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トピックス

ヒト ES/iPS 細胞から肝細胞への高効率分化誘導法の開発とその創薬応用

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要　旨

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

はじめに

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

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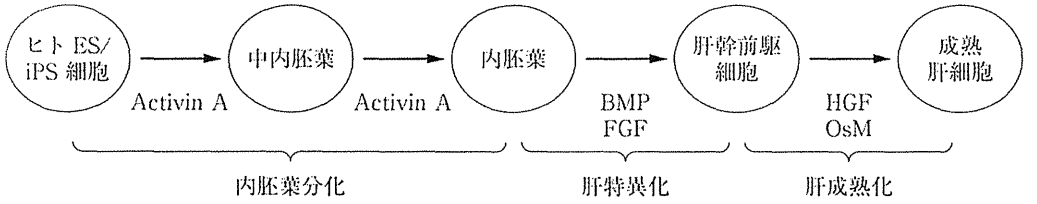
ヒト ES/iPS 細胞から肝細胞への分化誘導

１．液性因子の作用による従来の肝分化誘導法

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

ヒト ES/iPS 細胞から内胚葉への分化誘導ス

図1 ヒトES/iPS細胞から肝細胞への分化



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

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

2．肝分化関連遺伝子を導入する肝分化誘導法

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

3．分化誘導肝細胞と異種細胞との共培養法

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

ている．

4．肝分化させた細胞集団からの分化誘導肝細胞の抽出

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

分化誘導肝細胞の毒性評価系への応用

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

ま と め

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

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

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ヒトiPS細胞由来分化誘導肝細胞を用いた薬物毒性評価系の開発

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

川端健二[*1]　高山和雄[*2]　水口裕之[*3]

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

1. はじめに

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

2. 肝細胞の培養

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

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

3. ヒト iPS 細胞から肝細胞への分化誘導

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

3.1 ヒト iPS 細胞から内胚葉への分化誘導

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

3.2 内胚葉から肝細胞への特異化

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

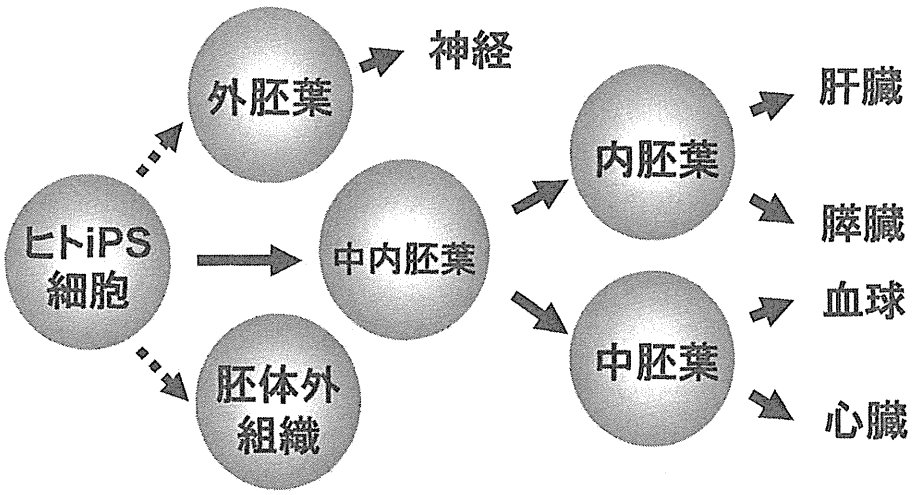

図 1　ヒト iPS 細胞から三胚葉への分化誘導
ヒト iPS 細胞はヒト ES 細胞とおなじく三胚葉に分化することができる。

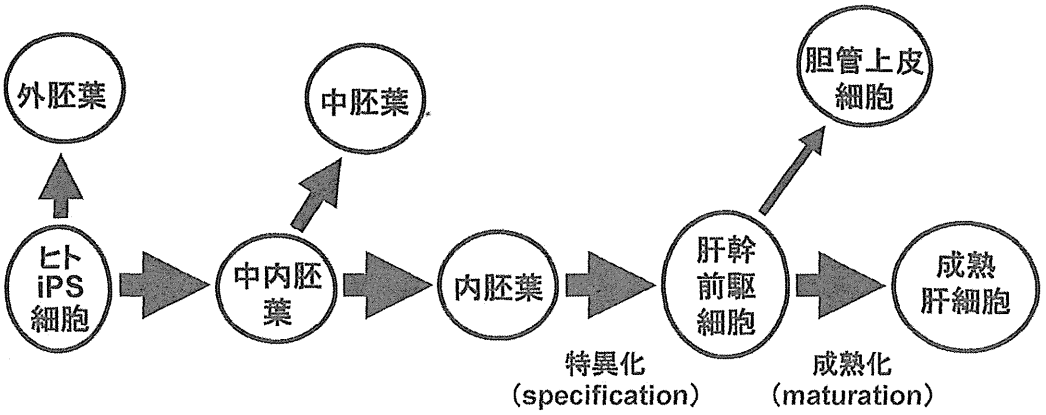

図2　ヒトiPS細胞から肝細胞への分化
ヒトiPS細胞から成熟肝細胞への分化は複数の過程に分けることができる。

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

3.3　肝幹前駆細胞から肝細胞への成熟化

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

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

3.4　遺伝子導入による肝細胞分化誘導

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

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

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

3.5　三次元培養技術による肝細胞の成熟化

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

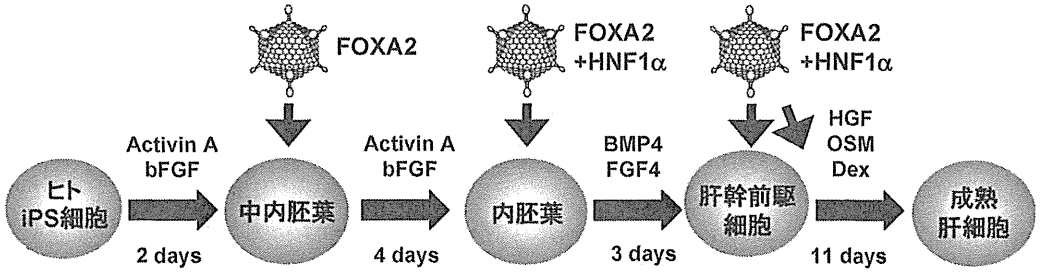

図3　遺伝子導入を用いたヒトiPS細胞から成熟肝細胞への分化誘導
分化の適切な時期に適切な遺伝子を一過性に発現させることにより，効率良く肝細胞を分化誘導できる。

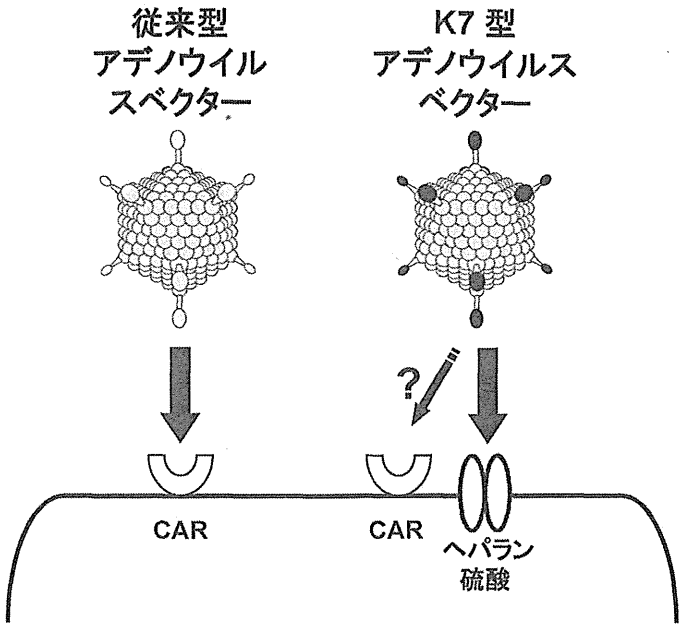

図4　改良型アデノウイルスベクター
改良型（K7型）アデノウイルスベクターはアデノウイルス受容体（CAR）だけでなく，ヘパラン硫酸も認識することにより，多くの細胞種に効率よく遺伝子導入が可能となる。

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

4. iPS細胞由来肝細胞を用いた薬物毒性評価系の開発

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

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

5. おわりに

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

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Placenta to cartilage: direct conversion of human placenta to chondrocytes with transformation by defined factors

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ABSTRACT **Cellular differentiation and lineage commitment are considered to be robust and irreversible processes during development. Recent work has shown that mouse and human fibroblasts can be reprogrammed to a pluripotent state with a combination of four transcription factors. We hypothesized that combinatorial expression of chondrocyte-specific transcription factors could directly convert human placental cells into chondrocytes. Starting from a pool of candidate genes, we identified a combination of only five genes (5F pool)—*BCL6, T* (also called *BRACHYURY*), *c-MYC, MITF*, and *BAF60C* (also called *SMARCD3*)—that rapidly and efficiently convert postnatal human chorion and decidual cells into chondrocytes. The cells generated expressed multiple cartilage-specific genes, such as *Collagen type II α1, LINK PROTEIN-1*, and *AGGRECAN*, and exhibited characteristics of cartilage both in vivo and in vitro. Expression of the endogenous genes for *T* and *MITF* was initiated, implying that the cell conversion is due to not only the forced expression of the transgenes, but also to cellular reprogramming by the transgenes. This direct conversion system from noncartilage tissue to cartilaginous tissue is a substantial advance toward understanding cartilage development, cell-based therapy, and oncogenesis of chondrocytes.**

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INTRODUCTION

The possibility of redirecting cell differentiation by overexpression of genes was suggested by H. Weintraub with the identification of the "master gene," *MyoD* (Davis *et al.*, 1987). The process was believed to involve reversion to a less differentiated state, a kind of dedifferentiation, before the new cell type is formed. Another process has since been introduced—the concept of direct conversion or direct reprogramming without dedifferentiation. This process is believed to be direct lineage switching rather than lineage switching back to a branch point and out again in a different direction (Hochedlinger and Jaenisch, 2006; Orkin and Zon, 2008). Direct conversion has been shown in β cells, cardiomyocytes, and neurons. A specific combination of three transcription factors (*Ngn3, Pdx1*, and *MafA*) reprograms differentiates pancreatic exocrine cells in adult mice into cells that closely resemble β cells (Zhou *et al.*, 2008); a combination of three factors (*Gata4, Tbx5*, and *Baf60c*) induces noncardiac mesoderm to differentiate directly into contractile cardiomyocytes (Ieda *et al.*, 2010); and a combination of three factors (*Ascl1, Brn2*, and *Myt1l*) converts mouse fibroblasts into functional neurons (Vierbuchen *et al.*, 2010). In this study, we used the strategy of direct conversion to generate chondrocytes from human somatic cells.

During skeletal development, chondrogenesis starts from condensed mesenchyme tissue, which differentiates into chondrocytes and begins secreting the molecules that form the extracellular matrix and leads to endochondral ossification. Cartilage is a stiff yet flexible connective tissue found in many areas in the bodies of humans and other animals. It is composed of chondrocytes, which

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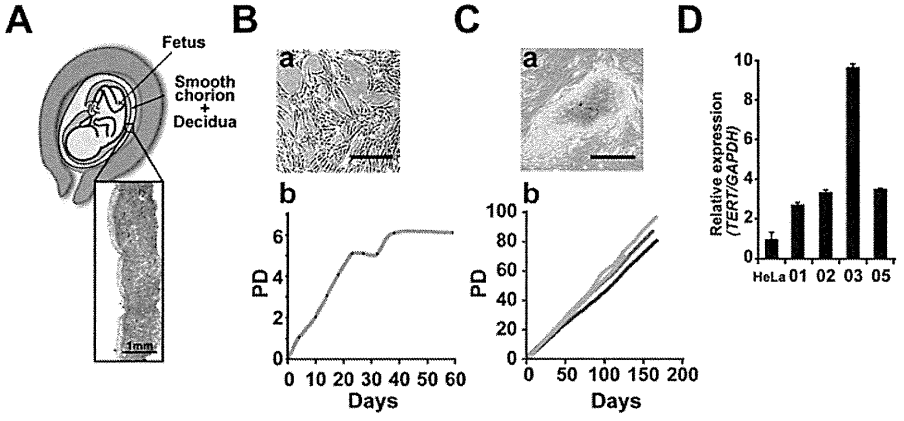
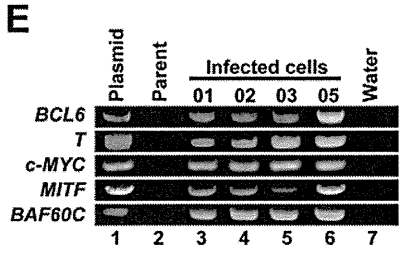
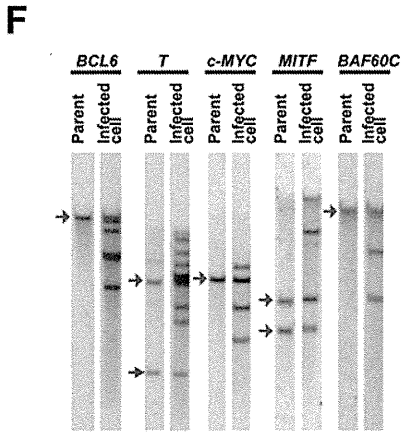
Abbreviations used: *ACAN, AGGRECAN*; *COL2A1, Collagen Type II α1*; *COL10A1, Collagen Type X α1*; *CRTL1, LINK PROTEIN-1*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; iCS, induced chondrosarcoma; MEFs, mouse embryonic fibroblasts; PCA, principal component analysis; PDs, population doublings; RT-PCR, reverse transcriptase PCR; siRNA, small interfering RNA; STRs, short tandem repeats.







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Locus	Parent		01		02		03	
D3S1358	15		15		15		15	
TH01	6		6		6		6	
D21S11	30	31.2	30	31.2	30	31.2	30	31.2
D18S51	14		14		14		14	
Penta_E	14	21	14	21	14	21	14	21
D5S818	10	11	10	11	10	11	10	11
D13S317	11		11		11		11	
D7S820	8	11	8	11	8	11	8	11
D16S539	12	13	12	13	12	13	12	13
CSF1PO	11	12	11	12	11	12	11	12
Penta_D	10	12	10	12	10	12	10	12
AMEL	X		X		X		X	
vWA	16		16		16		16	
D8S1179	10	13	10	13	10	13	10	13
TPOX	8	11	8	11	8	11	8	11
FGA	22	26	22	26	22	26	22	26

Locus	Parent		05	
D3S1358	15	16	15	16
TH01	6	9.3	6	9.3
D21S11	30	31.2	30	31.2
D18S51	14	19	14	19
Penta_E	15	21	15	21
D5S818	10		10	
D13S317	12		12	
D7S820	8	12	8	12
D16S539	9	12	9	12
CSF1PO	11	12	11	12
Penta_D	12		12	
AMEL	X	Y	X	Y
vWA	14	16	14	16
D8S1179	10	13	10	13
TPOX	11		11	
FGA	19	22	19	22

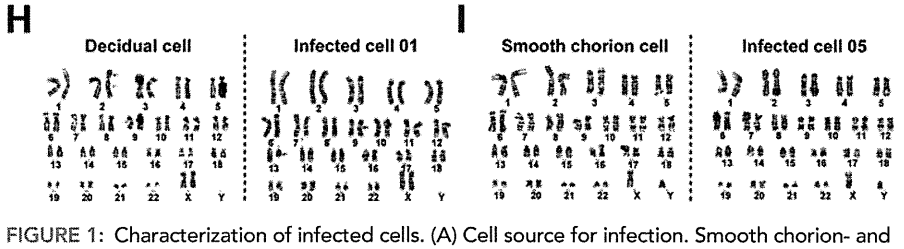

FIGURE 1: Characterization of infected cells. (A) Cell source for infection. Smooth chorion- and decidua-derived cells were used to investigate chondrogenesis by direct reprogramming. Bars, 1 mm. (B) Cell cultivation. (a) Phase contrast micrograph of parental cells. Bars indicate 200 μm. (b) Growth curve of parental cells. (C) Cells infected with five genes. (a) Phase contrast micrograph of infected cells. Bars, 200 μm. (b) Growth curve of infected cells. Orange, clone 01; red, clone 02; blue, clone 03; green, clone 05. Vertical axis indicates population doublings (PDs), and horizontal axis indicates days after infection. (D) Quantitative RT-PCR of *TERT* expression in the infected cell lines (clones 01, 02, 03, and 05). Individual RNA expression levels were normalized to respective *GAPDH* expression levels. HeLa cells were used for reference. Error bars, SD ($n = 3$). (E) Genomic DNA PCR analysis of uninfected and infected cells. To investigate chromosomal integration of the genes by retroviral infection, we performed genomic DNA PCR analysis, using transgene-specific primers of each gene. Five transgenes (*BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C*) were detected in all of the infected cell lines. (F) Southern blot analyses of the infected cells (clone 01). Genomic DNA was digested with *Spe*I, *Mfe*I, *Bgl*II, *Nco*I, and *Bam*HI and then probed for probes of the genes for *BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C*, respectively. The transgenes (*BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C*) were detected in all of the infected cell lines. Arrows indicate bands corresponding to the endogenous genes. (G) STR analysis of parental cells and the infected cells. All of the infected cells exhibited the same STR patterns as parental cells. (H) G-band chromosome analysis for parental cells with XX chromosomes and infected cells 01. (I) G-band chromosome analysis for parental cells with XY chromosomes and infected cells 05.

produce a large amount of collagen fiber, an abundant ground substance rich in proteoglycans, and elastin fibers. Developmentally, the undifferentiated mesenchymal cells migrate into the limb field and condense to form the cartilage anlage. Bone morphogenic proteins and transforming growth factor-β initiate the chondrogenic program and have significant effects on chondrogenesis through distinct mechanisms in a stage-specific manner. In addition to soluble factors, the high mobility group–domain transcription factors such as *Sox5*, *Sox6*, and *Sox9* control chondrogenic differentiation, maintain the chondrocyte phenotype, and regulate expression of extracellular matrix molecules, such as cartilage-specific collagen type II (Lefebvre *et al.*, 1997).

Murine chondrocytes can be converted from fetal fibroblasts by the direct reprogramming method using the cartilage-specific transcription factors *Sox9*, *c-Myc*, and *Klf4* (Hiramatsu *et al.*, 2011), but human chondrocytes converted from different types of cells have not yet been reported. In the present study, we generated chondrosarcoma cell lines derived from human placenta by the direct reprogramming method, using a different set of genes. Placental membrane can be obtained at every delivery and is usually discarded. Therefore it is an easily accessible cellular source without ethical problems.

RESULTS

Isolation of cells from smooth chorion and decidua

We used smooth chorion and decidua for a cell source by removing the amnion from the placental membrane and used the explant culture method in which the cells are outgrown from pieces of smooth chorion and decidua attached to dishes (Figure 1A and Supplemental Figure S1). The adherent chorion- and decidua-derived cells were passaged when the cells reached ~80% confluence. These placenta-derived cells continued to grow for 30 d, which was five population doublings (PDs), before reaching senescence (Figure 1B). The cells at four PDs were used as "parental cells" for conversion analysis.

Infection of transcription factors into placenta-derived cells

To select candidates for transcription factors that would be required to reprogram fibroblasts to a cartilage fate, we used microarray analyses to identify transcription factors and chromatin remodeling factors with greater expression in mouse embryonic stem cell that are differentiated into mesoderm. We started with a 14-gene set, that is, genes for mesoderm-specific transcription factors (*T*, *MITF*, *TBX5*, *TBX20*, *CSX/NKX2.5*, *GATA4*, *MEF2C*, *MESP1*, *ISL1*, *BCL6*, and *PRDM16*) and chromatin-remodeling/reprogramming factors (*BAF60C*, *c-MYC*, and *KLF4*). We generated individual retroviruses to efficiently express each gene. Viral infections were preceded by transfection of small interfering RNA (siRNA) to the *p53* gene (Supplemental Figure S2). Parallel experiments using retrovirus carrying the EGFP gene indicated that infection efficiency was nearly 100%. We investigated expression of cartilage-associated genes such as *Collagen Type II α1* (*COL2A1*), *Collagen Type X α1* (*COL10A1*), *LINK PROTEIN-1* (*CRTL1*), and *AGGRECAN* (*ACAN*) by reverse transcriptase (RT)-PCR and identified five genes (*BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C*) that induced chondrocyte gene expression. The induction levels of the cartilage-associated genes were greatly reduced by elimination of any one gene from the five-gene set. We thus decided to use the five-gene set for chondrogenic induction for subsequent experiments. After we seeded infected cells on mouse embryonic fibroblasts (MEFs), we detected a very large number of mouse embryonic stem cell–like colonies on MEFs 15 d after infection of the 5F pool (Figure 1C, a). Efficiency of colony formation (colony number per the number of cells infected) was 5.76 (±0.21) × 10^{-4}. We randomly picked four clones and analyzed cell growth rates. The cells replicated at a rate of once every 2 d and continued to grow for >150 d without reaching senescence (Figure 1C, b). All four clones expressed the *TERT* gene after establishment as a cell line (Figure 1D). The cells infected with the five genes exhibited a chondrogenic phenotype with malignant transformation, as shown by following results, and were thus designated induced chondrosarcoma (iCS) cells.

To determine chromosomal insertion of the genes, we performed genomic DNA PCR analysis (Figure 1E). The genes encoding BCL6, T, c-MYC, MITF, and BAF60C were detected in chromosomal genome of the four clones. Southern blot analysis with cDNA probes of each of the five genes (*BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C*) confirmed that each clone had chromosomal integration of the exogenously infected genes (Figure 1F and Supplemental Figure S3). The analysis of the 16 short tandem repeats (STRs) revealed that the infected clones were derived from parental cells: clones 1, 2 and 3 were derived from parental cells of the same donor with XX chromosomes, and clone 5 was derived from different parental cells with XY chromosome (Figure 1G). The STR patterns of the infected cells differed from those of any cell lines deposited on National Institutes of Health website (http://stemcells.nih.gov/research/nihresearch/scunit/genotyping.htm), implying that the cells generated are not a contamination of previously established cell lines. To determine the karyotypes of the iCS cell lines, karyotypic analysis was performed at different passages (P6– P23). Chromosomal G-band analyses showed that each clone had a normal karyotype with 46XX and 46XY (Figure 1, H and I, respectively). We then performed karyotypic analysis on iCS clones after prolonged passages (P15 and P23 for iCS-01; P13 and P21 for iCS-02; P12 and P21 for iCS-03; P7 and P23 for iCS-05, and did not detect any significant karyotypic change (Supplemental Figure S4).

In vitro chondrogenic phenotypes of the cells infected with the 5F pool

To investigate whether the infected cells exhibit a chondrogenic phenotype in vitro, we performed RT-PCR analysis using primers of the cartilage-specific genes (Figure 2A and Supplemental Table S1; Sekiya *et al.*, 2002; Shirasawa *et al.*, 2006). All the cell lines expressed the chondrocyte-specific/associated transcription factors (*SOX5*, *SOX6*, and *SOX9*), structural genes (*COL1A1*, *COL2A1*, *CRTL1*, and *ACAN*), and immortalizing gene (*TERT*). To see whether the endogenous genes for *BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C* were expressed by reprogramming, we performed RT-PCR analysis by the primers specific to the endogenous gene but not the transgenes (Supplemental Figure S5). Endogenous genes such as *T*, *MITF*, and *BAF60C* were induced (Figure 2B). To determine the surface markers of the cells, we performed flow cytometric analysis. All clones were positive for CD44, CD49c, CD151, and CD166 but not CD117 and CD133, suggesting that the cell marker pattern of iCS cells is compatible with that of chondrocytes (Figure 2C; Grogan *et al.*, 2007). Western blot analysis revealed that all the infected cells expressed COL2A1 and COL1A1 at the protein level (Figure 2D and Supplemental Figure S6). Comprehensive gene expression analysis showed that the expression pattern of the infected cells is similar to that of human adult chondrocytes and human fetal chondrocytes (Figure 2E). Expression of cartilage-specific genes such as *Sox9*, *Aggrecan*, and *Matrix Gla-protein* was detected in the infected cells and chondrocytes but not in the parent human smooth chorion and decidua cells (Figure 2F). Conversely, expression of placenta-associated genes such as *GATA3*, *CD200*, *PDCD1LG2*, *OLR1*, *TEK*, *HSD17B2*, and *FOXF1* was lost in the infected cells. Hierarchical clustering analysis revealed that the infected cells were grouped into the same category that includes chondrocytes obtained from human fetuses and adults (Figure 2G). In addition, principal component analysis (PCA) revealed that the infected cells and chondrocytes showed similar scores in the PC2 axis (Figure 2H). The representative genes (principal components) of the PC2 axis in Table 1 include cartilage-specific genes such as *Aggrecan*, *Fibromodulin*, and *Matrix Gla-protein* (Plaas and Wong-Palms, 1993; Yagami *et al.*, 1999; Sekiya *et al.*, 2002; Hjorten *et al.*, 2007; Surmann-Schmitt *et al.*, 2009).

Inhibition of five factors by small interfering RNA

To investigate the involvement of the five factors in chondrogenesis, we suppressed their expression by siRNA (Supplemental Table S2). The mRNAs for the five factors (*BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C*) were significantly decreased by siRNAs compared with control cells transfected with control siRNAs (Figure 3, A and B, and Supplemental Figures S7–S9). Morphological changes in the siRNA-treated cells were too variable to interpret. Gene expression of the chondrogenic-specific/associated transcription factors (*SOX5*, *SOX6*, and *SOX9*) and structural genes (*COL1A1*, *COL2A1*, *CRTL1*, and *ACAN*) decreased significantly in si*T* (siRNA to the *T* gene)-transfected cells compared with cells treated with control siRNA (Figure 3C and Supplemental Figure S10), suggesting that *T* is necessary for chondrogenic conversion (Hoffmann *et al.*, 2002). In addition, expression of the genes for *SOX5*, *SOX6*, *COL1A1*, and *COL2A1* decreased significantly in si*MITF*-transfected cells compared with cells transfected with control siRNA, suggesting that *MITF* is also necessary for chondrogenic conversion. In contrast, treatment of siRNA to *BCL6*, *c-MYC*, and *BAF60C*) did not alter cartilage-related genes (Zelzer and Olsen, 2003; Levy and Fisher, 2011). si*MITF* diminished the cobblestone appearance of iCS colonies and the cell lining at the periphery of iCS colonies and altered the appearance of the iCS cells to a fibroblast-like morphology, which may be related to decreased expression of the cartilage-associated genes.

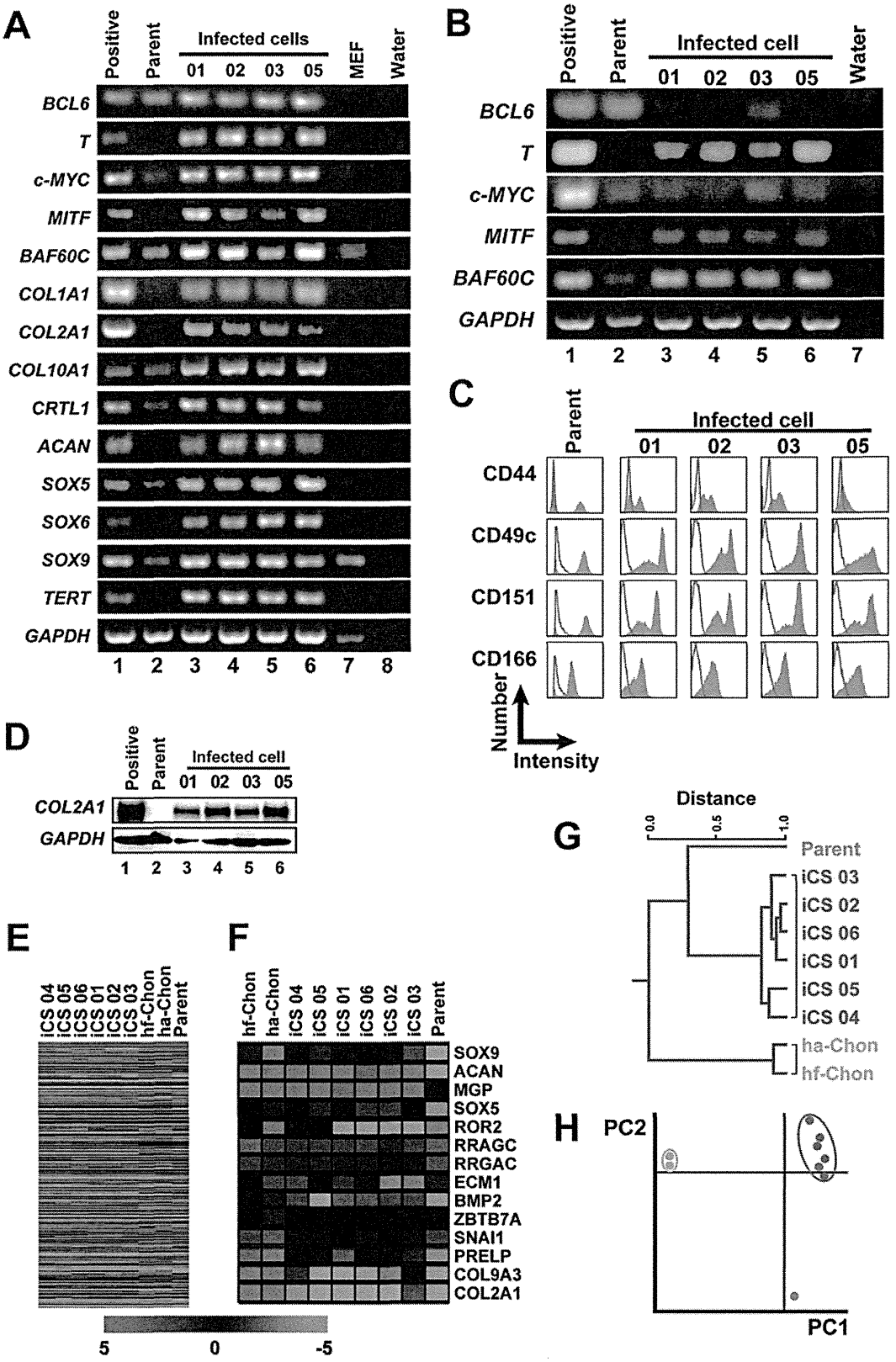

FIGURE 2: Chondrogenic phenotypes of infected cells. (A) RT-PCR analysis of the genes encoding cartilage-specific proteins (SOX5, SOX6, SOX9, COL1A1, COL2A1, COL10A1, CRTL1, and ACAN), immortalizing gene (TERT), and the infected genes (BCL6, T, c-MYC, MITF, and BAF60C). Primers that detect both the transgenes and endogenous genes for BCL6, T, c-MYC, MITF, and BAF60C were used (Supplemental Figure S5C). RNAs from the following sources were used for positive controls: heart for BCL6, MITF, BAF60C, and GAPDH; iPS cells for T, c-MYC, and TERT; and cartilage for COL1A1, COL2A1, COL10A1, CRTL1, ACAN, SOX5, SOX6, and SOX9. H_2O (water without RNA) served as a

Gene symbol	Description	Gene symbol	Description
ACAN	Aggrecan	CLEC4D	C-type lectin domain family 4, member D
FMOD	Fibromodulin	NRAP	Nebulin-related anchoring protein
MGP	Matrix Gla protein	OR2V2	Olfactory receptor, family 2, subfamily V, member 2
LRRC48	Leucine-rich repeat containing 48	KCNH7	Potassium voltage-gated channel, subfamily H (eag-related), member 7
SLPI	Secretory leukocyte peptidase inhibitor	KCNK17	Potassium channel, subfamily K, member 17
RAB11FIP4	RAB11 family interacting protein 4 (class II)	DRD1	Dopamine receptor D1
TLR5	Toll-like receptor 5	CTNNA2	Catenin (cadherin-associated protein), α2
NEBL	Nebulette	FMR1NB	Fragile X mental retardation 1 neighbor
RAB11FIP4	RAB11 family interacting protein 4 (class II)	ABCC12	ATP-binding cassette, subfamily C (CFTR/MRP), member 12
CAPG	Capping protein (actin filament), gelsolin-like	SLITRK3	SLIT and NTRK-like family, member 3
SLC26A4	Solute carrier family 26, member 4	CIITA	Class II, major histocompatibility complex, transactivator
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	GP2	Glycoprotein 2 (zymogen granule membrane)
CALR3	Calreticulin 3	OR12D3	Olfactory receptor, family 12, subfamily D, member 3
ESPN	Espin	GALNTL4	UDP-*N*-acetyl-α-D-galactosamine
SLC7A2	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	BRSK2	BR serine/threonine kinase 2
CHRNA4	Cholinergic receptor, nicotinic, α4	L08961	Transmembrane tyrosine kinase mRNA
ZBTB10	Zinc finger and BTB–domain containing 10	RAB33B	RAB33B, member RAS oncogene family
ND3	NADH-ubiquinone oxidoreductase chain 3(NADH dehydrogenase subunit 3)	ELA1	Elastase 1, pancreatic
EFNA1	Ephrin-A1	ASPA	Aspartoacylase (Canavan disease)
RGMA	RGM domain family, member A	IL18RAP	Interleukin 18 receptor accessory protein
ENST00000390243	Immunoglobulin κ light-chain V gene segment	EPHA8	EPH receptor A8
GPA33	Glycoprotein A33 (transmembrane)	CXCR6	Chemokine (C-X-C motif) receptor 6
CLMN	Calmin (calponin-like, transmembrane)	BAGE	B melanoma antigen
RAB11FIP4	RAB11 family interacting protein 4 (class II)	SIRPG	Signal-regulatory protein γ
KRT26	Keratin 26	AF083118	CATX-2 mRNA
YBX2	Y box–binding protein 2	TSPAN16	Tetraspanin 16
EEF1G	Eukaryotic translation elongation factor 1 γ	AF028840	Kruppel-associated box protein mRNA
NAG18	NAG18 protein on chromosome 19	WIF1	WNT inhibitory factor 1
CX62	Connexin 62	TTTY9A	Testis-specific transcript, Y-linked 9A (TTTY9A) on chromosome Y
KCNC2	Potassium voltage-gated channel, Shaw-related subfamily, member 2	LRRC50	Leucine-rich repeat containing 50
TSPAN33	Tetraspanin 33	ENST0000037416	Collagen, type XXVII, α1
PTCH1	Patched homologue 1 (*Drosophila*)	WFDC12	WAP four-disulfide c ore domain 12
DEFB126	Defensin, β126		
RAMP3	Receptor (G protein–coupled) activity–modifying protein 3		

TABLE 1: Representative genes in PC2 axis of the PCA.

negative control. (B) RT-PCR analysis of the endogenous genes encoding T, MITF, and BAF60C. The primers were prepared to amplify the endogenous genes but not the transgenes. RNAs from the following sources were used for positive controls: heart for BCL6, MITF, BAF60C, and GAPDH; and iPS cells for T and c-MYC. H_2O (water without RNA) served as a negative control. (C) Flow cytometric analysis of cell surface markers on the parental cells and infected cells. All of the results were compared with each isotype control. The *X*- and *Y*-axes indicate the intensity and the cell number, respectively. (D) Western blot analysis of COL2A1 protein in the infected cells and parental cells. (E, F) The heat map in the infected cells and parental cells. Each row represents a gene; each column represents a cell population. Expression levels of representative genes are shown in F. (G) Hierarchical clustering analysis (TIGR MeV; see *Materials and Methods*), based on expression levels of the cartilage-associated genes. (H) Principal component analysis of gene expression levels.

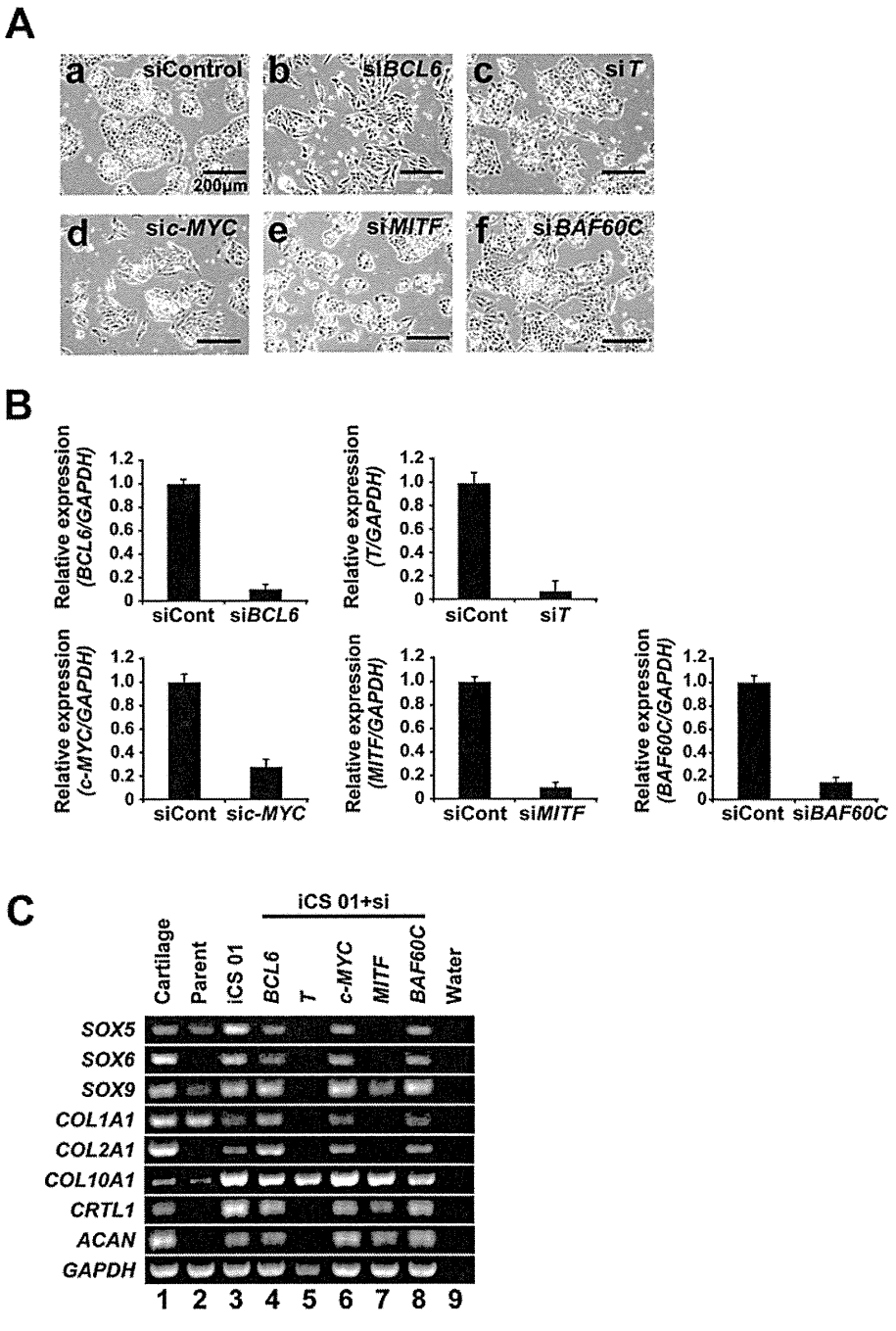

FIGURE 3: Functional effect of genes in iCS cells. (A) Phase contrast microscopic views. (a) Control siRNA–treated iCS cells. (b) *BCL6* siRNA–treated iCS cells. (c) *T* siRNA–treated iCS cells. (d) *c-MYC* siRNA–treated iCS cells. (e) *MITF* siRNA–treated iCS cells. (f) *BAF60C* siRNA–treated iCS cells. Bars, 200 μm. (B) Quantitative RT-PCR of each gene in siRNA-treated cells. Individual RNA expression levels were normalized to respective *GAPDH* expression levels. Error bars, mean ± SD ($n = 3$). (C) RT-PCR analysis of the genes encoding SOX5, SOX6, SOX9, COL1A1, COL2A1, COL10A1, CRTL1, and ACAN. Human cartilage and H_2O (water without RNA) served as positive and negative controls, respectively.

Cartilage formation after implantation of the cells infected with the 5F pool

To investigate whether iCS cells exhibit a chondrogenic phenotype in vivo, we intradermally injected the cells into dorsal flanks of immunodeficient Balb/c nu/nu mice. The masses generated underwent histopathological analysis 7 wk after injection. The injected cells generated cartilage that exhibited metachromasia by toluidine blue staining and were light blue when stained with Alcian blue (Figure 4A). In contrast, implantation of parental cells produced neither tumor nor cartilage (Figure 4E). RT-PCR analysis showed that iCS cartilage expressed genes for *COL1A1*, *COL2A1*, *COL10A1*, *CRTL1*, *ACAN*, CD44, CD49c, CD151, and CD166 (Figure 4B). Western blot analysis showed that iCS cartilage produced collagen type II at the protein level (Figure 4C). We also performed immunohistochemical analysis using antibodies to vimentin, collagen type II, and Ki-67. The antibody for vimentin that we used specifically reacts with human protein but not murine protein. The antibody for Ki-67 reacts with a human nuclear cell proliferation–associated antigen, and thus it does not react with differentiated chondrocytes. iCS cells stained positive for human vimentin, and extracellular matrix was positive for collagen type II, implying that the injected human cells generate cartilage (Figure 4D). Nearly 30% of iCS cells stained positive for Ki-67, indicating that iCS cells continued to replicate in cartilage at 7 wk after injection. iCS cells in the tumor had large nuclei with coarse chromatin structure and one or two nucleoli, and the ratio of nucleus/cytoplasm was large. The tumors generated by iCS cells were histopathologically diagnosed as chondrosarcoma by a certified pathologist (A.U.). Anchorage-independent colony formation is a hallmark of transformation and an in vitro correlate of tumorigenicity in vivo (Cremona and Lloyd, 2009). After cultivation in MethoCult H4034 medium, colony formation was evaluated (Figure 5). The colony-forming assay clearly revealed that iCS cells formed colonies but parental cells did not, indicating that iCS cells are transformed cells with chondrogenic potential.

Generation of chondrocytes from other human somatic cells

In addition to human smooth chorion, we used primary cultured cells from human menstrual blood and placental artery. We obtained 10 and 9 clones, respectively, from menstrual blood–derived cells and placental arterial endothelium. All of them proliferated as a chondrogenic cells with transformation and exhibited the same morphology with iCS in vitro (Figure 6). The growth rates of the clones generated from menstrual blood and placental artery were essentially the same as those of iCS cells. After implantation into the dermal tissue of nude mice, they generated chondrogenic tissue that showed metachromasia with the toluidine blue stain.

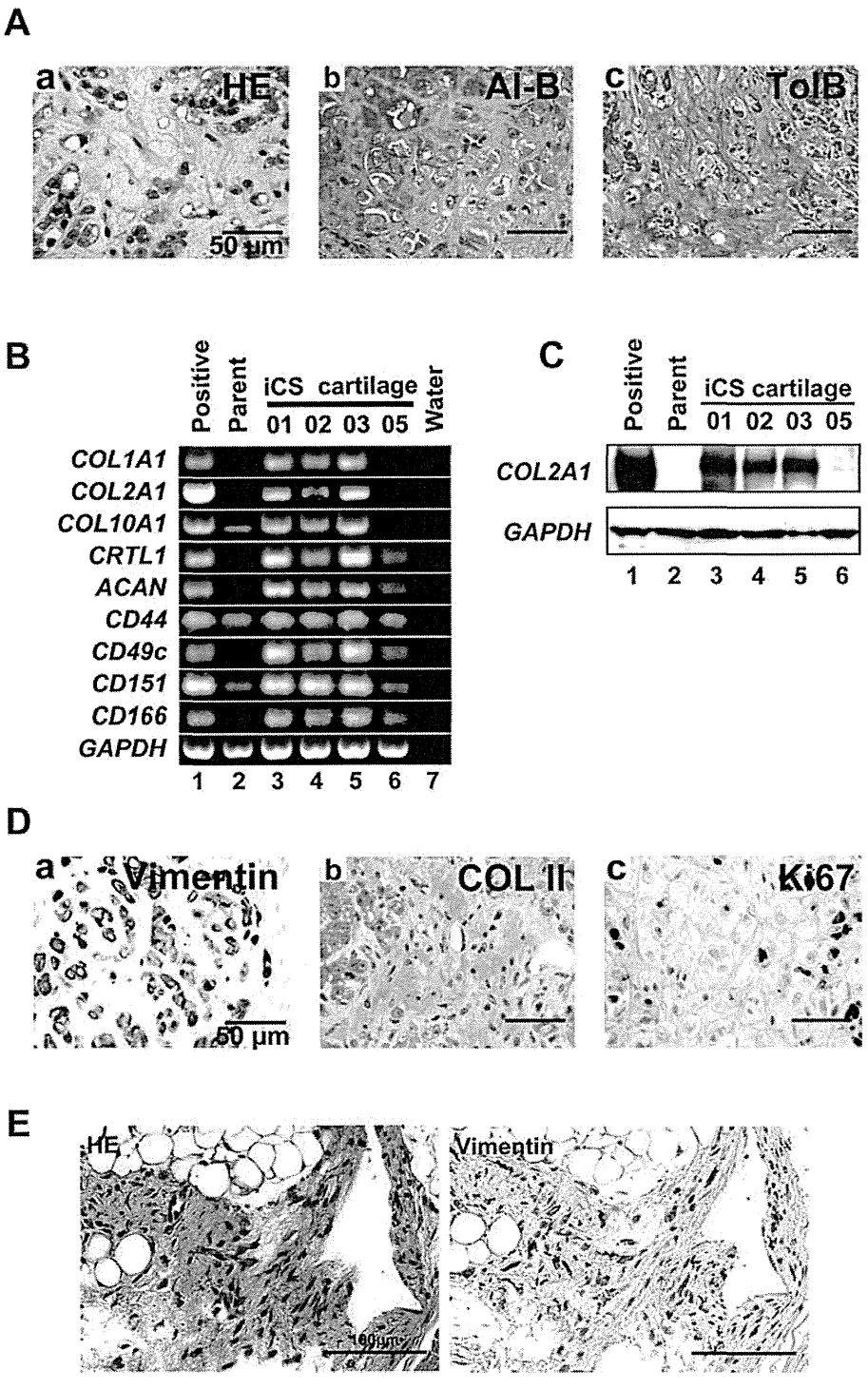

FIGURE 4: In vivo chondrogenic phenotypes of iCS cells. (A) Cartilage at 7 wk after injection of iCS cells. (a) HE stain, (b) Alcian blue, (c) toluidine blue. iCS cells at passage 3 were injected subcutaneously to the dorsal flank of athymic nude mice. Areas of extracellular matrix accumulation stain light to dark blue with Alcian blue (b) or light- to dark-red/purple with toluidine blue (c). Bars, 50 μm. These results are representative of five independent experiments. (B) RT-PCR analysis of the genes encoding SOX5, SOX6, SOX9, COL1A1, COL2A1, COL10A1, CRTL1, and ACAN in cartilage generated by iCS cells. Human cartilage and H_2O (water without RNA) served as positive and negative controls, respectively. Parental cells in culture serve for comparison. (C) Western blot analysis of COL2A1 protein in iCS cartilage at 7 wk after subcutaneous injection of iCS cells into athymic nude mice. Human cartilage serves a positive control. GAPDH was used as a loading control. (D) Immunohistochemical analysis of iCS cartilage. (a) Vimentin, (b) collagen type II (COL II), (c) Ki-67. (E) Implantation of the parental cells. We injected parental cells into athymic nude mice but did not detect any tumor formation. We performed histological analysis and immunohistochemical analysis using the human vimentin-specific antibody. The parental cells did not exhibit cartilage formation at the injected site. Left, HE stain. Right, immunohistochemistry using human-specific antibody to vimentin. Bars, 100 μm.

DISCUSSION

In mammals, cartilage does not regenerate in limb tissue, but cells that derive cartilage retain a strong memory of their embryonic origin in the axolotl (Kragl *et al.*, 2009). Cells are undergoing reprogramming that allows them to reenter embryonic programs of tissue formation, even if they do not revert back to the pluripotent state. Here we show that expression of five transcription factors can rapidly and efficiently convert nonchondrocytes (chorion- and decidua-derived cells) into chondrocytes. iCS cells displayed functional chondrogenic properties such as the generation of extracellular matrices. The possibility of redirecting cell differentiation by overexpression of genes was suggested by Weintraub with the identification of the *MyoD* "master" gene (Davis *et al.*, 1987). The process was believed to involve reversion to a less differentiated state, a kind of dedifferentiation, before the new cell type is formed. Another process has since been suggested, the concept of direct conversion or direct reprogramming without dedifferentiation. This process is believed to be direct lineage switching rather than lineage switching back to a branch point and out again in a different direction. Direct conversion has been shown in β cells, cardiomyocytes, and neurons. A specific combination of three transcription factors (*Ngn3*, *Pdx1*, and *MafA*) reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β cells (Zhou *et al.*, 2008); a combination of three factors (*Gata4*, *Tbx5*, and *Baf60c*) induces noncardiac mesoderm to differentiate directly into contractile cardiomyocytes (Ieda *et al.*, 2010); and a combination of three factors (*Ascl1*, *Brn2*, and *Myt1l*) converts mouse fibroblasts into functional neurons (Vierbuchen *et al.*, 2010). In this study, we used the strategy of direct conversion to generate chondrocytes from human extraembryonic somatic cells. Based on the same method, murine chondrocytes were generated from skin fibroblasts (Hiramatsu *et al.*, 2011) using the three transcription factors *Sox9*, *c-Myc*, and *Klf4*. *Sox9* is a determinant of chondrogenic lineage (Lefebvre *et al.*, 1997), *c-Myc* is a cell cycle driver (Schmidt, 1999), and *Klf4* is involved in the down-regulation of *p53* (Rowland