Spheroid culture methods permit the maintenance of liver-function of primary hepatocytes in comparison with the two-dimensional (2D)-culture.

The bioreactor method is also used for culturing primary hepatocytes. By studying various optimized conditions, flow conditions [92] and cell densities [93], this system has not only shown advantages in terms of maintaining the functions of primary hepatocytes *in vitro* in comparison with 2D-culture [94,95], but also has shown effects of spontaneous differentiation from ESCs into hepatocytes [96,97]. It has been reported that 3D culture using a bioreactor induces more functional maturation in hepatocytes differentiated from ESCs than 2D-culture [97]. The 3D culture methods using polymer scaffold systems have also demonstrated effectiveness both in culturing primary hepatocytes [98,99] and in differentiation from ESCs into hepatocytes *in vitro* [100-102]. These data showed that hepatocytes could be induced from ESCs on a polymer scaffold. ALB expression was detected earlier and the mRNA expression level of ALB was higher than in 2D culture. Furthermore, cell-sheet engineering has recently been reported [103,104]. Cell-sheet 3D culture was performed by using a culture dish coated with a temperature-responsive polymer, poly (N-isopropylacrylamide) [105-107]. Some groups have adopted culture methods with a combination of 3D culture and co-culture and showed that the liver function of primary hepatocytes could be maintained more strongly and longer than without co-culture conditions [108-110]. These combined methods will likely be a more effective differentiation condition to gain mature hepatocytes from ESCs and iPSCs.

Transplantation of Human ESC- or iPSC-derived Hepatocyte-like Cells

Because of the species differences between humans and other animals, it is difficult to apply biological phenomena of animals to humans in the early phase of drug screening [111]. It is known that chimera mice with human hepatocytes would be a powerful tool to predict drug toxicity and drug metabolism *in vivo* [112-115]. In addition, chimera mice are useful to investigate the molecular mechanisms involved in infection with human hepatitis B virus (HBV) and HCV, because there is no suitable small animal model for such study [116-118]. However, large amounts of human hepatocytes must

be prepared for these technologies, thus requiring large numbers of chimera mice. If it becomes possible to generate a robust chimera mouse model with hepatocyte-like cells differentiated from human ESCs or iPSCs, then chimera mice with humanized livers could be widely used in pharmaceutical development. To this end, several groups have reported the generation of chimera mice with hepatocyte-like cells differentiated from human ESCs and iPSCs. Cai et al. reported that human ESC-derived hepatocyte-like cells were transplanted into the carbon tetrachloride (CCl_4)-injured liver of severe combined immunodeficiency (SCID) mice and human alpha-1-antitrypsin (AAT) expression was detected in the liver [37]. Touboul et al. [119] showed that human ESC-derived hepatocyte-like cells can engraft and express human ALB and AAT in the liver of urokinase-type plasminogen activator-transgenic Rag2IL-2Rg$^{-/-}$ (uPA-Rag2IL-2Rg$^{-/-}$) mice. Duan et al. [120] reported that human ESC-derived hepatocyte-like cells were transplanted into the liver of NOD.CB17-Prkdc^{scid}/NcrCrl (NOD/SCID) mice and a significant level of human ALB was detected in the recipient mouse serum. Basma et al. [49] generated chimera mice and rats that secreted higher levels of human ALB than previously reported chimera mice. They sorted human ESC-derived hepatocyte-like cells based on surface asialoglycoprotein-receptor 1 (ASGPR1) expression and injected them into the spleen of uPA-SCID mice. Thereafter, they detected a much higher level of human ALB and human AAT in the mouse serum on day 75 after transplantation. They also performed transplantation into Nagase analbuminemic rats treated with both retrorsine, which can prevent proliferation of rat hepatocytes, and FK506, which can suppress immune response, after partial hepatectomy, demonstrating that large clusters of engrafted cells were observed in these rats and human ALB levels were reached at 20,000 ng/ml [49].

The growth speed of hepatocyte-like cells is slower than that of DE cells and hepatoblasts, both of which are immature stage cells as compared with hepatocyte-like cells [60]. It is likely that immature cells can proliferate better than mature cells in the mouse liver. Therefore, several groups have attempted to transplant DE cells or hepatoblasts. In one such attempt, human ESC-derived DE cells were successfully engrafted into the livers of NOD/SCID mice, which were treated with CCl_4 and retrorsine, and these mice expressed human AAT in the liver [57]. Recently, Liu et al. [121] compared the engraft efficiency of human ESC-derived multi-stage hepatic cells. They transplanted human DE, hepatoblasts and hepatocyte-like cells differentiated from human ESCs into the dimethylnitrosamine-injured liver of NOD/Lt-SCID/IL-2Rg$^{-/-}$ (NSG) mice, demonstrating that at low cell dosages, the engraftment efficiency of DE cells was slightly higher than that of hepatoblasts and hepatocyte-like cells differentiated from human ESCs. These results suggest that DE cells, which have proliferative capability, can regenerate liver better than hepatocyte-like cells, which have lower proliferative capability.

These technologies, which use ESC-derived cells, can be applied to iPSC-derived hepatocyte-like cells. Si-Tayeb et al. [59] injected human ESC- and iPSC-derived hepatocyte-like cells into the liver of neonatal mice and they detected human ALB expression clusters. Liu et al. [121] also transplanted human ESC- and iPSC-derived hepatocyte-like cells into mice, and achieved similar results. These findings indicate that human iPSC-derived hepatocyte-like cells can engraft into the rodent liver in a manner similar to human ESC-derived hepatocyte-like cells.

Although human ESC- or iPSC-derived hepatocyte-like cells can engraft in the mouse liver, the human ALB levels in chimera mice engrafted with human ESC- or iPSC-derived hepatocyte-like cells are much lower than those in chimera mice engrafted with human primary hepatocytes [49,112,117,121], suggesting that the efficiency of replacement in chimera mice generated with human ESC- or iPSC-derived hepatocyte-like cells would be low. Therefore, the chimerism of mice with human ESC or iPSC-derived hepatocyte-like cells should be improved to apply this technology to industrial applications.

Conclusions

In this review, we have described several protocols that could promote the differentiation of human ESCs or iPSCs into endodermal and hepatic cells. These methods are all based on the *in vivo* developmental process of embryos. In the future, by using a combination of these protocols or through the discovery of molecular findings about liver development, more efficient protocols for hepatic differentiation could be developed for regenerative medicine and drug development.

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トピックス

ヒト ES/iPS 細胞から肝細胞への高効率分化誘導法の開発とその創薬応用

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要旨

薬物誘発性肝障害は，医薬品候補化合物の開発中止や医薬品の市場撤退の主要な原因であり，医薬品開発研究の初期に肝毒性を精度高く予測することができれば，医薬品開発の効率化やコスト削減に繋がる．ヒト ES 細胞やヒト iPS 細胞からヒト初代培養肝細胞に類似した機能を有した肝細胞を作製できれば，*in vitro* での毒性評価において，ヒト初代培養肝細胞の代替ソースとなりうる．本稿では，ヒト ES/iPS 細胞から肝細胞への分化誘導技術と，毒性評価系への応用に関する現状と課題について概説する．

はじめに

薬物によって誘発される肝障害は，医薬品候補化合物の開発中止や医薬品の市場撤退の主な原因の１つである．現在は，ヒト初代培養肝細胞（本稿では，ヒト凍結肝細胞を含めてヒト初代培養肝細胞と表記する）を用いた *in vitro* 毒性評価系で肝毒性を起こす医薬品候補化合物を創薬研究の早期段階において同定し，スクリーニングすることが試みられている．しかしながら，ヒト初代培養肝細胞は高価であり，培養後急速に薬物代謝酵素をはじめとする肝機能が減弱すること，ロット差も大きいため（高機能な肝細胞ロットの）安定供給が困難であるといった問題点を有する．そこで，ヒト ES/iPS 細胞から分化誘導した肝細胞（分化誘導肝細胞）が，ヒト初代培養肝細胞の代替ソースとして期待されている．本稿では，これまでに検討されてきたヒト ES/iPS 細胞からの肝細胞分化誘導法とその課題について紹介するとともに，分化誘導肝細胞を薬物の毒性評価に応用する試みについても紹介する．

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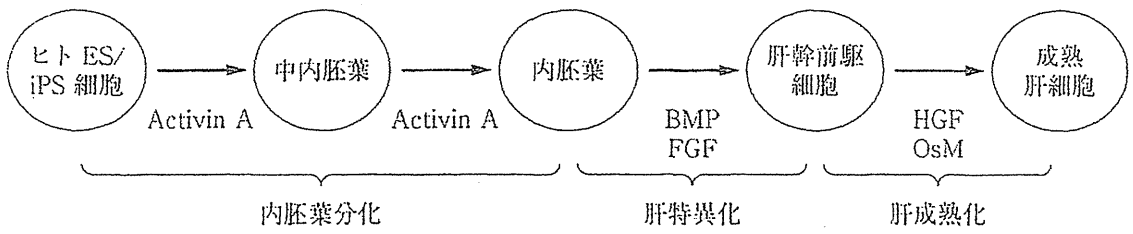
ヒト ES/iPS 細胞から肝細胞への分化誘導

1．液性因子の作用による従来の肝分化誘導法

ヒト ES 細胞から肝細胞への最初の分化誘導の報告では，胚様体（embryoid body：EB）を形成させた後，各種液性因子を作用させることで肝分化が試みられた[1]．しかしながら EB 形成法では細胞集団が不均一であり，分化がランダムに進行し，肝細胞への選択的な分化が制御できない．そこで効率よく肝細胞へ分化させるために，均一な分化誘導ができる平面培養で，生体内での肝発生・分化の環境を模倣してサイトカインや増殖因子などの各種液性因子を作用させることによって，中内胚葉，内胚葉，肝幹前駆細胞，肝細胞へと段階的に分化させる肝分化誘導法が開発された（図１）[2]．

ヒト ES/iPS 細胞から内胚葉への分化誘導ス

図1 ヒト ES/iPS 細胞から肝細胞への分化



ヒト ES/iPS 細胞は，中内胚葉，内胚葉，肝幹前駆細胞を介して肝細胞へと分化する．ヒト ES/iPS 細胞から内胚葉への分化には Activin A が使用される．内胚葉から肝幹前駆細胞への分化には BMP および FGF が併用される．肝幹前駆細胞から肝細胞への分化には HGF および OsM などが使用される．
略語：巻末の「今月の略語」参照

テップでは，Activin A がほぼすべてのプロトコールで使われている[3]．また，Wnt シグナルが内胚葉分化を促進するという報告もあるため，Activin A と Wnt を併用する内胚葉分化誘導法も報告されている[4]．肝特異化の分化誘導ステップ（内胚葉から肝幹前駆細胞への分化：図1）では，肝発生を模倣するように，FGF と BMP を組み合わせる方法が広く用いられている[5]．肝成熟化の分化誘導ステップ（肝幹前駆細胞から肝細胞への分化：図1）では，胎児肝細胞の増殖を支持する HGF[6] や，胎児肝細胞の肝成熟化を促進する Oncostatin M（OsM）[7] などが使用されている．しかしながら，これらの液性因子の作用のみからなる分化誘導法では肝細胞への分化効率は不十分であり，さらなる肝分化効率の向上が要求されている．

2．肝分化関連遺伝子を導入する肝分化誘導法

山中4因子と呼ばれる転写因子を体細胞に遺伝子導入すると細胞が初期化され iPS 細胞が樹立されるように，肝細胞への分化を含むあらゆる細胞の運命決定において遺伝子発現の制御は極めて重要なツールとなりうる．そこで筆者らは，ヒト ES/iPS 細胞から肝細胞への分化誘導の各ステップにおいても，各種液性因子の作用に加えて外来的に遺伝子発現を制御することによって肝分化の促進を試みた．ヒト ES/iPS 細胞から分化誘導された中内胚葉に，内胚葉形成に必須である SOX17（sry-related HMG box 17）遺伝子を導入した結果，約 80% の効率で内胚葉が分化誘導された[8]．また，分化誘導された内胚葉に，肝特異化に必須である HEX（hematopoietically expressed homeobox）遺伝子を導入することによって，肝幹前駆細胞への分化が促進された[9]．さらに，分化誘導された肝幹前駆細胞に HNF4α（hepatocyte nuclear factor 4 alpha）遺伝子を導入した結果，より高い肝機能を有した肝細胞を高効率に作製できた[10]．さらに筆者らは最近，FOXA2（forkhead box A2）と HNF1α（hepatocyte nuclear factor 1 alpha）遺伝子を組み合わせて各分化ステップの細胞に導入することによって，SOX17・HEX・HNF4α 遺伝子の導入を組み合わせた方法よりも高い cytochrome P450（CYP）代謝能を有する分化誘導肝細胞を作製することに成功した[11]．

3．分化誘導肝細胞と異種細胞との共培養法

胚発生過程では，肝幹前駆細胞は心臓中胚葉や横中隔間充織に接触しており，肝発生には隣接する中胚葉からのシグナルが重要である．そこで，ES 細胞からの肝分化過程において，胚発生を模倣するように中胚葉系の細胞との共培養が試みられた．ES 細胞を胎生中胚葉[12] や中胚葉由来の細胞株（M15）[13] と共培養することによって，肝分化が促進されることが報告され

ている．

4．肝分化させた細胞集団からの分化誘導肝細胞の抽出

上述したような肝分化誘導技術の改良によって，肝細胞への分化効率は飛躍的に向上したが，依然として最終的に分化させた細胞集団は分化度が不均一であり，分化が不十分な細胞が混在している状態である．そこで Basma らは，未分化な細胞や内胚葉では発現せず，肝細胞に特異的に発現する表面抗原として asialoglycoprotein receptor 1（ASGR1）に着目した[14]．肝分化させた細胞集団から ASGR1 陽性細胞をソートすることで，分化誘導肝細胞のみを抽出することに成功した．また Woo らは，indocyanine green（成熟した肝細胞が特異的に取り込むことが知られている色素）を取り込んだ細胞のみをソートすることによって，分化誘導肝細胞を選択的に抽出できることを報告した[15]．肝分化させた細胞集団から分化誘導肝細胞を高純度に抽出できる技術を活用することによって，均一な機能を有し，より成熟した肝細胞集団を供給できると期待される．

分化誘導肝細胞の毒性評価系への応用

薬物が引き起こす肝毒性の多くは，薬物が薬物代謝酵素で代謝されて生じる反応性代謝物が原因であるため，反応性代謝物による毒性を検出できる評価系の開発が必要である．筆者らは，トログリタゾン，アセトアミノフェンといった肝毒性を起こす薬物を，上述の遺伝子導入を組み合わせた分化誘導法で作製した分化誘導肝細胞に作用させたところ，細胞毒性が生じることを確認した[10]．また，肝毒性を生じる 20 種類以上の薬物を分化誘導肝細胞に作用させたところ，ほぼすべての薬物について，*in vitro* 肝毒性評価系として汎用される HepG2 細胞（肝がん細胞由来細胞株）よりも高感度に細胞毒性を検出することが可能であった[16]．さらに，薬物代謝酵素の阻害剤を併用して細胞毒性を評価したところ，薬物による細胞傷害性が一部減弱し，反応性代謝物による毒性も筆者らの分化誘導肝細胞で検出できることが明らかになった[10]．分化誘導肝細胞を用いた薬物の毒性評価はいまだ研究開発段階の技術ではあるが，本細胞を毒性評価系へ応用できる可能性が示唆された．

ま と め

肝発生の基礎研究で得られた知見をもとに，ヒト ES/iPS 細胞から薬物代謝能を有した肝細胞を分化誘導する研究が活発に行われ，肝分化誘導技術は確実に進歩してきた．しかしながら現在の肝分化誘導技術では，ヒト初代培養肝細胞の完全なる代替品を作出するまでには至っていない．今後，分化誘導肝細胞の創薬応用の実現を目指して，さらなる研究の進歩が期待される．

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Efficient Generation of Hepatocyte-like Cells from Human ES/iPS Cells for Drug Toxicity Screening

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ヒトiPS細胞由来分化誘導肝細胞を用いた薬物毒性評価系の開発

Evaluation of Drug Toxicity by Using Hepatocytes Derived from Human iPS Cells

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ヒトiPS細胞は再生医療だけではなく創薬への応用も強く期待されている。特に肝細胞をiPS細胞から効率良く分化誘導できれば，ハイスループットな薬物毒性評価系や薬物代謝評価系を新規に構築できると考えられる。本稿では，筆者らが考案したヒトiPS細胞由来肝細胞の分化誘導法とその薬物毒性評価への応用について紹介したい。

1. はじめに

現在の創薬プロセスにおいては，一つの医薬品が製品化されるまでに10～15年程度の期間および1,000億円を超える開発費が必要であるといわれており，研究開発費のうちの7割強は臨床試験以前の探索研究から前臨床研究までに投入されている。その過程で数万～100万件の候補化合物の中から薬効，毒性などの評価を経て一つが医薬品として承認を受ける。ここでしばしば問題となるのが薬物誘発性肝障害（肝毒性）であるが，医薬品の開発プロセスの早期に肝毒性を確度良く予測することは，創薬コスト削減・期間短縮・創薬シーズのヒット率の向上をもたらし，我が国の基幹産業のひとつである製薬産業の国際競争力向上に繋がると期待される。ヒト初代培養肝細胞の利用により肝毒性評価技術の向上が見込まれるものの，我が国においては入手が困難であり，安定供給や継続性の観点からその利用には限界がある為，より安定かつ容易に使用できる肝毒性評価系の確立が望まれている。近年，ヒト体細胞から分化多能性を有したiPS（induced pluripotent stem）細胞の樹立が報告され，iPS細胞由来分化誘導肝細胞は上記の問題点の克服が期待できることから大きな注目を集めている。本稿では，近年目覚ましい進歩を遂げているヒトiPS細胞から肝細胞への分化誘導法に関する知見を概説するとともに，それを利用した薬物毒性評価系への応用の可能性について筆者らの最新の結果を含めて紹介する。

2. 肝細胞の培養

肝臓は，炭水化物や脂質の代謝，グリコーゲンの貯蔵とグルコースの合成，尿素の生合成等，多くの機能を有する内胚葉由来の臓器である。肝臓を構成する細胞のうち，肝実質細胞（肝細胞）がこれらの主要な機能を担っており，*in vitro*で培養された肝細胞は，生物医学的研究だけでなく再生医療や薬物毒性評価系への応用も強く期待され

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ている。これまで肝組織の *in vitro* モデルとして初代培養肝細胞がしばしば用いられてきた。初代培養肝細胞は薬物代謝酵素や薬物トランスポーターを高発現していることから，現在でも *in vitro* での標準細胞として薬物毒性試験等で用いられている[1]。しかしながら，初代培養肝細胞は，高価であること，ドナーが制限されること，増殖しないために安定供給が難しいこと，培養後速やかにシトクロム P450 薬物代謝酵素の活性低下がみとめられること，等の問題点が指摘されている[2~4]。したがって，無限増殖能を有するヒト iPS 細胞から効率良く肝細胞が分化誘導できればこれらの問題点が解決できると期待されている。

3. ヒト iPS 細胞から肝細胞への分化誘導

ヒト iPS 細胞はヒト ES（embryonic stem）細胞と同様に分化多能性を有し，神経や皮膚，肝臓，血液，心筋等の三胚葉へ分化することができる[5,6]。ヒト iPS 細胞の分化誘導はヒト ES 細胞の分化誘導と基本的に同等であり，いずれも共通の手法を用いて分化誘導できる。したがって，以下にヒト iPS 細胞から肝細胞への分化誘導法について紹介するが，ヒト ES 細胞から肝細胞への分化誘導法の方がより多く報告されているため，ヒト ES 細胞に関する報告も混在していることに留意されたい。

3.1 ヒト iPS 細胞から内胚葉への分化誘導

ヒト iPS 細胞の分化誘導研究において，肝細胞等の内胚葉分化に関する研究は，神経細胞等の外胚葉分化に関する研究や心筋細胞・血液細胞等の中胚葉分化に関する研究よりも遅れてきた（図 1）。内胚葉分化誘導の研究が遅れてきた理由の一つとして，分化過程が複数の段階を経ることによるものと考えられる。肝細胞分化の場合，ヒト iPS 細胞は中内胚葉，内胚葉，肝幹前駆細胞を経由して成熟肝細胞へと分化し（図 2），この過程で種々の液性因子が必要とされる。このうち，内胚葉への分化誘導において最も頻繁に用いられている液性因子はアクチビン A である[7,8]。アクチビン A は TGF（transforming growth factor）-β ファミリーに属する増殖因子であり，受容体に結合した後，細胞内で Smad とよばれるアダプター分子群を活性化する[9]。アクチビン A 以外では，FGF（fibroblast growth factor）2 や Wnt3a も内胚葉分化誘導に用いられる。特に FGF2 については，アクチビン A と同時に作用させることにより，アクチビン A 単独作用時と比較し有意に内胚葉分化誘導効率が向上することが報告されている[10]。

3.2 内胚葉から肝細胞への特異化

内胚葉から肝細胞への分化は肝細胞特異化（specification）と肝細胞成熟化（maturation）の

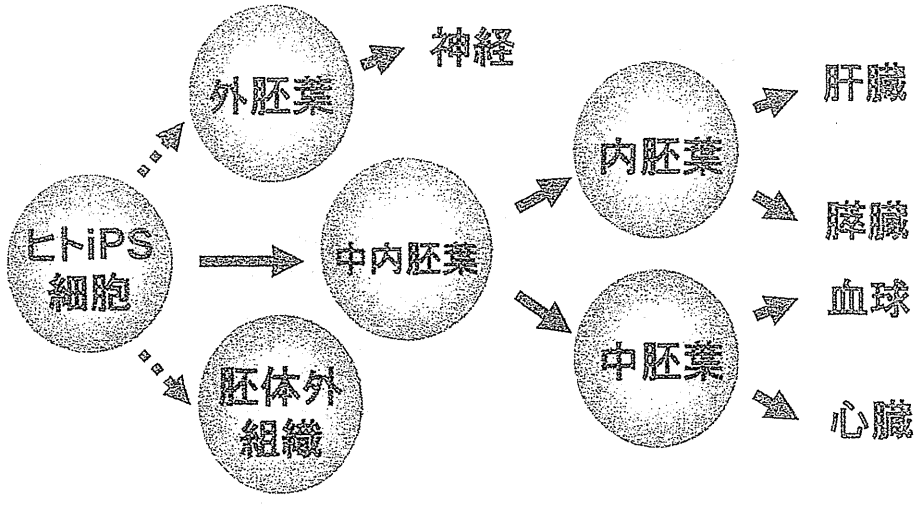

図 1 ヒト iPS 細胞から三胚葉への分化誘導
ヒト iPS 細胞はヒト ES 細胞とおなじく三胚葉に分化することができる。

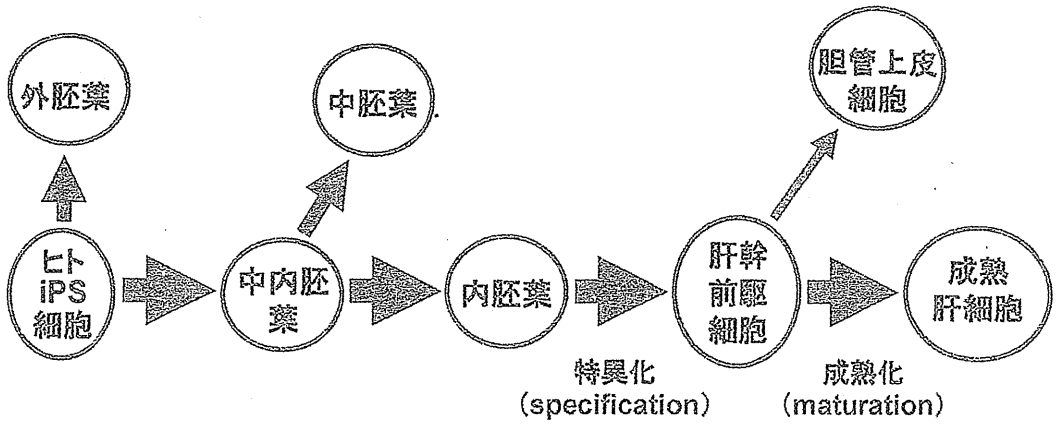

図2　ヒトiPS細胞から肝細胞への分化
ヒトiPS細胞から成熟肝細胞への分化は複数の過程に分けることができる。

2つのステップに分かれる（図2)。このうち，肝細胞特異化の過程では肝幹前駆細胞が分化誘導され，α-フェトプロテインやトランスサイレチンを発現するようになる[11,12]。この過程ではFGFシグナルとBMP（bone morphogenetic protein）シグナルが重要であることが知られており，FGF4とBMP2を作用させることにより肝特異化が著明に亢進することが報告されている[13]。またその他にも，FGF1/2/4とBMP2/4の組み合わせによって，内胚葉から肝細胞が分化誘導できるという報告もある[10]。

3.3　肝幹前駆細胞から肝細胞への成熟化

肝幹前駆細胞は肝実質細胞と胆管上皮細胞という2種類の系列に分化することが可能である（図2)。肝幹前駆細胞から肝実質細胞へ分化するにつれてα-フェトプロテインの発現量が低下し，代わってアルブミンの発現量が上昇してくる。この過程において重要な液性因子はHGF（hepatocyte growth factor）とオンコスタチンMである[14,15]。HGFは肝前駆細胞の増殖を促進させるとともに胆管への分化を阻害する。また，オンコスタチンMは肝前駆細胞の成熟化を促進する。

さらに各分化ステップで，培地や細胞外マトリックス（Ⅰ型コラーゲンやマトリゲルが汎用される）の種類，血清やフィーダー細胞の有無等が各プロトコールで工夫されている。ヒトiPS細胞由来分化誘導肝細胞を再生医療に利用する場合には，血清やフィーダー細胞等の異種動物由来成分を排除し，かつ組成の明らかな培地（chemically defined medium）で分化誘導する必要がある。一方，iPS細胞由来分化誘導肝細胞を創薬研究に応用する場合にはそのような制限は必要ではなく，むしろ創薬応用においては可能な限り成熟度が高い肝細胞を分化誘導する必要があるため，特に血清の使用は現時点では有用である。以前は，胚様体（embryoid body：EB）形成法を用いて肝細胞への分化が試みられてきたが，最近では，EB形成を介さず，上述のように直接分化させる方法が一般的である。しかしながら，これらの増殖因子やサイトカインの添加だけからなる分化誘導法は，肝細胞への分化効率もまだまだ不十分なのが現状であり，更なる分化効率の向上が必要となっている。

3.4　遺伝子導入による肝細胞分化誘導

先述したように，iPS細胞から肝細胞への分化誘導効率は未だ十分ではなく，薬物毒性評価系に応用するにはさらなる技術開発が必要である。筆者らや他のグループは，Sox17とよばれる内胚葉分化に重要な転写因子の遺伝子をヒトES細胞やiPS細胞に導入することにより，内胚葉への分化誘導効率が著明に向上することを明らかにした[16,17]。また，FoxA2とよばれる内胚葉で強く

発現している転写因子の遺伝子を導入することでも内胚葉分化は促進される[18]。肝特異化のステップでは，肝発生に重要な転写因子であるHex遺伝子を，iPS細胞由来内胚葉に導入することにより肝細胞分化が強く促進されることが筆者らと他のグループにより報告されている[19~21]。

また，筆者らは複数の遺伝子を分化の適切な時期に順次導入することにより，ヒトiPS細胞から成熟肝細胞までの分化誘導効率を向上させることを検討した。未分化iPS細胞からアクチビンA処理で分化させた中内胚葉にSOX17遺伝子を，内胚葉から肝幹前駆細胞への分化ステップではHEX遺伝子を，さらに肝幹前駆細胞から肝細胞への分化ステップではHNF4α遺伝子を導入することで，高いアルブミン産生能や薬物代謝機能を有した肝細胞を効率よく分化誘導することに成功した[17,20,22]。さらに最近では，ヒトiPS細胞から肝細胞への各分化ステップにおいて7種類の肝関連転写因子（FoxA2，SOX17，HEX，HNF1α，HNF1β，HNF4α，HNF6）を導入し，最も効率良く肝分化を促進できる転写因子を探索した結果，FoxA2およびHNF1α遺伝子を組み合わせて発現させることにより，さらに効率良く成熟肝細胞を分化誘導することに成功した（図3）[23]。なお，本分化誘導では，機能性に優れ，独自開発した改良型アデノウイルスベクターを用いた。iPS細胞から肝細胞への分化のように，分化の各ステップが階層的に起こる場合には，各分化ステップでだけ導入遺伝子が機能するように（後の細胞分化に影響を与えないように）遺伝子発現期間は一過性であること，そして効率よく細胞集団を分化させるためには，100%の遺伝子発現効率で遺伝子発現させることが必須となるが，改良型アデノウイルスベクターはこのような目的に唯一叶うベクターである。本研究で用いた改良型アデノウイルスベクターは，細胞への感染に関与するウイルス表面タンパク質のファイバータンパク質のC末端領域にポリリジン配列（KKKKKKK；リジン（K）が7つ続くのでK7と略称）を遺伝子工学的に付与しており，細胞表面のヘパラン硫酸を認識して多くの細胞種に効率よく遺伝子導入が可能となる（図4）。K7型アデノウイルスベクターは，未分化ヒトiPS細胞や，ヒトiPS細胞から分化した細胞に対しても100%の効率で遺伝子導入が可能であった[20]。

3.5 三次元培養技術による肝細胞の成熟化

肝細胞をハンギングドロップ法や浮遊培養法を用いてスフェロイド培養することにより成熟化することはよく知られている。筆者らは，細胞シート工学技術を用い，シート状に回収したマウスSwiss 3T3線維芽細胞とヒトiPS細胞由来分化誘導肝細胞とを積層三次元共培養し肝細胞の成熟化を検討した[24]。その結果，ヒトiPS細胞より分化誘導した単層の肝細胞と比較し，Swiss 3T3細胞と積層三次元共培養することによりアルブミンやHNF4α，CYP1A2などの肝細胞特異的な遺伝子発現量が上昇することが確認された。また，分化誘導した肝細胞の成熟化にはSwiss 3T3細胞の分泌する液性因子よりも，肝細胞とSwiss 3T3

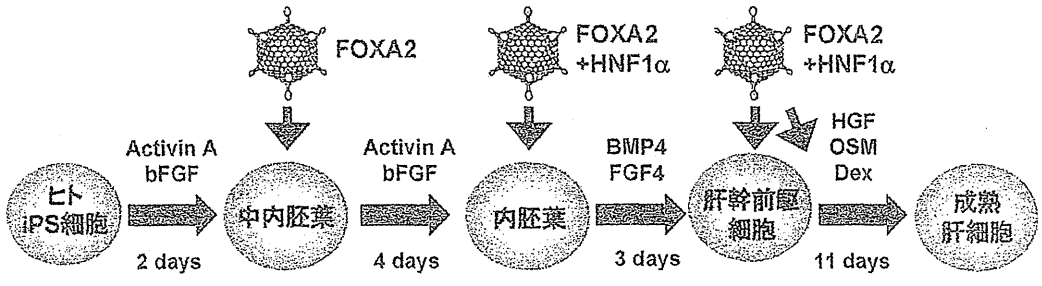

図3 遺伝子導入を用いたヒトiPS細胞から成熟肝細胞への分化誘導
分化の適切な時期に適切な遺伝子を一過性に発現させることにより，効率良く肝細胞を分化誘導できる。

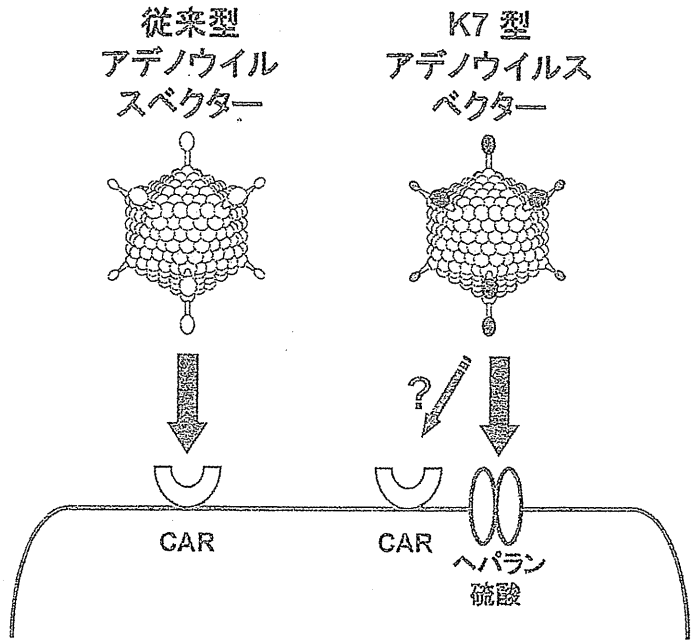

図4　改良型アデノウイルスベクター
改良型（K7型）アデノウイルスベクターはアデノウイルス受容体（CAR）だけでなく，ヘパラン硫酸も認識することにより，多くの細胞種に効率よく遺伝子導入が可能となる。

細胞との直接的な接触が重要であることを見いだし，Swiss 3T3細胞との積層三次元共培養により分化誘導した肝細胞は，成熟化がより促進されていることが明らかとなった。

4. iPS細胞由来肝細胞を用いた薬物毒性評価系の開発

このようにしてヒトiPS細胞から分化誘導した肝細胞は，形態学的には二核を有した成熟肝細胞の形状をしており，80～90%以上の細胞がアルブミン，アシアロ糖タンパク質受容体，LDL（low density lipoprotein）取り込み能，インドシアニングリーン取り込み能，薬物代謝酵素（CYP3A4, CYP7A1, CYP2D6等）陽性であり，ヒト初代培養肝細胞に匹敵する薬物代謝酵素の遺伝子発現レベルを示した。また，シトクロムP450酵素などで代謝される9種類の薬物の代謝プロファイルを調べたところ，分化誘導肝細胞の薬物代謝能はヒト初代培養肝細胞より低いものの（シトクロムP450酵素の種類により異なるが，分化誘導肝細胞はヒト初代培養肝細胞の1～40%程度の活性），いずれの薬物に対しても代謝能を有していることが確認された[23]。各シトクロムP450酵素の遺伝子発現と代謝能との間に，iPS細胞由来分化誘導肝細胞とヒト初代培養肝細胞で乖離が認められたが，この原因としては，そもそもシトクロムP450酵素の活性は個人差が大きいことが知られており（数十倍～千倍程度の個人差），低いシトクロムP450酵素活性の個人からiPS細胞が樹立されていた可能性や，シトクロムP450酵素の活性発現に必要な補酵素群の発現が未だ分化誘導肝細胞では十分でないこと等が考えられた。今後，異なった個人から樹立したヒトiPS細胞由来分化誘導肝細胞を用いて同様の検討する必要がある。また，Rashidらはα1-アンチトリプシン欠損症・家族性コレステロール血漿症・グリコーゲン貯蔵疾患症1αの患者の皮膚細胞からiPS細胞を作製し，肝細胞へ分化誘導させ，それぞれの病態を反映した肝細胞を作製できることを示した[25]。したがって，将来的には病態を反映したiPS細胞由来分化誘導肝細胞を用いた薬物毒性評価や代謝評価も可能となるであろう。

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

5. おわりに

これまでのヒトiPS細胞から分化誘導させた肝細胞は，機能面において初代培養肝細胞に比べて大きく劣っており，創薬研究への応用は困難であった。しかしながら，筆者らが開発した，遺伝子導入を駆使した分化誘導法により，創薬応用に向けてようやく最低限の解析が可能なレベルにまで分化した肝細胞を得ることが可能になった。一方で，ヒトiPS細胞由来分化誘導肝細胞を幅広く創薬研究に応用するためには，実験毎に3週間に及ぶ分化誘導を行う必要があり，これは細胞供給の観点から効率が悪いと考えられる。そこで現在筆者らは，分化途中の肝幹前駆細胞の段階で，凍結融解ができないか，あるいは分化細胞を大量に増幅できないかという課題にも取り組んでいる。今度，より一層高機能な（成熟度が高い）ヒトiPS細胞由来分化誘導肝細胞の作製法の開発を進めるとともに，本分化誘導肝細胞が創薬研究で広く活用されることを期待している。なお，本稿で紹介した分化誘導法で作製されたヒトiPS細胞由来分化誘導肝細胞は，㈱リプロセルよりReproHepatoとして市販されている。

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Supplemental Information

Using Chimeric Mice with Humanized Livers to Predict Human Drug Metabolism and a Drug-Drug Interaction

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***In vitro* characterization of clemizole metabolism**. To characterize the metabolic pathways and the basis for the inter-species differences, a detailed *in vitro* analysis of clemizole metabolism was performed. First, the metabolites formed after clemizole was incubated with mouse rat and human microsomes were determined as described in the supplemental information. Clemizole exhibited much greater stability in human microsomes, where it is largely metabolized on the pyrrolidine ring to form one major metabolite, the lactam, **M1**. Clemizole is more rapidly metabolized by rodent microsomes to multiple different metabolites (**M8, M9**), but very little **M1** was formed (**Figure S2, Tables S1, S2**). Since intact liver cells can generate more complex metabolites, the profile of clemizole metabolites formed after incubation with human and rat hepatocytes was next characterized (**Table S2**). M1 was the major metabolite formed after incubation with human hepatocytes; and smaller amounts of M14 (the glucuronide of M4) and M6, which is a ring-opened pyrrolidine oxidation product, were also formed. However, rat hepatocytes produced a very different pattern of clemizole metabolites; M15 (the glucuronide of M9) was the major metabolite and lesser amounts of other Phase 2 metabolites (M16-M19) were also formed. It is noteworthy that none of the rodent *in vitro* systems identified the major clemizole metabolites (M12, M14) that were produced by mice *in vivo*. Then, CYP450 reaction phenotyping indicated that clemizole could be metabolized by a variety of human CYP450 enzymes (described in the supplement and **Figure S3).** Taken together, the recombinant CYP450 enzyme and microsome data indicate that in human liver

clemizole is primarily converted to intermediate 'A' (Figure 2). Then, several CYP450 enzymes (CYP3A4, CYP2C19 or CYP2D6) could further oxidize this intermediate to M1; while CYP2C9 or CYP1A2 can produce M2, but they cannot produce M1; and CYP2C9 appears to be the only source for M4. After considering the relative CYP450 abundance and other factors (Proctor et al., 2004), the extrapolated data indicate that CYP3A4 and CYP2C9 account for 53% and 30%, respectively, of the human Phase 1 metabolism of clemizole; while CYP2D6 and CYP2C19 each contribute to ~5-10% of clemizole metabolism. The ability of ritonavir, which is an inhibitor of CYP3A4 activity, to inhibit clemizole metabolism *in vitro* is consistent with these results, and indicates that CYP3A4 plays a major role in clemizole metabolism (**Figure S4**). M1 is the predominant human drug metabolite because CYP3A4 is the most abundantly expressed (60% of total) hepatic CYP450 enzyme (Danielson, 2002). Although rodent CYP450 phenotyping was not performed, clemizole metabolism in rodents must be dominated by a CYP2C-type of aromatic oxidation reaction that produces the rodent predominant metabolites (M12, M14 and M15) shown in Figure 2.

Analysis of the structure of clemizole metabolites. Clemizole has three sites that are susceptible to Cytochrome P450 oxidation: the pyrrolidine, the benzimidazole and the linker between them. The chlorobenzyl group is stable to metabolism. The metabolites were identified based on their collision-induced dissociation (CID) behavior in tandem mass spectrometry, accurate mass and retention time. Table S1 lists the structures and relevant LC/MS/MS information. Figure 2 shows the structures of clemizole and its metabolites, and its metabolic pathways. All metabolites had an observed accurate mass within 2.8 ppm of the predicted accurate mass. Clemizole fragments upon CID to yield a predominate ion at m/z 255, due to loss of pyrrolidine. This ion is moved to m/z 271 in the aromatic ring oxidized metabolites. Clemizole also has a major CID fragment at m/z 125 that represents the chlorobenzyl cation and is unchanged in all metabolites. The predominant metabolite produced by human

microsomes M1 had a molecular ion 14 Da greater than clemizole, and the m/z 125 fragment ion with CID, which indicated the oxidation had occurred on the pyrrolidine; As M1 had a longer retention time than clemizole in reverse phase LC, this metabolite, is identified as the lactam. M2 has a molecular weight 2 Da less than clemizole so it is drawn as the dehydro metabolite. The aminol metabolite A initially formed via alpha-oxidation in the pyrrolidine was not observed here; it may be unstable during LC/MS to produce M2; or it may be further oxidized to form M1, M3 or M6 *in vitro* and *in vivo*. M3 is the primary amine formed via further oxidation of A or M6 possibly through unstable intermediate B. In CID M3 loses ammonia predominately to give m/z 255. M4 and M10 are mono-hydroxylation metabolites. M4, with its indicative CID fragment moved to m/z 271 and a short retention time, is drawn with oxidation on the benzimidazole although the exact regiochemistry is not certain. M10 is drawn as the N-oxide as it's retention time is similar to clemizole and it's CID main indicative fragment is at m/z 256/257 (N-oxides typically lead to altered CID patterns including radical species (Fitch et al., 2007).) M5 with an even-to-odd mass shift (due to nitrogen loss) is drawn as the primary alcohol that could be formed by reduction of an aldehyde, which would initially form if bridgehead oxidation and loss of pyrrolidine occurred. M6 is an amino acid likely formed via further oxidation of the unstable metabolite A. M6 is of higher abundance in hepatocyte incubations and *in vivo*. M7 and M9 are isomeric doubly oxidized metabolites with a net increase of +30 Da. M7 has dual oxidations on the pyrrolidine ring with its indicative fragment at m/z 255 along with a dominant water loss fragment. M9 combines the features of M1 and M4 with its indicative fragment at m/z 271. M8 combines the features of M4 and M5 with the characteristic m/z 271 ion due to water loss. M11 combines the features of M3 and M4 with the indicative fragment at m/z 271. M12 is a metabolite mainly observed in vivo; it has a molecular formula consistent with a complete loss of the pyrrolidinylmethyl side chain possibly via further oxidation of M3 or M5.

The identification of metabolites produced in hepatocyte incubations was conducted with an LTQ Orbitrap so MS^3 data is listed for these metabolites. MS^3 is very useful for identifying Phase 2 metabolites because in the first stage of CID the glucuronide (or sulfate) is removed so the MS^3 spectrum of a Phase 2 metabolite is identical to the MS/MS spectrum of the corresponding Phase 1 metabolite. M13 is a glucuronide of the parent drug, which was only observed after incubation with human hepatocytes. It is likely a quaternary N-glucuronide of the pyrrolidine, a pathway that is often lacking in rodents (Chiu and Huskey, 1998). M14 has the $[M+H]^+$ consistent with a mono-oxidation and a glucuronidation. In MS/MS M14 gave rise to fragment ions at m/z 342 and 271 consistent with it being the glucuronide of M4. M15 has the $[M+H]^+$ consistent with a double-oxidation and a glucuronidation. In MS/MS M15 gave rise to fragment ions at m/z 356 and 271 consistent with it being the glucuronide of M9. M16 is a glucuronide of M7 with the characteristic m/z 255 fragment ion. M17 is an acyl glucuronide of M6. M18 is a minor rat-specific glucuronide of a triply oxidized phase 1 metabolite (not observed unconjugated). M19 is a rat-specific glutathione conjugate with a mass consistent with glutathione trapping from an arene oxide reactive intermediate on the pathway to M9.

Recombinant Cyp450 reaction data. The data shown in Figure S3 indicates that multiple CYP450 are involved in the clemizole metabolism. Clemizole was rapidly metabolized after incubation with CYP2C9, CYP2C19, CYP2D6, or CYP3A4; and with CYP1A2 to a lesser extent. After other factors such as CYP450 abundance and differences in intrinsic activity between the recombinant system and human liver microsomes are incorporated (Proctor et al., 2004), the extrapolated data indicates that CYP3A4 and CYP2C9, are the major CYP450 isoforms that could account for 53% and 30%, respectively, of the Phase 1 metabolism of clemizole; while CYP2D6 and CYP2C19 are each responsible for ~5-10% of clemizole metabolism. A more detailed structural (QTRAP MRM-EPI) analysis of metabolites produced after incubation with 10 uM clemizole confirms that multiple metabolites were formed after clemizole was incubated with

each of the different CYP isoforms (**Table S3**). CYP3A4 is primarily responsible for production of metabolites M1 and M2; CYP2C9 for M2 and M4; CYP2D6 for M1 and M2; CYP2C19 for M2; and CYP1A2 for M2 and M4. Taken together, this data indicates that in human liver clemizole is converted to intermediate A (Figure 2) *in vitro*. CYP3A4, CYP2C19 and CYP2D6 can further oxidize this intermediate to M1; while in the presence of CYP2C9 or CYP1A2, M2 is generated, but they cannot produce M1. Cyp2C9 appears to be the only source of M4.

Supplemental Materials and Methods

Chemicals and reagents. For *in vitro* and animal experiments, clemizole hydrochloride and omeprazole were purchased from Sigma (St. Louis, MO); ritonavir was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pooled Human liver microsomes, male rat and mouse liver microsomes were purchased from BD Gentest (Woburn,MA). Cryopreserved human hepatocytes and recombinant CYP450 enzymes were purchased from BD Biosciences (San Jose, CA, USA). Rat hepatocytes were freshly isolated according to standard procedures. All other chemicals were purchased from commercial sources and were of the highest purity available.

Mouse pharmacokinetic studies. All animal experiments were performed using protocols approved by the Stanford Institutional Animal Care and Use Committee. Male C57BL6/J mice (8 weeks of age) were obtained from Jackson Labs and housed for 2 weeks prior to experimentation. NOG mice were obtained from In Vivo Sciences International (Sunnyvale, CA). Chimeric TK-NOG mice with humanized livers were prepared exactly as described (Hasegawa et al., 2011), except the Gancyclovir dose was increased to 25 mg/kg, which was administered 7 and 5 days prior to human liver cell transplantation. For transplantation, freshly isolated or cryopreserved human hepatocytes were obtained from Celsis In Vitro Inc. (Baltimore, MD) and