MODEL USING UPA/SCID MICE WITH HUMAN HEPATOCYTES.THE 62ND ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR THE STUDY OF LIVER DISEASES. Nov.4-8, 2011. San Francisco.

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（分担）研究報告書

日本人の細胞に由来するiPS細胞からの誘導ヒト肝細胞を用いたキメラマウス肝炎モデル開発とその前臨床応用に関する研究

研究分担者　中西　真　名古屋市立大学大学院医学研究科教授

研究要旨　本研究の目的は、日本人由来のiPS細胞から分化誘導し、ヒト肝細胞を得てuPA/SCIDマウスに移植することで、ヒト肝細胞保持キメラマウスを作製して日本人特異な肝炎ウイルス応答性を検討可能な系を確立することにある。この目的達成のため、安定的かつ効率的な肝細胞の分化のための分子基盤の解明および技術の開発と、肝炎ウイルス応答性における宿主側の要因を解析する目的で行われた。

A. 研究目的

平成23年度の研究目的は、研究班長の池田らが確立しつつある安定的かつ効率的なヒトiPS細胞からの肝細胞分化系を確立するために、分化誘導時における細胞周期停止が及ぼす影響について詳しく解析を行った。すなわち、iPS細胞に細胞周期停止遺伝子を導入して発現させた後、様々なマーカー遺伝子群の発現変化を解析するものである。NANOG等の未分化マーカーから、内胚葉、中胚葉、外胚葉マーカー、および分化肝細胞マーカー等の発現変動を解析し、大きく発現変動する遺伝子群の同定と、そのプロモーター領域におけるヒストン修飾およびDNAメチル化解析の基盤を確立することにある。

B. 研究方法

iPS細胞の細胞増殖を強制的に停止することが肝細胞への分化に如何なる影響を与えるかを明らかにする目的で、テトラサイクリン添加により誘導可能なp16、p53およびp21発現レンチウイルスベクターをヒトiPS細胞に導入し、上記遺伝子を発現誘導した後に各種分化、未分化マーカー遺伝子の発現変化を解析した。

（倫理面への配慮）

ヒト細胞として平成22年度は既に京都大学山中らにより株化されているヒト正常2倍体繊維芽細胞ゆらいヒトiPS細胞を用いたため、特に倫理面への配慮は必要ないと判断した。

C. 研究結果

iPS細胞に細胞周期停止機能をもつp16, p53, p21の発現を、テトラサイクリン存在下で誘導したところ、p16およびp53遺伝子を発現誘導したときのみ、強い増殖抑制効果を認めた。興味深いことに、p16を発現誘導した時のみ、未分化マーカーの発現が大きく低下し、また内胚葉、中胚葉、外胚葉マーカーの発現が大きく増加した。一方、これら未分化・分化マーカーの変動はp53遺伝子やp21遺伝子を発現誘導させても全く認められなかった。興味深いことに、これらの効果は、一過性（24時間）にp16遺伝子を発現誘導させても同様に認められた。以上の知見は、p16による分化誘導効果は細胞増殖停止によるものというよりは、特異な標的分子を介したものによると考えられた。

D. 考察

本研究は日本人独自の肝炎ウイルス応答性と薬剤感受性解析法を確立することを最終目的としている。この研究において、日本人由来のiPS細胞（将来的には患者由来の細胞を用いたiPS細胞）を安定的かつ効率的に肝細胞に分化する系を確立することが最も困難でありかつ重要なポイン

トであると考えられる。これを達成するための基盤整備として、1．iPS細胞の長期維持技術の確立、2．マウスおよびヒト細胞iPS化のための技術確立、3．iPS細胞を用いた効果的な肝細胞分化誘導法の確立が必要不可欠のものと考える。本年度は、iPS細胞からの効果的な肝細胞分化誘導系を確立する目的で、細胞周期停止制御遺伝子産物の細胞分化誘導に及ぼす影響を解析した。結果的に、p16遺伝子を発現誘導することで、効果的に分化方向にiPS細胞を誘導することが可能と考えられた。この結果により、これまでの肝細胞誘導法に加えて、p16遺伝子を導入することが効果的であること示唆された。

E　結論

iPS細胞に細胞周期停止機能をもつp16遺伝子を発現誘導したところ、未分化性質の喪失と、分化形質の獲得が確認された。この効果は、同様に細胞周期停止機能をもつp53やp21では見られないため、細胞周期停止によるものではなく、p16が直接分化誘導因子を調節することで制御されていると考えられた。今後この効果の詳細について検討を加えていきたい。

F. 研究発表

1.　論文発表

1. Sato S, Takahashi S, Asamoto M, Nakanishi M, Wakita T, Ogura Y, Yatabe Y, Shirai T. Histone H1 expression in human prostate cancer tissues and cell lines.***Pathol Int.*** 62(2):84-92 (2012) (査読有り)

2. Delhase M, Kim SY, Lee H, Naiki-Ito A, Chen Y, Ahn ER, Murata K, Kim SJ, Lautsch N, Kobayashi KS, Shirai T, Karin M, Nakanishi M. TANK-binding kinase 1 (TBK1) controls cell survival through PAI-2/serpinB2 and transglutaminase 2. ***Proc Natl Acad Sci U S A.*** 109(4):E177-86　(2012) (査読有り)

3. Ogawa K, Murasaki T, Sugiura S, Nakanishi M, Shirai T. Organ differences in the impact of p27(kip1) deficiency on carcinogenesis induced by N-methyl-N-nitrosourea. ***J Appl Toxicol.*** in press (2012) (査読有り)

4. Sugiyama M, Tanaka Y, Wakita T, Nakanishi M, Mizokami M. Genetic variation of the IL-28B promoter affecting gene expression.***PLoS One*** (2011) (査読有り)

5. Nakanishi M. Cell cycle. ***Rinsho Ketsueki.*** 52(10):1660-8(2011) (査読有り)

6. Sugiyama M, Inui A, Shin-I T, Komatsu H, Mukaide M, Masaki N, Murata K, Ito K, Nakanishi M, Fujisawa T, Mizokami M. Easy-to-use phylogenetic analysis system for hepatitis B virus infection. ***Hepatol Res.*** 41 (10) :936-945　(2011) (査読有り)

7. Harada N, Watanabe Y, Yoshimura Y, Sakumoto H, Makishima F, Tsuchiya M, Nakanishi K, Nakanishi M, Aoki Y. Identification of a checkpoint modulator with synthetic lethality to p53 mutants. ***Anticancer Drugs.*** 22 986-94 (2011) (査読有り)

8. Matsuyama M, Goto H, Kasahara K, Kawakami Y, Nakanishi M, Kiyono T, Goshima N, Inagaki M. Nuclear Chk1 prevents premature mitotic entry. ***J Cell Sci.*** 124　2113-9 (2011) (査読有り)

9. Mizutani E, Suzumori N, Ozaki Y, Oseto K, Yamada-Namikawa C, Nakanishi M, Sugiura-Ogasawara M. SYCP3 mutation may not be associated with recurrent miscarriage caused by aneuploidy. ***Hum Reprod.*** 26(5):1259-66 (2011) (査読有り)

10. Sugiyama M, Tanaka Y, Nakanishi M, Mizokami M. Novel findings for the development of drug therapy for various liver diseases: Genetic variation in IL-28B is associated with response to the therapy for chronic hepatitis C. ***J Pharmacol Sci.*** 115(3):263-9 (2011)(査読有り)

2. 学会発表

1. 中西 真
平成23年10月3日～5日
第70回日本癌学会総会

G. 知的財産権の出願・登録状況
特になし

厚生労働科学研究費補助金(肝炎等克服緊急対策研究事業)

分担研究報告書

ヒト iPS 細胞由来肝細胞を有したキメラマウスの作出

分担研究者　水口　裕之　大阪大学大学院薬学研究科

本研究では、ヒト iPS 細胞由来肝細胞を有したキメラマウスの作出を試みる。昨年度までにヒト iPS 細胞由来肝細胞の uPA 免疫不全マウスへの移植を実施し、マウス血中においてヒトアルブミンが産生されることを確認した。しかしながら、ヒトアルブミンが検出されたマウス個体の割合は低く、ヒトアルブミンが検出された個体においてもその濃度は低いものであった。我々は最近、iPS 細胞から肝細胞への分化誘導法を改良し、成熟度の高い肝細胞を作製可能な新たな分化誘導法の開発に成功した。そこで本年度は、新規分化誘導法により作製したヒト iPS 細胞由来肝細胞を uPA 免疫不全マウスへ移植し、キメラマウス作出が可能か否か検討した。

研究協力者

川端健二　(独)医薬基盤研究所
　　大阪大学大学院薬学研究科

櫻井文教　大阪大学大学院薬学研究科

田代克久　(独)医薬基盤研究所

高山和雄　大阪大学大学院薬学研究科
　　(独)医薬基盤研究所

長基康人　大阪大学大学院薬学研究科
　　(独)医薬基盤研究所

A. 研究目的

C 型肝炎感染者は世界で年間 200～300 万人ずつ感染者が増加しており、現在患者数は 2 億人、本邦には 200 万人いるとされている。日本人患者の多くは Ib 型 HCV に感染しており、難治性である 1b 型高ウイルス量患者に対する既存薬剤の奏効率は 50%に過ぎない。また、標準的治療法であるインターフェロン投与による副作用発現、各種薬剤耐性ウイルスの出現など、現在臨床で使用されている肝炎治療薬には克服すべき課題が多く、新規治療法の開発が望まれている。このため、創薬応用に適したヒト肝炎ウイルス感染モデルが必要とされている。

これまで、ヒト肝炎ウイルス感染モデルとしてはチンパンジーなどの霊長類動物が使用されてきたが、近年、uPA トランスジェニックマウスと、免疫不全マウスを交配して得られた uPA 免疫不全マウスに、ヒト肝細胞を移植することで作出されたキメラマウスが、ヒト肝炎ウイルス感染モデルとしての利用を期待されている。申請者は、独自開発した次世代アデノウイルス (Ad) ベクター技術を駆使することで、ヒト iPS 細胞から肝細胞を効率よく分化誘導させる技術の開発に成功している。そこで本研究では、フェニックスバイオ・立野知世博士との連携の元、申請者が作製したヒト iPS 細胞由来肝細胞を uPA 免疫不全マウスへ移植することにより、ヒト iPS 細胞由来肝細胞を有したキメラマウス作出のための基盤技術開発を試みる。ヒト iPS 細胞由来肝細胞を有したキメラマウスのを作出することで、肝炎ウイルスの生体内での動態解析や投薬による治療効果の検討

を簡便に実施することができる。また、移植する肝細胞がヒトiPS細胞由来なので、個人差も考慮したテーラーメイドの実験系も開発可能となり、副作用が軽減された新規ウイルス性肝炎治療法の開発につながることが期待される。

昨年度は、Adベクターにより3種類の遺伝子を導入することで作製したヒトiPS細胞由来肝細胞を用いてキメラマウスの作製に関する検討を行ったが、生着効率の点で改善すべき点が存在した。そこで、本年度では3遺伝子導入法を改良することでより機能の高いヒトiPS細胞由来肝細胞を作製し、フェニックスバイオ社・立野知世博士グループの協力の下、移植実験を実施した。また、本年度は分化誘導肝細胞に加え、分化段階の異なる細胞を移植することで、キメラマウス作出に有効な細胞の検討も行った。

B. 研究方法

B-1. ヒトiPS細胞由来肝細胞の生体移植実験

移植のレシピエントマウスには、ヒトアルブミンプロモーターによってuPA遺伝子を発現するuPAトランスジェニックマウスと、免疫不全マウスSCIDを交配して得られたuPA免疫不全マウスの2ー4週齢を用いた。今年度は、昨年の3遺伝子導入法を改良した分化誘導法でヒトiPS細胞から内胚葉系細胞、肝幹前駆細胞、肝細胞を誘導し、移植実験を実施した。改良法にて誘導した内胚葉系細胞、肝幹前駆細胞、肝細胞を、PBSにて洗浄後、0.025%トリプシン/EDTA溶液によって回収後、8.3% tryptose phosphate broth、10% FBS、10 μM hydrocortisone 21-hemisuccinate、1 μM insulin、25 mM $NaHCO_3$を添加したL15 medium培地へ懸濁した。uPA免疫不全マウスをエーテル吸入麻酔し、ヒトiPS細胞由来細胞懸濁液をハミルトンシリンジにより脾臓へ25μl（1×10^6細胞）注入した。移植マウスから毎週採血を実施し、血中ヒトアルブミン量をELISAにて検出を行った。また、移植後26日目に移植マウスの肝臓よりパラフィン切片を作製し、抗ヒトアルブミン抗体、抗ヒトCK8/18抗体を用いて免疫抗体染色を行った。

C. 研究結果

本年度は、昨年度の3遺伝子（SOX17＋HEX＋HNF4α）導入法を改良した新規分化誘導法にてヒトiPS細胞から肝細胞への分化誘導を行った。本プロトコールに従い作製された内胚葉系細胞および肝幹前駆細胞のほぼ全ての細胞はそれぞれCXCR4およびAFP（α-フェトプロテイン）陽性を示すことを確認している。また、最終的に得られた肝細胞のうち約80%の細胞がアルブミン陽性を示すだけでなく、CYP3A4、CYP2D6などの一部の薬物代謝酵素の遺伝子発現レベルがヒト初代培養肝細胞と同等であること、様々な薬剤を代謝可能であることを確認している。さらに、細胞の形態学的な観点からも、細胞間隙が明瞭で、多核の細胞が現れるなど、ヒト初代培養肝細胞の形態と酷似していることを確認している。また、本年度確立した改良法で誘導した肝細胞は、昨年度の3遺伝子導入法で誘導した肝細胞よりも高い成熟度を示すことも確認している。

続いて、フェニックスバイオ・立野知世博士グループにおいて、uPA免疫不全マウスへの移植を試みた（図1A）。ヒトiPS細胞由来の各種細胞1×10^6細胞を、それぞれ4匹のuPA免疫不全マウスへ移植した結果、ヒトiPS細胞由来肝細胞を移植したマウス4匹の血中において、移植後7日目よりヒトアルブミンが観察された。そのうち3匹はその後も血中ヒトアルブミン濃度が移植後26日目まで上昇した（図1B）。一方で、内胚葉系細胞や肝幹前駆細胞を移植したマウスではヒトアルブミン濃度の顕著な上昇はみとめられなかった（図1B）。さらに、血中ヒトアルブミンが検出されたマウス個体について、肝臓切片を作製して免疫染色を行ったところ、ヒトアルブミンやヒトCK8/18両陽性のコロニーが多数観察された(図1C, D)。

D. 考察

我々が開発した新規分化誘導系を駆使して誘導した肝細胞は、uPA 免疫不全マウスへの生着が可能であるだけでなく、マウス生体内で増殖し、マウス肝細胞を置換できることが明らかとなった。未熟で増殖活性が高い内胚葉系細胞や肝幹前駆細胞を移植したマウスの血中からはヒトアルブミンが検出されないか、非常に低い濃度であった。更なる検討は必要であるが、未熟な細胞よりも成熟した肝細胞の方が移植には適していると考えられる。したがって、ヒト iPS 細胞からより成熟した肝細胞を誘導・作成することが可能となれば、肝炎ウイルス感染実験にも使用可能なキメリズムの高いマウスを作出できるものと考えられる。今後、3 次元培養技術や更なる機能遺伝子の導入などを検討することで、ヒト iPS 細胞由来キメラマウスの作出に適した、成熟した肝細胞の分化誘導法の開発が必要であると考えられる。

E. 結論

Ad ベクターによる遺伝子導入技術を利用して作製したヒト iPS 細胞由来肝細胞は、uPA 免疫不全マウス移植することで、肝臓へ生着し、増殖することが示された。

G. 研究発表

1. 論文発表

1) Kawabata K., Inamura M., Mizuguchi H. Efficient hepatic differentiation of human iPS cells by gene transfer. ***Methods. Mol. Biol.***, 826: 115-124, 2012

2) Takayama K., Inamura M., Kawabata K., Katayama K., Higuchi M., Tashiro K., Nonaka A., Sakurai F., Hayakawa T., Furue MK., Mizuguchi H. Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4α transduction. ***Mol. Ther.***, 20: 127-137, 2012.

3) Yoshida T., Takayama K., Kondoh M., Sakurai F., Tani H., Sakamoto N., Matsuura Y., Mizuguchi H., Yagi K. Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection. ***Biochem. Biophys. Res. Commun.***, 416: 119-124, 2011.

4) Takayama K., Inamura M., Kawabata K., Tashiro K., Katayama K., Hayakawa T., Furue MK., Mizuguchi H. Efficient and selective generation of two distinct endoderm lineages from human ES and iPS cells by differentiation stage-specific SOX17 transduction. ***PLoS One***, 6, e21780, 2011.

5) Kawabata K., Takayama K., Nagamoto Y., Saldon M.S., Higuchi M., Mizuguchi H. Endodermal and hepatic differentiation from human embryonic stem cells and human induced pluripotent stem cells. ***J. Stem Cell Res. Ther.***, in press.

2. 学会発表

1) 高山和雄、稲村充、川端健二、菅原道子、菊池きよ美、櫻井文教、古江－楠田美保、水口裕之：FOXA2・HNF1α 遺伝子導入によるヒト多能性幹細胞から薬剤代謝能を有した肝細胞の分化誘導、日本薬学会第 132 年会、札幌、2012 年 3 月 28-31 日

2) 長基康人、田代克久、高山和雄、大橋一夫、櫻井文教、立花雅史、古江(楠田)美保、川端健二、

水口裕之：3次元共培養法によるヒトES･iPS細胞由来肝細胞の効率的な分化誘導法の開発、日本薬学会第132年会、札幌、2012年3月28-31日

3) 水口裕之：遺伝子導入技術を駆使したヒトES/iPS細胞から肝細胞への分化誘導技術開発と創薬応用、第26回長崎DDS・再生医療研究会、長崎、2011年12月9日

4) Kazuo Takayama, Mitsuru Inamura, Kenji Kawabata, Kazufumi Katayama, Katsuhisa Tashiro, Fuminori Sakurai, Miho Kusuda Furue, Hiroyuki Mizuguchi: Efficient generation of mature hepatocytes from human pluripotent stem cells by HNF4α transduction, 第26回日本薬物動態学会年会,2011年11月16-18日、長崎.

5) 田代克久、川端健二、櫻井文教、水口裕之：幹細胞への高効率遺伝子導入技術を利用した分化誘導法の開発、第61回日本薬学会近畿支部総会・大会、神戸、2011年10月22日

6) 水口裕之：遺伝子導入技術を駆使したヒトES/iPS細胞から肝臓細胞への分化誘導、第28回日本小児肝臓研究会、筑波、2011年7月16日

7) Kazuo Takayama, Mitsuru Inamura, Kenji Kawabata, Kazufumi Katayama, Katsuhisa Tashiro, Fuminori Sakurai, Miho Kusuda Furue, Hiroyuki Mizuguchi: HNF4α promotes hepatic maturation from human embryonic stem cell-derived hepatoblasts., The 6th Seoul-Kyoto-Osaka Joint Symposium on Pharmaceutical Sciences for Young Scientists, Seoul, Korea, June, 2011

8) Kazuo Takayama, Mitsuru Inamura, Kenji Kawabata, Kazufumi Katayama, Katsuhisa Tashiro, Fuminori Sakurai, Miho Kusuda Furue, Hiroyuki Mizuguchi: Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4α transduction., International Society for Stem Cell Research, Tronto, June, 2011

9) 高山和雄、稲村充、川端健二、田代克久、形山和史、櫻井文教、古江－楠田美保、水口裕之：HNF4α遺伝子導入によるヒトES・iPS細胞からの成熟肝細胞への高効率分化誘導, 第18回肝細胞研究会, 東京, 2011年6月24-25日

H. 知的財産権の出願・登録状況

1. 特許取得

該当事項なし

2. 実用新案登録

該当事項なし

3. その他

該当事項なし

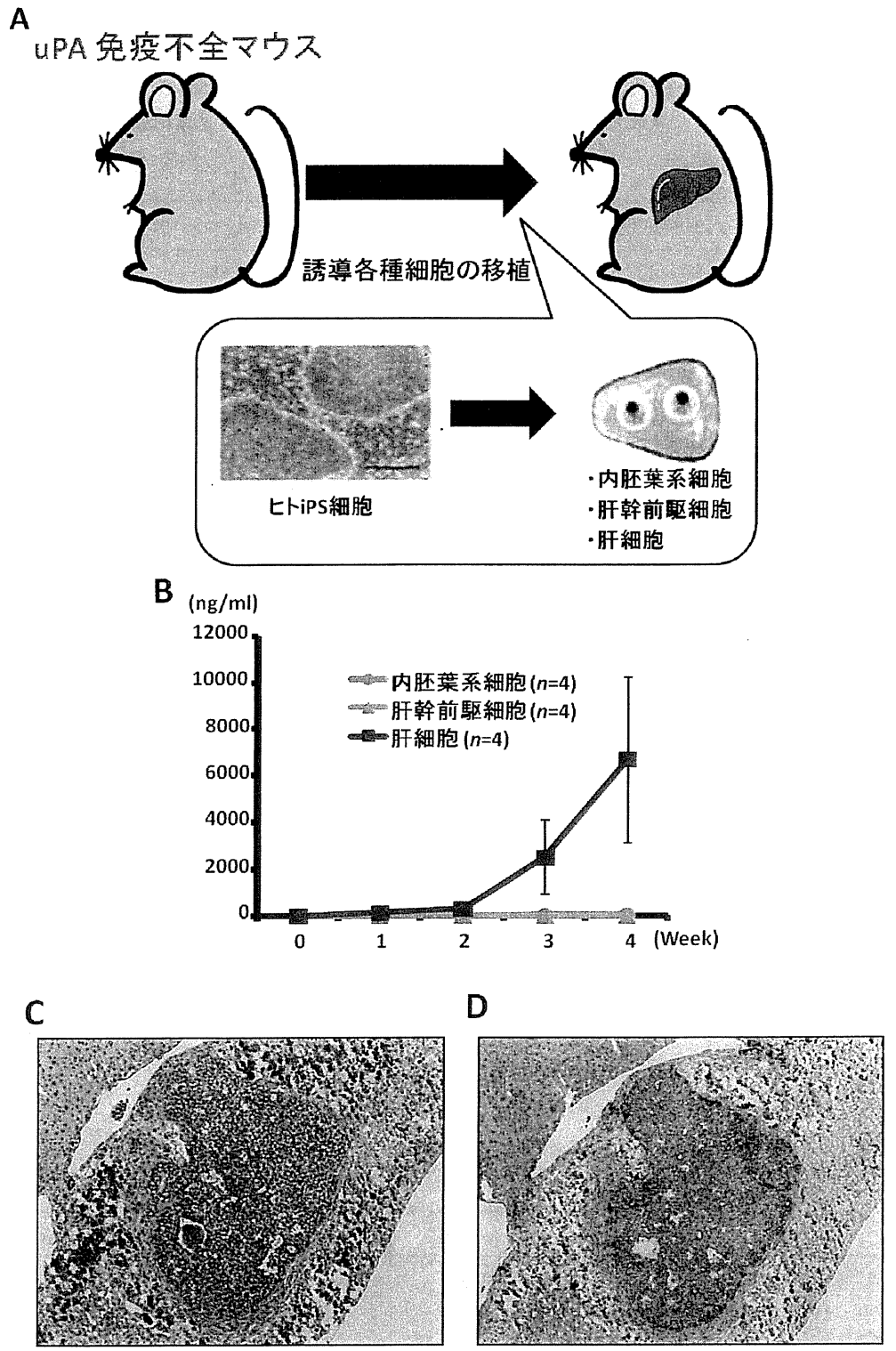

図1 ヒトiPS細胞由来肝細胞のuPA免疫不全マウスへの移植実験

(A) 2-4週齢のuPA免疫不全マウスへ、Adベクターを用いて誘導した、ヒトiPS細胞由来の各種細胞を脾臓経由で移植を行った。

(B) ヒトiPS細胞由来の各種細胞を移植後、経時的に採血を行い、血中ヒトアルブミン濃度をELISAによって測定した。

(C andD) ヒトiPS細胞由来の肝細胞を移植後、26日目に解剖し、肝臓切片を作製、免疫抗体染色によりヒトアルブミン(C)、ヒトCK8/18(D)陽性細胞をそれぞれ検出した。

研究成果の刊行に関する一覧表

研究代表者　池田　一雄

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ogawa T, Enomoto M, Fujii H, Sekiya Y, Yoshizato K, Ikeda K, Kawada N.	MicroRNA-221/222 upregulation indicates the activation of stellate cells and the progression of liver fibrosis.	Gut		In press	2012
Sekiya Y, Ogawa T, Yoshizato K, Ikeda K, Kawada N.	Suppression of hepatic stellate cell activation by microRNA-29b	Biochem Biophys Res Commun	412	74-79	2011
Sekiya Y, Ogawa T, Iizuka M, Yoshizato K, Ikeda K, Kawada N.	Down-regulation of cyclin E1 expression by microRNA-195 accounts for interferon-beta-induced inhibition of hepatic stellate cell proliferation.	J Cell Physiol.	226	2535-2542	2011
Thuy le TT, Morita T, Yoshida K, Wakasa K, Iizuka M, Ogawa T, Mori M, Sekiya Y, Momen S, Motoyama H, Ikeda K, Yoshizato K, Kawada N.	Promotion of liver and lung tumorigenesis in DEN-treated cytoglobin-deficient mice.	Am J Pathol.	179	1050-1060	2011

研究分担者　吉里　勝利

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okazaki A, Hiraga N, Imamura M, Nelson Hayes C, Tsuge M, Takahashi S, Aikata H, Abe H, Miki D, Ochi H, Tateno C, Yoshizato K, Ohdan H, Chayama K.	Severe necroinflammatory reaction caused by natural killer by Fas/FasL interaction and dendritic cells in human hepatocyte chimeric mouse.	Hepatology.			In press
Yoshizato K, Tateno C, Utoh R.	Mice with Liver Composed of Human Hepatocytes as an Animal Model for Drug Testing.	Curr Drug Discov Technol.	9	63-76	2012

Sekiya Y, Ogawa T, Yoshizato K, Ikeda K, Kawada N.	Suppression of hepatic stellate cell activation by microRNA-29b.	Biochem Biophys Res Commun.	Aug 19; 412(1)	74-9	2012
Sekiya Y, Ogawa T, Iizuka M, Yoshizato K, Ikeda K, Kawada N.	Down-regulation of cyclin E1 expression by microRNA-195 accounts for interferon-β-induced inhibition of hepatic stellate cell proliferation.	J Cell Physiol.	Oct; 226(10)	2535-42	2011
Thuy le TT, Morita T, Yoshida K, Wakasa K, Iizuka M, Ogawa T, Mori M, Sekiya Y, Momen S, Motoyama H, Ikeda K, Yoshizato K, Kawada N.	Promotion of liver and lung tumorigenesis in DEN-treated cytoglobin-deficient mice.	Am J Pathol.	Aug; 179(2)	1050-60	2011
Hiraga N, Imamura M, Abe H, Hayes CN, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, Yoshizato K, Matsui H, Kanai A, Inaba T, Tanaka S, Chayama K.	Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo.	Hepatology.	Sep 2;54(3)	781-8	2011
Hiraga N, Abe H, Imamura M, Tsuge M, Takahashi S, Hayes CN, Ochi H, Tateno C, Yoshizato K, Nakamura Y, Kamatani N, Chayama K.	Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus.	Hepatology.	Sep 2;54(3)	764-71	2011
Amano H, Hino H, Tateno C, Emoto K, Imaoka Y, Yamasaki C, Itamoto T, Tashiro H, Asahara T, Ohdan H, Yoshizato K.	Therapeutic potential of propagated hepatocyte transplantation in liver failure.	J Surg Res.	May 1;167(1)	e29-37	2011
Tateno C, Kataoka M, Utoh R, Tachibana A, Itamoto T, Asahara T, Miya F, Tsunoda T, Yoshizato K.	Growth hormone-dependent pathogenesis of human hepatic steatosis in a novel mouse model bearing a human hepatocyte-repopulated liver.	Endocrinology.	Apr;152 (4)	1479-91	2011

Abe H, Imamura M, Hiraga N, Tsuge M, Mitsui F, Kawaoka T, Takahashi S, Ochi H, Maekawa T, Hayes CN, Tateno C, Yoshizato K, Murakami S, Yamashita N, Matsuhira T, Asai K, Chayama K.	ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both in vitro and in vivo.	J Hepatol.	Jul;55(1)	11-8	2011
Ohara E, Hiraga N, Imamura M, Iwao E, Kamiya N, Yamada I, Kono T, Onishi M, Hirata D, Mitsui F, Kawaoka T, Tsuge M, Takahashi S, Abe H, Hayes CN, Ochi H, Tateno C, Yoshizato K, Tanaka S, Chayama K.	Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice.	J Hepatol.	May; 54(5)	872-8	2011

研究分担者　立野　知世

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sanoh S, Horiguchi A, Sugihara K, Kotake Y, Tayama Y, Ohshita H, Tateno C, Horie T, Kitamura S, Ohta S.	Prediction of In Vivo Hepatic Clearance and Half-life of Drug Candidates in Human using Chimeric Mice with Humanized Liver.	Drug Metab Dispos	40	322-328	2012
Hasegawa M, Tahara H, Inoue R, Kakuni M, Tateno C, Ushiki J.	Investigation of Drug-drug Interactions Caused by Human Pregnane X Receptor-mediated Induction of Cytochrome P450 3A4 and 2C Subfamilies in Chimeric Mice with a Humanized Liver.	Drug Metab Dispos	40	474-480	2012

研究分担者　寺岡　弘文

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
寺岡弘文	非肝臓源から肝細胞系譜への分化誘導―学内共同研究のまとめ（総説）	お茶の水医学雑誌	60(1)	7-17	2012
Ozeki R, et al.	Hepatic stellate cells mediate differentiation of dendritic cells from monocytes	Journal of Medical and Dental Sciences	59(1)	43-52	2012
Atsumi Y, et al.	Onset of quiescence following p53 mediated down-regulation of H2AX in normal cells	PLoS ONE	6(8)	e23432 (10 pages)	2011

研究分担者　田中　靖人

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Murayama A, Kato T, Akazawa D, Sugiyama N, Date T, Masaki T, Nakamoto S, Tanaka Y, Mizokami M, Yokosuka O, Nomoto A, Wakita T.	Production of infectious chimeric hepatitis C virus genotype 2b harboring minimal regions of JFH-1.	J Viro.	86(4)	2143-2152	2012
Date T, Morikawa K, Tanaka Y, Tanaka-Kaneko K, Sata T, Mizokami M, Wakita T.	Replication and Infectivity of a Novel Genotype 1b Hepatitis C Virus Clone. Microbiol Immunol.	in perss			2012
Matsuura K, Tanaka Y, Kusakabe A, Hige S, Inoue J, Komatsu M, Kuramitsu T, Hirano K, Ohno T, Hasegawa I, Kobashi H, Hino K, Hiasa Y, Nomura H, Sugauchi F, Nojiri S, Joh T, Mizokami M.	Recommendation of lamivudine-to-entecavir switching treatment in chronic hepatitis B responders: Randomized controlled trial.	Hepatol Res.	41(6)	505-511	2011

Wang J, Singh US, Rawal RK, Sugiyama M, Yoo J, Jha AK, Scroggin M, Huang Z, Murray MG, Govindarajan R, Tanaka Y, Korba B, Chu C K.	Antiviral activity of novel 2'-fluoro-6'-methylene-carbocyclic adenosine against wild-type and drug-resistant hepatitis B virus mutants.	Bioorg Med Chem Lett.	21(21)	6328-6331.	2011
Sa-Nguanmoo P, Tanaka Y, Ratanakorn P, Sugiyama M, Murakami S, Payungporn S, Sommanustweechai A, Mizokami M, Poovorawan Y.	Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes.	Virus Res.	158(1-2)	209-15.	2011

研究分担者　中西　真

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sato S, Takahashi S, Asamoto M, Nakanishi M, Wakita T, Ogura Y, Yatabe Y, Shirai T.	Histone H1 expression in human prostate cancer tissues and cell lines.	Pathol Int.	62	84-92	2012
Delhase M, Kim SY, Lee H, Naiki-Ito A, Chen Y, Ahn ER, Murata K, Kim SJ, Lautsch N, Kobayashi KS, Shirai T, Karin M, Nakanishi M.	TANK-binding kinase 1 (TBK1) controls cell survival through PAI-2/serpinB2 and transglutaminase 2.	Proc Natl Acad Sci U S A.	109	177-86	2012
Ogawa K, Murasaki T, Sugiura S, Nakanishi M, Shirai T.	Organ differences in the impact of p27(kip1) deficiency on carcinogenesis induced by N-methyl-N-nitrosourea.	J Appl Toxicol.	In press		2011
Sugiyama M, Tanaka Y, Wakita T, Nakanishi M, Mizokami M.	Genetic variation of the IL-28B promoter affecting gene expression.	PLoS One			2011
Nakanishi M.	Cell cycle	Rinsho Ketsueki.	52	1660-8	2011

Sugiyama M, Inui A, Shin-I T, Komatsu H, Mukaide M, Masaki N, Murata K, Ito K, Nakanishi M, Fujisawa T, Mizokami M.	Easy-to-use phylogenetic analysis system for hepatitis B virus infection.	Hepatol Res.	41	936-945	2011
Harada N, Watanabe Y, Yoshimura Y, Sakumoto H, Makishima F, Tsuchiya M, Nakanishi K, Nakanishi M, Aoki Y.	Identification of a checkpoint modulator with synthetic lethality to p53 mutants.	Anticancer Drugs.	22	986-94	2011
Matsuyama M, Goto H, Kasahara K, Kawakami Y, Nakanishi M, Kiyono T, Goshima N, Inagaki M.	Nuclear Chk1 prevents premature mitotic entry.	J Cell Sci.	124	2113-9	2011
Mizutani E, Suzumori N, Ozaki Y, Oseto K, Yamada-Namikawa C, Nakanishi M, Sugiura-Ogasawara M.	SYCP3 mutation may not be associated with recurrent miscarriage caused by aneuploidy.	Hum Reprod.	26	1259-66	2011
Sugiyama M, Tanaka Y, Nakanishi M, Mizokami M.	Novel findings for the development of drug therapy for various liver diseases: Genetic variation in IL-28B is associated with response to the therapy for chronic hepatitis C.	J Pharmacol Sci.	115	263-9	2011

研究分担者　水口　裕之

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kawabata K., Takayama K., Nagamoto Y., Saldon M.S., Higuchi M., Mizuguchi H.	Endodermal and hepatic differentiation from human embryonic stem cells and human induced pluripotent stem cells.	J. Stem Cell Res. Ther.	印刷中		

Kawabata K., Inamura M., Mizuguchi H.	Efficient hepatic differentiation of human iPS cells by gene transfer.	Methods. Mol. Biol.	326	115-124	2012
Takayama K., Inamura M., Kawabata K., Katayama K., Higuchi M., Tashiro K., Nonaka A., Sakurai F., Hayakawa T., Furue MK., Mizuguchi H.	Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4α transduction.	Mol.Ther.	20	127-137	2012
Yoshida T., Takayama K., Kondoh M., Sakurai F., Tani H., Sakamoto N., Matsuura Y., Mizuguchi H., Yagi K.	Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection.	Biochem. Biophys. Res. Commun.	416	119-124	2011
Takayama K., Inamura M., Kawabata K., Tashiro K., Katayama K., Hayakawa T., Furue MK., Mizuguchi H.	Efficient and selective generation of two distinct endoderm lineages from human ES and iPS cells by differentiation stage-specific SOX17 transduction.	PLoS One	6	e21780	2011

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ORIGINAL ARTICLE

MicroRNA-221/222 upregulation indicates the activation of stellate cells and the progression of liver fibrosis

Tomohiro Ogawa,[1,2,3] Masaru Enomoto,[1] Hideki Fujii,[1] Yumiko Sekiya,[1,2] Katsutoshi Yoshizato,[2,4] Kazuo Ikeda,[5] Norifumi Kawada[1,2]

[1]Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan
[2]Liver Research Center, Graduate School of Medicine, Osaka City University, Osaka, Japan
[3]Center for the Advancement of Higher Education, Faculty of Engineering, Kinki University, Hiroshima, Japan
[4]PhoenixBio Co Ltd., Hiroshima, Japan
[5]Department of Anatomy and Cell Biology, Graduate School of Medicine, Nagoya City University, Aichi, Japan

Correspondence to
Professor Norifumi Kawada, Department of Hepatology, Graduate School of Medicine, Osaka City University, 1-4-3, Asahimachi, Abeno, Osaka 545-8585, Japan; kawadanori@med.osaka-cu.ac.jp

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ABSTRACT

Background MicroRNAs (miRNAs) are important in hepatic pathophysiology and the development of liver cancer.

Objective To explore miRNAs that are regulated with the progression of liver fibrosis caused by chronic liver disease.

Design The regulated miRNAs in human livers infected with hepatitis C virus were identified by microarray analysis. Their expression in human livers with non-alcoholic steatohepatitis, mouse livers from two fibrosis models and cultured stellate cells was validated by real-time RT-PCR. The regulation of miR-222 expression in stellate cells by nuclear factor kappa B (NF-κB) was assayed. Finally, the effects of an miR-222 precursor or inhibitor on the expression of cyclin-dependent kinase inhibitor 1B (CDKN1B) and the growth of LX-2 cells were determined.

Results It was found that miR-199a-5p/199a-3p and miR-221/222 were upregulated in the human liver in a fibrosis progression–dependent manner. Among these miRNAs, miR-221/222 were upregulated in LX-2 cells and increased during the course of culture-dependent activation of mouse primary stellate cells, in a manner similar to the expression of α1(I) collagen and α-smooth muscle actin mRNAs. The expression of miR-221/222 increased in mouse models of liver fibrosis. In contrast, an NF-κB inhibitor significantly suppressed the miR-222 induction that was stimulated in culture by transforming growth factor α or tumour necrosis factor α. Although overexpression or downregulation of miR-222 failed to regulate the growth of LX-2 cells, miR-222 bound to the *CDKN1B* 3′UTR and regulated the expression of the corresponding protein.

Conclusion miR-221/222 may be new markers for stellate cell activation and liver fibrosis progression.

Significance of this study

What is already known about this subject?

- The abundance of miR-221/222 increases in human hepatocellular carcinoma (HCC).
- miR-221/222 elicit their oncogenic effects via the downregulation of tumour suppressors, such as p27, p57 and PTEN.
- The expression of miR-221/222 is induced by NF-κB activation in prostate carcinoma and glioblastoma cells.

What are the new findings?

- The expression of miR-221/222 increases with the progression of human liver fibrosis and is correlated with the expression levels of α1 (I) collagen and α-smooth muscle actin mRNAs.
- The expression of miR-221/222 is highly correlated with α1 (I) collagen mRNA expression in mouse stellate cells in culture.
- miR-222 expression is inhibited by an NF-κB inhibitor and upregulated by NF-κB activators, such as tumour necrosis factor α and transforming growth factor α.

How might they impact on clinical practice in the foreseeable future?

- miR-221/222 have the potential to be new markers for stellate cell activation and liver fibrosis progression in humans.
- The pattern of miR-221/222 expression can serve as a useful tool for understanding and investigating the mechanism of the progression of liver fibrosis.
- The miRNA profiling of human liver fibrosis contributes to the identification of predictors of disease prognosis and potential therapeutic targets.

INTRODUCTION

Hepatic fibrosis is a consequence of the accumulation of extracellular matrix (ECM) components in the liver. This process is caused by the persistent liver damage and wound healing reaction induced by chronic viral hepatitis, alcohol abuse, non-alcoholic steatohepatitis (NASH) and several other aetiologies and can progress to cirrhosis and hepatocellular carcinoma (HCC).[1] Hepatitis C virus (HCV) infection is one of the leading causes of end-stage liver diseases worldwide and the most common indication for liver transplantation in the USA and Europe. In addition, non-alcoholic fatty liver disease and its progressive form, NASH, have become urgent clinical problems owing to the increasing prevalence of metabolic syndrome.[2–4] Because fibrotic liver disease has thus become a global health problem, it is important to understand the molecular mechanisms of hepatic fibrosis, irrespective of the cause, to establish proper therapeutic strategies and to identify diagnostic markers of this disease.

It is generally accepted that excessive production of ECM components by activated hepatic stellate cells and myofibroblasts is responsible for hepatic fibrosis.[5 6] Hepatic stellate cells exist in Disse's space and store vitamin A under physiological conditions.[7] When liver injury occurs, these cells become activated in response to oxidative stress, growth factors and inflammatory stimuli that are produced by damaged hepatocytes, resident macrophages (Kupffer cells), infiltrating inflammatory cells and aggregated platelets. The hepatic stellate cells then undergo transformation into myofibroblast-like cells that express α-smooth muscle actin (αSMA).[5] Activated stellate cells deposit ECM components, including types I and III collagen, fibronectin and laminin, at the site of local tissue damage and secrete profibrogenic mediators, such as transforming growth factor β (TGFβ), connective tissue growth factor and platelet-derived growth factor, thereby playing a pivotal role in liver fibrogenesis.[6]

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs that interact with the 3′ untranslated region (UTR) of target mRNAs, resulting in the inhibition of translation or the promotion of mRNA degradation.[8 9] miRNAs are important in proliferation,[10] development[11] and differentiation[12] in many cell types and are involved in the development of many diseases, including cancer.[13–15] miR-122 has been the best studied miRNA with regard to liver pathophysiology. For example, miR-122 is highly abundant in the human liver and is essential for HCV replication.[16–19] Interferon β rapidly modulates the expression of miR-122, which has sequence-predicted targets within the HCV RNA.[20] In chronic hepatitis C, decreased miR-122 has been associated with an absence of virological response to interferon and ribavirin treatment.[21] miR-21, -34a, -93, -96, -221/222 and -519a increase and, in contrast, let-7c decreases in human HCC.[22] The expression levels of miR-21 and miR-122 correlate with the histological evaluation of HCV-induced liver disease.[23]

Here, we show that the expression of miR-221/222 increased with the progression of liver fibrosis and significantly correlated with the expression of α1 (I) collagen (Col1A1) and αSMA mRNAs in human fibrotic livers. The expression of miR-221/222 in human fibrotic livers was also reproduced in mouse models of hepatic fibrosis. Interestingly, miR-221/222 were more highly expressed in a human stellate cell line, LX-2, than in HCC cell lines and their expression was induced with the activation of mouse stellate cells. Finally, we show that the expression of miR-222 in stellate cells may be regulated by the activation of nuclear factor kappa B (NF-κB). Taken together, our findings indicate that miR-221/222 upregulation is a new marker for stellate cell activation and liver fibrosis progression that could be used for the clinical diagnosis of liver fibrosis.

MATERIALS AND METHODS

Materials

Precursors and inhibitors of miR-222 and the negative control miRNA were purchased from Ambion (Austin, Texas, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma Chemical Co (St Louis, Missouri, USA). The mouse monoclonal antibody against cyclin-dependent kinase inhibitor 1B (CDKN1B (p27, Kip1)) was from Cell Signaling Technology Inc (Beverly, Massachusetts, USA) and that against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Chemicon International Inc (Temecula, California, USA). Enhanced Chemiluminescence Plus detection reagent was from GE Healthcare (Buckinghamshire, UK). Immobilon P membranes were from Millipore Corp. (Bedford, Massachusetts, USA). Recombinant human TGFα and mouse tumour necrosis factor (TNF)α were from R&D Systems, Inc (Minneapolis, Minnesota, USA). 6-Amino-4-(4-phenoxyphenylethylamono)quinazoline (QNZ) was from EMD Chemicals, Inc (Gibbstown, New Jersey, USA). All other reagents were purchased from Sigma Chemical Co or Wako Pure Chemical Co (Osaka, Japan).

Liver biopsy specimens

Liver biopsy specimens were obtained from 35 patients with chronic hepatitis C genotype 1 infection and 26 patients with NASH using a 15-gauge Tru-Cut biopsy needle (Hakko Inc., Tokyo, Japan) under ultrasound guidance (table 1). Of the 26 patients with NASH, oral hypoglycaemic agents were given to four patients (sulphonylureas to three and metformin to one), antihypertensive agents to eight (angiotensin receptor blockers to five and calcium channel blockers to three) and antihyperlipidaemic agents to eight (statins to five and fibrates to three) at the time of liver biopsy. Informed consent was obtained from all patients before biopsy. All procedures were in accordance with the Helsinki Declaration of 1975 (2008 revision). Biopsied liver tissues were fixed in 10% formalin solution and then embedded in paraffin. The stage of liver fibrosis was evaluated according to the METAVIR scoring system in patients with chronic hepatitis C[24] and the Brunt classification in patients with NASH.[4] A portion of each biopsy sample was immediately placed in RNAlater (Qiagen, Valencia, California, USA), temporarily stored at −20°C and then used to extract total RNAs using the mirVana miRNA Isolation Kit (Applied

Table 1 Baseline characteristics of patients

Characteristics	All patients with CHC (n=35)	Patients with CHC undergoing microarray analysis (n=22)	Patients with NASH (n=26)
Age (years)*	59±9	58±6	58±12
Female sex (%)†	20 (57)	11 (50)	14 (54)
Interferon-naïve (%)†	24 (69)	15 (68)	
ALT (IU/l)‡	57 (34–99)	88 (51–171)	69 (28–226)
Albumin (g/dl)*	4.0±0.3	4.0±0.3	4.0±0.5
Platelet count ($\times 10^9$/l)*	179±53	169±46	184±54
HCV RNA ($\log_{10}$ copies/ml)*	6.1±1.0	6.0±1.2	
Grade of necroinflammation† (A0/A1/A2/A3)	3/23/8/1	2/13/6/1	0/10/10/6
Stage of fibrosis† (F1/F2/F3/F4)	19/7/7/2	11/4/5/2	7/8/8/3

*Mean±SD.
†Numbers of patients.
‡Median (IQR).
ALT, alanine aminotransferase; CHC, chronic hepatitis C; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis.

Biosystems, Foster City, California, USA). As controls, normal liver tissues were taken from four patients who underwent resection for metastatic liver tumours.

Microarray analysis

In 22 of the 35 patients with chronic hepatitis C and in four controls, microarray analysis was performed using 10 μg total RNA with the 3D-Gene Human miRNA Oligo chip v10.1 (Toray, Tokyo, Japan), as described in detail elsewhere.[25]

Mouse model of liver fibrosis

Male C57BL/6 mice, 7–10 weeks old, were purchased from Japan SLC, Inc (Shizuoka, Japan). All animals received humane care. The experimental protocol was approved by the Committee of Laboratory Animals according to institutional guidelines. Mice (n=5) were injected intraperitoneally with 200 μg/g body weight of thioacetamide (TAA, Sigma) diluted in saline three times a week for 4 or 8 weeks.[26] Control mice (n=5) were injected with saline. As another liver fibrosis model, mice were given either a methionine- and choline-deficient diet (MCDD, n=7) or methionine-choline control diet (MCCD, n=7) for 5 or 15 weeks, as previously described.[27] In addition, a similar model was generated in rats by giving them MCCD for 10 weeks, MCDD for 10 weeks, or MCDD for 8 weeks followed by MCCD for the last 2 weeks (recovery group).[27] The ingredients for these diets were purchased from MP Biomedicals (Solon, Ohio, USA).

Cells

Primary stellate cells were isolated from male C57BL/6 mice by the pronase-collagenase digestion method[28] and were cultured in DMEM supplemented with 10% FBS. Hepatocytes were isolated by collagenase digestion. One day after culturing, the cells were treated with TGFα (1–10 ng/ml), TNFα (0.1–1 ng/ml), or QNZ (10–100 nmol/l) for 24 or 72 h. LX-2 (donated by Dr Scott Friedman[29]), NIH3T3 and Huh7 cells were maintained on plastic culture plates in DMEM supplemented with 10% FBS. HepG2 cells (JCRB1054), obtained from the Health Science Research Resources Bank (Osaka, Japan), were maintained on plastic culture plates in Minimum Essential Medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% FBS, 1 mM sodium pyruvate (Invitrogen) and 1% non-essential amino acids (Invitrogen).

Quantitative real-time PCR

Total RNA was extracted from cells and liver tissues using the miRNeasy Mini Kit (Qiagen). cDNAs were synthesised using 0.5 μg of total RNA, a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and oligo(dT)$_{12-18}$ primers, according to the manufacturer's instructions. Gene expression was measured by real-time PCR using the cDNAs, THUNDERBIRD SYBR qPCR Mix Reagents (Toyobo) and gene-specific oligonucleotide primers (listed in table 2) with an ABI Prism 7500 Real-Time PCR System (Applied Biosystems). The GAPDH level was used to normalise the relative abundance of mRNAs. To detect miRNA expression, the RT reaction was performed using the TaqMan MicroRNA Assay (Applied Biosystems). Primers for PCR reactions in the miRNA assays were obtained from Applied Biosystems.

Immunoblotting

Proteins (20 μg) were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and then transferred onto Immobilon P membranes. After blocking, the membranes were treated with primary antibodies followed by peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualised by the enhanced chemiluminescence system using the Fujifilm Image Reader LAS-3000 (Fuji Medical Systems, Stamford, Connecticut, USA).

Table 2 List of primer sequences

Gene		Sequence (5′-3′)	Accession No
Human	Forward	CCCGGGTTTCAGAGACAACTTC	NM_000088
Col1A1	Reverse	TCCACATGCTTTATTCCAGCAATC	
Human	Forward	GACAATGGCTCTGGGCTCTGTAA	NM_001613
αSMA	Reverse	CTGTGCTTCGTCACCCACGTA	
Human	Forward	AGCTTGCCCGAGTTCTACTACAG	NM_004064
CDKN1B	Reverse	ACCAAATGCGTGTCCTCAGAGT	
Human	Forward	CTCTACTGGCGAAACCTGTATCC	NM_000089
Col1A2	Reverse	TCTCCTAGCCAGACGTGTTTCTT	
Human	Forward	CTGGCCACAACTGCCAAATG	NM_001145938
MMP1	Reverse	CTGTCCCTGAACAGCCCAGTACTTA	
Human	Forward	TGACATCAAGGGCATTCAGGAG	NM_001127891
MMP2	Reverse	TCTGAGCGATGCCATCAAATACA	
Human	Forward	TCGAACTTTGACAGCGACAAGAA	NM_004994
MMP9	Reverse	TCAGTGAAGCGGTACATAGGGTACA	
Human	Forward	GGATACTTCCACAGGTCCCACAA	NM_003254
TIMP1	Reverse	CTGCAGGTAGTGATGTGCAAGAGTC	
Human	Forward	GGAGCACTGTGTTTATGCTGGAA	NM_003255
TIMP2	Reverse	GACCGAGCGATTGCTCAAGA	
Human	Forward	AGCGACTCGCCAGAGTGGTTA	NM_000660
TGFβ1	Reverse	GCAGTGTGTTATCCCTGCTGTCA	
Human	Forward	GCACCGTCAAGGCTGAGAAC	NM_002046
GAPDH	Reverse	TGGTGAAGACGCCAGTGGA	
Mouse	Forward	CCTGGCAAAGACGGACTCAAC	NM_007742
Col1A1	Reverse	GCTGAAGTCATAACCGCCACTG	
Mouse	Forward	TCCCTGGAGAAGAGCTACGAACT	NM_007392
αSMA	Reverse	AAGCGTTCGTTTCCAATGGT	
Mouse	Forward	TGCACCACCAACTGCTTAG	NM_008084
GAPDH	Reverse	GGATGCAGGGATGATGTTC	

Col1A1, α1 (I) collagen; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; αSMA, α-smooth muscle actin; TIMP, tissue inhibitor of matrix metalloproteinase.

Transient transfection with miRNA precursors and inhibitors

Precursors or inhibitors of miR-222 and the negative control miRNA were transfected into human and mouse stellate cells using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) at a final concentration of 50 nmol/l, as previously described.[30 31] After 6 h, the culture medium was changed. Then, after 24 h, the cells were collected for total RNA and protein extraction.

Luciferase reporter assay

The interaction of the *CDKN1B* 3′UTR with miR-222 was assayed basically according to a previously described method.[30 31] The *CDKN1B* 3′UTR was obtained by PCR using human stellate cell cDNA as a template and the primer set forward 5′-TTCTCGAGGTTCTTGTCTTGATGTGTCACC-3′, reverse 5′-TTTCTAGAGAGAGCAGAGGCCTGAGAAG-3′. The Dual-Glo Luciferase Assay System (Promega, Madison, Wisconsin, USA) was used to analyse luciferase expression, according to the manufacturer's protocol.

Cell proliferation assay

LX-2 cells were plated at a density of 3×10^3 cells/well in 96-well plates for 24 h and were then transfected with the miR-222 precursor or inhibitor as described above. After 24 h, the medium was changed and culturing was continued for an additional

1–3 days before measuring cell proliferation by the WST-1 assay.[31]

Statistical analysis

Data, presented as box plots, are the median, IQR, minimum and maximum. The Mann–Whitney U test was used to analyse the distribution of continuous variables. The Jonckheere–Terpstra test for ordered alternatives was used to identify trends among classes. Correlation coefficients between parameters were evaluated by Spearman rank correlations. A two-tailed p value <0.05 was considered significant.

RESULTS

Patient characteristics

Table 1 shows the baseline characteristics of the patients infected with HCV and those with NASH who were included in this study. There were no significant differences in clinical, biochemical, haematological, virological, or histological characteristics between the 22 patients with HCV who were analysed by microarray and the 13 patients with HCV who were not.

miRNA expression profile in patients with HCV

We comprehensively compared miRNA expression profiles between cases of mild liver fibrosis (F1/F2) and advanced liver fibrosis (F3/F4) among the 22 patients with HCV using microarray analysis. As shown in the heat map in figure 1, 18 miRNAs were significantly upregulated (fold change, 1.21 to 2.59), whereas six miRNAs were significantly downregulated (fold change, −1.69 to −1.20) in livers with advanced fibrosis. Among the 18 overexpressed miRNAs, p values of <0.01 were found for miR-222 (fold change, 1.80), miR-214 (fold change, 1.84), miR-199a-3p (miR-199b-3p) (fold change, 1.90) and miR-199a-5p (fold change, 2.00). Among the six downregulated miRNAs, miR-422a (fold change, −1.69) showed a p value <0.01. Among them, the abundance of miR-199a-5p and miR-199a-3p has previously been reported to be increased in fibrotic liver disease.[32]

Next, we quantitatively confirmed our miRNA expression results in 35 patients with HCV (table 1) using real-time PCR. miR-199a-5p, miR-199a-3p and miR-222 were significantly upregulated in a stepwise manner according to the progression of liver fibrosis (figure 2A). miR-222 forms a cluster with miR-221 in the human and mouse genomes. In fact, miR-221 expression was significantly upregulated in patients with HCV with severe fibrosis (figure 2A). Col1A1 mRNA expression also significantly increased with increasing progression of liver fibrosis (figure 2B) and was significantly correlated with the expression of miR-222 ($r=0.843$, $p<0.001$) (figure 2C). Additionally, αSMA mRNA expression tended to increase according to the progression of fibrosis (figure 2B) and was significantly correlated with the expression of miR-222 ($r=0.701$, $p<0.001$) (figure 2C).

MicroRNA-221/222 expression in patients with NASH

We also validated miR-221 and miR-222 expression in 26 patients with biopsy-proven NASH (F1, seven; F2, eight; F3, eight; F4, three) by real-time PCR. The expression of these miRNAs was significantly upregulated in a manner dependent on the progression of liver fibrosis (figure 3A). Col1A1 mRNA expression was also significantly upregulated (figure 3B) and correlated with the expression of miR-222 ($r=0.626$, $p<0.001$) (figure 3C). In contrast, the expression level of αSMA mRNA tended towards a correlation with that of miR-222, but this result was not significant ($r=0.375$, $p=0.059$) (figure 3B,C). These results indicate the close correlation between miR-222 and Col1A1 mRNA expression in human liver fibrosis caused by NASH.

Expression of miR-221/222 in rodent models of liver fibrosis

Next, we studied miR-221 and miR-222 expression in mouse models of liver fibrosis. First, liver fibrosis was induced in mice by injecting TAA for 8 weeks. As shown in figure 4A, haematoxylin and eosin staining and Sirius red staining confirmed the occurrence of liver fibrosis, in particular around the central vein

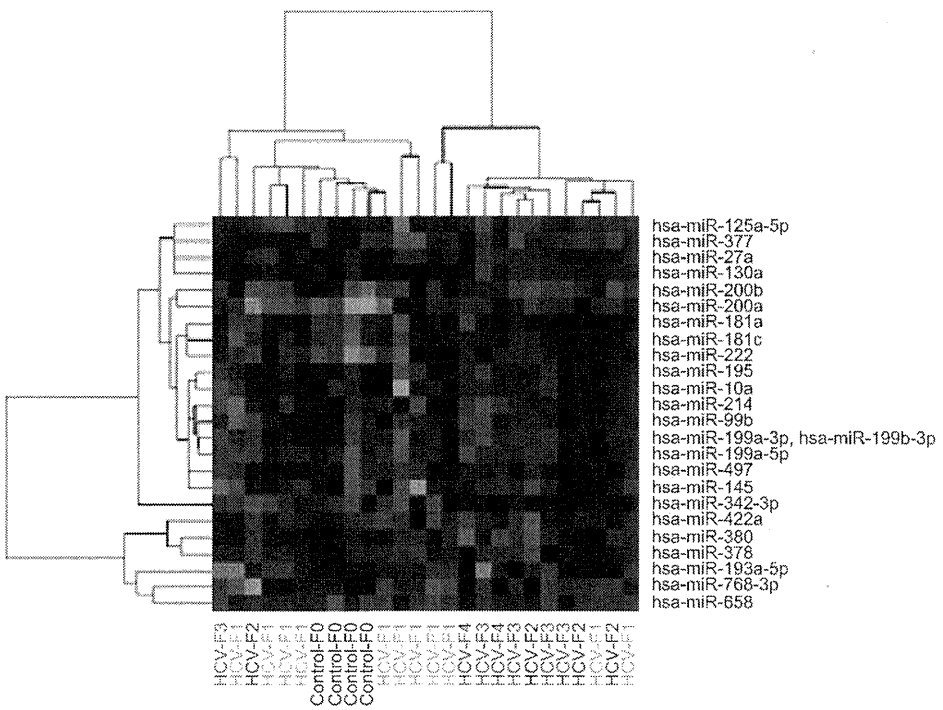

Figure 1 Heat map of microRNA (miRNA) expression in human liver tissue. Shown is the clustering of patients (n=22) with chronic hepatitis C by comprehensive analysis of intrahepatic miRNA expression according to the degree of hepatic fibrosis in biopsy specimens. Green and red denote downregulated and upregulated genes, respectively. F, fibrosis stage; HCV, hepatitis C virus.