d. ヒトにおける評価

a.～c.を経てようやくヒトへの応用が可能となると考えられる．実験的医療としての患者への細胞移植例や有効例などは比較的早く数年単位で報告されるかも知れない．しかし，1つの細胞治療法が安全性と有効性の確認を経て一般的に使用される治療法として確立されるまでには通常の薬剤と同等以上の多大な労力と時間を要する可能性がある．

2. 患者特異的モデル細胞

患者自身から細胞を採取し患者特異的なiPS細胞を樹立できるというiPS細胞にしかない特性は，移植免疫を回避した細胞治療ということだけでなく，全く新しい形で病態の解明や創薬への応用が可能である[15,16]．

a. 病態解明

心筋症，QT延長症候群，洞不全症候群等心臓を構成する細胞そのものに起因すると考えられる疾患が中心となると思われるが，患者自身の細胞からiPS細胞を樹立し，そこから該当すると考えられる細胞を分化誘導し種々のモデル細胞を構築できることは，病態解明に全く新しい手段を提供する．すなわち，これまではごく少量の生検サンプルの解析に限局されていたものが，個々の症例から生きた細胞を潤沢に得られることにより，標的細胞の遺伝子解析，機能解析や薬剤の効果判定などを実際の症例に関して繰り返し行うことが可能となる．原因遺伝子不明の症例においてもモデル細胞が構築できるので，モデル細胞を用いた原因遺伝子探索も可能となる．このように病態解明に向けたアプローチの方法は飛躍的に増大すると考えられる．

b. 創薬応用

iPS細胞の創薬応用には大きく，新規薬剤の探索と薬剤安全性試験への応用の2つが考えられる．疾患モデル細胞を用いて，同細胞の異常を改善する新規薬剤や疾患特異的に作用する薬剤などの探索が可能となる．また培養下における分化モデルを用いることにより，心筋分化促進物質などの新たな生理活性物質の探索も可能となる．筆者らは，マウスiPS細胞を用いて3次元培養下における血管構造形成モデルを構築し，新規海洋生物由来HDAC（ヒストン脱アセチル化酵素）阻害物質azumamideの血管形成抑制作用を示すことに成功した[17]．同モデルを用いた血管形成抑制または促進物質の探索が可能と考えられる．さらにこうしたシステムを患者特異的iPS細胞を用いて構築することにより，疾患特異的作用物質の探索などにも展開可能と考えられる．

受精卵を用意することが必要であるヒトES細胞と比べて，iPS細胞は数多くの細胞株を樹立しやすくiPS細胞バンクが構築しやすい．そこから細胞を誘導して並べることにより，種々のヒトモデル細胞パネルのようなものを構築することができる．こうしたヒトモデル細胞パネルは，薬剤の安全性試験に応用可能と考えられる．たとえば，千人分や1万人分などの心筋細胞や肝細胞を並べたパネルを用いて薬剤の細胞毒性をスクリーニングすることにより，まれに発生する心毒性や肝障害などを事前に検出できるかも知れない．さらには障害を起こす細胞を解析し原因を明らかにすることにより，副作用を起こすヒトを事前に特定し投薬を避ける「テーラーメード医療」に貢献しうる可能性もある．

3. その他動物モデルへの応用

循環器病関係のモデル動物には，マウスモデルばかりでなくマウス以外の動物種のものが数多くある（高血圧自然発症ラット，糖尿病モデルラット，心筋症ハムスター等）．これらモデル動物からのiPS細胞の樹立が可能となれば，モデル動物と同動物由来細胞を用いたin vitro実験を相互対応させながら新しい病態の解析を行うことなどが

可能となると考えられる．

D．iPS細胞研究の今後の展望

iPS細胞に関する研究は今後，iPS化（初期化）機構そのものに関するもの，iPS細胞を利用したもの，iPS化という現象を利用したものなど多岐にわたって進められると思われる．

1．iPS細胞の改良

現在，3～4個の遺伝子をレトロウイルスを用いて導入することによりiPS細胞は誘導されているが，細胞治療に用いることができるレベルのiPS細胞を樹立するためには，誘導法，誘導効率などの改良が必要である．

a．c-mycトランスジーンなしiPS細胞

iPS細胞誘導に用いた4因子の一つであるc-mycはがん遺伝子の一つであり，実際mycありiPS細胞由来のマウス個体では高率にがんが発生した．その後mycを除いた3因子でもiPS細胞誘導が可能となったが，mycなしiPS細胞由来マウス個体ではがんの発生がほとんど認められなくなった[18]．

b．レトロウイルスなしiPS細胞

現在iPS細胞誘導にはレトロウイルスによる遺伝子導入が行われている．レトロウイルスにより導入された遺伝子はゲノム上のどこかに組み込まれることになるので，導入遺伝子が組み込まれた場所によっては，がん化を含む種々の細胞の変異をもたらす可能性がある．レトロウイルスを用いずゲノムをintactに保ったままの遺伝子導入法の方がより安全なiPS細胞を樹立できると考えられる．

c．トランスジーンなしiPS細胞

レトロウイルスを用いない場合でも，遺伝子操作をした細胞はヒトへの移植応用は慎重に行われるべきと考えられる．遺伝子操作を行わず，タンパクを作用させたり低分子化合物を用いてiPS細胞を誘導しようとする試みが盛んに行われている．しかし，低分子化合物の多くが催がん性を有していることは古くから知られている．誘導に成功しても誘導された細胞のiPS細胞としての機能及び安全性は厳密に評価される必要がある．

d．誘導効率の改善

患者特異的iPS細胞バンクの構築など将来的には多数の安定したiPS細胞株を樹立できることが必要となる．そのために現在数%以下であるiPS細胞誘導効率をあげて，様々なヒトや組織からiPS細胞を樹立可能にしておく必要がある．

e．培養方法の改善

ヒトへの細胞移植応用に用いるiPS細胞の場合は，血清やフィーダー細胞を用いずに樹立し，GMP基準を満たすレベルのiPS細胞を用意する必要がある．

2．iPS化（リプログラミング）機構の解析

3個または4個の特定の遺伝子を導入することにより，線維芽細胞などからiPS細胞が誘導されるという事実は明らかとなったが，その分子メカニズムは全く不明である．遺伝子を導入された細胞のうち，iPS細胞化するのはいったいどの細胞で，iPS細胞化される細胞の条件は何か？，導入遺伝子の発現量やその組み合わせはどのように影響するのか？，実際導入遺伝子がどのように働いてiPS細胞化させているのか？，等々解決すべき問題は尽きない．

3．iPS細胞の医療応用

iPS細胞化のプロセスやメカニズムの解析とは全く独立して，iPS細胞として樹立させたものを出発点とし，これを様々な形で利用する方法の開発もiPS細胞研究の大きな柱である．上で述べた心血管細胞をはじめ，神経細胞，膵β細胞等々様様な臓器・組織がiPS細胞を用いた再生医学研究

のターゲットとなる.

4. その他

「いったん分化した細胞を簡便にリプログラミングしてもとに戻すことができる」という新しい技術は，様々な研究に新しいストラテジーを提供する．リプログラミングそのものに関する研究はもちろんのこと，分化/脱分化におけるエピジェネティクス研究やがん化メカニズムの解析などiPS細胞研究が新しい展開をもたらすと考えられる研究領域は数多く存在する.

むすび

哺乳類の成体の細胞がリプログラミングされて未分化なものに戻りうることはクローン羊ドリーによってすでに示されていたが，その後10年を経て報告されたヒトiPS細胞樹立の報告がそれを上回る反響をもって迎えられたのは，iPS細胞のもつ応用範囲の広さのせいであろう．「世界中どこでも施行可能な簡単な方法で成人由来の分化細胞から未分化幹細胞を誘導できる」ということが将来的に科学や社会に及ぼす影響は計り知れない．そこには当然功罪両面が生まれてくることになる．それはすべて科学者と社会が自ら責任を負うものである．極端な熱狂や批判に走ることなく冷静にかつ良識と叡知をもってiPS細胞の今後に対応していくことが必要と考えられる.

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3. 胚性幹細胞
1) ES 細胞による血管の分化再生

山下 潤

胚性幹細胞（ES 細胞）は，再生医療への応用のみならず分化の基礎研究においても不可欠な重要な役割を有する。われわれは血管構成細胞（内皮細胞および壁細胞）の分化および血管構造の形成過程を再現できる新しい ES 細胞分化系を開発した。この分化系を用いることにより，これまでにない包括的な血管発生機構の解析が可能となるとともに，血管再生治療への応用が期待される。血管の分化再生研究における ES 細胞の意義を考察する。

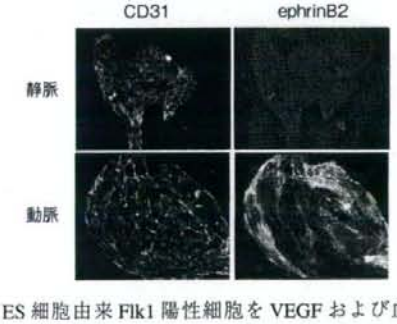
はじめに

未分化性と分化能を維持したまま無尽蔵に増殖することができる ES 細胞（胚性幹細胞：embryonic stem cells）は，再生医療への応用ということがその大きな使命であり研究ターゲットとされているが，日本ではまだヒト ES 細胞の臨床応用は認められていない。世界的にも ES 細胞由来細胞をヒトに用いた治療の報告はない。また，最近の体性幹細胞（成体内に存在する幹細胞）研究は，細胞移植に至るハードルが倫理面・安全面などで ES 細胞よりも低いため，再生医療応用において注目されている。にもかかわらず，ヒトを含めた ES 細胞研究は国内および世界的広がりを見せている。本稿では，ES 細胞研究，特に血管分野の現況と問題点，将来における可能性などについて概説する。

Ⅰ．ES 細胞からの血管細胞の分化多様化

筆者らは ES 細胞由来 2 型血管内皮増殖因子受容体（Flk1）陽性細胞が，血管を構成する細胞である血管内皮細胞と血管壁細胞（血管平滑筋細胞およびペリサイト）の共通の前駆細胞であり，Flk1 陽性細胞から内皮細胞および壁細胞の双方が分化誘導でき，毛細血管様の高次構造を培養下に形成できることを示した[1]。Flk1 陽性の血管前駆細胞は，VEGF の刺激により内皮細胞に，また主に PDGF-BB（血小板由来増殖因子）の刺激により壁細胞に分化すると考えられる。また，血流による物理的刺激である shear ストレスや拍動性進展刺激が Flk1 陽性細胞からの内皮細胞分化や壁細胞分化を誘導することも明らかにしている[2-4]。

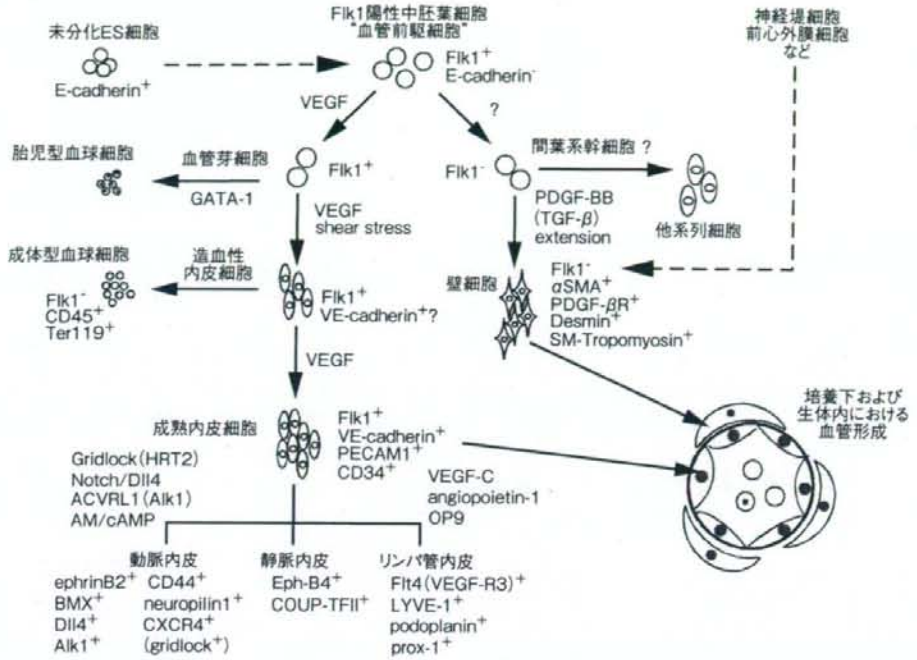
図❶ ES 細胞からの動静脈内皮分化



ES 細胞由来 Flk1 陽性細胞を VEGF および血清存在下に内皮細胞（CD31 陽性）へ分化誘導すると，ephrinB2 陰性の静脈内皮細胞（上段）が主に分化する。VEGF に加えて 8bromo-cAMP を添加すると ephrinB2 陽性の動脈内皮細胞（下段）が著しく増加する。（グラビア頁参照）

key words
ES 細胞，血管再生，動静脈分化，リンパ管分化，細胞移植，ヒト ES 細胞

図❷　Flk1 陽性細胞からの血管構成細胞の分化

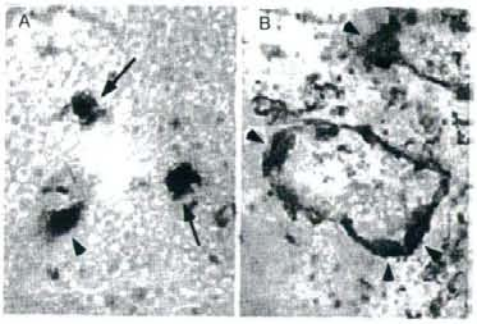


ES 細胞由来 Flk1 陽性細胞は，そこからすべての血管構成細胞（内皮細胞，壁細胞，血球細胞）が分化することのできる血管前駆細胞と考えられる。Flk1 陽性細胞から分化した内皮細胞は，一時的に血球形成能を有する時期があるが，その後，内皮細胞が成熟するにしたがって血球形成能は失われる。内皮細胞は成熟するとともに多様化も同時に行い，動脈，静脈，リンパ管内皮をはじめとした様々な内皮細胞に分化すると考えられる。VEGF が Flk1 に結合することによるシグナルが不十分であると Flk1 の発現は失われ，主に PDGF-BB の作用により壁細胞が誘導される。これら誘導された血管構成細胞は，培養下および生体内において血管構造を形成できる。

最近，動脈，静脈，リンパ管それぞれの内皮細胞特異的に発現している分子が数々報告され，内皮細胞の多様性に分子的根拠が与えられるようになってきた。筆者らは最近，Flk1 陽性細胞からの血管分化系を用い，ephrinB2 陽性（動脈）内皮，ephrinB2 陰性（静脈）内皮，および prox-1 陽性（リンパ管）内皮細胞と考えられる細胞の誘導と純化にそれぞれ成功した[5)6)]。すなわち，VEGFR2 陽性細胞を VEGF および血清存在下に内皮細胞に誘導すると，ほとんど（＞90～95％）の内皮細胞が ephrinB2 陰性の静脈内皮細胞となる。VEGF に加えて，cAMP アナログである 8bromo-cAMP または細胞内 cAMP を上昇させる液性因子の 1 つであるアドレノメデュリン（AM）を加え cAMP 経路を活性化することにより，内皮細胞において Notch シグナルの活性化が誘導され，ephrinB2 陽性の動脈内皮細胞が誘導される（図❶）。また一方，Flk1 陽性細胞を OP9 ストローマ細胞上で培養して内皮細胞を誘導したところ，prox-1 陽性リンパ管内皮細胞が出現した。この OP9 によるリンパ管誘導作用は，VEGF-C および angiopoietin の作用をブロックすることによりほぼ完全に阻害された。これらの結果により，ES 細胞を用いて，動脈，静脈，リンパ管内皮細胞のすべてを系統的に分化誘導することが可能になるとともにその新たな分化メカニズムが明らかになった[7)8)]。

以上の結果をまとめると ES 細胞からの血管細

図❸ ES 細胞由来血管細胞の分化ステージによる血管新生への寄与の仕方の違い（文献 8 より改変）



ヌードマウスに移植した C6 ラットグリオーマ周囲へ分化レベルの異なる ES 細胞由来血管細胞を直接注入。5 日後に摘出腫瘍を LacZ（青）および CD31（茶色）にて二重染色した。

A. Flk1 陽性血管前駆細胞の移植。LacZ/CD31 陽性の内皮細胞に分化した細胞（矢頭）に加え，LacZ 単独陽性の細胞を認める（矢印）。

B. Flk1 陽性細胞を 3 日間培養し分化した VE-カドヘリン陽性初期内皮細胞を移植。ほぼすべての LacZ 陽性細胞が CD31 陽性内皮細胞として血管に取り込まれている（矢頭）。 （グラビア頁参照）

胞分化は図❷のようになる。こうした新たなアプローチで血管分化多様化機構を解析することにより，動脈特異的血管新生やリンパ管特異的新生抑制による抗癌治療などの開発も期待される。さらに詳細に臓器特異的な血管の多様性を解析し理解することは，血管を介した臓器機能や病態の理解とそれに基づく様々な新しい治療戦略の開拓に結びつくと考えられる。

Ⅱ．ES 細胞による血管再生

筆者らは ES 細胞由来 Flk1 陽性細胞が生体内においても血管細胞に分化し，血管再生に寄与しうるかを検討するため，純化 Flk1 陽性細胞のニワトリ胎仔への移植実験を行った。心腔内注入により経管的に移植された Flk1 陽性細胞は，内皮細胞および壁細胞に分化するとともにニワトリ胎仔発生に伴って形成された新生血管に寄与した[1]。われわれはさらに，ES 細胞由来細胞の血管再生治療応用における可能性を検討するため，ES 細胞由来血管細胞の成体に対する移植効果を検討した[9]。すなわち，ES 細胞由来血管細胞をヌードマウスに移植した腫瘍周囲に注入し，移植細胞の新生血管への寄与を検討したところ，ES 細胞由来 Flk1 陽性細胞は，内皮細胞および壁細胞として新生血管へ寄与した。

図❹ ヒト ES 細胞からの血管細胞の分化誘導

ヒト ES 細胞から誘導された 2 型 VEGF 受容体陽性細胞を FACS により単離した後，IV 型コラーゲン上で，VEGF および血清存在下に培養すると，CD31 陽性内皮細胞（紫）と平滑筋 α アクチン陽性血管壁細胞（茶）が分化誘導される。 （グラビア頁参照）

次に，成体への移植に適切な細胞の分化段階を検討するため，分化段階の異なる血管細胞，すなわちソート直後の Flk1 陽性血管前駆細胞と，Flk1 陽性細胞をさらに 3 日間培養して初期内皮細胞に分化した細胞（VE-カドヘリン陽性）の移植を比較した。Flk1 陽性細胞を移植した群では，血管内皮細胞として寄与しているものの他に，それ以外の細胞として組織内に存在するものが多数（約 60%）認められた。一方，初期内皮を移植した群では，ほとんどすべての細胞（95%以上）が内皮細胞として血管に寄与していた（図❸）。また，Flk1 陽性細胞移植群では，細胞移植した腫瘍における血流増加は認められなかったが，分化させた血管細胞を移植した群では有意な血流増加が認められた。

これらの結果より，ES 細胞由来血管細胞の移植により，血管新生促進効果が認められるが，成体における血管新生をターゲットとした細胞移植

においては，血管前駆細胞のレベルの細胞よりも，やや血管に分化した初期内皮細胞のステージがより有効かつ特異的であると考えられた。このように，ES細胞由来細胞の移植においては，むやみに未分化細胞を移植すればよいわけではなく，ドナー細胞の分化段階とレシピエント側の状況を合わせた至適な分化段階の細胞を選択する必要があると考えられた。これらの知見は骨髄などの体性細胞の移植時においても考慮に入れる必要があると考えられる。

血管再生治療においては，倫理面・安全面・技術面でハードルが低い骨髄細胞や末梢血，G-CSFなどの薬剤を用いた血管新生治療が先行して行われ，優れた効果を上げている。心筋や神経と異なり，既存の組織からの新生が可能な血管においては，細胞による純粋な再生は必ずしも必要ではなく，ES細胞治療がこれらの治療を凌駕して有用であるという知見は今のところない。しかし将来にわたっては，直接的な細胞移植治療のみならず様々な血管再生治療のターゲットとなる新たなシーズを生み出し，血管特異的血管新生なども含め，さらに治療法を精緻に改善向上させていくうえでES細胞の血管再生研究における意義は大きいと考えられる。

Ⅲ．ヒトES細胞からの血管分化再生

ヒトES細胞を用いた血管細胞分化としては，胚様体を用いてCD31やVE-カドヘリン陽性内皮細胞の誘導と，フローサイトメトリーを用いての純化・再培養，培養下および免疫不全マウスに移植したゲル内における血管構造の形成が報告されている。京都大学のグループは，マウスES細胞と同様にサルES細胞においても2型VEGF受容体陽性細胞からの内皮細胞・壁細胞の分化[10]，培養下における血管構造形成に成功している。さらに同グループは，2002年より日本最初のヒトES細胞分化研究を輸入ヒトES細胞を用いて開始し，ヒトES細胞においても血管構成細胞の分化誘導と*in vitro*における管腔構造形成，さらにはマウス血管新生モデルにおける新生血管への移植細胞の寄与と血流改善効果を認めている（図❹）[11)12)]。ヒト細胞において血管分化再生機構が明らかになることにより，より治療応用に結びついた新たな知見が生まれることが期待される。

おわりに

このように，血管の発生・分化・再生機構に関して様々な知見が蓄積されてきているが，いまだ血管再生治療が明らかに有用な形で臨床応用されたといえるレベルには至っていない。臓器を構成する細胞を誘導して移植する，あるいは前駆細胞を移植するというだけで臓器の再生が進むというほど単純ではないことがようやく学習されてきたというのが実情に近いであろう。今後は，細胞そのものの分化メカニズムの解析（細胞外シグナルから細胞内環境の変化と安定化の過程をエピジェネティックな視点も含めて解明する）に加えて，細胞間および細胞-細胞外マトリクス相互作用や臓器・組織間相互作用など臓器としての機能を果たしうる機能ユニットを形成するために必要な要素すべてに関して理解を深め，それらを生体内でできるだけ再構成することが重要であろう。有効に分化しうる幹・前駆細胞（seed：種子）と，分化と機能発現を可能にする周囲環境（soil：土壌）の双方を整えた治療（Seed & Soil Therapy）をめざすことにより，再生医療はより実効性が期待されるものに近づくと考えられる。

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Biochemical and Biophysical Research Communications 379 (2009) 115–120

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cyclosporin-A potently induces highly cardiogenic progenitors from embryonic stem cells

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ARTICLE INFO

Article history:
Received 23 November 2008
Available online 16 December 2008

Keywords:
Cyclosporin-A
Cardiac progenitor
Cardiomyocytes
Differentiation
Embryonic stem cells

ABSTRACT

Though cardiac progenitor cells should be a suitable material for cardiac regeneration, efficient ways to induce cardiac progenitors from embryonic stem (ES) cells have not been established. Extending our systematic cardiovascular differentiation method of ES cells, here we show efficient and specific expansion of cardiomyocytes and highly cardiogenic progenitors from ES cells. An immunosuppressant, cyclosporin-A (CSA), showed a novel effect specifically acting on mesoderm cells to drastically increase cardiac progenitors as well as cardiomyocytes by 10–20 times. Approximately 200 cardiomyocytes could be induced from one mouse ES cell using this method. Expanded progenitors successfully integrated into scar tissue of infracted heart as cardiomyocytes after cell transplantation to rat myocardial infarction model. CSA elicited specific induction of cardiac lineage from mesoderm in a novel mesoderm-specific, NFAT independent fashion. This simple but efficient differentiation technology would be extended to induce pluripotent stem (iPS) cells and broadly contribute to cardiac regeneration.

Embryonic stem (ES) cell research has been providing various possibilities and strategies for regenerative medicine. Establishment of adult tissue derived, ES cell-like, stem cells, induced pluripotent stem (iPS) cells, is now further facilitating research for clinical application of stem cells [1–4]. Various ES cell studies showed induction of cardiomyocytes from ES cells and their contribution to cardiac tissues after transplantation [5]. Nevertheless, cell transplantation strategies of purified cardiomyocytes have not been fully established, mainly due to the limited proliferative potential and engraftment efficacy of differentiated cardiomyocytes [6,7]. Cardiac progenitors which can proliferate and give rise to cardiomyocytes should be suitable material to achieve efficient cardiac regeneration [8,9]. Methods to efficiently and specifically expand and purify cardiac progenitor cells, however, have not been reported.

Previously, we established a novel ES cell differentiation system that can reproduce the early process of cardiovascular development in vitro [8,10]. Endothelial cells (ECs), pericytes and vascular smooth muscle cells [10], and cardiomyocytes [8] are systematically induced from common mesodermal precursor, Flk1 (also designated as vascular endothelial growth factor receptor-2 (VEGFR2))-expressing cells. We also succeeded in identifying a cardiac progenitor population, FCV cells (Flk1$^+$/CXCR4$^+$/vascular endothelial cadherin$^-$ cell population) among the progeny of Flk1$^+$ mesoderm cells [8]. Though FCV cells, which are only a small subset of Flk1$^+$ cell progeny, showed highly cardiac specific progenitor activity, efficient expanding method of FCV cells remain unknown.

To explore a novel cardiac regenerative therapy, we investigated methods to efficiently induce cardiac progenitor cells and cardiomyocytes with the use of our ES cell differentiation system. Here we show that addition of an immunosuppressant, cyclosporine-A (CSA), to Flk1$^+$ mesoderm cells potently and specifically expand FCV progenitor population as well as cardiomyocytes. Expanded FCV cardiac progenitors showed differentiation potentials to cardiomyocytes in vivo after cell transplantation to rat myocardial infarction model. Discovery of the novel role of CSA

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doi:10.1016/j.bbrc.2008.12.019

with mesoderm-specific cardiogenic activity would provide a clue to explore cardiac regeneration strategies.

Materials and methods

Antibodies. Monoclonal antibodies (MoAbs) for murine E-cadherin (ECCD2), murine Flk1 (AVAS12) were prepared and labeled in our laboratory as described [8,10]. MoAb for cardiac troponin-T (cTnT) was purchased from NeoMarkers (Fremont, CA). Murine α-actinin was from Sigma (St. Louis, Mo). Phycoerythrin (PE)-conjugated AVAS12 was from eBioscience (San Diego, CA). MoAbs for mouse CD31 and biotinylated-CXCR4 were purchased from BD Pharmingen (San Diego, CA). Polyclonal rabbit antibody to GFP was from MBL (Nagoya, Japan).

Reagents. Cyclosporin-A (a gift from Novartis Pharma) was dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) at 30 mg/mL. Dilution of 1–3 μg/mL were made in differentiation medium (see below) at the time of use. FK506 (a gift from Astellas Pharma) was dissolved in DMSO at 10 mg/mL, dilution of 10 ng–1 μg/mL were made in differentiation medium. 11R-VIVIT was from Calbiochem (Darmstadt, Germany). PKH67 fluorescent dye was from Sigma.

Mouse ES cell culture. EMG7 mouse ES cells that carry mouse α-myosin heavy chain (MHC) promoter-driven EGFP gene were used for this study [8]. OP9 stroma cells were maintained as described [11].

Induction of cardiomyocyte differentiation. Induction and sorting of Flk1$^+$ cells were performed as previously described [8,10]. Briefly, mouse ES cells were cultured in differentiation medium (alpha minimum essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum) on type IV collagen-coated dishes (BIOCOAT™, Becton–Dickinson) or mitomycin C-treated confluent OP9 cell sheets (MMC-OP9) for 96–108 h. FACS-purified Flk1$^+$ cells were then plated onto MMC-OP9 and cultured in differentiation medium to induce cardiac differentiation. Medium was replaced every 2 days. Induction of FCV cells was performed as described [8]. Purified FCV progenitor cells were again plated onto MMC-OP9 cells. MMC-OP9 cells were pre-stained with PKH67 fluorescent dye (Sigma) before plating.

Flowcytometry and cell sorting. FACS for differentiating ES cells was performed as described [8,10]. After 96–108 h of ES cell differentiation, cultured cells were harvested and stained with allophycocyanin (APC)-conjugated AVAS12 and FITC-conjugated ECCD2. Viable Flk1$^+$/E-cadherin$^-$ cells, excluding propidium iodide (Sigma), were sorted by FACS Vantage (Becton–Dickinson). For FACS analysis of FCV progenitor cells, after 2 days differentiation of purified Flk1$^+$ cells on OP9 cells (Flk-d2), cells were harvested and stained with a combination of MoAbs of PE-conjugated AVAS12 and biotinylated CXCR4 followed by addition of streptavidin-conjugated APC. PKH-negative populations were analyzed as ES cell-derived cells. For cardiomyocytes, cells were harvested at Flk-d6. GFP$^+$ population was evaluated and sorted as differentiated cardiomyocytes.

Immunohistochemistry. Immunostaining for ECs and cardiomyocytes was performed as described [8]. Anti-mouse IgG antibody conjugated with horse radish peroxidase (HRP) (Invitrogen, Carlsbad, California) or anti-mouse, rat and Rabbit Ig Abs conjugated with Alexa 488 or 546 (Molecular Probes, Eugene, OR) were used for 2nd Abs. Nuclei were visualized with DAPI (Invitrogen). Preparation of rat heart sections was performed as previously described [12]. Double staining for cTnT and GFP was performed with mixture of anti-cTnT Ab labeled with Zenon Alexa Fluor 546 labeling kit (Molecular Probes) and anti-GFP Ab, followed by Alexa Fluor488-conjugated anti-Rabbit Ig Ab (Molecular Probes).

Quantification of cardiomyocyte differentiation. Cardiomyocyte differentiation was quantitatively evaluated by fluorescent intensity of cTnT staining using Alexa Fluor 546 as described [8].

Reverse-transcription polymerase chain reation (RT-PCR). RT-PCR was performed as previously described [8]. Primers that were used are indicated in supplemental Table I online.

Annexin-V FITC apoptosis assay. Cells were harvested at Flk-d2 and stained for Flk1 and CXCR4. After addition of Annexin-V FITC (Invitrogen), cells were incubated for 15 min at room temperature in the dark and subjected to FACS analysis.

EdU cell proliferation assay. EdU solution (Invitrogen) (10 μM) was added to the culture medium 2 h before sorting FCV cells by FACS. The sorted FCV cells were plated onto glass slides by Cytospin (Thermo Shandon) (Waltham, MA), fixed by 4% PFA and EdU was detected by incubating with Click-iT™ reaction cocktail (Invitrogen) according to the manufacturer's instructions. The numbers of EdU positive DAPI positive FCV cells were counted in 10 randomly selected fields.

Analysis of induced endothelial and blood cells. At Flk-d6, floating cells and attached cells were collected and stained with biotin-conjugated anti-CD45 Ab or anti-CD31 Ab (BD Pharmingen) followed by addition of streptavidin-APC.

Model of heart failure. Ligation of rat left coronary artery was performed as described [12] in accordance with the guidelines for Animal Experiments of Kyoto University, which conforms to the law of "Guide for the Care and Use of Laboratory Animals" in Japan.

ES cell transplantation. Four weeks after ligation, nude rats with moderate-sized myocardial infarction (MI) (infarct size: 20–40%) were used for cell transplantation experiment. Through left thoracotomy, after putting mattress sutures with 6.0 polypropylene thread at injection points to prevent leakage, 100 μL culture medium containing 4×10^5 cells were subepicardially injected into the center of the scar using a 27-gauge needle. Bulging over the MI area was confirmed after injection [12].

Electrophysiological studies. Action potentials of FACS-purified GFP positive cardiomyocytes were recorded as previously described [13]. All experiments were performed at 36–37 °C.

Statistical analysis. Values are reported as means ± SD. Comparisons among values were performed by ANOVA. At least three independent experiments were performed. $p < 0.05$ was considered significant.

Results

In our differentiation system, cells representing four different differentiation stages are sequentially induced, that is, undifferentiated ES cells, Flk1$^+$ mesoderm cells, FCV cardiac progenitor cells, and cardiomyocytes. When purified Flk1$^+$ cells are cultured on OP9 stroma cells, self-beating cardiomyocytes appear after 4 days of culture (Flk-d4) [8]. When we were performing transplantation experiments in which purified cardiomyocytes were injected into immune-competent mice treated with CSA to suppress immune rejection, we examined the direct effects of CSA on cardiomyocyte differentiation and survival in vitro in a control experiment. Surprisingly, addition of CSA (1–3 μg/mL) to Flk1$^+$ cells showed a striking effect to increase beating cells at Flk-d6 (experiment 1 in supplemental figure 1 online) (Fig. 1A, and supplemental video 1 and 2 online). Addition of CSA induced approximately 13 times increase in cardiac troponin-T (cTnT)-positive cardiomyocyte appearance than control (Fig. 1B and C). CSA-induced cardiomyocytes showed distinct expression of cTnT (Fig. 1D), and sarcomere formation (Fig. 1E). Action potential in purified cardiomyocytes showed existence of cells with pacemaker potential (Fig. 1F), as well as ventricular type cells lacking pacemaker potential and

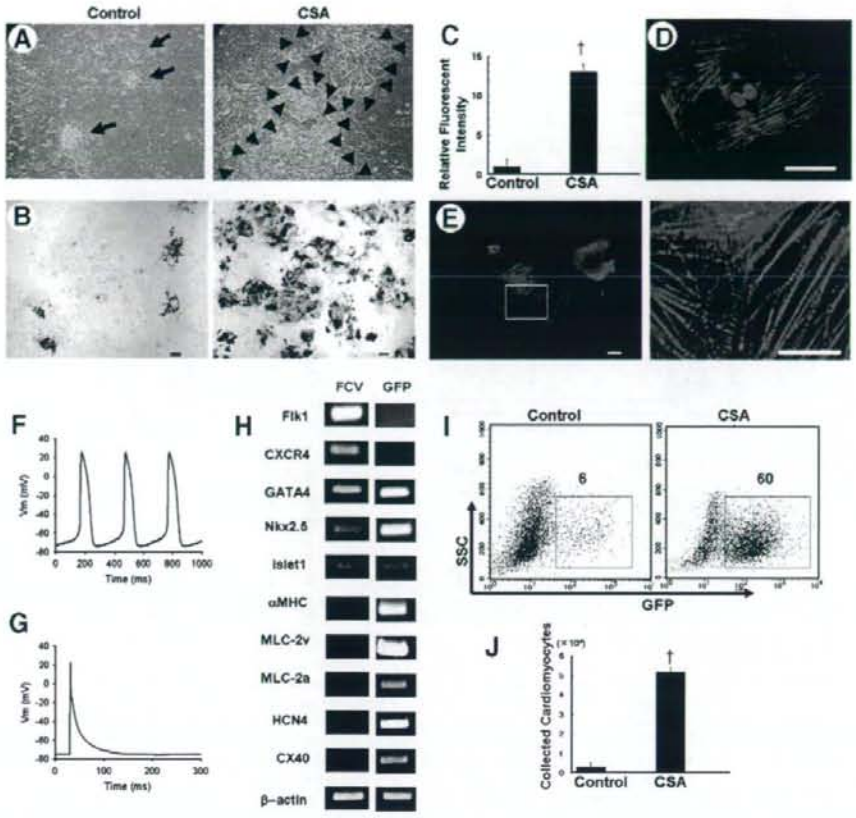

Fig. 1. Cardiomyocyte induction from Flk1$^+$ mesoderm by CSA (experiment 1). (A,B) Gross appearance of cardiomyocyte induction by CSA. Left panels: control. Right panels: CSA treatment. Scale bars = 100 μm. (A) Appearance of beating colony. Arrows indicate beating colonies. Arrow heads surround large beating area (see supplemental video 1 and 2 online). (B) Cardiomyocyte stained with cTnT (brown). (C) Quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is indicated ($n = 9$, $^{\dagger}p < 0.001$ vs control). (D,E) Appearance of isolated cardiomyocytes. (D) cTnT (red) and DAPI (blue) staining. Scale bar = 50 μm. (E) Actinin (red) staining. Right panel shows higher magnification of boxed area. Apparent sarcomere structures are observed. Scale bar = 25 μm. (F,G) Action potentials of induced cardiomyocytes. (F) Cell with pacemaker potential and spontaneous beating. (G) Quiescent ventricular type cell. (H) mRNA expressions in purified FCV progenitor cells and MHC promoter-driven GFP$^+$ cardiomyocytes induced by CSA. (I) FACS analysis for GFP$^+$ cardiomyocyte induction by CSA. Left panel: control. Right panel: CSA treatment. *X* axis: GFP. *Y* axis: side scatter. All Flk1$^+$ cell-derived population was analyzed. Percentages of GFP$^+$ cardiomyocytes are indicated. (J) Yield of purified cardiomyocytes. Cell number of obtained GFP$^+$ cardiomyocytes from 10^4 Flk1$^+$ cells ($n = 12$, $^{\dagger}p < 0.001$ vs control).

self-beating (Fig. 1G).Purified CSA-induced cardiomyocytes showed expression of various cardiac marker mRNAs, such as GATA4, Nkx2.5, islet1, alpha-myosin heavy chain (MHC), myosin light chain (MLC) 2v, and 2a, a pacemaker marker, HCN4, and conduction system marker, Cx40 (Fig. 1H). These results indicate that functional cardiomyocytes were successfully induced and expanded by CSA treatment. At optimal conditions, approximately 60% of Flk1$^+$ cell-derived cells became cardiomyocytes, positive for MHC promoter-driven GFP (GFP$^+$) (Fig. 1I). The CSA treatment resulted in approximately 17-fold increase in the yield of FACS-purified cardiomyocytes starting from the same number of Flk1$^+$ cells (CSA-treatment: $5.0 \pm 0.35 \times 10^4$ cells/10^4 Flk1$^+$ cells vs. control: $0.3 \pm 0.023 \times 10^4$ cells/10^4 Flk1$^+$ cells, $n = 12$, $^{\dagger}p < 0.001$) (Fig. 1J). As a result, approximately 200 cardiomyocytes could be obtained from one ES cell (Supplemental figure 2 online).

We further evaluated differentiation stage-specific effects of CSA. CSA did not have any influence on Flk1$^+$ mesoderm cell appearance from undifferentiated ES cells (Fig. 2A, experiment 2). No apparent difference in endoderm and ectoderm marker expression was induced by CSA treatment (Fig. 2B). Surprisingly, addition of CSA to Flk1$^+$ cells specifically increased FCV population to approximately 10–20 times more than control (experiment 3) (Fig. 2C). The maximum percentage of FCV cells within total Flk1$^+$ cell-derived cells was increased up to 40% by CSA. The yield of purified FCV progenitor cells was increased approximately 22 times by CSA treatment (CSA-treatment: $4.3 \pm 0.23 \times 10^3$ cells/10^4 Flk1$^+$ cells vs. control: $0.2 \pm 0.001 \times 10^3$ cells/10^4 Flk1$^+$ cells, $n = 12$, $^{\dagger}p < 0.001$) (Fig. 2D). Purified CSA-induced FCV cells showed expression of various cardiac progenitor marker mRNAs, such as Flk1, CXCR4, GATA4, Nkx2.5, islet1, but not differentiated cardiomyocyte markers, such as MHC, MLC-2v and 2a (Fig. 1H). Comparable number of cardiomyocytes was induced from the same number of control FCV cells and CSA-expanded FCV cells (data not shown). These results indicate that CSA-expanded FCV cells retained their high cardiogenic potentials. When CSA was added to purified FCV cells (experiment 4), slight increase (approximately 2.6 times) in cardiomyocytes was observed (Fig. 2E and F). These results indicate that the novel potent cardiomyocyte inducing activity of CSA is restricted to the period after mesoderm formation, and acts principally between the mesoderm and cardiac pro-

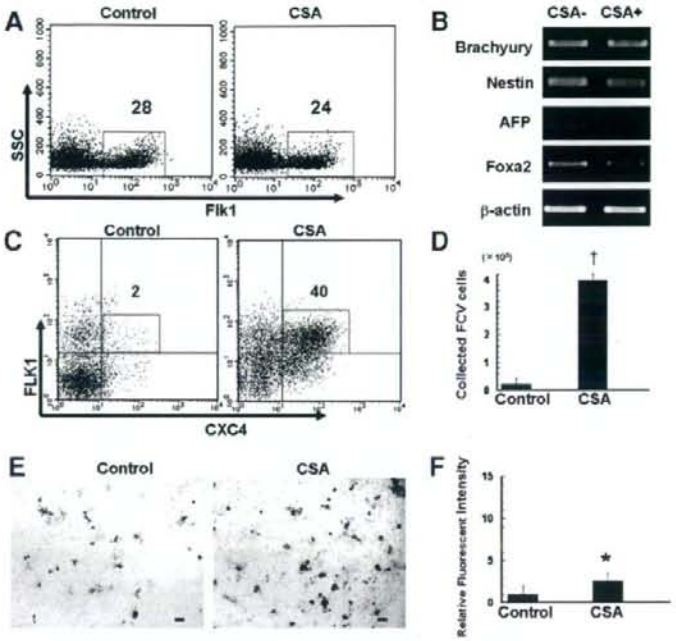

Fig. 2. Differentiation stage-specific effect of CSA. (A,B) Effects of CSA on early differentiation from undifferentiated ES cells (experiment 2). Undifferentiated ES cells were cultured in the absence of LIF for 4 days (ES-d4). (A) Induction of Flk1$^+$ mesoderm cells. FACS analysis for Flk1 and side scatter (SSC) in the absence (control) or presence (CSA) of CSA. (B) mRNA expressions for mesoendoderm marker, brachyury, ectoderm marker, Nestin, and endoderm markers, AFP and Foxa2 at ES-d4. (C,D) Induction of FCV progenitor cells from Flk1$^+$ mesoderm (experiment 3). (C) FACS analysis for cardiac progenitor induction by CSA. *X* axis: Flk1. *Y* axis: CXCR4. Percentages of FCV cells (double positive population) are indicated. (D) Yield of purified cardiac progenitor cells. Cell number of obtained FCV progenitor cells from 10^4 Flk1$^+$ cells ($n = 12$, $^\dagger p < 0.001$ vs control). (E,F) Cardiomyocyte induction from purified FCV cells by CSA (experiment 4). Purified FCV cells were recultured on OP9 cells for 4 days. (E) Gross appearance of cardiomyocytes stained with cTnT (brown). Left panel: control. Right panel: CSA treatment. Scale bars = 400 μm. (F) Quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is indicated ($n = 3$, $p < 0.05$ vs control).

genitor stages to induce specific and efficient expansion of the cardiac progenitor cells.

We further examined cellular and molecular mechanisms of CSA. EdU incorporation and annexin V expression in FCV cells were not affected by CSA treatment (Fig. 3A–C), indicating that the increase in FCV cells by CSA was not due to proliferation and/or survival of FCV cells. CSA treatment on Flk1$^+$ cells (Flk-d0-6: experiment 1) induced drastic increase in cardiomyocytes with reciprocal decrease in ECs or blood cells from Flk1$^+$ mesoderm cells (Fig. 3D and E). Even when CSA was added only in mesoderm stage (Flk-d0-2: experiment 5), similar effects were observed (Fig. 3F), suggesting that CSA should act on Flk1$^+$ cells and may shift the cell fate from ECs or blood cells to cardiomyocytes. Calcineurin inhibitors, CSA and FK506 exert their immunosuppressing effect through inhibition of nuclear factor of activated T-cells (NFAT) signaling [14]. Nevertheless, FK506 and a NFAT inhibitor, 11R-VIVIT, showed no significant effect on cardiomyocyte induction in our system (Fig. 3G), suggesting that the main cardiogenic effect of CSA should be NFAT independent. These results indicate that the potent cardiomyocyte inducing activity of CSA should be evoked through novel mechanisms to induce specific and efficient expansion of the cardiac progenitor cells with mesoderm-specific, NFAT-independent fashion. Future studies on precise mechanisms of CSA action should provide novel molecular understanding of cardiomyocyte differentiation and regeneration.

This efficient expansion of the rare FCV progenitors allowed us to confirm their cardiogenic potential in vivo. Finally, we examined in vivo cardiogenic potential of expanded FCV cells. We performed transplantation of CSA-expanded FCV cells to chronic myocardial infarction model of rat. At 2 weeks after the injection, transplanted FCV cells were successfully differentiated into cardiomyocytes and integrated in the infarct heart to form GFP$^+$/cTnT$^+$ donor cell-derived cardiomyocyte bundle in the scar tissue (Fig. 4A and B). This result indicates that CSA-expanded FCV cells can show highly cardiogenic potentials also in vivo after cell transplantation.

Discussion

Here, we show a novel technology for the specific and efficient expansion of highly cardiogenic progenitors as well as cardiomyocytes from ES cells with a newly discovered mesoderm-specific effect of CSA. The immunosuppressive action of the calcineurin inhibitor CSA stems from the inhibition of NFAT signaling in T cells [14]. Through calcineurin-NFAT signaling, CSA is also involved in various cellular processes, such as cardiac valve formation [15], cardiac hypertrophy [16], and hair growth [17]. Though a weak inductive effect of CSA on cardiomyocytes in embryoid bodies was reported [18], the effect on cardiac progenitors and its molecular mechanism were unclear. Our novel sequential cardiomyocyte differentiation system should succeed in distinctively digging out the potent mesoderm-specific and novel NFAT-independent effect of CSA, which has been buried in the cell mixture of embryoid bodies. This novel culture system would be amenable to screen and discover novel cardiac regenerative drugs from small molecules using chemical biology strategies.

FCV cells, which are detected at 6–6.5 days after the differentiation of mouse ES cells and at 1–2 days before cardiomyocyte

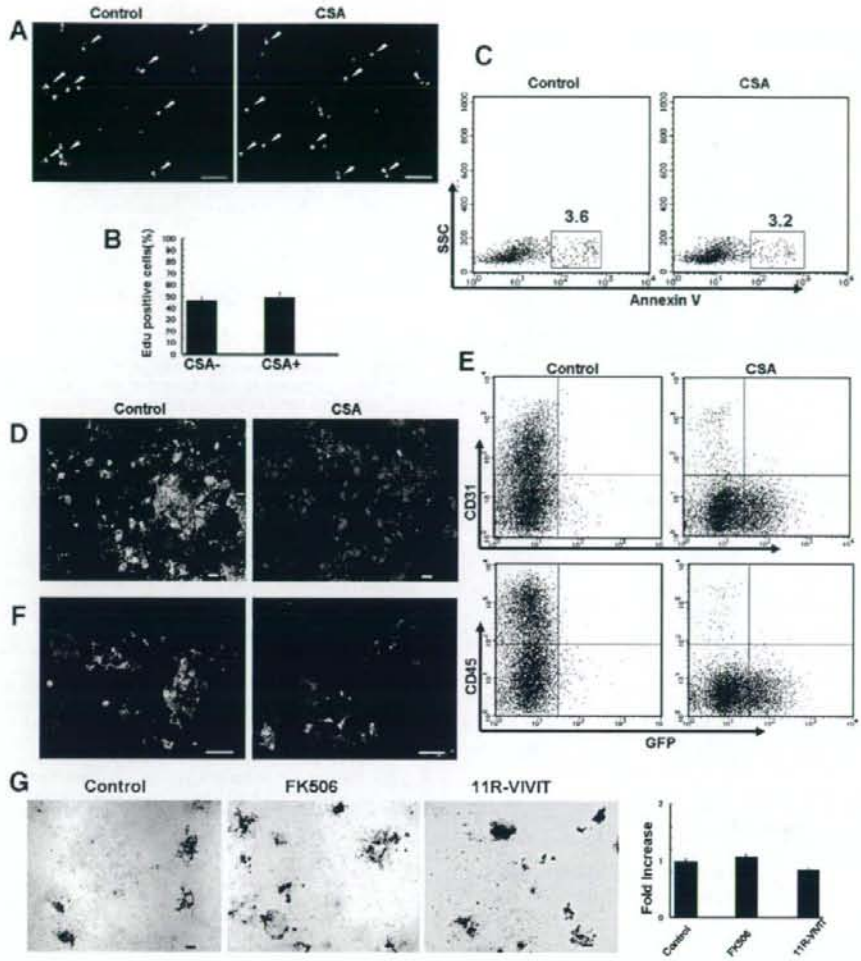

Fig. 3. Cellular and molecular mechanisms of CSA. (A,B) EdU incorporation. EdU-treated FCV cells were plated onto glass slides by cytocentrifugation. (A) Double staining for EdU (green) and DAPI (blue). Left panel: control. Right panel: CSA treatment. Double positive nuclei are indicated by arrowheads. Scale bar = 100 μm. (B) Quantitative evaluation of EdU positive FCV cells. Percentages of EdU positive cells in total cells are indicated (200 nuclei each, $n = 3$). (C) FACS analysis for annexin V expression. Gated FCV cell populations are shown. Percentages of apoptotic cells (annexin V$^+$, red box) are indicated. (D) Reciprocal appearance of ECs and cardiomyocytes by CSA treatment (experiment 1). Double immunostaining for CD31 (pan-ECs; green) and cTnT (red). Left panel: control. Right panel: CSA treatment. Scale bars = 400 μm. (E) Reciprocal appearance of ECs (upper panels) or blood cells (lower panels) with cardiomyocytes by CSA treatment. FACS analysis at Flk-d6 (experiment 1). Left panel: control. Right panel: CSA treatment. *X* axis: GFP (cardiomyocytes). *Y* axis: CD31 (pan-ECs), CD45 (pan-white blood cells). All Flk1$^+$ cell-derived population was analyzed. (F) Mesoderm-specific treatment of CSA (Flk-d0-2: experiment 5). Double immunostaining for CD31 (pan-ECs; green) and cTnT (red). Scale bars = 400 μm. (G) Effects of FK506 and NFAT inhibitor (11R-VIVIT) on cardiomyocyte differentiation (experiment 1). Left panels: gross appearance of cardiomyocytes stained with cTnT (brown), treated with vehicle (control), FK506, or 11R-VIVIT. Scale bars = 100 μm. Right panel: quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is indicated ($n = 3$).

appearance, are, to our knowledge, the first identified distinct cardiac progenitor population [8] and so far the nearest upstream cardiac progenitors to cardiomyocytes. Recently, several kinds of multipotent cardiac progenitor populations were reported [19–21]. Cardiac progenitors reported by Kattman et al. were identified at an earlier stage of differentiation (i.e. at 4.25 days after the differentiation) than FCV cells [19]. Other Nkx2.5$^+$ or islet1$^+$ cardiac progenitors were reported at 4–6 days of ES cell differentiation [20,21]. In our FCV population, approximately 42% of cells were Islet1$^+$ (42 ± 18%, 1000 cells each, $n = 3$), 24% were Nkx2.5$^+$ (24 ± 5%), and 14% were double positive for Islet1 and Nkx2.5 (14 ± 1%). FCV cells, thus, should be an overlapped population with Nkx2.5$^+$ and/or Islet1$^+$ cardiac progenitors.

Recently, novel ES cell-like pluripotent stem cells, iPS cells, were generated from adult somatic cells by transduction of defined transcription factors [1–4]. We have just succeeded in establishing an iPS cell differentiation system for various cardiovascular cells similar to the ES cell system [22]. This CSA-mediated cardiac cell induction method would be extended and applied to iPS cells, and that would broadly contribute to exploring cardiac regeneration strategies.

Competing interests statement

The authors declare that they have no competing financial interests.

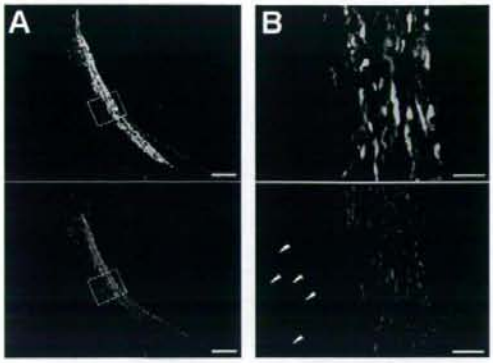

Fig. 4. Cardiagenic potential of expanded FCV cells in vivo. (A,B) Representative data of FCV cell transplantation (4×10^5 cells) to rat myocardial infarction model. Double immunostaining for GFP and cTnT. Upper panels: GFP (donor cell-derived cardiomyocytes, green) and DAPI (blue). Lower panels: cTnT (pan-cardiomyocytes, red) and DAPI. (A) Gross appearance of transplanted cell contribution to infarct area. Scale bars = 400 μm. (B) Higher magnification views of boxed area. Arrowheads show $cTnT^+/GFP^-$ endogenous cardiomyocytes. Scale bars = 50 μm.

Acknowledgments

We thank to Novartis Pharma for cyclosporin, Astellas Pharma for FK506, Dr. M. Takahashi for critical reading of the manuscript. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health, Labour and Welfare, the New Energy and Industrial Development Organization (NEDO) of Japan, the Project for Realization of Regenerative Medicine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.019.

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Circulation 2008;118;498-506; originally published online Jul 14, 2008;
DOI: 10.1161/CIRCULATIONAHA.108.769562
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Directed and Systematic Differentiation of Cardiovascular Cells From Mouse Induced Pluripotent Stem Cells

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Background—Induced pluripotent stem (iPS) cells are a novel stem cell population induced from mouse and human adult somatic cells through reprogramming by transduction of defined transcription factors. However, detailed differentiation properties and the directional differentiation system of iPS cells have not been demonstrated.

Methods and Results—Previously, we established a novel mouse embryonic stem (ES) cell differentiation system that can reproduce the early differentiation processes of cardiovascular cells. We applied our ES cell system to iPS cells and examined directional differentiation of mouse iPS cells to cardiovascular cells. Flk1 (also designated as vascular endothelial growth factor receptor-2)-expressing mesoderm cells were induced from iPS cells after ≈4-day culture for differentiation. Purified Flk1$^+$ cells gave rise to endothelial cells and mural cells by addition of vascular endothelial growth factor and serum. Arterial, venous, and lymphatic endothelial cells were also successfully induced. Self-beating cardiomyocytes could be induced from Flk1$^+$ cells by culture on OP9 stroma cells. Time course and efficiency of the differentiation were comparable to those of mouse ES cells. Occasionally, reexpression of transgene mRNAs, including c-myc, was observed in long-term differentiation cultures.

Conclusions—Various cardiovascular cells can be systematically induced from iPS cells. The differentiation properties of iPS cells are almost completely identical to those of ES cells. This system would greatly contribute to a novel understanding of iPS cell biology and the development of novel cardiovascular regenerative medicine. (***Circulation.* 2008;118:498-506.)**

Key Words: differentiation ■ endothelium ■ myocardium ■ stem cells

Embryonic stem (ES) cells have been considered potent candidates for regenerative medicine with their prominent properties of pluripotency and capacity for self-renewal. Novel ES cell–like pluripotent cells, termed *induced pluripotent stem* (iPS) *cells*, were generated from mouse skin fibroblasts by introducing 4 transcription factors (Oct3/4, Sox2, Klf4, c-myc),[1] and recently they were also successfully generated from human skin fibroblastsis.[2,3] These iPS cells opened a new gate for cell transplantation–based regenerative medicine by overcoming the ethical controversy over ES cells. Differentiation and selection methods for target cells were thus required for regenerative medicine with the use of iPS cells. However, the differentiation properties of iPS cells such as time course, potentials, and efficiency of the differentiation in vitro are still unclear, and a directed differentiation method for iPS cells has not been demonstrated.

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Clinical Perspective p 506

Previously, we established a novel ES cell differentiation system that can reproduce the early process of cardiovascular development in vitro.[4–6] Flk1 (also designated as vascular endothelial growth factor [VEGF] receptor-2) is the earliest differentiation marker for endothelial cells (ECs) and blood cells and a marker for lateral plate mesoderm.[7,8] We induced Flk1$^+$ cells from ES cells, purified them by fluorescence-activated cell sorting (FACS), and recultured the purified cells.[9] We succeeded in inducing cardiovascular cells such as vascular ECs, mural cells (pericytes and vascular smooth muscle cells),[4,10] and cardiomyocytes[5] from common progenitors, Flk1$^+$ cells. We also identified a novel cardiac progenitor population during ES cell differentiation.[5] More recently,

Received January 28, 2008; accepted May 6, 2008.

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The online-only Data Supplement, which contains Figures I through V, Movies I and II, and a supplemental Methods section, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.769562/DC1.

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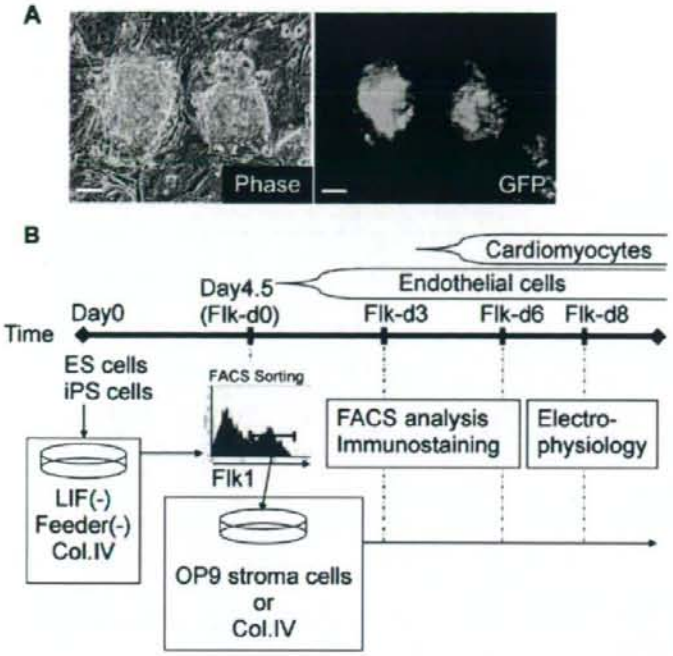

Figure 1. Experimental design of this study. A, Appearance of undifferentiated mouse Nanog-iPS cells. Left, Phase contrast. Right, Nanog promoter-driven GFP. Bars=100 μm. B, Procedure and time course of cardiomyocyte differentiation. Undifferentiated ES or iPS cells were cultured for 4.5 days on collagen (Col.) IV–coated dishes in differentiation medium (see Methods) to induce differentiation to Flk1$^+$ cells. FACS-purified Flk1$^+$ cells were plated onto collagen IV dish or OP9 stroma cells to induce further differentiation to ECs and cardiomyocytes (designated as Flk1-d0). ECs and cardiomyocytes appeared from Flk-d1 and Flk-d4-5, respectively. Differentiation of ECs and cardiomyocytes was evaluated by immunostaining and/or FACS analysis at Flk-d3 and Flk-d6. Electrophysiological study was done at Flk-d8 with the use of TMRM-purified cardiomyocytes (see Methods).

we succeeded in inducing arterial, venous, and lymphatic ECs from Flk1$^+$ cells.[11,12] This system is therefore useful to systematically induce various cardiovascular cells from common progenitors and dissect their differentiation processes.[6,10,13]

We applied this system to iPS cells and examined cardiovascular cell differentiation of iPS cells. In the present report, we show that all of the cardiovascular cells can be systematically induced from iPS cells and that the differentiation properties of iPS cells are largely comparable to those of ES cells.

Methods

Antibodies

Monoclonal antibodies for murine Flk1 (AVAS12) and murine vascular endothelial (VE)–cadherin (VECD1, for FACS) were described previously.[8,14] Monoclonal antibodies for murine CD31 (1:500), VE-cadherin (for immunostaining, 1:200), and CXCR4 were purchased from Pharmingen (San Diego, Calif). Monoclonal antibodies for murine α-smooth muscle actin (SMA) (1:1000) and α-actinin (sarcomeric) (1:800) were from Sigma (St Louis, Mo). The antibody for cardiac troponin-T (cTnT) (1:200) was from NeoMarkers (Fremont, Calif). Polyclonal antibodies for murine LYVE1 (1:500) and prox1 (1:50) were from Angiobio (Del Mar, Calif) and Reliatech (Braunschweig, Germany), respectively. Antibodies for SM22α (1:400) and calponin (1:500) were from Abcam (Cambridge, UK). Anti-HCN4 (1:200) and anti-Cav3.2 (1:200) antibodies were from Chemicon (Temecula, Calif). Anti-Kir2.1 (1:200) and anti-connexin 43 (1:200) antibodies were from Alomone (Israel) and Invitrogen (Carlsbad, Calif), respectively.

Cell Culture

Murine ES cell lines EB5, EMG7, and D3 and OP9 stroma cells were maintained as described.[5,11,15] EMG7 ES cells were generated by introduction of α-myosin heavy chain (MHC) promoter-driven EGFP gene to EB5 ES cells.[5] Cardiomyocytes induced from EMG7 cells could be detected by GFP expression (MHC-GFP). Germline-competent mouse iPS cell lines 20D17, 38C2, and 38D2, carrying Nanog promoter-driven GFP/IRES/puromycin-resistant gene (Nanog-iPS cells), were established and maintained as described previously.[16] Briefly, iPS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% FCS, nonessential amino acids, 1 mmol/L sodium pyruvate, 5.5 mmol/L 2-mercaptoethanol, 50 U/mL penicillin, and 50 mg/mL streptomycin on feeder layers of mitomycin-C–treated mouse embryonic fibroblast cells carrying stably incorporated puromycin-resistance gene. All experiments were performed with the use of 3 Nanog-iPS cell lines. 20D17 was used as the iPS cell representative in all experiments unless stated otherwise.

Induction of cell differentiation was performed as described with the use of differentiation medium[4] (DM) (α-minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum and 5×10^{-5} mol/L 2-mercaptoethanol). Flk1$^+$ mesoderm cells were induced by 96- to 108-hour culture of ES or iPS cells (plated at 1.7×10^3 cells/cm^2) in DM in the absence of leukemia inhibitory factor (LIF) on type IV collagen–coated dishes (BD Biosciences, San Jose, Calif). To induce vascular ECs, FACS-purified Flk1$^+$ cells (1×10^4 cells/cm^2) were cultured on type IV collagen–coated dishes with DM supplemented with 100 ng/mL human $VEGF_{165}$ (R&D Systems, Minneapolis, Minn) in the presence or absence of 8-bromoadenosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cAMP) (0.5 mmol/L) (Nacalai Tesque, Kyoto, Japan).[11] Lymphatic endothelial cell was induced by coculture of Flk1$^+$ cells (5×10^3 cells/cm^2) on confluent OP9 stroma cells[12] with DM. After 3-day culture of Flk1$^+$ cells, induced ECs were subjected to FACS or immunostaining. Cardiomyocytes were induced by coculture of Flk1$^+$ cells (1.5×10^4 cells/cm^2) on OP9 cells for 4 to 6 days[5] with DM.

Three-Dimensional Culture

Three-dimensional culture was performed as described previously.[4] Briefly, Flk1$^+$ cells (4×10^5 cells/mL) were incubated in DM containing 100 ng/mL VEGF on uncoated petri dishes for 12 hours to induce aggregation. Aggregates were resuspended in 2× DM and mixed with an isovolume of collagen I-A gel (3 mg/mL) (Nitta Gelatin, Osaka, Japan). We plated 250 μL of this mixture onto a

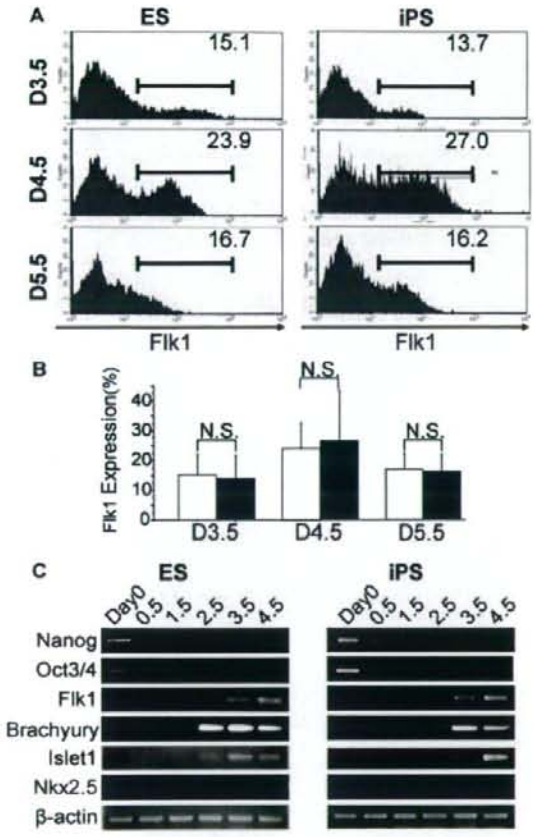

Figure 2. Differentiation of ES and iPS cells to mesoderm. A, Representative results of expression profiles of Flk1 on differentiation day 3.5 to 5.5 by FACS analysis. ES cells (EB5; left) and iPS cells (20D17; right) are shown. Percentages of Flk1$^+$ cells in total cells are indicated. B, Quantitative evaluation of Flk1$^+$ cell differentiation from ES cells (open column) and iPS cells (closed column) (n=4). N.S. indicates not significant. P=0.79 (D3.5), P=0.96 (D4.5), P=0.90 (D5.5), ES cells (EB5) vs iPS cells (20D17). C, Reverse transcription polymerase chain reaction analysis for gene expression of early differentiation markers during mesoderm differentiation (differentiation day 0 to 4.5). ES cells (EB5; left) and iPS cells (20D17; right) are shown.

lucent insert disk, Cell Disk (Sumitomo Bakelite, Tokyo, Japan), in 24-well dishes. After 30 minutes at 37°C to allow polymerization, we added 500 μL DM with VEGF (final, 100 ng/mL). To monitor vascular formation, collagen-embedded Flk1$^+$ cell aggregates were cultured in a temperature- and gas-controlled chamber (37°C, 5% CO_2), and phase-contrast images were acquired every 15 minutes with Metamorph software (Molecular Devices, Tokyo, Japan) for up to 4 days.

Flow Cytometry and Cell Sorting

FACS analysis was performed as described previously.[4,5,11] After induction of Flk1$^+$ cells, cultured cells were harvested and stained with allophycocyanin-conjugated AVAS12. Living Flk1$^+$ cells excluding propidium iodide (Sigma) were sorted by FACS Vantage (Becton Dickinson). After 3 days of Flk1$^+$ cell differentiation (Flk-d3), cultured cells were harvested and stained with a combination of monoclonal antibodies for CD31 and CXCR4 or VE-cadherin, then subjected to FACS analysis. Induced cardiomyocytes were purified with the use of tetramethyl rhodamine methyl ester (TMRM) (Invitrogen),[17,18] a fluorescent probe to monitor the membrane potential of mitochondria. In brief, cells were dissociated with 0.25% trypsin/EDTA, then incubated in DM with 50 nmol/L TMRM at 37°C for 15 minutes. Stained cells were washed twice and subjected to FACS sorting. A TMRM-high population was considered as purified cardiomyocytes in iPS cells (Figures I and II in the online-only Data Supplement).

Immunostaining

Immunostaining for ECs and cardiomyocytes was performed as described previously.[4,5,11] Briefly, 4% paraformaldehyde-fixed cells were blocked by 2% skim milk (BD Biosciences) and incubated overnight with primary antibodies at 4°C. For immunohistochemistry, anti-mouse IgG conjugated with alkaline phosphatase and anti-rabbit IgG horseradish peroxidase (Invitrogen) were used as secondary antibodies. For immunofluorescent staining, anti-mouse, -rat, -rabbit, or -goat IgG antibodies conjugated with Alexa488 or Alexa546 (Invitrogen) were used for secondary antibodies. Nuclei were visualized with DAPI (Invitrogen). EphrinB2 expression was examined by the binding of EphB4-Fc chimeric protein (R&D).[11] Stained cells were photographed with inverted fluorescence microscopy with Eclipse TE2000-U (Nikon, Tokyo, Japan) and the digital camera system AxioCam HRc with the use of AxioVision Software (Carl Zeiss, Jena, Germany) or confocal microscopy (LSM510; Carl Zeiss). To quantify the numbers of induced EC or cardiomyocyte colonies, positively staining colonies were counted in 8 randomly selected fields.

Immunostaining for 3-Dimensional Cultured Vascular Structures

Immunostaining for vascular structures in type I collagen gel was performed after the whole-mount immunostaining procedure. Briefly, gels were fixed with 4% paraformaldehyde and blocked by 1% skim milk/0.1% Triton X/PBS solution and incubated with anti-CD31 and SMA antibodies. Alexa488-conjugated anti-rat IgG and Alexa546-conjugated anti-mouse IgG were used as secondary antibodies. Stained cells were photographed with a confocal microscope (LSM510; Carl Zeiss).

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from various kinds of cell populations with the use of RNeasy Mini or Micro Kit (QIAGEN, Valencia, Calif). cDNA was synthesized by the SuperScript III First-strand Synthesis System (Invitrogen). Polymerase chain reaction was performed with the use of Taq polymerase or KOD Plus (Toyobo, Tokyo, Japan). Primers that were used are indicated in Table I in the online-only Data Supplement.

Electrophysiological Studies

The FACS-purified TMRM-high population was seeded on gelatin-coated coverslips. The myocytes were cultured for 2 days under this condition before use. The coverslips were then transferred to a patch clamp recording chamber, and electrophysiological measurements were performed with the use of Axopatch200B (Axon Instruments/Molecular Devices Corp, Union City, Calif).

Composition of Solutions

Physiological bathing solution contained the following (in mmol/L): 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.5 $MgCl_2$, 1.8 $CaCl_2$, and 5 HEPES (pH=7.4 with NaOH). Standard high-K^+ pipette solution contained the following (in mmol/L): 110 L-aspartic acid, 30 KCl, 5 MgATP, 0.1 NaGTP, 5 K_2 creatine phosphate, 2 EGTA, 10 HEPES, and 10 NaOH (pH=7.2 with KOH). All experiments were performed at 37°C.

Statistical Analysis

All data were obtained from at least 3 independent experiments. Statistical analysis of the data was performed with Student t test or ANOVA. $P<0.05$ was considered significant. All data are shown as mean±SD.

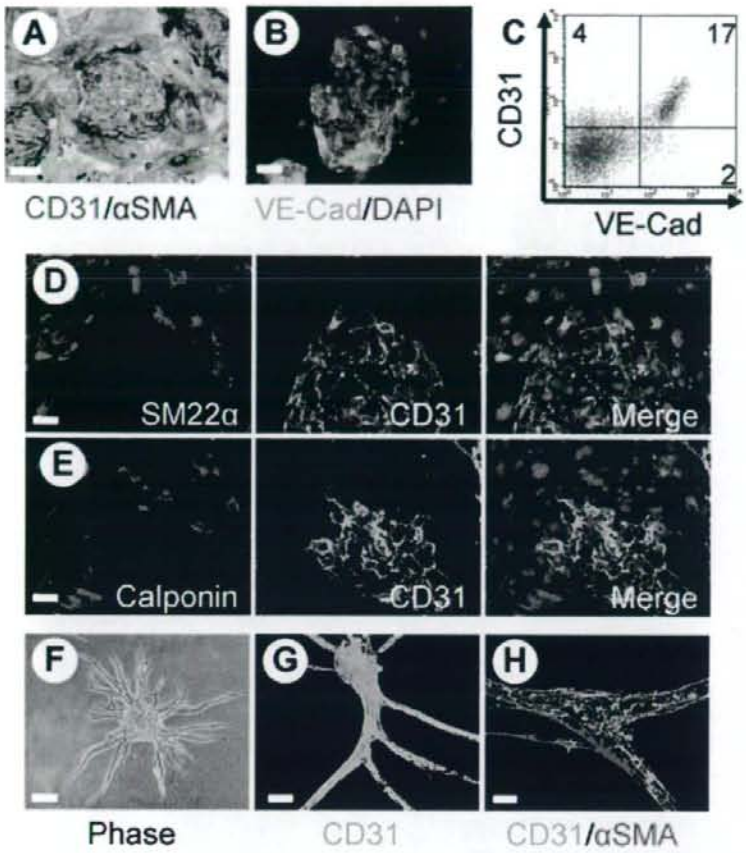

Figure 3. Differentiation of iPS cells to vascular cells. A to E, Two-dimensional culture after 3-day culture of iPS cell–derived Flk1$^+$ cells on collagen IV–coated dishes. A, Double immunostaining for CD31 (pan-ECs; purple) and SMA (mural cells; brown). B, Immunostaining for VE-cadherin (VE-Cad) (green) and DAPI (nuclei, blue). C, FACS analysis for CD31 and VE-cadherin. Percentages of quadrant populations are indicated. D, E, Double immunostaining for mural cell markers SM22α (D) and calponin (E) (left panels, red) and CD31 (middle panels, green). Right panels, Merged images with DAPI (blue). F to H, Three-dimensional culture. Flk1$^+$ cell aggregates were 3-dimensionally cultured in type I collagen gel for 3 days. F, Gross appearance of vascular-like structure. G, In-gel immunostaining for CD31 (green). H, Double in-gel staining for CD31 (green) and SMA (red). Attachments of mural cells (red) were observed. Bar=100 μm (A, F), 50 μm (B, D, E, G), 25 μm (H).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

iPS Cell Differentiation to Mesoderm Cells

Undifferentiated mouse iPS cell colonies maintained on a feeder layer showed an appearance similar to that of mouse ES cells and coexpressed Nanog promoter-driven EGFP (Figure 1A). Figure 1B schematically summarizes our differentiation methods to induce cardiovascular lineage cells. We applied this system to iPS cells and examined the directional differentiation of iPS cells to cardiovascular cells.

First, we induced mesoderm cell differentiation from ES and iPS cells. Undifferentiated ES or iPS cells were cultured on type IV collagen–coated dishes with DM (see Methods) to induce mesoderm differentiation (Figure 1B). We examined the time course and efficiency of Flk1$^+$ mesoderm cell appearance. As demonstrated previously,[9] ES cell–derived Flk1$^+$ cells appeared from ≈3 days of differentiation and reached a maximum at day 4.5 of differentiation (Figure 2A). Similarly, Flk1$^+$ cells were induced from iPS cells under the same culture conditions. Time course and efficiency of Flk1$^+$ cell induction were comparable between ES and iPS cells (Figure 2A and 2B). As shown in Figure 2C, undifferentiated ES cell markers Nanog and Oct3/4[19–21] were expressed in both ES and iPS cells and started to decrease after differentiation. The mesoendoderm marker Brachyury[22] started to be observed from differentiation day 2.5. The mesoderm marker Flk1 appeared from day 3.5. Islet1, a cardiac progenitor marker,[23] was expressed from day 3.5, whereas another cardiac marker, Nkx2.5,[24,25] was not observed until day 4.5. These results were compatible with our previous results.[5] iPS cells therefore differentiate into mesoderm cells with a time course and efficiency similar to those of ES cells.

iPS Cell Differentiation to Vascular Cells

Next we induced ECs from Flk1$^+$ cells. FACS-purified Flk1$^+$ cells were recultured on type IV collagen–coated dishes with VEGF and serum (designated as Flk-d0) (Figure 1B). Three days after the differentiation (Flk-d3), CD31$^+$ ECs and SMA$^+$ mural cells were selectively induced from Flk1$^+$ cells (Figure 3A). Almost all of CD31$^+$ ECs were also positive for another EC marker, VE-cadherin (Figure 3B and 3C). Induced SMA$^+$ mural cells, which were negative for CD31, expressed other smooth muscle markers, SM22α (Figure 3D) and calponin (Figure 3E). When iPS cell–derived Flk1$^+$ cell aggregates were cultured 3-dimensionally in type I collagen gel, a vascular-like structure was formed successfully (Figure 3F; Movie I in the online-only Data Supplement). The vascular-like structure con-