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日本におけるヒトES、iPS細胞研究標準化：その3　品質管理

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要旨　ヒト胚性幹（ES）細胞や人工多能性幹（iPS）細胞を用いて、発生過程におけるメカニズムの解明、あるいは再生医療や創薬などへの応用にむけて、国内外で研究が盛んに進められている。しかし、ヒトES/iPS細胞の形質は不安定であり、培地やフィーダー細胞のロットなどや、継代や培地交換のタイミングによっても簡単に変化する。倍加速度が早い異常クローンが出現した場合、5継代でほとんどの細胞集団が入れ替わる可能性が予測されている。研究のツールとして使う際にも随時品質管理が必要である。この総説では、研究室内でできるヒトES/iPS細胞の品質管理について概説する。

キーワード：　ヒトES細胞、ヒトiPS細胞、品質検査

序　　文

1998年 Thomson ら[1)] によりヒトES細胞が樹立され、2006年山中らのグループ[2)] によりマウス人工多能性幹（iPS）細胞が発表され、世界中で多能性幹細胞の研究が盛んに行われるようになった。これまでの総説でも述べたが[3,4)]、ヒトES/iPS細胞は従来の一般的な培養細胞とは異なる点が多く、また、研究室間による研究結果や技術の差も大きく、株間の差も大きい。ヒトES細胞研究の基礎知識が培われていない国内においては培養に苦労している研究者も多い。英国シェフィールド大学 Andrews 教授がリーダーとして推進している International human stem cell initiatives（ISCI）プロジェクトでは、日本を含めた世界11カ国の研究者らが共同でヒトES細胞株の特徴を比較し、ヒトES細胞研究の標準化が進められている[5)]。同プロジェクトでは59株のヒトES細胞を集めて、フローサイトメトリーを用いた表面抗原の発現プロフィール、PCR-アレイを用いた未分化マーカー遺伝子発現、胚様体作成法により分化させた際の遺伝子発現、インプリンティング遺伝子、X染色体不活性化について、解析方法とその結果

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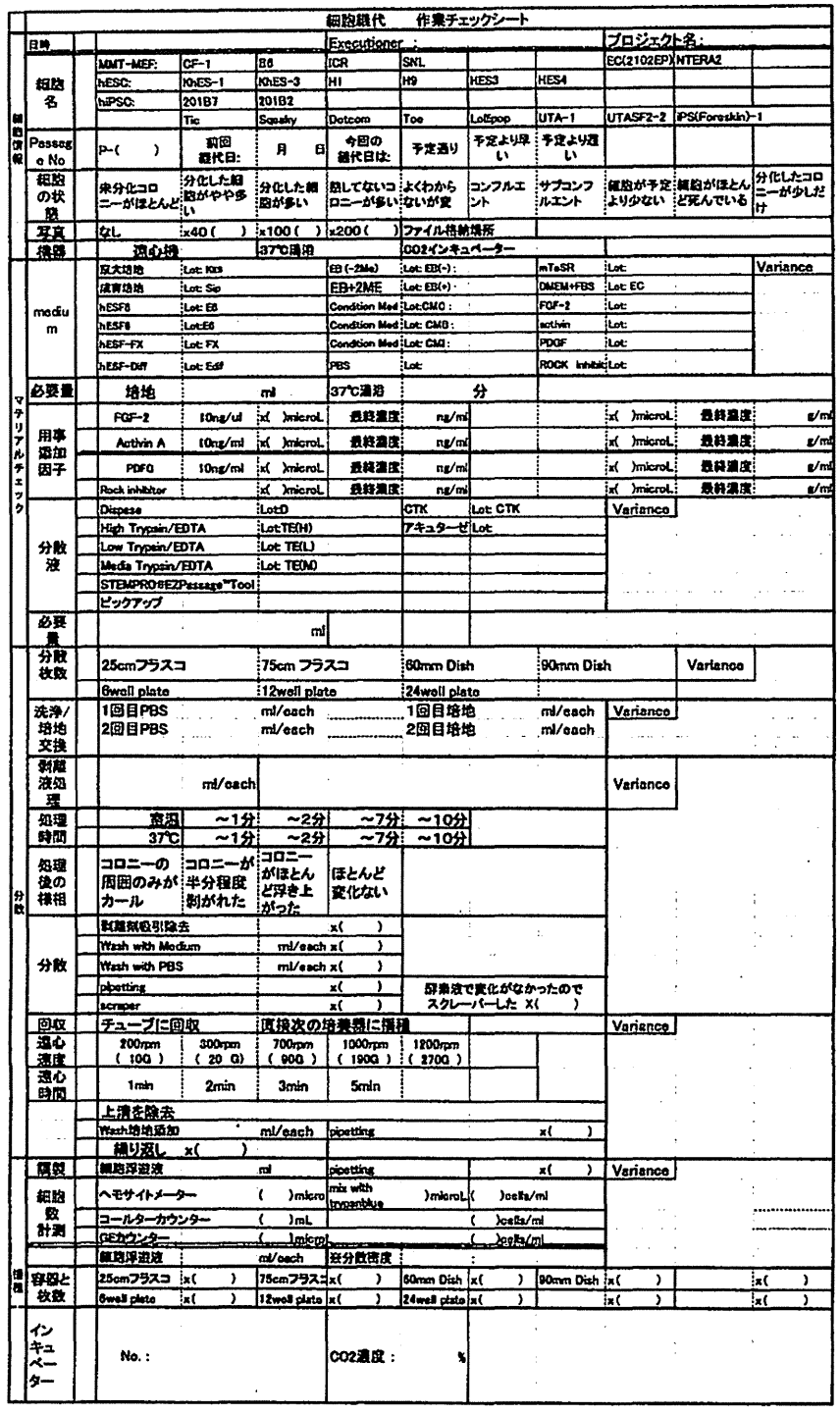

細胞現代　作業チェックシート

細胞情報	日時				Executioner：				プロジェクト名：		
	細胞名	MMT-MEF:	CF-1	B6	ICR	SNL			EC(2102EP)	NTERA2	
		hESC:	KhES-1	KhES-3	H1	H9	HES3	HES4			
		hiPSC:	201B7	201B2							
			Tic	Squaky	Dotcom	Toe	Lollipop	UTA-1	UTASF2-2	iPS(Foreskin)-1	
	Passage No	P-(　)	前回継代日:	月　日	今回の継代日は:	予定通り	予定より早い	予定より遅い			
	細胞の状態	未分化コロニーがほとんど	分化した細胞がやや多い	分化した細胞が多い	despてないコロニーが多い	よくわからないが変	コンフルエント	サブコンフルエント	細胞が予定より少ない	細胞がほとんど死んでいる	分化したコロニーが少しだけ
	写真	なし	x40(　)	x100(　)	x200(　)	ファイル格納場所					
	機器	遠心機		37℃湯浴		CO2インキュベーター					
マテリアルチェック	medium	反大培地	Lot: KO3		EB(-2Me)	Lot: EB(-)：		mTeSR	Lot:		Variance
		維育培地	Lot: Sip		EB+2ME	Lot: EB(+)：		DMEM+FBS	Lot: EC		
		hESF8	Lot: E8		Condition Med	Lot:CM0：		FGF-2	Lot:		
		hESF8	Lot:E8		Condition Med	Lot: CM0：		activin	Lot:		
		hESF-FX	Lot: FX		Condition Med	Lot: CM0：		PDGF	Lot:		
		hESF-Diff	Lot: Esf		PBS	Lot:		ROCK Inhibitor	Lot:		
	必要量	培地		ml	37℃湯浴	分					
	用事添加因子	FGF-2	10ng/ul	x(　)microL	最終濃度	ng/ml			x(　)microL	最終濃度	g/ml
		Activin A	10ng/ml	x(　)microL	最終濃度	ng/ml			x(　)microL	最終濃度	g/ml
		PDFG	10ng/ml	x(　)microL	最終濃度	ng/ml			x(　)microL	最終濃度	g/ml
		Rock inhibitor		x(　)microL	最終濃度	ng/ml			x(　)microL	最終濃度	g/ml
	分散液	Dispese		Lot:D		CTK	Lot: CTK		Variance		
		High Trypsin/EDTA		Lot:TE(H)		アキュターゼ	Lot:				
		Low Trypsin/EDTA		Lot: TE(L)							
		Media Trypsin/EDTA		Lot: TE(M)							
		STEMPRO®EZPassage™Tool									
		ピックアップ									
	必要量			ml							
分散	分散枚数	25cmフラスコ		75cm フラスコ		60mm Dish		90mm Dish		Variance	
		6well plate		12well plate		24well plate					
	洗浄/培地交換	1回目PBS		ml/each		1回目培地		ml/each	Variance		
		2回目PBS		ml/each		2回目培地		ml/each			
	剥離液処理		ml/each						Variance		
	処理時間	室温	～1分	～2分	～7分	～10分					
		37℃	～1分	～2分	～7分	～10分					
	処理後の様相	コロニーの周囲のみがカール	コロニーが半分程度剥がれた	コロニーがほとんど浮き上がった	ほとんど変化ない						
	分散	剥離液吸引除去			x(　)						
		Wash with Medium			ml/each x(　)						
		Wash with PBS			ml/each x(　)						
		pipetting			x(　)	酵素液で変化がなかったのでスクレーパーした x(　)					
		scraper			x(　)						
	回収	チューブに回収		直接次の培養器に播種					Variance		
	遠心速度	200rpm (100)	300rpm (200)	700rpm (900)	1000rpm (1900)	1200rpm (2700)					
	遠心時間	1min	2min	3min	5min						
		上清を除去									
		Wash培地追加		ml/each	pipetting			x(　)			
		繰り返し x(　)									
	懸製	細胞浮遊液		ml	pipetting			x(　)	Variance		
	細胞数計測	ヘモサイトメーター		(　)micro	mix with trypanblue	)microL	(　)cells/ml				
		コールターカウンター		(　)mL			(　)cells/ml				
		CEカウンター		(　)micro			(　)cells/ml				
		細胞浮遊液		ml/each	目分散密度		:				
播種	容器と枚数	25cmフラスコ	x(　)	75cmフラスコ	x(　)	60mm Dish	x(　)	90mm Dish	x(　)		x(　)
		6well plate	x(　)	12well plate	x(　)	24well plate	x(　)		x(　)		x(　)
	インキュベーター	No.：			CO2濃度：	%					

図1．ヒトES/iPS細胞用培養記録用紙　(継代用)
継代用、培地交換用ともに培養記録用のファイルは培養資源研究室・幹細胞研究チームのホームページ http://www.nibio.go.jp/baiyou/index.html に掲載予定。

を2007年に発表している[6]。また、その結果を受けて各国の幹細胞バンクや細胞バンクが参画するInternational stem cell banking initiative（ISCBI）では、研究用幹細胞のバンキングの国際ガイドラインを発表した[7]。日本語訳は京都大学再生医科学研究所・高田らが日本再生医療学会雑誌「再生医療」に掲載している[8]。さらに、2011年には、19カ国38研究室からヒトES細胞125株とヒトiPS細胞11株の樹立早期と長期継代後のサンプルを集め、ゲノム安定性の比較分析を実施し、ゲノムの変化などについて発表した。特に1、12、17、20番染色体の部位に起きやすいことが明らかとなり、その中でも、20番染色体の一定部位のコピー数増幅が特に起きやすいことが示された。これまでもヒトES細胞株のゲノム不安定については報告があり[9-20]、また、ヒトiPS細胞株についても、ゲノム不安定性やそのメカニズムなどが報告されている[21-24]。

以上のように、ヒトES/iPS細胞は形質が不安定であり、培地やフィーダー細胞のロットなどや、継代や培地交換のタイミングによっても簡単に変化する。倍加速度が早い異常クローンが出現した場合、5継代でほとんどの細胞集団が入れ替わる可能性が予測されている[25]。従って、ES/iPS細胞の評価は、細胞バンクなどの特定の機関のみで行われるものではなく、研究室においても各自が使用している細胞の品質に問題がないかどうか、管理として行うことが望まれる。ISCIのようにすべての項目を検査することは難しい。そこで、研究室で日常的に行える品質管理について概説する。

1．培養記録

基本的な事ではあるが、維持培養などの記載がおろそかになりがちである。経験的な事例であるが、ヒトES/iPS細胞の場合、継代のタイミングが

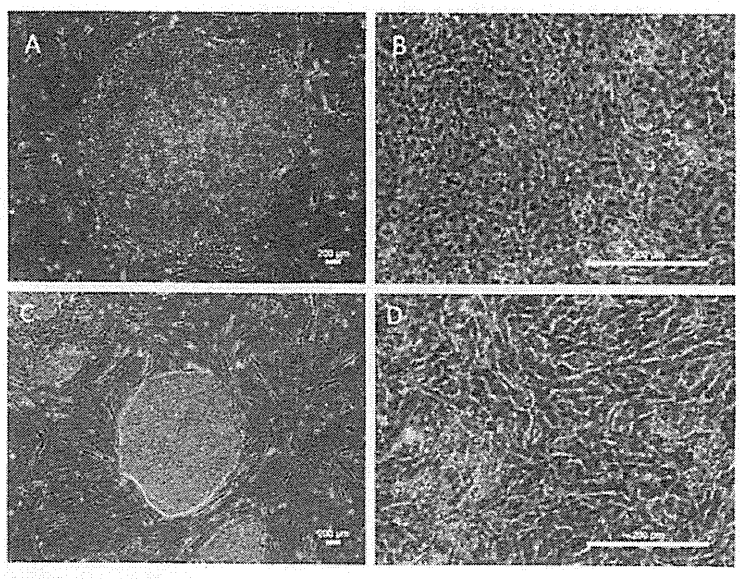

図2．ヒトES細胞の位相差顕微鏡像
A：ヒトES細胞 KhES-1（京都大学再生医科学研究所より分配）の未分化性の良い状態の弱拡大写真。
B：Aの強拡大写真。
C：Aの培養時とは異なるロットのフィーダー細胞を用いて未分化性があまり高くない状態の弱拡大写真。
D：Cの強拡大写真。

悪い場合 3 代継代後に影響がでることが多い。3 継代前にどのような作業を行ったか、正確に記録されていることにより、ヒト ES/iPS 細胞の未分化性の低下に与えた影響を同定できることも多い。一般的には、ヒト ES/iPS 細胞はフィーダー細胞と代替血清で培養が行われているが、昨今、新しい培養条件が次々と開発されており、研究室内でも複数の培養条件で継代維持されていることも少なくない。同じフォーマットに記録することにより、記録漏れをなくすことができるため、当研究室では複数の培養条件であっても共有に使用できる培養記録用紙を作成した（図 1）。現在、このフォーマットを他の研究室などにも試用をお願いしている。これにより、培養に関する問題について共通のプラットフォームで意見交換する一助となればと考える。

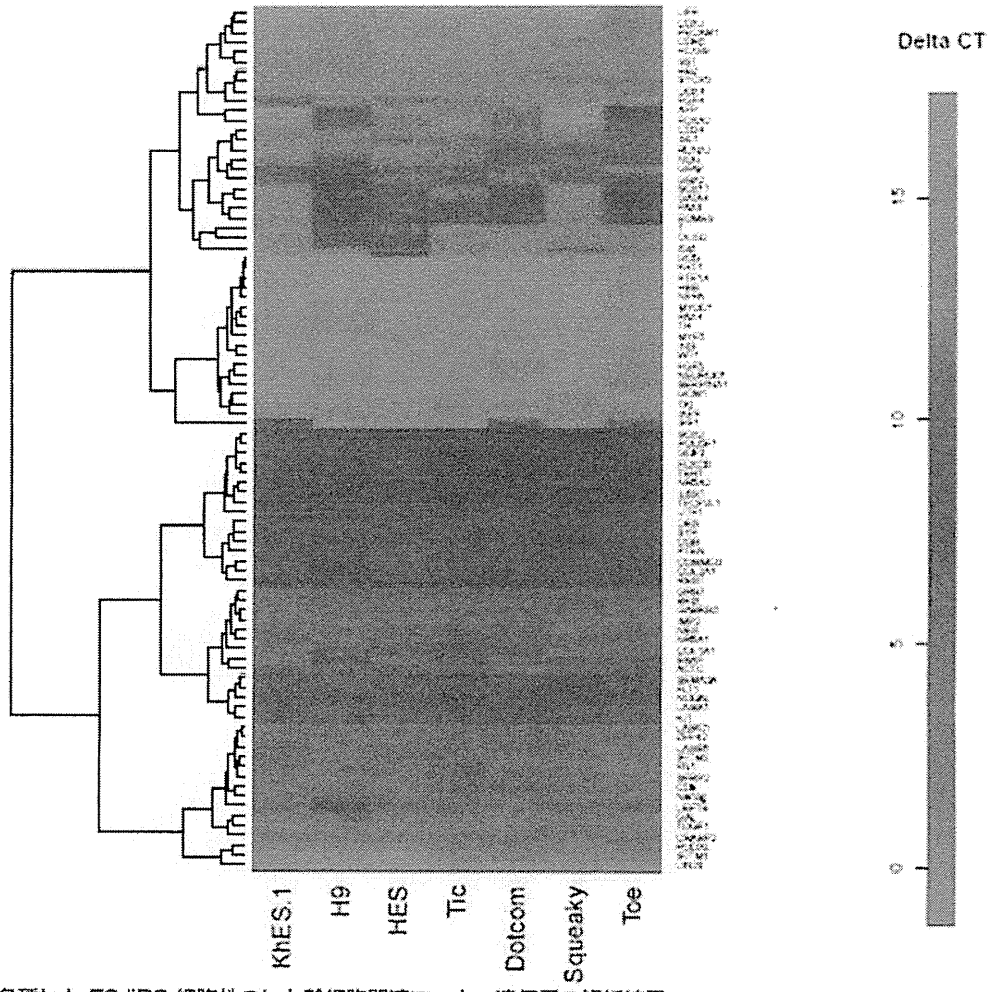

図 3．各種ヒト ES/iPS 細胞株のヒト幹細胞関連マーカー遺伝子の解析結果
MEF 上で培養した各種 ES 細胞（KhES-1: 京都大学再生医科学研究所より分配、H9、HES3: WiCell バンクより分譲）および iPS 細胞（国立成育医療センター梅澤らにより樹立され、JCRB 細胞バンクに寄託された iPS 細胞：Tic: JCRB1331、Dotcom: 1327、Sqeuaky: JCRB1329、Toe: JCRB1338）のヒト幹細胞関連マーカー遺伝子の発現率。赤：高発現、青：低発現

2．位相差顕微鏡像

現状では多能性を示す絶対的なマーカーは見つかっておらず、いくつかの解析方法を用いて未分化性や多能性を同定する必要がある。その中で、形態は重要な評価基準のひとつである[7]。継代直後のES/iPS細胞は、やや扁平な形態をしている株が多いが、継代後数日後の未分化なES/iPS細胞は、細胞質がほとんどなく、丸い核を持った細胞がコンパクトに集合したコロニーを形成し、細胞間境が明瞭でない特徴的な様相を呈する。英国の幹細胞研究者らは、コンパクトに集合したコロニー形態になることを「熟す」と表現することが多い。継代直前や凍結直前に、位相差顕微鏡写真を必ず撮るようにし、コロニーの形態がわかるように弱拡大と、細胞質や核の状態がわかるように強拡大の画像を取得する（図2）。

3．幹細胞関連遺伝子発現検査

2007年にISCIの報告によれば[6]、未分化なヒトES細胞に発現している遺伝子は、*NANOG*、*POU5F1*（*OCT4*）、*TDGF1*、*DNMT3B*、*GABRB3*や*GDF3*であった。このプロジェクトで使われた幹細胞遺伝子PCRアレイが市販されており、当研究室においても使用している。新しい株を入手して継代開始後できるだけ早いタイミングと、半年毎にRNAをサンプリングし、株間の比較を行っている。具体的には、細胞からRNA（必要RNA量1 pg ～ 2 μg）を抽出し、High Capacity RNA-to-cDNA Kit（ABI社、品番：4387406）を用いて逆転写し、PCRアレイにアプライする。PCRアレイ解析にはTaqMan Array Human Stem Cell Pluripotency Card（ABI社、品番：4385344）を使用し、AB7900HTリアルタイムPCRシステムで測定することで、ヒト幹細胞関連マーカー遺伝子の発現変化を解析している（図3）。

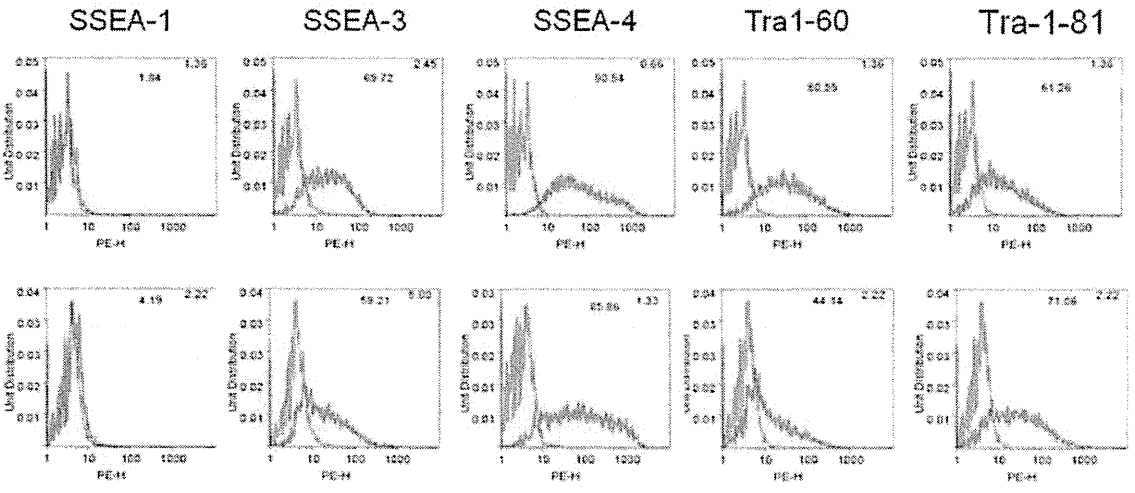

図4．フローサイトメトリー解析結果
上段：図2のA、Bの状態の細胞を各種抗体にて解析した結果。
下段：図2のC、Dの状態の細胞を上記と同様に解析した結果。
良い状態の上段に比べて、悪い状態の下段では、SSEA-1は発現していないものの、SSEA-3やTra-1-60のピークが左にシフトしているのがわかる。

4．フローサイトメトリー検査ならびに免疫染色検査

上記の幹細胞関連遺伝子発現は、それほど大きく変化することは少ないが、細胞表面抗原発現プロフィールは、ヒト ES/iPS 細胞を培養しているその培地やフィーダーのロットにより変化することも多い（図4）。位相差顕微鏡像を見て普段の状態とは異なると思われるような場合には、フローサイトメトリーを行って確認することをお薦めする。抗体2種による二重染色を行うのは効率的ではあるが、実際にはバックが高くなるなどのトラブルも多い。そのため、当研究室においては基本的にはすべてシングルで染色を行っている。免疫染色の場合には、6ウェルプレート2枚に播種し、6種類の抗体による染色を行っている。また、フローサイトメトリーと免疫染色は同じ抗体を用いている。その方法を表2−5に記載した。

5．染色体数計測

かつて日本組織培養学会が樹立細胞の登録を行っていた頃は、その細胞の染色体写真が必須であったが、近年は外部に委託することが多く、検査方法を知らない研究者も多い。G バンド分染法による核型解析は知識と経験を有するが、染色体数の計測であれば、日本組織培養学会編の「組織培養の技術」にその方法が記載されており、それほどの技術を要しない。ただ、ヒト ES/iPS 細胞においては、コルセミドに対する感受性が異なる株もあり、フィーダー細胞が邪魔をすることもあるので、若干の工夫が必要である。フィーダー細胞を除去するためにマトリジェル上に播種する方法もあるが、筆者らは EDTA にて ES/iPS 細胞のみを分散させる方法を行っている。その方法を表6、7に記載した。

表1．フローサイトメトリーと免疫染色の共通抗体

一次抗体	会社名	No.	希釈
SSEA-1	Santa cruz	SC-21702	1:100
SSEA-3	R & D	MAB1434	1:100
SSEA-4	Santa cruz	SC21704	1:50
TRA-1-60	Santa cruz	SC21705	1:100
Oct3/4	Santa cruz	SC-5279	1:50
Nanog	Cell Signaling	3580	1:400
ネガティブコントロール	**会社名**	**No.**	**希釈**
Mouse IgM	BioLegend	401601	1:100
Rat IgM	BioLegend	400801	1:100
Mouse IgG1	BioLegend	401401	1:100
Mouse IgG2a	BioLegend	400201	1:100
Mouse IgG3	BioLegend	401301	1:100
二次抗体	**会社名**	**No.**	**希釈**
Anti-Mouse IgM Alex647	Invitrogen	A21238	1:500
Anti-rat IgM Alex647	Invitrogen	A21248	1:500
Anti-Mouse IgG Alex647	Invitrogen	A21236	1:300
Anti-Rabbit IgG Alex555	Invitrogen	A21429	1:200

抗体は1%FBS/PBS で希釈している。

表2．フローサイトメトリー解析の準備

材　　料	メーカー	品　番
細胞：検体、陽性コントロール細胞（2102Ep）	−	−
各種抗体（一次抗体、二次抗体）	表1参照	−
15 ml 用　遠心管	greiner	188271
96 well round plate	nunc	268152
シール	Diversified Biotech	BEM-1
PBS (−)	GIBCO	10010-23
0.05%Trypsin/1mMEDTA	GIBCO Invitrogen	15090 E-6511
Medium：DMEM (High glucose)+10%FBS	GIBCO	11965
4%PFA	WAKO	163-20145
cell strainer キャップ付き 5 mL ラウンドチューブ	BD	352235

表3．フローサイトメトリーのための作業手順

1	ベンチ	96 well round plate に、指定の一次抗体希釈液を 10 μL ずつ添加する。
2		使用する細胞を PBS で洗浄する。
3		0.05%Trypsin/1mMEDTA を 1 mL 加え、乾かない程度に吸引する。
4		3−5 min インキュベータに戻す。
5		フラスコをたたいて分散し、細胞の分散状態を検鏡する。
		【細胞がはがれていた場合】Medium を 10 mL 加え、ピペッティング。
		【細胞がはがれていない場合】上清をアスピレートし、Medium を 10 mL 加え、ピペッティング。
6		single cell になっているか確認する為、再度検鏡する。
7		細胞数を測定する。
8	ベンチ	細胞懸濁液を遠心し、上清をアスピレートした後、Medium で 1−4×10^6 cells/mL に調整する（クリーンベンチ内で実施）。
9		96 well round plate に 10 μL ずつ分注する。
10		plate をシールし、4°C で 60 min 振盪する。
11		300G　(1,260 rpm) 3 min 遠心する。
12		ペレットを確認した後、デカントで上清を捨て、Medium を 150 μL/well 加え、遠心洗浄。
13		上記を二度繰り返す。
14		Medium を 45 μL/well 加え、指定の二次抗体希釈液を 5 μL ずつ添加する。
15		plate をシールし、4°C で 30 min 振盪する。
16		300G (1,260 rpm) 3 min 遠心する。
17		上記同様に、遠心洗浄を三度行う。
18		ペレットを確認した後、上清を捨て、PBS を 150 μL/well 加え、遠心洗浄する。
19		上清を捨て、5×10^5 cells/mL となるよう 4% PFA を加え、strainer を通した後、FACS 用 sample とする（ドラフト内で実施）（FACS にかける際、液量は最低 200 μL 以上必要）。

表4．免疫染色のための準備

材　　料	メーカー	品　番
細胞：検体、陽性コントロール細胞（2102Ep）	–	–
各種抗体（一次抗体、二次抗体）	表1参照	–
50 mL 用　遠心管	greiner	227261
6 well plate	nunc	268152
PBS (–)	GIBCO	10010-23
4% PFA	WAKO	163-20145
FBS	–	–
Triton X-100	SIGMA-ALDRICH	T8787
Tween 20	SIGMA-ALDRICH	P2287
Hoechst 33342	Invitrogen	H1399
5 mL　エッペンドルフチューブ	イナ・オプティカ	ST-500

表5．免疫染色の手順

1．細胞の準備

1	6 well plate に細胞を播種する（クリーンベンチ内で実施）。
2	継代日となったら、培地を吸引除去（クリーンベンチ内で実施）。
3	PBS を 5 mL 加え、吸引除去（クリーンベンチ内で実施）。
4	4% PFA を 1 mL/well で加え、15 min 静置（固定）（ドラフト内で実施）。
5	4% PFA を PFA 廃液用ボトルにデカントで廃棄する（ドラフト内で実施）。
6	PBS を約 5 ml 手早く加え、PFA 廃液用ボトルにデカントにて廃棄する（ドラフト内で実施）。
7	PBS を約 5 ml 手早く加え、デカントにて廃棄する（洗浄）。
8	上記 7 を同様に 1 回（洗浄）。
	直ちに染色を行わない場合は PBS を 5 mL/well で満たし、プレートの蓋を閉め、パラフィルムでシールし、4°C で保存する。

2．免疫染色

【細胞表面抗原の場合】

1	3% FBS in PBS 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）。
2	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）。
3	PBS を 7 mL/well 加え、5 min 静置（洗浄）。
4	上記を同様に 2 回（洗浄）。
5	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）。
6	PBS を 7 mL/well 加え、すぐにデカントで廃棄する。
7	PBS を 7 mL/well 加え、室温 10 min 静置（洗浄）。
8	上記を同様に 1 回（洗浄）。
9	PBS で500倍希釈した Hoechst を 1 mL/well 加え、室温で 3 min（シェイカー上で反応させる）。
10	PBS を 7 mL/well 加え、すぐにデカントにて液をのぞく（洗浄）。
11	PBS を少量入れて、検鏡する。
12	4% PFA を 1 mL/well 加え、室温 10 min 処理し、後固定をする（ドラフト内で実施）。
13	PBS で手早く 3 回洗浄する（ドラフト内で実施）。
14	PBS を 5 mL/well 加え、観察する。
	直ちに観察しない場合は、プレートの蓋を閉め、パラフィルムでシールし、アルミ箔で遮光して、4°C で保存する。

【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		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）。
	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消拭し、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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文 献

1) Thomson, J. A., Itskovitz-Eldor, J., et al.: Embryonic stem cell lines derived from human blastocysts. Science, 282, 1145–1147, (1998)

2) Takahashi, K. and Yamanaka, S.: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 126, 663–676, (2006)
3) Furue, M. K.: Standardization of human embryonic stem (ES) cell and induced pulripoent stem (iPS) cell research in Japan. Tissue Culture Research Communications, 27, 139–147, (2008)
4) Furue, M. K.: Standardization of human embryonic stem (ES) cell and induced pulripoent stem (iPS) cell research in Japan: How to detect differentiation potency of human ES/iPS cells. Tissue Culture Research Communications, 28, 129–133, (2009)
5) Andrews, P. W., Benvenisty, N., et al.: The International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. Nat Biotechnol, 23, 795–797, (2005)
6) Adewumi, O., Aflatoonian, B., et al.: Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol, (2007)
7) Consensus guidance for banking and supply of human embryonic stem cell lines for research purposes. Stem Cell Rev, 5, 301–314, (2009)
8) 高田 圭：研究使用を目的としたヒト胚性幹細胞株のバンキングと分配のための総合指針、再生医学、10、(2011)
9) Draper, J. S., Smith, K., et al.: Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol, 22, 53–54, (2004)
10) Baker, D. E., Harrison, N. J., et al.: Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotechnol, 25, 207–215, (2007)
11) Mitalipova, M. M., Rao, R. R., et al.: Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol, 23, 19–20, (2005)
12) Hoffman, L. M. and Carpenter, M. K.: Characterization and culture of human embryonic stem cells. Nat Biotechnol, 23, 699–708, (2005)
13) Maitra, A., Arking, D. E., et al.: Genomic alterations in cultured human embryonic stem cells. Nat Genet, 37, 1099–1103, (2005)
14) Buzzard, J. J., Gough, N. M., et al.: Karyotype of human ES cells during extended culture. Nat Biotechnol, 22, 381–382; author reply 382, (2004)
15) Caisander, G., Park, H., et al.: Chromosomal integrity maintained in five human embryonic stem cell lines after prolonged in vitro culture. Chromosome Res, 14, 131–137, (2006)
16) Inzunza, J., Sahlen, S., et al.: Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. Mol Hum Reprod, 10, 461–466, (2004)
17) Rosler, E. S., Fisk, G. J., et al.: Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn, 229, 259–274, (2004)
18) Lefort, N., Feyeux, M., et al.: Human embryonic stem cells reveal recurrent genomic instability at 20q11. 21. Nat Biotechnol, 26, 1364–1366, (2008)
19) Spits, C., Mateizel, I., et al.: Recurrent chromosomal abnormalities in human embryonic stem cells. Nat Biotechnol, 26, 1361–1363, (2008)
20) Mayshar, Y., Ben-David, U., et al.: Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. Cell Stem Cell, 7, 521–531, (2010)
21) Ramos-Mejia, V., Munoz-Lopez, M., et al.: iPSC lines that do not silence the expression of the ectopic reprogramming factors may display enhanced propensity to genomic instability. Cell Res, 20, 1092–1095, (2010)
22) Blasco, M. A., Serrano, M., et al.: Genomic instability in iPS: time for a break. Embo J, 30, 991–993, (2011)
23) Kinoshita, T., Nagamatsu, G., et al.: Ataxia-telangiectasia mutated (ATM) deficiency decreases reprogramming efficiency and leads to genomic instability in iPS cells. Biochem Biophys Res Commun, 407, 321–326, (2011)
24) Prigione, A., Hossini, A. M., et al.: Mitochondrial-associated cell death mechanisms are reset to an embryonic-like state in aged donor-derived iPS cells harboring chromosomal aberrations. PLoS One, 6, e27352, (2011)
25) Olariu, V., Harrison, N. J., et al.: Modeling the evolution of culture-adapted human embryonic stem cells. Stem Cell, Res, 4, 50–56, (2010)
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日本におけるヒトES、iPS細胞研究標準化

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

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.

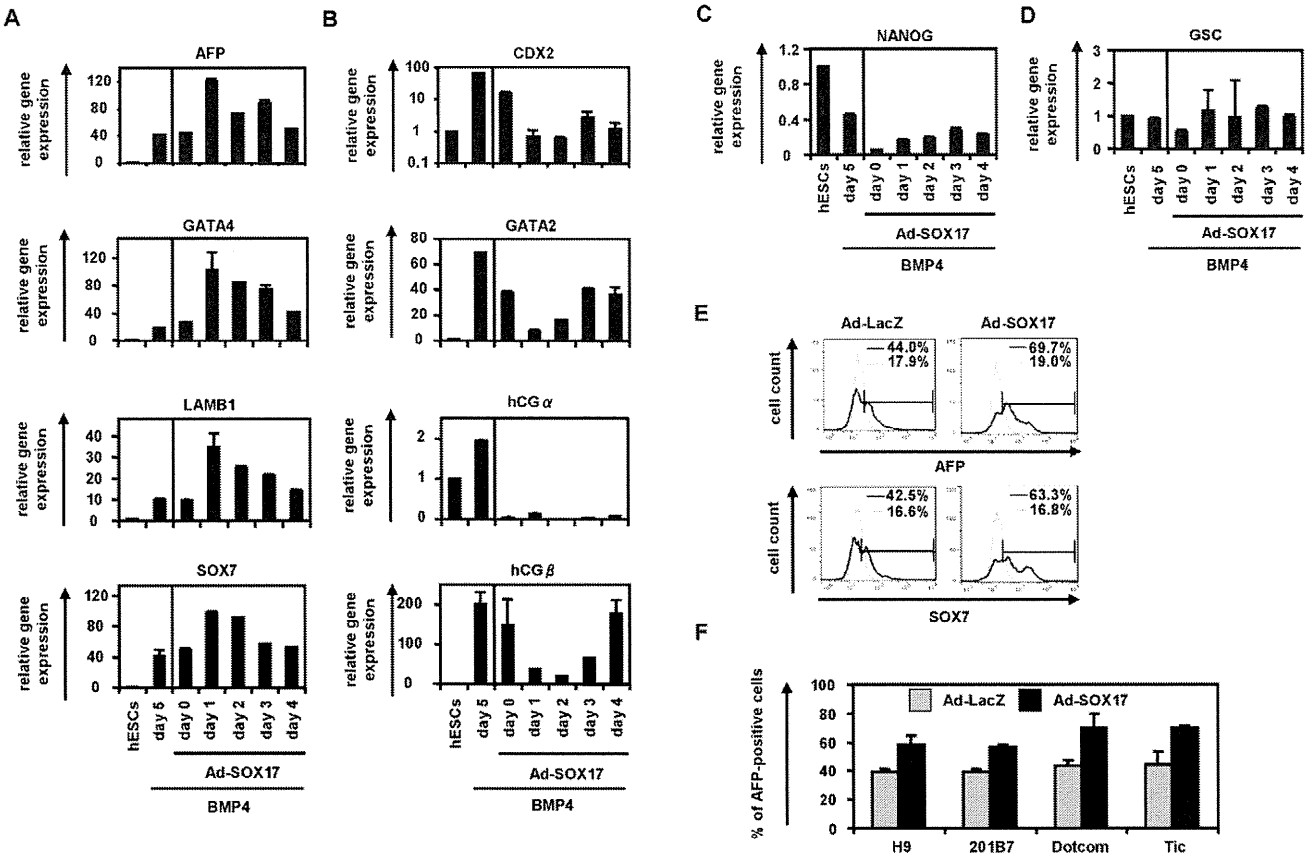

Figure 1. Efficient ExEn differentiation from human ESC- and iPSC-derived PrE cells by SOX17 transduction. (A–D) Undifferentiated human ESCs (H9) and BMP4-induced human ESC-derived cells, which were cultured with the medium containing BMP4 (20 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 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

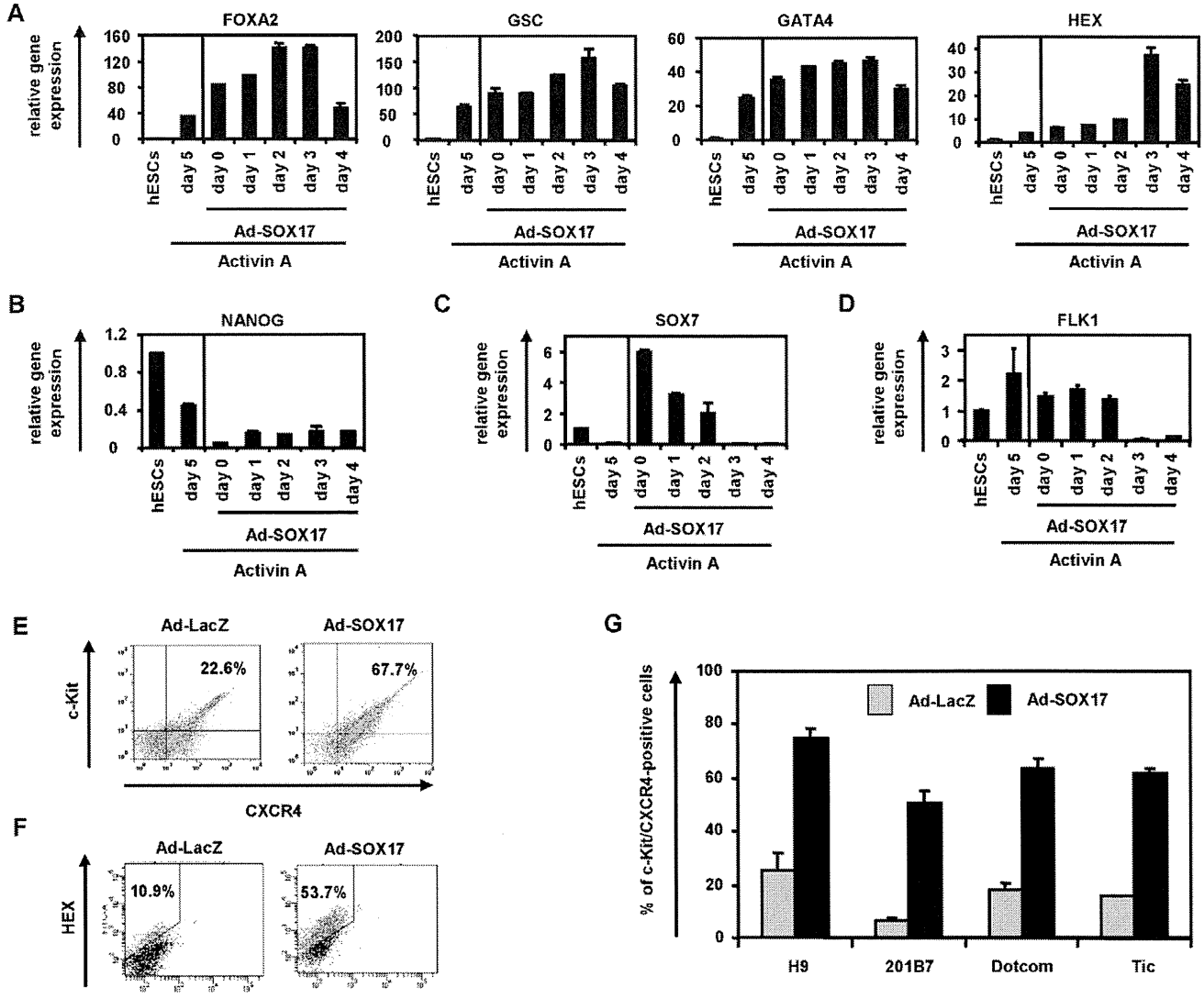

Figure 2. Efficient DE differentiation from human ESC- and iPSC-derived mesendoderm cells by SOX17 transduction. (A–D) Undifferentiated human ESCs (H9) and Activin A-induced human ESC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were 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.

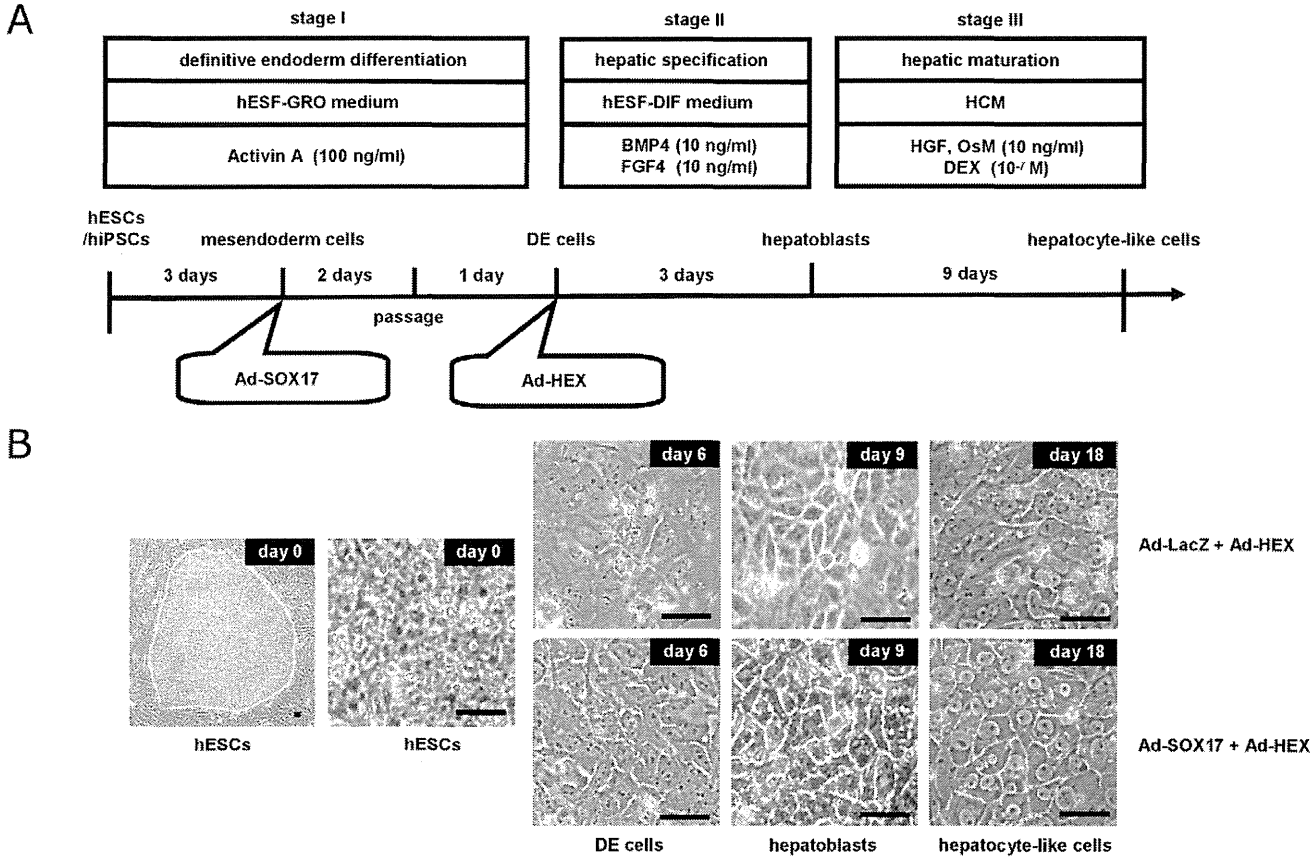

Figure 3. Hepatic Differentiation of Human ESC- and iPSC-Derived DE Cells Transduced with Ad-HEX. (A) The procedure for differentiation of human ESCs and iPSCs into hepatoblasts and hepatocyte-like cells is presented schematically. Both hESF-GRO and hESF-DIF medium were supplemented with 5 factors and 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

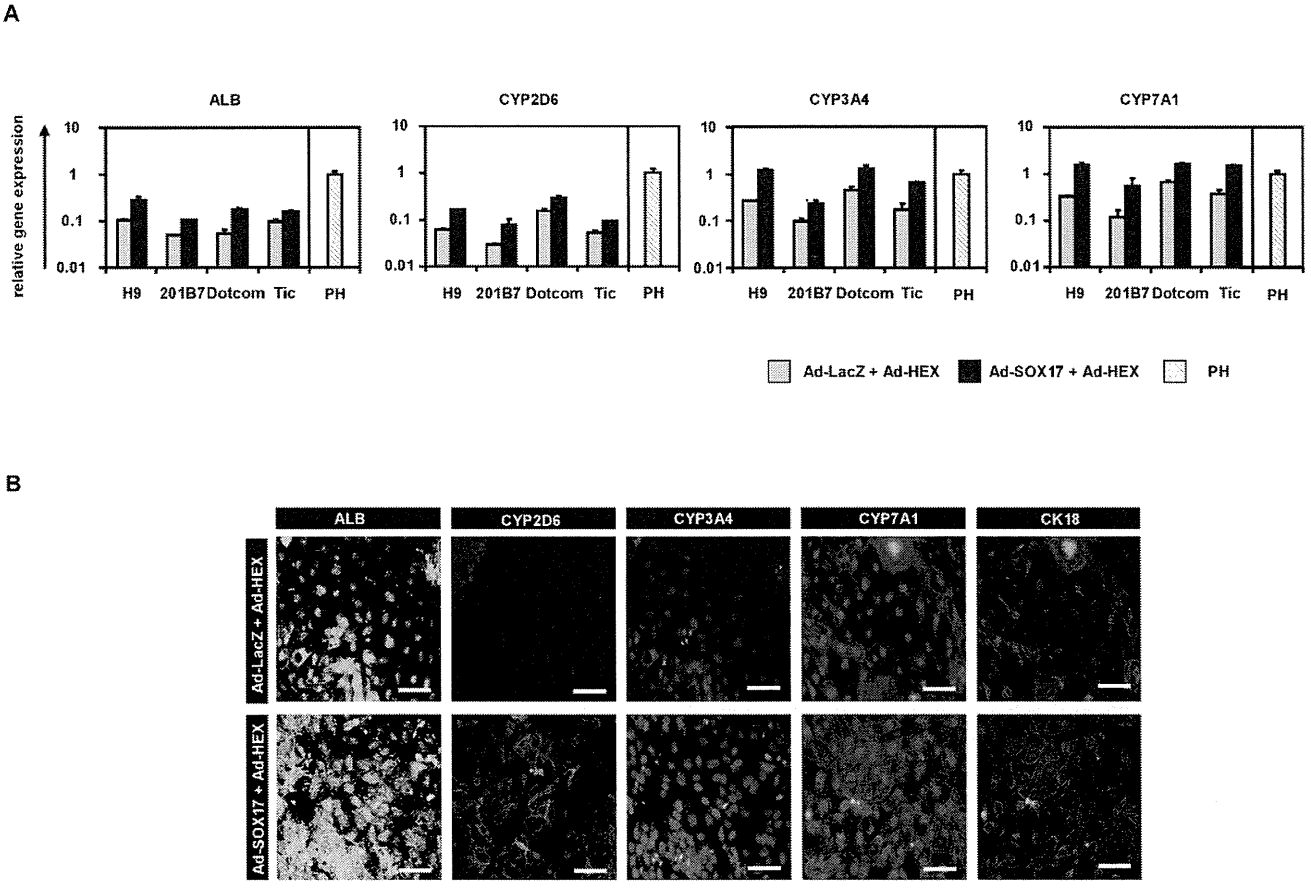

Figure 4. Characterization of hepatocyte-like cells from human ESC- and iPSC-derived DE cells. (A) The Ad-LacZ-transduced cells and Ad-SOX17-transduced cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 h on day 6. On day 18 of differentiation, the levels of expression of the hepatocyte markers (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