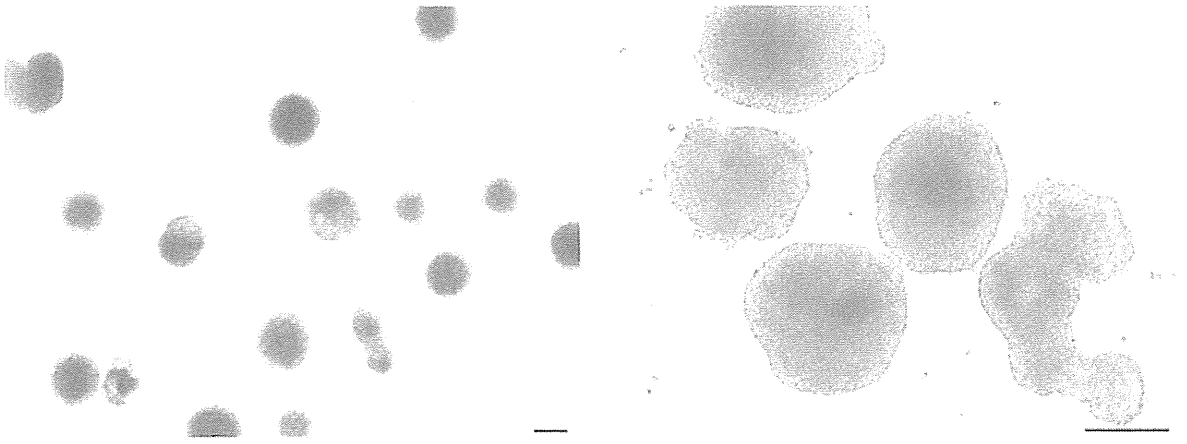


図2　胚様体
Scale bar 200 μm

い培地を10 mL加える．ペトリディッシュは，フィーダー細胞の残存などが付着してきたら新しいものと交換する（1週間に1度程度交換するとよい）．

結果判定

培養後，胚様体の塊ができることを確認する（図2）．画像を記録する．当研究室ではRNAを抽出してPCR法にて分化マーカーの上昇（GATA4, Brachyury, PAX6, EOMESのような三胚葉性マーカーと栄養外胚葉マーカー）と未分化マーカー（NANOG, OCT3/4など）の低下を確認している．未分化マーカーは2週間程度の培養では検出される場合ある．

A-6. テラトーマアッセイ

準　備

□ SCIDマウス

移植実施時に6～8週齢となるようにする．

□ 細胞数の調製の目安

マウス1匹あたり，1 ×10^6 cells程度の細胞を0.5 mL程度の培地に懸濁する．

□ 細胞注入用キャピラリ（図3 A）

プロトコール

精巣への細胞の移植

❶ マウスに麻酔をかける

❷ 麻酔が効いたら，マウスの足の付け根付近をハサミで縦方向に切開する．1 cmほど切開すれば充分である（図3B）

❸ 手術用ピンセットを使用し，切開した部位から精巣を取り出す（図3C）
はじめに脂肪を取り出し，さらに引き出すと精巣が出てくる．精巣は6 mmくらいの楕円上の形状をしている．

❹ 18G注射針を使用して，精巣にキャピラリーを差し込むための穴をあける（図3D）

❺ インジェクション用のキャピラリーに，細胞を吸い込む（図3E）
❹で開けた穴にキャピラリーの先端を差し込み，少しずつ細胞を注入する（図3F）．

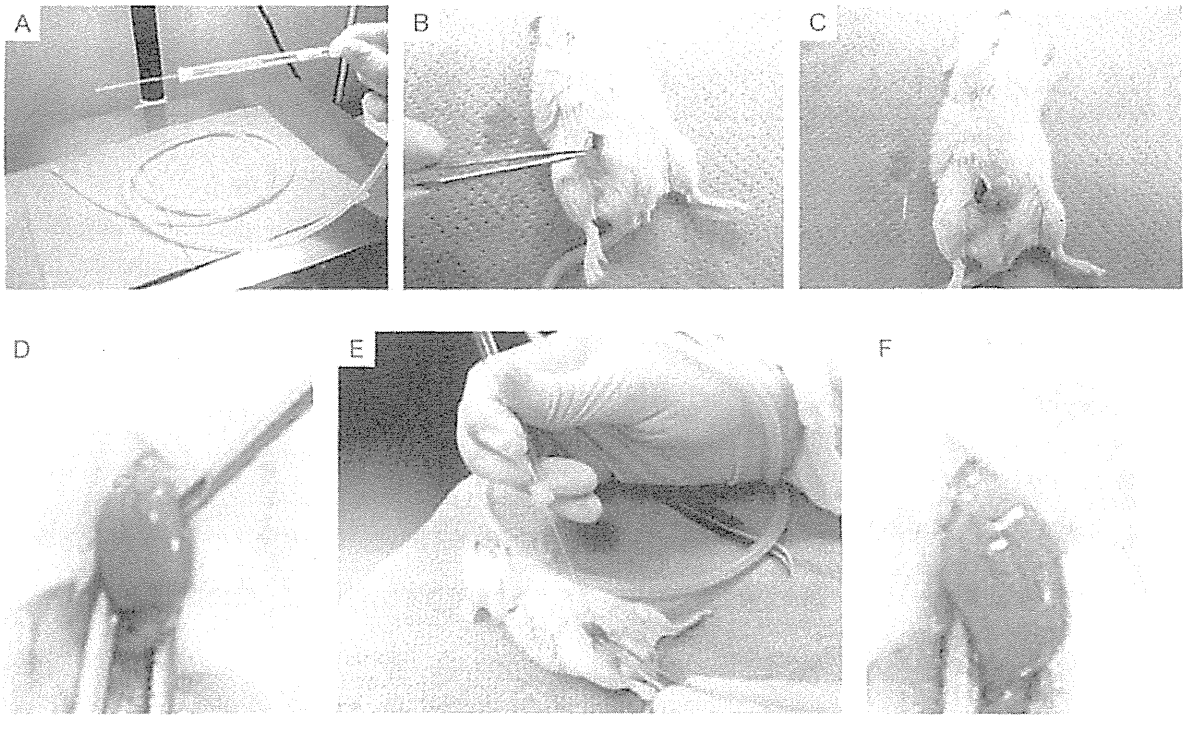


図3　精巣への細胞移植

❻ 全量の細胞を注入したら，精巣や脂肪などを腹膜の中に戻す

❼ 手術用糸付き針を使用し，腹膜および皮をそれぞれ縫合する

❽ 加温プレートの上でマウスを温めた後ケージに戻す

❾ 移植後８週間マウスを飼育し，テラトーマを形成させる
移植後６週程度で，テラトーマの形成が確認される．

テラトーマの摘出

❶ 頸椎脱臼により，マウスを安楽死させる

❷ 手術用ハサミにより開腹し，形成したテラトーマを腹腔内より取り出す

❸ テラトーマをPBSの入った100 mmペトリディッシュに移す

❹ マウス由来の臓器と思われる部分などをハサミで除去し，テラトーマをもう一度PBSで洗浄する

❺ テラトーマを写真にとる

テラトーマの固定から染色まで

❶ テラトーマを１ cm以下の大きさに切り分ける

❷ ホルマリン固定後，パラフィン包埋後ミクロトーム５ μm程度に薄切する

❸ 加温したスライドグラスの上で縮んだ切片を伸展させ，乾燥させて標本を作製する

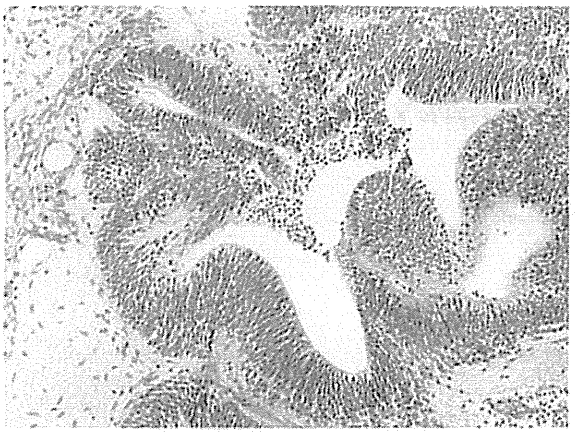
❹ その後HE（ヘマトキシリン・エオジン）染色などを行う

結果の判定

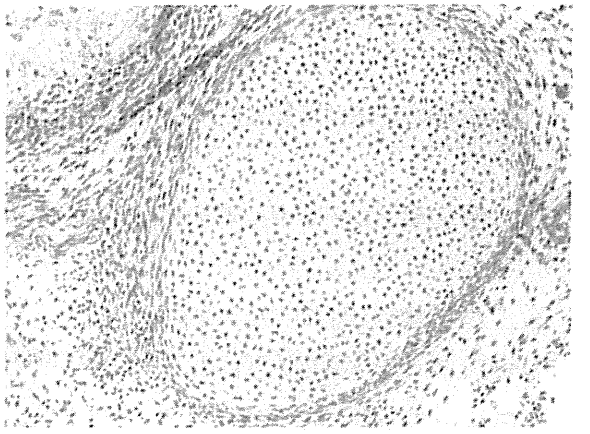
HE染色した標本を顕微鏡で，三胚葉（内胚葉，外胚葉，中胚葉）に分化していることを確認する．特徴的な組織部位を図４に示す．

テラトーマの組織学的解析を正確に行うためには相当程度の経験を要するため，専門家

神経上皮（外胚葉）



軟骨（中胚葉）



消化管上皮（内胚葉）

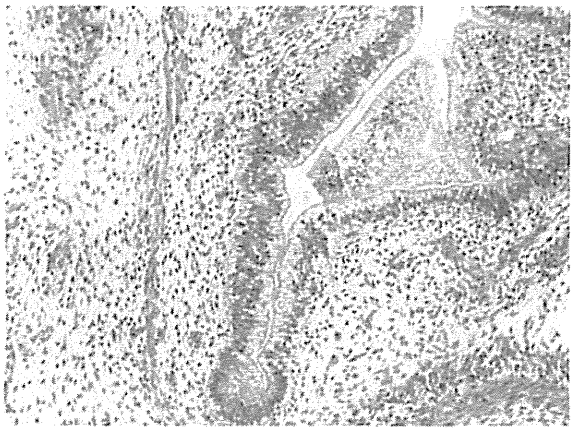


図4　テラトーマ組織のヘマトキシリン・エオジン染色

の助言を求めることが推奨されるが，比較的容易に判別できるものについて図に示した．外胚葉性の組織としては神経上皮が管状の組織をつくった神経管様の構造がしばしば観察される．中胚葉性組織では軟骨や筋組織が見つけやすいだろう．このほか消化管上皮の特徴的な構造は内胚葉性組織の代表とするのに適している．脂肪や，血管の形成が認められる場合も多いがこれらはマウス由来の組織である場合もあり注意が必要である．確定するにはヒト特異的なこれらの細胞に対する抗体での染色を併用するとよい．

A-7. 出荷基準

特性解析として上記のように多くの検査を行っているが，定量性がないものもあり結果の判断は特に難しい．未分化性，多分化能については複数の検査を行い結果を総合的に判断することが重要である．当研究室では表2のような出荷基準を作成している．

表2　出荷基準

試験項目	検査方法	判定基準
未分化性確認検査	アルカリホスファターゼ染色	染色後，5視野の画像をランダムに撮影し記録を残す．コロニーを100個カウントして90個以上のコロニーが陽性であること（90％以上）
	免疫染色	染色後，5視野の画像をランダムに撮影し記録を残す．コロニーを100個カウントして90個以上のコロニーが陽性であること（90％以上）
	フローサイトメトリー法	SSEA-1が陰性であること（陽性率10％以下），SSEA-3，SSEA-4，TRA-1-60，TRA-1-81，NANOG，OCT 3/4，Sox2は陽性であること（陽性率70％以上）
	PCR法	NANOG，OCT 3/4，DNMT 3B，TDGF，GABRB3，GDF3の発現が確認されること
多分化能確認検査	胚葉体形成確認	胚様体の形成を確認すること．培養後胚様体からRNAを抽出し分化・未分化マーカーの値をPCRで確認．培養前と比較して，未分化マーカーの低下および分化マーカーの上昇がみられること
	テラトーマアッセイ	SCIDマウスに細胞を接種しマウス内にテラトーマの形成を確認すること．テラトーマを摘出し，ヘマトキシリン・エオジン染色にて三胚葉に分化していることが確認できること
核型解析検査	ギムザ染色，G-バンディング法	30個の分裂中期でG-バンディング解析を行い，クローナルな核型異常が5％以下であること
マイコプラズマ検査	培養法，リアルタイムPCR法	＜培養＞菌の発育を認めないこと ＜PCR＞細胞及び上清についてPCRを行い陰性であること
無菌検査（薬局方準拠）	培養ボトル検査法	細胞培養上清を培養ボトルに播種し菌の発育を認めないこと
ウイルス検査	PCR法	HIV，HBV，HCV，HTLV-1，CMV，HP19，EB，HHV-6，-7，HSV，EBVについて陰性であること

B. 核型解析

染色体解析は臨床検査にて行われており，疾患を特定する有用な検査法の1つである．しかし培養細胞から得られる核型の異常は通常の臨床検体で起こりづらい現象もみられ，判定が難しいこともある．ヒト多能性幹細胞は長期培養すると染色体異常を起こすことがある．ここでは，細胞の品質管理という面から，スクリーニング的な核型解析検査について述べる．具体的にはギムザ染色とG-バンディング法を取り上げる．

準　備

細胞調製用試薬

□ 0.075M KCl（低張液）

1 M KClをMilliQで希釈する．

□ 固定液

メタノールと酢酸を3：1の割合で混ぜる．用事調製．

□ 50％エタノール

□ コルセミド（ライフテクノロジーズ社，#15212-012）

□ ヒーター

ホットプレート，パラフィン伸展器，ヒートブロックなど．

ギムザ染色用試薬

□ 100％ メタノール

□ 4％ ギムザ液

リン酸緩衝液（pH6.8）にギムザ原液（MERCK cat#HX263488）を加える．

G-バンディング用試薬

□ 100％ メタノール

□ 0.025％トリプシン液

2.5％トリプシンをMilliQで希釈する．

□ 5％ 血清加PBS 溶液，10％ 血清加PBS溶液

FBSなどの血清を5％，10％になるように加える．

□ 3％ ギムザ液

リン酸緩衝液（pH6.8）にギムザ原液を加える．2本作製し，A液，B液とする．

□ マウント液（Fisher Scientific社，#SP15-100）

プロトコール

細胞調製

❶ 60～70％程度コンフレントな状態の培養ES/iPS細胞にコルセミドを最終濃度0.1 μg/mLになるように添加し，37 ℃で2～3時間培養する

❷ コルセミドを添加した上清を除去し，PBSで洗浄する

❸ 0.25 ％トリプシン溶液やディスパーゼ，EDTAなどの解離液を用いて，細胞を培養プレートから解離する

❹ 1,000 rpm（190G），5分間遠心後，上清を捨てる

❺ 0.075 M KClを1 mLを加え1 mLピペットで塊がなくなるまでよく混ぜる

❻ さらに0.075 M KClを3 mLを加えピペッティングする

❼ 37 ℃で13～15分間低張処理する．固定液を1 mL加えピペットで混ぜる

❽ 1,000 rpm（190G），5 分間遠心し上清を捨てる

❾ 固定液を 4 mL加えピペットでまぜる

❿ ❽〜❾を３回繰り返す

⓫ 1,000 rpm，５分間遠心し上清を捨てる

⓬ 固定液を 4 mL以上加え−20 ℃で保存する*1

*1 固定液を 10 mL（4 mL以上）入れ半年ぐらい保存可能である．その際は１回置換してから続きを行う．

ホットプレートを用いた標本作製

❶ あらかじめ細胞量にあわせて適量の固定液を加えた細胞浮遊液を作製する

❷ ホットプレートを 37 ℃に温めておき，ヒーターと作業台に湿らしたキムワイプを敷く

❸ あらかじめスライドグラスを洗浄し 50 % エタノール中に保存しておく

❹ スライドグラスをキムワイプで軽くふく

❺ 作業台のキムワイプの上に置き，素早く細胞浮遊液を１滴，滴下する
エタノールが乾く前に素早く滴下すること．

❻ 滴下した浮遊液の広がりを確認してヒーターに乗せる

❼ 乾いたら取り出して顕微鏡で観察し，細胞量，展開具合を確認する

ギムザ染色

❶ 展開したスライドグラスが乾いてから作業を行う

❷ 100％メタノールに２〜３秒浸す

❸ 4％ギムザ液に10～15分間浸す

❹ 水道水でスライドの表面を洗浄する

❺ 水気を切って風乾させる

❻ マウント液で封入する

G-バンディング（トリプシン-ギムザ染色）

❶ 展開したスライドガラスをよく乾燥させる

❷ 0.025％ トリプシン溶液に約20秒浸す

❸ 10％ 血清加PBS溶液で洗浄する

❹ 5％ 血清加PBS溶液で洗浄する

❺ 3％ ギムザ液(A)内にスライドを浸し，数回スライドを上げ下げする

❻ 3％ ギムザ液(B)で5分染色する

❼ 2～3秒，水洗いし乾燥させる

❽ マウント液で封入する

! トラブルへの対応

■ギムザ染色がうまくいかない

G-バンディング時ギムザ染色のトリプシン液添加時間は，季節や室温によって調整することがすすめられる．染色体検査は季節や実験室の気温湿度の状態によっても標本の状態が左右されるので，何度か予備実験を行い適切な条件を見つける必要がある．コルセミドの添加時間もサンプルによって調整するなど，工夫が必要である．

表3　核型検査表

標準 G-バンディング法	最低8核体の分裂中期の染色体を解析．20個の分裂中期の染色体数を計数	
クローン性異常の確認手法	クローナルな染色体異常はその意義をさらに解釈するために，継代後の再検査で確認する必要がある．	
	単一細胞の異常（例えば染色体異数性，転移）は，場合によっては染色体のモザイクを排除するためにさらなる検査を必要とする．	
単一細胞で観察された異常の確認方法	染色体番号1，8，12，14，17，20およびXでの正倍数性（不均衡再配列を含む）	初期培養では最低30個の細胞についてG-バンディング計数を行う．培養後期には30個の細胞についてG-バンディング計数を行うと共に間期細胞100個についてFISH解析を行う．
	他の異数性と構造上再配列	初期培養から少なくとも30個の細胞についてG-band計数を行う．
最低品質スコア	ISCN400バンドレベルはG-band解析で最低限必要なレベルであり，ISCN500バンドレベル以上での細胞解析が望ましい．	
低水準分析	もしISCN400バンドレベルの解析ができない場合には，その方法を通常使用するとしても「低水準の分析」であり再検査が必要かもしれないと警告，明示すべきである．	
報告	報告に含むべき内容： ・核型の名称．使用可能な最新のISCN命名2009を用いる． ・分析法（例えば核型，FISH，CGH，特殊なバンド形成など） ・バンド形成レベルの平均値．単細胞での染色体番号1，8，12，14，17，20およびX（このリストは検討中）における異数性または構造異常．	
用語定義 文献3を元に作成	解析：分析中期の染色体を計数し，各染色体のバンドの相同性を比較するとともに男性核型のXおよびY染色体のバンド形成パターンを確認． 計数：分裂中期において明白な構造異常が検出された染色体の数を提示する．または，FISH解析における間期の核でのシグナル数を提示する． スコア：細胞または分裂中期における異常の有無について完全な解析なしに確認． クローン：単一細胞から得られた細胞集団． このような細胞は同一の染色体構成をもつ．もし3つの細胞が同じ染色体を失っているならば，あるいは2つの細胞が同じ過剰な染色体，あるいは構造的な組換え染色体が含まれていれば，クローンといえる．	

解析，結果判定

まず初めに，ギムザ染色した標本の各メタフェーズについて染色体数をカウントする．スライドの端から順を追ってメタフェーズを観察することによりバイアスのかからない視点で全体的な数の異常について確認できる．通常スライド1枚あたり30～300位のメタフェーズが確認できる．培養細胞の場合，染色体の数本がメタフェーズから飛んでしまい数が減っていることも多い．そのサンプルの全体像を知ることができ，後々の検査で有用なことがある．次にギムザ染色でG-バンディングを行う．当研究室ではIkaros画像解析システム（Zeiss社）を使用している．30個のメタフェーズについて染色体を注意深く解析し，欠失，転座，挿入，増加，消失などについて確認している（表3）．クローナルな異常が5％以上生じた場合はさらに観察数を増やして確認を行う．必要に応じて詳細検査（FISH，CGH，SNPs，SKYなど）も行う．

G-バンディングの解析判定には熟練した技術が必要である．専門家や外部委託業者に依頼することも考慮されたい．

C. 感染性因子の制御

ES・iPS細胞を臨床利用する際に制御の必要性がある感染性因子としては，細菌/真菌類，ウイルス，マイコプラズマが主なものとしてあげられる．これらが培養細胞に混入する経路としてはドナーに由来するものと培養工程に由来するものに分けられる．ウイルスについては感染症を含めた病歴や各種検査によりその適格性が判断され，さらにES・iPS細胞を供給するバンクにおいて充分な検査が行われるため，適切な機関から入手した細胞ではウイルスが混入している可能性は少ないものと考えてよい．

さらに培養工程では，細菌/真菌類およびマイコプラズマの混入の可能性が懸念される．臨床目的での培養では通常抗生物質を使用しないため多くの細菌類の混入は目視により確認できる．一方でマイコプラズマの混入は細胞に一見してわかる変化がみられないため注意が必要である．

現在細胞培養に用いられる培地などの資材については充分な品質管理が行われているため，これらを介しての混入の可能性はほぼなく，感染性因子の混入は実験者の操作，あるいは実験室の環境管理が不適切であることによると考えてよい．したがって，感染性因子の制御は「もち込まない，拡散させない」が重要な対策となる．

ここでは感染性因子のうち培養工程での混入の可能性が高いマイコプラズマの検査法について解説する．すでに述べたようにマイコプラズマの感染は実験者に由来して起こる．そのため，マイコプラズマ検査は工程管理の1つの方法であるともいえる．最終製品だけでなく，製造過程での検査も有効である．

1. 局方のマイコプラズマ検査法

現在，日本薬局方（局方）のマイコプラズマ否定試験には，培養法と指示細胞を用いたDNA染色法（以下染色法），PCR法の3法が記載されている．基本的には，培養法か染色法による検出法を行い，染色法で陽性だった場合に，PCR法での否定試験を行うことが可能である．

培養法は平板培地，液体培地を併用し14日間の培養を行い，マイコプラズマ特有の目玉焼き状のコロニーの発育を確認するものである．染色法は指示細胞を播種したプレートにサンプルを散布し3～6日間培養後，蛍光色素で染色し顕微鏡で観察するものである．細胞核を囲むように微小な核外蛍光斑点が0.5％以上あれば陽性となる．両方法とも，陽性対象として100CFU以下のマイコプラズマ生菌を用意する必要があり，研究室では生菌を常に保持することは難しく，さらに両検査とも検査に日数がかかり，即時判断には適さない．PCR法は感度と特異性を高めるため2段階PCR法（ネステッドPCR法）を用いることが推奨されているが，局方に記載されているプライマーでは検出が難しいとの報告がある．

2. 酵素法

研究室内でスクリーニング的に簡単に行える検査法としてマイコアラート（MycoAlert，ロンザ社）による検出法がある．マイコプラズマが有する酵素の生物化学反応を利用し培

養物中のマイコプラズマの汚染を検出する方法である．生きているマイコプラズマが存在する場合，マイコプラズマの膜を溶解し放出した酵素を基質と反応させる．酵素はADPのATP変換を促進させるため，基質を加える前後のATPの変化により，マイコプラズマ汚染の有無を確認する方法である．

3. リアルタイムPCRによる検出法

前述したように，局方によるマイコプラズマ否定試験は簡便に高感度な結果を得ることが難しい．近年リアルタイムPCRによるマイコプラズマ検出キットが販売されている〔MycoTOOL PCR Mycoplasma Detection Kit（ロシュ・ダイアグノスティクス社），MycoSEQ Mycoplasma Detection System（ライフテクノロジーズ社）〕．これらのキットは，欧州薬局方，米国薬局方などに準拠したマイコプラズマ亜属に対する否定試験を行うことができ，日本の局方にて否定すべきと記されているマイコプラズマ亜属はすべて網羅している．さらに高感度（検出感度：＜1～10 CFU/mL）なうえ，DNA抽出から結果判定まで5時間程度と迅速性も高い．使用する検体についてのプロトコールは，細胞のみ，細胞上清のみ，細胞と上清の混合と3種類のプロトコールがあり，検査をするタイミングや状況に応じて選択することができる．現在はまだキットが高額だという問題があるが，今後広く普及していく方法だと考えられる．

上に述べたようにいずれの方法も検出感度，検出可能な菌種，時間などの点で一長一短があり，それぞれの検出方法の特性をふまえたうえで目的に応じて複数の方法を組み合わせて利用することが望ましい．

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A synthetic nanofibrillar matrix promotes *in vitro* hepatic differentiation of embryonic stem cells and induced pluripotent stem cells

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Summary

Embryonic stem (ES) cells recapitulate normal developmental processes and serve as an attractive source for routine access to a large number of cells for research and therapies. We previously reported that ES cells cultured on M15 cells, or a synthesized basement membrane (sBM) substratum, efficiently differentiated into an endodermal fate and subsequently adopted fates of various digestive organs, such as the pancreas and liver. Here, we established a novel hepatic differentiation procedure using the synthetic nanofiber (sNF) as a cell culture scaffold. We first compared endoderm induction and hepatic differentiation between murine ES cells grown on sNF and several other substrata. The functional assays for hepatocytes reveal that the ES cells grown on sNF were directed into hepatic differentiation. To clarify the mechanisms for the promotion of ES cell differentiation in the sNF system, we focused on the function of Rac1, which is a Rho family member protein known to regulate the actin cytoskeleton. We observed the activation of Rac1 in undifferentiated and differentiated ES cells cultured on sNF plates, but not in those cultured on normal plastic plates. We also show that inhibition of Rac1 blocked the potentiating effects of sNF on endoderm and hepatic differentiation throughout the whole differentiation stages. Taken together, our results suggest that morphological changes result in cellular differentiation controlled by Rac1 activation, and that motility is not only the consequence, but is also able to trigger differentiation. In conclusion, we believe that sNF is a promising material that might contribute to tissue engineering and drug delivery.

Key words: Hepatic differentiation, *In vitro* differentiation, Embryonic stem cells, Induced pluripotent stem cells

Introduction

The liver is an important organ that performs many complex functions, including the metabolism of carbohydrates, proteins and lipids, as well as storage of essential nutrients and biotransformation of drugs. Drug biotransformation involves detoxification and bioactivation, where the metabolite becomes more toxic. Therefore, drug biotransformation plays an important role in the early stages of drug discovery processes. Primary hepatocyte cultures are often used for pharmacological assays, but they are short-lived and cannot be maintained in long-term culture. In addition, there are considerable donor-dependent variations. By contrast, embryonic stem (ES) cells or induced pluripotent stem (iPS) cells can proliferate infinitely and maintain their pluripotent ability to differentiate into various cell types. There is evidence that ES or iPS cells recapitulate normal developmental processes, and can serve as an alternative resource for hepatological researches, drug development and clinical uses. Through our present knowledge of developmental biology, efficient induction of hepatic lineage cells has been established. For example, based on the evidence that TGFβ–activin–Smad2 signaling is involved in definitive endoderm formation in the mouse (Tremblay et al., 2000), the activation of Activin–Nodal signaling was used for endoderm induction (D'Amour et al., 2005; Kubo et al., 2004). Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) were added for the specification of liver lineages (Jung, 1999; Mfopou et al., 2010; Shiraki et al., 2008a); this helped to mimic the mesodermal signals from the septum transversum mesenchyme in normal development (Katsumoto et al., 2010; Shin et al., 2007; Rossi et al., 2001). Because hepatocyte growth factors are known to be important effectors in the specification of cell fate and organogenesis of the liver (Schmidt et al., 1995; Sonnenberg et al., 1993), hepatocyte growth factor (HGF), dexamethasone and oncostatin M have been used for induction of hepatocyte maturation (Basma et al., 2009; Kamiya et al., 1999; Si-Tayeb et al., 2010). Compared with the factors described above that direct hepatic differentiation, the role of extracellular matrices (ECMs) and scaffolds remains unclear.

We have previously reported that culturing ES/iPS cells on the mesonephric M15 cell line, in the presence of specific growth factors, resulted in an efficient induction of endoderm-derived tissues, such as the liver or pancreas (Shiraki et al., 2008a; Shiraki et al., 2008b; Umeda et al., 2013). We further suggested that the basement membrane components, including *lama5*, play an important role in guiding the differentiation of ES cells into regional-specific lineages of the definitive endoderm (Higuchi et al., 2010). We also successfully established an alternative hepatic differentiation procedure without using feeder cells, but with a synthesized basement membrane (sBM) substratum (Higuchi et al., 2010; Shiraki et al., 2011). Together, these results revealed the importance of the ECM for differentiation of ES cells.

The basement membrane, a highly integrated three-dimensional structure composed of ECM molecules, is known to regulate various cellular processes. It is known that electrospun nanofibers provide not only three-dimensional microenvironments mimicking the ECM, but also appropriate guidance cues to modulate cell behavior. Here, we tested the effects of synthetic nanofiber (sNF) matrices on ES/iPS cell differentiation. We found that ES/iPS cells grown on the sNF were induced into endoderm and then hepatic fates. Overall, we conclude that the sNF is more potent in promoting hepatic differentiation, compared with the traditional two-dimensional culture surfaces, and is able to substitute for the sBM or M15 cells.

Results

Differentiation of murine and human ES cells into the hepatic lineages on the sNF matrix

We first tested the sNF matrix for its potency to mimic the basement membrane substratum of the cells. Murine SK7 ES cells (Shiraki et al., 2008a) were seeded onto the sNF matrix, and allowed to differentiate into the hepatic lineage by sequential changes of medium containing specific growth factors (Fig. 1A). We found that the expression of the pluripotent marker *Oct3/4* was downregulated, whereas the mesendoderm marker *Gsc* and definitive endoderm markers *Sox17* and *Foxa2* were expressed at day 4 (d4) of differentiation (Fig. 1B). Whereas *Gsc* was downregulated rapidly, *Sox17* and *Foxa2* showed peak expressions around d7 and were downregulated afterwards. Notably, the hepatic progenitor marker gene, alpha-fetoprotein (*Afp*), and the mature hepatocyte marker, albumin (*Alb1*) were detectable from d12 and d16, respectively, and their expression levels were increased with time. Although the *Afp* transcript level was decreased after d22, the *Alb1* expression continued increasing beyond d22. The immunocytochemical analysis further confirms that ALB and AFP were present in the cytoplasm of differentiated ES cells (Fig. 1C). In addition, periodic-acid–Schiff (PAS) staining and the Indocyanin Green (ICG) test were also conducted to investigate the hepatocyte functions of differentiated ES cells. The former reflects glycogen storage by showing positive populations as magenta in the cytoplasm, and the latter is used to examine cellular uptake activities, which are regarded as a hepatocyte detoxification function. As shown in Fig. 1D, glycogen storage was observed as the accumulation of magenta staining in the cytoplasm of the differentiated cells (top panel) and the ICG test also shows a similar result (bottom panel).

We next investigated whether human ES or iPS cells could differentiate in the sNF system. We used khES3 human ES cells

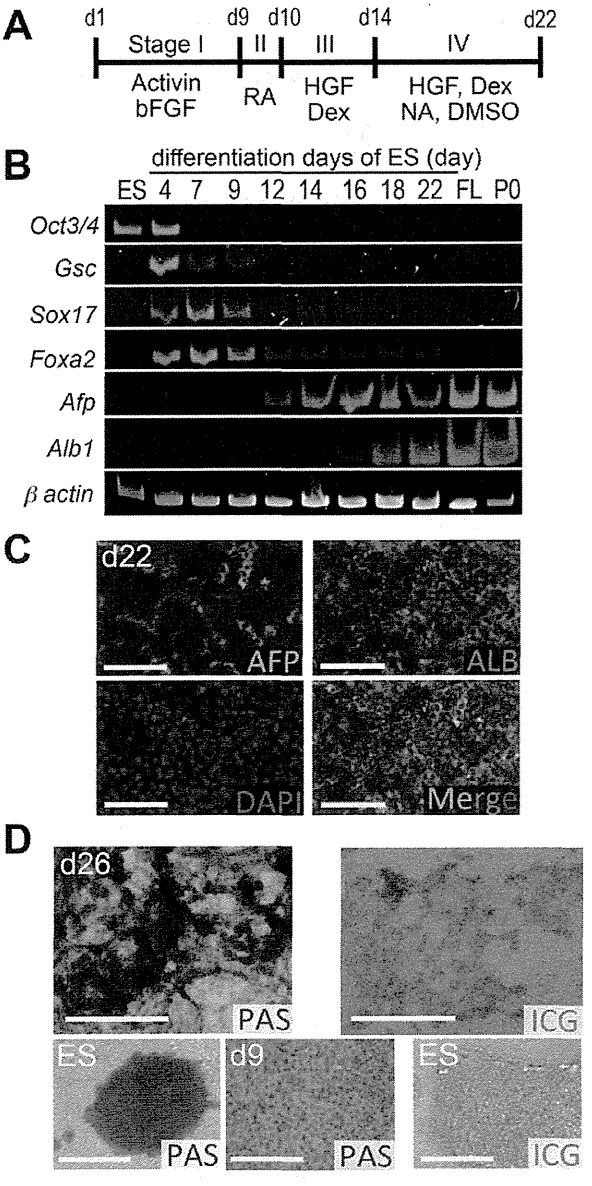

Fig. 1. Differentiation of murine ES cells into the hepatocyte lineage on nanofiber scaffolds. (A) Schematic diagram of the differentiation procedure for mouse ES cells. (B) Time-dependent expression levels of endoderm and hepatic marker genes. β-actin was used as a control. FL, fetal liver on embryonic day 12.5; P0, neonatal liver on postnatal day 0. (C) The immunochemical analysis of differentiated ES cells on day 22 (d22) for α-fetoprotein (AFP, green) and albumin (ALB, red) with nuclear counterstaining (DAPI). (D) Hepatocyte functional tests for PAS and ICG on d26 differentiated ES cells (top panels) and undifferentiated (bottom left and right panels) and d9 differentiated (bottom middle panel) ES cells as negative controls (bottom panels). Nuclei are counterstained with hematoxylin (blue). Scale bars: 250 μm.

(supplementary material Fig. S1A–D), as well as human iPS cell lines, such as Toe (supplementary material Fig. S1E,G) and 201B7 (supplementary material Fig. S1F), and found that these cells were able to differentiate into hepatocyte-like cells, thereby

producing ALB and taking up ICG (supplementary material Fig. S1D–G). Together, these results indicate that sNF is a suitable matrix for potentiating hepatic differentiation, not only in murine cells, but also in human ES cells and iPS cells.

sNF is more potent than normal plates precoated with other matrices

To compare the supportive effects of NFs and other substrata, we seeded murine ES cells onto either the sNF matrix or normal plates precoated with other stubstrata, including collagen I, Matrigel, gelatin and fibronectin and then performed the differentiation experiment as described in Fig. 1A. On differentiation day 22 (d22), quantitative PCR analyses were carried out to quantify the expression profiles of hepatic function marker genes in differentiated cells. Our results indicate that ES cells grown on sNF showed higher expression levels of proteins secreted by hepatocytes, such as serine peptidase inhibitor a1 (*Serpina1*), *Ttr* and *Alb1*, compared with those grown on other substrata (Fig. 2). Similar results were observed for the expression of several other genes, including glucose 6-phosphatase (*G6p*) and fatty-acid

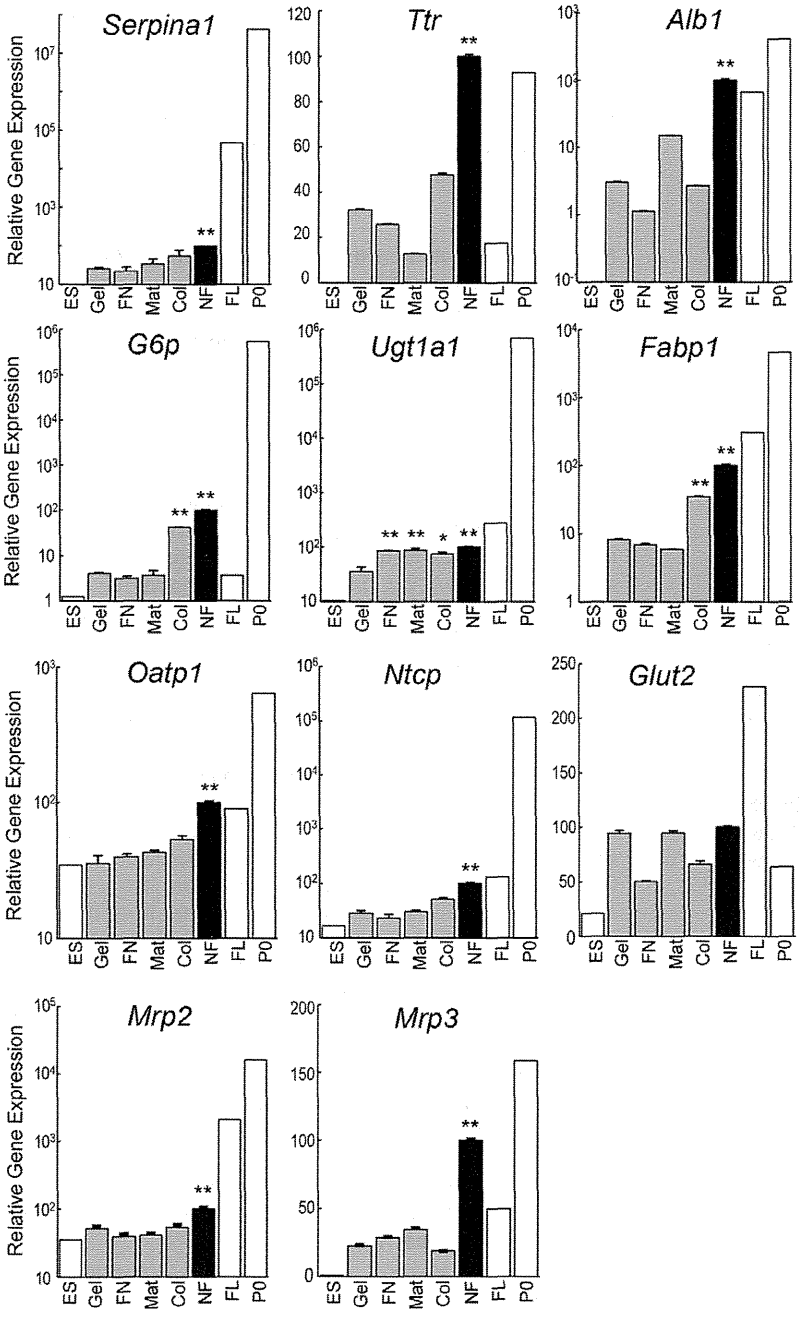

Fig. 2. Expression of hepatic markers in differentiated murine ES cells on sNF or other substrata. Expression levels of various gene transcripts quantified by real time PCR in d22 differentiated ES cells cultured on sNF, collagen I (Col), Matrigel (Mat), fibronectin (FN) or gelatin (Gel). ES, undifferentiated ES cells. FL (E12.5 fetal liver) and P0 (neonatal liver on postnatal day 0) are used as references. For differentiated ES cells, values represent mean ± s.e.m. (n=3). *P<0.05 and **P<0.01, by one-way ANOVA with the post-hoc Dunnett's test.

binding protein (*Fabp1*), or transporters, such as organic anion-transporting polypeptide 1 (*Oatp1*), Na^+-taurocholate cotransporting polypeptide (*Ntcp*) and UDP-glucuronosyltransferase (*Ugt1a1*), as well as multidrug resistance-associated protein family proteins 2 and 3 (*Mrp2* and *Mrp3*) (Fig. 2). By contrast, little change was found in the expression of glucose transporter 2 (*Glut2*).

To determine the hepatic functions of differentiated ES cells grown on Matrigel, collagen I or sNF, we next measured their ALB secretions, ICG uptake and cytochrome p450 (CYP) activities. Our results show that ES cells grown on sNF secreted ALB at a higher level compared with those on Matrigel or collagen I (Fig. 3A). By day 26, the percentage of ICG-positive ES cells on sNF was also higher than in ES cells grown on the other two substrates (Fig. 3B,C).

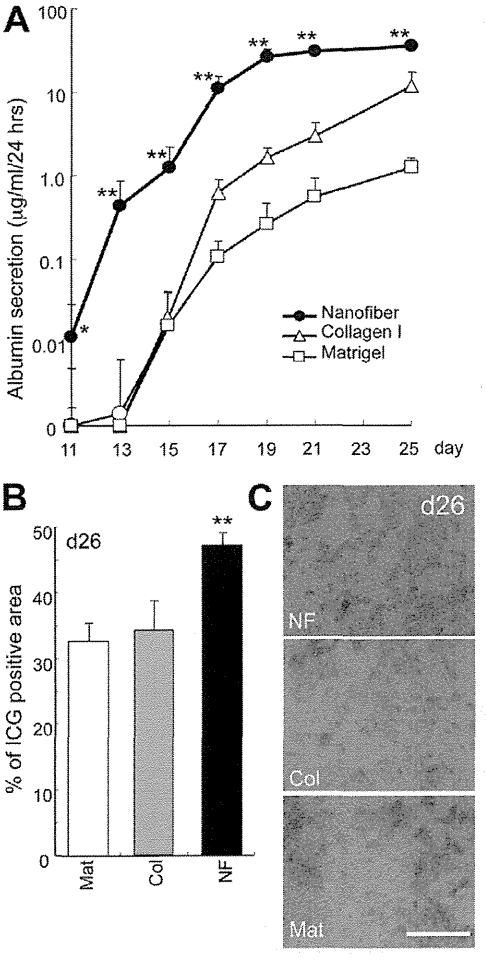

Fig. 3. Liver functional assays of differentiated murine ES cells grown on nanofiber scaffolds versus other substrata. (A) ELISA analysis of time-dependent albumin secretion for 24 hours by ES cells grown on Matrigel (Mat), collagen I (Col) or NF. (B,C) ICG tests performed on d26 differentiatied ES cells. The percentage of cells taking up ICG in culture was calculated (B) and representative images are shown (C). Values represent means ± s.e.m. (n=6). *P<0.05, **P<0.01, by two-tailed Student's t-test. Scale bar: 250 μm.

To measure CYP activities, the differentiated ES cells were treated with a CYP1A inducer, 3-methylcholantrene (3MC), for 48 hours during d22–d24 or d64–d66, as shown in supplementary material Fig. S2A. We found that the differentiated cells cultured on sNF had higher CYP1A1 activities and responses to the inducer than those cultured on fibronectin, Matrigel or collagen I (supplementary material Fig. S2B). We also assayed the effects of sNF on the maintenance of the mature hepatic cells. ES cells cultured on sNF were able to maintain their CYP1A1 activities and responses to 3MC even on d66, whereas those cultured on other matrices did not survive in long-term cultures (supplementary material Fig. S2C). It is also worth noting that the differentiated cells on sNF could be maintained in culture for more than 100 days. Specifically, we show that the ES cells cultured on sNF for 129 days were able to uptake and secrete ICG (supplementary material Fig. S2D). Based on these findings, we conclude that sNF is an excellent matrix, not only for the differentiation of ES cells into the hepatic lineage but also for maintaining the mature state of ES-cell-derived hepatocytes.

High Rac1 activities in undifferentiated and differentiated ES cells grown on sNF

Next, we investigated the effects of sNF on hepatic differentiation of ES cells. It was previously reported that the undifferentiated murine ES cells cultured on sNF exhibited spheroid morphologies and formed dome-like structures, and proliferated well (Nur-E-Kamal et al., 2006). Therefore, we checked the morphological changes between the undifferentiated and differentiated states of the murine ES cells. To exclude the effect of the extracellular matrix, we compared gelatin-coated normal two-dimensional (2D) plates with either gelatin-coated or uncoated sNF. We found that the undifferentiated ES cells grown on gelatin-coated 2D plates, with cytoplasmic spreading morphologies and attached to the plate surface in large areas (Fig. 4A, left). By contrast, the ES cells grown on sNF showed aggregated morphologies (Fig. 4A, middle and right).

In the differentiated state, the ES cells were found to form a monolayer on sNF. However, the morphological differences were still observed between ES cells cultured on sNF or normal 2D plates (Fig. 4B). Because these morphological changes are known to be regulated by cytoskeletal molecules, such as small Rho GTPase family member proteins, we next examined Rac1 activities in undifferentiated and differentiated ES cells. Our western blot analyses demonstrate that the GTP-bound active form of Rac1 was expressed at a higher level in ES cells cultured on sNF than those cultured on normal 2D plates, even though total Rac1 expression levels were similar (Fig. 4C,D). Interestingly, both the undifferentiated (Fig. 4C) and the differentiated ES cells on d8 (Fig. 4D) showed higher Rac-GTP activities. These results not only agree with the morphological differences of the ES cells, but also suggest that activated Rac1 plays a crucial role in potentiating the differentiation activity of ES cells cultured on sNF into hepatic lineages.

A crucial role of Rac activation in potentiating the differentiation of ES cells into the definitive endoderm and hepatocyte lineages

NSC23766 is a selective inhibitor of Rac1 activation that is mediated by the Rac-specific guanine nucleotide exchange factors (GEFs) TrioN and Tiam1, without affecting other Rho

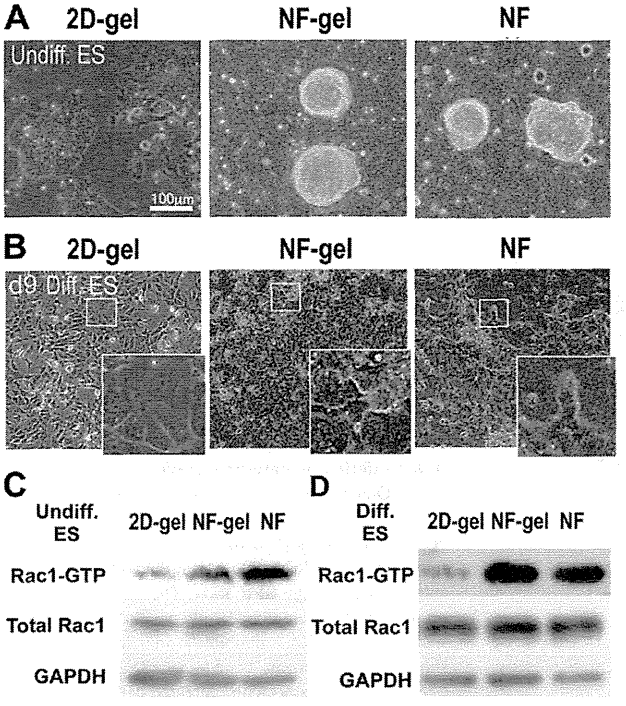

Fig. 4. NF induces Rac1 hydroxylation in both undifferentiated and day 9 differentiated murine ES cells. (A,B) Representative images of undifferentiated (A) and day 9 (d9) differentiated (B) ES cells grown on gelatin-precoated normal plates (2D-gel), gelatin-coated NF plates (NF-gel) and uncoated NF plates (NF). Insets are higher magnifications of the boxed regions. (C,D) Western blot analysis of GTP-bound active Rac1, total Rac1 and GAPDH expression in undifferentiated (C) or differentiated (D) ES cells described in A,B.

family members, such as RhoA or Cdc42 (Gao et al., 2004). We confirmed that 100 μM NSC23766 inhibited Rac1 hydroxylation (supplementary material Fig. 3A). To test whether sNF potentiates the differentiation of ES cells into the hepatic lineages through Rac1 activation, we treated murine ES cells with 100 μM NSC23766 at various stages and then determined the expression of stage-specific markers (Fig. 5A–C). We first added NSC23766 for 4 days at stage I to examine the effect of Rac1 activation on endoderm induction (Fig. 5A). We found that *Foxa2* expression was downregulated by the Rac1 inhibitor in ES cells cultured on sNF (Fig. 5A). We next examined the effects of Rac1 inhibition on hepatic differentiation. Our results show that the Rac1 inhibitor added at stage II (Fig. 5B) or stage III (Fig. 5C) downregulated the expression of hepatic markers, *Afp* or *Alb1*, on d10 or d14, respectively.

These results suggested that Rac1 activation is crucial for endoderm and hepatic differentiation. We subsequently examined the stage dependency of hepatic differentiation on Rac1 activities. The Rac1 inhibitor was added at different stages (I, II, III or IV) and *Alb1* expression was assayed on day 18 (Fig. 5D). We found that Rac1 inhibition at all four stages blocked the potentiating effects of sNF, and resulted in decreases in *Alb1* expression. These results further confirm the important role of Rac1 and demonstrate that continuous activation of Rac1 is crucial for the potentiation of hepatic differentiation.

Then we confirmed whether NSC23766 had any effect on the proliferation of ES cells. NSC23766 decreased the proportion of EdU-positive cells in stages I, II and III, particularly in stage I, without apparent toxicity (supplementary material Fig. S3C). Interestingly, the total numbers of cells in the NSC23766-treated groups was smaller in stages I and II, which became greater than that of control groups used in stages III and IV (supplementary material Fig. S3B). Taken together, these findings suggest that Rac1 differentially contributes to proliferation in the early differentiation stages and promotes differentiation in the late stage.

Discussion

Our previous study suggested that although addition of soluble growth factors is sufficient to promote the differentiation of ES cells into the definitive endoderm, further differentiation from the definitive endoderm into hepatic and pancreatic fates appears to require a direct contact with M15 cells (Shiraki et al., 2008a). We previously showed the importance of basement membrane substratum by culturing ES cells on sBM (Higuchi et al., 2010; Shiraki et al., 2011). Specifically, ES cells grown on sBM were able to differentiate into hepatic and pancreatic lineages. These results imply that the basement membrane structure plays a major role in the differentiation of ES cells. Although the sBM used previously was constructed by overexpressing recombinant laminin-511 (laminin α5, laminin β1 and laminin γ1) in H293 cells (Doi et al., 2002), ES cells or iPS cells could be induced into the hepatic and pancreatic lineages. The efficacy of such an sBM for differentiation was high, and the differentiated cells could perform liver-specific functions, such as protein secretion, detoxification and glycogen storage (Higuchi et al., 2010; Shiraki et al., 2011).

The nanofiber produced by the electrospinning technique is a chemically and physically stable synthetic three-dimensional surface that mimics the structural geometry and porosity of the basement membrane or ECM (Schindler et al., 2005; Schindler et al., 2006). NF scaffolds have been shown to recapitulate the structural features of stem cell niche (Lim and Mao, 2009) and have been used for the *ex vivo* expansion of various types of stem cells such as murine ES cells (Hashemi et al., 2011; Nur-E-Kamal et al., 2006) and human tissue stem cells. In addition, the ECM was found to deposit as an extensive scaffold on the basal surface of the cells attached to NFs (Shih et al., 2006; Chua et al., 2007; Ma et al., 2008). Importantly, the sNF system has been reported to enhance not only the differentiation of murine ES cells into neural lineages (Lim et al., 2010; Purcell et al., 2012; Xie et al., 2009), but also differentiation from human MSCs into hepatoblasts (Ghaedi et al., 2012; Kazemnejad et al., 2009).

Taken together, these previous observations revealed that the sNF matrix is useful as a substratum to replace feeder cells and that it has the ability to potentiate hepatic differentiation. In this study, we show that both murine and human ES cells, as well as human iPS cells, could differentiate on sNF and exhibit liver-specific functions. Furthermore, we demonstrate that Rac1 activation was involved in hepatic differentiation. Rac1, a member of the Rho family GTP-binding proteins, including Rho and Cdc42 (Heasman and Ridley, 2008), functions by activating actin-rich lamellipodial protrusion and membrane ruffling, which are thought to be a major part of the driving force for cell movement (Nobes and Hall, 1995; Ridley et al., 1992). Although Rho family proteins were reported to be

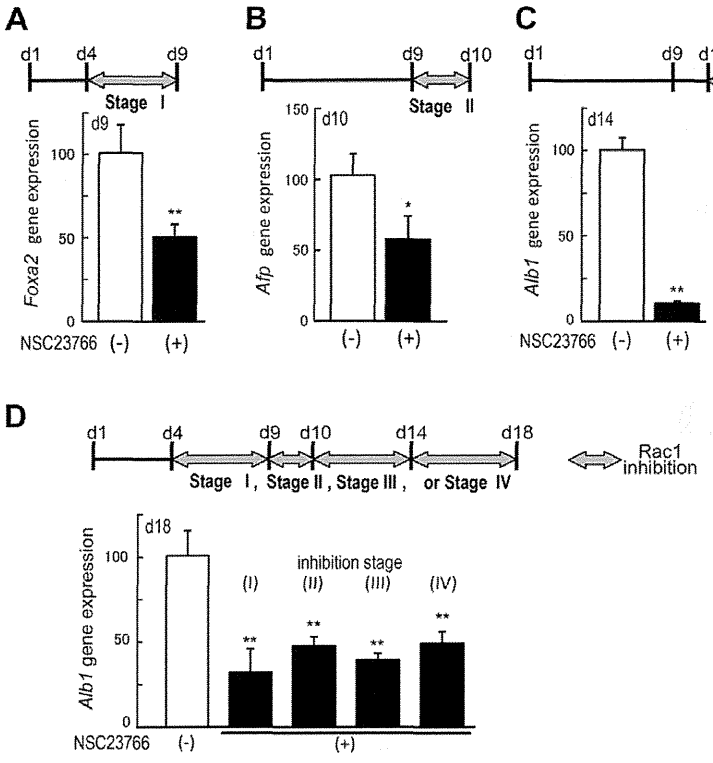

Fig. 5. Inhibition of the Rac1 pathway blocks the differentiation-potentiating activity of NF. (A–C) Quantitative PCR analysis of the gene expression of *Foxa2* (A), *Afp* (B) and *Alb1* (C), in differentiated cells cultured on sNF with (+) or without (−) the Rac1 inhibitor NSC23766 (100 μM), at the end of stage I (A), stage II (B) or stage III (C). (D) The expression of *Alb1* on day 18 in differentiated cells, treated with (+) or without (−) the Rac1 inhibitor at indicated stages. Data shown represent mean ± s.e.m. (n=3); *P<0.05 and **P<0.01, compared with untreated cells on sNF with the Rac1 inhibitor by two-tailed Student's t-test or one-way ANOVA with the post-hoc Dunnett's test.

expressed by ES cells cultured on sNF (Nur-E-Kamal et al., 2005; Nur-E-Kamal et al., 2006; Schindler et al., 2006), their roles have never been investigated.

In vivo developmental processes occurring in the endoderm and its derivatives cause dynamic migration during gastrulation and later stages of organogenesis (Woo et al., 2012), suggesting that motility and differentiation are closely inter-related. In this study, we observed that ES cells cultured on sNF showed greater Rac1 activation than did cells cultured on the normal 2D surface. Indeed, Rac1 is known to be involved in not only endoderm induction but also hepatic specification and maturation. In particular, *Rac1* mutant mice died by mouse embryonic day 9.5 (E9.5) because of severe developmental abnormalities, and *Rac1*-deficient embryos showed numerous cell deaths in the space between the ectoderm and endoderm at the primitive streak stage (Sugihara et al., 1998). Rac1 is also important for cellular differentiation, for example, epithelial differentiation in the small intestine (Stappenbeck and Gordon, 2000), pancreatic islet morphogenesis (Greiner et al., 2009), myogenic differentiation (Heller et al., 2001), maintenance of the thymic epithelial cells (Hunziker et al., 2011), formation of the lens (Maddala et al., 2011) and neuronal development (Corbetta et al., 2009; Leone et al., 2010). In addition, Rac1 has been shown to crosstalk with many downstream signaling pathways such as Wnt (Clarke, 2006; Malliri et al., 2006), TGF-β1 (Varon et al., 2008), Nodal (Woo et al., 2012), retinoic acid (Lee et al., 2008) and Myc (Hunziker et al., 2011; Nikolova et al., 2008). Interestingly, Rac1 is also known to mediate stem cell-shape-dependent regulation of differentiation to a chondrogenic versus myogenic fate (Gao et al., 2010). On the basis of these studies, we postulate that the sNF system might potentiate ES cells to differentiate into hepatic lineages by interacting downstream of certain growth factors during differentiation processes.

In conclusion, we show that Rac1 was activated in both undifferentiated and differentiated ES cells cultured on sNF plates and that Rac1 inhibition blocked the potentiating effects of sNF on endoderm and hepatic differentiation. These results suggest that continuous activation of Rac1 throughout the differentiation stage is crucial for potentiating differentiation. Our results also highlight the morphological changes during differentiation along the Rac1 pathway, which controls cellular morphology, motility and differentiation into the hepatic lineage. Here, we established a completely chemically defined method that requires no serum or no xenogenic substrata, thereby eliminating the risk of contamination with unknown factors. We believe that this novel method could be an attractive culture model for pharmacological research and research on stem cell biology and therapeutic strategies.

Materials and Methods

ES and iPS cell lines

The murine ES cell line, SK7 (Shiraki et al., 2008a) was maintained on mouse embryonic fibroblast (MEF) feeders in Glasgow minimum essential medium (Invitrogen, Glasgow, UK) supplemented with 1000 units/ml leukemia inhibitory factor (LIF; Chemicon, Temecula, CA), 15% knocked-out serum replacement (KSR; Invitrogen), 1% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 μM nonessential amino acids (NEAA; Invitrogen), 2 mM L-glutamine (L-Gln; Invitrogen), 1 mM sodium pyruvate (Invitrogen), 50 units/ml penicillin and 50 μg/ml streptomycin (PS; Invitrogen) and 100 μM β-mercaptoethanol (β-ME; Sigma-Aldrich, St Louis, MO).

Human ES cells (KhES-3) (Suemori et al., 2006) were from Dr Norio Nakatsuji and Dr Hirofumi Suemori (Kyoto University, Kyoto, Japan). They were used in accordance with the human ES cell guidelines of the Japanese government. This human ES work was approved by Kumamoto University institutional review board. Human iPS 201B7 cells were a gift from Dr Yamanaka (Kyoto University, Kyoto, Japan). The human iPS Toe cell line was established by M. Toyoda and

colleagues (National Institute for Child Health and Development, Tokyo, Japan). Undifferentiated human ES and iPS cells were maintained as described previously (Shiraki et al., 2008b).

Culture plates

Synthetic nanofiber (sNF) matrices were purchased from Corning Coster (Ultra-Web Synthetic Polyamine Surface #3873XX1; Cambridge, MA). Plate surfaces were coated with electrospun polyamide nanofibers. sNF matrices consisted of two kinds of polyamide polymers, A $(C_{28}O_4N_4H_{47})_n$ and B $(C_{28}O_{4.4}N_4H_{47})_n$, which were crosslinked in the presence of an acid catalyst, and were 200–400 nm in diameter (average 280 nm). Pore sizes, similar to those of the cell basement membrane, were ~700 nm. For comparison, Corning 96-well plates were pretreated for 3 hours at 37℃ with 0.1% gelatin (Sigma-Aldrich), Matrigel (BD, Franklin Lakes, NJ) or CellStart (Invitrogen). Collagen I (Nitta Gelatin, Japan) was diluted with Dulbecco's modified Eagle's medium (DMEM; Invitrogen) at a concentration of 1 mg/ml and plate surfaces were treated for 15 minutes, then dried until use.

Differentiation of murine ES cells into hepatic lineages on sNF

Murine ES cells plated at a density of 1.5×10^4 cells/cm^2 in culture plates described above were grown for 8 days in DMEM containing 4,500 mg/l glucose, s NEAA, L-Gln, PS, β-ME, 10 mg/ml insulin, 5.5 mg/ml transferrin, 6.7 pg/ml selenium (Insulin-Transferrin-Selenium-G Supplement; ITS, Invitrogen), 0.25% AlbuMAX II (Invitrogen), 10 ng/ml recombinant human activin-A (R&D Systems, Minneapolis, MN), 5 ng/ml; recombinant human bFGF, and cultured for 8 days. On day 9 (d9), the medium was changed to RPMI-1640 (Invitrogen) containing 10^{-6} M retinoic acid (RA; Stemolecule all-trans retinoic acid; Stemgent, Cambridge, MA) and B27 supplement (Invitrogen). On d10, medium was switched to 2000 mg/l glucose DMEM (Invitrogen), 10% KSR, 10 ng/ml recombinant human hepatocyte growth factor (Peprotech, Rocky Hill, NJ) and 10 μM dexamethasone (Sigma-Aldrich), and cultured until d14. Next, 1 mM nicotinamide (NA; Sigma-Aldrich) and 1% dimethylsufoxide (DMSO; Sigma-Aldrich), were added to medium and KSR was removed. Medium was replaced every 2 days with fresh medium and growth factors.

Human ES/iPS cells were pretreated with the ROCK inhibitor Y27632 (Wako, Japan) 1 day before trypsinization. Cells were plated at a density of 3×10^5 cells/cm^2 on Matrigel-coated sNF matrices with Y27632. The following two procedures were subsequently used to induce hepatic differentiation of various human ES/iPS cells; simplified (two-step) protocol, KhES3 and 201B7 cells; or conventional (three-step) protocol, Toe cells. In the simplified protocol, medium used at first contained B27 and 100 ng/ml activin-A in RPMI-1640, which was then, switched to 10 ng/ml HGF, 10 μM dexamethasone, 0.5% DMSO, 0.5 mM NA. In the conventional protocol, medium used first was the same as that in the simplified protocol, followed by 1% DMSO and 20% KSR in knockout DMEM/F12 (Invitrogen) for 6 days and, then DMEM containing HGF, dexamethasone and 10% KSR. Finally, the above medium was added with 0.5 mM NA. Medium was replaced every 2 days with fresh medium and growth factors. KhES3 and 201B7 cells were induced hepatic differentiation with simplified protocol, and Toe cells were treated with conventional protocol.

Periodic-acid–Schiff's staining

For detection of glycogen storage in the differentiated cells, periodic-acid–Schiff's (PAS) staining kit (Muto Pure Chemicals, Tokyo, Japan) was used. Cells cultured for 9 and 26 days, and undifferentiated ES cells were fixed in 3.3% formalin for 10 minutes, and stained following the manufacturer's instructions, then nuclear counterstaining with hematoxylin (blue) was performed.

Albumin secretion assay

The culture medium was replaced with fresh medium every 2 days, and supernatants were collected 24 hours after replacing the medium. The mouse (human) albumin secreted in the supernatant was determined using a mouse (human) ELISA quantification kit (Bethyl, Montgomery, TX).

Indocyanine Green (ICG) test

Indocyanine Green (Daiichi-Sankyo Pharm., Japan) was diluted with the above culture medium to a final concentration of 1 mg/dl. The ICG test solution was added to the differentiated ES cells after the appropriate culture periods and undifferentiated ES cells were used as controls, and incubated at 37℃ for 30 minutes. Then, after three washes with phosphate-buffered saline (PBS), the cellular uptake of ICG was examined by microscopy. The percentage ICG-positive areas represent the proportion of ICG-positive area versus total cell area, which were determined using ImageJ software (US National Institutes of Health, Bethesda, MD).

CYP inductions

To check the inducibilities of cytochrome P450 activities in response to inducers, we used the P450-Glo CYP Assay Kit (Promega, Madison, WI). The differentiated ES cells were treated with 5 μM 3-methylcholantrene as inducers of CYP1A. The medium containing the inducers was changed every 24 hours. 48 hours after treatment, we changed the medium and used the appropriate luminogenic CYP substrates (Luciferine-CEE for CYP1A). The cells were incubated at 37℃ for 3 hours, and then the supernatants were mixed with equal amount of detection reagent, according to the manufacturer's instructions. The luminescence was measured using a GloMax 96 microplate luminometer (Promega), and luminometer settings were as in the manufacturer's instructions. Cell numbers were calculated using CellTiter-Glo luminescent cell viability assays (Promega) to normalize P450-Glo assay values to cell number.

Immunocytochemistry

After culture for the appropriate times, cells were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. After removal of paraformaldehyde solution, the fixed cells were permeabilized with 0.1% Triton X-100 for 10 minutes. The permeabilized cells were rinsed several times with PBS and were then incubated with 20% Blocking One (Nacalai Tesque, Japan) in PBST (0.1% Tween-20 in PBS) for blocking. After blocking, the cells were incubated with the diluted antibody in 20% Blocking One in PBST (0.1% Tween-20 in PBS) in a humidified chamber overnight at 4℃. After washing the cells in PBST, cells were incubated with the secondary antibody in 20% Blocking One for 2 hours at room temperature in the dark. After washing off the secondary antibody in PBST, cells were counterstained with 6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics, Switzerland). The following antibodies were used as primary antibodies: rabbit anti-alphafeto protein (Dako, Denmark), goat anti-albumin (Sigma-Aldrich), goat anti-Sox17, mouse anti-FoxA2 (R&D systems); secondary antibodies used were conjugated to Alexa Fluor 568, Alexa Fluor 488 and Alexa Fluor 633 (Invitrogen). For human ES cell cultures, goat antibodies against human albumin (Bethyl) were used as primary antibodies.

Cell proliferation assay

Cell proliferation was evaluated using Click-iT EdU assay kit (invitrogen). The cells cultured with or without NSC23766 were exposed to 10 μM of 5-ethnyl-2'-deoxyuridine (EdU) for 1 hour at 37℃ before fixation. The fixed cells were processed for immunocytochemistry as described above, with an additional step for EdU detection. Before incubation with secondary antibodies, the cells were incubated with EdU in the Click-iT reaction cocktail and Alexa Fluor 488 for 30 minutes at room temperature, following the manufacturer's instructions. Images were collected using ImageXpress Micro (Molecular Devices) and EdU-positive nuclei per total number of nuclei were counted.

RT-PCR analysis

RNA was extracted from ES cells or mouse liver using an RNeasy mini-kit (Qiagen, Germany) and then treated with DNase (Qiagen). For reverse transcription reactions, 3 μg RNA was reverse-transcribed using ReverTra Ace (Toyobo, Japan) and oligo dT primers (Toyobo). One μl of fivefold-diluted cDNA (1% of the RT product) was used for PCR analyses. The primer sequences for each primer set are shown in supplementary material Table S1. For real-time PCR analysis, mRNA expression was quantified with SyberGreen on an ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: denaturation at 95℃ for 15 seconds, annealing and extention at 60℃ for 60 seconds, for up to 40 cycles. Each measurement was normalized to β-actin (mouse) and *GAPDH* (human) for each sample by subtracting the average β-actin (mouse) and *GAPDH* (human) C_t values (Threshold Cycle) from the average C_t for each gene. Target mRNA levels, expressed as arbitrary units, were determined using a standard curve method.

Rac pull-down assay

Murine ES cells were trypsinized and suspended at a density of 5×10^4 cells/ml. Cells were then plated onto sNF either with or without 0.1% gelatin pretreatment; control plates were pretreated with 0.1% gelatin. Undifferentiated cells were harvested 48 hours after incubation under ES cell maintenance culture conditions at 37℃, whereas differentiated cells were harvested 9 days after hepatic differentiation started. The activation of Rac was determined using a Rac1 Activation Assay Kit purchased from Millipore. Briefly, cells were washed with PBS and suspended in lysis buffer provided by the supplier. Aliquots were taken from each cell lysate, and the amount of GAPDH proteins present in the lysates was determined and used for normalization. GTP-bound forms of Rac were then pulled down from lysates using reagents provided by the supplier, following the recommended instructions. Proteins present in total cell lysates or Rac pull-down samples were separated by SDS-PAGE (12%) and transferred onto a nylon membrane. Western blotting was performed using antibodies against Rac1, according to the ECL protocol provided by the suppliers. Luminescence of Rac1 bands was quantified using the GE ImageQuant LAS 4000 (GE Healthcare Life Science, Sweden).

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Author contributions

T.Y. performed cellular and biochemical analyses; T.Y. and N.S. established the ES cell differentiation system; M.T., N.K., H.O., Y.M., H.A. and A.U. established human iPS Toe cell line; Y.S., K.K. and S.K. provided technical advice, designed the experiments and wrote the paper. All authors discussed the results and commented on the manuscript.

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