【核内抗原の場合】

1	0.1% Triton X in 1% FBS-PBS を 2 mL/well 加え、室温 10 min（パーミライズ）（シェイカー上で反応させる）。
2	3% FBS in PBS-T を 1 mL/well 加え、室温 30 min（ブロッキング）（シェイカー上で反応させる）。
3	希釈した一次抗体を 500 μL/well 加え、4°C で O/N（シェイカー上で反応させる）。
4	0.1% FBS in PBS-T を 7 mL/well 加え、室温 5 min 静置（洗浄）。
5	4 の作業を同様に 2 回（洗浄）。
6	希釈した二次抗体を 500 μL/well 加え、室温 30 min（シェイカー上で反応させる）。
7	PBS を 7 mL/well 加えて、すぐにデカントにて液をのぞく（洗浄）。
8	PBS を 7 mL/well 加え、室温 10 min 静置（洗浄）。
9	上記を同様に1回（洗浄）。
10	PBS で500倍希釈した Hoechst を 1 mL/well 加え、室温 3 min（核染）（シェイカー上で反応させる）。
11	PBS を 7 mL/well 加え、すぐにデカントにて液をのぞく（洗浄）。
12	PBS を少量加えて、検鏡する。
13	4% PFA を 1 mL/well 加え、室温 10 min 処理（後固定）（ドラフト内で実施）。
14	PBS で手早く 3 回洗浄する（ドラフト内で実施）。
15	PBS を 5 mL/well で加え、観察する。
	直ちに観察しない場合は、パラフィルムとアルミで巻いて、4°C で保存する。

表6．染色体検査の準備

使用試薬・器具

		メーカー	カタログ No.	備　考
代替コルセミド	Metaphase Arresting Solution	フナコシ	GGS-JL008	
0.2%　EDTA・4Na in CMF-DPBS				
CMF-DPBS (×1)		GIBCO	14190	
0.075M KCL				
カルノア固定液**				用事調製
プレパラート	メタノール（特級）	SIGMA		用事調製
	脱脂済みプレパラート			
100% エタノール				
ギムザ染色液		MERK		
染色バット大				
染色バット大金具				

** 酢酸：メタノール＝ 1：3

表7．染色体数計測の手順

1．サンプルの準備

ステップ		作業場所	作業内容
播種	1	安全キャビネット内	通常継代時の約 2 ～ 3 倍の密度で細胞を播種する（コロニーは小さめがよい）。
処理	2	安全キャビネット内	対数増殖期の細胞に代替コルセミドを添加（* 培地 5 mL の場合 5 μL）。
処理	3	CO_2 インキュベーター	** 2 ～ 3 時間培養（丸く光る細胞が多く見られる）。

回収	4	安全キャビネット内	培養上清を 15 mL 遠心管に回収。
	5		0.2%EDTA・4Na を 2 mL 添加する。
	7	顕微鏡下	室温にて約 5 分静置（コロニーにひびがはいり各細胞間の境界が明瞭になるまで）。
	8	安全キャビネット内	培養容器をタッピング（コロニーのみがはがれる）。
	9		先にとっておいた培養上清を加える。
	10		4 回程度ピペッティングし、single cell にする。
	12		すべての溶液を回収し、最初の培養上清と合わせる。
	13		1,500 rpm 5 min 遠心。
	14		上清を除去。
	15		タッピング。
	16		PBS を 5 mL 添加。
	17		よくピペッティングする。
	18	遠心機	1,500 rpm 5 min 遠心。
低調処理	19	安全キャビネット内	上清除去。
	20		タッピング。
	21	実験台	0.075M KCl を 3 mL 添加。
	22		先太のピペットでよくピペッティングする。
	23		30 min 静置（低張処理。株によっては45分）。
固定	24	ドラフト	
	25		細胞浮遊させている KCL の 2 倍量（6 ml）のカルノア固定液をゆっくり加える（最初の 1 ml はゆっくり一滴ずつ加える）。
	26	遠心機	1,600 rpm 7 min
	27	ドラフト	全量の2/3の上清除去。
	28		軽くタッピング。
	29		先太のピペットでゆるやかにピペッティング。
	30		全量の 2 倍量のカルノアを添加。
	31		先太のピペットでゆるやかにピペッティング。
	32	遠心機	1,600 rpm 7 min
	33	ドラフト	全量の2/3の上清除去。
	34		タッピング。
	35		先太のピペットでゆるやかにピペッティング。
	36		全量の 2 倍量のカルノアを添加。
	37	遠心機	1,600 rpm 7 min
	38	ドラフト	全量の上清を吸引。
	39		タッピング。
	40		カルノア約 1 ml 添加。 （濃度はわずかに混濁する程度とし，スライドガラス上に展開した細胞数が100～200個／弱拡大（100倍）となるように固定液で調整する。）
	41		ゆるやかにピペッティング（＝核浮遊液）。
	42	冷凍庫	すぐに展開しない場合は、－20°C で保存する。

2. 展　開

ステップ	作業場所	作業内容	チェック
	すぐに展開せず保存した場合：	（ドラフト）	（カルノア液作成）
		（遠心機）	（1,600 rpm 7min）
		（ドラフト）	（全量の上清を吸引）
		（ドラフト）	（タッピング）

		(ドラフト)	(カルノア約 1 ml 添加 (展開した際にちょうどよい密度になるような量に調整する) (=核浮遊液))。	
展開	43	ドラフト	染色バット大に新しいエタノールを 160 ml 入れる。	
	44	ドラフト	スライドグラス (−20°C エタノールに保存してあったもの) を新しいエタノールに浸漬する。	
	45	実験台	スライドガラスをエタノールから出して、ガーゼで表面を虹がでなくなるまで清拭し、No. を書く。	
	46	実験台	水に濡らしたキムタオルの上にスライドグラスを置く。	
	47	実験台	核浮遊液をやや約 2 cm 上から、スライドガラス中央に 1 滴 (約20マイクロリットル) たらす。 標本の出来は滴下した細胞浮遊液の広がる速度と、乾燥する速度の両方に左右されるため、恒温槽の温度, 滴下までの時間、細胞浮遊液の温度と濃度を調節する。	
	48	実験台	5 分程度静置。	
	49	顕微鏡	位相差レンズを用いて検鏡して、展開されていることを確認。	
エイジング	50	伸展機あるいはインキュベーター	37°C で 3 時間乾燥。	
	(50)	(専用箱など)	(G-band 分染する場合には、さらに室温にて 3 日間静置)	

* コルセミドの処理濃度は株によるので初回は複数濃度を検討する。

** 処理時間は 2 ～ 4 時間程度になるようにコルセミドの濃度を決定するのが望ましい。Overnight で処理すると染色体が短くなってしまい、G バンディング解析が難しくなる。また、対数増殖期でない時期に作用させると時間が必要となる。

ギムザ染色

ステップ		作業場所	作業内容	チェック
	51	ドラフト	染色バットにギムザ液を入れる。	
	52	ドラフト	染色バットにメタノールを入れる。	
	53	ドラフト	標本をメタノールに 1 分浸漬する。	
	54	ドラフト	取り出し、3% ギムザ液 /PBS に 5 min. 浸漬。	
	55	流し	流水にて水洗。	
	56	実験台	自然乾燥。	
	57	実験台	Eukitt で封入する。	

ま と め

以上簡単ではあるが、ヒト ES、iPS 細胞の品質管理についての概要をまとめた。本総説が多くの研究者によるより良い成果産出の一助となることを願う。

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Quality control for human embryonic stem (ES) cell and induced pluripotent stem (iPS) cells on the bench

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Abstract: In 1998, human embryonic stem (hES) cells have been established. In 2006, mouse induced pluripotent stem (hiPS) cells have been developed. Since then, the hES/iPS cells have been used as a tool for understanding the mechanisms in human development and regeneration application research in the world. However, quality of these human pluripotent stem cells vary because batches of medium or feeder cells vary. If abnormal mutant cells with a growth advantage appear, they will be selectively amplified in only 5 passages. Therefore, quality controls of these cells are required as an experimental tool. In this review, we have summarized the method of basic quality control of human ES/iPS cells on the bench.

Key words: human ES cells, human iPS cells, quality control

投稿論文

ヒト多能性幹細胞の命名法の国際統一規格案について

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Keywords：embryonic stem cells　induced pluripotent stem cells　standardization

summary

In a few years, thousands of human embryonic stem (ES) / induced pluripotent stem (iPS) cell lines have been established in laboratories around the world. To date, confusions have arisen due to duplicate or redundant naming of cell lines. In addition, not all the important information such as provenance, derivation method and characterization are provided by researchers. To address these issues, a convention for naming and reporting human ES/iPS cell lines is urgently called. Recently Stem Cell Banks and researchers in the US, UK, China, Australia and the other countries proposed a new nomenclature system and a minimum set of criteria for reporting newly generated human ES/iPS cell lines. In this review, we have introduced their recommendations for developing a rule for naming and reporting of human ES/iPS cell lines.

はじめに

1998年にヒト胚性幹細胞(embryonic stem cell：ES細胞)[1)]が樹立され，2007年には，ヒト人工多能性幹細胞(induced pluripotent stem cell：iPS細胞)[2)]が開発された。これらの多能性幹細胞は，発生や疾患メカニズム解明など基礎研究のみならず，再生医療や創薬，毒性評価，ワクチン作製などへ応用の期待が高まっている。ヒトES/iPS細胞の株数は急ピッチで増加しており，すでに数千株にも及ぶ。実用化への研究を進めるために，国際幹細胞バンキングイニシアティブ(International Stem Cell Banking Initiative：ISCBI)では各国の細胞バンクや樹立機関が協力して世界中の研究者が相互に利用できる環境の整備を推進している。ところが，ヒトES/iPS細胞株の命名法について整備されておらず，混乱が生じている。このような現状から，2011年4月に，米国，英国，オーストラリア，中国などの幹細胞バンクや幹細胞研究者らから，「ヒトES/iPS細胞株の命名法および発表に関する標準化」[3)]が提案され，さらにその提案に対する意見[4)5)]が寄せられた。ISCBIや国際細胞バンク・ワーキンググループに参加する筆者らが，その内容を概説したい。

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ヒトES/iPS細胞の国際的な相互利用に向けて

2003年に設置された日本を含む22ヵ国からなる国際幹細胞フォーラム(http://www.stem-cell-forum.net/ISCF/)からの助成を受けて，2005年から英国シェフィールド大学Andrews教授が中心となって推進しているInternational Stem Cell Initiatives (ISCI)プロジェクトでは，日本(京都大学再生医科学研究所・中辻憲夫教授)を含めた世界11ヵ国のヒトES細胞樹立研究者らが連携して，ヒトES細胞株を登録し，樹立の方法，未分化/分化マーカーの発現などの解析方法とその結果を公表し[6)7)]，ヒトES細胞研究の標準化を進めてきた(http://www.stem-cell-forum.net/ISCF/initiatives/)。ISCIワークショップには筆者らも加わり標準化についての議論を行った。2008年からは，ヒトiPS細胞も含めて検討されている。さらに，ヒトES/iPS細胞株を各国間で相互に利用する体制を構築する必要があるとの認識のもとに，2007年から英国UK Stem cell Bankをはじめとする世界各国の細胞バンクが連携し，ISCBIプロジェクトが開始され，筆者らが参加している。このプロジェクトにおいては，ヒトES細胞のドナーの情報管理，資源化，品質管理法や分譲について，国際的にコンセンサスを図ってヒトES細胞を資源化するためのガイドラインを作成している[8)9)](和訳は，京都大学再生医科学研究所・細胞プロセシング・高田らより本誌Vol.10 No.4，p79-96，2011に掲載されているので参照されたい)。さらに，相互利用するためには不可欠な細胞登録における「細胞株の命名法」に関しても統一規定を設けることが現在の重要課題であり，国際的に活発な議論が展開されている。

これまでの現状

国内において細胞バンクが整備される1984年以前は，日本組織培養学会が細胞株を認定してJTCの番号を付与して登録する事業を実施していた。現在は，細胞バンクが整備され，JCRB(医薬基盤研究所細胞バンク，旧国立医薬品食品衛生研究所細胞バンク)，RCB(理化学研究所バイオリソースセンター細胞バンク)に，研究者が細胞株を寄託し，バンクの略称とともに登録番号で管理され，データベース上で公開されている。海外においても，米国のATCC，国立がん研究所(National Cancer Institute：NCI)，欧州細胞培養コレクション(European Collection of Animal Cell Cultures：ECACC)などの細胞バンクが各機関の略称や独自の登録番号(カタログ番号)を用いて管理し，情報を公開している。このように整備されていても，細胞株の情報や原著論文を検索するときに不都合が起こる。たとえば，“3T3 Swiss Albino”“3T3-Swiss albino”“Swiss-3T3”は同種の細胞株名であるが，データベースや論文での記載方法は他にも何通りも存在する。3T3と入力して検索すると，“3T3 (+3)”，“3T3-L1”，“3T3-SV40”など，別種の細胞株やサブクローンも検索にかかる。

まだ歴史の浅いヒトES/iPS細胞株においても，異なる研究機関で樹立された別個の細胞に全く同じ名前がつくといった問題がすでに生じている。たとえば，全く別の患者から採取した羊水(amniotic fluid：AF)に由来する2つのiPS細胞株の両方ともが“AF-iPS”と命名されたり[10)11)]，ジストロフィン遺伝子に異なる箇所に変異をもつ2人のデュシェンヌ型筋ジストロフィー(Duchenne muscular dystrophy：DMD)患者から樹立した全く別のiPS細胞であるにも関わらず，両方ともが“DMD-iPS1”と命名されたりしている[12)13)]。“iPS-1”や“iPS-WT”といった名称は汎用され，その名称のみから細胞株を特定することはできない[4)]。また，“KhES-1”“KhES-3”“HES-3”など，ヒトES細胞の名称に汎用される“HES”は，ヒト胎児皮膚(human embryonic skin：HES)由来線維芽細胞の株名“HES 5”[14)]などとも同じ表記であるため混同されやすい。細胞株を混同してしまえば，研究成果の妥当性，

重要性を正当に評価できなくなる。このように細胞命名法の国際的な統一規定がなかったことがデータベースの管理・利用を不便なものにしている。

ヒトES/iPS細胞の命名法の提案

2010年の国際幹細胞学会(International Society for Stem Cell Research：ISSCR，2010年7月15日開催)，およびISCI(2010年9月15日開催)のワークショップで議論された内容に準拠して，米国マサチューセッツ医科大学ヒト幹細胞バンクのInternational stem cell registry(ISCR)が代表として提案する「ヒトES/iPS細胞株の命名法および細胞登録に関する統一規定の案」が米国科学誌「Cell Stem Cell」2011年4月8日号[3]に掲載された。これに対し，京都大学iPS細胞研究所(CiRA)山中伸弥所長らの意見[4]と米国細胞バンクAmerican Type Culture Collection(ATCC) Brian Pollok所長らの意見[5]が同誌の6月3日号に掲載された。両者ともISCRの提案に大筋で同意した上で，幹細胞研究の将来展望をもとに想定される問題を提起し，改善案を提示した。

ヒトES/iPS細胞の命名法についての統一規定案

ISCRによる命名法の統一規定案(**図**)[3]は，特に次に示す5点に配慮したものである。①独自の識別方法(樹立機関IDと細胞株シリアル番号)を採用し，細胞株間で混同しないようにすること。②細胞株に関する情報が直感的に認識できること。③既存の細胞株名の表記方法(例：KhES-1，KhES3，CT4，B124-2)と同じフォーマットを採用すること。④異なる系統の細胞株であること(例：TSRI68iとSHEF4e-ALS)や，同じ系統の細胞株であること(例：SHEF3とSHEF5)を容易に認識できること。⑤柔軟性のあるルールにすること。

その細胞の名称の表記方法は**図**[3]に示すような4つの構成要素からなるものであり，(a)細胞株の樹立機関のID，(b)細胞株のシリアル番号(ID)，(c)ES細胞またはiPS細胞を区別する略号，および(d)細胞の特徴を示す情報を記載する。さらに，データベース上で処理するため，規定された場所にハイフンを使用する，文字数と数字の桁数を規定する，スペースを使用しない，ハイフンを含めて14桁に統一することを提案[3]している。

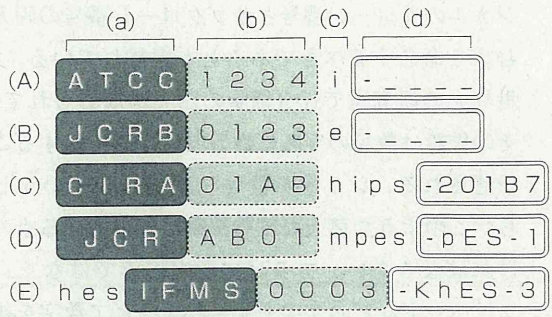

図　ヒトES/iPS細胞株の命名法の案

(A)(B) ISCR[3]，(C)(D)山中所長ら[4]，(E)筆者らの案による細胞株名表記の例

(a)細胞株を樹立した研究機関(研究室または研究所)のID

(b)細胞株のID

(c)細胞種や由来を識別する記号。ISCRの提案[3]によると“iPS細胞”を“i”，“ES細胞”を“e”で表す。

(d)“-(ハイフン)”とその後に続くアルファベットまたは数字で細胞株の特徴やクローン番号などを表す。ISCR[3]は，(c)と(d)の部分は任意とし，(a)と(b)のみで細胞名を表すことも考慮している。それぞれの要素を表す部分に使用する文字数と数字の桁数に自由度をもたせることも可能だが，データベース管理および検索の便宜上，①スペースを含まない，②アルファベットの大文字や小文字の表記法，数字の桁数，ハイフンの位置なども統一し，③全体で14文字に限定したものが望ましいとしている。細胞株名の表記法については，さらに議論が必要である。(文献3より引用改変)

ヒトES/iPS細胞サブクローン株の数への対応

iPS細胞は1種類のドナー細胞から100種類以上のクローンを作製することもある。山中所長らは，細胞株を識別するためのIDの表記(**図**(b)の部分)は増大する株数に対応し得る方式でなければならないと提言[4]している。また，サブクローンを作製した場合，オリ

ジナルのクローン番号とサブクローン番号の両方を含むよう命名するべきであるとも提言している。現在，世界中の研究室でiPS細胞の樹立が進められており，その株数は数年のうちに数万という値に達することが予想される。シリアル番号やIDをつけていくとしたら，このような莫大な細胞株数に対応できるものでなければならない。アラビア数字のみではなく，アルファベットなどの文字や記号とアラビア数字を組み合わせてIDを表記すれば，細胞株数が膨大になっても対応できるのではないかと山中所長ら[4]は提案している。

ヒトES/iPS細胞の既存の細胞株について

山中所長ら[4]は，すでに世界中に広く知られている細胞株(例：201B7, hFIB2-iPS2)に新規にシリアル番号などで設定し直す場合にも，オリジナルの名称およびクローンIDを継承することができるような柔軟性をもたせるべきであると提言している。オリジナルの名称から細胞株の情報や原著論文を簡単に収集できるなど，研究者にとって都合が良い点が多いとしている[4]。すでに独自の方式で命名し，細胞株を管理している研究機関は多いため，すべての研究機関の樹立細胞株に対して公平にIDを分配するには多くの困難が予想される。しかし，集積された細胞情報や研究成果を活用するためにも，国際規格のIDを公平に付与できるよう整備し，細胞株のデータベース化を推進していく必要があるのではないだろうか。

すべての多能性幹細胞へ適応

ISCRの提案は，**図**の(c)の部分には，“i”あるいは“e”を表記することによりその細胞株がヒトiPS細胞株とヒトES細胞株のいずれかであることを識別できるようにするというものである[3]。この点に関して，山中所長らとPollok所長ら両者ともに，マウスiPS細胞，体細胞核移植ES細胞(NT-ESC)，単為発生胚由来ES細胞(parthenogenetic-ESC)，胚性腫瘍細胞(embryonal carcinoma cell：ECC)，胚性生殖系細胞(embryonal germ cell：EGC)，エピブラスト幹細胞(epiblast stem cell：EpiSC)などの多能性細胞をすべてこの命名法規定の対象に含めるべきであり，これらを正確に識別できるよう動物種や由来細胞を表すコードを**図**の(c)に表記することを提案している[4][5]。しかし，筆者らは，細胞種を識別するコードを頭につけたほうが分類しやすいのではないかと考える(**図**)。

ヒトES/iPS細胞において特定の病名を表すのは適当ではない

名称に病名を含めることに関して，Pollok所長ら[5]は懸念を抱いている。多くのヒトiPS細胞は，新生児表皮線維芽細胞(neonatal foreskin fibroblasts)から人工的に誘導され，“正常(non-diseased)”な指標細胞としても使用されている。しかしながら，組織を採取する段階でドナーの異常を検出できることは難しく，その匿名性からドナーの病歴の追跡は不可能である。現段階では，ヒトES/iPS細胞や分化させた細胞の病気に対する感受性を明らかにすること(どのような病気になりやすいかを予測すること)も不可能である。細胞登録の際にはドナーの病歴などに関する情報もわかっている範囲で報告すべきであるが，遺伝子の変異や欠失などの確定された情報の表記を提案している。

ヒトES/iPS細胞を培養する現場での作業

山中所長ら[4]とPollok所長ら[5]は両者とも，細胞株の名称に使用する文字数はできるだけ短くするよう主張している。データベース上で管理する際の利便性も重要だが，現場での作業も考慮するべきである。培養デッシュや凍結チューブにグローブをした手で書きやすく，読み取りやすくすることが重要である。ATCCで1.5mLチューブを用いる場合，細胞株の名称が10文字以下であることが理想的であるとPollok所長ら[5]は述べている。山中所長ら[4]も，ISCRの提案した14文字[3]は不便を感じる長さであり，簡略化した名称を使用し始めるようになることを危惧する。簡略化した名

表1　海外の細胞登録サイト

	機関	アドレス
Stem Cell Registry	ISCI	http://www.stem-cell-forum.net/ISCF/initiatives/isci/stem-cell-registry/
ISCR	UMass	http://www.umassmed.edu/iscr/index.aspx
hESCreg	EU連携	http://www.hescreg.eu/
NIH Human Embryonic Stem Cell Registry	NIH	http://grants.nih.gov/stem_cells/registry/current.htm

ISCI：The International Stem Cell Initiative
ISCR：The International Stem Cell Registry
UMass：The University of Massachusetts Medical School, Human Stem Cell Bank and Registry
hESCreg：European Human Embryonic Stem Cell Registry

称の使用は，細胞の混同のリスクにつながる。医薬基盤研JCRB細胞バンクや理化学研究所バイオリソースセンター細胞バンクでは，場合によってバーコードラベルを用いて管理している。最近は安価なバーコードリーダーもあり，研究室レベルにおいても利用が可能ではないだろうか。

これまでに命名法が規定され，広く活用されている例がある[3]。分化抗原群は，“CD42a”“CD42b”のようにCD番号で表記され，個別の抗原が認識される。また，遺伝子や蛋白質などについてはさまざまな名称が使用されるが，データベースに登録されたアクセッション番号によって識別され，容易にその原著論文まで確認できる。利便性の高い命名法の策定とデータベースの構築を行い，現場のニーズに対応する鍵となるのが，やはり細胞登録システムの整備である。細胞株の名称やIDとともに細胞情報を登録し，管理していくことが必要であろう。

ヒトES/iPS細胞の登録

ヒトES/iPS細胞を樹立した際，具体的な報告方法に関する国際的な統一規定はなく，新規の細胞の樹立を含めた研究成果の報告項目などは研究者やジャーナルの査読者に任されている。今後，産業応用される可能性があることから，各国の倫理規定を尊重して共有できるよう倫理的妥当性および科学的合理性を将来にわたって確保することが肝要である。**表1**の記載の通り，海外のヒトES細胞については，ISCBI，EUヒトES細胞登録(European Human Embryonic Stem Cell Registry：hESCreg)が連携して，それぞれのホームページで公開をしている。また，NIHヒトES細胞登録(NIH Human Embryonic Stem Cell Registry)では，NIHの研究費を使用して研究が可能な細胞が掲載されている。細胞登録に必要な情報として，**表2**に示す5つの項目が提案されている[3)8)]。このようなヒトES/iPS細胞の情報整備は，新規細胞株の樹立に必要な基本データ作成とその情報公開の推進につながると思われる。

おわりに

幹細胞研究者の方々には，細胞樹立の際に前記の問題をご一考いただければ幸いである。一方で，幹細胞研究者らの声をさらに集約し，想定される問題を回避し，かつ利便性の高い命名法を早期に確立することが望まれる。ES/iPS細胞株を含む多能性幹細胞の命名法および発表に関するルールを設け，情報をデータベース化し，世界中で共有することは，幹細胞研究の推進につながる。本総説が日本の幹細胞研究推進の一助となれば幸いである。

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表2　ES/iPS細胞の登録や研究成果の報告の際に必要な情報

細胞株の由来（source） 細胞のタイプ，由来組織，継代数など ドナーから採取された場合：ドナーの年齢，性別，人種（自己報告または解析結果）* 細胞バンクや民間企業から入手した場合：細胞株のアクセッション番号
樹立方法（derivation method） 細胞の株化までの方法，培地および添加物，培養期間，継代数など詳細な培養方法 ES細胞の場合：胚の取り扱い方法，胚盤胞を得るための透明帯除去方法，胚盤胞からの内部細胞塊の単離方法 iPS細胞の場合：リプログラミングに用いたベクターシステム，低分子，蛋白質，mRNAやmiRNAとその導入・誘導方法
細胞特性（characterization） 未分化状態の確認（免疫染色，フローサイトメトリー，遺伝子発現プロファイリングなど） 多能性の確認（*in vitro*分化，テラトーマ形成，遺伝子発現プロファイリングなど） 核型，SNP（一塩基多型）によるゲノム解析結果*
細胞同一確認（genetic identity）と無菌性（sterility） STR（short tandem repeat）やSNP解析による細胞認証試験結果* 無菌試験結果およびマイコプラズマ否定試験結果
細胞の来歴（provenance） ドナーに対する説明および同意（インフォームド・コンセント），利益相反についての確認

研究者から提供される細胞株の情報を名称・登録番号と合わせて管理していくべきだが，特に個人を特定できる情報（*）は，各国の倫理規定を尊重し，慎重に管理されなければならない。

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Efficient and Directive Generation of Two Distinct Endoderm Lineages from Human ESCs and iPSCs by Differentiation Stage-Specific SOX17 Transduction

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Abstract

The establishment of methods for directive differentiation from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is important for regenerative medicine. Although Sry-related HMG box 17 (SOX17) overexpression in ESCs leads to differentiation of either extraembryonic or definitive endoderm cells, respectively, the mechanism of these distinct results remains unknown. Therefore, we utilized a transient adenovirus vector-mediated overexpression system to mimic the SOX17 expression pattern of embryogenesis. The number of alpha-fetoprotein-positive extraembryonic endoderm (ExEn) cells was increased by transient SOX17 transduction in human ESC- and iPSC-derived primitive endoderm cells. In contrast, the number of hematopoietically expressed homeobox (HEX)-positive definitive endoderm (DE) cells, which correspond to the anterior DE *in vivo*, was increased by transient adenovirus vector-mediated SOX17 expression in human ESC- and iPSC-derived mesendoderm cells. Moreover, hepatocyte-like cells were efficiently generated by sequential transduction of SOX17 and HEX. Our findings show that a stage-specific transduction of SOX17 in the primitive endoderm or mesendoderm promotes directive ExEn or DE differentiation by SOX17 transduction, respectively.

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Introduction

There are two distinct endoderm lineages in early embryogenesis, the extraembryonic endoderm (ExEn) and the definitive endoderm (DE). The first of these lineages, the ExEn plays crucial roles in mammalian development, although it does not contribute to the formation of body cells. In early embryogenesis, a part of the inner cell mass of the blastocyst differentiates into the primitive endoderm (PrE). The PrE differentiates into the ExEn that composes the parietal endoderm, which contributes to the primary yolk sac, and the visceral endoderm, which overlies the epiblast [1,2]. In contrast, the second of the endoderm lineages, the DE arises from the primitive streak (PS), which is called the mesendoderm [3]. The DE has the ability to differentiate into the hepatic and pancreatic tissue [4].

The establishment of human embryonic stem cells (ESCs) [5] and human induced pluripotent stem cells (iPSCs) [6,7] has opened up new opportunities for basic research and regenerative medicine. To exploit the potential of human ESCs and iPSCs, it is necessary to understand the mechanisms of their differentiation. Although growth factor-mediated ExEn or DE differentiation is widely performed, it leads to a heterogeneous population [8,9,10,11]. Several studies have utilized not only growth factors but also modulation of transcription factors to control downstream signaling cascades [10,12,13]. Sox17, an Sry-related HMG box transcription factor, is required for development of both the ExEn and DE. In mice, during ExEn and DE development, Sox17 expression is first observed in the PrE and in the anterior PS, respectively [14]. Previous study showed that stable Sox17 overexpression promotes ExEn differentiation from mouse ESCs [12]. On the other hand, another previous study has demonstrated that DE progenitors can be established from human ESCs by stable expression of SOX17 [10]. The mechanism of these discrepancies which occurs in SOX17 transduction still remains unknown. Also, the role of SOX17 in human ExEn differentiation still remains unknown. Therefore, it is quite difficult to promote directive differentiation into either ExEn or DE cells by SOX17 transduction.

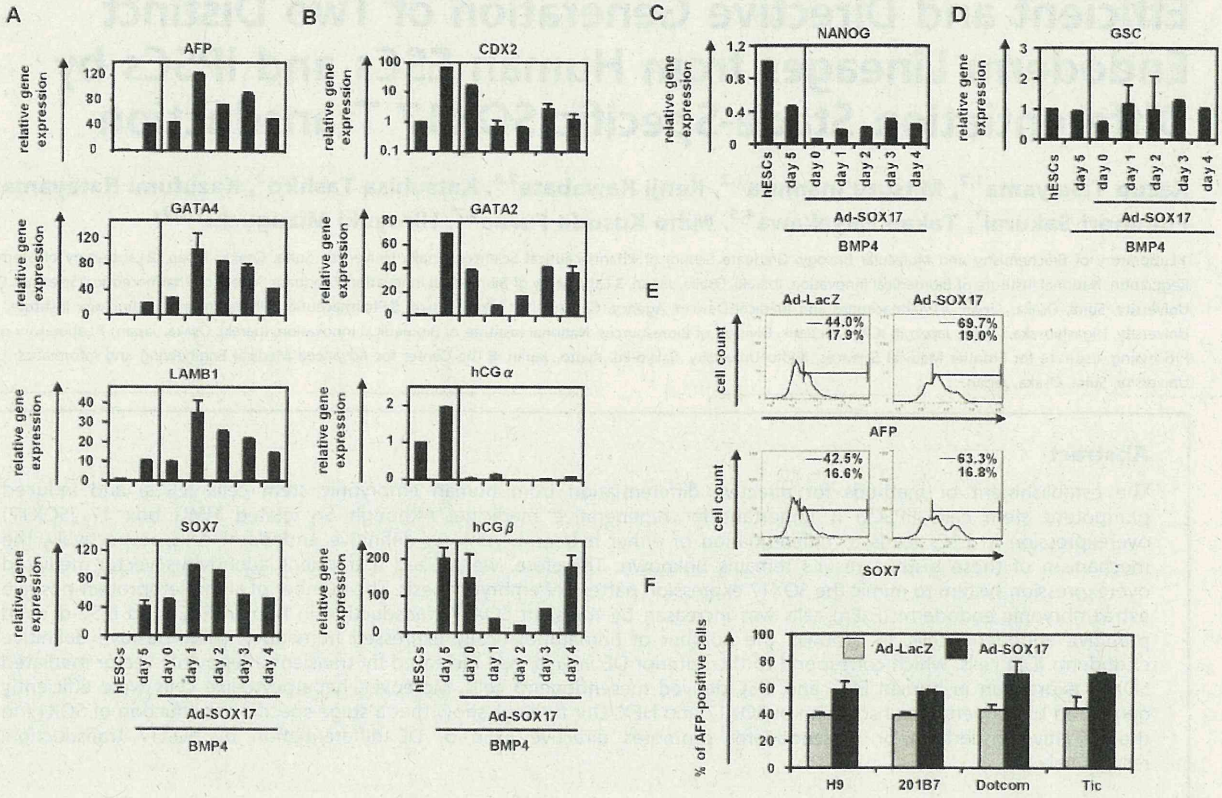

Figure 1. Efficient ExEn differentiation from human ESC- and iPSC-derived PrE cells by SOX17 transduction. (A–D) Undifferentiated human ESCs (H9) and BMP4-induced human ESC-derived cells, which were cultured with the medium containing BMP4 (20 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with 20 ng/ml of BMP4, and then the gene expression levels of *(A)* the ExEn markers (AFP, GATA4, LAMB1, and SOX7), (B) the trophectoderm markers (CDX2, GATA2, hCGα, and hCGβ), (C) the pluripotent marker (NANOG), and (D) the DE marker (GSC) were examined by real-time RT-PCR on day 5 of differentiation. The horizontal axis represents the day on which the cells were transduced with Ad-SOX17. The expression levels of undifferentiated human ESCs on day 0 were defined 1.0. (E) On day 1, human ESC-derived PrE cells, which were cultured with the medium containing BMP4 for 1 day, were transduced with Ad-LacZ or Ad-SOX17 and cultured until day 5. The ExEn cells were subjected to immunostaining with anti-AFP or anti-SOX7 antibodies, and then analyzed by flow cytometry. (F) After Ad-LacZ or Ad-SOX17 transduction, the efficacies of ExEn differentiation from the human ES cell line (H9) and the three human iPS cell lines (201B7, Dotcom, and Tic) were compared on day 5 of differentiation. All data are represented as the means±SD ($n=3$).
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In this study, we utilized SOX17 as a stage-specific regulator of ExEn and DE differentiation from human ESCs and iPSCs. The human ESC- and iPSC-derived cells were transduced with SOX17-expressing adenovirus vector (Ad-SOX17), and the resulting phenotypes were assessed for their ability to differentiate into ExEn and DE cells *in vitro*. In addition, we examined whether SOX17-transduced cells have the ability to differentiate into the hepatic lineage. The results showed that stage-specific overexpression of the SOX17 transcription factor promotes directive differentiation into either ExEn or DE cells.

Results

The induction of human ESC-derived PrE cells and human ESC-derived mesendoderm cells

To determine the appropriate stage for SOX17 transduction, ExEn or DE cells were differentiated from human ESCs by a conventional method using BMP4 (20 ng/ml) or Activin A (100 ng/ml), respectively (Figures S1 and S2). Experiments for bidirectional differentiation using BMP4 and Activin A indicated that PrE cells were obtained on day 1 (Figure S1) and mesendoderm cells were obtained on day 3 (Figure S2). We expected that stage-specific SOX17 transduction into PrE cells or mesendoderm cells could promote ExEn or DE differentiation, because the time period of intiation of SOX17 expression was correlated with the time period of formation of PrE cells (day 1) (Figure S1C) and mesendoderm cells (day 3) (Figure S2C), respectively.

PrE stage-specific SOX17 overexpression promotes directive ExEn differentiation from human ESCs

To examine the effect of forced and transient expression of SOX17 on the differentiation of human ESC- and iPSC-derived cells, we used a fiber-modified adenovirus (Ad) vector containing the EF-1α promoter and a stretch of lysine residues (KKKKKKK, K7) peptides in the C-terminal region of the fiber knob. The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing the K7 peptides has been shown to be efficient for transduction into many kinds of cells [15,16].

Because the time period of initiation of SOX17 expression was correlated with the time period of formation of PrE cells (day 1) (Figure S1), we expected that stage-specific SOX17 transduction

into PrE cells would promote ExEn differentiation. Therefore, we examined the stage-specific role of SOX17 in ExEn differentiation. Ad-SOX17 transduction was performed in human ESCs treated with BMP4 for 0, 1, 2, 3, or 4 days, and the Ad-SOX17-transduced cells were cultured with medium containing BMP4 until day 5 (Figures 1A–1D). We confirmed the expression of exogenous SOX17 in the human ESC-derived mesendoderm cells transduced with Ad-SOX17 (Figure S3). Since BMP4 is known for its capability to induce both ExEn and trophectoderm [8,9], we analyzed not only the expression levels of ExEn markers but also those of trophectoderm markers by real-time RT-PCR after 5 days of differentiation (Figures 1A and 1B). The transduction of Ad-SOX17 on day 1 led to the highest expression levels of ExEn markers, alpha-fetoprotein (AFP), GATA4, laminin B1 (LAMB1), and SOX7 [17,18,19]. In contrast, the expression levels of the trophectoderm markers CDX2, GATA2, hCGα (human chorionic gonadotropin), and hCGβ [20] were down-regulated in Ad-SOX17-transduced cells as compared with non-transduced cells (Figure 1B). The expression levels of the pluripotent marker NANOG and DE marker GSC were not increased by SOX17 transduction (Figures 1C and 1D). We confirmed that there were no differences between non-transduced cells and Ad-LacZ-transduced cells in gene expression levels of all the markers investigated in Figures 1A–1D (data not shown). Therefore, we concluded that ExEn cells were efficiently induced from Ad-SOX17-transduced PrE cells.

The effects of SOX17 transduction on the ExEn differentiation from human ESC-derived PrE cells were also assessed by quantifying AFP- or SOX7-positive ExEn cells. The percentage of AFP- or SOX7-positive cells was significantly increased in Ad-SOX17-transduced cells (69.7% and 63.3%, respectively) (Figure 1E). Similar results were observed in the human iPS cell lines (201B7, Dotcom, and Tic) (Figure 1F). These findings indicated that stage-specific SOX17 overexpression in human ESC-derived PrE cells enhances ExEn differentiation.

Mesendoderm stage-specific SOX17 overexpression promotes directive DE differentiation from human ESCs

To examine the effects of transient SOX17 overexpression on DE differentiation from human ESCs, we optimized the timing of the Ad-SOX17 transduction. Ad-SOX17 transduction was performed in human ESCs treated with Activin A (100 ng/ml) for 0, 1, 2, 3, or 4 days, and the Ad-SOX17-transduced cells were cultured with medium containing Activin A (100 ng/ml) until day 5 (Figures 2A–2C). Using a fiber-modified Ad vector, both undifferentiated human ESCs and Activin A-induced human ESC-derived cells were efficiently transduced (Figure S4). The transduction of SOX17 on day 3 led to the highest expression levels of the DE markers FOXA2 [21], GSC [22], GATA4 [17], and HEX [23] (Figure 2A). In contrast to the DE markers, the expression levels of the pluripotent marker NANOG [24] were down-regulated in Ad-SOX17-transduced cells as compared with non-transduced cells (Figure 2B). The expression levels of the ExEn marker SOX7 [14] were up-regulated, when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 0, 1, or 2 days (Figure 2C). On the other hand, the expression levels of the ExEn marker SOX7 were significantly down-regulated, when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 3 or 4 days, indicating that SOX17 overexpression prior to mesendoderm formation (day 0, 1, and 2) promoted not only DE differentiation but also ExEn differentiation. Similar results were obtained with the human iPS cell line (Tic) (Figure S5). Although the expression levels of the mesoderm marker FLK1 [25] did not exhibit any change when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 0, 1, or 2 days (Figure 2D), their expression levels were significantly down-regulated when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 3 or 4 days. These results suggest that SOX17 overexpression promotes directive differentiation from mesendoderm cells into the DE cells, but not into mesoderm cells. We also confirmed that Ad-vector mediated gene expression in the human ESC-derived mesendoderm cells (day 3) continued until day 6 and disappeared on day 10 (Figure S6). SOX17 transduction in the human ESC-derived cells on day 3 and 4 had no effect on cell viability, while that in the cells on day 0, 1, and 2 resulted in severely impaired cell viability (Figure S7), probably because SOX17 transduction directed the cells on day 0, 1, and 2 to differentiate into ExEn cells but the medium containing Activin A (100 ng/ml) was inappropriate for ExEn cells. We confirmed that there were no differences between non-transduced cells and Ad-LacZ-transduced cells in gene expression levels of all the markers investigated in Figures 2A–2D (data not shown). These results indicated that stage-specific SOX17 overexpression in human ESC-derived mesendoderm cells is essential for promoting efficient DE differentiation.

It has been previously reported that human ESC-derived mesendoderm cells and DE cells became CXCR4-positive (>80%) by culturing human ESCs with Activin A (100 ng/ml) [26]. However, Activin A is not sufficient for homogenous differentiation of c-Kit/CXCR4-double-positive DE cells [10,11] or HEX-positive anterior DE cells [23]. Seguin et al. and Morrison et al. reported that the differentiation efficiency of c-Kit/CXCR4-double-positive DE cells was approximately 30% in the absence of stable Sox17 expression and that of HEX-positive anterior DE cells was only about 10% [10,23]. Therefore, we next examined whether Ad-SOX17 transduction improves the differentiation efficiency of c-Kit/CXCR4-double-positive DE cells and HEX-positive anterior DE cells. Human ESC-derived mesendoderm cells were transduced with Ad-SOX17, and the number of CXCR4/c-Kit-double-positive cells was analyzed by using a flow cytometer. The percentage of CXCR4/c-Kit-double-positive cells was significantly increased in Ad-SOX17-transduced cells (67.7%), while that in Ad-LacZ-transduced cells was only 22% (Figure 2E). The percentage of HEX-positive cells was also significantly increased in Ad-SOX17-transduced cells (53.7%), while that in Ad-LacZ-transduced cells was approximately 11% (Figure 2F). Similar results were also observed in the three human iPS cell lines (201B7, Dotcom, and Tic) (Figure 2G). These findings indicated that stage-specific SOX17 overexpression in human ESC-derived mesendoderm cells promotes efficient differentiation of DE cells.

Ad-SOX17-transduced cells tend to differentiate into the hepatic lineage

To investigate whether Ad-SOX17-transduced cells have the ability to differentiate into hepatoblasts and hepatocyte-like cells, Ad-SOX17-transduced cells were differentiated according to our previously described method [13]. Our previous report demonstrated that transient HEX transduction efficiently generates hepatoblasts from human ESC- and iPSC-derived DE cells. The hepatic differentiation protocol used in this study is illustrated in Figure 3A. After the hepatic differentiation, the morphology of human ESCs transduced with Ad-SOX17 followed by Ad-HEX was gradually changed into a hepatocyte morphology: polygonal in shape with distinct round nuclei by day 18 (Figure 3B). We also

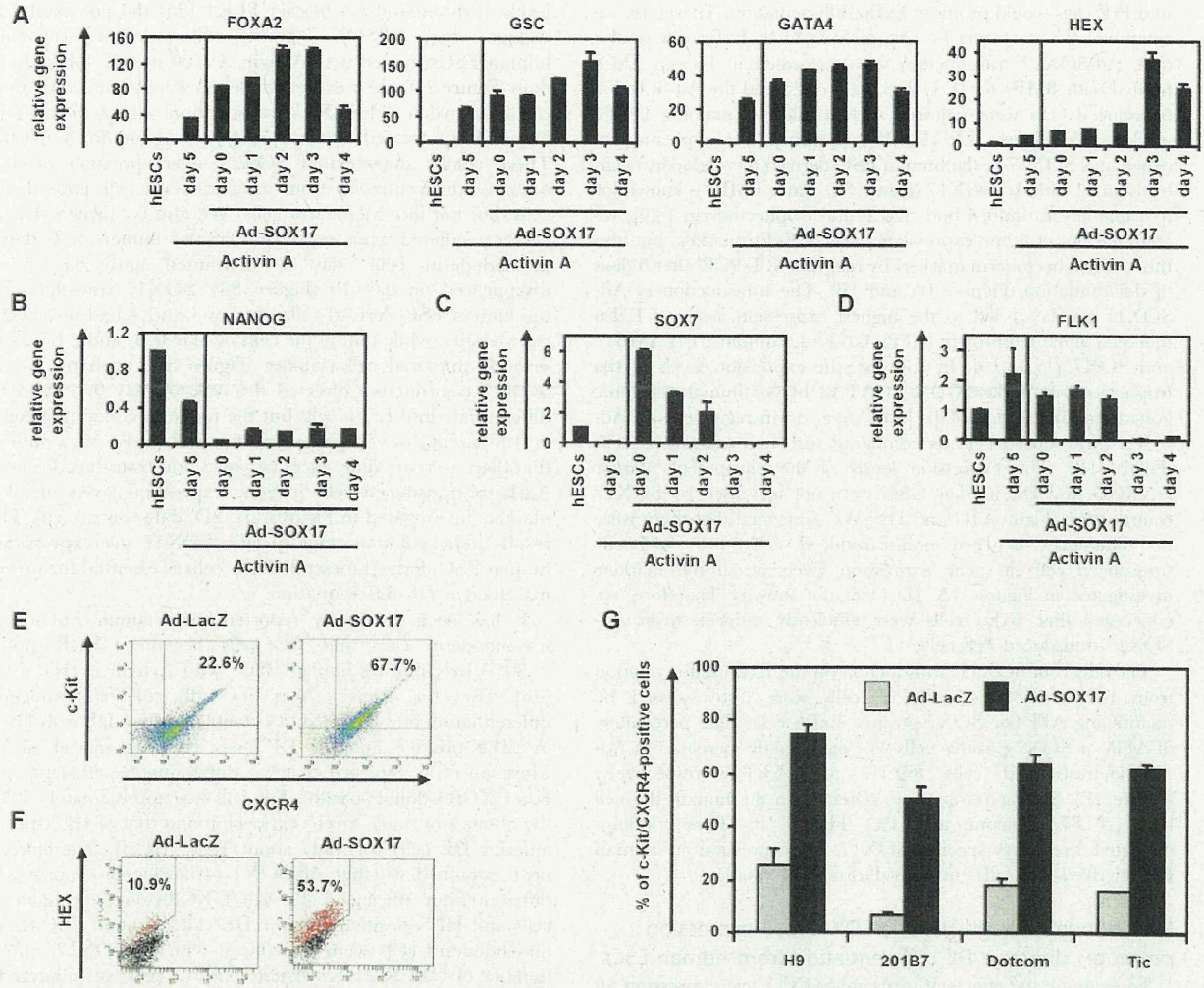

Figure 2. Efficient DE differentiation from human ESC- and iPSC-derived mesendoderm cells by SOX17 transduction. (A–D) Undifferentiated human ESCs (H9) and Activin A-induced human ESC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with 100 ng/ml of Activin A, and the gene expression levels of (A) the DE markers (FOXA2, GSC, and GATA4) and anterior DE marker (HEX), (B) the pluripotent marker (NANOG), (C) the ExEn marker (SOX7), and (D) the mesoderm marker (FLK1) were examined by real-time RT-PCR on day 5 of differentiation. The horizontal axis represents the day on which the cells were transduced with Ad-SOX17. The expression levels of human ESCs on day 0 were defined 1.0. (E, F) After human ESCs were cultured with 100 ng/ml of Activin A for 3 days, human ESC-derived mesendoderm cells were transduced with Ad-LacZ or Ad-SOX17 and cultured until day 5. Ad-LacZ- or Ad-SOX17-transduced DE cells were subjected to immunostaining with anti-c-Kit, anti-CXCR4 (E) and anti-HEX antibodies (F) and then analyzed by flow cytometry. (G) After Ad-LacZ or Ad-SOX17 transduction, the DE differentiation efficacies of the human ES cell line (H9) and three human iPS cell lines (201B7, Dotcom, and Tic) were compared at day 5 of differentiation. All data are represented as the means±SD ($n=3$).
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examined hepatic gene and protein expression levels on day18 of differentiation. For this purpose, we used a human ES cell line (H9) and three human iPS cell lines (201B7, Dotcom, Tic). On day 18 of differentiation, the gene and protein expression analysis showed up-regulation of the hepatic markers albumin (ALB) [27], cytochrome P450 2D6 (CYP2D6), CYP3A4, and CYP7A1 [28] mRNA and ALB, CYP2D6, CYP3A4, CYP7A1, and cytokeratin (CK)18 proteins in both Ad-SOX17- and Ad-HEX-transduced cells transduced cells as compared with both Ad-LacZ- and Ad-HEX-transduced cells (Figures 4A and 4B). These results indicated that Ad-SOX17-transduced cells were more committed to the hepatic lineage than non-transduced cells.

Discussion

The directed differentiation from human ESCs and iPSCs is a useful model system for studying mammalian development as well as a powerful tool for regenerative medicine [29]. In the present study, we elucidated the bidirectional role of SOX17 on either ExEn or DE differentiation from human ESCs and iPSCs. We initially confirmed that initiation of SOX17 expression was consistent with the time period of PrE or mesendoderm cells formation (Figures S1 and S2). We speculated that stage-specific transient SOX17 transduction in PrE or mesendoderm could enhance ExEn or DE differentiation from human ESCs and iPSCs, respectively.

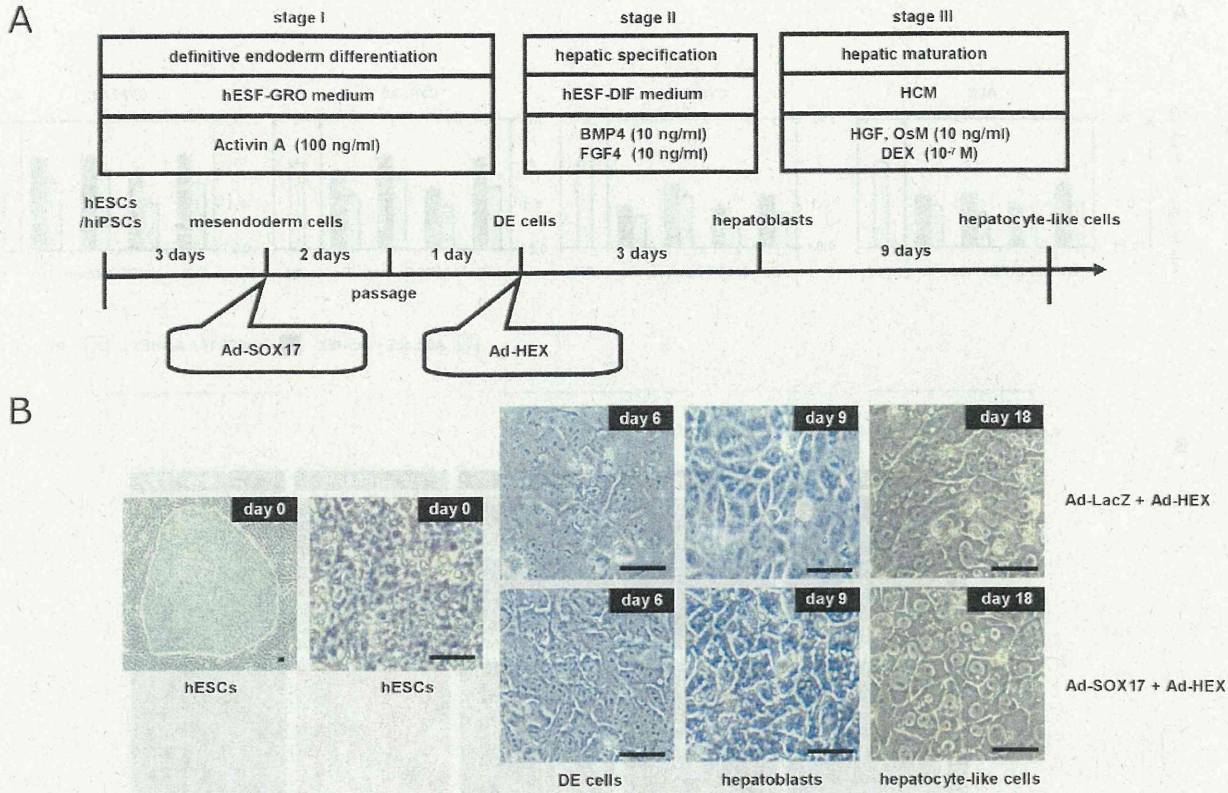

Figure 3. Hepatic Differentiation of Human ESC- and iPSC-Derived DE Cells Transduced with Ad-HEX. (A) The procedure for differentiation of human ESCs and iPSCs into hepatoblasts and hepatocyte-like cells is presented schematically. Both hESF-GRO and hESF-DIF medium were supplemented with 5 factors and 0.5 mg/ml fatty acid-free BSA, as described in the Materials and Methods section. (B) Sequential morphological changes (day 0–18) of human ESCs (H9) differentiated into hepatocyte-like cells via the DE cells and the hepatoblasts are shown. The scale bar represents 50 μm.
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SOX17 transduction at the pluripotent stage promoted random differentiation giving heterogeneous populations containing both ExEn and DE cells were obtained (Figures 2A–2C). Qu et al. reported that SOX17 promotes random differentiation of mouse ESCs into PrE cells and DE cells *in vitro* [30], which is in consistent with the present study. Previously, Niakan et al. and Seguin et al. respectively demonstrated that ESCs could promote either ExEn or DE differentiation by stable SOX17 expression, respectively [10,12]. Although these discrepancies might be attributable to differences in the species used in the experiments (i.e., human versus mice), SOX17 might have distinct functions according to the appropriate differentiation stage. To elucidate these discrepancies, we examined the stage-specific roles of SOX17 in the present study, and found that human ESCs and iPSCs could differentiate into either ExEn or DE cells when SOX17 was overexpressed at the PrE or mesendoderm stage, respectively, but not when it was overexpressed at the pluripotent stage (Figures 1 and 2). This is because endogenous SOX17 is strongly expressed in the PrE and primitive streak tissues but only slightly expressed in the inner cell mass, our system might adequately reflect the early embryogenesis [14,31].

In ExEn differentiation from human ESCs, stage-specific SOX17 overexpression in human ESC-derived PrE cells promoted efficient ExEn differentiation and repressed trophectoderm differentiation (Figures 1A and 1B), although SOX17 transduction at the pluripotent stage did not induce the efficient differentiation of ExEn cells. In our protocol, the stage-specific overexpression of SOX17 could elevate the efficacy of AFP-positive or SOX7-positive ExEn differentiation from human ESCs and iPSCs. The reason for the efficient ExEn differentiation by SOX17 transduction might be due to the fact that SOX17 lies downstream from GATA6 and directly regulates the expression of GATA4 and GATA6 [12]. Although it was previously been reported that Sox17 plays a substantial role in late-stage differentiation of ExEn cells *in vitro* [32], those reports utilized embryoid body formation, in which other types of cells, including endoderm, mesoderm, and ectoderm cells, might have influences on cellular differentiation. The present study showed the role of SOX17 in a homogeneous differentiation system by utilizing a mono-layer culture system.

In DE differentiation from human ESCs, we found that DE cells were efficiently differentiated from the human ESC-derived mesendoderm cells by stage-specific SOX17 overexpression (Figure 2). Therefore, we concluded that SOX17 plays a significant role in the differentiation of mesendoderm cells to DE cells. Although SOX17 overexpression before the formation of mesendoderm cells did not affect mesoderm differentiation, SOX17 transduction at the mesendoderm stage selectively promoted DE differentiation and repressed mesoderm differentiation (Figures 2A and 2D). These results show that SOX17 plays a crucial role in decision of DE differentiation from mesendoderm cells, as previous studies suggested [33,34]. Interestingly, SOX17 transduction at the pluripotent stage promoted not only DE

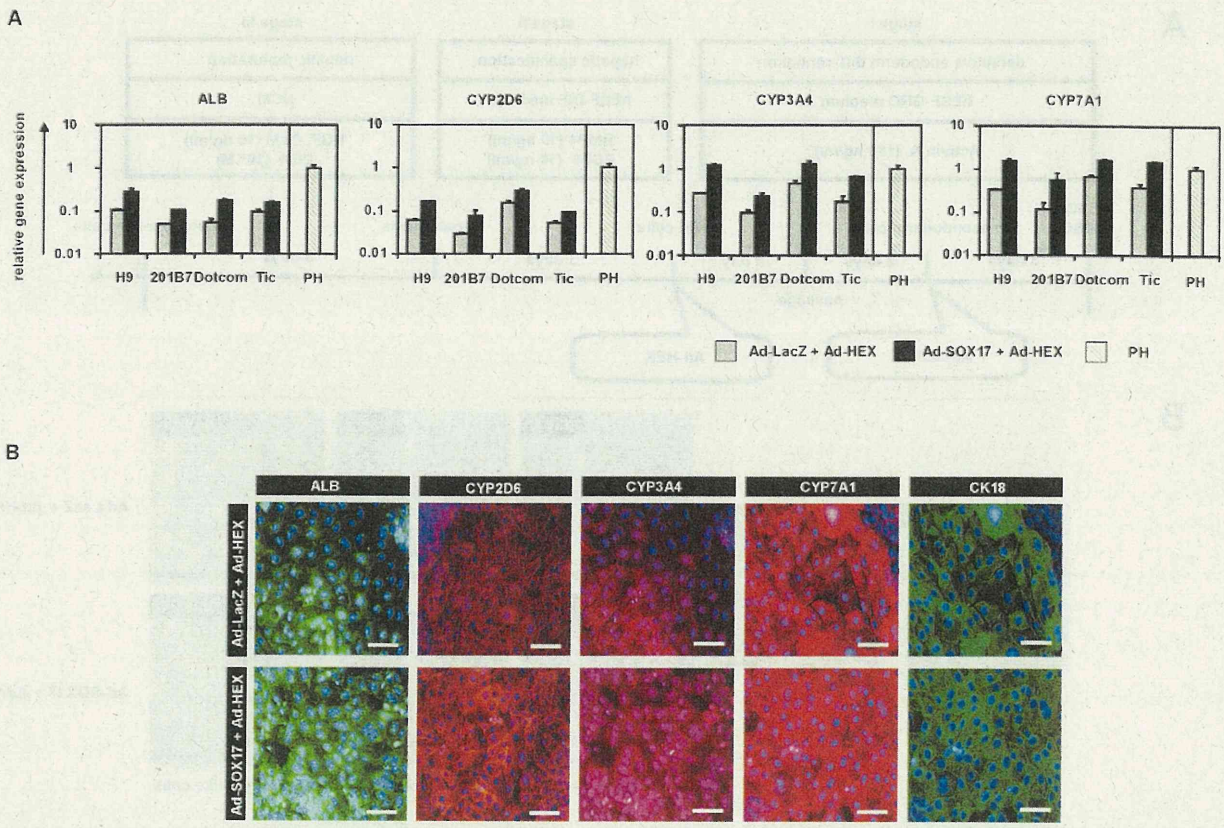

Figure 4. Characterization of hepatocyte-like cells from human ESC- and iPSC-derived DE cells. (A) The Ad-LacZ-transduced cells and Ad-SOX17-transduced cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 h on day 6. On day 18 of differentiation, the levels of expression of the hepatocyte markers (ALB, CYP2D6, CYP3A4, and CYP7A1) were examined by real-time RT-PCR in human ESC (H9)-derived hepatocyte-like cells and human iPSC (201B7, Dotcom, or Tic)-derived hepatocyte-like cells. The gene expression profiles of cells transduced with both Ad-SOX17 and Ad-HEX (black bar) were compared with those of cells transduced with both Ad-LacZ and Ad-HEX (gray bar). The expression level of primary human hepatocytes (PH, hatched bar), which were cultured 48 h after plating the cells, were defined as 1.0. All data are represented as the means±SD ($n = 3$). (B) The expression of the hepatocyte markers ALB (green), CYP2D6 (red), CYP3A4 (red), CYP7A1 (red), and CK18 (green) was also examined by immunohistochemistry on day 18 of differentiation. Nuclei were counterstained with DAPI (blue). The scale bar represents 50 μm.
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differentiation but also ExEn differentiation even in the presence of Activin A (Figures 2A and 2C), demonstrating that transduction at an inappropriate stage of differentiation prevents directed differentiation. These results suggest that stage-specific SOX17 transduction mimicking the gene expression pattern in embryogenesis could selectively promote DE differentiation.

Another important finding about DE differentiation is that the protocol in the present study was sufficient for nearly homogeneous DE and anterior DE differentiation by mesendoderm stage-specific SOX17 overexpression; the differentiation efficacies of c-Kit/CXCR4-double-positive DE cells and HEX-positive anterior DE cells were approximately 70% and 54%, respectively (Figures 2E and 2F). The conventional differentiation protocols without gene transfer were not sufficient for homogenous DE and anterior DE differentiation; the differentiation efficacies of DE and anterior DE were approximately 30% and 10%, respectively [10,11,23]. One of the reasons for the efficient DE differentiation by SOX17 transduction might be the activation of the FOXA2 gene which could regulate many endoderm-associated genes [35]. Moreover, SOX17-transduced cells were more committed to the hepatic lineage (Figure 4). This might be because the number of HEX-positive anterior DE cell populations was increased by SOX17 transduction. Recent studies have shown that the conditional expression of Sox17 in the pancreas at E12.5, when it is not normally expressed, is sufficient to promote biliary differentiation at the expense of endocrine cells [36]. Therefore, we reconfirmed that our protocol in which SOX17 was transiently transduced at the appropriate stage of differentiation was useful for DE and hepatic differentiation from human ESCs and iPSCs.

Using human iPSCs as well as human ESCs, we confirmed that stage-specific overexpression of SOX17 could promote directive differentiation of either ExEn or DE cells (Figures 1F, 2G, and 4A). Interestingly, a difference of DE and hepatic differentiation efficacy among human iPS cell lines was observed (Figures 1F and 2G). Therefore, it would be necessary to select a human iPS cell line that is suitable for hepatic differentiation in the case of medical applications, such as liver transplantation.

To control cellular differentiation mimicking embryogenesis, we employed Ad vectors, which are one of the most efficient transient gene delivery vehicles and have been widely used in both experimental studies and clinical trials [37]. Recently, we have also demonstrated that ectopic HEX expression by Ad vectors in human ESC-derived DE cells markedly enhances the hepatic differentiation [13]. Thus, Ad vector-mediated transient gene

transfer should be a powerful tool for regulating cellular differentiation.

In summary, the findings presented here demonstrate a stage-specific role of SOX17 in the ExEn and DE differentiation from human ESCs and iPSCs (Figure S8). Although previous reports showed that SOX17 overexpression in ESCs leads to differentiation of either ExEn or DE cells, we established a novel method to promote directive differentiation by SOX17 transduction. Because we utilized a stage-specific overexpression system, our findings provide further evidence that the lineage commitment in this method seems to reflects what is observed in embryonic development. In the present study, both human ESCs and iPSCs (3 lines) were used and all cell lines showed efficient ExEn or DE differentiation, indicating that our novel protocol is a powerful tool for efficient and cell line-independent endoderm differentiation. Moreover, the establishing methods for efficient hepatic differentiation by sequential SOX17 and HEX transduction would be useful for *in vitro* applications such as screening of pharmacological compounds as well as for regenerative therapy.

Materials and Methods

In vitro Differentiation

Before the initiation of cellular differentiation, the medium of human ESCs and iPSCs was exchanged for a defined serum-free medium hESF9 [38] and cultured as we previously reported. hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute) supplemented with 5 factors (10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, and 10 μM sodium selenite), oleic acid conjugated with fatty acid free bovine albumin, 10 ng/ml FGF2, and 100 ng/ml heparin (all from Sigma).

To induce, ExEn cells, human ESCs and iPSCs were cultured for 5 days on a gelatin-coated plate in mouse embryonic conditioned-medium supplemented with 20 ng/ml BMP4 (R&D system) and 1% FCS (GIBCO-BRL).

The differentiation protocol for induction of DE cells, hepatoblasts, and hepatocyte-like cells was based on our previous report with some modifications [13]. Briefly, in DE differentiation, human ESCs and iPSCs were cultured for 5 days on a Matrigel (BD)-coated plate in hESF-DIF medium (Cell Science & Technology Institute) supplemented with the above-described 5 factors, 0.5 mg/ml BSA, and 100 ng/ml Activin A (R&D Systems). For induction of hepatoblasts, the DE cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 h and cultured in hESF-DIF (Cell Science & Technology Institute) medium supplemented with the above-described 5 factors, 0.5 mg/ml BSA, 10 ng/ml bone morphology protein 4 (BMP4) (R&D Systems), and 10 ng/ml FGF4 (R&D systems). In hepatic differentiation, the cells were cultured in hepatocyte culture medium (HCM) supplemented with SingleQuots (Lonza), 10 ng/ml hepatocyte growth factor (HGF) (R&D Systems), 10 ng/ml Oncostatin M (OsM) (R&D Systems), and 10^{-7} M dexamethasone (DEX) (Sigma).

Human ESC and iPSC Culture

A human ES cell line, H9 (WiCell Research Institute), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with Repro Stem (Repro CELL), supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (Sigma). Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps, and subcultured every 4 or 5 days. Two human iPS cell lines generated from the human embryonic lung fibroblast cell line MCR5 were provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331; and Dotcom, JCRB Number: JCRB1327) [39,40]. These human iPS cell lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with iPSellon (Cardio), supplemented with 10 ng /ml FGF2. Another human iPS cell line, 201B7, generated from human dermal fibroblasts (HDF) was kindly provided by Dr. S. Yamanaka (Kyoto University) [6]. The human iPS cell line 201B7 was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with Repro Stem (Repro CELL), supplemented with 5 ng/ml FGF2 (Sigma). Human iPSCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps, and subcultured every 5 or 6 days.

Adenovirus (Ad) Vectors

Ad vectors were constructed by an improved *in vitro* ligation method [41,42]. The human SOX17 gene (accession number NM_022454) was amplified by PCR using primers designed to incorporate the 5′ BamHI and 3′ XbaI restriction enzyme sites: Fwd 5′-gcagggatccagcgccatgagcagcccgg-3′ and Rev 5′-cttctagagatcagggacctgtcacacgtc-3′. The human SOX17 gene was inserted into pcDNA3 (Invitrogen), resulting in pcDNA-SOX17, and then the human SOX17 gene was inserted into pHMEF5 [15], which contains the human EF-1α promoter, resulting in pHMEF-SOX17. The pHMEF-SOX17 was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7 [16], resulting in pAd-SOX17. The human elongation factor-1α (EF-1α) promoter-driven LacZ- or HEX-expressing Ad vectors, Ad-LacZ or Ad-HEX, were constructed previously. [13,43]. Ad-SOX17, Ad-HEX, and Ad-LacZ, which contain a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of human ESCs, iPSCs, and DE cells, were generated and purified as described previously [13,15,43]. The vector particle (VP) titer was determined by using a spectrophotometric method [44].

Flow Cytometry

Single-cell suspensions of human ESCs, iPSCs, and their derivatives were fixed with methanol at 4°C for 20 min, then incubated with the primary antibody, followed by the secondary antibody. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (Becton Dickinson).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from human ESCs, iPSCs, and their derivatives using ISOGENE (Nippon Gene) according to the manufacture's instructions. Primary human hepatocytes were purchased from CellzDirect. cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed with Taqman gene expression assays (Applied Biosystems) or SYBR Premix Ex Taq (TaKaRa) using an ABI PRISM 7000 Sequence Detector (Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used in this study are described in Table S1.

Immunohistochemistry

The cells were fixed with methanol or 4% PFA. After blocking with PBS containing 2% BSA and 0.2% Triton X-100 (Sigma), the cells were incubated with primary antibody at 4°C for 16 h, followed by incubation with a secondary antibody that was labeled

with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) at room temperature for 1 h. All the antibodies are listed in Table S2.

Crystal Violet Staining

The human ESC-derived cells that had adhered to the wells were stained with 200 µl of 0.3% crystal violet solution at room temperature for 15 min. Excess crystal violet was then removed and the wells were washed three times. Fixed crystal violet was solubilized in 200 µl of 100% ethanol at room temperature for 15 min. Cell viability was estimated by measuring the absorbance at 595 nm of each well using a microtiter plate reader (Sunrise, Tecan).

LacZ Assay

The human ESC- and iPSC-derived cells were transduced with Ad-LacZ at 3,000 VP/cell for 1.5 h. After culturing for the indicated number of days, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining was performed as described previously [15].

Supporting Information

Table S1 List of Taqman probes and primers used in this study.
(DOC)

Table S2 List of antibodies used in this study.
(DOC)

Figure S1 PrE cells formation from human ESCs on day 1 of differentiation. (A) The procedure for differentiation of human ESCs and iPSCs to ExEn cells by treatment with BMP4 (20 ng/ml) is presented schematically. (B) Human ESCs (H9) were morphologically changed during ExEn differentiation; when human ESCs were cultured with the medium containing BMP4 (20 ng/ml) for 5 days, the cells began to show flattened epithelial morphology. The scale bar represents 50 µm. (C–E) The tTemporal protein expression analysis during ExEn differentiation was performed by immunohistochemistry. The PrE markers COUP-TF1 [21] (red), SOX17 [14] (red), and SOX7 [14] (red) were detected on day 1. In contrast to the PS markers, the expression of the DE marker GSC [22] (red) was not detected and the level of the pluripotent marker NANOG (green) declined between day 0 and day 1. Nuclei were counterstained with DAPI (blue). The scale bar represents 50 µm.
(PDF)

Figure S2 Mesendoderm cells formation from human ESCs on day 3 of differentiation. (A) The procedure for differentiation of human ESCs and iPSCs to DE cells by treatment with Activin A (100 ng/ml) is presented schematically. hESF-GRO medium was supplemented with 5 factors and 0.5 mg/ml fatty acid free BSA, as described in the Materials and Methods. (B) Human ESCs (H9) were morphologically changed during DE differentiation; when human ESCs were cultured with the medium containing Activin A (100 ng/ml) for 5 days, the morphology of the cells began to show visible cell-cell boundaries. The scale bar represents 50 µm. (C–E) The tTemporal protein expression analysis during DE differentiation was performed by immunohistochemistry. The anterior PS markers FOXA2 [21] (red), GSC [22] (red), and SOX17 [14] (red) were adequately detected on day 3. The PS marker T [45] (red) was detected until day 3. In contrast to the PS markers, the expression of the pluripotent marker NANOG [24] (green) declined between day 2 and day 3. Nuclei were counterstained with DAPI (blue). The scale bar represents 50 µm.
(PDF)

Figure S3 Overexpression of SOX17 mRNA in human ESC (H9)-derived PS cells by Ad-SOX17 transduction. Human ESC-derived PS cells (day 1) were transduced with 3,000VP/cell of Ad-SOX17 for 1.5 h. On day 3 of differentiation, real-time RT-PCR analysis of the SOX17 expression was performed in Ad-LacZ-transduced cells and Ad-SOX17-transduced cells. On the y axis, the expression levels of undifferentiated human ESCs on day 0 was were taken defined as 1.0. All data are represented as the means±SD ($n = 3$).
(PDF)

Figure S4 Efficient transduction in Activin A-induced human ESC (H9)-derived cells by using a fiber-modified Ad vector containing the EF-1α promoter. Undifferentiated human ESCs and Activin A-induced human ESC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 vector particles (VP)/cell of Ad-LacZ for 1.5 h. The day after transduction, X-gal staining was performed. The scale bar represents 100 µm. Similar results were obtained in two independent experiments.
(PDF)

Figure S5 Optimization of the time period for Ad-SOX17 transduction to promote DE differentiation from human iPSCs (Tic). Undifferentiated human iPSCs and Activin A-induced human iPSC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with Activin A (100 ng/ml) until day 5, and then real-time RT-PCR analysis was performed. The horizontal axis represents the day on which the cells were transduced with Ad-SOX17. On the y axis, the expression levels of undifferentiated cells on day 0 was were taken defined as 1.0. All data are represented as the means±SD ($n = 3$).
(PDF)

Figure S6 Time course of LacZ expression in human ESC (H9)-derived mesendoderm cells transduced with Ad-LacZ. The hHuman ESC-derived mesendoderm cells (day 3) were transduced with 3,000 VP/cell of Ad-LacZ for 1.5 h. On days 4, 5, 6, 8, and 10, X-gal staining was performed. Note that human ESC-derived cells were passaged on day 5. The scale bar represents 100 µm. Similar results were obtained in two independent experiments.
(PDF)

Figure S7 Optimization of the time period for Ad-SOX17 transduction into Activin A-induced human ESC (H9)-derived cells. Undifferentiated human ESCs and Activin A-induced hESC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-LacZ or Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with Activin A (100 ng/ml) until day 5, then the cell viability was evaluated with crystal violet staining. The horizontal axis represents the day on which the cells were transduced with Ad-SOX17. On the y axis, the level of non-transduced cells was taken defined as 1.0. All data are represented as the means±SD (n = 3).
(PDF)

Figure S8 Model of differentiation of human ESCs and iPSCs into ExEn and DE cells by stage-specific SOX17 transduction. The ExEn and DE differentiation process is divided into at least two stages. In the first stage, human ESCs differentiate into either PrE cells by treatment with BMP4 (20 ng/ml) or mesendoderm cells by treatment with Activin A (100 ng/

ml). In the second stage, SOX17 promotes the further differentiation of each precursor cell into ExEn and DE cells, respectively. We have demonstrated that the efficient differentiation of these two distinct endoderm lineages is accomplished by stage-specific SOX17 transduction.
(PDF)

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