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Commentary

Unconscious Exposure to Radiation

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When we consider the risk of radiation caused by the Fukushima Daiichi Nuclear Plant accident, we may feel the situation to be much like the formation of rain spots on a car. The dirty spots are difficult to tolerate by the owner of a brand-new car but can be accepted by a used car owner who does not clean his or her car frequently. In the course of collecting information to prepare a webpage concerning radiation risk on the Japanese Environmental Mutagen Society (JEMS) homepage following the Fukushima accident, I have learned that we have already unconsciously been exposed to an unexpected level of radiation. Therefore, our body is not like that brand-new car affected by rain spots or, in this case, radioactive contamination. We are internally exposed to ^{40}K radiation through the foods we eat on a daily basis, and we have already been exposed to the 1,000–10,000 times higher background of the nuclear fallout that occurred during the 1960s because of world-wide nuclear bomb experiments. It is important to know these facts to consider the excess risk derived from the Fukushima accident and thereby learn to be more cautious. Obtaining a proper answer scientifically about the health effects of low-level radiation exposure is very difficult when using available data on radiation biology. Increasing risk awareness and communication is also important together with proving the real risk of low-level radiation. Radiation risk should be considered in a relative manner by comparing it with other confounding factors, which together can be treated as a total risk. The increased risk posed by radiation exposure can be traded-off by reducing other risk factors affecting our lifestyle. The most important task for us is to transfer available scientific knowledge to the public such that the information is more understandable to help people make their own decisions on how to face radiation risk.

Key words: radiation risk, risk communication, risk assessment, Fukushima nuclear accident, low dose radiation

Introduction

Following the accident at the Fukushima Daiichi Nuclear Plant on March 11, 2011, public concern was focused on radiation risk. Because of the tragedies of Hiroshima/Nagasaki and Chernobyl, people fear the invisible risk of radiation. The pacifying comments (hiding of truth) provided by radiation specialists regarding the safety of nuclear plants after the accident compromised their reliability. In addition, there is a conflict even among radiation biologists regarding the estimation of the radiation risk caused by the accident. Therefore, an important task for scientists is to explain the level of radiation risk that actually exists in a more understandable manner.

When the accident occurred, I was a member of the public relation office of the Japanese Environmental Mutagen Society (JEMS). I then decided to create a webpage on the risk of radiation to provide useful information to the public. Throughout the course of preparing the page, I have learned many important facts about radiation exposure that I did not know before because I am not a radiation biologist. The most striking piece of information for me was the high background of the nuclear fallout that occurred during the 1960s.

Unconscious External Exposure

During the late 1950s to early 1960s, many nuclear bomb experiments were carried out worldwide by the US, the Soviet Union, and the UK, followed by France and China. The experiments were initially performed in the atmosphere and released an enormous amount of radioactive nuclides all over the world. In Tokyo, the nuclear fallout level reached its maximum in 1963, which was 1,000–10,000 times higher than the normal background before the Fukushima accident. The high fallout level has gradually decreased but has persisted over the decades.

Data on the environmental radiation level in the past and present are available at the Japan Chemical Analysis Center, which is directed by the Nuclear Regulation Authority (1). Although the species of nuclear fallouts from the nuclear bomb or the nuclear plant accident are different, Cs-137 (^{137}Cs) is a common concern for a long-lived radioactivity. Figure 1 shows the annual changes

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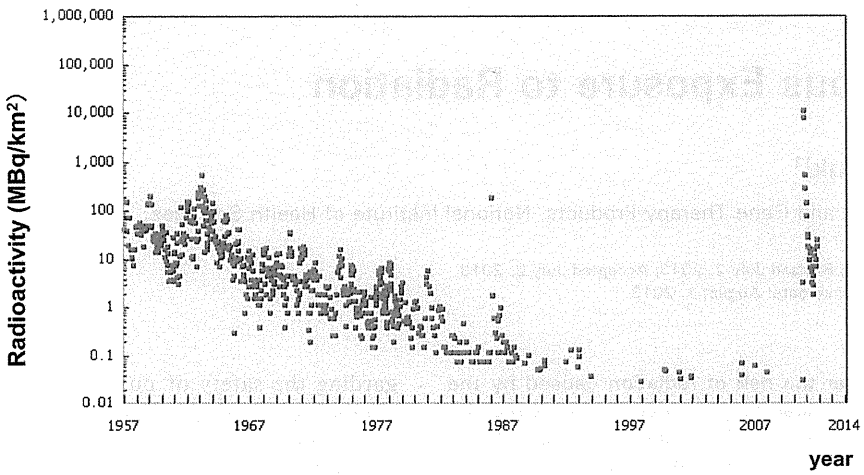

Fig. 1. (Color online) Annual changes in monthly nuclear fallout (^{137}Cs) level in Tokyo. The graph was created at the database site of the Nuclear Regulation Authority at http://search.kankyo-hoshano.go.jp/servlet/search.top (on June 5, 2013). Data are available for the period 1957–2012. Peaks in radioactivity are shown to have occurred in 1963 (atmospheric nuclear bomb experiments), 1986 (Chernobyl accident), and 2011 (Fukushima accident).

in the monthly nuclear fallout (^{137}Cs) from 1957 to 2011 in Tokyo. The data show that the peak after the Chernobyl accident (1986) is very similar to that in 1963, when the fallout from nuclear bomb experiments reached its maximum. However, after the Fukushima accident in 2011, the peak reached a level more than 10 times higher. Therefore, we have been exposed to the highest level of fallout in history in the wake of the Fukushima accident. This fact should have been announced at the time of the accident to reduce outdoor exposure as much as possible, especially during rain. Although the same data for Fukushima in 2011 were not available on the website of the Japan Chemical Analysis Center, the fallout level is considered to be at least higher than that in Tokyo. Fallout in Fukushima has nearly stopped now but the high level of fallout has already settled down in the area surrounding the nuclear plant, making it difficult for residents living within 20 km of the plant to return to their homes. Removing these sedimented radioactive nuclides remains an important task for the Japanese government.

I was born in 1962, when the nuclear fallout reached its maximum level. Therefore, I grew up under the high background radio contamination during the so-called sensitive younger generation. At that time, unfortunately, environmental pollution from industries was much worse than it is now in Japan. Thus, our generation has grown up in a dangerous environment but without any apparent health defects, such as increased incidence of cancer. There is a chance that a careful epidemiological survey according to annual birth groups to compare health defects in relation to radiation exposure may provide informative data on the effects of nuclear fallout in future.

In addition to artificial nuclear fallouts, we are also exposed externally to natural radiation sources such as radon, radium, and potassium from the ground and cosmic radiation from space. People believe that hot springs containing radon or radium are good for their health, but these springs exhibit a certain level of radioactivity (0.1–10 μSv/h). Annually, we are exposed to 0.48 mSv of radiation from the ground (natural rock). The radiation background is high at the Japanese parliament building due to the potassium-rich feldspar containing granite used for its construction. A transcontinental flight by aircraft causes approximately 0.1 mSv of exposure. In total, ordinary people are exposed externally to 2.1 mSv of radiation annually (world average), which exceeds the recommended maximum annual additional exposure level of 1 mSv set by the International Commission on Radiological Protection (ICRP) (2). Residents living in the high-radiation-background area in Kerala, India, are exposed to up to 5 mSv of radiation annually, but there is no evidence for increased incidence of cancer among the residents.

In addition, we are exposed to radiation when undergoing X-ray and computed tomography (CT) scans. A single chest CT scan causes 6.9 mSv of radiation and an average medical exposure in Japan is about 4 mSv, which is far beyond the recommended maximum annual exposure limit. The risk of medical radiation exposure should be a greater public concern because the dose level is relatively high.

Unconscious Internal Exposure

In addition to external exposure, we are exposed internally to natural sources of radiation. Potassium is a one of the essential elements for humans and one of

three of the most important nutrients for plants. Therefore, virtually every food contains potassium. Importantly, potassium has a radioactive isotope, potassium-40 (^{40}K), that is found naturally with an incidence of 0.0117%. Generally, we take more than 1 g of potassium from everyday foods, and a standard adult weighing 60 kg has approximately 4,000 Bq of radioactivity due to ^{40}K in his or her body, where the unit Bq denotes nuclear decay events per second; therefore, we are exposed to 4,000 ^{40}K decays every second. Approximately 89% of ^{40}K forms ^{40}Ca by beta decay, and the remaining 11% decays to ^{40}Ar by electron capture and the emission of gamma rays (Fig. 2). Therefore, our body is always internally exposed to a certain level of radiation.

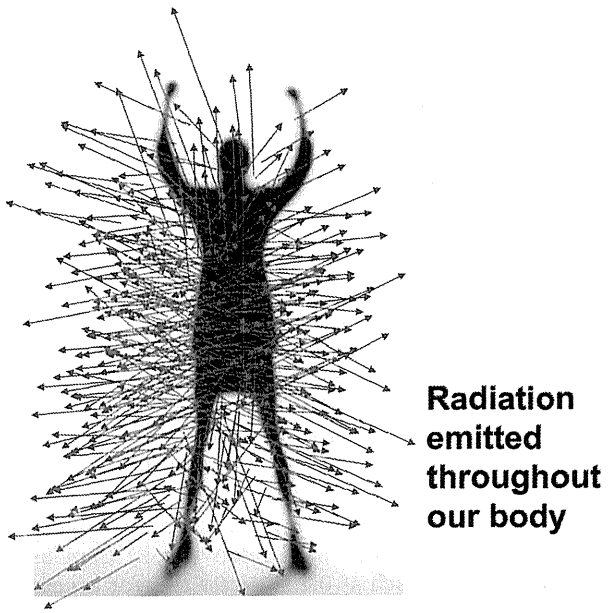

Fig. 2. (Color online) An illustration of gamma rays released throughout the human body. Because of internal exposure of ^{40}K, 4000 Bq radiation in human body (60 kg) is releasing. Among them, approximately 10% (400 Bq) is the γray which passes and released from the body as illustrated although they are not visible.

In addition to natural sources of radiation, we have been exposed to artificial radiation through foods contaminated by nuclear fallout. Data regarding the radioactivity of foods are available at the same Nuclear Regulation Authority Database site (http://search.kankyo-hoshano.go.jp/food). When the annual changes in the radioactivity (^{137}Cs) of tea leaves in Shizuoka is plotted (Fig. 3), for example, it is clearly seen that the level of radioactivity was high when the level of nuclear fallout was high. It exceeded the current food regulation level of 100 Bq/kg for ^{137}Cs in 1963, which is similar to the levels observed in the aftermath of the Chernobyl and Fukushima accidents. In contrast, the radioactivity of ^{40}K has been constant at approximately 100 Bq/kg. It is more difficult to suppress the radioactivity level in dried food by per kg unit than in normal foods. Considerations should be made based on the amount of food consumed.

In conclusion, we are exposed internally to ^{40}K radiation through everyday foods.

Relative Risk

In my presentation at the JEMS open symposium, 2013, I introduced an antique wine glass composed of what is called "uranium glass" as an example of familiar radiation sources. The glass contains uranium, which fluoresces under UV light (Fig. 4). Uranium glass

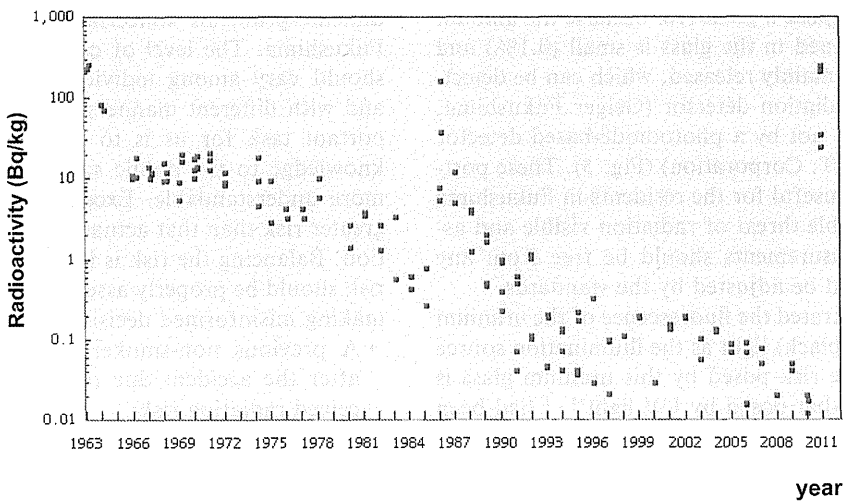

Fig. 3. (Color online) Annual changes in ^{137}Cs in tea leaves in Shizuoka. The graph was created at the database site of the Nuclear Regulation Authority at http://search.kankyo-hoshano.go.jp/servlet/search.top (on June 5, 2013). Data are available for the period 1963–2011.

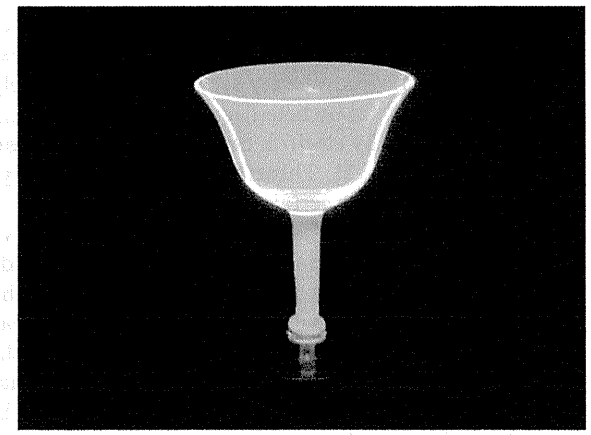

Fig. 4. (Color online) Uranium glass which illuminating fluorescence under UV light exposure.

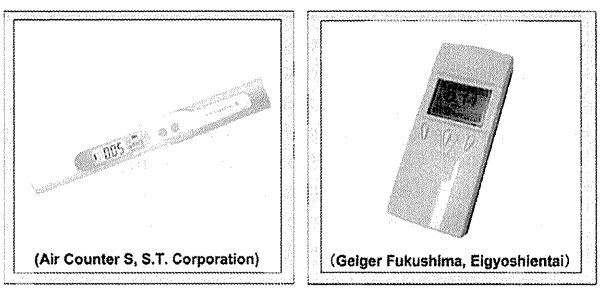

Fig. 5. (Color online) Portable radiation counters: (left) photodiode type (Air Counter S, S.T. Corporation) and (right) Geiger type (Geiger Fukushima, Eigyoshientai).

is popular among antique collectors and has been used frequently in the past. Uranium itself presents a real hazard by releasing beta and gamma rays. Using uranium glass does not pose a great risk because the amount of uranium contained in the glass is small (0.1%) and only beta rays are mainly released, which can be detected by a Geiger radiation detector (Geiger Fukushima, Eigyoshientai) but not by a photodiode-based detector (Air Counter S, S.T. Corporation) (Fig. 5). These portable detectors are useful for the residents in Fukushima to make the invisible threat of radiation visible and assessable. The measurements should be free from any miss-operation and be adjusted by the standards.

When I demonstrated the fluorescence of the uranium glass, I used UV (black) light as the illumination source and said that "the risk posed by this uranium glass is much lower than that posed by UV light". I had been involved in research using transgenic mouse mutation assays and understand that UV is a more powerful inducer of gene mutations than X-rays. Ono *et al.* reported on the mutagenicity of X-ray and UVB radiation in the transgenic MutaMouse (3). An increased mutation

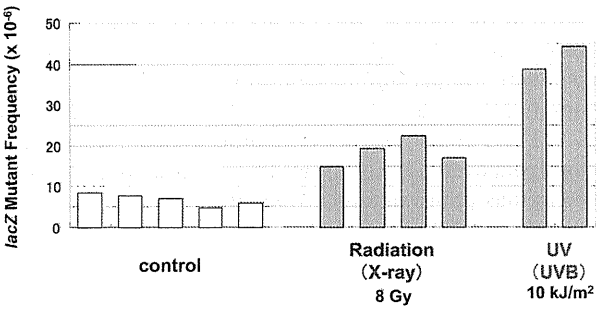

Fig. 6. (Color online) *LacZ* mutant frequency in skin of MutaMouse after X-ray (8 Gy) or UVB (10 kJ/m^2) radiation. The mutant frequency of *lacZ* gene was analyzed 1 week after exposure. The graph was adopted from data obtained by Ono *et al.* (3).

frequency (MF) (a few times) was observed in skin after applying a lethal dose (8 Gy) of X-ray radiation; however, an approximately 6-fold increase in MF was observed in skin after applying 10 kJ/m^2 of UVB radiation (Fig. 6), a dose level easily achieved by sunlight exposure for a few hours in summer. Therefore, the gene mutations induced by UVB light is much higher than that induced by X-ray radiation. However, not much attention is paid to the risk for the genotoxicity posed by UV light compared with that posed by X-ray radiation. The risk of low dose levels is more understandable when compared with other risks (relative risk) than as an absolute value, such as 0.1% excess risk for cancer.

Way of Considering Risk

After understanding the limitation of the current level of science in making a conclusion about the real risk of low-level radiation, I feel that specialists in risk assessment and communication or regulatory science are more important than radiation biologists in solving the difficult problems concerning radio contamination in Fukushima. The level of concern about the same risk should vary among individuals in different situations and with different manners of thinking. The most important task for us is to transfer available scientific knowledge to the public such that the information is more understandable. Excessive fear of radiation is a greater risk than that actually posed by low-level radiation. Balancing the risk is important (4), and radiation risk should be properly assessed to prevent people from making misinformed decisions, for example,

- A previous non-smoker who has started smoking after the accident due to stress caused by the perceived radiation risk;
- Pregnant women who choose to have abortions because of a fear of birth defects;
- Hospitalized patients in a serious condition who are forced to move away from the contaminated area.

Because the population in Fukushima is approximately

2 million, 2000 deaths are expected if an excess risk of mortality is 0.1% (although it is impossible to prove scientifically). If the risk is considered in this manner, the excess risk may not be considered acceptable. It is recommended that the situation be thought in a different way: 0.1% excess risk of mortality, for example, shortens the lifespan of a 40-year-old person by 2 weeks on average (although it may still not acceptable for a part of persons). The risk should be considered together with other risk factors. Therefore, the way in which risk is considered and communicated is important. In addition, the importance of risk education should be emphasized. We do not learn about the basics of radiation risk and biology at school. Thus, we are not good at considering and managing risk. The real risk of low-level radiation should be taught in school to be able to manage radiation risk in the future.

After the accident, I was embarrassed to see an article in a weekly magazine titled "20 years later in Japan, cancers, malformations, strange diseases, and mental retardations". Mass media in Japan has frequently overemphasized radiation risk and causing anxiety among citizens. Sohei Kondo said in his book (5) about the problem in mass media of aggravating the perceived risk of radiation, "However, I do not agree to punish the mass media for their exaggerated reports that caused a radiation phobia. The primary cause of the radiation phobia is a concept 'lowest level of radiation still has toxicity,' which got to be a common sense. This is a fundamental thinking for a specialist for radiation protection, for which mass media broadcasts extravagantly. Radiation specialists are lacking their efforts on telling exposed residents that there is no scientific evidence on the miss-concept and real information on the safety of the low level radiation by a plain and precise manner".

Unresolved Questions on Radiation Risk

What level of radiation risk was posed to those who grew up in the 1960s?: Evidence shows that there was no apparent adverse effect among children due to the global nuclear fallout, but an excess incidence of cancer was reported for residents living near the Nevada test site (6). Although the incidence of leukemia, which can be detected early, did not clearly show an increase due to the global nuclear fallout, solid cancers, which are late-onset, can only be analyzed among individuals who were exposed to the higher background during their childhood in the 1960s because they will enter cancer-prone ages in the coming years. The results of such analysis may provide a scientific basis for conducting a risk assessment of low-dose radiation in relation to the Fukushima accident.

It has also been recently reported that there was a 12 % excess relative risk of childhood leukemia per millisievert of cumulative red bone marrow dose from gamma radiation (including radon) in a control case study of natural background radiation in Great Britain (7).

What is the scientific evidence that supports the ICRP recommendation of 1 mSv as the acceptable annual effective dose for radiation exposure?: A Japanese regulatory decision against radiation protection was made based on the recommendation of the ICRP. The recommendation was made based on available scientific data. However, it should be noted that there is scientific uncertainty involved, such as that associated with the linear non-threshold model (LNT). People tend to believe the value to be an absolute and authoritative one, but the scientific uncertainty involved should be explained and resolved. This is an important task for regulatory scientists.

When a resident in Fukushima develops cancer, can we say that it has no relation to the excess radiation cause by the accident?: Because the incidence of cancer in normal Japanese is approximately 50%, one out of two persons in Fukushima develops cancer. Even if the involvement of excess radiation dosage is very low, it is impossible to be considered zero. It seems natural that residents in Fukushima feel that the radiation caused their cancer. We need to have a scientific tool to determine the involvement of radiation in the development of their tumors, such as a molecular signature of radiation damage such as a specific gain of chromosome band 7q11 (8), or sequence specific deletions.

Concluding Remarks

- A paradigm shift in risk assessment from "zero" to "acceptable" risk is necessary.
- Radiation risk should not be considered alone but as a total risk involving several factors.
- Relief and safety are independent. The concept of acceptable risk varies among individuals. Therefore, relief is more desired than safety.
- We should consider the unconscious exposure to radiation mentioned in this article.
- The most important task for us is to transfer available scientific knowledge to the public such that the information provided is more understandable and provides people the opportunity to make their own decisions.
- Within the 10-km area surrounding the nuclear plant, the ambient radiation dose is 10- to 100-fold higher than it was before the accident, with the area showing hot spots 1,000 times higher in intensity (June 2014). Continuous efforts should be undertaken to reduce ground contamination, possibly by inverting soil or covering it with concrete.

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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

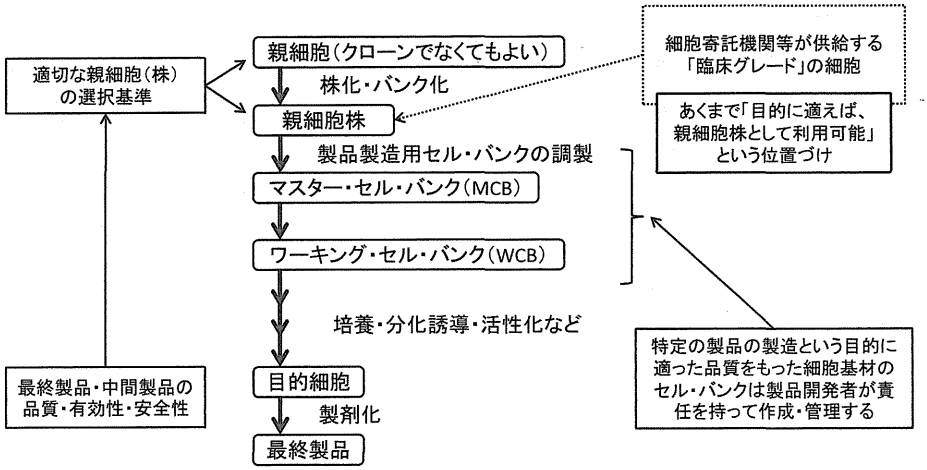

図1　再生医療製品の製造の概略

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．

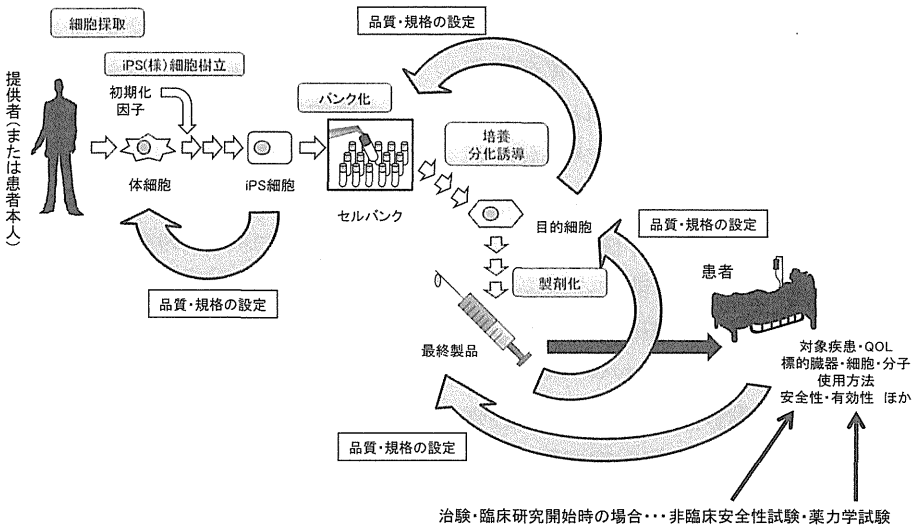

図2　再生医療製品とその素材の品質（iPS細胞由来製品の例）
最終製品としての再生医療製品の品質をもとに中間製品の品質，中間製品の品質から出発原料の品質，と逆行性に規定される．

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⑥ES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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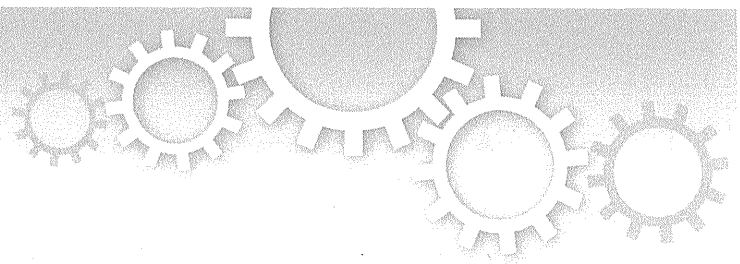

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Pigment Epithelium-Derived Factor Secreted from Retinal Pigment Epithelium Facilitates Apoptotic Cell Death of iPSC

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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

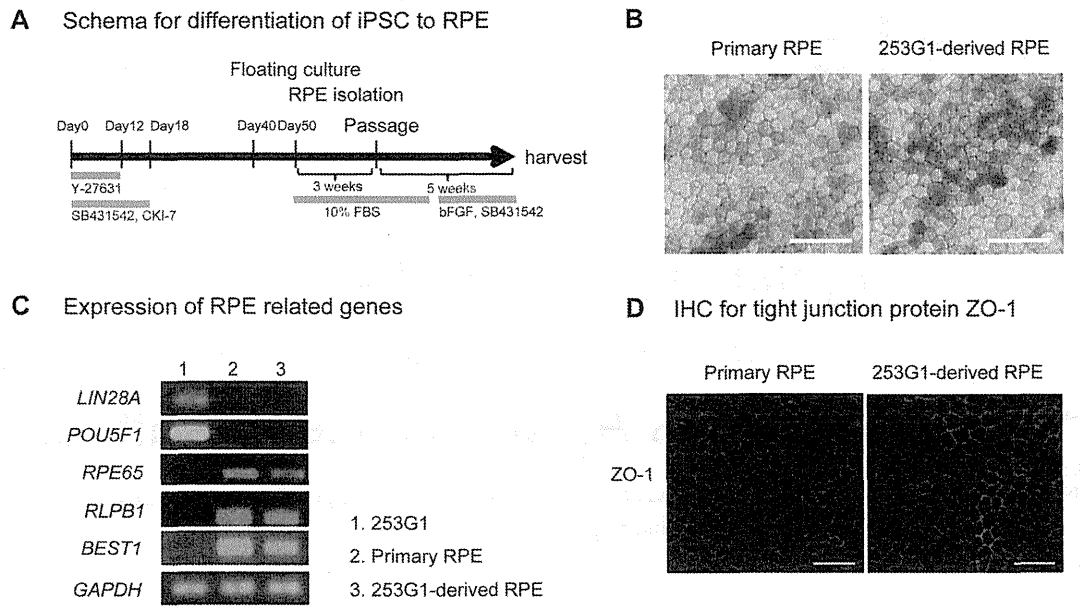

Figure 1 | Characterization of pigment epithelial cells derived from iPSC. (A) Protocol for differentiation to RPE from iPSC clone. (B) Phase contrast images of primary RPE (left panel) and iPSC clone 253G1-derived RPE (right panel). Scale bar = 50 μm. (C) Expression of pluripotency-related undifferentiated 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

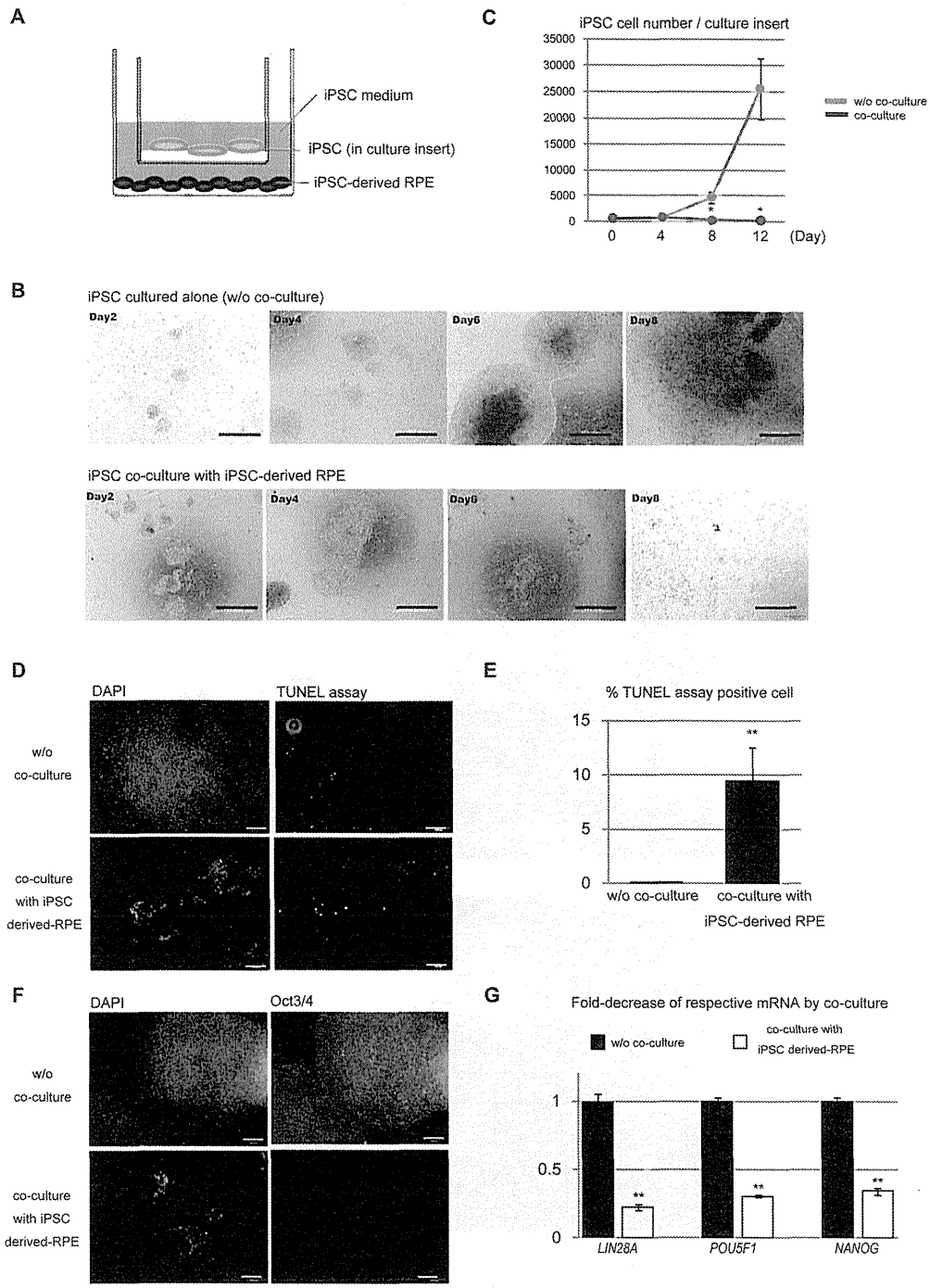

Figure 2 | Cell growth of iPSCs co-cultured with iPSC-derived RPE was perturbed. (A) Schema for co-culturing iPSC with RPE. iPSCs were maintained in culture inserts coated with Matrigel and co-cultured with iPSC-derived RPE seeded on the bottom of the dishes in iPSC culture medium. (B) Phase contrast 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.

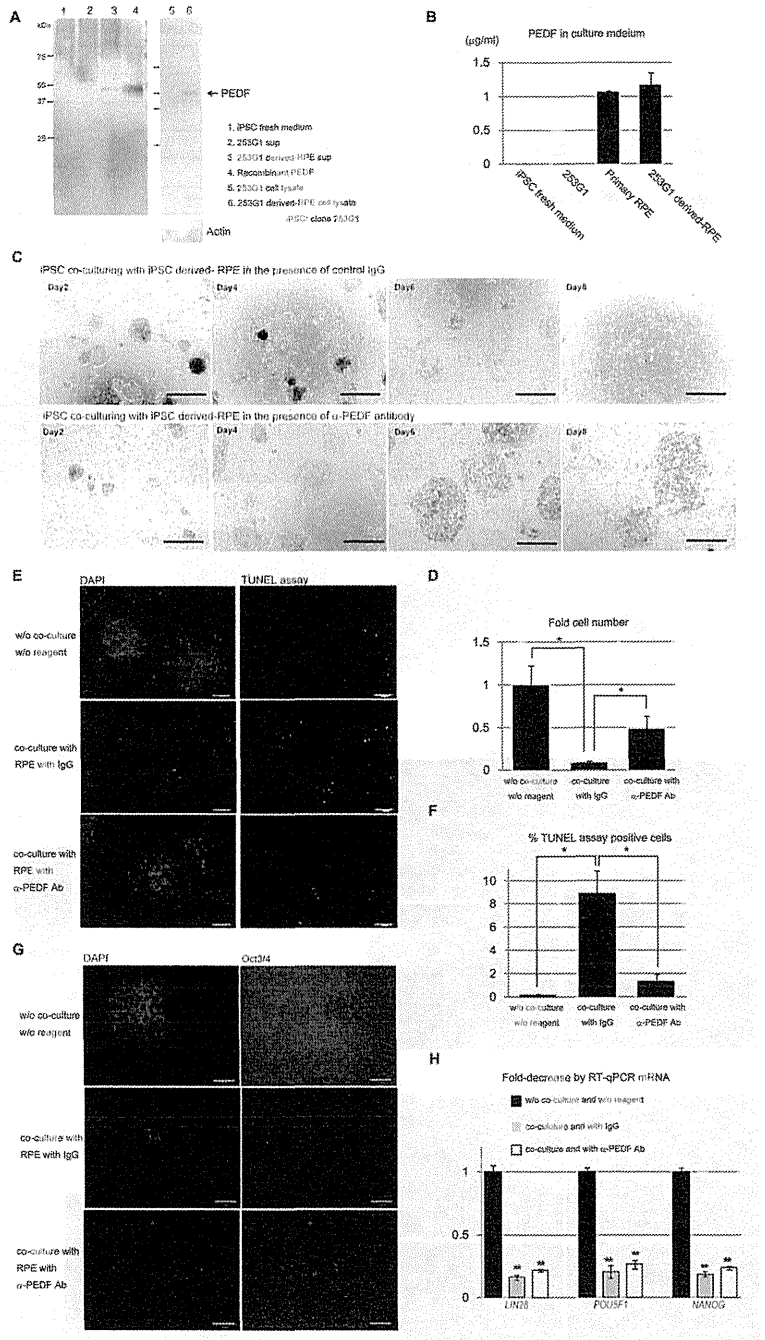

Figure 3 | Addition of antibody against PEDF blocked apoptotic cell death in iPSC induced by co-culture with RPE. (A) Western blot of iPS fresh culture medium, 253G1 sup, 253G1-derived RPE sup and recombinant PEDF, or cell lysates of 253G1 and 253G1-derived RPE using an anti-PEDF specific antibody. Detection 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.

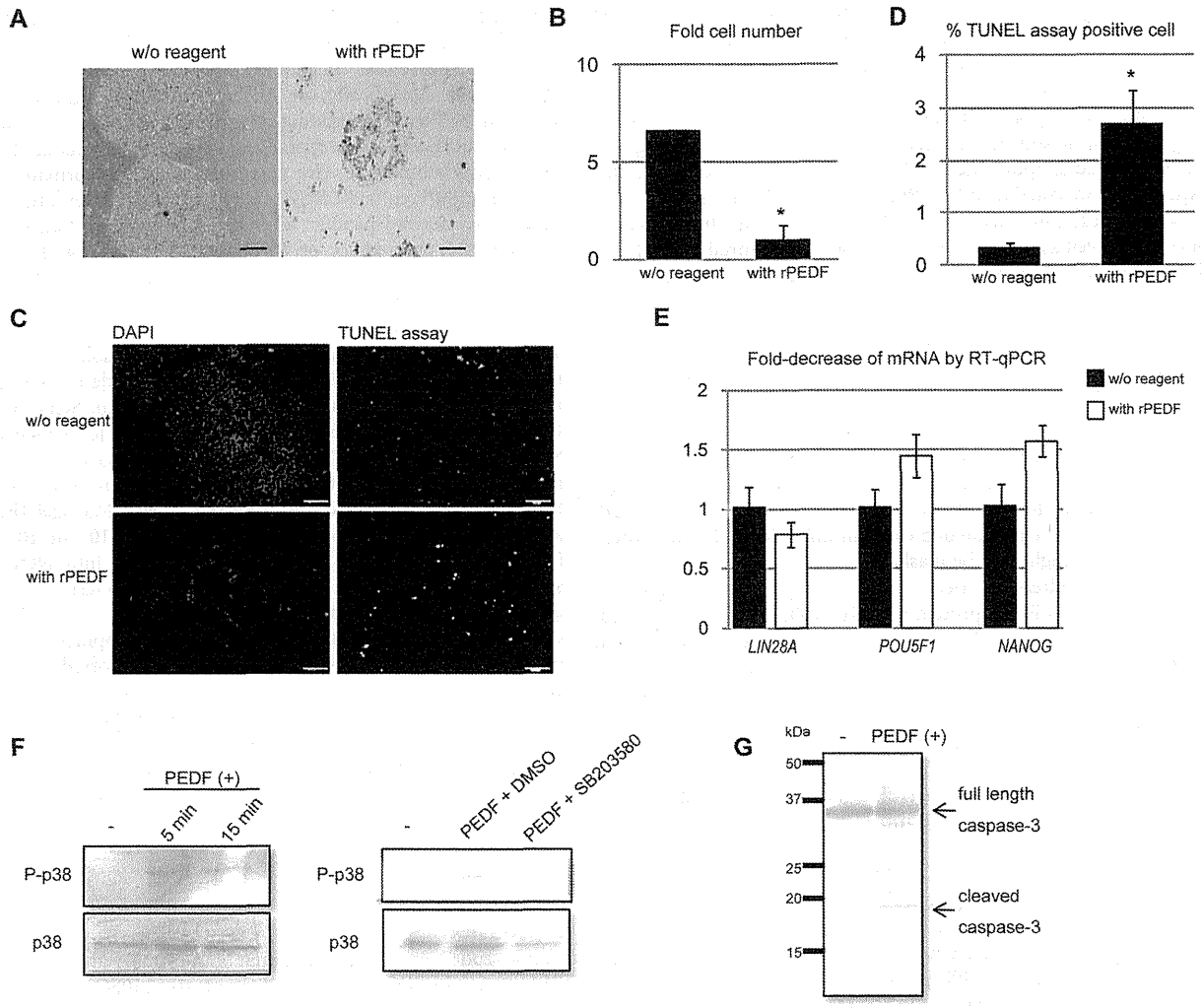

Figure 4 | Recombinant PEDF (rPEDF) induced apoptotic death in iPSCs. (A) Phase-contrast images of iPSC clone 253G1 without or with rPEDF (50 μg/mL) after four days of culture. Scale bar = 200 μm. (B) Fold-change in the number of 253G1 cells cultured with rPEDF (50 μg/mL) after four days of culture, 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

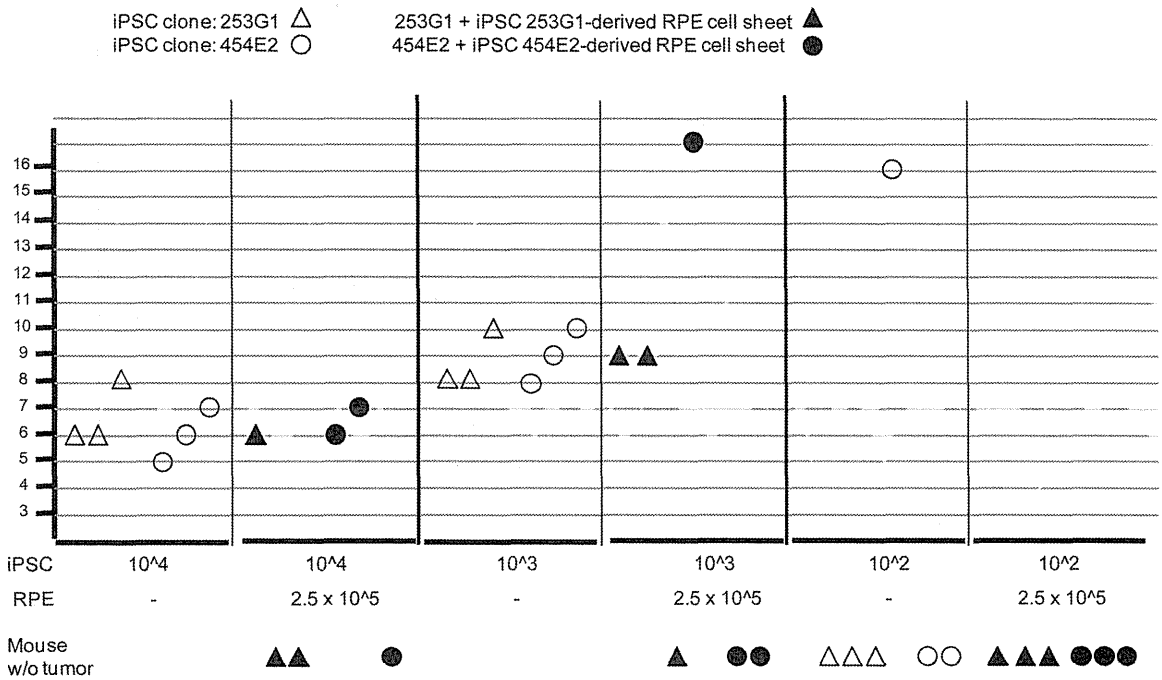

Figure 5 | iPSC clones 253G1 or 454E2 (10^2, 10^3 and 10^4 cells) were subcutaneously transplanted to NOG mice (three per group) without or with a 253G1-derived RPE sheet or a 454E2-derived RPE sheet, respectively. The sheets consisted of (approximately) 2.5×10^5 RPE cells. The Y-axis shows the week 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