sheets might accelerate a rapid differentiation of MSCs into chondrocytes without the help of chondro-differentiation reagents. These 3D tissue models using cell sheets would give new insights to stem cell differentiation in 3D environment and contribute to the future application of the stem cells to cartilage regenerative therapy.

Authors' Contribution

Waki Sekine and Yuji Haraguchi contributed equally to this study.

Disclosure

T. Shimizu and M. Yamato are consultants for CellSeed, Inc. T. Okano is an investor in CellSeed, Inc., and an investor/developer designated on the patent for temperature-responsive culture surfaces.

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シリーズ（医薬品評価をめぐる最近の話題）

再生医療製品の素材としての多能性幹細胞（ES/iPS 細胞）の品質

Quality of Pluripotent Stem Cells as Substrates Used for Production of Cell-Processed Therapeutic Products

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Abstract

Pluripotent stem cells (PSCs), including embryonic stem cells and induced pluripotent stem cells, have opened new avenues for regenerative medicine/cell therapy. PSCs are expected to be new materials used for production of cell-processed therapeutic products (CTPs), especially for the treatment of serious or life-threatening diseases/conditions, for which no adequate therapy is currently available. At present, many attempts are being made to develop various types of PSC-derived CTPs. The most ideal base camp in the stable manufacture of a CTP is a cell bank that has been well-characterized, is stable, possesses the ability to propagate, can be regenerated and has a stable supply, and finally can differentiate into the desired cells. In most cases of the manufacture of PSC-derived CTPs, PSC bank systems are developed to supply substrates for stable production of the final products with reproducible quality. To establish quality management systems for ensuring quality, safety and efficacy of CTPs, it is necessary to well understand the quality of the cell banks/substrates, which have significant impacts on the characteristics of the final products. From a view point of manufacturing biologics, based on the concept of ICH Harmonized Tripartite Guideline Q5D (ICH-Q5D), this minireview provides a perspective on the meaning and specification method of the quality of pluripotent stem cells as cell banks/substrates for production of CTPs.

抄　録

胚性幹細胞や人工多能性幹細胞などの，いわゆる多能性幹細胞は，再生医療／細胞治療に新たな展開をもたらしている．多能性幹細胞は，特に従来十分な治療法が存在しなかった重篤ないし生命を脅かす疾患を対象とした再生医療製品を製造するために用いる新しい素材として期待されている．現在，多能性幹細胞を分化させることによって様々な種類の再生医療製品の開発の取り組みが数多くなされている．再生医療製品の安定な製造における最も理想的な起点は，十分に解析され，安定で，増殖能力を持ち，再生可能かつ安定供給可能で，その上，目的細胞に分化する能力をもつセル・バンクである．ほとんどの多能性幹細胞由来再生医療製品の製造においては，再現性のある品質を持った最終製品を安定的に製造するための細胞基材の供給源として，多能性幹細胞のセル・バンクが樹立される．セル・バンク／細

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胞基材の品質は，最終製品の特性に大きな影響を与えるものであり，最終製品の品質・安全性・有効性を確保するための品質マネジメントシステムを構築するためには，セル・バンク／細胞基材の品質を十分に把握することが必須である．本稿では，バイオロジクスの製造という観点から，ICH-Q5D の考え方をもとに，再生医療製品のセル・バンク／細胞基材としての多能性幹細胞の品質の意味合いとその設定のありかたについて概説する．

Key words: pluripotent stem cell, ES cell, iPS cell, cell bank, quality

1．はじめに

胚性幹細胞（ES 細胞，embryonic stem cell）や人工多能性幹細胞（iPS 細胞，induced pluripotent stem cell）などの「多能性幹細胞」は，その幅広い多能性ゆえに，いままで入手が困難であった各種細胞を作製することのできる素材となることが期待され，またその無限の自己複製能ゆえに，ひとたび目的細胞への効率的分化誘導方法が確立すれば，再生医療・細胞治療に利用できる細胞，すなわち，いわゆる再生医療製品の成分となる細胞を大量かつ安定に供給することが可能となることが期待されている．既に2011年１月に米国では，ヒト ES 細胞を加工した医薬品の再生医療における活用例として，世界初の治験（脊髄損傷治療）が開始され，2011年７月には同じく米国で網膜疾患治療を目的としたヒト ES 細胞加工製品の治験が開始されている（ただし，前者の治験は2011年11月に経済的理由により中断）．また，2007年に世界初のヒト iPS 細胞が樹立されたことを契機に，細胞のプログラミングを人為的に操作，制御できる時代が到来し，新規細胞基材，新規製造関連資材，新規製造方法，新規適用法等，新たなイノベーションを推進し，再生医療・細胞治療へ応用しようとする研究展開が国内外できわめて活発化している．この中に実用化に有望と考えられるシーズも数多くあり，例えば，2013年夏にはわが国においてヒト iPS 細胞を加工して作製した網膜色素上皮細胞を加齢黄斑変性の患者らに対して臨床応用する研究が開始されるに至っている．

再生医療製品の品質管理において重要なこととして，最終製品としての再生医療製品の規格及び試験方法の設定，製造工程の妥当性の検証と一定性の維持管理などに加えて，素材（原材料・中間製品）の品質管理を適正に行うことが挙げられる．従って，多能性幹細胞由来再生医療製品のような，一昔前には実現が想定されていなかった再生医療製品の開発では，製品に特有の品質・安全性確保のための基盤技術（例えば最終製品に残存する多能性幹細胞の造腫瘍性に起因する安全性上のリスクの評価法など）の確立が必要となると同時に，最終製品を再現性の高い品質で継続的に生産する方策，特に多能性幹細胞のような新しい素材の品質・規格の設定のあり方を理解することが重要となる．

多能性幹細胞を，幹細胞生物学や発生学の対象（客体的存在，Vorhandensein）としてではなく，再生医療製品の素材（道具的存在，Zuhandensein）として見た場合，その位置づけは，「対象とする特定の治療目的に適う，品質・有効性・安全性を備えた最終製品」を製造するのに適したもの，ということになる．一般的に，バイオロジクス（生物薬品）の製造においては，その製造管理の出発点を「ICH-Q5D 的な意味でのセル・バンク」（後述）に設定すること，つまり，解析が十分で，形質が安定で，増殖性を有し，更新も，安定供給も可能で，最終目的製品を高い再現性で効率よく生産することが可能なセル・バンクを製造工程上の起点として設定することが基本とされる．再生医療製品はバイオロジクスの一種と考えられ，また，多能性幹細胞は通常，細胞株として樹立され，チューブないしアンプルに分注した状態で保

存（バンク化）される．そこで本稿では，再生医療製品の素材としての多能性幹細胞の品質のあり方について，バイオロジクス製造のためのセル・バンクの品質という観点から概説することにする．

2．セル・バンクの定義

「セル・バンク」（ないし「細胞バンク」）という言葉はアカデミアから産業界まで幅広く用いられているが，その定義は，立場や目的によって複数あり，それぞれ意味合いが異なる．例えば，①「研究目的または体の損傷部位の外科的再建を目的とした凍結組織標本を保管する貯蔵施設」のように定義されていることもあるし[1]，②「提供されたヒトの細胞（中略）等について，研究用資源として品質管理を実施して，不特定多数の研究者に分譲する非営利的事業」と定義されることもある[2]．しかしバイオロジクスの製造においては，日米EU医薬品規制調和国際会議（ICH）のガイドラインQ5D（ICH-Q5D）に従い，セル・バンクの定義は③「均一な組成の内容物をそれぞれに含む相当数の容器を集めた状態で，一定の条件下で保存しているもの．個々の容器には，単一の細胞プールから分注された細胞が含まれている．」とされ，チューブないしアンプルに入った凍結細胞という実体を指す[3]．また，ICH-Q5Dで扱っているのは，特定のバイオロジクスを生産する目的で使用される細胞基材（後述）としてのセル・バンクである．

本稿では，ヒト多能性幹細胞加工製品等の再生医療製品をバイオロジクスの一種ととらえ，「セル・バンク」という言葉を，特に断らない限り，ICH-Q5Dに従い，「細胞基材としてのセル・バンク」という意味で用いることとする．

3．セル・バンク・システム構築の目的

ICH-Q5Dに基づくセル・バンク・システムは大抵の場合2段階のシステムから成り立っている．即ち，大本の細胞を一定の培養条件下で最低限の継代数を経て増殖させることにより調製したセル・バンクを「マスター・セル・バンク」と呼び，マスター・セル・バンクから一定の条件で培養して得られる均質な細胞懸濁液を分注して調製した，実際の製造に使用されるセル・バンクを「ワーキング・セル・バンク」と呼ぶ．なお，「微生物細胞あるいはヒト又は動物由来の細胞で，ヒトを対象に *in vivo* 又は *in vitro* で投与されるバイオロジクスを生産する上で必要な能力を有するもの」は「細胞基材」と呼ばれる．ヒト多能性幹細胞加工製品をはじめとする再生医療製品の素材となる細胞はすべて細胞基材である．従って，セル・バンクも細胞基材の一種である．また，マスター・セル・バンクを調製する元になる親細胞株や親細胞株を樹立するために使用される親細胞も細胞基材である（図1）．

バイオロジクスの製造におけるセル・バンク・システム構築の目的は，「一定の品質の特定の最終製品を安定的かつ継続的に製造する」ということにある．逆に言えば，一定の品質の最終目的製品を安定的かつ継続的に製造する上で重要かつ科学的に合理的な場合に，セル・バンク・システムの構築またはその他の細胞基材の調製が必要となる．従って，再生医療製品の製造において全ての種類の細胞基材が必須であるというわけでない．

4．セル・バンクの品質

ICH-Q5Dにおける「セル・バンク」の意味合いの中では必ず細胞の具体的な臨床用途・最終製品が特定されているのに対し，先述の第1・第2の定義では細胞の具体的用途は特定されない[*1]．この違いによってセル・バンクの品質の意味合いも大きく異なる．

第1・第2の定義，すなわち「具体的臨床用途が未特定のセル・バンク」[*2]における品質上の注意点は4つある．その一つは，①感染因子混入などの汚染が無いことの保証である．これは作業従事者の安全性の確保（および臨床グレードの細胞の場合にはさらに患者の安全性の確保）の意味合いがある．もう一点は，②学問的定義（一般的定

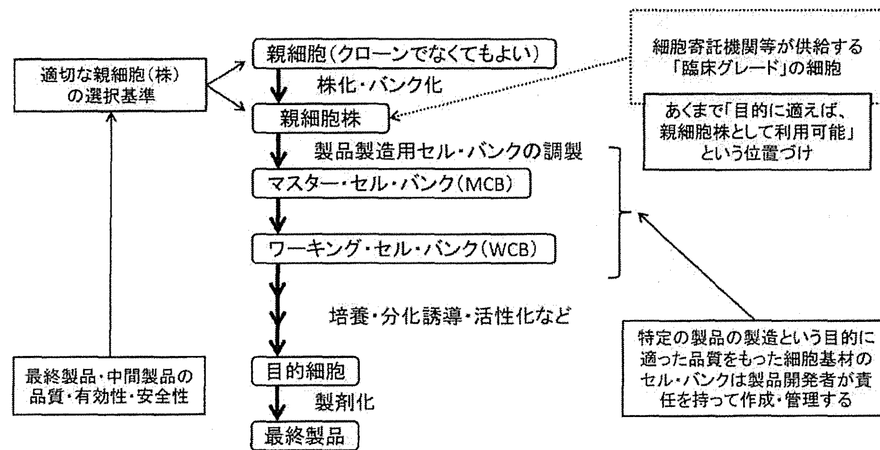

図１　再生医療製品の製造の概略

義）に基づく細胞種としての同一性・純度とその安定性を保証することである．例えば，リプログラミングされた「iPS細胞様細胞」を「iPS細胞」としてバンク化する場合には，三胚葉系への多分化能を確認することが必須である．

先の第３の定義（ICH-Q5D）に従った「特定の臨床用途・最終製品製造のためのセル・バンク」すなわち「細胞基材としてのセル・バンク」における品質上の注意点の１つは上の場合と同様に①感染因子混入などの汚染が無いことの保証である．ただし，臨床用であることから，患者の安全性の確保の意味合いがより強い．もう一つの注意点は具体的臨床用途が未特定のセル・バンクの場合とは異なり，②患者に投与される最終製品の品質・有効性・安全性の再現性を確保するための素材としての特性とその安定性である．例えば，リプログラミングされた「iPS細胞様細胞」を特定の分化細胞製造用の素材としてバンク化する場合には，目的とする細胞への分化効率の高さやその再現性の高さの方が多分化能よりも重要となる．

つまり，再生医療製品の製造における，素材としての細胞基材（セル・バンク等）の品質・規格については，製造プロセス全体として最終製品の有効性・安全性が確保できるように設定することが原則となる．

再生医療製品の製造においては，細胞という極めて複雑な構造と不確実性の高い特性を持つ要素が存在するために，素材の品質をもとに最終製品の品質を設計･デザインすることが不可能である．従って，再生医療製品の場合には，対象疾患，患者のQOL（Quality of Life），標的となる臓器・細胞・分子，製品の使用方法，製品の安全性・有効性（First-in-Humanの場合には，非臨床安全性試験や非臨床Proof-of-Concept試験（非臨床薬力学試験）等のデータ）などをもとに，最終製品の品質・規格が設定され，最終製品の品質・規格から目的細胞の品質･規格が決定される．同様に，目的細胞の品質・規格からセル・バンクの品質・規格が決定され，セル・バンクの品質・規格から親細胞の品質・規格が決定されることになる（図２）．

５．細胞基材としての多能性幹細胞の品質

ICH-Q5Dガイドラインの構成は，①細胞基材（細胞株）の起源．履歴・調製（すなわちドナー情報・培養歴及び株化の方法など），②細胞のバンク化の手法，および③セル・バンクの特性解析となっている．なお，セル・バンクの特性解析としては，特性解析試験，純度試験，細胞基材の安定性，核型分析･造腫瘍性試験が挙げられている．

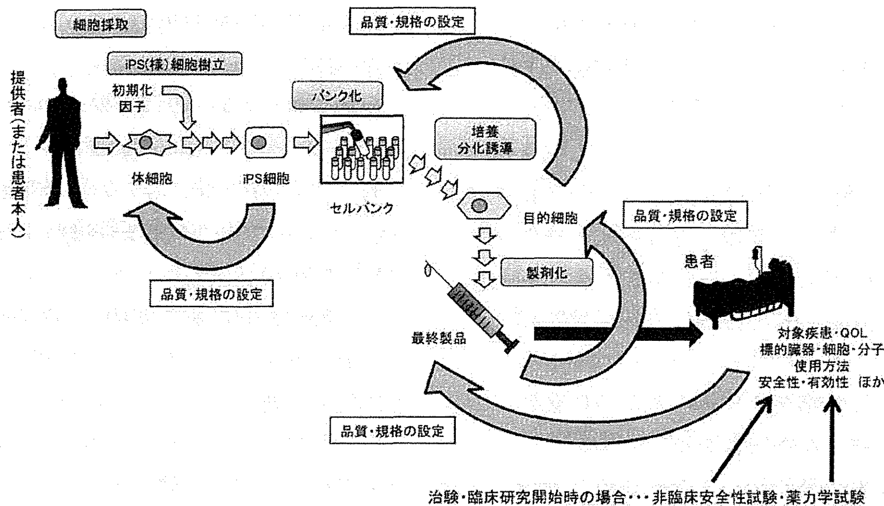

図2　再生医療製品とその素材の品質（iPS 細胞由来製品の例）
最終製品としての再生医療製品の品質をもとに中間製品の品質，中間製品の品質から出発原料の品質，と逆行性に規定される．

これらをまとめると，バイオロジクス製造用細胞基材の主な留意事項が，「汚染がないことの保証」と，「同一性・均質性の確認・維持」であることが分かる．

例えば，米国 WiCell 研究所の保有する「臨床グレード」のヒト ES 細胞株（WA09株）の品質管理においては，①主要なウイルス・感染因子のチェックが実施されると同時に②フィーダー細胞フリー培養系（TeSR™，マトリゲル™）を使用し，感染因子の混入を防ぎ，また，③融解後生存率，④同一性試験（縦列型反復配列（short tandem repeat; STR）検査），⑤染色体異常検査（G バンド，比較ゲノムハイブリダイゼーション（Comparative genomic hybridization, CGH）検査），および⑥ ES 細胞マーカーおよび分化マーカーの発現の検査等によって，同一性・均質性の確認・維持が行われている[4]．

ここで注意しなければならないのは，WA09株のような，臨床用途・最終製品が未特定な「臨床グレード」の多能性幹細胞における上記①～⑥のような留意事項と，特定の再生医療製品の製造という目的に適った ICH-Q5D 的なセル・バンクの品質管理における留意事項とは同じとは限らないということである．

2011年にハーバード大学の Bock らは，20株の ES 細胞と12株の iPS 細胞について，様々な細胞系譜への分化傾向（プロペンシティ）を評価する目的で，各株の細胞を用いて形成させた胚葉体中の細胞種マーカー，胚葉マーカーの発現を検討したデータを報告している[5]．この報告では，各多能性細胞株は確かに多能性を保有するものの，株間で分化プロペンシティのプロファイルが様々であることが示されている．即ち，ヒト ES/iPS 細胞株のセル・バンクを多能性幹細胞の一般的／学問的定義に従い「未分化度」や「多能性」のみで品質管理した場合，目的とする細胞への分化効率に細胞株間で大きなバラツキが生じる恐れがある．従って，多能性幹細胞加工製品の細胞基材としてのセル・バンクにおいては，「目的に適った分化プロペンシティ」すなわち「目的細胞への高い分化効率とその高い再現性」を品質特性とする必要性がある．2011年12月，動物由来成分を全く使用せずに「臨床グレード」のヒト ES 細胞が樹立され，UK Stem Cell Bank に寄託されたとのニュースが Nature News で報道されたが，同報道には「実際にヒトに投与されるまでには何年も

かかるかもしれない」「細胞株間で組織形成能力は様々であり，心筋を作りやすい株や軟骨を作りやすい株などが存在するので，臨床グレードの株の一連のセットが必要だ」というコメントも記されている[6]．ヒト多能性幹細胞株／バンクの分化プロペンシティの予測と管理は，今後のヒト多能性幹細胞加工製品の実用化の上で非常に重要な課題となると予想される．例えば既に米国では2013年３月に，ライフテクノロジー社がハーバード大学と契約を結び，目的細胞を作成するために必要な特性をもつ最も有望な細胞株を迅速に選抜するための分析ツールの開発を行うことを発表しており[7]，こうした研究の成果として今後出てくると想定される分析ツールや関連特許は，将来，iPS細胞樹立技術の基本特許が失効した後のわが国の多能性幹細胞由来再生医療製品のビジネス展開に大きな影響を及ぼす可能性がある．

6．多能性幹細胞のセル・バンクの樹立・管理

多能性幹細胞由来再生医療製品をはじめとする各種再生医療製品の製造においては，一定の品質の最終製品を安定的かつ継続的に製造する上で重要かつ科学的に合理的な場合に，セル・バンク・システムの構築またはその他の細胞基材の調製が必要となる．わが国の「ヒト幹細胞臨床研究」のように小規模かつ非継続的な医療ならば，多能性幹細胞の供給は細胞寄託機関等からの一時的なものでも済んでしまうかもしれない．しかしそれ以外の場合には，即ち産業・医療の一環として多能性幹細胞由来再生医療製品を安定的かつ継続的に供給するためには，抗体医薬や組換えタンパク質医薬品等の他のすべてのバイオロジクスと同様に，特定の製品の製造という目的に適った品質の細胞基材としてのセル・バンクを製品の開発者が自らの責任において樹立・管理するのが基本だと考えるべきである（**図１**）．その際には，最終製品または中間製品の品質・安全性・有効性を基に，適切な親細胞（株）を選択する必要がある．

細胞寄託機関等が供給する「臨床グレードの多能性幹細胞」は，最終製品の品質を安定的かつ継続的に確保するために重要かつ科学的に合理的である場合，つまり製品製造という目的に適う場合において利用可能であるが，細胞寄託機関等の「臨床グレードの多能性幹細胞」を利用することが製品製造の必須条件だというわけではない．むしろ，特定の製品を効率的かつ再現性良く製造するためのセル・バンクを，感染因子・免疫原性因子の混入を避けつつ，いかに効率的かつ安価に樹立・選別できるかどうかの方が再生医療製品の製造・実用化・継続的供給という目的のためには重要である．

7．おわりに

上で述べたように，細胞基材としての多能性幹細胞のセル・バンクの品質は，個々の最終製品の品質・態様・適用法・対象疾患等で決まる．細胞株／セル・バンク・システムの「標準化」はデータの相互参照性という意味において学問的には重要である．しかし，多能性幹細胞に由来する再生医療製品の製造においては「はじめにセル・バンクの品質（もしくは標準化）ありき」ということはありえず，特定の再生医療製品を一定の品質で再現性良く製造するという目的を達成するためにセル・バンクの品質・規格が決定される．標準化された部品・原材料から最終製品の品質が設計可能な多くの工業製品（建築，機械からコンピュータープログラムまで）の開発手法と同様な発想を，再生医療製品の開発に当てはめることはできない．むしろ再生医療製品の素材の品質についての考え方は，生きた素材であると言う意味で，醸造のそれに近い．ビールの製造の場合，酵母は例えば「芳醇な香り」という最終製品の品質を実現するために素材として厳選され，その上で酵母の品質・規格が決定されるのであり，標準化された酵母の品質をもとにビールの味や香りをデザインすることはありえない．また，選び抜かれた酵母はセル・バンクとして開発者が管理する，あるい

はビジネス戦略によっては囲い込んで門外不出とするものである.

一般的留意事項（必要条件）のみを満たした「臨床グレードの多能性幹細胞」から特定の再生医療製品を製造する場合には，それまで管理されていなかった幾つかの細胞の特性のバラツキにより，目的とする最終製品の品質が十分に確保できない恐れがある．従って，製品ごとに具体的目的に適った品質の多能性幹細胞のセル・バンクが必要となる．もちろん，細胞寄託機関等が供給する「臨床グレードの多能性幹細胞」のセル・バンクは，安価で簡単にアクセス可能な整理された細胞基材供給源（親細胞株）として非常に有用となる可能性はある．ただしその場合でも開発者はそこから改めて特定の製品製造に適う品質のセル・バンクを作成することが必要とされると考えるべきである.

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注

＊1 第1の定義によるセル・バンクは「研究目的または体の損傷部位の外科的再建を目的とした」ものではあるが，これはあくまで漠然とした目的であり，また，その目的に使用するものを貯蔵する「施設」である（例：理研セルバンク，American Type Culture Collection, Wisconsin International Stem Cell Bank, UK Stem Cell Bank）．予め「具体的な臨床用途・最終製品」を特定してから樹立されるものでなく，また細胞（を含むチューブ・アンプル）自体を指すものでもない点で，ICH-Q5D の言う「細胞基材のセル・バンク」とは異なる.

＊2 具体的臨床用途・最終製品を予め特定することはせず，漠然と外科的再建・再生医療等での臨床利用を想定して樹立される，いわゆる「臨床グレード」と呼ばれる多能性幹細胞が国内外に存在する．これらは感染因子混入防止のための厳重な管理に加え，免疫原性を示す恐れのある動物由来成分等を含んだ試薬を細胞の樹立・維持に使用しないなど，より厳密な規格の下に製造された細胞であることを意味している.

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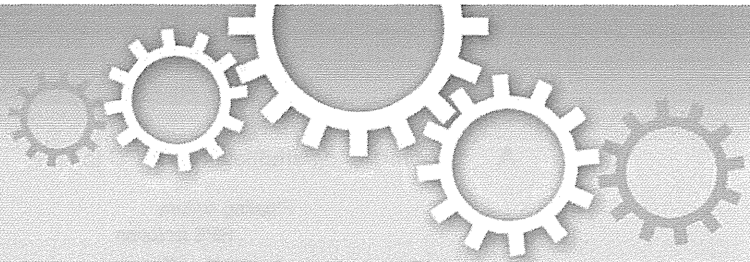

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Pigment Epithelium-Derived Factor Secreted from Retinal Pigment Epithelium Facilitates Apoptotic Cell Death of iPSC

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We show that pigment epithelium-derived factor (PEDF), which is secreted from primary or iPSC-derived retinal pigment epithelium (RPE), dramatically inhibits the growth of iPSCs. PEDF is detected abundantly in culture supernatants of primary or iPSC-derived RPE. Apoptotic cell death is induced in iPSC when co-cultured with RPE, a process that is significantly blocked by addition of antibody against PEDF. Indeed, addition of recombinant PEDF to the iPSC cell culture induces apoptotic cell death in iPSCs, but the expression of pluripotency related-genes is maintained, suggesting that PEDF causes cell death, not differentiation, of iPSCs. To recapitulate this event *in vivo*, we examined tumor formation in NOG mice after subcutaneous injection of iPSCs with or without an iPSC-derived RPE sheet (2.5×10^5 RPE cells). We observed that the tumor forming potential of iPSCs was significantly suppressed by simultaneous transplantation with an iPSC-derived RPE sheet.

Cell therapy using embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) has already entered the scope of clinical application. Indeed, a clinical trial using ESC derived-RPE cells for Stargardt's disease and the dry type of age-related macular degeneration (dry AMD) has been initiated[1]. Clinical trials using autologous iPSC-derived RPE for the wet type of age-related macular degeneration (wet AMD) are also being planned by several groups[2].

However, tumor formation from residual undifferentiated iPSCs or ESCs is an issue to be evaluated carefully in the transplantation of pluripotent stem cell-derived tissue products. This issue becomes more serious in the case of transplanting autologous iPSC-derived cells or tissues at sites lacking an immune barrier. The tumor forming potential of the remaining undifferentiated iPSCs in iPSC derived-cell products should be examined by taking into account the number of iPSC-derived cells to be transplanted, and the micro-environment of the transplantation site. The method and its sensitivity to detect the remaining iPSCs are also key issues to assure the safety of transplantation of iPSC-derived cell products.

We recently reported a method that was highly sensitive for the detection of residual iPSCs in iPSC-derived retinal pigment epithelium (RPE). It relied on qRT-PCR using primers for the *LIN28A* transcript[3]. With this method, we could theoretically detect iPSCs equivalent to 0.01% of the total cell product. Considering the fact that we plan to transplant $4 - 8 \times 10^4$ iPSC-derived RPE cells in a clinical setting, we should be able to detect the few residual iPSCs in the iPSC-derived RPE prior to transplant. Apart from the development of a sensitive residual iPSC detection method, it is important to explore the paracrine effects originating from differentiated iPSCs and/or host tissues on residual iPSCs. Secreted factors could have profound effects on iPSCs and their derived products after transplant. For example, RPE is known to secrete a variety of cytokines, connective tissue proteins, extracellular matrix proteins, complement factors, proteases, and protease inhibitors[4]. In this report, we studied the non-autonomous trans-effects of RPE on iPSCs and discuss the safety concerns for tumor formation from residual iPSCs in iPSC-derived RPE.

Results

Differentiation of iPSC into RPE cells. In an effort to establish a robust differentiation protocol for pluripotent stem cells into retinal pigment epithelium (RPE), the differentiation protocol shown in Figure 1A was used. In this

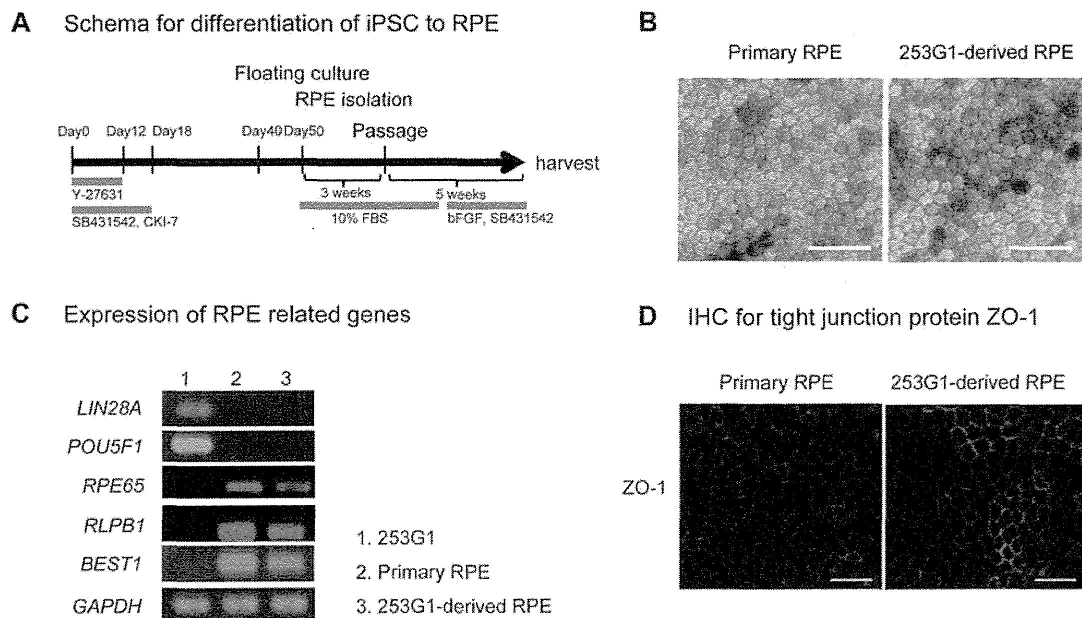

Figure 1 | Characterization of pigment epithelial cells derived from iPSC. (A) Protocol for differentiation to RPE from iPSC clone. (B) Phase contrast images of primary RPE (left panel) and iPSC clone 253G1-derived RPE (right panel). Scale bar = 50 μm. (C) Expression of pluripotency-related undifferentiated marker genes (*LIN28A* and *POU5F1*) and RPE-specific genes (*RPE65*, *RLPB1*, and *BEST1*) detected by qRT-PCR. *GAPDH* was used for internal gene expression control. (D) Immunofluorescence staining of tight junction protein ZO-1. Secondary antibody Alexa 488 was used to visualize the staining. Scale bar = 50 μm.

report, we used a commercially available iPSC clone 253G1[5] (Riken Bio Resource Center, Tsukuba Japan) as a cell source for RPE differentiation to present a reproducible profile of iPSC-derived RPE. RPEs are sporadically pigmented, polygonal in shape, and grow in monolayers when cultured in dishes. iPSC clone 253G1 derived-RPE and primary RPE showed the same morphology in microscopic observation (Fig. 1B). To determine whether iPSC-derived RPE cells possessed the characteristic gene expression of primary RPE, the expression of *RPE65*, *RLPB1*, and *BEST1* was analysed by RT-PCR. 253G1-derived RPE cells expressed the *RPE65*, *RLPB1*, and *BEST1* messages, but not pluripotency-related genes such as *LIN28A* and *POU5F1* (Fig. 1C). Tight junction specific protein, ZO-1 was also detected both in 253G1-derived RPE and primary RPE by immunofluorescent staining (Fig. 1D).

Cell growth of iPS cells co-cultured with iPSC-derived RPE was drastically perturbed. To explore the effect of factors secreted by iPSC-derived RPE on iPSCs *in vitro*, we conducted co-culture experiments (Fig. 2A). iPSCs seeded on Matrigel-coated culture (Transwell) inserts were co-cultured with iPSC-derived RPE seeded on CELL start-coated dishes in iPS medium (ReproFF2 supplemented with bFGF). The iPSCs in the culture insert were harvested every four days and the cell number was scored. We found that the proliferation of iPSC was significantly inhibited by co-culturing with iPSC-derived RPE (Fig. 2B,C). It is notable that a similar trans-effect was observed when iPSCs were co-cultured with primary RPE (Supplementary Fig. 1A–C). Marked inhibition of the growth of iPSCs co-cultured with iPSC-derived RPE was, at least partly, mediated by apoptotic cell death, as shown by the presence of TUNEL-positive cells (Fig. 2D, E). Furthermore, immunostaining and qRT-PCR study of the remaining iPSCs in co-culture showed that the expression of pluripotent-related genes (such as *LIN28A*, *POU5F1*, and *NANOG*) was markedly reduced, suggesting that the conditioned medium from iPSC-derived RPE induced cell death and at the same time promoted differentiation of iPSCs (Fig. 2F,G).

This observation prompted us to explore the factors from iPSC-derived RPE and primary RPE that had a trans-effect on iPSC cell proliferation. We performed microarray analysis with the GeneChip® system (Affymetrix), studying primary RPE, the 253G1-derived RPE and the parent iPSC clone 253G1. Several secreted factors were identified, with high message expression in both primary RPE and iPSC-derived RPE but only low/no expression in iPSC. For example, pigment epithelium-derived factor (PEDF), vascular endothelium growth factor (VEGF), bone morphogenetic protein 4 (BMP4), microsomal glutathione S-transferase (MGST), and glutathione S-transferase mu3 (GSTM3) showed high levels of message [Supplementary Table 1 and Data Set in GEO http://www.ncbi.nlm.nih.gov/gds (GEO number: GSE43257)]. Among those molecules, PEDF, VEGF, BMP4 have been reported to affect differentiation, proliferation, migration, and apoptosis[9,10,11] Thus, they were extracted and examined for a trans-effect on iPSCs.

Apoptotic cell death of iPSC is partly mediated by PEDF. Using a specific anti-PEDF antibody, PEDF protein (with a size of 50 kDa) was detected by Western blotting (Fig. 3A) in the conditioned medium of iPSC-derived RPE, and in cell lysates of both iPSC and iPSC-derived RPE. Fresh iPSC medium (medium without co-culturing) was used as a control sample. The amount of PEDF present after 24 h of cell culture (24 hours after changing with fresh medium) was measured by ELISA. The conditioned media from both primary RPE and iPSC-derived RPE contained a considerable amount of PEDF (more than 1 μg/mL) (Figure 3B).

VEGF and BMP4 in the conditioned media from primary RPE or iPSC-derived RPE cell cultures were detected by ELISA (Supplementary Fig. 2A,B). However, addition of 0.1 μg/mL, 5 μg/mL, or 20 μg/mL of recombinant VEGF (rVEGF, Peprotech), or 0.02 μg/mL, 1 μg/mL, or 4 μg/mL of rBMP4 (Peprotech) failed to alter cell growth markedly (Supplementary Fig. 2C). Then, we examined the effect of PEDF on the growth of iPSCs. To address this, a specific neutralizing antibody for PEDF (BioProducts, MD) was added to the co-culturing system and the proliferation of iPSCs in the culture

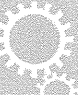

Figure 2 | Cell growth of iPSCs co-cultured with iPSC-derived RPE was perturbed. (A) Schema for co-culturing iPSC with RPE. iPSCs were maintained in culture inserts coated with Matrigel and co-cultured with iPSC-derived RPE seeded on the bottom of the dishes in iPSC culture medium. (B) Phase contrast photos of iPSC clone 253G1 in 12-well Transwells either cultured alone or co-cultured with 253G1-derived RPE on the designated day of culture. Scale bar = 500 μm. (C) Growth curve of iPSC clone 253G1 co-cultured with 253G1-derived RPE or cultured alone (w/o co-culture). The number of iPSC clone 253G1 cells in 12-well Transwells at the designated day of culture was scored. Means of three independent experiments are plotted on a linear graph with standard deviation (SD). *, $P < 0.05$, compared as indicated. (D) Apoptotic cell death was examined (or analyzed) by TUNEL assays and visualized as white spots on day six of culture. Scale bar = 200 μm. (E) Ratio of TUNEL-positive cells to DAPI positive 253G1 cells (as a percentage) either cultured alone (w/o co-culture) or co-cultured with 253G1-derived RPE. Mean results (with SD) from four independent experiments. **, $P < 0.005$, compared as indicated. (F) 253G1 cells co-cultured with 253G1-derived RPE markedly lost the expression of undifferentiated marker Oct3/4 (POU5F1) after six days of culture. Cells were stained with antibody for Oct3/4 (POU5F1), and then visualized with secondary antibody Alexa 488 (green, right panels). Nuclei were stained with DAPI (blue, left panels). Scale bar = 200 μm. (G) Fold-decrease of indicated mRNAs in iPSC resulting from co-culturing with iPSC-derived RPE. mRNA levels of *LIN28A, POU5F1* or *NANOG* in 235G1 were measured by quantitative RT-PCR. *GAPDH* was used as an internal control to normalize the mRNA levels of these genes. Mean results (with SD) derived from three independent experiments. **, $P < 0.005$, compared as indicated.

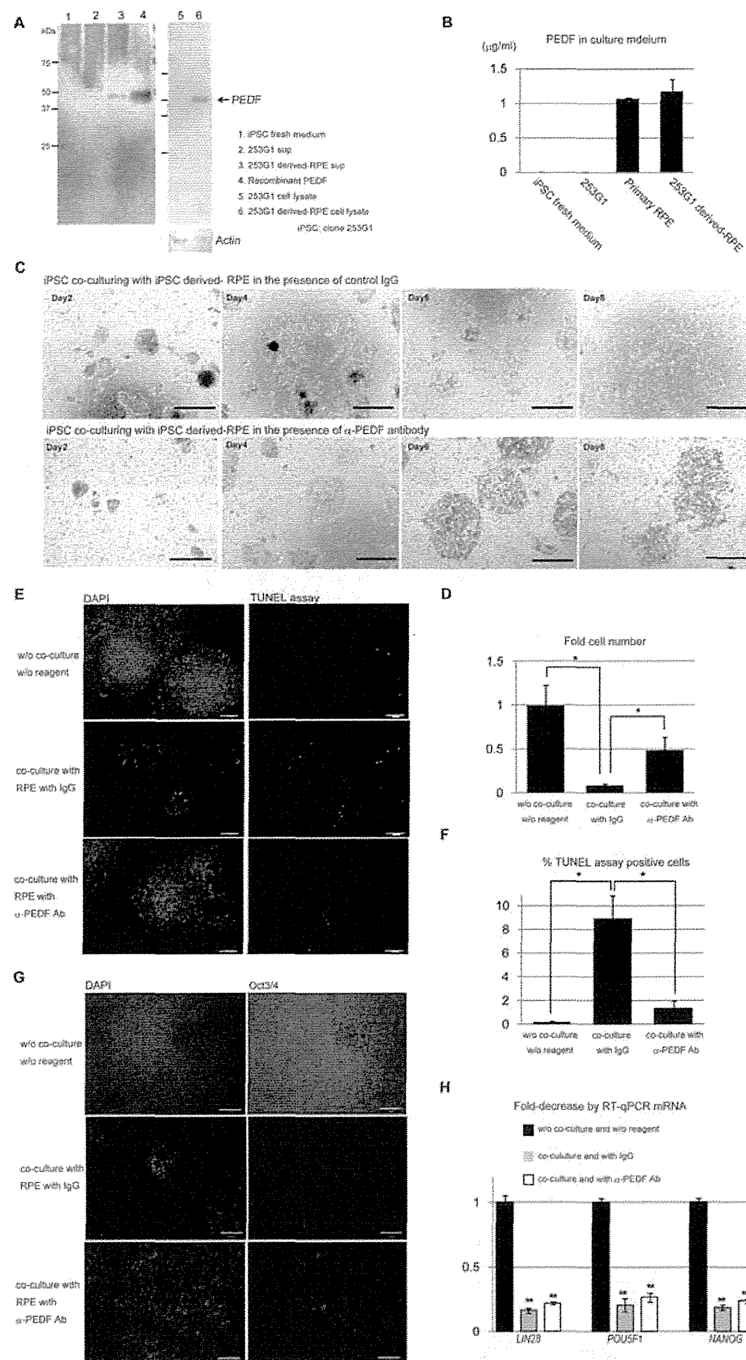

Figure 3 | Addition of antibody against PEDF blocked apoptotic cell death in iPSC induced by co-culture with RPE. (A) Western blot of iPS fresh culture medium, 253G1 sup, 253G1-derived RPE sup and recombinant PEDF, or cell lysates of 253G1 and 253G1-derived RPE using an anti-PEDF specific antibody. Detection of actin was used as a loading control for cell lysates. (B) Quantitation of PEDF in iPSC fresh culture medium, 253G1 sup, primary RPE or 253G1-derived RPE conditioned medium by ELISA. Mean results of three independent experiments (with SD). (C) Phase contrast images of 235G1 co-cultured with 253G1-derived RPE in the presence of control IgG1 or anti-PEDF antibody on the designated day of culture. Scale bar = 500 μm. *(D)* Fold-change in the number of 253G1 cells co-cultured with 253G1-derived RPE in the presence of IgG1 or anti-PEDF antibody after six days of incubation. Cell counts were compared to 253G1 cultured alone without reagent. Mean results of four independent experiments (with SD). *, $P < 0.05$. (E) 235G1 co-cultured with 253G1-derived RPE in the presence of control IgG1 or anti-PEDF antibody after six days of culture were examined by TUNEL assay visualized as white spots. 253G1 cultured alone without reagent was used as the control. Scale bar = 200 μm. (F) Ratio of TUNEL assay-positive 253G1 cells to DAPI positive cells when 253G1 cells were co-cultured with 253G1-derived RPE in the presence of control IgG1 or anti-PEDF antibody after six days of incubation. Mean results (with SD) from four independent experiments. *, $P < 0.05$, compared as indicated. (G) 253G1 cells co-cultured with 253G1-derived RPE markedly lost the expression of undifferentiated marker Oct3/4 (POU5F1) after six days of incubation. Cells were stained with antibody for Oct3/4, and then visualized with secondary antibody Alexa 488. Nuclei were stained with DAPI. Scale bar = 200 μm. (H) Fold-decrease of indicated mRNAs in iPSC resulting from co-culturing with 253G1-derived RPE. mRNA levels of *LIN28A, POU5F1* and *NANOG* were measured by quantitative RT-PCR. *GAPDH* was used as an internal control to normalize the mRNA expression levels. Mean results of three independent experiments (with SD). **, $P < 0.005$.

inserts was examined. Growth inhibition of 253G1 cells co-cultured with 253G1-derived RPE was observed in the presence of control IgG. However, growth inhibition was efficiently blocked by anti-PEDF antibody (Fig. 3C). Almost half of the iPSCs were rescued by addition of 5 μg/mL polyclonal anti-PEDF neutralizing antibody (Fig. 3D). Specifically, it appeared that neutralizing antibody against PEDF reduced apoptotic death of iPSCs (Fig. 3E, 3F). Based on this experiment, we concluded that PEDF induced cell death of iPSCs.

Next, we examined whether PEDF could promote differentiation of iPSCs as well as induce cell death. iPSCs co-cultured with RPE in the presence of control IgG initiated differentiation as evidenced by a decrease of *LIN28A, POU5F1* and *NANOG* message levels. This message reduction was not attenuated by the addition of anti-PEDF antibody (Fig. 3G, 3H), suggesting that PEDF contributed to the induction of iPSC death but not to iPSC differentiation. VEGF and BMP4, known to induce pluripotent stem cell differentiation, were also detected in the RPE-conditioned medium by ELISA (Supplementary Fig. 2 A, B). We hypothesize that those factors could contribute to the differentiation of iPSCs. However, most iPSCs are subject to cell death by PEDF in RPE-conditioned medium (Fig. 2B, C). Thus, the differentiation of the remaining iPSCs induced by these factors, if any, might well be masked.

To directly address the effects of PEDF on the growth of iPSC, we used recombinant PEDF protein (rPEDF, Millipore). The biological activity of procured rPEDF was titered with human umbilical vein endothelial cells (HUVEC), as PEDF reportedly has anti-angiogenic function[12]. Indeed, the conditioned medium from RPE showed a cell growth inhibitory effect on HUVEC (Supplementary Fig. 3A). Thus, we examined several doses of rPEDF (Supplementary Fig. 3B) for its growth inhibitory effect on HUVEC. We found that 50 μg/mL PEDF possessed a biological effect on HUVEC comparable to that of 1/4 volume of conditioned medium mixed with HUVEC medium (M-200 supplemented with LSGS). There was no cell growth inhibitory effect under 50 μg/mL of rPEDF. Therefore, we used 50 μg/mL of rPEDF for further examination of the effect of rPEDF. At 50 μg/mL rPEDF, we observed increased apoptosis in HUVECs (Supplementary Fig 3C), as well as a growth inhibitory effect (Supplementary Fig 3D). To rule out the possibility that the high dose of recombinant protein contained various non-specific factors that might have non-specifically induced cell death, neuroblastoma SK-N-BE (2) and primary RPE cells were cultured with 50 μg/mL of rPEDF. We found that 50 μg/mL rPEDF did not change either the morphology or reduce the number of neuroblastoma cells (Supplementary Fig. 3E) or primary RPE (Supplementary Fig. 3F).

One plausible explanation for the marked gap in dosage between the amount of PEDF in the conditioned medium and the biologically relevant dose of rPEDF would be low biological activity of rPEDF due to altered post-transcriptional modification of PEDF when it is produced in Baby Hamster Kidney cells. Fifty μg/mL rPEDF inhibited cell growth of iPSC (Fig. 4A,B) and induced apoptotic cell death as evidenced by TUNEL assay (Fig. 4C,D). It is interesting to note that 50 μg/mL of rPEDF also induced apoptotic cell death in human ES cells (khES01) (Supplementary Fig.4). The morphology of the remaining iPSCs after rPEDF addition was the same as untreated iPSC (Fig. 4A). Moreover, reductions in the messages of pluripotency related-genes *LIN28A, POU5F1*, and *NANOG* in the remaining cells were not observed (Fig. 4E). The cell number counted by scoring DAPI-positive cells after rPEDF treatment was not constant. That may account for the up-regulation of message of pluripotency related-genes after addition of rPEDF.

We next explored the PEDF-mediated signal pathway leading to apoptosis in iPSCs. Western blotting detected phosphorylation of p38 mitogen-activated protein kinase (MAPK) and cleaved caspase-3 after rPEDF stimulation of iPSC (Fig. 4F,G). Taken together, it is conceivable that PEDF induced the apoptotic death of iPSC, but did not induce differentiation of iPSC.

RPE cell sheet suppressed tumor formation potential of iPSC when co-transplanted *in vivo*. We plan to transplant a cell sheet of RPE to the retinas of patients who suffer from aged macular degeneration. Specifically, we will use one to two RPE cell sheets (1.3 mm × 3 mm), consisting of approximately $2 - 5 \times 10^4$ RPE cells. The RPE sheet is prepared on a collagen gel [Kamao H, et al. manuscript submitted]. The possibility of tumor formation from residual undifferentiated iPSCs or incompletely differentiated cells in an iPSC-derived product after transplant remains an issue. To evaluate the trans-effect of RPE on the remaining iPSCs after transplant to the retina, we set up a series of iPSC "spike tests" in the presence of RPE sheets using immunosuppressed animals. We tested the tumor formation potential in several immunosuppressed animals by injecting several doses of iPSCs either subcutaneously or in the retina. Recipient animals included rat (nude rat: F344/NJcl-rnu/rnu) and mouse (Nude: BALB/cA, JCl-nu/nu; SCID: C.B-17/Icr-scid/scid, Jcl; NOD-SCID: NOD/ShiJic-scid, Jcl; NOG: NOD/ShiJic-scid, IL-2Rγ KO Jic). We found the NOG mouse was the most sensitive animal in terms of tumor formation from iPSCs and HeLa cells when injected subcutaneously with Matrigel (BD), in agreement with a previous report[13]. Then, 10^2, 10^3, or 10^4 iPSCs (clone 253G1 or 454E2) were co-transplanted into NOG mice subcutaneously without or with iPSC clone 253G1 or a 454E2 derived-RPE sheet consisting of approximately 2.5×10^5 RPE cells. The mice were monitored for tumor development at the site of injection for 30 weeks. Three-way ANOVA (factors: dose of iPSCs, clone of iPSCs, presence of RPE cell sheet) and the post-hoc Student-Neuman-Keuls test for latency of tumor formation indicated that tumors appeared significantly earlier in the groups inoculated with 10^3 or 10^4 iPSCs, compared with that inoculated with 10^2 iPSCs. (Fig. 5, $P < 0.001$). More importantly, the statistical analysis indicated a significant difference between the groups with and without RPE sheet ($P < 0.01$), whereas there was no difference between the iPSC clones.

Discussion

In this report, we demonstrated that both primary and iPSC-derived RPE secreted PEDF that induced apoptosis in iPSC.

To elucidate the mechanism by which the tumor (teratoma) forming capacity of iPSCs was suppressed when the RPE cell sheet was co-transplanted, additional studies are required. It is possible that nonspecific effects of the transplanted RPE sheet could compete with the tumor for endogenous growth substrates. Alternatively, it might induce the host immune system to attack co-transplanted cells or reduce the size of the tumor through an anti-angiogenic effect of PEDF as reported[16,17]. However, we have some suggestive data pertinent to this issue. Effect of PEDF on cell growth *in vitro* varies depending on cell type. Indeed, reduction of HeLa cell number was not drastic compared with that of iPSC cell number after the RPE conditioned medium treatment (Supplementary Fig. 5A). In this context, HeLa cells formed tumors when as few as one hundred cells were injected into the retinas of nude rats ($TPD_{50} = 32$). In contrast, injection of as many as one thousand hiPSCs into nude rat retinas did not generate teratomas ($TPD_{50} = 31623$) (Supplementary Fig. 5B). These experiments suggested that a non-autonomous effect of RPE *in vivo*, if any, is cell-type specific, and that RPE selectively suppress the growth of iPSCs when iPSCs are transplanted in RPE or co-transplanted with RPE.

Considering the fact that we plan to transplant $4 - 8 \times 10^4$ iPSC-derived RPE cells in a clinical setting and have developed a highly sensitive iPSCs detection system using qRT-PCR[3] (theoretically capable of detecting iPSCs in RPE cells when iPSCs constitute only 0.01% of the total cell product), the chances of a tumor formation from the undetectably low number of residual iPSCs in iPSC-derived RPE cell sheet following the transplantation should be extremely low in the presence of PEDF secreted from RPE.

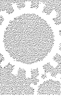

Figure 4 | Recombinant PEDF (rPEDF) induced apoptotic death in iPSCs. (A) Phase-contrast images of iPSC clone 253G1 without or with rPEDF (50 μg/mL) after four days of culture. Scale bar = 200 μm. (B) Fold-change in the number of 253G1 cells cultured with rPEDF (50 μg/mL) after four days of culture, compared with the number of 253G1 cells cultured without rPEDF. Mean results of three independent experiments (with SD). *, $P < 0.05$, compared as indicated. (C) Apoptotic death of 253G1 cells was examined by the TUNEL assay and visualized as white spots (right) after four days of culture. Nuclear staining with DAPI is shown on the left. Scale bar = 200 μm. (D) Ratio of TUNEL positive cells to DAPI positive cells, as a percentage, when cultured with or without recombinant PEDF. Mean results of three independent experiments (with SD). *, $P < 0.05$, compared as indicated. (E) mRNA levels of *LIN28A, POU5F1* and *NANOG* in 253G1 cells after four days of culture without or with rPEDF (50 μg/mL) were measured by qRT-PCR. *GAPDH* was used as an internal control to normalize mRNA expression levels. Fold-decrease or increase of respective mRNAs in iPSC. Mean results of three independent experiments (with SD). (F) Left panels: phosphorylated p38 MAPK (P-p38) or p38 MAPK (p38) after six hr serum starvation of iPSCs (−), and five min (5 min) or 15 min (15 min) after addition of PEDF (50 μg/mL) [PEDF(+)]. Proteins were detected by Western blotting with specific antibody. p38 was used as an internal control. Right panels: phosphorylated p38 MAPK (P-p38) in serum starved iPSCs (-), 10 min after addition of PEDF (50 μg/mL) in the absence (PEDF + DMSO) or presence of p38 inhibitor SB203580 (PEDF + SB203580). Proteins were detected by Western blotting. (G) Cleaved Caspase-3 after six hr serum starvation of iPSCs [−], or ten min after addition of PEDF (50 μg/mL) [PEDF(+)] was detected by Western blotting with specific antibody.

PEDF is a 50 kDa secreted protein that is also known as serpin F1[14,15]. PEDF is reported to possess various biological functions including inhibition of endothelial proliferation[9,11] and angiogenesis[16,17], as well as neurophilic functions[18,19,20] and induction of apoptosis[21,22], after binding to its receptor[23]. PEDF enhances gamma secretase activities leading to cleavage of VEGF receptor-1[24] and VEGF receptor-2[25], and induces an anti-angiogenic protein, thrombospondin[26]. With regard to angiogenesis and endothelial cell proliferation, RPE secretes both counter-acting PEDF and VEGF. RPE, however, maintains the microenvironment and the structure of the retina by secreting these factors into a different side of the retinal membrane. These facts necessitate RPE sheet transplantation in the proper orientation rather than as single RPE cells to ensure the function of retina. PEDF is reported to promote the differentiation of primitive retinal cells[27]and retinoblastoma cells[19], but the effect of PEDF on iPSC seems to be limited to induction of apoptotic cell death, not neural differentiation of iPSC.

PEDF reportedly stimulates several signal pathways including activation of Ras, NF-kB[18], FAS/FASL[12], PPAR-gamma, and the p53-mediated pathway[21]. The p38 MAPK-mediated cleavage of caspases is also reported in endothelial cells[22]. In this study, we showed the activation of p38 and cleavage of caspase-3 after

Figure 5 | iPSC clones 253G1 or 454E2 (10^2, 10^3 and 10^4 cells) were subcutaneously transplanted to NOG mice (three per group) without or with a 253G1-derived RPE sheet or a 454E2-derived RPE sheet, respectively. The sheets consisted of (approximately) 2.5×10^5 RPE cells. The Y-axis shows the week when the tumors were first detected in each case. The number of iPSC cells and iPSC-derived RPE are shown on the X-axis. The numbers of mice without tumor formation are shown as symbols below the X-axis.

rPEDF stimulation in iPSC (Fig. 4F). Therefore, it is conceivable that p38 MAPK-dependent cleavage of multiple caspases could lead to apoptosis in iPSC after PEDF stimulation. Recently, it was reported that PEDF activated ERK1/2 and maintained growth of hESC[28]. They used 100 ng/mL rPEDF to show activation of ERK1/2 in serum-starved hESC, and ERK1/2 inhibitor PD98059 inhibited growth of hESC. Based on these experiments, they concluded that PEDF maintained cell growth of hESC via ERK1/2 activation. ERK1/2, key signal molecules, activate multiple signals leading to various biological responses. Blocking ERK1/2 activities will inevitably suppress multiple critical cellular responses and not necessarily address a PEDF specific-signal event. In our experiments, we did not observe a biological process resulting from rPEDF stimulation below 50 μg/mL (Supplementary Fig. 3B). Thus, we believe 100 ng/mL rPEDF might be enough to initiate ERK1/2 signaling, but not enough to initiate cellular events in hiPSC or hESC.

In summary, we showed a novel effect of PEDF on the survival of remaining iPSCs in iPSC-derived RPE and suggest further application of PEDF in pluripotent stem cell-based cell therapy in the future.

Methods

All the experiments using human samples and animal studies were approved by the IRB of the Foundation for Biomedical Research and Innovation (FBRI) and Riken Center for Developmental Biology (Riken CDB), and the committee for animal experiments of the FBRI.

Cell culture. Human primary retinal pigment epithelium (RPE, Lonza) was maintained in Retinal Pigment Epithelial Cell Basal Medium (Lonza Biologics, Basel, Switzerland) containing supplements (L-glutamine, GA-1000, and bFGF; Lonza). Human iPS cell (iPSC) lines 253G1[5] [Riken Bio Resource Center (Tsukuba, Japan)] and 454E2[6] were maintained on feeder cell SNL[7] in human ES cell culture medium and 5 ng/mL bFGF (Peprotech). iPSCs were cultured in ReproFF2 (ReproCELL) supplemented with 5 ng/mL bFGF medium. iPSC-derived RPE[3,8] was maintained in RPE maintenance medium [DMEM:F12 (7 : 3) (Sigma-Aldrich) containing B-27 supplement (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.5 mM SB431542 (Sigma-Aldrich) and 10 ng/mL bFGF (Wako)] . HUVECs (BD™) were maintained in M-200 supplemented with LSGS and neuroblastoma cells (SK-N-BE (2), ATCC) were cultured in DMEM containing 10% FBS.

Cell growth of iPSCs, HUVECs and neuroblastoma cells in the absence or presence of recombinant PEDF or anti-PEDF antibody. 253G1 cells were seeded in Matrigel (BD Bioscience)-coated 12-well Transwell cell culture inserts with an 8 μm pore size (BD). They were co-cultured with primary RPE or 253G1 derived-RPE seeded on the bottom of dishes in ReproFF2 medium supplemented with bFGF in the absence or presence of one to 50 μg/mL rPEDF (Millipore, cat # GF134 lot: DAM 1821182) or 5 μg/mL polyclonal anti-PEDF blocking antibody (cat # AB-PEDF1, BioProducts MD)[29,30,31,32] or 5 μg/mL non-functional control rabbit IgG (Santa Cruz). Cell growth of 253G1, HUVEC, neuroblastoma and primary RPE in the absence and presence of 50 μg/mL rPEDF was evaluated after four to 6 days of culture (without co-culture).

Chip analysis. Total RNA from 253G1 or 253G1-derived RPE was isolated with a RNeasy Plus Mini Kit (Qiagen) in accordance with the manufacturer's instruction and hybridized with Gene Chip Human Genome U133 Plus ver. 2.0 (Affymetrix). Hybridized microarray data were scanned with a GeneChip Scanner 3000 7 G. Analyzed data can be retrieved from the GEO http://www.ncbi.nlm.nih.gov/gds/. Our GEO data set number is GSE43257.

ELISA. Levels of PEDF, VEGF or BMP4 in primary RPE or iPSC (253G1)-derived RPE culture medium (conditioned medium) collected after 24 h of culture were determined with human ELISA kits (PEDF, BioVendor; VEGF, eBioscience; BMP4, RayBiotech) in accordance with the manufacturers' instructions.

qRT-PCR. Total RNA was isolated with the RNeasy plus Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Contaminating genomic DNA was removed using a gDNA Eliminator spin column. cDNA was generated from one μg of total RNA using PrimeScript RT Master Mix (Takara Bio) and PrimeSTAR MAX DNA Polymerase (TaKaRa Bio). Real-time PCR was then performed with an ABI 7000 Sequence Detection System (Applied-Biosystems) and SYBR-green in accordance with the manufacturer's instruction. The primers designed for real-time PCR were as follows: for *LIN28A*, forward primer, 5'- CTGTCCAAATGCAA GTGAGG-3', reverse primer, 5'-GCAGGTTGTAGGGTGATTCC-3'; for *POU5F1*, forward primer, 5'- GAAGGTATTCAGCCAAACGAC-3', reverse primer, 5'- GTT ACAGAACCACACTCGGA-3'; for *NANOG*, forward primer, 5'- CTCAGCTACA AACAGGTGAAGAC-3', reverse primer, 5'- TCCCTGGTGGTAGGAAGAG TAAA-3'; for *RPE65*, forward primer, 5'- ATGGACTTGGCTTGAATCACTT-3', reverse primer, 5'-GAACAGTCCATGAAAGGTGACA-3'; for *BEST1*, forward primer, 5'-ATCAGAGGCCAGGCTACTACAG-3', reverse primer, 5'-TCCACAG TTTTCCTCCTCACTT-3'; for *RLPB1*, forward primer, 5'-GACTGGGG

TTAAATCTCACAGC-3′, reverse primer, 5′-TGACATGTTGCCTATGGAAGAC-3′; for *GAPDH*, forward primer, 5′- CGATGCTGGCGCTGAGTAC-3′, reverse primer, 5′-CCACCACTGACACGTTGGC3′. Respective gene expression levels were normalized to that of GAPDH.

TUNEL staining and Immunohistochemistry. Apoptotic cells were detected with the *In situ* Cell Death detection kit (fluorescein, Roche Diagnostics) in accordance with the manufacturer's instructions. The percentage of TUNEL-positive cells was calculated by scoring TUNEL-positive cells divided by total DAPI-positive cells in three non-overlapping areas (two mm^2 per well).

For immunochemical staining, cells were fixed with 4% paraformaldehyde followed by staining with antibodies against Oct3/4 (POU5F1) (1 : 100 dilution; sc-5279; Santa Cruz), or ZO-1 (1 : 200 dilution; Invitrogen). Antibodies were visualized with Alexa Fluor 488 goat anti-mouse (1 : 1,000; Invitrogen) or Alexa Flour 488 goat anti-rabbit (1 : 1,000; Invitrogen). Fluorescent microscopic images were captured with a fluorescent microscope (Olympus BX51, IX71; Tokyo, Japan).

Western blotting. Cell culture supernatants (conditioned media) or recombinant protein samples were loaded onto a 5 – 20% gradient SDS-polyacrylamide gel, subjected to electrophoresis under reducing conditions and blotted onto a PVDF membrane (BioRad). Blots were blocked with a solution of 3% nonfat dry milk/PBS/0.1% Tween-20 at room temperature, rinsed twice with PBS/0.1% Tween-20 and incubated with 1 : 200 diluted polyclonal anti-PEDF antibody (BioProducts MD), followed by 1 : 5000 diluted anti-rabbit IgG-HRP (Amersham). Detection of actin by anti-actin antibody (Santa Cruz I-19) was used as a loading control. Membranes were rinsed three times in PBS/0.1% Tween-20. Signals were detected with horseradish peroxidase using an ECL kit (Promega). Cell lysates were made from iPSCs that were serum-starved for six h (−) or five min or 15 min after addition of PEDF in the absence or presence of p38 MAPK inhibitor SB203580 (Cell Signaling). Lysates were blotted onto PVDF membranes, and anti-phospho-p38 antibody (Cell Signaling), anti-p38 antibody (Cell Signaling) or anti-caspase 3 antibody (Cell Signaling) was used to detect the respective molecules.

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Author contributions

H. Kanemura conducted all of the biological assays and prepared the manuscript; M.G. designed the research; N.N. analyzed the gene chip data; N.S. supplied the iPSC and iPSC-derived RPE; H. Kamao supplied the RPE sheets; Y.S. performed the statistical analysis of transplantation experiments; M.T. performed the QC for the RPE sheets and interpreted the *in vivo* experiments; S.K. supervised all the experimental results and edited the manuscript.

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再生医療製品の造腫瘍性・悪性腫瘍形成能の評価

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再生医療または細胞治療に用いる目的で，ヒト体細胞，体性幹細胞，ES細胞，iPS細胞などに培養などの加工を施して製造・調製されるものは再生医療製品(または細胞・組織加工製品)とよばれている．再生医療製品のリスクのひとつとして“造腫瘍性”があげられる．一般的には“造腫瘍性のリスク”と一口にいわれることが多いが，安全性上の視点から大きく2つ，すなわち“腫瘍による物理的障害のリスク”と“悪性腫瘍形成のリスク”に分けられる．“腫瘍による物理的障害”とは腫瘍形成により周辺組織が圧迫などを受けることによる障害で，関節再生・脊髄再生などのケースで問題となる．この場合はたとえ良性であっても腫瘍自体がリスクファクターとなる．一方，“悪性腫瘍形成”は腫瘍の悪性度がリスクファクターとなる．

ヒト体細胞・体性幹細胞由来製品

移植医療の現場では細胞・組織の造腫瘍性の評価がほとんど行われていない．つまり，再生医療製品の原材料となるような未加工のヒト体細胞・体性幹細胞には一般的に造腫瘍性がないと考えられていることになる．また，世界各地でヒト体細胞・体性幹細胞に由来する再生医療製品の臨床応用が進んでいるが，これらの製品の投与を原因とする腫瘍形成の学術論文としての報告は，ヒト胎児由来神経幹細胞を用いた毛細血管拡張性運動失調症の治療により脳腫瘍が形成されたとするもの1件しかない[1]．成人由来の体細胞または体性幹細胞を原材料とした再生医療製品に限れば，患者への投与による腫瘍形成の報告は著者らの知るところでは存在しない．

過去にヒト間葉系幹細胞の *in vitro* 培養時の自発的な悪性形質転換が4件報告されているが，このうち2件[2,3]は癌細胞株のクロスコンタミネーションによるものであることが後に判明している．また，残りの2件[4,5]では *in vitro* 培養時に細胞の不死化が確認されている．これらのことは，最終製品への悪性腫瘍細胞のクロスコンタミネーション防止および細胞増殖特性の把握が重要であることを示している．したがって，十分な工程管理の下に培養・加工され，細胞増殖特性解析で異常がないことを確認した成人体細胞・体性幹細胞由来の再生医療製品については，非臨床安全性試験として免疫不全マウスを用いた造腫瘍性試験を行う必要性はあまり高くないと考えられる．

ヒトES/iPS細胞由来製品

ヒトES細胞またはiPS細胞に由来する再生医療製品には未分化ES/iPS細胞の残留の可能性がある．したがって，残存未分化ES/iPS細胞に起因する奇形腫形成能(造腫瘍性)の評価・管理が重要となる．ヒトES/iPS細胞が増殖分化して形成する奇形腫は多くの場合，良性であり，正常二倍体のヒトES細胞を免疫不全マウスに移植して悪性腫瘍が発生したという報告はない．一方，ヒト由来iPS細胞に関しては免疫不全マウスに投与した場合に悪性腫瘍が形成されたという報告が存在する[6]．また，iPS細胞樹立時の細胞初期化過程は悪性形質転換の研究で従来用いられてきた発癌フォーカス形成試験(*in vitro* での遺伝子導入による悪性肉腫形成試験)との間での類似性が指摘され，共通の機序の存在が提唱されている[7]．

ヒトiPS細胞由来の再生医療製品のなかに残存する未分化iPS細胞の造腫瘍性にはさまざまな要素，すなわち目的細胞への分化の難しさ，ヒトiPS細胞の原材料となる体細胞の種類，ヒトiPS細胞中における初期化因子残存の有無[6]など，ヒトiPS細胞に付随する要因と，投与部位，投与細胞数，マトリゲルなどの共投与物の有無といった外部要因とが影響する．再生医療製品の原材料としてのヒトiPS細胞に内在する奇形腫悪性化にかかわる因子・機序の詳細は明らかではなく，悪性腫瘍を形成しやすい細胞株の判定方法および使用防止策を明らかにすることは今後の課題である．ただし，ヒトES/iPS細胞由来製品の造腫瘍性を評価するうえでは“原材料となる幹細胞の造腫瘍

性と最終製品の造腫瘍性との相関・因果関係は未解明である"という点に最大の注意が必要である．すなわち，臨床適用に際しては原材料となるヒトES/iPS細胞ではなくあくまで最終製品としてのヒトES/iPS細胞由来製品の造腫瘍性評価がもっとも重要であることにつねに留意しなければならない．

新技術による造腫瘍性評価の可能性

最近，ヒトiPS細胞由来の再生医療製品および原材料としてのヒトiPS細胞の造腫瘍性・悪性腫瘍形成能の評価に先端的解析技術である次世代シーケンサーを利用できないかという議論がある．ただし，原材料のiPS細胞またはiPS細胞由来製品に含まれる細胞の遺伝子変異による造腫瘍性の変化の評価を目的として全ゲノムシーケンス解析や全エクソンシーケンス解析を行うことは，現実的にはあまり用をなさない．その理由は再生医療製品の安全性との因果関係が明瞭な遺伝子変異の具体例は非常に乏しく，個々の製品の安全性の指標としての各変異の有用性がいまのところまだほとんど明らかでないからである．あえて行うのならばむしろ，発癌リスクと非常に高い相関があることが既知である少数の遺伝子変異について何らかの高精度・高感度な方法で解析し，わずかな変異の出現を検出する努力をする方が有用と考えられる．再生医療製品の開発における次世代シーケンサーの用途としてはほかに，縦列型反復配列(short tandem repeat：STR)解析などの代替として原材料iPS細胞の同一性評価を目的とした利用や，比較ゲノムハイブリダイゼーション(comparative genomic hybridization：CGH)解析などの代替として細胞のゲノム不安定性の評価を目的とした利用も考えられるが，その際には各目的に応じた試験系の性能と限界についての科学的検討が事前に必要である．

新しい技術が開発されても，それがただちに製品の安全性評価に適用できるとは限らない．すなわち，その技術による試験の結果を受け，製品開発，製造および臨床の場において具体的にどういう判断が可能なのかが明らかでなければ安全対策としての試験の意味をなさないということに注意しなければならない．要するに，すくなくとも現段階では細胞の造腫瘍性の変化，不死化，悪性形質転換を評価するには，たとえば*in vivo*造腫瘍性試験，軟寒天コロニー形成試験，*in vitro*細胞増殖速度評価など，直接的に細胞増殖特性を評価する試験の実施を考えることが先決であることには変わりないということである．

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細胞・組織加工製品の開発環境整備に向けたレギュラトリーサイエンス研究

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Regulatory science research to facilitate the development of cell/tissue-processed products

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Regenerative medicine is regarded as innovative therapy for severe diseases and damages caused by tissue loss and functional impairment. In Japan, regenerative medicine is one of the most important subjects issued by Council for Science and Technology Policy and also referred to in Medical Innovation of New Growth Strategy. Cell/tissue-processed products are living cells, which have been manipulated or processed for the purpose of regenerative medicine, and are extensively developing. Human somatic cells, somatic stem cells, embryonic stem cells, and induced pluripotent stem cells are cell sources used for regenerative medicine. Since we lack in experiences with cell/tissue-processed products, technical development of safety and quality assessment is urgently needed. National Institute of Health Sciences has carried out a mission of Regulatory Science and worked on safety assessment of pharmaceuticals and medical devices and their guideline development. The objective of our study is to develop safety and quality assessment methods for cell/tissue-processed products derived from stem cells, based on recent progresses in life science. We are currently developing methods to evaluate products as follows; a) useful and quantitative tumorigenicity tests to detect contamination of undifferentiated and/or abnormal cells in products, b) quality assessment by gene expression analysis and detection of genetic stability in a manufacturing process, and c) analysis of quality attributes associated with propensity of undifferentiated cells to set acceptable criteria of cell banks. We will be able to provide indicators to control the quality, efficacy and safety of stem cell-processed products and support efficient and economical promotion of the products. Especially, this study would help translate stem cell science into therapeutic products to patients with severe and life-threatening diseases, consequently contributing to administrative policy of Ministry of Health, Labor and Welfare.

Keywords: cell/tissue-processed products, induced pluripotent stem (iPS) cells, regenerative medicine

研究目的

再生医療は，身体の一部の機能不全や欠損による重篤な疾患や障害を治療できる革新的な方法として注目されており，総合科学技術会議の提言や「新成長戦略」のメディカルイノベーションなどにおいても最重要課題とされている．平成25年1月11日閣議決定の『日本経済再生に向けた緊急経済対策』でも，iPS細胞等を用いた再生医療等に係る研究開発・実用化を支援する環境整備に取り組むことが明記されている．平成25年2月には再生医療等の新規医療産業の国際競争力を高める司令塔機能として，内閣官房に『健康・医療戦略室』が設置された．また，平成25年4月26日成立の『再生医療推進法』には，再生医療の迅速かつ安全な研究開発及び提供並びに普及の促進に関する施策を総合的に策定及び実施する責務を国が有することが示されている．

再生医療（や細胞治療）に使用することを目的に生きた細胞を加工して製造される製品は細胞・組織加工製品と呼ばれ，国内外で活発に研究・開発が行われている．

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