研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
酒井佳夫 金子周一	肝硬変に対する再生医療の臨床試験評価		再生医療における臨床研究と製品開発	技術情報協会	東京	2013	427-431

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再生医療における臨床研究と製品開発

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「肝硬変に対する再生医療の臨床試験評価」　抜刷

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＜4＞　肝硬変に対する再生医療の臨床試験評価

はじめに

幹細胞学の発展により，その多分化能および抗炎症効果などのさまざまな生物学的作用が明らかになるにつれて，機能障害をきたしている臓器に対する再生療法への応用が試みられようとしている。肝硬変に対しても，幹細胞を含む細胞を用いる非臨床試験での有用性の報告，ならびに肝再生療法開発への臨床試験が進んでいる。本稿では，肝硬変の病態からの再生療法の目標，再生療法開発に用いられる可能性のある細胞を考察し，肝再生療法確立に向けた臨床試験における評価について概説する。

1.　肝硬変の病態からみた肝再生治療の目標

1.1　肝硬変の病態の特徴

肝硬変の原因は，慢性肝疾患であり，B型，C型肝炎ウイルスの持続感染による慢性肝炎，アルコール性肝炎，非アルコール性脂肪肝炎，自己免疫性肝炎および原発性胆汁性肝硬変等がある。慢性肝疾患において原因が除去されない場合，肝内に炎症が持続し肝細胞の壊死と再生が繰り返される。これに伴って，肝組織の小葉構造が破壊され，高度の線維化と再生結節が形成された状態が肝硬変である。肝硬変に至ると，肝臓が有するさまざまな機能の障害，低下があきらかとなってくる。肝臓は，生体内で最も多くの生化学反応を行う人体の生化学工場であるが，肝硬変ではこの生化学機能が低下する。また，肝臓への血流は，約4分の3が門脈から供給されているが，肝硬変状態にいたった高度の肝線維化状態では，門脈における圧が上昇した門脈圧亢進状態となる。こうした肝予備能の低下，門脈圧亢進状態によって，低アルブミン血症，腹水，浮腫，黄疸，胃食道静脈瘤，高アンモニア血症，肝性脳症等のさまざまな合併症が生じる[1]。肝硬変では，持続する肝内炎症をきたす原因が除去されない場合，これらの合併症を伴いながら肝不全に至る。腹水，黄疸，肝性脳症の臨床症状を呈する非代償期の肝硬変状態では，著しく予後が不良である[2]。重篤な肝不全にいたった場合，その治療法は唯一肝移植のみであるが，ドナー数は限られている。特に日本では脳死ドナーの深刻な不足があり[3]，生体肝移植に頼らざるを得ないが，この場合，生体ドナーへの侵襲も大きな問題である。また，肝硬変状態は高癌化の状態は高危険状態であり[4]，C型肝硬変の場合では，年率6～7％の発生頻度であることが知られているが，これには高度に進展した肝線維化状態が深く関与する[5]。このため，肝予備能のさらなる低下による肝不全への進展を防ぐ，また，進展した肝線維化状態を改善し，合併症である門脈圧亢進症を軽減し，さらに，肝細胞癌の発生率の軽減させることが，肝硬変患者の生活の質の向上と予後を改善することにつながる。すなわち，肝実質細胞機能の補填効果に加え，肝内に持続する炎症を抑制，消失させ，肝細胞の壊死を防ぐ，また，高度の肝線維化状態の改善を可能とする抗炎症修復効果を有する治療法が必要とされる。

1.2　肝再生療法開発に用いられる可能性のある細胞とその特徴

幹細胞に関する技術，知見の発展に伴って，さまざまな幹細胞について，臓器再生療法の実現化に向けた検討がなされようとしている。幹細胞には，全能性幹細胞である胚性幹細胞（ES細胞），生体内に元来存在する間葉系幹細胞，各臓器に存在する組織幹細胞が知られている[6]。また，人工的に線維芽細胞等の体細胞よりあらゆる細胞に分化しうる幹細胞（iPS細胞）を作製する技術が確立された[7]。ES細胞は，発生段階にあるヒト胚を扱うため，倫理上の問題があり，かつ非自己の細胞である，iPS細胞は，自己の細胞から作製可能なあらゆる細胞へ分化する全能性をもつ画期的な幹細胞であるが，作製効率，作製時間の短縮等が技術的に改善が必要であり，また，発生段階の全能性幹細胞と完全に同じか，造腫瘍性が否定できるかが課題である。生体内に元来存在する間葉系幹細胞は，骨髄，脂肪組織，臍帯組織に豊富に含まれる。特に骨髄，脂肪組織は，自己由来のものの採取が可能である。間葉系幹細胞は，さまざまな細胞への分化能力を有し，また，抗炎症効果など生体内の恒常性維持に有益な生体内反応をもたらす可能性があるため，障害をきたした臓器の再生療法への応用が期待されている。

2. 臨床試験と安全性・有効性評価

表1 再生療法開発に用いる細胞が加工製品に相当する場合の各ガイドライン
（医薬品医療機器総合機構（PMDA）「再生医療製品の実用化にむけたPMDAの取組み」より抜粋）

原材料管理	生物由来原料基準	平成15年5月20日	厚生労働省告示第210号
	ヒト・動物由来成分原料	平成12年12月26日	医薬発第1314号
製造管理・品質管理	ヒト（自己）由来細胞・組織加工製品	平成20年3月27日	薬食監麻発第0327025号
細胞・組織由来別の品質・安全性確保	ヒト（自己）由来細胞・組織加工製品	平成20年2月8日	薬食発第0208003号
	ヒト（同種）由来細胞・組織加工製品	平成20年9月12日	薬食発第0912006号
	ヒト（自己）体性幹細胞加工製品	平成24年9月7日	薬食発0907第2号
	ヒト（同種）体性幹細胞加工製品	平成24年9月7日	薬食発0907第3号
	ヒト（自己）iPS（様）細胞加工製品	平成24年9月7日	薬食発0907第4号
	ヒト（同種）iPS（様）細胞加工製品	平成24年9月7日	薬食発0907第5号
	ヒト（同種）iPS（様）細胞加工製品	平成24年9月7日	薬食発0907第5号
	ヒトES細胞加工製品	平成24年9月7日	薬食発0907第6号

2.1 再生医療開発における安全性評価

再生療法開発における臨床実用化に向けては，他の新しい治療法開発と同様に，まず安全性確認が必要とされる。幹細胞を用いる場合の再生療法の臨床研究では，「ヒト幹細胞を用いる臨床研究に関する指針（平成２２年厚生労働省告示第３８０号）」に従い，各施設倫理委員会での審議，厚生労働省科学技術部会における臨床研究計画の審査，厚生労働大臣意見が必要とされる。再生療法への応用開発として用いる幹細胞には，上述の如く，ES細胞，iPS細胞，間葉系幹細胞等，さまざまな幹細胞の可能性があるが，それぞれの特性に応じた評価が必要とされる。医薬品・医療機器に該当する製剤としての細胞を用いる再生療法開発の臨床試験では，取り扱う細胞について，原材料，製造管理・品質管理，および細胞・組織由来別の品質・安全性確保の観点から，関連するガイドラインを考慮しながらの検討が必要とされる（表1）。自己由来の細胞か，他家由来の細胞か，製造・加工を行った細胞か，等により，その安全性評価が異なると考えられる。

2.2 肝硬変の病態から考えられる有効性評価の項目

上述の如く，肝硬変は，高度の肝線維化状態に陥った慢性肝疾患の終末状態であり，原因が除去されない限り，肝内の慢性炎症，肝細胞の壊死と再生が続く。C型肝硬変の場合，原因となるC型肝炎ウイルスが除去された場合でも，肝硬変状態の本態である高度の線維化状態が改善することが知られているが，その改善速度は極めて遅い[8]。すなわち，短期間では線維化の明らかな改善のみを評価項目として有効性を判断するのは困難と考えられる。肝硬変の病態を考慮すると，肝予備能，門脈圧亢進にともない生じる症状，および肝炎の程度を反映する項目が，再生療法の効果判定の評価に用いられる（図1）。具体的には，肝細胞壊死の指標として，肝細胞が破壊された際に血中に逸脱する酵素であるアスパラギンサントランスフェラーゼ（AST），アラニントランスフェラーゼ（ALT）の血清中の活性値，肝予備能の指標として，肝臓の生合成能，代謝能力を反映する血清総タンパク濃度，アルブミン濃度，総ビリルビン値，および凝固検査におけるプロトロンビン時間が評価されうる。また，腹部超音波検査，CT等，画像での腹水の有無，程度の評価，臨床症状として，肝性脳症，浮腫，が評価項目として考えられる。

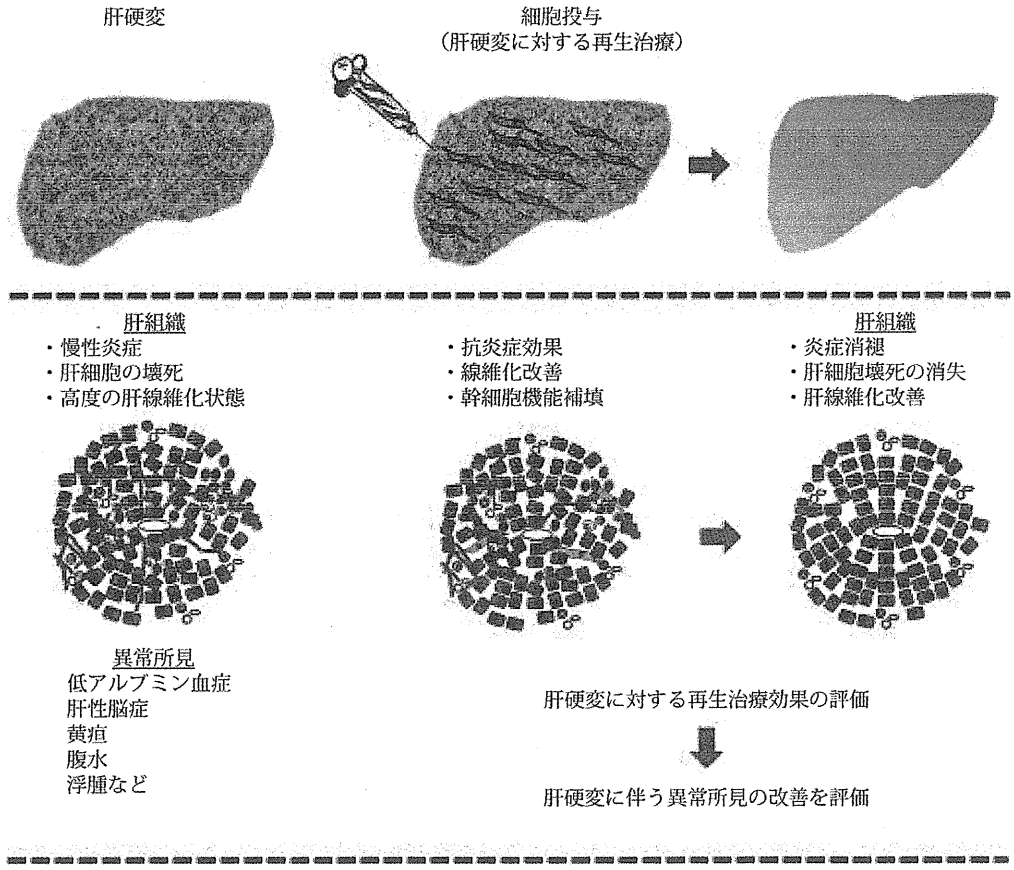

図1　肝硬変の病態からみた再生療法効果の評価

2.3　肝硬変に対する肝再生療法の臨床試験の実際について

表2に ClinicalTrials.gov に登録されている，肝硬変を対象とした再生療法臨床試験と考えられ，治療効果に関する評価項目が記されている主な臨床試験を示した。用いる細胞には，骨髄細胞，末梢血幹細胞，臍帯由来細胞などがあり，自己由来細胞，同種細胞，培養細胞を用いる臨床試験が登録されている。ほとんどの臨床試験は第Ⅰ相，Ⅱ相相当の試験であり，安全性と有効性探索を目的としている。評価項目として，生存率での評価のほか，肝線維化，肝炎活動性の指標である AST，ALT を評価項目としている臨床試験がある。試験評価期間については多くは6か月であり，2年の臨床試験もある。

表２　clnicaltrials.gov に登録されている治療効果に関する評価項目が記されている主な肝再生療法の臨床試験

臨床試験タイトル	登録番号	スポンサー	投与細胞	Phase	投与方法	選択条件における肝硬変重症度	評価期間	主要評価項目	副次評価項目
Autologous Bone Marrow Stem Cells in Cirrhosis Patients	NCT00713934	Royan Institute	骨髄由来 CD133 陽性細胞	Phase 1, 2	portal vein infusiion	C-P* B or C	6か月	Liver function test, MELD* score	Cirrhosis mortality
The Effectiveness and Safety for Mesenchymal Stem Cell for Alcoholic Liver Cirrhosis	NCT01741090	Yonsei University	骨髄由来間葉系幹細胞	Phase 2	Intraarterial（Hepatic）	C-P B or C	6か月	The improvement of Liver Histologic grade according to Metavir and Laennec fibrosis scoring system	The evaluation of hepatic dendritic cells activity by immunohistochemistry, Liver fibrosis quantitative analysis using Hydroxyproline contents in liver tissue, Real-Time PCR for relative mRNA expression of TGF-beta, collagen, procollagen, MMP2 or 9, HVPG*, Hepatic vein arrival time using microbubble contrast enhanced ultrasonography, Liver stiffness measurement with transient elastography, C-P score, MELD score
Dose Finding Study to Assess Safety and Efficacy of Stem Cells in Liver Cirrhosis	NCT01591200	Stempeutics Research Pvt Ltd	間葉系幹細胞（培養）	Phase 2	Intraarterial（Hepatic）	C-P B or C	2年	Safety	Liver function tests, CT scan of abdomen, Change in MELD score, Improvement in quality of life as assessed by SF 36 questionnaire, Histological evaluation of liver biopsy by immunohistochemical staining for AFP, PCNA, SMA, C-P score
Safety and Efficacy of Stem Cell Transplantation for Treatment of Liver Cirrhosis	NCT01491165	General Hospital of Chinese Armed Police Forces	臍帯由来間葉系幹細胞	Phase 2, 3	through interventional procedures	compensted cirrhosis	1年	liver volume calculated by MR	liver biopsy, gastroscopy（Observe and photograph the related varicose veins）, blood biochemistry, blood test, liver enzyme fiber spectrum, coagulation, portal vein and splenic vein measure
Safety and Efficacy of Human Bone Marrow Stem Cells for Treatment of HBV-related Liver Cirrhosis	NCT01724697	Fourth Military Medical University	自己骨髄間葉系幹細胞	Phase 1, 2	via hepatic artery	C-P score 9-15	1年	one year survival rate	MELD, AFP, renal function, C-P score
Safety and Efficacy of Human Umbilical Cord Derived Mesenchymal Stem Cells for Treatment of HBV-related Liver Cirrhosis	NCT01728727	Fourth Military Medical University	臍帯由来間葉系幹細胞	Phase 1, 2	via hepatic artery	C-P score 9-15	1年	one year survival rate	MELD, AFP, renal function, C-P score
Improvement of Liver Function in Liver Cirrhosis Patients After Autologous Mesenchymal Stem Cell Injection:a Phase I-II Clinical Trial	NCT00420134	Shaheed Beheshti Medical University	自己間葉系幹細胞由来前駆肝細胞	Phase 1, 2	portal vein under ultrasound guide	MELD over 10,	6か月	Liver function test, MELD score	Cirrhosis mortality
ABMSC Infusion Through Hepatic Artery in Portal Hypertension Surgery for the Treatment of Liver Cirrhosis	NCT01560845	Wenzhou Medical College	自己骨髄由来幹細胞	Phase 2, 3	hepatic artery	Advanced liver cirrhosis after hepatitis B resulted in bleeding from esophageal varices and hypersplenism, and needed open abdominal portal hypertension surgery;	1か月	C-P score	Incidence of complications, Mortality, blood test（hypersplenism）, liver volume calculated by CT, Indocyanine green（ICG）retention（clearance）, blood biochemistry
Mesenchymal Stem Cells Treat Liver Cirrhosis	NCT01233102	Chinese Academy of Sciences	臍帯由来間葉系幹細胞	Phase 1, 2	Hepatic artery infusion or Intravenous infusion	C-P more than 8, MELD more than 20	1年	ALT, T-Bil, PT, prealbumin, albumin, overall survival	
HIV Liver Regeneration Project for HIV Patients With Cirrhosis by Autologous Bone Marrow Transplantation	NCT01309594	National Center for Global Health and Medicine, Japan	自己骨髄細胞		intravenous	have cirrhosis with 7 or higher C-P Score in C-P Score B	24週	Post-transplantation prognosis for cirrhosis, C-P score, albumin, erum fibrosis markers, Transient Elastography , ascites imagery, SF-36v2（TM）Health Survey.	
Evaluate Safety and Efficacy of Autologous Bone Marrow-derived Endothelial Progenitor Cells in Advanced Liver Cirrhosis	NCT01333228	Clinica Universidad de Navarra, Universidad de Navarra, Spain	自己骨髄由来内皮前駆細胞	Phase 1, 2	hepatic artery	C-P 8 or above	12週	Number of Participants with Adverse Events as a Measure of Safety and tolerability	AST, ALT, albumin, PT, MELD, HVPG, ascites grade, episodes of upper gastrointestinal bleeding, episodes of hepatic encephalopathy
Safety and Efficacy of Human Autologous Peripheral Blood Stem Cells for Treatment of HBV-related Liver Cirrhosis	NCT01728688	Fourth Military Medical University, China	末梢血液幹細胞	Phase 1, 2	hepatic artery	C-P score 9-15	1年	one-year survival rate	MELD score, C-P score, alpha fetoprotein, renal function
The Effectiveness and Safety for Mesenchymal Stem Cell for Alcoholic Liver Cirrhosis	NCT01741090	Yonsei University, Korea	骨髄由来間葉系幹細胞（培養）			C-P class B or C, ≥ 7 scores	6か月	The improvement of Liver Histologic grade	The evaluation of hepatic dendritic cells activity by immunohistochemistry, Liver fibrosis quantitative analysis using Hydroxyproline contents in liver tissue, Real-Time Polymerase Chain Reaction for relative mRNA expression of TGF-beta, collagen, procollagen, MMP2 or 9, Hepatic venous pressure gradient（HVPG）, Hepatic vein arrival time using microbubble contrast enhanced ultrasonography, Liver stiffness measurement with transient elastography, C-P score, MELD score
Human Menstrual Blood-derived Mesenchymal Stem Cells for Patients With Liver Cirrhosis	NCT01483248	S-Evans Biosciences Co.,Ltd., China	月経血由来間葉系幹細胞	Phase 1, 2	intravenous	not specified	48週	Overall Survival	Liver function improvement, complications such as fever,anaphylaxis,cough,chest distress,and dyspnea,et al, The improvement of ascites after 12-week treatment, C-P score, MELD score, SF36-quality of life
Human Umbilical Cord Mesenchymal Stem Cells Transplantation for Patients With Decompensated Liver Cirrhosis	NCT01342250	S-Evans Biosciences Co.,Ltd., China	臍帯血細胞	Phase 1,2	not described	C-P B/C（7-12 points）; or Meld score ≦ 21	1年	Overall Survival	Liver function improvement, The size of liver and the width of portal venous, Incidence of hepatocellular carcinoma within 1 year, C-P score, MELD score,SF36-quality of life（SF36-QOL）, The clinical symptom improvement（including appetite, debilitation, abdominal distension, edema of lower limbs, et al ）

*C-P:Child-Pugh, HVPG:Hepatic Venous Pressure Gradient , MELD :model for endstage liver disease

3. 肝再生療法の確立，実用化にむけて

上述のごとく，海外ではさまざまな幹細胞および幹細胞を含む細胞，前駆細胞を用いた肝硬変に対する再生療法開発の臨床試験が計画，実施されているが，我が国において，安全で有効な肝再生療法を確立し，実用化するためには，関連する規制，法規に従い臨床試験を行う必要がある。国民皆保険の我が国において標準治療として認められるには，治療法の保険適応が必要である。また，細胞あるいは細胞製剤等について薬事承認が必要な医薬品・医療機器を扱う再生療法開発においては，治験による開発が必要となる。再生療法に用いられる可能性のある幹細胞，前駆細胞は，培養しない自己由来の細胞，培養した細胞，同種細胞，さらに人工的に製造されるiPS細胞とさまざまであり，実用化にむけた臨床試験では，早期から規制当局と相談を行い，評価項目，方法を確認し，臨床研究，臨床試験を実施していくことが極めて重要である。前術の如く，肝硬変の病態は，肝機能の低下のみならず，生活の質を低下させるさまざまな合併症を生じる慢性疾患の終末状態である。肝再生療法の有効性の評価には，短い期間では，肝予備能低下，門脈圧亢進症，炎症の程度が臨床試験での評価項目と考えられるが，長期間における生存率の改善に，生活の質を低下させる合併症の改善効果も重要な指標と考えられ，実用化後においての評価の視点となる。

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REGULAR ARTICLE

Improvement of liver fibrosis by infusion of cultured cells derived from human bone marrow

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Abstract We develop "autologous bone marrow cell infusion (ABM*i*) therapy" for the treatment of human decompensated liver cirrhosis and confirm the efficacy and safety of this treatment in multicenter clinical studies. With the goal of further expanding the applications of ABM*i*, we first cultured human bone marrow cells and then determined whether a cell fraction found to be effective in improving liver fibrosis can be amplified. Cells harvested after two passages (P2 cells) consistently contained approximately 94 % mesenchymal stem cells (MSCs); conversely, the cells harvested after only medium change (P0 cells) contained many macrophages. MSCs (2.8×10^8) in P2 cells were harvested from 3.8×10^8 bone marrow-derived mononuclear cells after 22 days. DNA-chip analysis also showed during the culturing step that bone marrow-derived cells decreased with macrophage phenotype. The infused 5×10^5 P2 cells significantly improved liver fibrosis in the nonobese diabetic/severe combined immunodeficient (NOD-SCID) mouse carbon tetrachloride (CCl_4) liver cirrhosis model and induced the expression of matrix metalloproteinase (MMP)-9 and suppressed expressions of alpha smooth muscle actin (αSMA), tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ) in the liver. Cultured human bone marrow-derived cells (P2 cells) significantly inhibited liver fibrosis. The increase of MMP-9 and suppressed activation of hepatic stellate cells (HSCs) through the regulation of humoral factors (TNFα and TGFβ) contribute to the improvement of liver fibrosis by MSCs comprising about 94 % of P2 cells. MSCs in cultured human bone marrow-derived mononuclear cells (BM-MNCs) proliferate sufficiently in cell therapy, so we believe our cultured bone marrow-derived cell therapy can lead to expanded clinical applications and enable outpatient therapy.

Keywords Autologous bone marrow cell infusion · Nonobese diabetic/severe combined immunodeficient mouse · Carbon tetrachloride · Mesenchymal stem cell · Matrix metalloproteinase

Abbreviations

αSMA	Alpha smooth muscle actin
ABMi	Autologous bone marrow cell infusion
BM-MNC	Bone marrow-derived mononuclear cell
CCl_4	Carbon tetrachloride
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HSC	Hepatic stellate cell
IHC	Immunohistochemistry
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NOD-SCID	Nonobese diabetic/severe combined immunodeficient
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription polymerase chain reaction
TNFα	Tumor necrosis factor alpha
TGFβ	Transforming growth factor beta

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Introduction

In the past, fluorescence in situ hybridization has been used to confirm the presence of the Y chromosome in liver and gastrointestinal tissues in autopsies of patients (XX) with blood disorders who had undergone bone marrow grafts (XY) and these findings suggested the presence of pluripotent stem cells in bone marrow cells (Alison et al. 2000; Theise et al. 2000). In addition, adherent cells ($CD90^+$, $CD44^+$ $CD14^-$, $CD34^-$ and $CD45^-$) have been isolated from human bone marrow aspirate, which suggests that these pluripotent cells are MSCs (Pittenger et al. 1999).

In our laboratory, we began basic research in mice concerning autologous bone marrow cell infusion (ABM*i*) therapy for liver cirrhosis by focusing on the presence in bone marrow of these pluripotent cells that engraft in the liver. We established a "murine green fluorescent protein (GFP)/CCl_4 model" to evaluate bone marrow cell differentiation and proliferation in liver cirrhosis (Terai et al. 2003). We found that liver fibrosis was improved in the same liver cirrhosis model because the infused bone marrow cells produce MMP-9 and other substances (Sakaida et al. 2004). Using the results of this basic research in mice as a foundation, in 2003, we began a clinical study entitled "ABM*i* therapy for decompensated liver cirrhosis" and we were the first to report on the efficacy and safety of this therapy (Terai et al. 2006). In joint research with this laboratory, researchers at Yonsei University in Korea have recently found that the therapeutic effect of ABM*i* lasts for at least 1 year in patients with hepatitis B-induced liver cirrhosis (Kim et al. 2010). In addition, in joint research with Yamagata University, we found that improved liver function in patients with alcoholic liver cirrhosis continues for 6 months after ABM*i* therapy (Saito et al. 2011). Cell therapy using bone marrow cells is a promising therapy for liver cirrhosis (Terai et al. 2012; Takami et al. 2012).

However, current ABM*i* therapy requires the collection of bone marrow aspirate under general anesthesia, so there are strict usage criteria regarding the general health condition of patients. Therefore, with the goal of expanding the therapeutic applications of ABM*i*, in our laboratory, we cultured human bone marrow cells and evaluated whether the cell fraction that has an improving effect on liver fibrosis can be amplified. We found that, from the standpoint of growth capability and pluripotency, the second passage (P2) cells comprise a fraction that is clearly more important for liver cirrhosis therapy. In addition, we set out to analyze the mechanism of liver fibrosis improvement using mouse livers in which an improvement in fibrosis brought about by cell infusion had already been confirmed. Here, we report our findings on the effect on liver fibrosis of cultured human bone marrow-derived cells.

Materials and methods

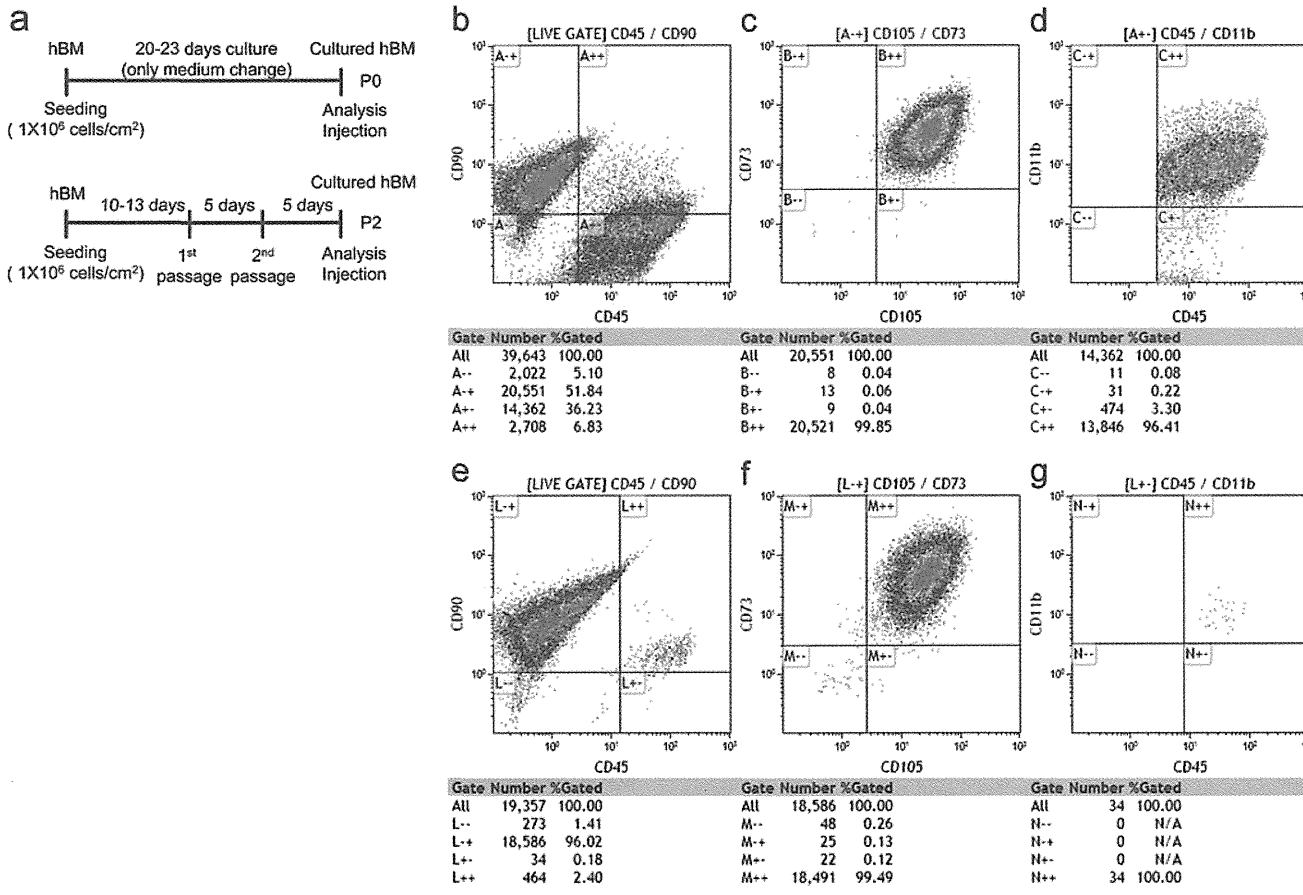
Preparation of culture of human BM-MNCs and human MSCs

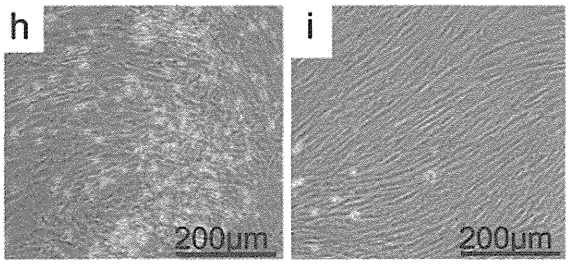
Human BM-MNCs (Code:2M-125A, male, HIV/HBV/HCV-negative) were purchased from Lonza (Basel, Switzerland). Human BM-MNCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (GIBCO) and penicillin/streptomycin (GIBCO) on non-coated dishes (Becton and Dickinson, NJ, USA). Human BM-MNCs were plated at a density of 1×10^6 cells/cm^2 and incubated at 37 °C with 5 % CO_2. After 20–23 days culture, proliferated cells grown under only medium change every 3 days (P0 cells) or through two successive passages on days 10–13 and 5 additional days before confluence (P2 cells) were harvested (Fig. 1a). The cells were detached with trypsin-EDTA (0.05 % trypsin, 0.53 mM EDTA-4Na) (GIBCO) for 5 min at 37 °C. Human MSCs were cultured in the same manner and 2–3 successive passages were performed. Before infusion, these cells were trypsinized and washed twice with phosphate-buffered saline (PBS) (GIBCO).

Characterization of cultured human BM-MNCs

The cell marker expressions of P0 and P2 cells were analyzed by flow cytometry (FACS Calibur; Becton and Dickinson). Cells were stained using the following preconjugated antibodies: CD45, CD90, CD105 (eBioscience, CA, USA), CD73 and

Fig. 1 The majority of cultured human bone marrow-derived mononuclear cells (BM-MNCs) (P2 cells) were phenotypically mesenchymal stem cells (MSCs). **a** Human BM-MNCs (hBM) were plated at a density of 1×10^6 cells/cm^2 on non-coated dishes in 10 % FBS-DMEM and incubated. After 20–23 days of culture, proliferated cells with only medium change every 3 days (P0 cells) or two successive passages on days 10–13 and 5 additional days before confluence (P2 cells) were harvested. **b–d** Cellular characteristics of P0 cells. Typical data from analysis by flow cytometry are shown. P0 cells were approximately fractionated into two subgroups: MSCs and macrophages. CD45 (+) cells (hematopoietic cells) accounted for 43.1 % of P0 cells and macrophages (CD45 and CD11b positive and CD90 (−) cells) accounted for 34.9 %; conversely, 51.8 % were MSCs (CD45 (−) and CD90, CD105 and CD73 positive). **e–g** Cellular characteristics of P2 cells. In contrast to P0 cells, the majority of cultured human BM-MNCs after two passages (P2) were MSCs (95.5 %), 2.6 % were CD45 (+) cells and 0.2 % were macrophages. **h** On the photomicrograph (×100), P0 cells were contaminated with many round hematopoietic cells. Conversely, **i** on the photomicrograph (×100), P2 cells were homogenous fibroblastic-shaped cells. **j** The mean MSC percentage of P2 cells from four healthy men was 94.1±2.6 % and individual differences were small. **k** After 13 days of plating of human BM-MNCs at a density of 1×10^6 cells/cm^2 (3.8×10^6 cells/3.8 cm^2) and incubation in 10 % FBS-DMEM, adhesive cells had proliferated to $0.7\pm0.3\times10^5$ cells/3.8 cm^2 ($n=5$) and under two successive passages (P2) grew to $28.3\pm15.9\times10^5$ cells/3.8 cm^2 ($n=5$)





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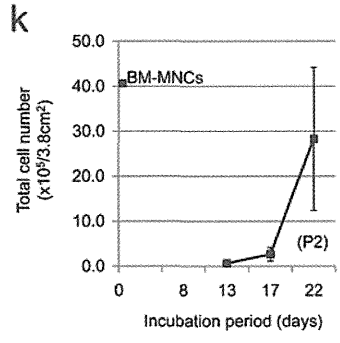
	BM-MNCs	day13 (pre passage)	day17 (1st passage)	day22 (P2) (2nd passage)
Alived cell number (x10⁵/3.8cm²/well) (n=5)	38.0	0.66±0.31	2.7±1.5	28.3±15.9



	P0	P2
MSC(%) (CD90+/CD105+/CD73+/CD45-) (n=4)	66.2±11.7	94.1±2.6

CD11b (Beckman Coulter, CA, USA). Through fluorescence-activated cell sorting analysis, the phenotypical MSC ratio (%) was examined. P2 cells were stained with additional antibodies to confirm MSC phenotypically: CD34, CD13, CD45, CD73, CD90, HLA-DR, HLA-ABC, iso IgG (BD Pharmingen, CA, USA), CD44, CD105, CD11b (Beckman Coulter) and CD117 (Beckton and Dickinson).

Differentiation and DNA-chip analysis

P2 cells were cultured with each differentiation medium and evaluated for adipogenesis (Oil-Red O, anti-mouse FABP-4 antibody), osteogenesis (Alizarin Red) and chondrogenesis (anti-human aggrecan antibody) with a human MSC functional identification kit (Invitrogen, NY, USA). We compared DNA expression between P0 and P2 cells using the DNA-chip system (Agilent Technology, CA, USA). We next also analyzed the expression pattern using the IPA software system (Ingenuity Systems, CA, USA).

Experimental protocol for the NOD-SCID mouse CCl_4 liver cirrhosis model

All animals received humane care and the experiments were approved by the Animal Experiment Committee of Yamaguchi University School of Medicine according to the National Institutes of Health criteria. NOD.CB17-Prkdc^{scid}/J female mice 5 weeks of age purchased from Charles River Laboratories (MA, USA) were properly anesthetized during the experiments.

NOD.CB17-Prkdc^{scid}/J female mice 6 weeks of age were treated with CCl_4 (Wako, Tokyo, Japan) dissolved in corn oil (Wako) (1:3) twice a week for 6 weeks, only once with 0.5 mL/kg body (0.25 μg/g) CCl_4, from the second time with 1.0 mL/kg body weight (0.5 μg/g), and for the last 4 weeks with 1.5 mL/kg body weight (0.75 μg/g). These were used as the control group of the NOD-SCID mouse CCl_4 liver cirrhosis model. Treatment with 1.5 mL/kg body weight CCl_4 was continued for a further 4 weeks. Then, 5.0×10^5 P2 cells for the P2-administration group ($n=18$) and 5.0×10^5 P0 cells for the P0-administration group ($n=7$) were infused through the tail vein, while in the control group ($n=13$), only PBS was injected.

Quantitative analysis of liver fibrosis

After 4 weeks of administration of P2 or P0 cells, the livers of the NOD-SCID mouse CCl_4 liver cirrhosis model were fixed in 4 % formaldehyde and 3-μm paraffin sections were used for analysis. The liver fibrosis area was quantified with Sirius red staining and assessed by application software (BIOREVO microscope BZ-9000, BZ-II; KEYENCE, Osaka, Japan) at a magnification of ×100. The mean value of 10 randomly selected areas per sample was used as the expressed percent area of fibrosis.

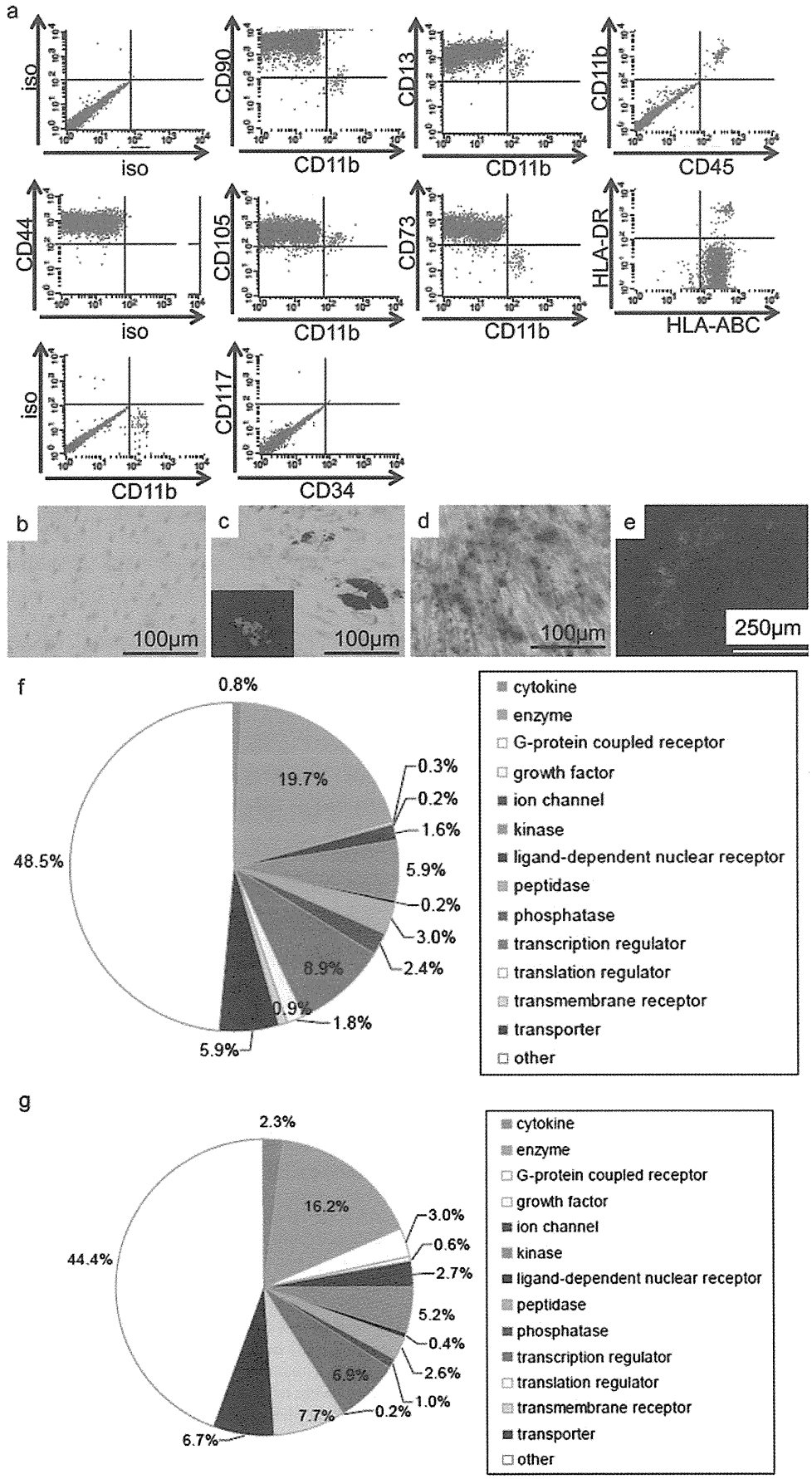
Fig. 2 P2 cells were phenotypically and functionally mesenchymal stem cells (MSCs). **a** The phenotypical character of P2 cells was consistent with that of MSCs. Hematopoietic stem cell marker (CD34)-positive cells in P2 cells were only 0.06 %. **b**–**e** P2 cells (×200) (**b**) differentiated into adipocytes (×200) (**c**), osteocytes(×200) (**d**) and chondrocytes (× 40) (**e**). The control group cells (**b**) were cultured in only 10 % FBS-DMEM. **f** Category of up-regulated genes in P2 cells than those in P0 cells. **g** Category of down-regulated genes in P0 cells than those in P2 cells

Immunohistochemistry of MMP-9 and αSMA

Three-micrometer paraffin sections of livers 2 or 4 weeks after administration of P2 cells were used for immunostaining. MMP-9 and αSMA detection required antigen retrieval with Vector Antigen Unmasking Solution (Vector Laboratories, CA, USA) and the bound antibodies were detected using the avidin-biotin complex method staining kit (Vector Laboratories). Primary antibodies were used at the following dilutions: 1:100 for MMP-9 (R and D Systems, MN, USA) and 1:300 for αSMA (Abcam, Cambridge, UK). Biotinylated antibody was used as the secondary antibody. The number of MMP-9(+) cells was counted at a magnification of ×200 and the mean value of six randomly selected areas per sample was assessed. The αSMA(+) area (%) was quantified at a magnification of ×100 and the mean value of ten randomly selected areas per sample was assessed. The same application software described above was used.

Quantification of messenger RNA levels by real-time reverse-transcription polymerase chain reaction (PCR)

Total RNA was extracted from the liver of mice 4 weeks after P2 cell infusion using RNA extraction solution (ISOGEN; Nippon Gene, Tokyo, Japan) and complementary DNA was generated from 500 ng of RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, IN, USA). Primers for the messenger RNA (mRNA) expression of MMP-9, αSMA, TNFα and TGFβ were evaluated using real-time PCR. Real-time PCR was performed with SYBR Green Master Mix (Roche Diagnostic, Basel, Switzerland). The primers used for MMP-9 were 5'-TCT CTA CGG CCG GCT TTG CT-3' (forward) and 5'-GGC AAG TCT TCA GAG TAG TT-3' (reverse), those for αSMA were 5'-ACT CTC TTC CAG CCA TCT TTC A-3' (forward) and 5'-ATA GGT GGT TTC GTG GAT GC-3' (reverse), those for TNFα were 5'-CAG GTT CTG TCC CTT TCA CTC ACT-3' (forward) and 5'-GTT CAG TAG ACA GAA GAG CGT GGT-3' (reverse), those for TGFβ1 were 5'-TGG AGC AAC ATG TGG AAC TC-3' (forward) and 5'-CAG CAG CCG GTT ACC AAG-3' (reverse) and those for β-actin were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enzyme
G-protein coupled receptor
growth factor
ion channel
kinase
ligand-dependent nuclear receptor
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kinase
ligand-dependent nuclear receptor
peptidase
phosphatase
transcription regulator
translation regulator
transmembrane receptor
transporter
other

5'-TGA CAG GAT GCA GAA GGA GA-3' (forward) and 5'-GCT GGA AGG TGG ACA GTG AG-3' (reverse).

Statistics

Data are presented as mean ± standard error of the mean. The two-tailed Student's *t* test was used to analyze parametric data.

Results

The majority of P2 cells were phenotypically and functionally MSCs

Cultured human BM-MNCs without passage (P0 cells) were approximately fractionated into two subgroups: MSCs and macrophages. CD45 (+) cells (hematopoietic cells) accounted for 43.1 % of P0 cells and macrophages [CD45 and CD11b positive and CD90 (−) cells] accounted for 34.9 %; conversely, 51.8 % were MSCs [CD45 (−) and CD90, CD105 and CD73 positive] (Fig. 1b–d). In contrast to P0 cells, the majority of cultured human BM-MNCs under two passages (P2 cells) were MSCs (95.5 %), 2.6 % were CD45 (+) cells and only 0.2 % were macrophages (Fig. 1e–g). On the photomicrograph (×100), P0 cells were contaminated with many round hematopoietic cells; conversely, P2 cells were homogenous fibroblastic-shaped cells (Fig. 1h–i). The mean MSC percentage of P2 cells from four healthy men were 94.1±2.6 % and individual differences were small (Fig. 1j). After 13 days of plating of human BM-MNCs at a density of 1×10^6 cells/cm^2 (3.8×10^6 cells/3.8 cm^2) and incubation in 10 % FBS-DMEM, adhesive cells grew to $0.7\pm0.3\times10^5$ cells/3.8 cm^2 ($n=5$) and, under two successive passages, P2 cells grew to $28.3\pm15.9\times10^5$ cells/3.8 cm^2 ($n=5$) (Fig. 1k). The phenotypical character of P2 cells was consistent with that of MSCs. Hematopoietic stem cell marker (CD34)-positive cells in P2 cells accounted for only 0.06 % (Fig. 2a). P2 cells differentiated into adipocytes, osteocytes and chondrocytes (Fig. 2b–e).

Comparison of DNA expressions between P2 cells and P0 cells

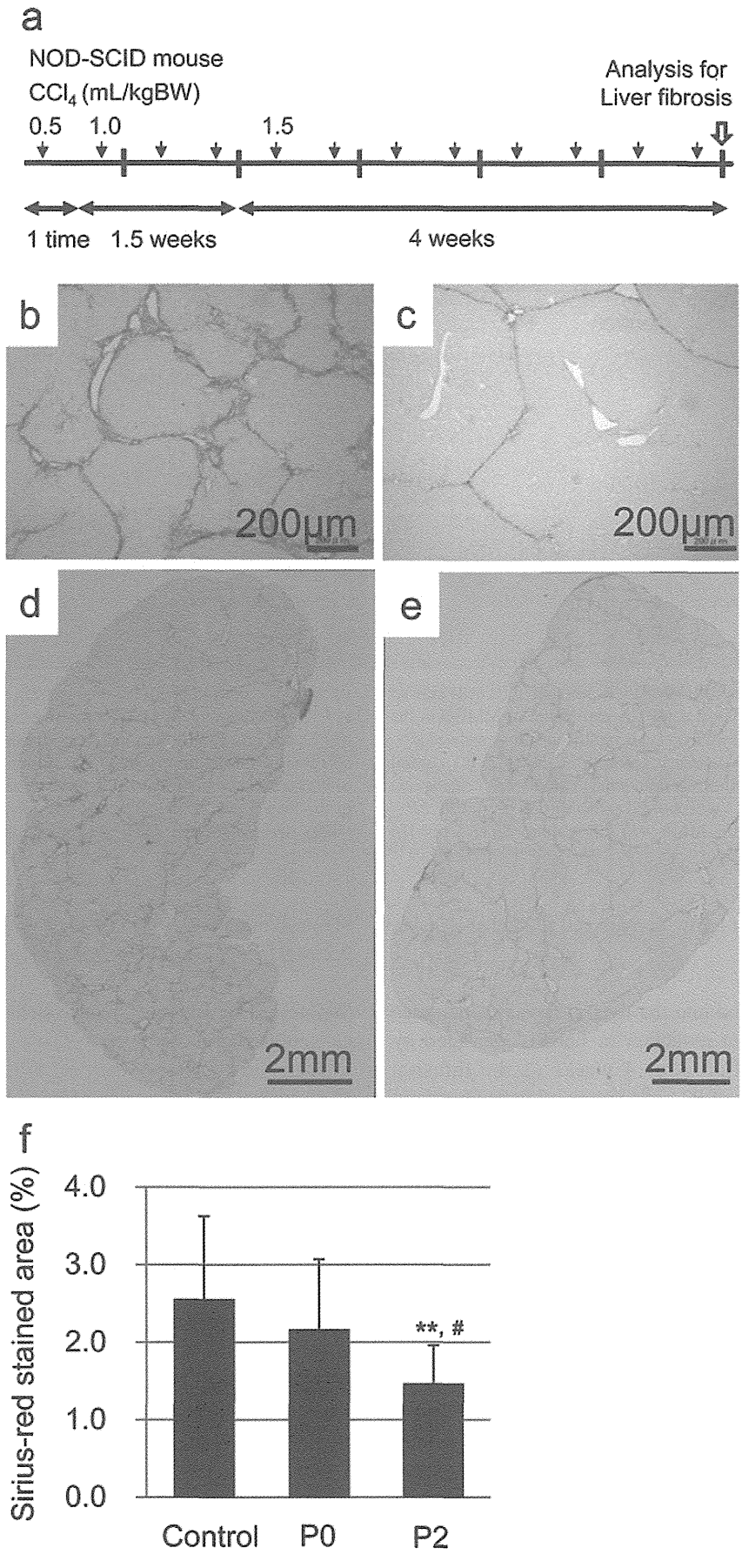
We performed DNA-chip analysis using an IPA system. The majority gene that is upregulated is 1,569 probe. In P2 cells, genes related with cell cycle, G2/M DNA damage checkpoint regulated genes were up-regulated. On the other hand, genes related with function of blood cells, proliferation of blood cells and leukocyte migration were down-regulated, indicating that the macrophage fraction was decreased (Fig. 2f, g). Moreover, in P2 cells, we also confirmed that many clusters of differentiation (CD) markers related to monocytes and lymphocytes were down-regulated, consistent with the decreased macrophage phenotype in P2 cells (Table 1).

Table 1 Down-regulated CD markers in P2 cells versus P0 cells

Symbol	Ratio (log_2)	*P* value	Entrez gene ID	Type of cells
CD2	−2.937	1.63E-02	914	Subcortical/cortical/ medullary thymocyte
CD9	−1.308	4.31E-02	928	Pre-B cell
CD14	−3.379	9.57E-03	929	Promonocyte
CD19	−2.839	3.72E-02	930	Pro-B cell, pre-pre-B cell, pre-B cell, early B cell
CD33	−4.022	1.04E-03	945	CFU-GEMM myeloid stem cell, BFU-E, CFU-M, CFU-G, CFU-Eo, promonocyte, myelocyte
CD36	−3.025	1.65E-02	948	CFU-E
CD37	−3.454	2.02E-02	951	Early B cell
CD52	−4.706	3.00E-03	1,043	Early B cell, cortical/ medullary thymocyte
CD53	−3.348	1.30E-02	963	Monocyte, B cell
CD68	−2.351	1.06E-02	968	Monocyte, DC, granulocyte, B subset
CD74	−3.092	2.21E-03	972	Pre-B cell, early B cell
CD82	−0.743	2.35E-02	3,732	Many hematopoietic cells except RBC
CD83	−1.762	3.24E-02	9,308	MatDC, langerhans cell
CD84	−3.063	3.07E-02	8,832	B cell, monocyte, macrophage, platelet
CD163	−4.059	1.21E-03	9,332	Monocyte, macrophage
CD209	−3.706	6.98E-03	30,835	Monocyte, macrophage
CD302	−1.753	2.05E-02	9,936	DC
CD163L1	−3.496	3.04E-02	283,316	Unknown
CD300A	−3.220	2.20E-02	11,314	Unknown
CD300C	−2.264	3.00E-03	10,871	Unknown

Many CD markers related to monocytes and lymphocytes were down-regulated

Fig. 3 Advanced liver fibrosis was induced in NOD.CB17-Prkdc^{scid}/J mice by chronic administration of CCl_4 (NOD-SCID mouse CCl_4 liver cirrhosis model). Liver fibrosis in this model was improved by infusion of P2 cells. **a** NOD.CB17-Prkdc^{scid}/J female mice 6 weeks of age were treated with CCl_4 dissolved in corn oil (1:3) twice a week for 6 weeks, only once with 0.5 mL/kg body weight CCl_4, from the second time with 1.0 mL/kg body weight and for the last 4 weeks with 1.5 mL/kg body weight. In the NOD-SCID mouse CCl_4 liver cirrhosis model, P2 or P0 cells were infused. Treatment with 1.5 mL/kg body weight CCl_4 was continued further for 4 weeks. **b**–**c** Photomicrograph showing Sirius red staining for hepatic collagens after 4 weeks 5×10^5 P2 cells infusion into the liver cirrhosis model mouse (×100) (**c**) and control PBS-infusion (×100) (**b**). **d**–**e** Photomicrograph of the right lobe of the liver (control group **d** and P2 cells infused group **e**) (original magnification ×100). **f** P2 cells (5×10^5) infused in the liver cirrhosis model mouse resulted in a significant reduction in fibrosis measured by Sirius red quantification after 4 weeks of infusion. The infusion of P2 cells improved liver fibrosis in this liver fibrosis model [$P=0.009$, $^{**}P<0.01$, 1.5±0.5 % ($n=11$) vs. control 2.6±1.1 % ($n=7$); $P=0.048$, $^{\#}P<0.05$ vs. P0-administration group 2.2±0.9 % ($n=7$)]


a
NOD-SCID mouse
CCl4 (mL/kgBW)
0.5 1.0 1.5
Analysis for
Liver fibrosis
1 time 1.5 weeks
4 weeks
b
200μm
c
200μm
d
2mm
e
2mm
f
Sirius-red stained area (%)
4.0
3.0
2.0
1.0
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Control
P0
P2

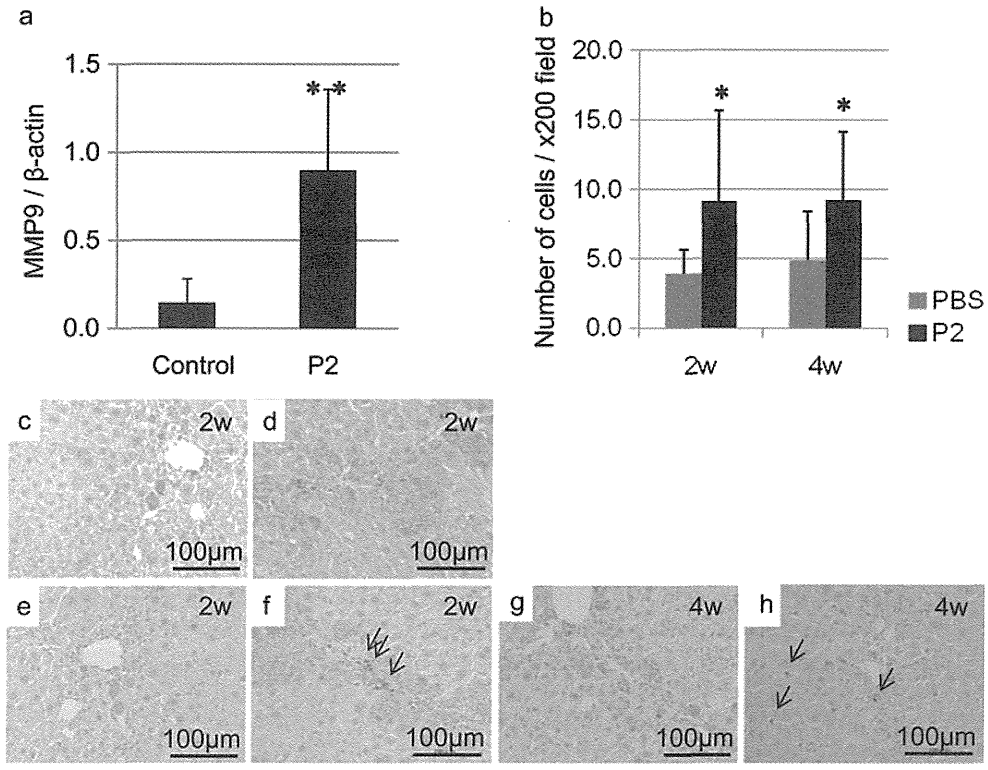

Fig. 4 Matrix metalloproteinase (MMP)-9 expression in P2 cells infused cirrhosis liver was up-regulated. **a** mRNA expression of MMP-9 in the liver after 4 weeks of P2 cells infusion was significantly up-regulated [$P=0.003$, $^{**}P<0.01$, MMP-9/β-actin 0.9±0.5 ($n=6$) vs. 0.2±0.1 control ($n=6$)]. **b** P2 cells also significantly up-regulated MMP-9 protein expression in the liver after 2 and 4 weeks of P2 cells-infusion [$P=0.031$, $^{*}P<0.05$ and $P=0.047$, $^{*}P<0.05$, MMP-9(+) cell number 9.2±6.5 ($n=10$) vs. 3.9±1.7 control ($n=9$), 9.2±4.9 ($n=12$) vs. 4.9±3.5 control ($n=8$)]. **c**–**f** Photomicrograph of MMP-9-positive cells in the liver after 2 weeks of P2 cells infusion (**f**) (original magnification ×400). *Arrow* indicated. IgG control of PBS group (**c**) and of P2 group (**d**) and MMP-9 expression of PBS control group (**e**). **g**–**h** Photomicrograph of MMP-9-positive cells in the liver 4 weeks after P2 cells infusion (**h**) (original magnification ×400). *Arrow* indicated. MMP-9 expression of PBS control (**g**)

Cultured human bone marrow-derived cell (P2 cells) infusion improves liver fibrosis

P2 cells (5×10^5) infused into the liver cirrhosis model mice resulted in a significant reduction in fibrosis measured by Sirius red quantification after 4 weeks of the infusion (Fig. 3a). The infusion of P2 cells improved liver fibrosis in this liver fibrosis model [$P=0.009$, $^{**}P<0.01$, 1.5±0.5 % ($n=11$) vs. control 2.6±1.1 % ($n=7$); $P=0.048$, $^{\#}P<0.05$ vs. P0-administration group 2.2±0.9 % ($n=7$)] (Fig. 3b–f).

Up-regulation of MMP-9 expression in P2 cells-infused cirrhosis liver

Messenger RNA expression of MMP-9, which degrades the extracellular matrix, in the liver after 4 weeks of infusion of P2 cells was significantly up-regulated [$P=0.003$, $^{**}P<0.01$, MMP-9/β-actin 0.9±0.5 ($n=6$) vs. 0.2±0.1 control ($n=6$)] (Fig. 4a). P2 cells also significantly up-regulated MMP-9 protein expression in the liver after 2 and 4 weeks of P2 cell infusion [$P=0.031$, $^{*}P<0.05$ and $P=0.047$, $^{*}P<0.05$, MMP-9(+) cell number 9.2±6.5 ($n=10$) vs. 3.9±1.7 control ($n=9$), 9.2±4.9 ($n=12$) vs. 4.9±3.5 control ($n=8$), ×200] (Fig. 4b–h).

Suppressed activation of hepatic stellate cells (HSCs) in P2 cells-infused cirrhosis liver

Messenger RNA expression of αSMA, a marker of activated HSCs, in the liver after 4 weeks of infusion of P2 cells was significantly reduced [$P=0.045$, $^{*}P<0.05$, αSMA/β-actin 0.6;0.2 ($n=4$) vs. 0.9;0.2 control ($n=6$)] (Fig. 5a). The amount of αSMA staining [αSMA(+) area(%)] in the P2 cells infusion group decreased [$P=0.048$, $^{*}P<0.05$, 1.0±0.5 % ($n=8$) vs. 2.0±1.1 % control ($n=6$), ×100] (Fig. 5b–d).

Reduction of TNFα and TGFβ in P2 cells recipients

Messenger RNA expression of TNFα, an inflammatory cytokine, in the liver after 4 weeks of P2 cells infusion was significantly reduced [$P=0.019$, $^{*}P<0.05$, TNFα/β-actin 0.2±0.1 ($n=8$) vs. 2.3±2.3 control ($n=9$)] (Fig. 6a). Messenger RNA expression of TGFβ, which activates HSCs, in the liver after 4 weeks of P2 cells infusion was significantly

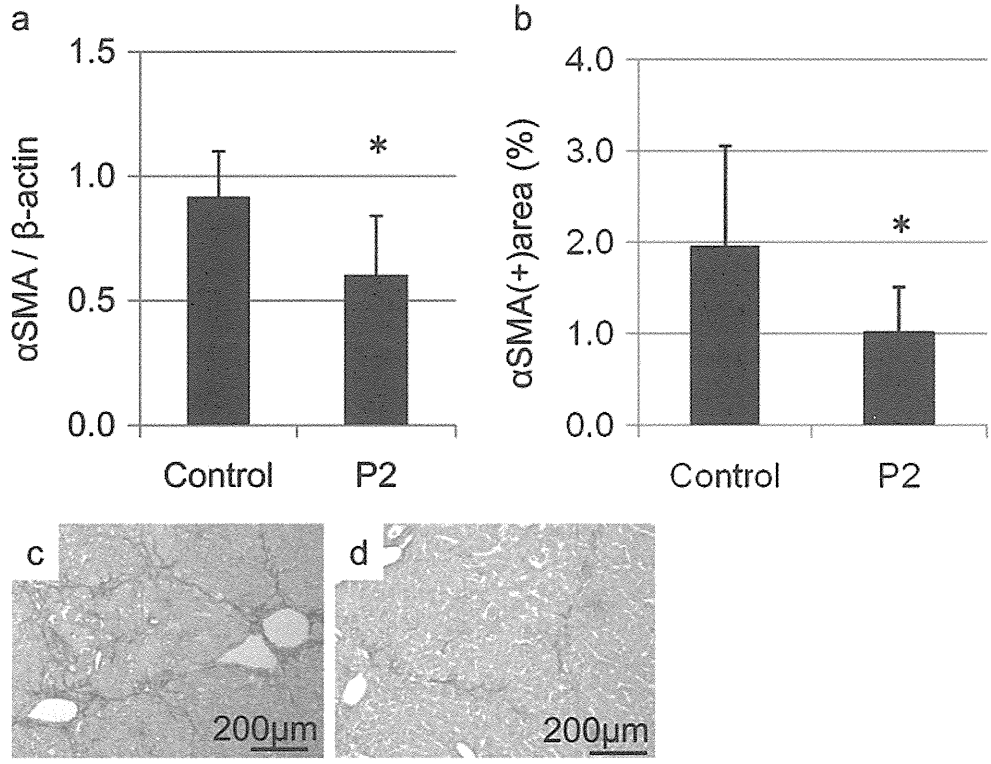

Fig. 5 P2 cells delivery causes a reduction of alpha smooth muscle actin (αSMA)-positive hepatic stellate cells (HSCs). **a** mRNA expression of αSMA in the liver after 4 weeks of P2 cells infusion was significantly reduced [$P=0.045$, $^*P<0.05$, αSMA/β-actin 0.6±0.2 ($n=4$) vs. 0.9±0.2 control ($n=6$)]. **b** The amount of αSMA staining [αSMA(+) area(%)] in the P2 cells-infused group decreased [$P=0.048$, $^*P<0.05$, 1.0±0.5 % ($n=8$) vs. 2.0±1.1 % control ($n=6$)]. **c**–**d** Photomicrographs demonstrate the reduction in αSMA(+) HSCs after 4 weeks of P2 cells delivery (**d**) and PBS control (**c**) (original magnification ×100)

reduced [$P=0.049$, $^*P<0.05$, TGFβ/β-actin 0.9±0.6 ($n=13$) vs. 1.5±1.0 control ($n=12$)] (Fig. 6b).

Discussion

We reported in 2004 that we had administered whole bone marrow cells from a GFP transgenic mouse to model mice with cirrhosis induced by repeated administration of CCl_4 and that an improvement in liver fibrosis, accompanied by improvements in liver function and survival, was obtained with donor-derived bone marrow cells that adhered to the fibrotic regions of the cirrhotic livers and produced fibrolytic enzymes including MMP-9 (Sakaida et al. 2004). With regard to clinical research, we were the first in the world to begin ABM*i* therapy, in 2003 and we demonstrated that it improved liver function in patients with cirrhosis without serious adverse events. At that time, we also found an increase in the number of proliferating

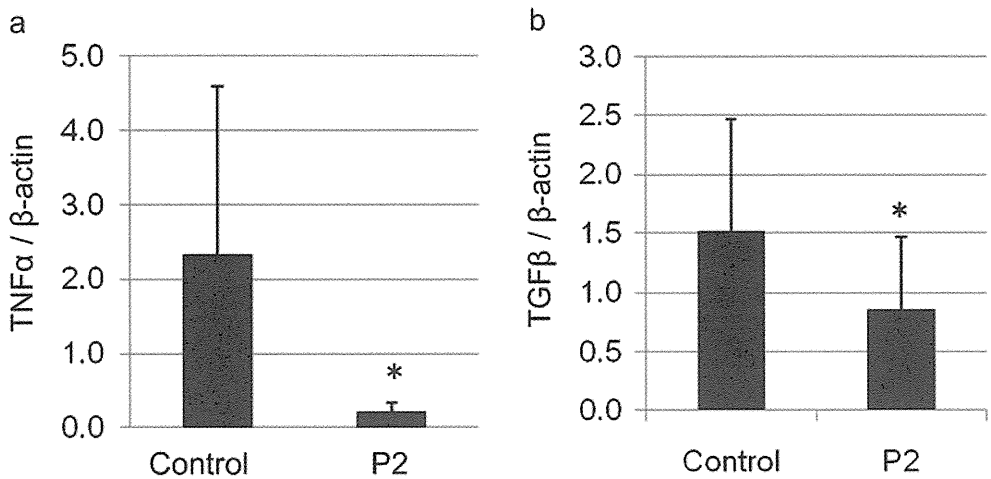

Fig. 6 P2 cells delivery causes a reduction of tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ). **a** mRNA expression of TNFα, an inflammatory cytokine, in the liver after 4 weeks of P2 cells infusion was significantly reduced [$P=0.019$, $^*P<0.05$, TNFα/β-actin 0.2±0.1 ($n=8$) vs. 2.3±2.3 control ($n=9$)]. **b** mRNA expression of TGFβ in the liver, which activates HSCs, after 4 weeks of P2 cells infusion was significantly reduced [$P=0.049$, $^*P<0.05$, TGFβ/β-actin 0.9±0.6 ($n=13$) vs. 1.5±1.0 control ($n=12$)]

cell nuclear antigen-positive cells after infusion of the bone marrow (Terai et al. 2006). Furthermore, based on joint research with our laboratory, Kim et al. of Yonsei University reported that the efficacy of ABM*i* therapy continued for at least 1 year and that, in liver biopsies taken over time, activation of the hepatic progenitor cell fraction was confirmed (Kim et al. 2010). Additionally, based on joint research with our laboratory, Saito et al. of Yamagata University reported that, in patients with alcohol-induced liver cirrhosis, ABM*i* was effective in improving liver function and their findings indicated that bone marrow may activate this process (Saito et al. 2011). As noted above, ABM*i* therapy has been shown to improve the pathological condition of cirrhosis in human clinical research but the cell fraction necessary for this therapeutic effect is still unknown. Meanwhile, ABM*i* therapy has been limited in its application because it requires the collection of 400 mL of bone marrow aspirate under general anesthesia. In reports from other laboratories on cultured bone marrow cells, bone marrow-derived macrophages have been shown to improve liver fibrosis in studies using murine bone marrow cells (Thomas et al. 2011). The proportion of macrophages in murine bone marrow is inherently large and is also large in cultured bone marrow cells. It is possible that the liver fibrosis-improving effect of cultured murine bone marrow cells comes mainly from macrophages. We also performed analyses using a mice model and found that bone marrow cells easily differentiated macrophages and the infusion of the macrophage fraction improved liver fibrosis (Iwamoto et al. 2013; Phinney et al. 1999). Basically, in the mice model, macrophages are easily cultured (Phinney et al. 1999). Huang et al. showed that MSC is also effective in improving liver fibrosis (Huang et al. 2013). So, we believe that both macrophages and MSC might be important for improving liver fibrosis. These are mice data, so we analyzed and cultured human bone marrow cells and then determined whether a cell fraction with this fibrosis-improving effect could be amplified to set up a clinical study.

In anticipation of clinical use in humans, we cultured human BM-MNCs in a medium that contained only 10 % FBS without the addition of growth factors. We carried out subculturing twice and, after approximately 3 weeks, we obtained a sufficient number of cells to expect an effect on liver fibrosis. We collected populations of P2 cells with almost no individual differences, in which the cell fractions were stable and contained roughly 94 % MSCs, a few percent hematopoietic cells consisting mainly in macrophages and less than 0.1 % hematopoietic stem cells (Fig. 1b–i). We found that, in cultured human bone marrow cells, the main component of the P2 cells were MSCs and that there was a clear difference in the proportion of MSCs between P2 and P0. DNA-chip analysis also showed that bone marrow-derived cells also decreased the macrophage fraction. Therefore, the characteristics of culturable bone marrow-derived cells may differ between mice and humans.

Next, P2 cells were infused via the caudal vein and they significantly improved liver fibrosis in an immunodeficient liver cirrhosis mouse model (NOD-SCID mouse CCl_4 liver cirrhosis model) that we developed for this study. Furthermore, we were also able to confirm that cultured human MSCs brought about a significant improvement in liver fibrosis using the same mouse model. Therefore, this study demonstrates that the liver fibrosis-improving effect in the cultured human bone marrow-derived cells originates in MSCs.

In human clinical research, Mohamadnejad and Kharaziha have shown that MSC from bone marrow can improve the pathological condition of liver cirrhosis by infusion both intravenously and via the portal vein (Mohamadnejad et al. 2007; Kharaziha et al. 2009). In addition, Pai et al. have demonstrated that liver function is improved when CD34-positive cells induced from bone marrow cells by granulocyte colony-stimulating factor are grown in vitro and then administered via the hepatic vein (Pai et al. 2008). In contrast, our research has demonstrated that a sufficient number of homogenous cells with almost no individual differences can be recovered after two passages of human BM-MNCs to which only serum was added without the involvement of growth factors, as well as that cultured cell infusion therapy, that can be expected to improve liver fibrosis, is possible through intravenous infusion. In addition, we developed an animal model that enables the evaluation of human cell function, proved the liver fibrosis-improving effect of MSCs in cultured human bone marrow-derived cells (P2) and demonstrated cultured cell collection conditions that are simple and provide a stable effect. In the future, we plan to assess cultured bone marrow-derived cell fractions from patients with liver cirrhosis and evaluate the effect on improving liver fibrosis in this animal model to enable the collection and infusion of even better cells and to determine the prognosis of therapeutic efficacy in patients.

In this study, we have shown that the mechanism of the improvement in liver fibrosis brought about by cultured human bone marrow-derived cells occurs via the enhanced expression of MMP-9, which is important for fibrolysis and a decrease in fiber production brought about by a decrease in HSC activity. Moreover, we have shown that this mechanism acts by controlling the production of cytokines such as TGFβ and TNFα. As shown in Fig. 6a, b, TNFα and TGFβ expressions were decreased. MSC infusion might induce the decrease of these cytokines and improve liver fibrosis. These results might be similar with our previous analysis and show rapid cytokine change after autologous bone marrow cell infusion (Mizunaga et al. 2012). We also report that TNFα signal is important to regulate the improvement of liver fibrosis after bone marrow cell infusion (Hisanaga et al. 2011). These cytokine changes after cultured human bone marrow cell infusion might be important to improve liver fibrosis.

In our ABM*i* therapy, we collected 400 mL of autologous bone marrow aspirate (BM-MNC fraction average, 7.8×10^9