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肝硬変・肝がん治療への応用を目的とした β-catenin依存性シグナルによる肝代謝機能制御機構の基礎的研究

(H22-政策創薬--一般-011)

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研究代表者　関根　茂樹

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肝硬変・肝がん治療への応用を目的とした β-catenin依存性シグナルによる肝代謝機能制御機構の基礎的研究

（H22-政策創薬-一般-011）

研究代表者　関根　茂樹　　国立がん研究センター研究所

研究要旨

肝臓は多彩な代謝機能を担う臓器であり、これらの機能は多くのシグナル経路によって複雑に制御されている。申請者はこれまで主に肝細胞特異的β-cateninノックアウトマウスを用いてβ-cateninシグナルによる肝代謝機能制御を解析し、この経路が肝予備能に影響を与えている事、そして外来異物を含む種々の物質の代謝を制御している事が明らかにしつつある。さらに、β-cateninは肝小葉内で領域特異的な遺伝子発現制御に重要な働きを果たしている事から、肝小葉構造の破壊を伴う肝硬変組織においては、このシグナル経路の異常の存在が予想される。これまで肝硬変は主に肝実質細胞の減少により機能不全を来す疾患と考えられてきたが、肝小葉構築の破壊に伴うβ-cateninシグナル異常が相乗的に関わっているとすれば、このシグナルの制御による肝機能改善の可能性が期待される。一方、このシグナル経路は肝発がんにおいても重要な役割を果たしている事が報告されている。約30%の肝細胞がんにおいてβ-cateninをコードする*CTNNB1*遺伝子変異が見られ、その頻度は特にC型肝炎患者に発生する腫瘍で高率である事が知られている。このシグナル経路が代謝機能に与える役割を考慮すると、これらの腫瘍発生の背景となっているC型肝炎および、その発がん過程においてβ-cateninシグナルの異常が関わっている事、さらにβ-catenin遺伝子変異陽性の腫瘍は特徴的な代謝特性を有している事が予想される。本研究では、肝臓におけるβ-cateninシグナルの生理的機能と肝硬変、肝細胞がんにおける異常を総合的に明らかにし、このシグナル経路の制御を通じた肝機能の改善の可能性を探る。また、肝細胞がんにおけるβ-catenin遺伝子異常に伴う腫瘍特性を明らかにし、これを診断および個別改良への応用の可能性を検討する。

研究分担者

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ユニット長

A．研究目的

ウイルス性肝炎の終末像である肝硬変や肝細胞がんの治療については未だ対症療法や外科切除等が治療の重要な部分を占めており、新たな薬物治療の開発が望まれる。β-cateninは肝小葉内で領域特異的な遺伝子発現制御に重要な働きを果たしており、この制御を通じて種々の代謝機能の制御に関わっている事が明らかにされつつある。さらに、

30-40%の肝細胞がんではβ-cateninの変異の存在が知られている。本研究では肝細胞の代謝機能調節に関わるβ-cateninの役割に注目し、その肝硬変、肝細胞がんにおけるβ-cateninシグナル異常の病態への関わりを明らかにし、このシグナル制御を通じた、肝硬変における肝代謝能の改善や肝細胞がんの診断・個別化治療への応用のための基礎を築く事を目標とする。

B．研究方法

本研究では、マウスモデルの解析によりβ-catenin の生体肝における生理的機能を明らかにし、これを基盤として、肝硬変および肝細胞がん臨床検体の解析によるヒト疾患でのβ-catenin シグナル異常の病態への関わりを検索する。

遺伝子改変マウスとしては、肝細胞特異的β-catenin ノックアウトマウスおよびβ-catenin 依存性転写活性レポーターマウスを用いる。ノックアウトマウスは主に生体肝におけるβ-catenin による遺伝子発現制御と機能の解析に用いる。レポーターマウスは種々の病態における肝組織中のβ-catenin シグナルの変化の解析に用いる。

ヒト肝硬変および肝がん臨床検体におけるβ-catenin 下流遺伝子の発現を定量PCR および免疫組織化学染色で検索する。これに用いる肝硬変および肝がん臨床検体は既にcDNA 合成済みのものが約50症例、組織化学染色用の検体が約70症例確保されており、肝細胞がん検体についてはβ-cateninの遺伝子変異解析を終えたところである。これらの蒐集された検体のうち、約80%はウイルス性肝炎患者から得られたものである。

マウスモデルや臨床検体で明らかになったβ-catenin による下流遺伝子発現制御機構を明らかにする目的で、必要に応じて培養細胞を用いてin vitro の実験を行う。

（倫理面への配慮）

肝がん臨床検体の解析に関しては既に国立がんセンター倫理審査委員会の審査と承認を受けている。実施に当たっては「疫学研究に関する倫理指針」に基づいて行う。研究対象となる臨床検体については国立がんセンター中央病院での肝切除材料のうち、摘出標本のがん研究への利用に関して文書による患者の同意が得られている検体のみを用いる。解析にあたっては適切に匿名化を行い、患者情報の取り扱いに留意する。

動物実験に関しては既に国立がんセンター動物実験倫理委員会の審査を受け、認可を受けている。実施に当たっては「国立がんセンターにおける動物実験に関する指針」ならびに関連規定を遵守する。

C．研究結果

β-catenin依存性シグナルの肝臓における胆汁酸代謝制御への役割を明らかにする目的で、肝細胞特異的β-cateninノックアウトマウスにおいて、肝臓、血液、糞便などにおける胆汁酸およびビリルビンの動態の変化を検索した。この結果、ノックアウトマウスでは胆汁酸の産生が低下しており、さらに胆汁酸およびビリルビンの血中からの排泄能が低下していることを見いだした。これらの胆汁酸およびビリルビン代謝機構の原因を調べるため、これらの物質の代謝に関わる分子の発現を検索したところ、Cyp7a1, Cyp27a1をはじめとする複数の胆汁産合成に関わる分子、およびSlco1b2等の血中から肝細胞への胆汁酸取り込みに関わるトランスポーターの著明な発現低下が認められた。以上の所見から、β-catenin依存性シグナルは肝臓において胆汁酸の産生と排泄を促進的に制御していることが示唆された。

この結果に基づいて、マウス肝臓において発現の変化が認められた胆汁酸代謝に関わる分子の発現をヒト肝細胞がんおよび非腫瘍肝組織で検索した。肝細胞がんにおいては*CTNNB1*遺伝子変異の存在は胆汁産生に関わる分子の発現や胆汁酸シグナルの変化との相関は認められなかった。しかし、胆汁酸の産生に関わる分子のうちAMACRの発現が*CTNNB1*遺伝子変異と強く相関していた。また、SLCO1B3の発現は*CTNNB1*遺伝子が野生型の腫瘍では著明な発現低下を示すものの、*CTNNB1*遺伝子変異の存在する肝細胞がんにおいて発現が保たれている事を見いだした。さらに、胆汁色素沈着は*CTNNB1*の存在よりもSLCO1B3の発現に、より強く相関していた。

現在、胆汁酸以外の代謝経路の変化に関しても検索を進めている。肝細胞特異的マウスの解析から、β-cateninシグナルが脂質代謝、糖代謝に与える影響に関して基礎的な所見を得ており、今後この解析を進めていく。

D．考察

β-cateninは肝臓において胆汁酸およびビリルビンの代謝制御に重要な役割を果たしている。その制御機能の少なくとも一部は肝細胞がんにおいても保たれており、肝細胞がんの腫瘍の特性に影響を与えていると考えられる。

AMACRは前立腺がんの大半で高発現していることが知られており、分枝鎖脂肪酸の代謝を通じて腫瘍の悪性度の関わっている事が示唆されている。今後、AMACRがβ-catenin下流遺伝子として肝細胞がん発生に寄与しているか、主にマウスモデルを使って検討を行う。

近年、*CTNNB1*遺伝子変異を伴う肝細胞がんは胆汁色素の沈着を伴うことが報告されているが、我々の検討では胆汁色素沈着は*CTNNB1*の存在よりもSLCO1B3の発現に、より強く相関していた。SLCO1B3はビリルビンの肝細胞への取り込みに関わっていることが知られており、この所見は肝細胞がんにおけるSLCO1B3の機能性を反映していると考えられる。

興味深い事に、SLCO1B3は肝細胞特異的MRI造影剤であるGd-EOB-DTPAの主要なトランスポーターでもある。SLCO1B3の発現がβ-cateninにより制御されていることを考慮すると、Gd-EOB-DTPAによるMRI造影画像を、β-catenin依存性シグナルの変化として理解できる可能性が示唆される。現在、β-cateninシグナル，SLCO1B3発現とGd-EOB-DTPAによる造影像の相関について肝がんを含む肝占拠性病変について手術切除標本を用いて検索を進めている。

E．結論

β-cateninシグナルは肝臓において胆汁酸の産生と、胆汁酸・ビリルビンの肝細胞への取り込みに促進的に働いている。この制御の一部は肝細胞がんでも認められ、*CTNNB1*遺伝子変異陽性肝細胞がんはAMACR，SLCO1B3の高発現を示した。*CTNNB1*遺伝子変異はこれらの分子の誘導を通じて肝細胞がんの代謝特性に影響を与えていることが予測される。特にSLCO1B3については、肝造影剤の取り込みにも関わっている、これまでに蓄積された種々の肝疾患におけるWnt/β-cateninシグナルの異常に関連する知見をGd-EOB-DTPAによる造影の理解へ応用することが可能になると期待される。

F．健康危険情報

該当なし

G．研究発表

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2. Sekine S, Ogawa R, Ojima H, Kanai Y. Overexpression of α-methylacyl-CoA racemase is associated with CTNNB1 mutations in hepatocellular carcinomas. Histopathology. in press

2．学会発表

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H．知的財産権の出願・登録状況

（予定を含む）

1．特許取得　　該当なし
2．実用新案登録　該当なし
3．その他　　該当なし

別添4

厚生労働科学研究費補助金（創薬基盤推進研究事業）

分担研究報告書

肝硬変・肝がん治療への応用を目的とした β-catenin依存性シグナルによる肝代謝機能制御機構の基礎的研究

（H22-政策創薬-一般-011）

研究分担者　関根　茂樹　　国立がん研究センター研究所

研究要旨

肝臓は多彩な代謝機能を担う臓器であり、これらの機能は多くのシグナル経路によって複雑に制御されている。申請者はこれまで主に肝細胞特異的β-cateninノックアウトマウスを用いてβ-cateninシグナルによる肝代謝機能制御を解析し、この経路が肝予備能に影響を与えている事、そして外来異物を含む種々の物質の代謝を制御している事が明らかにしつつある。さらに、β-cateninは肝小葉内で領域特異的な遺伝子発現制御に重要な働きを果たしている事から、肝小葉構造の破壊を伴う肝硬変組織においては、このシグナル経路の異常の存在が予想される。これまで肝硬変は主に肝実質細胞の減少により機能不全を来す疾患と考えられてきたが、肝小葉構築の破壊に伴うβ-cateninシグナル異常が相乗的に関わっているとすれば、このシグナルの制御による肝機能改善の可能性が期待される。一方、このシグナル経路は肝発がんにおいても重要な役割を果たしている事が報告されている。約30%の肝細胞がんにおいてβ-cateninをコードする*CTNNB1*遺伝子変異が見られ、その頻度は特にC型肝炎患者に発生する腫瘍で高率である事が知られている。このシグナル経路が代謝機能に与える役割を考慮すると、これらの腫瘍発生の背景となっているC型肝炎および、その発がん過程においてβ-cateninシグナルの異常が関わっている事、さらにβ-catenin遺伝子変異陽性の腫瘍は特徴的な代謝特性を有している事が予想される。本研究では、肝臓におけるβ-cateninシグナルの生理的機能と肝硬変、肝細胞がんにおける異常を総合的に明らかにし、このシグナル経路の制御を通じた肝機能の改善の可能性を探る。また、肝細胞がんにおけるβ-catenin遺伝子異常に伴う腫瘍特性を明らかにし、これを診断および個別改良への応用の可能性を検討する。

A．研究目的

ウイルス性肝炎の終末像である肝硬変や肝細胞がんの治療については未だ対症療法や外科切除等が治療の重要な部分を占めており、新たな薬物治療の開発が望まれる。β-cateninは肝小葉内で領域特異的な遺伝子発現制御に重要な働きを果たしており、この制御を通じて種々の代謝機能の制御に関わっている事が明らかにされつつある。さらに、30-40%の肝細胞がんではβ-cateninの変異の存在が知られている。本研究では肝細胞の代謝機能調節に関わるβ-cateninの役割に注目し、その肝硬変、肝細胞がんにおけるβ-cateninシグナル異常の病態への関わりを明らかにし、このシグナル制御を通じた、肝硬変にお

ける肝代謝能の改善や肝細胞がんの診断・個別化治療への応用のための基礎を築く事を目標とする。

B．研究方法

本研究では、マウスモデルの解析によりβ-catenin の生体肝における生理的機能を明らかにし、これを基盤として、肝硬変および肝細胞がん臨床検体の解析によるヒト疾患でのβ-catenin シグナル異常の病態への関わりを検索する。

遺伝子改変マウスとしては、肝細胞特異的β-catenin ノックアウトマウスおよびβ-catenin 依存性転写活性レポーターマウスを用いる。ノックアウトマウスは主に生体肝におけるβ-catenin による遺伝子発現制御と機能の解析に用いる。レポーターマウスは種々の病態における肝組織中のβ-catenin シグナルの変化の解析に用いる。

ヒト肝硬変および肝がん臨床検体におけるβ-catenin 下流遺伝子の発現を定量PCR および免疫組織化学染色で検索する。これに用いる肝硬変および肝がん臨床検体は既にcDNA 合成済みのものが約50症例、組織化学染色用の検体が約70症例確保されており、肝細胞がん検体についてはβ-cateninの遺伝子変異解析を終えたところである。これらの蒐集された検体のうち、約80%はウイルス性肝炎患者から得られたものである。

マウスモデルや臨床検体で明らかになったβ-catenin による下流遺伝子発現制御機構を明らかにする目的で、必要に応じて培養細胞を用いてin vitro の実験を行う。

（倫理面への配慮）

肝がん臨床検体の解析に関しては既に国立がんセンター倫理審査委員会の審査と承認を受けている。実施に当たっては「疫学研究に関する倫理指針」に基づいて行う。研究対象となる臨床検体については国立がんセンター中央病院での肝切除材料のうち、摘出標本のがん研究への利用に関して文書による患者の同意が得られている検体のみを用いる。解析にあたっては適切に匿名化を行い、患者情報の取り扱いに留意する。

動物実験に関しては既に国立がんセンター動物実験倫理委員会の審査を受け、認可を受けている。実施に当たっては「国立がんセンターにおける動物実験に関する指針」ならびに関連規定を遵守する。

C．研究結果

β-catenin依存性シグナルの肝臓における胆汁酸代謝制御への役割を明らかにする目的で、肝細胞特異的β-cateninノックアウトマウスにおいて、肝臓、血液、糞便などにおける胆汁酸およびビリルビンの動態の変化を検索した。この結果、ノックアウトマウスでは胆汁酸の産生が低下しており、さらに胆汁酸およびビリルビンの血中からの排泄能が低下していることを見いだした。これらの胆汁酸およびビリルビン代謝機構の原因を調べるため、これらの物質の代謝に関わる分子の発現を検索したところ、Cyp7a1, Cyp27a1をはじめとする複数の胆汁産合成に関わる分子、およびSlco1b2等の血中から肝細胞への胆汁酸取り込みに関わるトランスポーターの著明な発現低下が認められた。以上の所見から、β-catenin依存性シグナルは肝臓において胆汁酸の産生と排泄を促進的に制御していることが示唆された。

この結果に基づいて、マウス肝臓において発現の変化が認められた胆汁酸代謝に関わる分子の発現をヒト肝細胞がんおよび非腫瘍肝組織で検索した。肝細胞がんにおいては*CTNNB1*遺伝子変異の存在は胆汁産生に関わる分子の発現や胆汁酸シグナルの変化との相関は認められなかった。しかし、胆汁酸の産生に関わる分子のうちAMACRの発現が*CTNNB1*遺伝子変異と強く相関していた。また、SLCO1B3の発現は*CTNNB1*遺伝子が野生型の腫瘍では著明な発現低下を示すものの、*CTNNB1*遺伝子変異の存在する肝細胞がんにおいて発現が保たれている事を見いだした。さらに、胆汁色素沈着は*CTNNB1*の存在よりもSLCO1B3の発現に、より強く相関していた。

現在、胆汁酸以外の代謝経路の変化に関しても検索を進めている。肝細胞特異的マウスの解析から、β-cateninシグナルが脂質代謝、糖代謝に与える影響に関して基礎的な所見を得ており、今後この解析を進めていく。

D．考察

β-cateninは肝臓において胆汁酸およびビリルビンの代謝制御に重要な役割を果たしている。その制御機能の少なくとも一部は肝細胞がんにおいても保た

れており、肝細胞がんの腫瘍の特性に影響を与えていると考えられる。

AMACRは前立腺がんの大半で高発現していることが知られており、分枝鎖脂肪酸の代謝を通じて腫瘍の悪性度の関わっている事が示唆されている。今後、AMACRがβ-catenin下流遺伝子として肝細胞がん発生に寄与しているか、主にマウスモデルを使って検討を行う。

近年、*CTNNB1*遺伝子変異を伴う肝細胞がんは胆汁色素の沈着を伴うことが報告されているが、我々の検討では胆汁色素沈着は*CTNNB1*の存在よりもSLCO1B3の発現に、より強く相関していた。SLCO1B3はビリルビンの肝細胞への取り込みに関わっていることが知られており、この所見は肝細胞がんにおけるSLCO1B3の機能性を反映していると考えられる。

興味深い事に、SLCO1B3は肝細胞特異的MRI造影剤であるGd-EOB-DTPAの主要なトランスポーターでもある。SLCO1B3の発現がβ-cateninにより制御されていることを考慮すると、Gd-EOB-DTPAによるMRI造影画像を、β-catenin依存性シグナルの変化として理解できる可能性が示唆される。現在、β-cateninシグナル, SLCO1B3発現とGd-EOB-DTPAによる造影像の相関について肝がんを含む肝占拠性病変について手術切除標本を用いて検索を進めている。

E．結論

β-cateninシグナルは肝臓において胆汁酸の産生と、胆汁酸・ビリルビンの肝細胞への取り込みに促進的に働いている。この制御の一部は肝細胞がんでも認められ、*CTNNB1*遺伝子変異陽性肝細胞がんはAMACR, SLCO1B3の高発現を示した。*CTNNB1*遺伝子変異はこれらの分子の誘導を通じて肝細胞がんの代謝特性に影響を与えていることが予測される。特にSLCO1B3については、肝造影剤の取り込みにも関わっている、これまでに蓄積された種々の肝疾患におけるWnt/β-cateninシグナルの異常に関連する知見をGd-EOB-DTPAによる造影の理解へ応用することが可能になると期待される。

F．健康危険情報

総括研究報告書に記入。

G．研究発表

1. 論文発表

1. Sekine S, Ogawa R, Kanai Y. Hepatomas with activating Ctnnb1 mutations in "Ctnnb1-deficient" livers: a tricky aspect of a conditional knockout mouse model. Carcinogenesis. 2011;32:622-8
2. Sekine S, Ogawa R, Ojima H, Kanai Y. Overexpression of α-methylacyl-CoA racemase is associated with CTNNB1 mutations in hepatocellular carcinomas. Histopathology. in press

2. 学会発表

関根茂樹、小川玲子、金井弥栄. β-cateninによる胆汁酸代謝制御、第17回肝細胞研究会、2010

H．知的財産権の出願・登録状況

（予定を含む）

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

別添５

研究成果の刊行に関する一覧表

雑誌

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Sekine S, Ogawa R, Kanai Y.	Hepatomas with activating Ctnnb1 mutations in "Ctnnb1-deficient" livers: a tricky aspect of a conditional knockout mouse model.	Carcinogenesis	32	622-8	2011
Sekine S, Ogawa R, Ojima H, Kanai Y	Overexpression of α -methylacyl-CoA racemase is associated with *CTNNB1* mutations in hepatocellular carcinomas.	Histopathology		in press	2011

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Hepatomas with activating *Ctnnb1* mutations in '*Ctnnb1*-deficient' livers: a tricky aspect of a conditional knockout mouse model

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Conditional knockout mice, based on the Cre-loxP system, are a widely used model for examining organ-specific gene functions. To date, efficient hepatocyte-specific knockout has been reported in many different models, but little attention has been paid to the long-term stability of the recombination efficiency. In the present study, we characterized *Alb-Cre;Ctnnb1*$^{flox/flox}$ 'hepatocyte-specific *Ctnnb1* knockout' mice of different ages to test whether efficient recombination is maintained over time. At 2 months of age, the knockout mouse livers achieved efficient deletions of β-catenin in hepatocytes. However, as the mice aged, the reappearance and expansion of β-catenin-expressing hepatocytes were observed. In 1-year-old mice, a significant proportion of the pericentral hepatocytes in the knockout mouse livers were replaced with β-catenin-positive hepatocytes, whereas the periportal hepatocytes mostly remained β-catenin-negative. Furthermore, most of the 1-year-old mice spontaneously developed hepatocellular adenomas and carcinomas that were positive for β-catenin and overexpressed glutamine synthetase and Slc1a2, both of which are hallmarks of active β-catenin signaling. Sequencing analysis revealed that the *Ctnnb1* alleles were not inactivated but had activating mutations in these tumors. The present study suggests that recombination efficiency should be carefully examined when hepatocyte-specific knockout mice of different ages are analyzed. In addition, illegitimate deletion mutations should be recognized as potential adverse effects of the Cre-loxP system.

Introduction

Knockout mouse models are an important tool for investigating gene functions *in vivo*. Although systemic knockout is the most straightforward strategy, conditional knockout models based on the Cre-loxP system are also widely used when systemic knockout results in a lethal outcome or an organ-specific gene function needs to be determined (1,2). Several Cre transgenic lines have been generated to achieve the hepatocyte-specific recombination of conditional alleles. Among them, *Alb-Cre* mice are widely used to achieve recombination in the adult liver, and the use of this strain reportedly allows the virtually complete deletion of conditional alleles in adult hepatocytes (3,4).

β-Catenin, encoded by *Ctnnb1*, is involved in two distinct processes in cells: cell adhesion and the transduction of Wnt signaling. In the absence of active Wnt signaling, β-catenin is mostly localized to the membrane in a complex with cadherins that mediates cell–cell adhesion. When the Wnt signaling pathway is activated, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it activates T cell factor (TCF)-dependent transcription (5). Recent studies have revealed multiple physiological roles of β-catenin in hepatocytes, including the regulation of metabolism and proliferation. In addition to these physiologic functions, β-catenin also plays a role in tumorigenesis. The acquisition of oncogenic *Ctnnb1* mutations leads to constitutively active TCF-dependent transcription, and the dysregulated expression of β-catenin/TCF target genes is thought to promote tumorigenesis (6). Oncogenic *CTNNB1* mutations have been identified in a variety of human tumors, and ~30% of hepatocellular carcinomas harbor *CTNNB1* mutations (7,8).

We previously generated *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice, in which β-catenin was efficiently eliminated from adult hepatocytes, to examine the functions of β-catenin in the liver (9,10). However, we noted that the efficient disruption of β-catenin was not stably maintained: the mutant livers were gradually repopulated with wild-type hepatocytes as the mice aged. Furthermore, the majority of 1-year-old mice unexpectedly developed hepatocellular adenomas and carcinomas. Our observations revealed some critical adverse effects of the Cre-loxP system in hepatocyte-specific knockout models.

Abbreviations: PCR, polymerase chain reaction; RT, reverse transcription; TCF, T cell factor.

Materials and methods

Mice

Alb-Cre (3,4), *Ctnnb1*$^{flox/flox}$ (11) and *Alb-Cre;Ctnnb1*$^{flox/flox}$ (10) mice were on a C57 background and generated as described previously. The mice used in the present study were maintained in barrier facilities according to the protocols approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center, Japan.

Reverse transcription–polymerase chain reaction

RNA extraction, reverse transcription (RT) and conventional polymerase chain reaction (PCR) were performed using standard protocols (12). For conventional PCR, the PCR products were electrophoresed in an agarose gel and visualized under ultraviolet light with ethidium bromide staining. Quantitative RT–PCR reactions were performed using FastStart Universal Probe Master (Roche Applied Science, Penzberg, Germany). The expression level of each gene was determined using *Gusb* as a standard, as described previously (10). The primer sequences and probes used in the present study are shown in supplementary Table 1, available at *Carcinogenesis* Online.

Histology and immunohistochemistry

Liver tissue samples were fixed in 10% buffered formalin and embedded in paraffin; sections were then subjected to hematoxylin and eosin and immunohistochemical staining. Immunohistochemistry was performed using an indirect immunoperoxidase method with peroxidase-labeled anti-mouse, anti-rabbit or anti-rat polymers (Histofine Simple Stain; Nichirei, Tokyo, Japan), as described previously (13). The primary antibodies used in the present study were anti-β-catenin (clone 14; 1:250 dilution; BD Bioscience, San Diego, CA), anti-Cyp2e1 (polyclonal, 1:1000 dilution; gift from Dr Magnus Ingelman-Sundberg), anti-GLT-1 (polyclonal, 1:500 dilution; gift from Masahiko Watanabe), anti-glutamine synthetase (clone 6; 1:500 dilution; Becton Dickinson, Franklin Lakes, NJ) and anti-Ki-67 (clone TEC-3; 1:200 dilution; DAKO, Glostrup, Denmark).

Double immunohistochemical staining was performed using glycine buffer treatment following the first antibody reaction as described previously (14). 3,3′-Diaminobenzidine tetrahydrochloride and Vector VIP (Vector Laboratories, Burlingame, CA) were used as chromogens.

Mutational analysis

Paraffin embedded or frozen tissue samples were used for DNA extraction. The samples were incubated in a DNA extraction buffer (50 mmol/l Tris–HCl, pH 8.0, 1 mmol/l ethylenediaminetetraacetic acid, 0.5% (vol/vol) Tween 20, 200 μg/ml proteinase K) at 55°C overnight. Proteinase K was inactivated by heating at 100°C for 10 min. The digested DNA samples were directly subjected to PCR (15). Complementary DNA samples, prepared as described above, were also used for sequencing analysis. The primers used for the mutational analysis are listed in supplementary Table 2, available at *Carcinogenesis* Online. The PCR products were electrophoresed in an agarose gel, visualized under ultraviolet light with ethidium bromide staining and recovered using a QIAquick Gel Extraction Kit (Qiagen Hilden, Germany). When PCR products of different sizes were detected, they were separately isolated. Isolated PCR products were sequenced bidirectionally using the same primers that were used for amplification.

Western blotting

Tissue samples were homogenized in Radioimmunoprecipitation assay buffer [50 mM Tris–HCl, pH 7.4, 1% (vol/vol) NP40, 0.1% (wt/vol) sodium dodecyl

sulfate, 0.25% (wt/vol) Na-deoxycholate and 1 mM ethylenediaminetetraacetic acid] with protease inhibitors (Complete; Roche Applied Science). Equal amounts of proteins were electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gels and transferred to a polyvinylidene difluoride membrane, then processed for immunoblotting with antibodies for β-catenin (clone 14; 1:2000 dilution) and glutamine synthetase (clone 6; 1:5000 dilution). Horseradish peroxidase-conjugated anti-mouse secondary antibody was used at a dilution of 1:5000 and was detected using enhanced chemiluminescence (GE Healthcare, Pittsburg, CA). The protein-transferred membrane was stained with Coomassie brilliant blue as a loading control.

Statistical analysis

The results are presented as the mean ± standard deviation. P value of <0.05 by Student's two-tailed t test was considered to be significant.

Results

Efficient disruption of β-catenin in Alb-Cre;Ctnnb1$^{flox/flox}$ mouse livers is not maintained in elderly mice

We previously showed that young adult *Alb-Cre;Ctnnb1$^{flox/flox}$* mouse livers exhibited the efficient disruption of *Ctnnb1* in hepatocytes (9,10). However, whether efficient recombination was stably maintained over a long time period was unclear. To test this issue, we used quantitative RT–PCR to examine the expression of *Ctnnb1* in the livers of 2-, 4-, 8- and 12-month-old *Alb-Cre;Ctnnb1$^{flox/flox}$* mice. In the 2-month-old *Alb-Cre;Ctnnb1$^{flox/flox}$* mice, the expression of *Ctnnb1* was reduced to 10% of that in the controls (Figure 1A). *Ctnnb1* expression was repressed to a similar level in the 4-month-old mice but significantly recovered in the 8- and 12-month-old mice. Although the repression of *Ctnnb1* was not complete even in the 2-month-old mice, this effect was probably due to the presence of a non-parenchymal cell population that retained *Ctnnb1* expression (10).

To estimate the recombination efficiency of *Ctnnb1* alleles specifically in hepatocytes, we also quantified the expressions of *Glul* and *Cyp2e1* (Figure 1B and C). These two genes are exclusively expressed in hepatocytes in the liver and their expressions require β-catenin (10). Although the expressions of *Glul* and *Cyp2e1* were almost completely diminished in the 2-month-old mutant mice, the expressions gradually recovered as the mice aged. In the 1-year-old mice, the *Glul* and *Cyp2e1* expression levels were about a quarter of that observed in the controls. These findings indicated that the *Ctnnb1* alleles were efficiently deleted in the hepatocytes of 2-month-old *Alb-Cre;Ctnnb1$^{flox/flox}$* mice, but that this deletion efficiency was not stably maintained over a long time period.

β-Catenin-positive hepatocytes repopulate pericentral areas of elderly Alb-Cre;Ctnnb1$^{flox/flox}$ mouse livers

We next performed immunohistochemistry to determine the localization of β-catenin (encoded by *Ctnnb1*)-positive hepatocytes in these mice. Control mouse livers exhibited the membranous expression of β-catenin in hepatocytes (Figure 2A). In contrast, the 2-month-old *Alb-Cre;Ctnnb1$^{flox/flox}$* mouse livers exhibited the virtually complete loss of β-catenin in the hepatocytes, whereas the sinusoidal endothelial cells retained β-catenin expression (Figure 2D). In the 4-month-old *Alb-Cre;Ctnnb1$^{flox/flox}$* mice, a few β-catenin-positive hepatocytes were detected (Figure 2G). The β-catenin-positive hepatocytes had expanded and formed clusters in the 8-month-old mice (Figure 2J); in the 12-month-old mice, the β-catenin-positive hepatocytes had predominantly repopulated the pericentral areas within the liver lobules, whereas the periportal areas mostly remained negative for β-catenin (Figure 2M).

Immunohistochemistry for two β-catenin-regulated gene products, glutamine synthetase (encoded by *Glul*) and Cyp2e1, produced findings that were consistent with β-catenin immunohistochemistry results. In the control mice, glutamine synthetase was expressed in a few layers of hepatocytes surrounding the central veins (Figure 2B), whereas Cyp2e1 was expressed in broader pericentral areas (Figure 2C). In the 2-month-old *Alb-Cre;Ctnnb1$^{flox/flox}$* mice, the expressions of

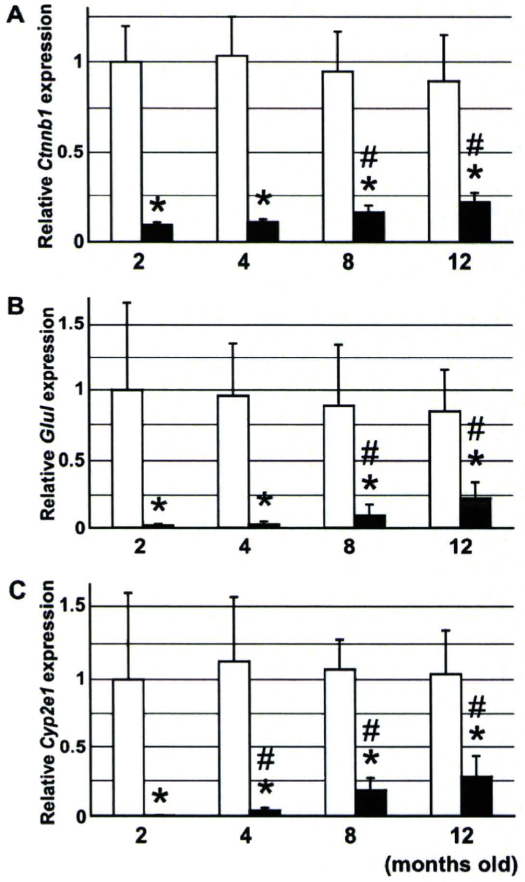

Fig. 1. Expression of *Ctnnb1*, *Glul* and *Cyp2e1* in *Alb-Cre;Ctnnb1$^{flox/flox}$* mouse livers at different ages. The expression levels of *Ctnnb1* (**A**), *Glul* (**B**) and *Cyp2e1* (**C**) in livers of *Alb-Cre;Ctnnb1$^{flox/flox}$* and control *Ctnnb1$^{flox/flox}$* mouse livers of different ages were determined using quantitative RT–PCR. White bars: controls; Black bars: *Alb-Cre;Ctnnb1$^{flox/flox}$* mice. n = 6–9 per group. Values are presented as the means ± standard deviations. $^{*}P < 0.05$ compared with controls of the same age. $\#P < 0.05$ compared with 2-month-old mice of the same genotype.

glutamine synthetase and Cyp2e1 were detected only in a few hepatocytes in the liver (Figure 2E and F). A small, but increased number of glutamine synthetase- and Cyp2e1-positive hepatocytes appeared in the 4-month-old mice (Figure 2H and I), and this cell population had further expanded in the 8-month-old mice (Figure 2K and L). In the 12-month-old mice, the pericentral expression patterns of these proteins were readily recognizable (Figure 2N and O). Interestingly, in 2–8-month-old mice, some glutamine synthetase- and Cyp2e1-positive hepatocytes were also observed in periportal areas, consistent with a finding reported by Braeuning *et al.*. (16). However, these cells were mostly localized to pericentral areas in 12-month-old mice.

Since the number of β-catenin-expressing hepatocytes increased with age, we assumed that these cells had a growth advantage over β-catenin-deficient hepatocytes. To test this hypothesis, we quantified the numbers of proliferating hepatocytes in β-catenin-positive and-deficient cell populations using immunohistochemistry for Ki-67. As a result, the proliferation of β-catenin-positive hepatocytes in *Alb-Cre;Ctnnb1$^{flox/flox}$* mice was significantly increased proliferation, compared with the proliferation of β-catenin-deficient hepatocytes and hepatocytes in wild-type mice (Figure 3). Since β-catenin-deficient hepatocytes were replaced by β-catenin-positive hepatocytes over time, we suspected that the β-catenin-deficient hepatocytes might be lost to apoptosis. However, we did not observe a significant

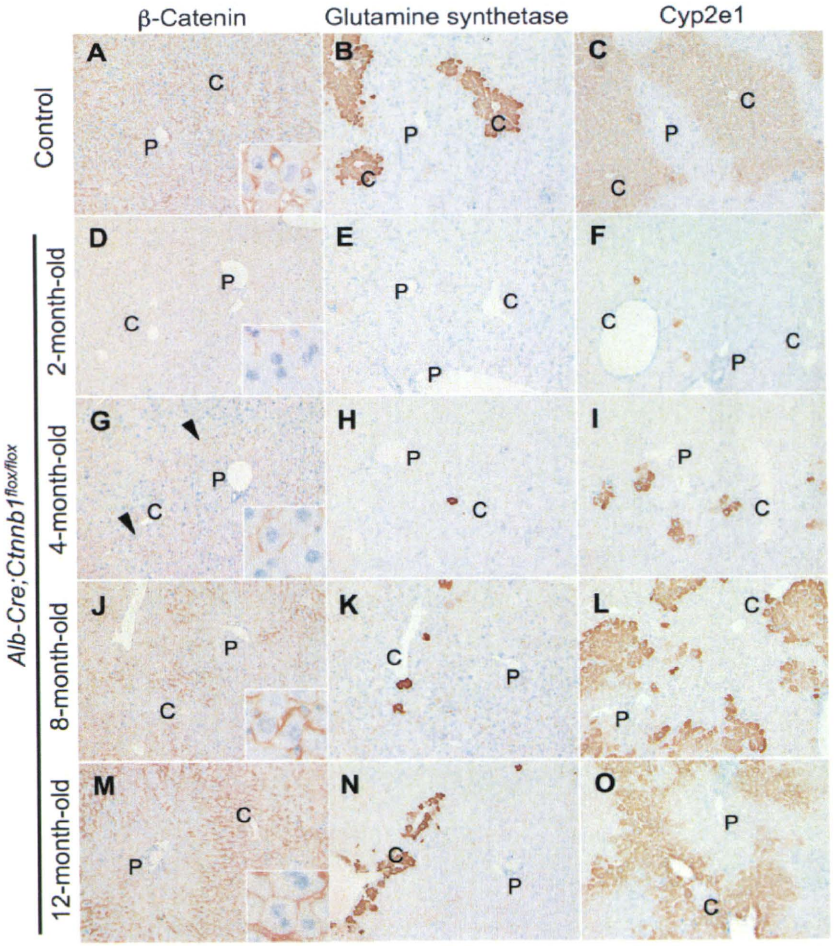

Fig. 2. Expression of β-catenin, glutamine synthetase and Cyp2e1 in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers at different ages. Expressions of β-catenin (**A**, **D**, **G**, **J** and **M**), glutamine synthetase (**B**, **E**, **H**, **K** and **N**) and Cyp2e1 (**C**, **F**, **I**, **L** and **O**) in control (A–C) and *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers (D–O), as determined using immunohistochemistry. Insets show high-magnification views of β-catenin staining (A, D, G, J and M). A few β-catenin-positive hepatocytes are indicated by the arrowheads (G). At least four mice were examined per group and representative results are presented. C, central veins; P, portal veins.

elevation in apoptotic activity in β-catenin-deficient hepatocytes using a TUNEL assay or immunohistochemistry for cleaved caspase-3 (data not shown), although an increase in apoptosis might not have been detectable because the replacement process progressed very slowly over a period of months.

Together, these results indicate that the β-catenin is efficiently eliminated in 2-month-old *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse hepatocytes; however, a gradual repopulation with β-catenin-positive hepatocytes occurred in older mice. This process is achieved through the expansion of a few residual β-catenin-positive hepatocytes in young *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers. Notably, β-catenin-positive hepatocytes predominantly repopulated the pericentral areas within the liver lobules in elderly mice, suggesting that the retention of wild-type β-catenin confers survival advantages to hepatocytes in the pericentral areas within the liver lobule.

Development of hepatocellular adenomas and carcinomas in Alb-Cre;Ctnnb1$^{flox/flox}$ *mouse livers*

Unexpectedly, we noted that 11 of the 13 *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice used in this study spontaneously developed liver tumors at an age of 1 year (Figure 4A). Most of the mice had one to five tumors, but two mice developed numerous tumors involving almost the entire liver. Among the tumors that were histologically examined, 17 lesions were hepatocellular adenomas and consisted of tumor cells without apparent atypia arranged in thin trabecule (Figure 4B and C; supplementary Table 3 is available at *Carcinogenesis* Online) (17). Eight lesions showed significant structural and/or cytological atypia enabling a diagnosis of hepatocellular carcinoma (Figure 4D). Surprisingly, immunohistochemistry demonstrated the membranous expression of β-catenin in 24 of the 25 tumors, including both adenomas and carcinomas (Figure 4E). Among these, 10 lesions also exhibited the nuclear and cytoplasmic accumulation of β-catenin (Figure 4F). Furthermore, all the β-catenin-positive tumors expressed glutamine synthetase and Slc1a2, which are hallmarks of active Wnt/β-catenin signaling (Figure 4G and H) (18,19), regardless of the presence of the nuclear/cytoplasmic accumulation of β-catenin. β-Catenin, glutamine synthetase and Slc1a2 were not expressed in one adenoma (Figure 4I and J).

Tumors developed in Alb-Cre;Ctnnb1$^{flox/flox}$ *mouse livers harbor activating Ctnnb1 mutations*

The expression of glutamine synthetase and Slc1a2 in a hepatocellular tumor implies the presence of active Wnt/β-catenin signaling in these tumors. Since genetic alterations of *Ctnnb1* are the most common mechanism responsible for the activation of this pathway during tumorigenesis, we analyzed the presence of *Ctnnb1* gene mutations.

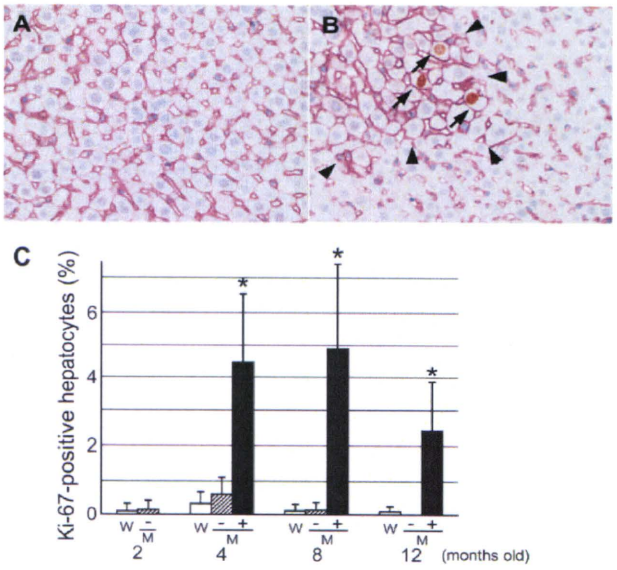

Fig. 3. Proliferative activity of β-catenin-positive hepatocytes in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers (**A** and **B**) Double staining immunohistochemistry for β-catenin (purple) and Ki-67 (brown) in control (A) and *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers (B) from 8-month-old. Hepatocytes in the control mouse liver show the membranous expression of β-catenin, but no Ki-67-positive cells are visible (A). Three hepatocytes within a cluster of β-catenin-positive hepatocytes (arrowheads) express Ki-67 (arrows) (B). Note that the sinusoidal endothelial cells retain β-catenin expression in the areas of the β-catenin-deficient hepatocytes. (**C**) Quantitative analysis of proliferative activity. At least 300 hepatocytes were counted when analysing the Ki-67-positive cells in each population. The values are presented as the mean ± standard deviation. The numbers of β-catenin-positive hepatocytes in 2-month-old *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers were too small to be quantified. n = 4–6 per group. W, wild-type controls; M, *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice; −, β-catenin-deficient hepatocytes; +, β-catenin-positive hepatocytes; $^{*}P < 0.05$ compared with wild-type controls of the same age.

Sequencing analyses identified deletion mutations within *Ctnnb1* in 18 of the 23 lesions that were examined (Figure 5A–E; supplementary Table 3 is available at *Carcinogenesis* Online). Of note, 10 mutations involved the first loxP site at their breakpoints. Furthermore, deletions in the messenger RNA transcripts were also identified in 13 of the 17 tumors that were examined. These deletions involved sequences encoding the N-terminal region of β-catenin, which is required for proteasomal degradation. On the other hand, analysis of messenger RNA samples of non-neoplastic tissue showed wild-type sequences of *Ctnnb1*.

Consistent with the presence of the deletion mutations, truncated protein products were identified using western blotting in all 13 samples that were examined (Figure 5F; supplementary Table 3 is available at *Carcinogenesis* Online). Even though the expression levels of truncated β-catenin were not significantly increased compared with the corresponding levels of wild-type β-catenin in non-neoplastic liver tissues, this observation is consistent with previous studies on *Ctnnb1*-mutated mouse liver tumors (20,21). The overexpression of glutamine synthetase was also confirmed using western blotting in tumors. We also examined *Cre* transgene expression in these tumors using RT–PCR and found that 15 of 18 tumors examined retained *Cre* expression (Figure 5G). Expression of *Cre* was significantly reduced in the remaining three tumors. Together, these findings suggest that most of the liver tumors in the *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice-harbored *Ctnnb1* mutations that resulted in the truncation of β-catenin, and the expression of the *Cre* transgene was not silenced in most of the tumors.

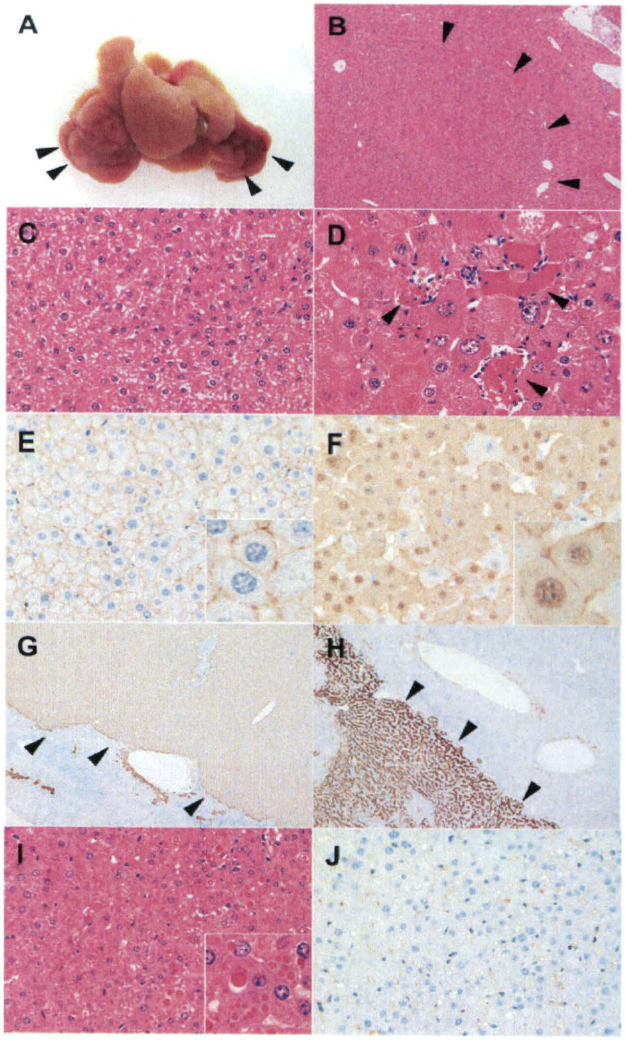

Fig. 4. Development of liver tumors in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers (**A**) Gross morphology of liver tumors in a 1-year-old *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse (arrowheads). (**B**) Low power magnification of a hepatocellular adenoma exhibiting expansive growth (arrowheads). (**C**) Hepatocellular adenoma. Well-differentiated hepatocytes without apparent cellular atypia are arranged in a thin trabecular pattern. (**D**) Hepatocellular carcinoma. Tumor cells are variable in size and exhibit prominent nuclear atypia. Foci of single cell necrosis are visible (arrowheads). (**E**) Hepatocellular adenoma exhibiting membranous β-catenin expression (inset: high power magnification). (**F**) Hepatocellular adenoma showing the cytoplasmic and nuclear accumulation, in addition to membranous expression, of β-catenin (inset: high power magnification). (**G**) Expression of glutamine synthetase in a hepatocellular adenoma (arrowheads). Non-neoplastic hepatocytes surrounding the central veins are also stained positive. (**H**) Expression of Slc1a2 in a hepatocellular adenoma (arrowheads). The pericentral hepatocytes also express Slc1a2. (**I** and **J**) Histology of a β-catenin-negative adenoma. The tumor cells contain numerous hyaline globules (I, inset: high power magnification) and lack β-catenin expression (J).

Discussion

Alb-Cre transgenic mice have been widely used to achieve hepatocyte-specific recombination in adult mice (3,4). The use of this strain enables the virtually complete recombination of conditional alleles flanked by loxP sites in adult hepatocytes since the *Alb* promoter is

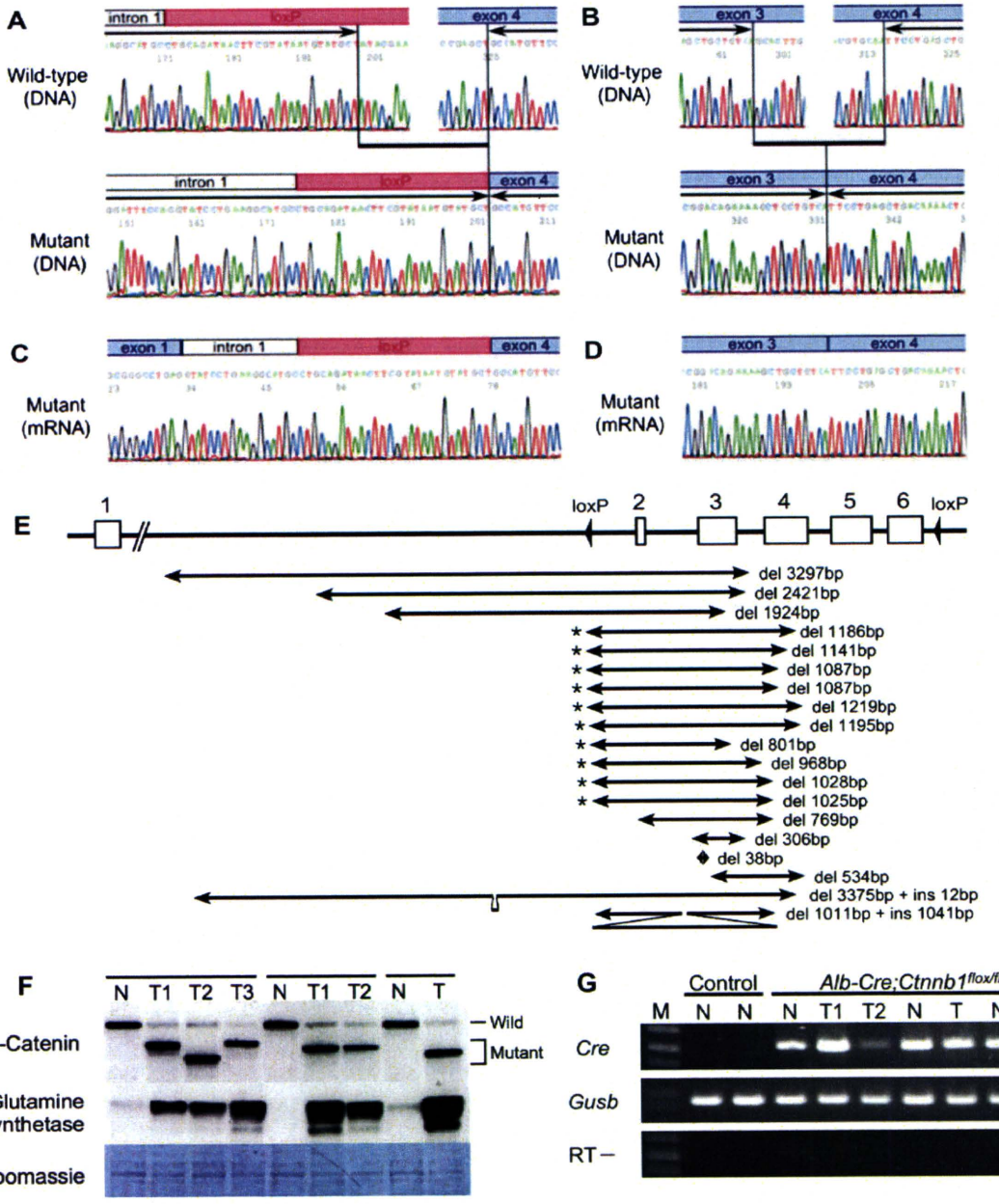

Fig. 5. Liver tumors in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice harbor *Ctnnb1* mutations (**A** and **C**) An example of a deletion mutation involving the first loxP site. The deletion mutation spans the first loxP sequence to exon 4 (A). This deletion mutation resulted in aberrant splicing and parts of intron 1 and loxP were inserted between exon 1 and a truncated exon 4 (C). (**B** and **D**) Another example of a deletion mutation spanning exons 3 and 4 (B). This deletion resulted in a 390 bp in-frame deletion at the messenger RNA level (D). (**E**) Deletion mutations in liver tumors in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice. The deletions detected in the liver tumors are indicated by the arrows. Ten of the nineteen deletions involved the first loxP site at their breakpoints (indicated by the asterisks). Two mutations were associated with insertions. Open boxes indicate exons of *Ctnnb1*. (**F**) Truncated β-catenin proteins in the liver tumors. All the tumors that were examined contained truncated β-catenin protein. The tumors overexpressed glutamine synthetase, consistent with the results of the immunohistochemistry analysis. (**G**) RT–PCR analysis of *Cre* transgene expression in liver tumors. Most of the tumors and all non-neoplastic liver tissues obtained from *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice-retained *Cre* expression, but one tumor sample showed significantly reduced *Cre* expression. *Gusb* served as a positive control for the RT reactions. RT−, RNA samples without RT reactions served as negative controls; Control, control *Ctnnb1*$^{flox/flox}$ mice; M, DNA size marker; T, tumor; N, non-neoplastic liver tissue.

active in mature hepatocytes. As we previously reported, the efficient deletion of the conditional allele was also achieved in adult mice when crossed with *Ctnnb1*$^{flox/flox}$ mice. However, the present study showed that the efficient deletion was not maintained in elderly mice because of repopulation with wild-type hepatocytes that had escaped Cre-mediated recombination. Indeed, we previously reported a similar repopulation process in *Alb-Cre;Dicer*$^{flox/flox}$ mice although the repopulation took place at a much earlier time point (12). Our previous and present observations imply that repopulation with wild-type hepatocytes might not be an exceptional event and might represent a potential artifact in the analysis of hepatocyte-specific knockout mouse models.

Liver repopulation is a process in which a small number of phenotypically distinct hepatocytes gradually expand and replace the host hepatocytes *in vivo* (22,23). This phenomenon has been well documented in therapeutic liver repopulation, where wild-type hepatocytes are transplanted into metabolically defective livers and gradually replace the host hepatocytes (24). Because of the enormous proliferative capacity of hepatocytes, the introduction of a small number of wild-type hepatocytes is sufficient to repopulate a significant proportion of the mutant livers over time. This process takes place when the transplanted hepatocytes exhibit a growth advantage and continuous damage is occurring to the host hepatocytes.

Interestingly, wild-type hepatocytes predominantly replaced the pericentral areas, whereas β-catenin-deficient hepatocytes persistently resided in the periportal areas within the liver lobules of elderly *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice. Wnt/β-catenin signaling is physiologically active in pericentral, but not periportal, hepatocytes (9,25). Accordingly, in the pericentral areas, the retention of β-catenin allows the proper transduction of Wnt/β-catenin signaling in wild-type hepatocytes, although Wnt/β-catenin signaling is blocked in β-catenin-deficient hepatocytes. As a result, wild-type hepatocytes selectively achieve the proper expression of β-catenin target genes and exhibit a survival advantage over β-catenin-deficient hepatocytes, as demonstrated in the present study. On the other hand, periportal hepatocytes do not express β-catenin target genes regardless of the status of the *Ctnnb1* alleles. Thus, wild-type hepatocytes are phenotypically indifferent from β-catenin-deficient hepatocytes in the periportal areas and do not exhibit a growth advantage.

Recent studies have shown the involvement of Wnt/β-catenin signaling in zonal gene expression within the liver lobule (25,26). Many β-catenin-regulated genes are related to the metabolic functions of hepatocytes; consequently, the region-specific activation of Wnt/β-catenin signaling is thought to be the basis for the metabolic heterogeneity of hepatocytes (25). In addition, the present observation implies that intact Wnt/β-catenin signaling confers a survival advantage to pericentral hepatocytes but not to periportal hepatocytes. Any of the β-catenin-regulated genes may be responsible for the survival advantage, but the exact mechanism remains to be elucidated.

Recently, Braeuning *et al.* (16) reported the presence of residual β-catenin-positive hepatocytes in young adult *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice, consistent with our observation. A potentially discrepant finding is that the residual β-catenin-positive hepatocytes did not exhibit a growth advantage over β-catenin-deficient hepatocytes unless the mice were subjected to phenobarbital treatment. However, they examined mice that were up to 5 months old and that had been raised under normal breeding conditions. Indeed, in our gene expression analysis as well, 4-month-old mice exhibited no significant increases in the expressions of *Ctnnb1* and *Glul*, compared with the levels of 2-month-old mice. The expansion of the β-catenin-expressing hepatocytes became more obvious in elderly mice. Furthermore, the increased expression of Ki-67 further supports the growth advantage of β-catenin-positive hepatocytes over β-catenin-deficient hepatocytes.

At a glance, the spontaneous tumor development in 'β-catenin-deficient' livers seems paradoxical, since β-catenin is a major oncogene product. However, immunohistochemical and mutational analyses have revealed that these tumors did not actually lose β-catenin expression but rather acquired oncogenic mutations in their *Ctnnb1* alleles. Notably, the mutational analysis showed a characteristic mutation spectrum for the hepatomas that developed in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice. A significant proportion of the identified deletions involved loxP sites, and most of the tumors retained the expression of the *Cre* transgene. These observations suggest that these mutations were probably caused by the misrecombination of the loxP-flanked region. It might be possible that expression of Cre recombinase might be also responsible for the other mutations since the spectrum of *Ctnnb1* mutations in the current model is quite distinct from that of previously reported spontaneous mutations. All the mutations identified here were deletion mutations, whereas missense mutations are more common among spontaneous *Ctnnb1* mutations in mouse models of hepatocarcinogenesis (7,27). Indeed, illegitimate recombination arising from the transgenic expression of Cre or CreER^{T2} has been reported in some models in the absence of loxP-flanked conditional alleles (28,29). On the other hand, there were three tumors that showed significant reduction of *Cre* transgene expression. These tumors could be developed by acquisition of *Ctnnb1* mutations in β-catenin-positive hepatocytes that were escaped from *Cre*-mediated recombination although it is not a major pathway of tumorigenesis in this model.

The introduction of mutant β-catenin to hepatocytes is not sufficient to induce hepatocyte proliferation or to initiate tumorigenesis by itself in wild-type mice (30,31). Therefore, some factor promoting tumorigenesis probably exists in this model. Indeed, the process of liver repopulation has been suggested to increase the risk of tumorigenesis in several mouse models (32,33). The repopulation process allows the sustained clonal expansion of wild-type hepatocytes as well as hepatocytes with oncogenic alterations (34), and this process probably increases the risk of tumor progression (Figure 6). Together, these findings suggest that the concurrent occurrence of misrecombination and liver repopulation by wild-type hepatocytes resulted in tumorigenesis in the *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice.

A recent study has reported an increase in tumor formation in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice treated with *N*-nitrosodiethylamine (35). However, the present study showed that *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice actually develop liver tumors even in the absence of carcinogen treatment. Indeed, Rignall (36) reported that two of six tumors in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice treated by *N*-nitrosodiethylamine and phenobarbital exhibited *Ctnnb1* mutations. Of note, the authors only performed analysis of exon 3 of *Ctnnb1*, which would not have identified most of the large deletion mutations detected in our analysis. The potential occurrence of illegitimate recombination involving *Ctnnb1* and secondary effects by liver repopulation should be taken into consideration to accurately evaluate the effect of β-catenin-loss on hepatocarcinogenesis using this mouse model.

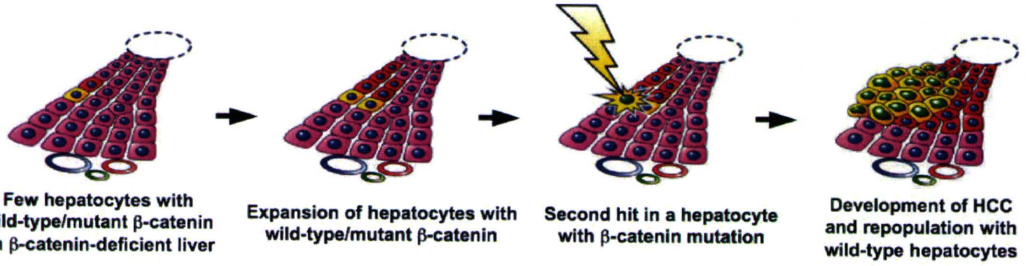

Fig. 6. Model of tumorigenesis in *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers At 2 weeks of age, the *Alb-Cre;Ctnnb1*$^{flox/flox}$ mouse livers are mostly composed of β-catenin-deficient hepatocytes (pink cells); however, a few residual β-catenin-positive hepatocytes that have escaped Cre-mediated recombination (red cells) and hepatocytes with activating *Ctnnb1* mutations as a result of illegitimate recombination (yellow cells) are present. Hepatocytes with wild-type or mutant β-catenin exhibit a survival advantage and gradually repopulate the pericentral areas. During this process, a 'second-hit', presumably an additional genetic alteration, initiates tumorigenesis in co-operation with the *Ctnnb1* mutation.

The present model probably represents an extreme example of artifacts related to the Cre-loxP system. However, the potential occurrence of repopulation should always be considered when analysing results obtained using hepatocyte-specific conditional knockout mouse models, particularly when mice of different ages are compared. Also, the formation of biologically active deletion mutants, such as dominant negative or constitutively active products, should be recognized as a potential adverse effect arising from illegitimate recombination in a manner that is not limited to the current model. Additionally, our findings demonstrated that active Wnt/β-catenin signaling confers a survival advantage to pericentral hepatocytes but not to periportal hepatocytes. Wnt/β-catenin signaling might act as a region-specific survival signal within the liver lobule.

Supplementary material

Supplementary Tables 1–3 can be found at http://carcin.oxfordjournals.org/

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