合のプロトコール支援は無料である．

ATMPに関する場合には，SAWPを通じた相談以外に，より非公式な制度として技術革新タスクフォース（ITF, Innovation Task Force）との相談も利用可能である．ITFはEMA内の多部局から成るグループで，法律・ガイドライン等が未整備な先端的治療・技術に関して規制面での問題点を議論することを目的としている．したがって，既存のガイドラインではカバーしきれないケースの多いATMPのような新規の製品については，開発者から規制面での疑問点をITFに投げかけることができる．この制度はITFから助言を受けるというよりもむしろ意見交換の意味合いが強い．ITFとの相談は無料であるが論議内容の法的拘束力はない．

更にこれらの制度とは別に，EMAの先端医療委員会（CAT, Committee for Advanced Therapies）は，開発者の品目がATMPに該当するか否かの助言を無料で行うとともに，SMEの非臨床試験・品質試験のデータの科学性に関する暫定認証を無料で行っている（後述）．

4.2　ATMPの中央審査

EU内の国境を越えたATMPの流通に関しては，EMAがECからの委任を受けて一括して承認審査を行っており，そこで品質・安全性・有効性に関する科学的評価が行われている．EMA内でヒト向けの医薬品の販売承認審査を行うのは，ヒト用医薬品委員会（CHMP; Committee for Human Medicinal Products）であるが，ATMPについては従来の医薬品・医療機器よりも専門的かつ多分野にわたる評価を要することから，CHMPの下部諮問組織として先端医療委員会（CAT）が2008年12月末に設置され，CATでの品質・有効性・安全性の評価意見書案をもとにしてCHMPが承認審査を行い，CHMPが作成した評価意見書をもとにしてECが承認の判断をする，という体制が取られている．ATMPの品質・安全性・有効性確保に関する要件・評価をEU内で調和させ，直接的で迅速な流通を図る目的から，ATMPはEU加盟国内での審査を経ることなく，直接CATでの評価を受けることになった．

4.3　経過措置

2008年12月30日以前にEU内で流通が承認されたATMPに関しては，経過措置が取られる．組織工学製品ではないATMPの場合には3年の移行期間（2008年12月31日～2011年12月30日），組織工学製品である場合には，4年の移行期間（2008年12月31日～2012年12月30日）が与えられており，それまでにATMPとしての再承認を受ける必要がある．期間内に再承認を受けない場合には，EU市場での承認は取り消される．

4.4　先端医療委員会（CAT）の構成と任務

4.4.1　構成

先端医療委員会（CAT）は，EU加盟国から各1名（副委員各1名），患者団体から2名（副委員2名），臨床医が2名（副委員2名）の，正副合計66名で構成され，会議は毎月1回開催される．患者団体及び臨床医の代表者としての委員はECが選定する．現在は，患者団体としてEGAN（欧州遺伝病連帯ネットワークEuropean Genetic Alliances' Network）及びEurordi（欧州希少疾病機構European Organisation for Rare Diseases），臨床医の代表者としてESGCT（欧州遺伝子細胞治療学会European Society of Gene and Cell Therapy）及びEBMT（欧州血液骨髄移植グループEuropean Group for Blood and Marrow Transplantation）のメンバーがCATに参加している．なお，CHMPとの連携の必要性から，加盟国代表の委員うち5名はCHMPの委員である必要がある．

ATMPの評価において必要な学問領域としては，医療機器・組織工学・遺伝子治療・細胞治療・バイオテクノロジー・外科学・ファーマコビジランス・リスクマネージメント及び倫理学が挙げられており，委員会全体で必要な領域がカバーできるようにアレンジされている．その内訳は，遺伝子治療専門家が19%，細胞治療専門家が21%，組織工学の専門家が17%，バイオテクノロジー専門家が24%，倫理学専門家が8%，ファーマコビジランス専門家が5%，医療機器専門家5%，外科学専門家1%となっている．

4.4.2　CATの任務

CATの任務には，①ATMPの科学的評価，②ATMP該当性に関する助言，③SMEのATMP品質・非臨床データの暫定認証，④SAWPへの協力，そのほか，ATMP以外の製品についてのCHMPとの相談，及びECへの助言などがある．

4.4.2.1　ATMPの科学的評価

CATの任務の中でも主要なのは，ATMPの科学的評価である．個別のATMPについて，CATは品質・安全性・有効性に関する科学的評価結果を意見書案としてCHMPに提出する．評価意見書案の提出は，正式な承認申請日から数えて約200作業日以内に行う．なお，CHMPは正式な承認申請日から数えて210作業日以内に承認に関する評価意見書を確定する．なお，これら作業日には土日祝日を含む．また，CATの質問事項リストが出された時から申請者がこれに回答するまでの間は作業日に勘定しない．ATMPが医療機器との複合製品

の場合には，CATは医療機器認証機関との情報交換も行う．

4.4.2.2　ATMP該当性に関する助言

CATは特定の品目がATMPに該当するか否かについて，科学的な基準に基づいた検討・判断を行う．製品の分類に関する助言要請は，治験届や承認申請の有無に係らず随時受け付けられており，手数料もかからない．正式な助言要請から60日以内で回答されることになっている．CATの回答は，製品の内容・治療対象・CATによる検討結果について，秘匿事項を除いた後に公開される．また，ATMPのファーマコビジランス及びリスクマネージメントシステムの計画及び実施に関しても，承認申請者・承認取得者からの要請に応じて助言を行う．

4.4.2.3　SMEのATMP品質･非臨床データの暫定認証

中小ベンチャー企業等（SME）はATMPの品質・非臨床データに関し，CATによる科学的評価に基づく暫定認証を受けることができる．暫定認証の審査は治験開始・承認申請の有無に係らず，SMEから申請があった場合に随時行われる．あくまで品質・非臨床データの科学的評価の結果のみを認証するものであって，治験届や承認申請とは独立したものとみなされている．すなわち，認証書は法的には治験届や承認申請の際に提出すべきデータの代用として使うことはできない．ただしECとしては，同じデータを用いて将来，治験あるいは承認の申請が行われる際には，申請の評価が行いやすくなることも期待している．

4.4.2.4　SAWPへの協力

CATはSAWPに協力することにより，ATMPの科学的助言にも関与している．ただし，CATのSAWPへの関わり方の詳細については試行錯誤が続いている．

4.5　ATMP承認審査におけるEMA各組織の役割

4.5.1　CATとCHMPの共同作業

従来の医薬品の場合はCATに諮問されることなく，CHMPラポーターとCHMP副ラポーターがそれぞれ専門家チームを構成して評価し，その評価結果をCHMPで議論する．結論がCHMPで了承されると，それを受けたECが承認をすることになる．一方，ATMPの評価はCATラポーターとCHMPコーディネーター及び品質・安全性・有効性の各専門家からなるチームと，CAT副ラポーターとCHMP副コーディネーター及び品質・安全性・有効性の各専門家からなるチームの2チームで行う．2チームが作成した評価レポートをCHMPのメンバー1名とCATのメンバー1名以上が査読し，その結果をCATの全体会議で議論する．CATは議論した内容を評価意見書案としてCHMPに提出する．CHMPは評価意見書案をもとに承認審査を行って評価意見書を作成し，更にこれをもとにECが承認の可否を判断する．

4.5.2　CATの役割

先述のようにCATはATMPの科学的評価を行うことになっているが，具体的作業としては，ATMPの評価に関して質問事項のリスト，解決すべき問題点のリスト，及び評価意見書案の内容を議論する．また，必要となれば会議中にEMAのワーキングパーティーメンバー等の外部専門家にもスライドと電話でのプレゼンテーションをさせ，議論を行う．CAT正副ラポーターは，CATの全体会議における評価の過程・議論をコーディネートするとともに，評価レポート，質問事項リスト，問題点リスト等の作成を担当し，またEMAのワーキングパーティーメンバー等の外部専門家との相談の必要性があるかどうかの判断を行う．

4.5.3　CHMPの役割

CHMPはATMPの評価を行う2チームの任命を行うともに，CATの評価意見書案をもとにした評価意見書を作成する．また，CATでの評価過程でコメントを加えることもできる．全体会議で主なATMPについての科学的意見や議論について情報を共有し，必要であれば審査期間（正式な承認申請日から数えて210日作業日）の最後に問題点リストの作成及び口頭での説明の機会設定を行うことができる．

CHMP正副コーディネーターは，CATの上部組織であるCHMPとCATとの間の情報のパイプ役となるとともに，CHMPにおいてCATの意見についての討議・採択を担当する．また，審査期間中にEMAのワーキングパーティーメンバー等の外部専門家との相談の必要性があるかどうかの判断を行う．

4.5.4　EMA事務局の役割

EMAはCATの評価意見書案及びCHMPの評価意見書がそれぞれ決められた期間内に作成されることをチェックすると同時に，CAT及びCHMPの評価の透明性を確保する．CAT事務局は，CAT正副ラポーターの評価レポートの科学的面及び規制の面での整合性を確保すると同時に，CHMPでの最終承認を受けるための評価意見書案の準備を行う．更に，CAT事務局はATMPの評価や回収に関する情報収集・提供を行う．

5.　市販後安全対策

Regulation (EC) No 1394/2007には，ATMP市販後における安全対策として，トレーサビリティの確保と市販後における安全性監視（ファーマコビジランス）が挙げられている．ATMPのドナー・原材料・製品・製造工程

及び患者のトレーサビリティの確保は従来の関連Directiveに従うことになるが，先述のように，現在ATMPに特化した指針についても検討中である．

ファーマコビジランスについては，ATMPに特化した指針[14]が出され，2008年12月末より発効している．EUでは従来，ファーマコビジランスはファーマコビジランスシステムとリスクマネージメントシステムとで構成されているが，このATMP向け指針では有効性フォローアップシステムの構築が要求されている点が特徴的である．また，リスクマネージメントの実施に当たってのATMPに特有のリスクの例，ファーマコビジランスの実施における注意点，リスクを最小化するための方策なども示されている．

ATMPは生きている細胞・組織を含む．したがって，患者への投与後，長期間の間には細胞・組織の性質に変化が生じる可能性があり，これと同時にATMPとしての有効性にも変化が生じ得る．一方，そうした変化が患者にどのような影響をもたらすか，という点については販売承認前には十分には理解し得ない．ATMPに対する患者の免疫応答性及び反復投与による免疫獲得等も，有効性・安全性に影響する可能性がある．また，ATMPの投与の様式（手術時の患者の状態・前処理，手術及び手術後の処置などまで含む）によっても有効性・安全性は変わり得る．更に，ATMPは作用期間が限定的なものから終生埋植され続けるものまで様々である．これらの理由から，ATMPに関しては有効性のフォローアップが重視されることになる．

ATMPの市販後安全対策の課題としては，構築したファーマコビジランスシステム，リスクマネージメントシステム及び有効性フォローアップシステムに関する不透明性が挙げられている．すなわち，データが非公開で，要旨のみが公開されることになっており，新たなATMPの開発促進・安全性確保の上で問題視されている．また，データの保管及びトレーサビリティシステムの担い手が承認申請者である点も，そのままでよいのかという議論がある．

6. 例外規定─ホスピタルエグゼンプション─

ATMPの中央審査の原則の例外として，Regulation (EC) No 1394/2007のArticle 28には，①特定の一患者向けの特注品の処方箋に従って，②固有の品質基準に基づき，③非反復的に製造され，④医療従事者の職務責任の下，⑤同一加盟国内で，⑥単一病院において使用されるという条件すべてを満たす場合には中央審査とはならない，という規定がある．これをホスピタルエグゼンプション（病院特例，Hospital Exemption）と言う．ただし，ホスピタルエグゼンプションに該当する品目の場合も，生産国において製造工程と品質に関する承認を受ける必要があり，またファーマコビジランス実施とトレーサビリティの確保が必要となる．特に自己由来細胞を用いたATMPの場合，患者ごとのオーダーメードであることから「非反復的生産」と考えがちだが，通常EUでは，一定の標準化された製造工程で工業的（産業的）に製造される場合には，自己細胞を原材料としても患者ごとに互いに別個の製品とはならず，反復的製造と見なされる[20]．これは製造工程中にあるリスクが多くの製品・患者に拡散するのを防ぐためである．

おわりに

ATMPは目覚ましい進展を見せ，EUでも次々と新たな開発品が出現しているが，細胞・組織・遺伝子といった，これまでにない複雑な構成成分を含むと同時に，その臨床応用に関しては非常に限られた経験と知識しか存在せず，明確な科学的根拠に基づいた品質や安全性等の確保が課題であった．これを克服するための取り組みとしてRegulation (EC) No 1394/2007が発出されたが，その取り組みの中にもまだ問題点は多い．例えば，中小ベンチャー企業向けのATMP品質・非臨床データの暫定認証は，臨床試験審査や販売承認審査とは正式な法的繋がりがないため，その意義付け，位置付けはまだ明確ではない．開発の早い段階で暫定認証が行われてもデータ自体が最終的な製品の規格と乖離したものとなりかねず，逆に遅ければ大企業への技術移転等が進まない．適切なタイミングについての判断もまだ難しい．また，ATMPに関するホスピタルエグゼンプションの要件中の単語の解釈の違いから，EU地域内でも特定の先端治療が受けられる国と受けられない国が生じ，実施国に患者が集中する，いわゆる「医療難民」が発生することが危惧され，CATでも「非反復的」「単一の病院」などの単語の定義についてハーモナイゼーションの必要性が説かれている．ATMP向けのGCPやGMP及びトレーサビリティに関する詳細な指針等もまだ確定されていない．

こうした問題はあるものの，EUの規制当局は，ATMPに対して品質や安全性等の確保及びリスク-ベネフィットのバランスを図りつつ，実用化を促進するために試行錯誤をいくつも繰り返しながら，着実に規制の枠組み作りを進めている．既に2009年6月には培養軟骨製品が，新たな審査体制の下での初のATMP品目として販売承認を受けているが，即座にCATはその審査経験をもとに，培養軟骨製品の承認審査における留意点をまとめた

文書[21]を公表している．また，研究開発が進むiPS細胞等の多能性幹細胞に由来するATMPに関する特別な留意点をまとめた文書[22]を公表するなど，EUの医薬品産業の強化に必要な新技術の開発支援に積極的な姿勢を示している．

細胞・組織加工製品を医薬品か医療機器かに分類するのではなく，ATMPという医薬品カテゴリーに括って特別な規制をかける，というEUの非常に大胆な取り組みは，従来の医薬品・医療機器の二分法に拘泥されずに先端医療製品そのものと率直に向き合いつつ品質・安全性・有効性の評価を行うことができる可能性を持っている．あらゆる医療製品や医療技術が究極的には患者あるいは将来，患者になりうる人々のために制度上も最も効果的，合理的なアプローチをとるという視点で考えれば，むしろ必然的な帰結であるかも知れない．我が国における先端医療の実用化促進施策，及び規制の国際協調のためにも参考とすべきものと考えられる．

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Transplantation of Human Adipose Tissue-Derived Multilineage Progenitor Cells Reduces Serum Cholesterol in Hyperlipidemic Watanabe Rabbits

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Familial hypercholesterolemia (FH) is an autosomal codominant disease characterized by high concentrations of proatherogenic lipoproteins and premature atherosclerosis secondary to low-density lipoprotein (LDL) receptor deficiency. We examined a novel cell therapy strategy for the treatment of FH in the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH. We delivered human adipose tissue-derived multilineage progenitor cells (hADMPCs) via portal vein and followed by immunosuppressive regimen to avoid xenogenic rejection. Transplantation of hADMPCs resulted in significant reductions in total cholesterol, and the reductions were observed within 4 weeks and maintained for 12 weeks. ^{125}I-LDL turnover study showed that the rate of LDL clearance was significantly higher in the WHHL rabbits with transplanted hADMPCs than those without transplanted. After transplantation hADMPCs were localized in the portal triad, subsequently integrated into the hepatic parenchyma. The integrated cells expressed human albumin, human alpha-1-antitrypsin, human Factor IX, human LDL receptors, and human bile salt export pump, indicating that the transplanted hADMPCs resided, survived, and showed hepatocytic differentiation *in vivo* and lowered serum cholesterol in the WHHL rabbits. These results suggested that hADMPC transplantation could correct the metabolic defects and be a novel therapy for inherited liver diseases.

Introduction

FAMILIAL HYPERCHOLESTEROLEMIA (FH) IS characterized by premature and accelerated development of atherosclerotic lesions caused by elevated levels of cholesterol-rich lipoproteins in plasma. The disease is caused by mutations in the low-density lipoprotein (LDL) receptor gene that result in a significant decrease in receptor-mediated uptake of lipoproteins from the circulation.[1–3] Patients homozygous for defects in LDL receptors have serum cholesterol levels 5–10 times those of normal and suffer as early as the first two decades of life from complications such as coronary artery disease.[4,5] In homozygous FH patients, conventional drug therapy cannot treat the condition, and therapeutic recourses are limited to chronic plasmapheresis or orthotopic liver transplantation.[1] Although liver transplants lower LDL levels, the procedure is life threatening; in addition, donor livers are in short supply. Cellular transplantation has been proposed to provide functional LDL receptors for the treatment of hypercholesterolemia. Transplantation of allogenic and xenogenic hepatocytes has been shown to be effective in lowering serum cholesterol in the Watanabe heritable hyperlipidemic (WHHL) rabbit,[6–9] which is an animal model for homozygous FH. Further, a number of gene therapy approaches have shown some promises in animal models and human,[10–13] and the therapies will cure a number of patients with FH in near future. As an alternative to whole-organ transplantation and/or gene therapy, we have investigated the ability of human adipose tissue-derived multilineage progenitor cells (hADMPCs) to differentiate into hepatocytes *in vitro* and to replace critical liver functions[14] as well as previous reports,[15,16] because the *in vitro* differentiation of hADMPCs into various kinds of cell types in now well reported and hADMPCs can be easily and safely obtained in large

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quantities without serious ethics issues.[17,18] In this study, we are investigating whether hADMPCs could differentiate into hepatocytes *in vivo* and replace critical liver functions as considerable therapeutic potential for cellular replacement.

Materials and Methods

Cells

hADMPCs were prepared as described previously[19] with some modifications.[14,17,18] Adipose tissues from human subjects were resected during plastic surgery in five subjects (four males and one female, age, 20–60 years) as excess discards. Ten to 50 g of subcutaneous adipose tissue was collected from each subject. All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine, and Foundation for Biomedical Research and Innovation. After five to six passages, the hADMPCs were used for transplantation. Human cryopreserved hepatocytes were purchased from Invitrogen (Lot number: HuP81) and cultured as indicated by the manufacturer's protocol. Human adipose tissue-derived fibroblastic cells were obtained according to previous report.[20]

Flow cytometric analysis

hADMPCs isolated from adipose tissue were characterized by flow cytometry. Cells were detached from culture dishes by 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and suspended in Dulbecco's phosphate-buffered saline (DPBS; Nacalai Tesque) containing 0.1% fetal bovine serum. Aliquots (5×10^5 cells) were incubated for 30 min at 4°C with fluorescein isothiocyanate-conjugated mouse monoclonal antibodies to human CD31 (BD PharMingen), CD105 (Ancell Corporation), CD133 (R&D Systems), phycoerythrin-conjugated mouse monoclonal antibodies to human CD29, CD34, CD45, CD73 (BD PharMingen), CD44, or CD166 (Ancell). Isotype-identical antibodies served as controls. Further, the cells were incubated with mouse monoclonal antibodies against human stage-specific embryonic antigen-4 (from Chemicon International, Inc.), ABCG-2, or CD117 (BD PharMingen) with nonspecific mouse antibody used as a negative control. After washing with DPBS, cells were incubated with phycoerythrin-labeled goat anti-mouse Ig antibody (BD PharMingen) for 30 min at 4°C. After three washes, cells were resuspended in DPBS and analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

Adipogenic, osteogenic, and chondrogenic differentiation procedure

For adipogenic differentiation, cells were cultured in the differentiation medium (Zen-Bio, Inc.). After 3 days, half of the medium was changed with adipocyte medium (Zen-Bio) every 2 days. Five days after differentiation, adipocytes were characterized by microscopic observation of intracellular lipid droplets by Oil Red O staining. Osteogenic differentiation was induced by culturing the cells in Dulbecco's modified Eagle's medium containing 10 nM dexamethasone, 50 mg/dL ascorbic acid 2-phosphate, 10 mM β-glycerophosphate (Sigma), and 10% fetal bovine serum. Differentiation was examined by Alizarin red staining. For Alizarin red staining, the cells were washed three times and fixed with dehydrated ethanol. After fixation, the cells were stained with 1% Alizarin red S in 0.1% NH_4OH (pH 6.5) for 5 min and then washed with H_2O. For chondrogenic differentiation, hADMPCs were first trypsinized and 2×10^5 cells were centrifuged at 400 *g* for 10 min. The resulting pellets were cultured in the chondrogenic medium (alpha-minimum essential medium (alpha-MEM) supplemented with 10 ng/mL transforming growth factor-β, 10 nM dexamethasone, 100 μM ascorbate, and 10 μL/mL 100×ITS Solution) for 14 days. For Alcian Blue staining, nuclear counterstaining with Weigert's hematoxylin was followed by 0.5% Alcian Blue 8GX for proteoglycan-rich cartilage matrix.

hADMPC transplantation and immunosuppression regimen

WHHL rabbits (8 weeks old; purchased from Kitayama-labes, Inc.) were anesthetized with pentobarbital (50 mg/kg). An incision distal and parallel to the lower end of the ribcage was made. The peritoneum was incised, and hADMPCs ($n = 5$) or human adipose tissue-derived fibroblastic cells ($n = 3$) (3×10^7 cells) suspended in 3 mL of Hanks' balanced salt solutions (HBSS) (20°C) or 3 mL of control saline ($n = 6$) were infused in 5 min into the portal vein via a 18-gauge Angiocath™ (BD). The immunosuppression regimen (Fig. 1A) consisted of the following: (1) intramuscular injection of cyclosporin A (6 mg/kg/day) daily from the day before surgery to sacrifice; (2) intramuscular injection of rapamycin (0.05 mg/kg/day) daily from the day before surgery to sacrifice; (3) methylprednisolone at 3 mg/kg/day (days 1–7), followed by tapering to 2 mg/kg/day (days 8–14), 1 mg/kg/day (days 15–21) and 0.5 mg/kg/day (day 22 to the time at sacrifice); (4) intravenous injection of cyclophosphamide (20 mg/kg/day) at days 0, 2, 5, and 7; (5) ganciclovir (2.5 mg/kg/day intramuscular injection (i.m.)) was also administrated to avoid viral infection in the immunocompromised host.

DNA extraction and quantification of human-derived cells

Total DNA of WHHL rabbit liver, which was obtained at the time just after hADMPC transplantation, and 2, 4, 8, and 12 weeks after transplantation, were isolated using a NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions. hADMPCs and rabbit hepatocytes were mixed at the ratios of 100:0 (100%), 10:90 (10%), 1:99 (1%), 0.1:99.9 (0.1%), 0.01:99.99 (0.01%), and 0.001:99.999 (0.001%), and DNA was isolated. Seven hundred nanograms of each samples of extracted DNA was quantified by real-time polymerase chain reaction (PCR) using the ABI Prism 7900 Sequence Detection System (Applied Biosystems), primers for the 82 bp *Alu* amplicon (forward, 5′-GTCAGGAGATCGA GACCATCCC; reverse, 5′-CCACTACGCCCGGCTAATT), and SYBR Green (TOYOBO) dye using a previously published protocol.[21,22] Reactions were performed in quadruplicate and the *Alu* levels were calculated by the standard curve.

Assay for lipid profiling

Serum samples were obtained from nonfasting rabbits before and after transplantation. Serum total cholesterol was measured in each sample using assay kits from Wako Pure Chemical Industries. Serum lipoproteins were analyzed by an on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides by high-performance

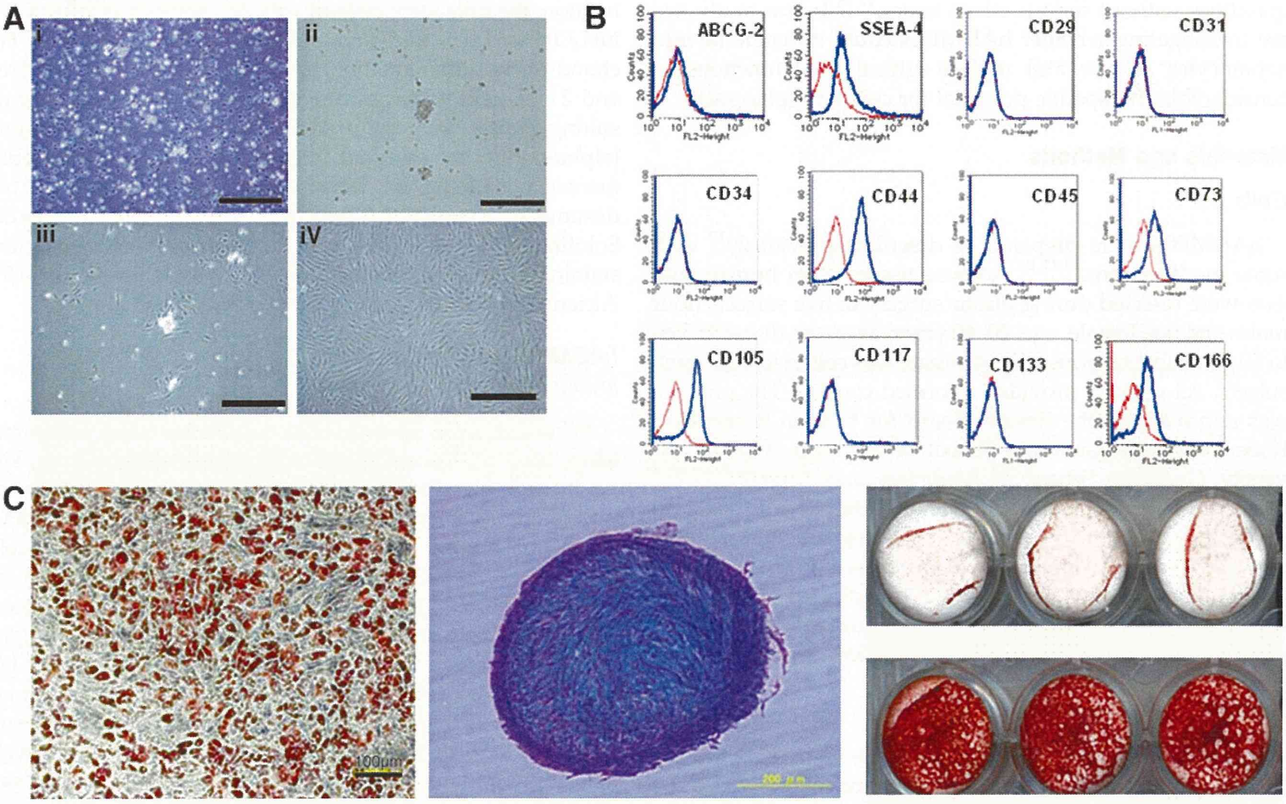


FIG. 1. **(A)** Morphological characters of human adipose tissue-derived multilineage progenitor cells (hADMPCs). The cells obtained from adipose tissue were seeded and incubated for 24 h **(i)**. After incubation, the adherent cells were treated with ethylenediaminetetraacetic acid solution, and the resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin-coated dishes (BD BioCoat) **(ii, iii)**. Within two to three passages after the initial plating of the primary culture, hADMPCs appeared as a monolayer of large flat cells (25–30 μm in diameter). As the cells approached confluence, they assumed a more spindle-shaped, fibroblastic morphology **(iv)**. i) Bar = 499 μm, ii) bar = 201 μm, iii) bar = 502 μm and iv) bar = 202 μm. **(B)** Cell surface markers expressed on hADMPCs. The cells were negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells, ABCG-2, CD34, and CD133. They were also negative for CD31, an endothelial cell-associated marker, and the surface antigen c-Kit (CD117). However, they stained positively for a number of surface markers characteristic of mesenchymal and/or neural stem cells, but not embryonic stem (ES) cells, including CD29, CD44 (hyaluronan receptor), CD73, CD105 (endoglin), and CD166. hADMPCs also were positive for stage-specific embryonic antigen (SSEA)-4. **(C)** Adipocytic, chondrocytic, and osteocytic differentiation potentials of hADMPCs. Adipocytic differentiation potential of hADMPCs was confirmed by Oil Red O staining (the left panel) (bar = 100 μm). Chondrocytic differentiation potential of hADMPCs was estimated by extracellular matrices with Alcian Blue staining (the middle panel). Osteogenic differentiation potential of hADMPCs was confirmed by Alizarin red S staining for mineralized nodules (the right panel).

liquid chromatography at Skylight Biotech, according to the procedure as described.[23]

Immunohistochemical staining of WHHL rabbit liver sections

The WHHL livers were harvested and fixed immediately with 10% formalin. They were placed into optimal cutting temperature compound (Sakura Finetechnical Co.), frozen immediately, and then sectioned at 7 μm thickness. The sections were then incubated with blocking solution (Blocking one; Nacalai Tesque) for 1 h. The samples were incubated with rabbit anti-human-specific albumin antibody (MBL), rabbit anti-human-specific alpha 1 anti-trypsin antibody, and rabbit anti-LDL receptor antibody, followed by Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes). To show the colocalization of human CD90 and albumin, the samples were incubated with the rabbit anti-human CD90 monoclonal antibody (Epitomics, Inc.) and then with Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes), and washed extensively. Then, the specimens were incubated with rabbit anti-human-specific albumin antibody (MBL), followed by Alexa Fluor 546-labeled goat anti-rabbit IgG (Molecular Probes). The treated sample was examined with a BioZero laser scanning microscope (Keyence).

PCR analysis of WHHL rabbit liver for human liver-specific genes

Total RNAs of WHHL rabbit liver, hADMPCs, and human hepatocytes were isolated using an RNAeasy kit (Qiagen). After treatment with DNase, the cDNA was synthesized using Superscript III RNase H-minus Reverse Transcriptase (Invitrogen). Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). About 20×Assays-on-Demand™ Gene Expression Assay Mix for human alpha-1-antitrypsin (Hs01097800_m1), human albumin (Hs00609411_m1), human factor 9, human GATA-binding protein 4 (GATA4) (Hs00171403_m1), human hepatocyte nuclear factor 3 beta (Hs00232764_m1), human LDL receptor (Hs00181192_m1), and human glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1) were obtained from Applied Biosystems. It was confirmed that human detectors and rabbit detectors do not cross-react with the other species. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2×), was also purchased from Applied Biosystems. Reactions were performed in quadruplicate and the mRNA levels were normalized relative to human glyceraldehyde-3-phosphate dehy-

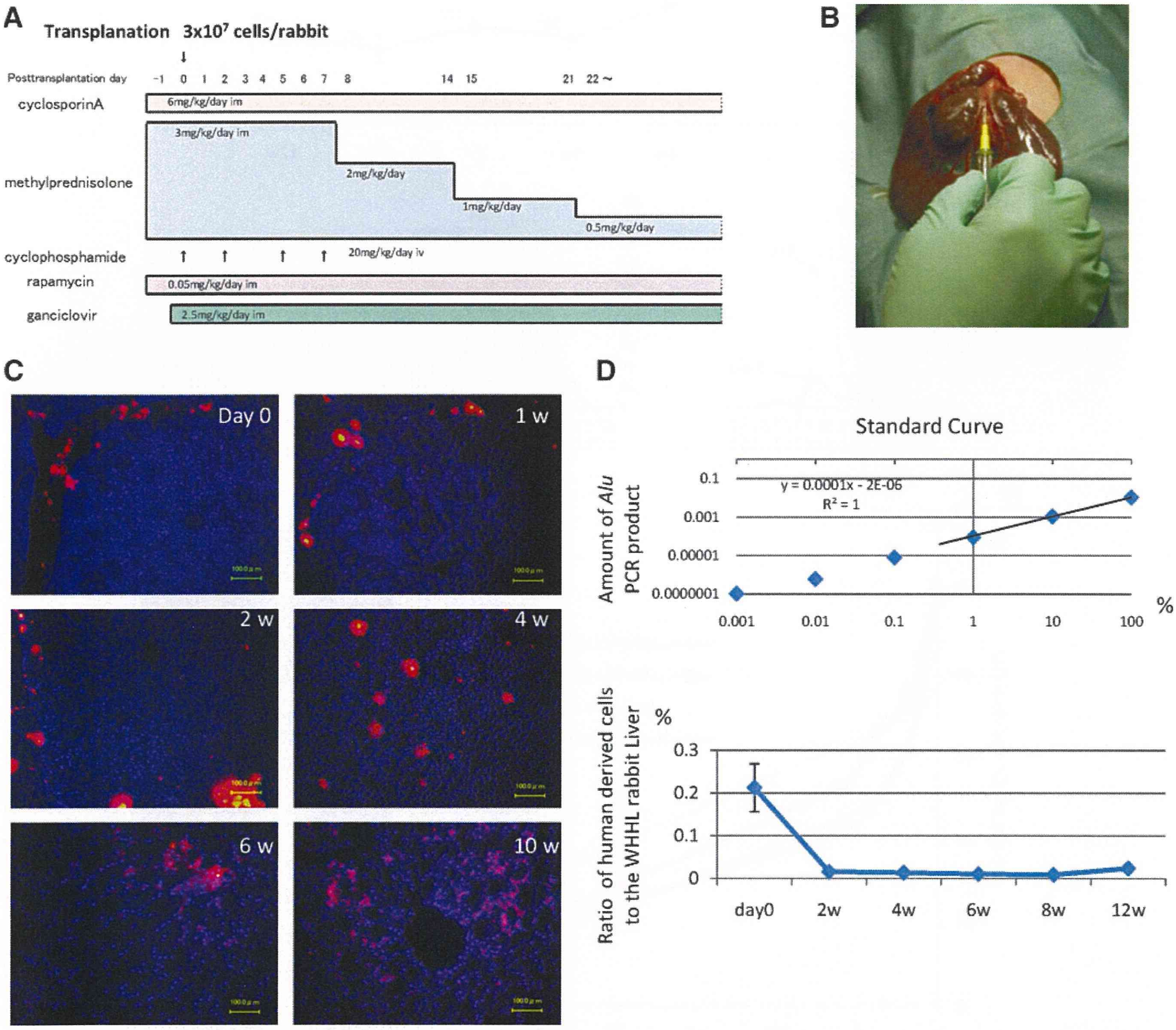


FIG. 2. **(A)** Immunosuppression regimen. Cyclosporin A (6 mg/kg/day) and rapamycin (0.05 mg/kg/day) were administered intramuscularly daily from the day before surgery to sacrifice. Methylprednisolone was administered at 3 mg/kg/day (days 1–7), 2 mg/kg/day (days 8–14), 1 mg/kg/day (days 15–21), and 0.5 mg/kg/day (day 22 to sacrifice). Cyclophosphamide (20 mg/kg/day) was injected intravenously at days 0, 2, 5, and 7. Ganciclovir (2.5 mg/kg/day) was also injected intramuscularly to avoid viral infection in the immunocompromised host. **(B)** Surgical procedure. Watanabe heritable hyperlipidemic (WHHL) rabbits were anesthetized with pentobarbital. An incision was made distal and parallel to the lower end of the ribcage. The peritoneum was incised and hADMPCs, and human adipose tissue-derived fibroblastic cells (hADFCs) (3×10^7 cells/rabbit) or controls were infused into the portal vein using an 18-gauge Angiocath. **(C)** Localization of transplanted hADMPCs in the WHHL liver. At the day of and 1, 2, 4, 6, and 10 weeks after transplantation of DiI-labeled hADMPCs via the portal vein, the WHHL rabbit liver was examined histologically. DiI-fluorescent labeled-hADMPCs resided and distributed in the portal area at the day of transplantation. One to 2 weeks after transplantation, the DiI-stained hADMPCs-derived cells were localized near the portal areas. Four weeks after transplantation some of the DiI-stained cells resembled innate hepatocytes morphologically. Six and 10 weeks after transplantation, DiI-positive transplanted cells were dispersed in a centrilobular direction, resembling the mature innate hepatocytes. Bars = 100 µm. **(D)** Quantification of repopulation of the transplanted cells in the liver. The ratios of human-derived cell repopulation were examined by analyzing an *Alu* repetitive DNA sequence at the day of and 2, 4, 8, and 12 weeks after transplantation. In upper panel the standard curve was indicated, and in lower panel the ratio of repopulation of human cells was shown in time course after transplantation of hADMPCs.

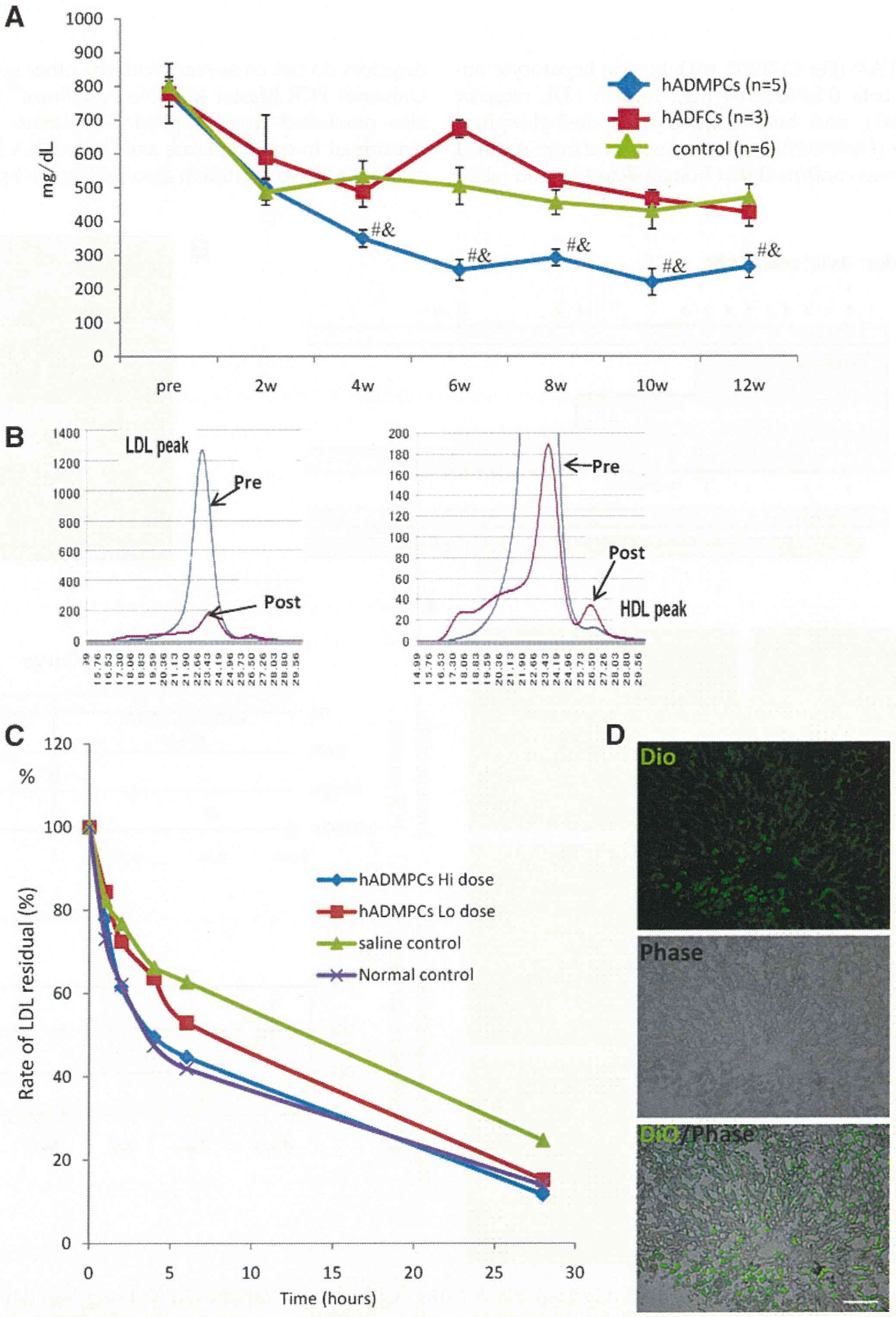
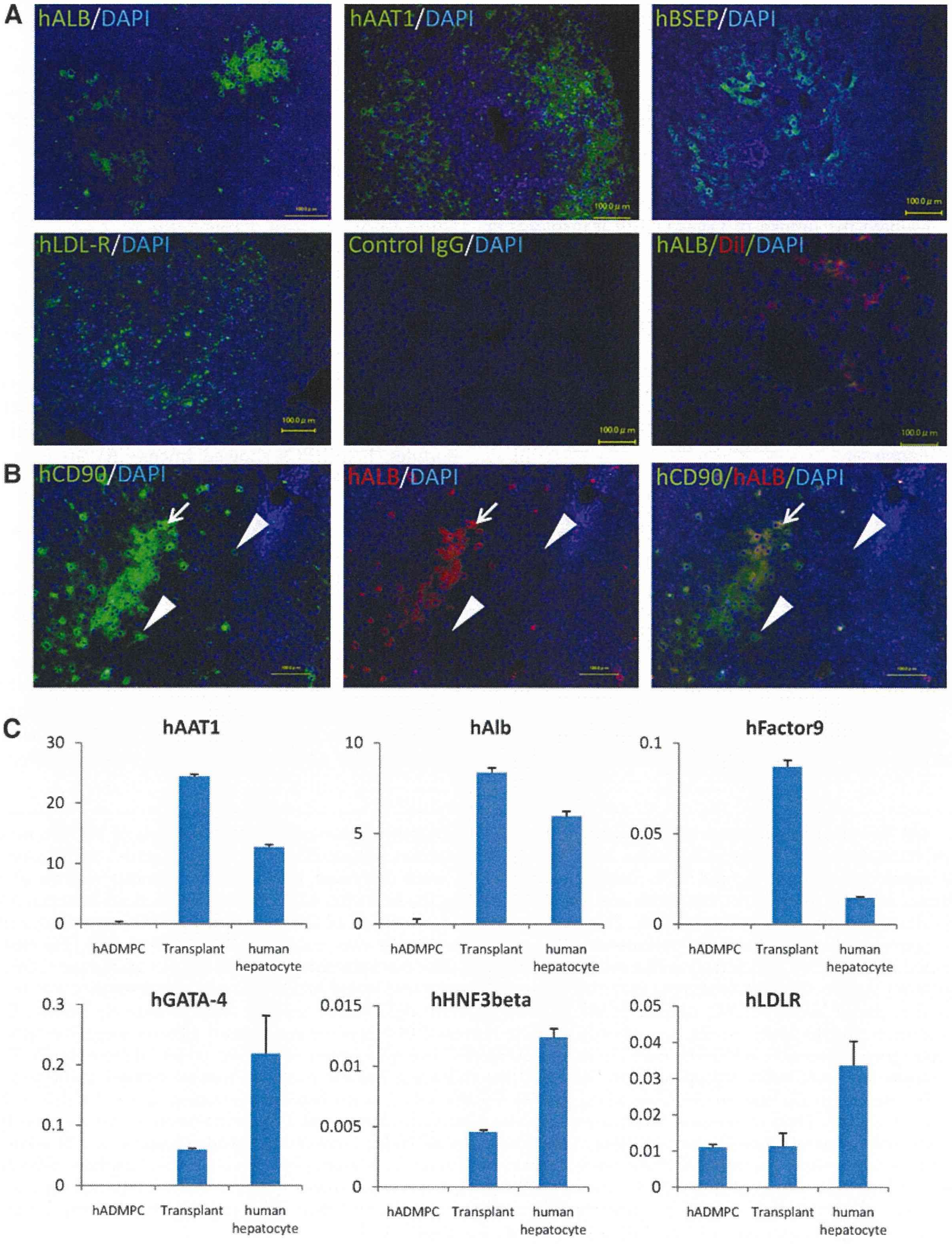


FIG. 3. **(A)** Total serum cholesterol levels. hADMPC transplantation in WHHL rabbits was followed for 12 weeks. Total serum cholesterol was measured in five rabbits that each received 3×10^7 hADMPCs, three rabbits that each received 3×10^7 hADFCs, and in six rabbits that received saline (control). Bars indicated mean ± standard error of the mean (SEM) ($^{\#}p < 0.05$; control vs. the hADMPC-transplanted WHHL rabbit; $^{\&}p < 0.05$; the hADFC-transplanted WHHL rabbit vs. the hADMPC-transplanted WHHL rabbit). **(B)** Lipoprotein profiles in a representative WHHL rabbit with hADMPC transplantation after gel filtration. Serum samples from the WHHL rabbit before and 4 weeks after transplantation were fractionated. Note the marked reduction in low-density lipoprotein (LDL) peak and appearance of high-density lipoprotein (HDL) peak. **(C)** Rate of clearance of LDL from the serum of rabbits with and without transplantation of hADMPCs. Animals were injected with ^{125}I-labeled human LDL, and the time course of clearance was monitored following trichroloacetic acid precipitation of serum at time 5 min, 1 h, 2 h, 4 h, 6 h, and 28 h. Residual ^{125}I-LDL was expressed as percentages of that at 5 min. $^{\#}p < 0.05$ (control vs. the hADMPC-transplanted WHHL rabbit [low dose]) and $^{*}p < 0.05$ (control vs. the hADMPC-transplanted WHHL rabbit [high dose]). **(D)** DiO-LDL uptake into hADMPC-derived hepatocytes in the WHHL rabbi liver. Thin-sliced recipient liver was incubated with DiO-labeled LDL in the serum-free medium for 24 h. After washing and fixation, the incubated slices were applied for fluorescent microscopy. DiO-LDL uptake cells (green) and no uptake parenchymal cells were observed in the section. Bar = 100 µm.

drogenase expression. To confirm that hADMPCs differentiated into hepatocytes *in vivo*, the cells before transplantation and human primary hepatocytes (Invitrogen, Lot number; HuP81) were applied for quantitative PCR as control.

Clearance of ^{125}I-LDL from rabbit serum

WHHL rabbits (8 weeks old) were anesthetized with pentobarbital (50 mg/kg). The peritoneum was incised and hADMPCs (high-dose; 3×10^7 cells/rabbit, $n=2$, low-dose; 5×10^6 cells/rabbit, $n=2$) suspended in 3 mL of HBSS (20°C) ($n=5$) or 3 mL of control saline ($n=2$) were infused into the portal vein via a 18-gauge Angiocath (BD). The rabbits were immunosuppressed using the protocol illustrated in Figure 1A. Eight weeks later, the animals were tested by the LDL turnover assay. ^{125}I human LDL (BT-913R, Lot No. 9130709; Biomedical Technologies Inc.) was delivered via the marginal ear vein of the WHHL rabbits and normal control



rabbits in physiological saline containing 2 mg/mL bovine serum albumin. Blood was collected from the opposite ear after injection at 5 min, 1 h, 2 h, 4 h, 6 h, and 28 h. ^{125}I-labeled apolipoprotein B-containing LDL was precipitated with 20% of trichloroacetic acid (Wako Pure Chemical Industries) (serum; 320 μL, 100% w/v trichloroacetic acid (TCA) 80 μL), and then the precipitants were applied for counting.

Uptake of DiO-labeled LDL by transplants ex vivo

Human LDL (1.019–1.063 g/mL) was isolated by sequential ultracentrifugation from normolipidemic donors as previously described,[24] dialyzed against saline-EDTA, and then sterilized by filtration through a 0.2 μm filter. Lipoproteins were labeled with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO; Sigma) by incubating the LDL in 0.5% bovine serum albumin/PBS with 100 mL DiO in dimethyl sulfoxide (3 mg/mL) for 8 h at 37°C. The lipoproteins were obtained by sequential ultra centrifugation (1.019–1.063 g/mL) as described,[14] and then dialyzed against PBS and filtered before use. To evaluate the uptake of DiO-LDL by transplants *ex vivo*, thin-sliced WHHL rabbit liver tissue were incubated with serum-free Dulbecco's modified Eagle's medium containing 10 μg/mL DiO-LDL for 24 h at 37°C. Finally, the incubated slices were rinsed, fixed with 10% formalin, sectioned into 5 μm thickness, and mounted with Perma-Flour (Japan Tanner Corporation). The slides were examined using a BioZero laser scanning microscope (Kyence).

Statistical analysis

Values were expressed as mean ± standard error of the mean. Differences between mean values of treated and untreated groups were evaluated using the Student's *t*-test. A $p\text{-value} < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS Statistics 17.0 package (SPSS Inc.).

Results

Characteristics of hADMPCs

The cells obtained from adipose tissue were seeded and incubated for 24 h (Fig. 1Ai). After incubation, the adherent cells were treated with EDTA solution, and the resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin-coated dishes (BD BioCoat) (Fig. 1Aii and 1Aiii). Within two to three passages after the initial plating of the primary culture, hADMPCs appeared as a monolayer of large flat cells (25–30 μm in diameter). As the cells approached confluence, they assumed a more spindle-shaped, fibroblastic morphology (Fig. 1Aiv). After passaging five to six times, the hADMPCs were applied for transplantation. We used flow cytometry to assess markers expressed by hADMPCs (Fig. 1B). The cells were negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells, ABCG-2, CD34, and CD133. They were also negative for CD31, an endothelial cell-associated marker and the surface antigen c-Kit (CD117). However, they stained positively for a number of surface markers characteristic of mesenchymal and/or neural stem cells, but not embryonic stem cells, including CD29, CD44 (hyaluronan receptor), CD73, CD105 (endoglin), and CD166. hADMPCs also were positive for stage-specific embryonic antigen-4. Next, adipogenic, osteogenic, and chondrogenic differentiation potential of hADMPCs were examined (Fig. 1C). Adipogenic differentiation was induced by culture with differentiation medium containing 1-methyl-3-isobutylxanthine (a peroxisome proliferator-activated receptor γ agonist), dexamethasone, and insulin. Induction was confirmed by the accumulation of intracellular lipid droplets that were stained with Oil Red O. After 7-day induction for osteogenesis, hADMPCs were stained with Alizarin red S for mineralized nodules. hADMPCs showed intense Alcian Blue staining, indicating chondrogenic induction capability of hADMPCs.

Serum cholesterol in WHHL rabbit with transplants

hADMPCs were separated from human subcutaneous adipose tissues, cultured for five to seven passages, and applied for transplantation into WHHL rabbits. WHHL rabbits received immunosuppressants and an antiviral agent as illustrated in Figure 2A, and then were transplanted 3×10^7 hADMPCs by portal vein infusion (Fig. 2B). At the day of and 1, 2, 4, 6, and 10 weeks after transplantation of hADMPCs via the portal vein, we examined whether the cells reside or not in the liver after transplantation. Typical

FIG. 4. **(A)** Immunohistochemical identification of human hepatocytic marker cells in liver sections of WHHL rabbits after hADMPC transplantation. Twelve weeks after hADMPC transplantation, human albumin-, human alpha-1-antitrypsin-, human bile salt export pump (BSEP)-, and LDL-receptor-positive cells were dispersed within the perivenous regions of the liver parenchyma, where they made contact with and integrated among the host cells with cell–cell interactions between hADMPC-derived cells and diseased hepatocytes pair. Ten weeks after transplantation of DiI-stained hADMPCs, copresence of human albumin (green) and pretreated DiI-fluorescence (red) on the same cells was observed. Bar = 100 μm. **(B)** Differentiation of transplanted hADMPCs into hepatocyte-like cells. Twelve weeks after transplantation, almost but not all human CD90-positive cells expressed human albumin, indicating that major population of transplanted hADMPCs could differentiate into hepatocyte-like cells (left panel: human CD90; middle panel: human albumin; right panel: merge). Arrows indicate human CD90 and human albumin double-positive cells; arrowheads indicate human CD90-positive but human albumin-negative cells. **(C)** Human hepatic gene expression in WHHL rabbit liver after hADMPC transplantation. RNA was prepared from the WHHL rabbit liver 12 weeks after hADMPC transplantation. We used the following hepatic markers: human alpha-1-antitrypsin, human albumin, human factor IX, human GATA-binding protein 4 (GATA-4), human hepatocyte nuclear factor 3 (HNF-3) beta, and human LDL-receptor. Their expression levels were examined by quantitative real time-polymerase chain reaction (RT-PCR) using Assays-on-Demand Gene Expression Assay Mix. The livers of WHHL rabbits that received saline ($n = 3$) were negative for human hepatic genes. The mRNA levels were normalized based on human glyceraldehyde-3-phosphate dehydrogenase expression as housekeeping gene and data are mean ± SEM of triplicate experiments. The livers of WHHL rabbits that received hADMPC transplantation ($n = 3$) were positive for human hepatic genes, and their expression levels were similar to those of human primary hepatocytes but not hADMPCs *per se*. Data are mean ± SEM.

distribution patterns of transplanted hADMPCs were followed in Figure 2C. DiI-fluorescent labeled-hADMPCs resided and distributed in the portal area at the day of transplantation. Six and 10 weeks after transplantation, DiI-positive transplanted cells migrated into centrilobular direction. Next, to demonstrate certain percentage of repopulation of the transplanted cells in the liver, the ratios of human-derived cell repopulation were examined by analyzing a repetitive DNA sequence at the day of and 2, 4, 6, and 12 weeks after transplantation (Fig. 2D). To indicate standard curve, we mixed the indicated percentage of hADMPCs with rabbit hepatocytes and plotted the obtained the amount of *Alu* PCR products, and estimated the amount of repopulation of the transplanted cells in the liver. At the day of transplantation, the ratio of hADMPCs to whole WHHL rabbit liver cells was 0.21% ± 0.056% (mean ± standard error of the mean) and the ratio decreased to 0.016% ± 0.002%, 0.011% ± 0.001%, and 0.009% ± 0.0001% after 2, 4, and 8 weeks of transplantation, respectively. After 12 weeks of transplantation, the ratio was increased to 0.024% ± 0.00005% as indicated (Fig. 2D).

To reveal the effects of hADMPC transplantation onto the lipid profiles of the WHHL rabbit, serum cholesterol levels were monitored over 12 weeks (Fig. 3A). Significant reductions in total serum cholesterol were observed within 4 weeks of the transplantation, and the reductions were maintained for the entire period. The reduction in serum cholesterol in the animals that received hADMPC transplantation was significantly greater than that of the control group. To determine the effects of hADMPC transplantation on the fractions of high-density lipoprotein and LDL in recipient animals, fractionation by fast protein liquid chromatography was performed (Fig. 3B). Transplantation of hADMPCs resulted in marked reduction of the peak LDL-cholesterol and increment of high-density lipoprotein cholesterol fraction (right panel).

Next, clearance experiments were performed with human LDL to confirm that the transplanted hADMPCs contributed the fall in serum cholesterol through uptake of LDL via LDL receptors. The rate of LDL clearance was significantly higher in the WHHL rabbits with transplanted hADMPCs than WHHL rabbits without transplanted hADMPCs (Fig. 3C). Rabbits with hADMPC transplants showed ~2.4-fold (high-dose; 3×10^7 cells/rabbit) and 1.4-fold (low-dose; 5×10^6 cells/rabbit) increase in the rate of LDL cholesterol clearance.

To evaluate the uptake of DiO-LDL by transplants *ex vivo*, thin-sliced WHHL rabbit liver was incubated with DiO-labeled LDL for 24 h and the uptake was examined as clearance experiment (Fig. 3D). DiO-LDL was uptaken by some but not all of the cells in the WHHL rabbit liver transplanted with hADMPCs. The DiO-LDL-uptaking cells were seen dispersed, contacted, and integrated among the nonuptaking parenchymal cells, suggesting that hADMPCs differentiated into hepatocytes *in vivo*, lowered of serum cholesterol via LDL uptake.

hADMPCs reside, survive, and differentiate into hepatocytes in vivo

After establishment of the graft as indicated by long-term lowering of serum cholesterol, human-specific hepatocytic proteins, such as albumin, alpha-1-antitrypsin, bile salt export pump, and LDL-receptor, positive cells were identified dispersed within perivenous regions of the liver parenchyma, where they have contacted and integrated among the host cells (Fig. 4A), with cell–cell interactions conserved between hADMPC-derived hepatocytes and diseased hepatocytes pair. Ten weeks after transplantation of DiI-prestained hADMPCs, copresence of human albumin (green) and pretreated DiI-fluorescence (red) on the same cells was observed (Fig. 4A), indicating the transplanted hADMPCs might differentiate into hepatocyte-like cells. To confirm transplanted hADMPCs might differentiate into hepatocyte-like cells and to reveal the efficacy of differentiation, the colocalization of human CD90 and human albumin was examined. As shown in Figure 4B, almost but not all human CD90-positive cells expressed human albumin, indicating that about 80% or more of transplanted hADMPCs could differentiated into human albumin-positive hepatocyte-like cells 12 weeks after transplantation. Next, to confirm the differentiation of hADMPCs into hepatocytes *in vivo*, expression of hepatocyte markers was analyzed by quantitative RT-PCR. The WHHL rabbit liver that was transplanted with hADMPCs expressed higher levels of human-specific alpha-1-antitrypsin, albumin, and coagulation factor IX than hADMPCs (Fig. 4C). The expression levels of human GATA-4, human hepatocyte nuclear factor 3 beta, and LDL-receptor were also higher in the WHHL rabbit liver than hADMPCs (Fig. 4C). These results indicate that hADMPCs differentiate into mature hepatocytes *in vivo*.

Discussion

We have used the WHHL rabbit to study the ability of hADMPC-derived hepatocytes to lower serum cholesterol in an animal model of FH. Our results have shown that hADMPCs transplanted into the rabbit liver differentiate into hepatocytes *in vivo* and effectively clear LDL from the circulation.

The reductions in cholesterol brought about by the engrafted hADMPC-derived hepatocytes suggest that human LDL receptors can act as replacement for the mutant LDL receptors in the WHHL rabbit. This capacity of hADMPC-derive hepatocytes is not unexpected, as the liver is the most important site of LDL uptake, accounting for >50% of total removal from the circulation, and the liver is only organ capable of converting cholesterol to bile for excretion. The substantial decrease in serum cholesterol achieved suggests that the hADMPC-derived hepatocytes both internalize LDL and metabolize the cholesterol to bile for excretion. The correlation between cholesterol and coronary heart disease has been well documented, and decreases in serum cholesterol of the magnitude that we have demonstrated would be expected to decrease morbidity and mortality in the patients with severe FH.[25]

The appearance of the hADMPC-derived hepatocytes as revealed by immunohistochemistry and RT-PCR indicated that the hADMPCs differentiated into hepatocytes and integrated into the liver parenchyma. The perivenous migration of the differentiated hepatocytes derived from hADMPCs along the portal-venous axis and suggests that hADMPCs recognize conserved signals on host cells and matrix. There are some reports describing the hepatogenic differentiation potential of hADMPCs.[15,16] These studies described that hepatocytes differentiated from hADMPCs *ex vivo* engrafted in the liver and functioned, and that the hADMPCs could be resided and changed their characters into hepatocyte-like cells only in the chemically damaged liver. These reports, revealing that hADMPCs have capabilities to differentiate into hepatocytes, hinted us that hADMPCs might differentiate into hepatocytes in liver. Hepatogenic signals from the microenvironment such as cell-to-cell connections or intermediates are probably important factors that dictate the type of functional hepatocytes in hepatic differentiation.[26] We are currently investigating the mechanism for the differentiation hADMPCs into hepatocytes.

The choice of cell source is critical for realizing success in cellular therapy. Liposuction surgeries yield a massive amount of lipoaspirate adipose tissue from 100 mL to >3 L as cell sources.[27] A major advantage of hADMPCs is their availability in safe and easy with few ethical issues, as compared with the shortage of human livers for orthotopic transplantation, which has been shown to be effective for the treatment of FH.[25] Our serum cholesterol reduction studies and *in vitro* studies demonstrated that human LDL binds to the hADMPC-derived hepatocytes receptor, indicating that this therapy will be useful in humans. Previous attempts to study the efficacy of hepatocyte transplantation in the WHHL rabbit model have employed allogenic hepatocytes, xenogenic hepatocytes, or hepatocytes transduced *ex vivo* with a recombinant retrovirus containing the LDL receptor cDNA.[6–13] The lowering effects of hepatocyte transplantation on serum cholesterol have been reported, but there was some problems. First, hepatocytes could not be expanded *ex vivo* with functional potentials; second, the cell viability reduced after cryopreservation; third, the many injected hepatocytes are supposed to be cleared by the reticuloendothelial system or lose viability during early phase. The rate of LDL clearance was returned to normal in LDL receptor knockout mice by introduction of an adenoviral construct containing an LDL receptor cDNA, and similar approaches have lowered serum cholesterol levels in the WHHL rabbit.[10,12,13] However, sustained expression of the LDL receptor from viral vectors can be difficult to achieve.[11,13] Moreover, hepatocytes derived from hADMPCs have the advantage that the LDL receptor is expressed from an endogenous gene with intact regulatory sequences. Such control of LDL receptor levels would not be expected after treatment of hypercholesterolemia with LDL receptor cDNA construct that lack the regulatory regions of the gene.[28]

Our experiments have shown that the hADMPCs expressed hepatocyte markers after transplantation *in vivo* and the integrated cells into parenchyma provide functional LDL receptors, indicating that they differentiated into hepatocytes and might lower serum cholesterol in the WHHL rabbit. These results suggested that hADMPC transplantation via portal vein could correct the metabolic defects of FH patients and that hADMPC-derived hepatocytes could function as supplier with plasma proteins derived from liver, giving us an idea that hADMPC-transplantation might be a novel cell therapy for hemophilia, alpha-1 antitrypsin deficiency, mucolipidosis, and other diseases caused by genetic defects for liver function. In near future, the therapy will be a novel therapy for kinds of inherited liver diseases.

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Disclosure Statement

All of the authors stated no conflict of interest.

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