【研究方法】

細胞あるいは組織から抽出したRNAを基に遺伝子網羅解析を行い、その遺伝子情報からiPS細胞の品質をフィードバックすることが可能について評価検討した。まず、253G1由来のRPE細胞とPrimary RPE細胞からRNAを抽出し、Human Genome U133 plus 2.0を用いて網羅的遺伝子解析データを行った。これらの遺伝子情報を代謝情報に変換しヒートマップを作成した。

【結果】

Primary RPE細胞は、Protein Synthesis代謝に関する遺伝子が多く発現していることが分かり、RPE細胞自体が様々なタンパク質を分泌している特性を有することが確認できた。一方、iPS細胞由来RPE細胞においても、Primary細胞と同様に多くのProtein Synthesis代謝の結果が見られた。その他、遺伝子発現からCell Death and SurvivalやCellular Growth and Proliferationに関する代謝発現もPrimary RPEとiPS細胞由来RPE細胞で同様の結果が得られた。（図5）これらの結果より、iPS細胞由来RPE細胞とPrimaryRPE細胞の細胞内代謝状況は、非常に性質の近い細胞であることが確認された。

以上、移殖細胞や組織よりRNAを抽出し、遺伝子網羅解析を行う事により、iPS細胞由来分化細胞のさらなる細胞評価を行った。

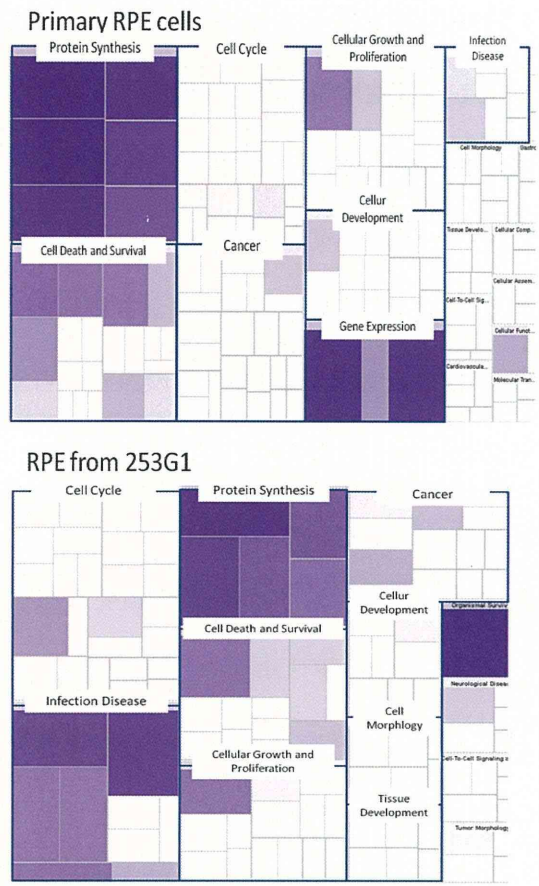

図5. Primary RPEおよび253G1由来RPE細胞のオミックス解析での細胞内代謝のHeatmap

【研究目的】

細胞保管業務では、当初予想していた移殖細胞の保管だけでは有害事例発生時における細胞検査で十分な原因追究ができず再生医療の安全に推進するに繋がらない可能性が示唆されたことから、昨年度に作成した細胞情報管理、細胞保管場所管理、保管機器の監視、細胞保管施設の維持などに関する運用規定を、多種細胞の保管/管理する事を想定した運用規定および細胞保管/管理規定を改正し、多検体の保管が可能な運用方法の改正を行った。

【研究方法】

保管業務を遂行するために必要な契約書の管理やデータ・ホームページ管理、広報活動等の業務を支援するために事務局を設置し、必要人員を確保することでヒト幹細胞アーカイブの全体的な運用体制を確定した。

【結果】

細胞寄託に関する情報発信および細胞寄託時の個人情報管理等のヒューマンエラー防止、国民への広報活動のためヒト幹細胞アーカイブ【Archive of Human Stemcell in Clinical research; AHSC】用ホームページ (http://stemcell-archive.fbri.org) を作成し、次年度より医療機関ネットワークへの幹細胞の寄託依頼の手続きを開始する状況を整えた。また、細胞提供機関と寄託を受ける財団双方間で実運用のための契約締結準備中である。2014年3月には、臨床用細胞検体の保管シュミュレーションを実施し、運用マニュアル、管理マニュアルの最終確認を行った。ヒト幹細胞アーカイブ広報活動としては、細胞保管事業目的および細胞保管に対する契約内容（案）を理化学研究所 高橋政代先生らの細胞製造グループとMeetingし、細胞寄託について確認を頂いた(2012年11月11日)。

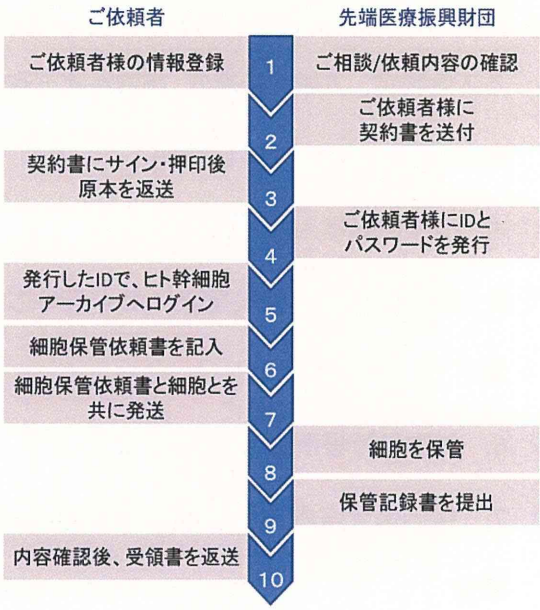

図6. ヒト幹細胞アーカイブを活用した細胞保管業務のフローチャート*

*財団内のサーバーで公開型と非公開型に分類してセキュリティ面を担保しているため、細胞情報管理等の流れに関しては非公開しない。

細胞保管事務局としては、細胞提供機関と細胞寄託に関する契約書の作成や細胞寄託に対する広報活動、ヒト幹細胞アーカイブ用ホームページの立ち上げを行い、ホームページ上に、細胞寄託者が細胞寄託するまでの手順をフローチャートとして明記し、容易に寄託依頼できるよう情報発信している。（図6）

細胞寄託までの操作

1. 細胞保管依頼者（以下；依頼者）にヒト幹細胞アーカイブ HP にアクセスして頂き、依頼者情報等を入力。
2. 細胞保管事務局で依頼登録内容に問題がない事を確認後、依頼者に細胞保管業務委託契約書を発送する。
3. 依頼者が契約書に捺印後、財団に返送。
4. 契約締結を確認後、財団側で ID・パスワードを発行。
5. 依頼者に ID・パスワードを送付する。
6. 依頼者は、ID・パスワードでログインし、専用フォームの細胞情報記入欄を記入して、細胞発送日を指定する。
7. 細胞保管事務局は、細胞受取り可能であることを確認し連絡する。
8. 細胞を受け取り、細胞保管作業手順書に準じ細胞を保管する。
9. 細胞情報に ID を付与し保管場所と細胞保管記録書として保管する。
10. 細胞を受取り
11. 依頼者に細胞保管受領書を送る。

細胞保管施設の整備状況は、ES細胞/ iPS細胞の細胞保存用液体窒素タンク・超低温フリーザー・温度管理システム・室内酸素濃度センサー・検体管理システム整備を構築し、細胞保管機器の運転状況などの管理体制を整えた。(図7)

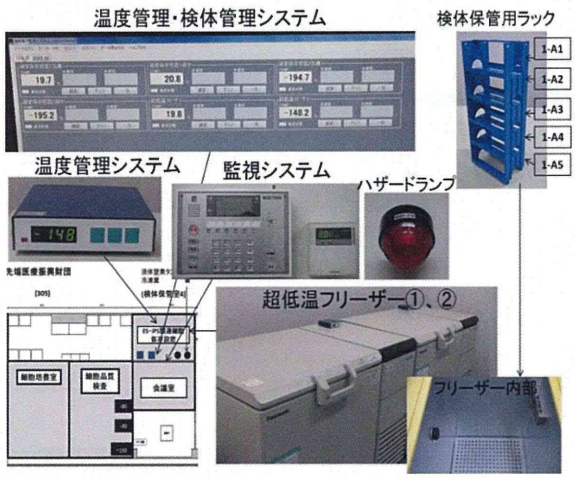

図7. 細胞保管施設の整備状況

その他、Table 2 に示した各種規定書・手順書を作成した。

Table 2. 細胞保管保管情報の管理規定、手順書

1. 作業者の教育等の管理規定を設定。
2. 細胞保管事業に関する業務および契約規定、
3. 検体保管室管理規定 (受入細胞に関する入庫判定、入庫記録管理, 細胞保管不適合時の処置、細胞の取出し管理)
4. 検体保管室入室記録
5. 検体保管室の警戒発報に対する対応規定
6. 細胞保管場所アドレス入力規定
7. 検体保管室内細胞保管機器の作動監視規定
8. 検体保管依頼者の個人情報保護法の取り扱い規定
9. 検体保管依頼書の作成
10. 依頼蛮行発行規定
11. 検体情報の管理規定
12. 検体発送手順書の作成
13. 検体保管手順書の作成
14. 検体保管記録書の作成
15. 検体管理ID発行規定
16. 検体保管記録書の管理規定
17. セキュリティ侵害時の対応規定
18. 細胞返還依頼書の作成
19. ヒト幹細胞アーカイブのHP運用規定

【今後の予定】次年度には、慶応義塾大学中村雅也先生や京都大学　高橋淳先生らの

研究進捗に応じて、本事業への細胞寄託にご協力頂けるよう広報を行う予定である。具体的には、ヒト幹細胞アーカイブHPを活用し広報を行い細胞寄託を開始する。(図8)

広報活動としては、学術会議・シンポジウム・展示会等への出展を行い将来、臨床研究を実施される先生方への細胞寄託協力を求める。加えて、次年度に広報活動用としてヒト幹細胞アーカイブ用配布資料のA4 x 1 頁程度を作成する予定である。文部科学省「橋渡し研究」等の臨床研究実施、実施予定の先生方に配布資料を送付予定である。臨床研究 に使用されたiPS細胞由来RPE細胞の細胞寄託を開始。

次年度より、CGH array等を用いたCopy Numbers Variation (CNV)も染色体解析技術に導入し、gene stabilityと造腫瘍性に関する移植後検査法の開発に着手した。

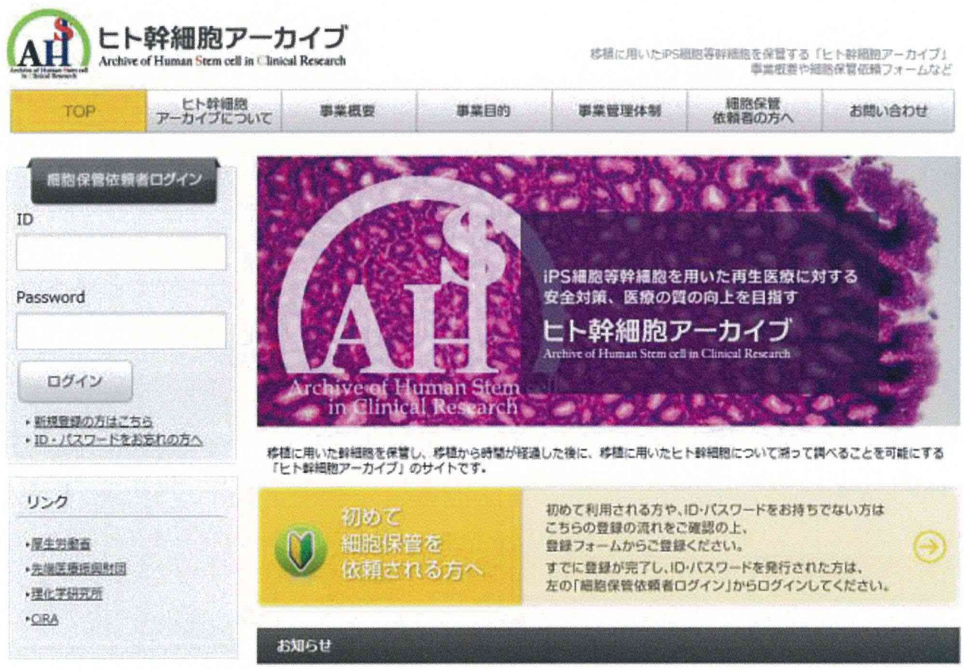

図 8. ヒト幹細胞アーカイブの HP TOP 画面

Ⅳ. 会 議 記 録

平成25年度　厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業）

（再生医療関係研究分野）細胞保管事務局

細胞保管ホームページ立上に関する打合せ等

月日	時間	場所	参加者							主な内容
			(有)エムリンクス 中氏	(有)エムリンクス 山下氏	細胞療法開発事業部門 西下	細胞療法開発事業推進課 清水	細胞療法開発事業推進課 ジェイコブス	細胞療法開発事業推進課 竹内	情報システム課 久後	情報システム課 大前	
H25年											
11月7日	14:00~15:30	TRI-2F 事務室	○	○	○		○	○			事業内容、HPの構想を(有)エムリンクに説明
11月15日	15:00~16:30	TRI-2F 事務室	○	○	○		○	○			(有)エムリンクよりHPコンテンツ概略(案)の提示
11月25日	13:30~14:30	IBR臨床棟3F 財団事務室			○		○	○	○	○	システム構成について。サーバ2台設置(公開用、DB用)。
12月5日	15:00~16:30	TRI-2F 西会議室	○	○		○	○	○	○	○	(有)エムリンクよりHPコンテンツ詳細(案)の提示。システム構成の確認
12月25日	16:00~16:30	TRI-2F 西会議室			○			○			事前打合せ
12月25日	16:30~17:30	TRI-2F 西会議室	○	○	○	○	○	○	○	○	(有)エムリンクよりHPコンテンツ修正(案)の提示。システム構成の再確認
H26年											
2月21日	10:00~11:00	TRI-2F 西会議室			○		○	○			HPコンテンツ。事務局と弊の役割分担
3月5日	11:30~12:30	TRI-2F 西会議室			○		○	○			HPコンテンツ確認→(有)エムリンクに修正依頼
3月10日	11:00~12:30	TRI-2F 西会議室			○		○	○			HPコンテンツ確認→(有)エムリンクに修正依頼
3月14日	10:00~11:00	TRI-2F 西会議室			○		○	○			HPコンテンツ確認→(有)エムリンクに修正依頼

Ⅴ．研究成果の刊行物・印刷物

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PLOS | ONE

Tumorigenicity Studies of Induced Pluripotent Stem Cell (iPSC)-Derived Retinal Pigment Epithelium (RPE) for the Treatment of Age-Related Macular Degeneration

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Abstract

Basic studies of human pluripotential stem cells have advanced rapidly and stem cell products are now seeing therapeutic applications. However, questions remain regarding the tumorigenic potential of such cells. Here, we report the tumorigenic potential of induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) for the treatment of wet-type, age-related macular degeneration (AMD). First, immunodeficient mouse strains (nude, SCID, NOD-SCID and NOG) were tested for HeLa cells' tumor-forming capacity by transplanting various cell doses subcutaneously with or without Matrigel. The 50% Tumor Producing Dose (TPD_{50} value) is the minimal dose of transplanted cells that generated tumors in 50% of animals. For HeLa cells, the TPD_{50} was the lowest when cells were embedded in Matrigel and transplanted into NOG mice ($TPD_{50} = 10^{1.1}$, n = 75). The TPD_{50} for undifferentiated iPSCs transplanted subcutaneously to NOG mice in Matrigel was $10^{2.12}$; (n = 30). Based on these experiments, 1×10^6 iPSC-derived RPE were transplanted subcutaneously with Matrigel, and no tumor was found during 15 months of monitoring (n = 65). Next, to model clinical application, we assessed the tumor-forming potential of HeLa cells and iPSC 201B7 cells following subretinal transplantation of nude rats. The TPD_{50} for iPSCs was $10^{4.73}$ (n = 20) and for HeLa cells $10^{1.32}$ (n = 37) respectively. Next, the tumorigenicity of iPSC-derived RPE was tested in the subretinal space of nude rats by transplanting $0.8–1.5 \times 10^4$ iPSC-derived RPE in a collagen-lined (1 mm × 1 mm) sheet. No tumor was found with iPSC-derived RPE sheets during 6–12 months of monitoring (n = 26). Considering the number of rodents used, the monitoring period, the sensitivity of detecting tumors via subcutaneous and subretinal administration routes and the incidence of tumor formation from the iPSC-derived RPE, we conclude that the tumorigenic potential of the iPSC-derived RPE was negligible.

Citation: Kanemura H, Go MJ, Shikamura M, Nishishita N, Sakai N, et al. (2014) Tumorigenicity Studies of Induced Pluripotent Stem Cell (iPSC)-Derived Retinal Pigment Epithelium (RPE) for the Treatment of Age-Related Macular Degeneration. PLoS ONE 9(1): e85336. doi:10.1371/journal.pone.0085336

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Clinical cell therapy trials were recently initiated for treatment of Stargardt's disease and the dry type of age-related macular degeneration (dry AMD). The trials have used human embryonic stem cell (hESC)-derived retinal pigment epithelium (RPE) [1–4]. In addition, several groups are planning clinical trials with autologous human induced pluripotent stem cell (hiPSC)-derived RPE for the wet type of AMD. Thus, cell therapy using human pluripotent stem cells (hPSCs) has reached clinical application. However, in contrast to tissue stem cells that have a limited proliferation potential, tumor formation from residual undifferentiated or incompletely differentiated hPSCs in hPSC-derived cell products is an issue that must be carefully analyzed. This issue is particularly important when transplanting autologous hiPSC-derived cells.

We recently reported a highly sensitive residual hiPSC detection method based on qRT-PCR using primers for the *LIN28A* transcript [5] in hiPSC-derived RPE. This method enables us to detect residual hiPSCs down to 0.002% of differentiated RPE cells. As we plan to transplant $4–8 \times 10^4$ hiPSC-derived RPE cells into the subretinal space of patients, this method is sensitive enough to detect a few residual hiPSCs, if any, in a clinical setting.

The tumorigenic potential of hiPSC-derived RPE cells is attributable to contamination by undifferentiated hiPSCs, intermediate products having proliferation potentials and/or tumorigenic transformed cells. Contamination by these cells should be assessed by nonclinical testing using suitable animal models [6,7]. However, there is no internationally recognized guideline for tumorigenicity testing in cell therapy products. The most relevant guideline is the WHO TRS 878, "Recommendation for the evaluation of animal cell cultures as substrates for the manufacture of cell banks" [8,9]. The guideline recommends transplanting 1×10^7 test cells subcutaneously to 10 nude mice and monitoring tumor formation for more than 16 weeks. Transplantation of the same dose of a well-known tumorigenic cell line such as HeLa in parallel is suggested as a tumor-forming positive control. The WHO guideline covers animal cell substrates for the production of

biological medicinal products and specifically excludes viable animal cells that are intended for therapeutic transplantation into patients. To examine the tumorigenicity of hiPSC-derived cells intended for administration to patients, several teratoma-forming tests exploring dose and administration route were studied using immuno-deficient mice [6],[10]. However, discussions how we can interpret and extrapolate the results of tumorigenicity testing with immuno-deficient or immuno-suppressant animals to human patients continue [6,7]. Recently a commentary report from FDA/CBER pointed out the issues to be considered for cell-based products and associated challenges for preclinical animal study [11]. The report stated that although the nature of cells used for cellular therapy is diverse, tumorigenic test results from the administration of cells through nonclinical routes would not be considered relevant as it would not track the behavior of transplanted cells in a micro-environment. When tumorigenicity testing of ESC-derived cellular products is undertaken, the study design should include groups of animals that have received undifferentiated ESCs, serial dilutions of undifferentiated ESCs combined with ESC-derived final products and the final intended clinical products. This approach would thereby address the tumor-forming potential of these cell groups in animal models.

Tumorigenicity testing via the clinical route of administration could recapitulate the fate of transplanted cells in a microenvironment of host tissue and could be fairly extrapolated to human application. However, elaborate surgical intervention requires skills that greatly influence the outcome of transplantation. For example, it is difficult to determine whether the cells were transplanted into the right location or organ in small rodents. These concerns can be overcome by conducting a subcutaneous tumorigenicity test in addition to testing via the clinical route.

In this report, we conducted 2 types of *in vivo* tumorigenicity tests by transplanting hiPSC-derived RPE cells into subcutaneous and sub-retinal spaces in immuno-deficient animals. The results and limits of these tests are discussed.

Results

Tumorigenicity Tests with Several Types of Immuno-deficient Mice

The tumor-forming potential of human iPSC-derived cell products should be examined using a suitable animal transplantation model. One should take into account the number of cells to be transplanted, the method of transplantation, the microenvironment of the transplantation site, the monitoring period and the status of the immune-deficient animals.

First, we checked the tumor-forming potential of several immune-deficient animals by subcutaneously transplanting HeLa cells over a wide range of doses (1 to 1×10^6 in 10-fold increments) with or without Matrigel (BD) and observed tumor formation every day for up to 36 weeks on a daily basis. Matrigel is known to enhance the tumor-forming potential of transplanted cells [16]. Recipient animals included immune-deficient nude, SCID [10], NOD-SCID [17], and NOG [18] mice. The minimal dose of transplanted cells that generated tumors in 50% of the transplanted animal (TPD_{50}) was calculated statistically to evaluate the sensitivities of tumor formation in each animal model [19]. We found the NOG mouse was most susceptible to tumors. That is, when transplanted subcutaneously with Matrigel, tumors were generated by the lowest number of HeLa cells. The TPD_{50} for HeLa was $10^{1.1}$ (n = 75), in agreement with a previous report [20], (Figure 1, Table 1). It is interesting to note that among the conditions tested, the highest number of HeLa cells was required to form tumors in nude mice without Matrigel. TPD_{50} for nude mice without Matrigel was $10^{4.9}$ (n = 120), which is also in agreement with a previous report [19] (Figure 1, Table 1). Therefore, we selected NOG mice and Matrigel for embedding the test cells for further assays as it provided sensitive tumor detection using small numbers of transplanted cells. The tumor-formation potential of iPSCs was assessed by subcutaneously transplanting several doses of the iPSC cell line 201B7 with Matrigel into NOG mice. The TPD_{50} for iPSC was $10^{2.12}$ (n = 30) over 12 months' monitoring (Table 2). The TPD_{50} value for iPSCs in subcutaneous transplantation provided a reference cell number for the contamination of iPSCs in iPSC-derived RPE cells.

Characterization of Established hiPSCs and hiPSC-derived RPE

hiPSC lines 59-G3, 101-EV3, K11-EV9, K21-EV15 K21-G18, 101-G25, RNT9-2-8, and RNT10–24 were established from dermal fibroblasts of 6 patients (59, K11, K21, 101, RNT9, RNT10) with retinitis pigmentosa. Quality control tests for established iPSCs were as follows. (1) Cells form colonies and must show human ESC-like morphology by microscopic observation. (2) Cells must express SSEA-4, TRA-1-60, POU5F1 (OCT3/4) and NANOG proteins as determined by immunostaining. (3) Cells must not express EBNA plasmid fragment by PCR or qRT-PCR. (4). Cells must possess a normal karyotype by the G-band method.

Retinal differentiation was subsequently initiated. The resulting RPE cell lines were established as follows. 59-G3 RPE was derived from hiPSC clone 59-G3; 101-EV3 RPE was from 101-EV3; K11-EV9 RPE was from K11-EV9; K21-EV15 RPE was from K21-EV15; K21-G18 RPE was from K21-G18; 101-G25 RPE and RNT9 RPE were from RNT9-2-8; and, RNT10 RPE was from RNT10-24. The protocol for RPE differentiation from hiPSC was shown in our recent report [21]. It requires 3 months for RPE differentiation and another 2 months to prepare the RPE sheet. The following quality control tests for the hiPSC-derived RPE cell lines were conducted. (1) The EBNA plasmid fragment was not detectable by PCR. (2) The cells showed the characteristic morphology and pigmentation of RPE with a single or double layer cell structure. (3) BEST1 and PAX6 molecules were detected by immunohistochemistry in over 95% of final hiPSC-derived RPE cells. (4) RPE-specific markers *RPE65, CRALBP, MERTK* and *BEST1* were confirmed by RT-PCR. (5) *LIN28A* was not detected by qRT-PCR. (6) Migration of non-RPE cells into the collagen layer lining the hiPSC-derived RPE cell sheet shall be below 0.1% of the total RPE cells. (7) The RPE cell sheet shall consist of over 70% viable cells with a density of over 4500 cells/mm^2. Items (6) and (7) were quality control tests for the RPE cell sheet. All the cell culture processes including establishment of hiPSCs from a patient's fibroblasts and differentiation to RPE were conducted in a GMP-grade cell processing facility. The morphology and immunostaining of hiPSC-derived RPE cell lines 59-G3 RPE, K21-G18 RPE and 101-G25 RPE are shown in Figure 2A, B. The other hiPSC-derived RPE cell lines showed the same phenotype. The gene expression patterns of these cell lines are shown in Figure 2C. Primary RPE was used as a reference. It is notable that neither *LIN28A* nor *POU5F1 (OCT3/4)* was detected above background levels in hiPSC-derived RPE cells[5]. This finding serves as a useful criterion to eliminate immature hiPSCs in hiPSC-derived RPE (Figure 2D, 2E).