Metabolic Plasticity in Cell State Homeostasis and Differentiation of Cultured Human Corneal Endothelial Cells

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Purpose: To clarify whether cultured human corneal endothelial cells (cHCECs), heterogeneous in their differentiation state, exhibit distinctive energy metabolism with the aim to develop a reliable method to sort cHCECs applicable for regenerative medicine.

Methods: The presence of cHCEC subpopulations (SPs) was verified via surface cluster-of-differentiation (CD) marker expression. Cultured HCEC metabolic extracts or corresponding culture supernatants with distinctive cellular phenotypes in regard to energy-metabolism-related functional markers c-Myc and CD44 were prepared and analyzed via capillary electrophoresis–tandem mass spectrometry. The metabolic requirements of heterogeneous SPs of cHCECs were also investigated.

Results: After successfully discriminating SPs, as verified via surface CD markers in terms of their secretory metabolites, we found that the CD44++ SP with cell-state transition (CST) exhibited disposition for anaerobic glycolysis, whereas the CD44− SP without CST was disposed to mitochondria-dependent oxidative phosphorylation (OXPHOS). These results raised the possibility of establishing effective culture conditions to selectively expand mature cHCECs with a hexagonal cobblestone shape and inclination for mitochondria-dependent OXPHOS.

Conclusions: The findings of this study open a pathway for monitoring the disposition of cHCECs via their energy metabolism, thus leading to safe and stable regenerative medicine by use of metabolically defined cHCECs in cell-suspension form.

Keywords: cultured corneal endothelial cells, metabolome, cultured cell subpopulation, mitochondria, glycolysis

Corneal endothelium includes a monolayer of hexagonal cobblestone-shaped cells and is indispensable for the maintenance of corneal transparency. Thus, corneal endothelial dysfunction results in corneal turbidity, and a marked deterioration of visual acuity. Human corneal endothelial functions as a barrier between the aqueous humor and corneal stroma, characterized by focal tight junctions,1 a “pump” with Na+, K+ ATPase activity, and the presence of HCO3−, Cl−, and carbonic anhydrase activity that regulate corneal hydration2–4 to maintain transparency.

Since the proliferative potential of human corneal endothelial cells (HCECs) is extremely limited,5–7 severe damage to the corneal endothelium resulting from pathologic conditions leads to corneal endothelial dysfunction, and ultimately to the loss of corneal transparency.8–10 To restore the injured tissue, the residual HCECs tend to migrate and spread over the damaged area.

Endothelial keratoplasty involving dissection of the Descemet’s membrane with the endothelium layer has been performed worldwide to restore the function of the damaged tissue. Our group, together with others, has been investigating the possibility of cultured HCEC (cHCEC) transplantation in the form of a cell suspension.11–17 Of note, Koizumi et al.13 have developed a new corneal-cell transplantation method involving the intraocular injection of substrate-free cHCECs, and the efficacy of those cells has been confirmed in a cell-transplantation study using a cynomolgus-monkey corneal endothelial dysfunction model.

To date, most researchers conceptualize cHCECs only as deriving from corneal endothelium tissue and disregard details pertaining to the refinement of the biochemical features. As pointed out by Okumura et al.18 and Cheong et al.,19 cHCECs are indeed heterogeneous in their surface CD markers. Moreover, Miyai et al.20 have pointed out the presence of highly frequent chromosomal aneuploidy in “cultured human corneal cells,” thus indicating the presence of heterogeneous populations with or without aneuploidy.

Mitochondria are responsible for coordinating cellular energy production in most somatic cells, and every cell type in a specific state can have a distinct metabolic signature.21 Proliferating cells, such as stem cells, tend to prefer glycolysis, while mature differentiated cells, such as HCECs, are under...
regulation by a greater oxidative phosphorylation (OXPHOS) metabolism. Acquisition of, and departure from, stemness are reportedly governed by genetic and epigenetic conductors, with modulation of energy metabolism.\(^{22}\)

An understanding of the metabolic profile, either to increased dependence on OXPHOS activity during differentiation or a shift to glycolytic metabolism in cell proliferation, reportedly should support the optimization of culture conditions.\(^{23}\) Cultured HCECs have a tendency for cell-state transition (CST) into a senescent phenotype, epithelial-mesenchymal transition (EMT), and transformed fibroblastic cell morphology. To detail molecular mechanisms underlying the impaired proliferation of HCECs, we attempted to clarify the presence of functionally heterogeneous SPs in cHCECs, distinct in their energy metabolism.

One of the most notable obstacles in the application of cHCECs in the clinical setting is the lack of a standardized method to monitor the quality of cHCECs, as they are intrinsically heterogeneous in their composite cell populations. Our findings in another study (unpublished data) have demonstrated that the average area of cHCECs fully differentiated to CD44\(^{+}\) mature, hexagonal, cobblestone-shaped-type cells is approximately 200 to 220 \(\mu\)m\(^2\), while that of CD44\(^{+}\) senescent-type nonhexagonal cells forming island-like clusters or CD44\(^{+}\) fibroblastic-like cells is approximately double that area. More details on the functional heterogeneity will be provided in a separate paper (manuscript submitted and currently under review).

With that knowledge in hand, we sought to establish a noninvasive quality-control method for characterizing the cellular phenotype of cHCECs, instead of the combination of surface CD markers. On the basis of our observation that cHCECs are composed of diverse SPs in regard to the expression of CD44, CD24, and CD26, and that c-Myc expression is restricted to a certain SP with CST, we became interested in investigating in detail the secretory metabolites in and from these SPs.

The Warburg effect, the phenomenon in which cancer cells exhibit rapid glucose consumption with secretion of lactate despite abundant oxygen availability, has been recognized since the 1930s.\(^{24–26}\) Studies have shown that c-Myc is critically involved in the glucose metabolism genes, including GLUT1, hexokinase 2, and pyruvate kinase muscle,\(^{27,28}\) and that through the upregulation of these genes, c-Myc contributes directly to the Warburg effect.\(^{28,29}\)

The aim of this study was to clarify whether cHCECs, heterogeneous in their differentiation state, also exhibit a unique energy metabolism among distinct SPs.

### Materials and Methods

#### Human Corneal Endothelial Cell Donors

The human tissue used in this study was handled in accordance with the tenets set forth in the Declaration of Helsinki. Human corneal endothelial cells were obtained from 20 human donor corneas obtained from SightLife (Seattle, WA, USA) eye bank, and were cultured before performing karyotyping analysis. Informed written consent for eye donation for research was obtained from the next of kin of all deceased donors. All tissues were recovered under the tenets of the Uniform Anatomical Gift Act of the particular state in which the donor consent was obtained and the tissue was recovered.

The 9 male and 11 female donors ranged in age from 2 to 75 years (mean age: 43.7 ± 26.4 years). All donor corneas were preserved in Optisol GS (Chiron Vision, Inc., Irvine, CA, USA) and imported via international air transport for research purposes. Donor information accompanying the donor corneas showed that they were all considered healthy and absent of any corneal disease, and that all donors had no past history of chromosomal abnormality.

#### Cell Cultures of HCECs

The HCECs were cultured according to published protocols, with some modifications.\(^{17}\) Briefly, the Descemet’s membranes with the CECs were stripped from donor corneas and digested at 37°C with 1 mg/mL collagenase A (Roche Applied Science, Penzberg, Germany) for 2 hours. The HCECs obtained from a single donor cornea were seeded in one well of a Type-I collagen-coated six-well plate (Corning, Inc., Corning, NY, USA). The culture medium was prepared according to published protocols. The HCECs were passaged at ratios of 1:3 by using 10x TrypLE Select (Life Technologies Corp., Carlsbad, CA, USA) at 37°C for 12 minutes when they reached confluence. The HCECs at passages 2 through 5 were used for all experiments. The cultures were performed by using corneal endothelium from 20 distinctive donors, and the experiments were carried out in triplicate and repeated at least two times.

#### Flow Cytometry Analysis of cHCECs

Human corneal endothelial cells were collected from the culture dish as described above and suspended at a concentration of \(4 \times 10^6\) cells/mL in FACS buffer (PBS containing 1% bovine serum albumin [BSA] and 0.05% NaN\(_3\)). Next, an equal volume of antibody solution was added and incubated at 4°C for 2 hours. After washing with FACS buffer, the HCECs were analyzed by use of a BD FACSCanto II Flow Cytometry System (BD Biosciences, San Jose, CA, USA).

#### Immunocytochemical Evaluation of c-Myc Expression

Human corneal endothelial cells cultured in six-well plates were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. The cells were then washed again with PBS, followed by treatment with 0.1% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO, USA) in PBS at room temperature (RT) for 5 minutes. Blocking was carried out with 1% BSA in PBS for 5 minutes, and the cells were then stained with rabbit anti-c-Myc antibody (Ab) (No. 5605; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. Next, after washing three times with PBS, the cells were stained with Alexa488-conjugated secondary Ab anti-rabbit IgG (1:1000) (A11034; Invitrogen, Carlsbad, CA, USA) as second Ab. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA).

#### Fluorescence Analysis of 2-NBDG Uptake

Glucose uptake by bulk-cultured HCECs was conducted by flow cytometry analysis. Briefly, cHCECs were incubated with 600 \(\mu\)M 2-NBDG at 37°C for 5, 10, and 30 minutes, 15 minutes after culture medium replacement by glucose-deprived medium. The cells were then washed two times with cold FACS buffer (PBS containing 1% BSA), resuspended in ice-cold FACS buffer, and subjected to flow cytometry. The mean fluorescence intensity of different groups was analyzed by BD FACSDiva (BD Biosciences) software and corrected for autofluorescence from unlabeled cells.
Glucose Starvation

Human corneal endothelial cells obtained from a single donor cornea were seeded in one well of a Type-I collagen–coated six-well plate (Corning). Cultured HCECs were exposed for different periods to glucose-depleted culture conditions supplemented with or without lactate (10 mM, unless otherwise stated). The exposed HCECs were then evaluated in terms of morphology, surface markers, and the gene expression of HCEC-related functional markers such as collagen 4A1, 4A2, and 8A2.

**Na+/K+-ATPase**

Immunohistochemical staining of Na+/K+-ATPase was performed before and after glucose starvation. Cultured HCECs were fixed with ice-cold methanol for 10 minutes, and then permeabilized with PBS(−) containing 0.1% Triton X-100 at RT for 15 minutes. After blocking nonspecific reactivity with 1% BSA in PBS(−) at RT for 1 hour, Na+/K+-ATPase staining was performed with 2 μg/mL Na+/K+-ATPase monoclonal antibody (EMD Millipore Corporation, Temecula, CA, USA), followed by N-Histofine MAX-PO (MULTI) (Nichirei Biosciences, Inc., Tokyo, Japan) detection reagent. After washing with PBS(−) containing 0.1% Triton X-100, cells were developed with N-Histofine Simple Stain DAB Solution (Nichirei Biosciences) and counterstained with hematoxylin (Merck KGaA, Darmstadt, Germany). Finally, cells were mounted with N-Histofine Aqueous Mounting Medium (Nichirei Biosciences) and observed under a bright-field microscope.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from cHCECs by use of the miRNeasy Mini Kit (QIAGEN, Hilden, Germany). The cDNA was synthesized by use of the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction was performed via TaqMan Fast Advanced Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (inventoried) (Applied Biosystems) under the following conditions: activation of enzyme at 95°C for 20 seconds, 40 cycles of denaturation at 95°C for 1 second, and annealing/elongation at 60°C for 20 seconds. The StepOnePlus Real-Time PCR system (Applied Biosystems) was used for PCR amplification and analysis.

The levels of MMP1, MMP2, MMP4, TIMP1, BMP2, SPARC, TGFβ1, TGFβ2, FN1, SERPINB2, CD44, CD166, CD105, CD24, Col3A1, Col4A1, Col4A2, Col8A2, CDH2, VIM, CDKN1B, CDKN1C, IGFBP3, SNAIL1, SNAIL2, and IL1B were normalized to that of 18S rRNA. The results were presented as ΔΔCt (relative units of expression).

**Statistical Analysis**

Student’s t test was used to determine the statistical significance (P value) of mean values for 2-sample comparisons. Dunnett’s multiple-comparisons test was used to determine the statistical significance for the comparison of multiple sample sets. Values shown on the graphs represent the mean ± SE.

**Measurement of Metabolites in cHCECs**

Metabolic extracts of intracellular metabolites were prepared from HCECs cultured in 6-well or 24-well plates with methanol containing Internal Standard Solution (Human Metabolome Technologies, Inc., Tsuruoka, Japan). The culture medium was aspirated from the well, and the cells were washed two times with 5% mannitol solution as follows: 1.5 mL followed by 0.5 mL (6-well plates) or 0.3 mL followed by 0.1 mL (24-well plates). The cells were then treated with 600 μL (6-well plates) or 200 μL (24-well plates) of methanol and left at rest for 30 seconds in order to inactivate enzymes. Next, the cell extract was treated with 410 μL (6-well plates) or 140 μL (24-well plates) Milli-Q (EMD Millipore)–purified water containing internal standards (H3304+1002; Human Metabolome Technologies) and left at rest for another 30 seconds. The obtained extract was then centrifuged at 2300g at 4°C for 5 minutes, and all of the upper aqueous layer was centrifugally filtered through a 5-kDa cutoff filter (EMD Millipore) at 9100g and 4°C for 120 minutes to remove proteins. The filtrate was then centrifugally concentrated and resuspended in 50 μL Milli-Q–purified water for capillary electrophoresis–mass spectrometry (CE-MS) analysis.

**Measurement of Metabolites in Culture Medium**

For the measurement of metabolites in the culture medium, 20 μL of the medium and 80 μL Milli-Q–purified water containing internal standards (H3304+1002; Human Metabolome Technologies) were thoroughly mixed. The mixture was centrifugally filtered through a 5-kDa cutoff filter at 9100g and 4°C for 120 minutes to remove proteins and macromolecules. The filtrate was then diluted five times by Milli-Q–purified water for CE-MS analysis.

**Analysis of the Metabolome Data**

Cationic compounds were measured in the positive mode of CE time-of-flight mass spectrometry (CE-TOFMS), and anionic compounds were measured in the positive and negative modes of CE tandem MS (CE-MS/MS) according to the methods developed by Yoga et al. Peaks detected by CE-TOFMS and CE-MS/MS were extracted by using automatic integration software (MasterHands, Keio University, Tsuruoka, Japan, and MassHunter Quantitative Analysis B.04.00, Agilent Technologies, Santa Clara, CA, USA, respectively) in order to obtain peak information including m/z, migration time (MT), and peak area. The peaks were annotated with putative metabolites from the Human Metabolome Technologies metabolite database based on their MTs in CE and m/z values determined by TOFMS. The tolerance range for the peak annotation was configured at ±0.5 minutes for MT and ±10 ppm for m/z. In addition, concentrations of metabolites were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and by using standard curves, which were obtained by three-point calibrations.

Hierarchical cluster analysis (HCA) and principal component analysis were performed by use of our proprietary software, that is, “PeakStat” and “SampleStat,” respectively. Detected metabolites were plotted on metabolic pathway maps with Visualization and Analysis of Networks containing Experimental Data (VANTED) software. Metabolome measurements were carried out through a facility service at Human Metabolome Technologies.

**Results**

C-Myc Expression and Glucose Uptake by Bulk-Cultured cHCECs

Bulk-cultured cHCECs with no assignment of the SP presence were immunocytochemically stained with anti-c-Myc antibody. C-Myc expression was confirmed at the position of morpho-
logically transformed cell-like shapes in phase contrast microscopy (Fig. 1A). This indicated that at certain culture conditions, at least two SPs exist with or without c-Myc expression in cHCECs. Considering the role of c-Myc in glycolysis, the uptake of glucose in bulk-cultured cHCECs was investigated and the considerable uptake in cHCECs was confirmed, although no difference in the degree of glucose uptake was confirmed (single peaks of 2-NBDG uptake at all incubation time points; Fig. 1B).

**Phenotype and Morphologic Changes by Glucose Starvation**

To evaluate the selective deletion of SPs of cHCECs in bulk cultures, cHCECs were cultured in glucose-starved medium (Dulbecco’s modified Eagle’s medium, DMEM) without fetal bovine serum in the presence of lactate. After passages to P3, the cultured cells were incubated in DMEM with glutamine, but without glucose, in the absence or presence of up to 10 mM lactate for 72 hours. Then, the resultant cHCECs were further cultured at three distinct dilution passages (1:3, 1:9, and 1:30) under normal conditions for 4 weeks. Morphologic changes (Fig. 2A) demonstrated the deletion of some cHCEC SPs, indicating the presence of glycolytic energy metabolism even with uniform glucose uptake (Fig. 1B). The efficacy of recovery was found to be clearly dependent on the concentration of lactate added (Fig. 2B).

**Alteration of EMT, Cell Senescence, and Fibrosis-Related Genes by Glucose Starvation**

The partial selective deletion of cHCEC SPs by glucose starvation was reproducibly confirmed, indicating the presence of SPs distinct in their glycolytic energy metabolism. To further elucidate the deletion effects, RNA was extracted from the prestarved cHCECs and cHCECs starved for 72 hours. The expression of genes related to CST, such as EMT, cell senescence, and fibrosis, was analyzed (Fig. 3). The most impressive upregulation after glucose starvation was assigned to MMP1, MMP2, BMP2, and TGFβ1, while TGFβ2 showed decrease (Fig. 3A).

In the same experiments with other cultures and starvation conditions, upregulation was confirmed for MMP1, MMP2, MMP4, BMP2, and TGFβ1, while TGFβ2 showed decrease. On the contrary, most of the genes related to EMT and fibrosis were uniformly downregulated. The downregulated genes included SPARC, TGFβ2, IL-1β, CD44, CD24, CDH2, VIM, SNAI1, SNAI2, and IGFBP 3, 4, and 7 (part of the data shown in Fig. 3B). Several isoforms of collagen were also downregulated (i.e., 3A1, 4A1, and 8A2; data not shown) and CDKN1s showed a tendency to decrease.
Metabolomic Profile Clustering Among Heterogeneous cHCECs

Three lots of cHCECs (C16P6, C21P3, and 164P1) were analyzed, and proteins were extracted 2 or 3 days after medium changes at subconfluent cell density (7.22 × 10⁵, 9.85 × 10⁵, and 3 × 10⁵, respectively). Unsupervised HCA of the normalized metabolite intensities showed clear separation among the three lots of cHCECs (Fig. 4A). The morphologic differences of these cHCECs are shown together with the flow cytometry analysis data in Figure 4B. Despite the superficially similar microscopic features, the HCA differed greatly, especially between C16, C164, and C21. The distinction observed by flow cytometry analysis coincided well with the results. Nevertheless, metabolite profiles were distinct between C164 and C16, possibly due to the presence of CD44⁺⁺⁺ cell populations in C16 (Fig. 4B).

Lactate to pyruvate ratios were higher in C164 than in C16 and C21, and the content of GSH, GSSG, and total GSH, the markers of intracellular redox status, were dramatically lower in C16 and C21 than in C164 (Fig. 4C).

Metabolomic Profile Clustering of Secreted Metabolites

In our aim to develop a practical and noninvasive method for monitoring the conformity of chCEC quality for regenerative therapy, as opposed to the invasive way of following surface CD markers, we comprehensively surveyed the secretory metabolites in culture medium precisely assigned to the cHCEC SPs distinct in the expression levels of surface CD44 antigens. The metabolite changes in conditioned media are detailed in Figure 5. Hierarchical cluster analysis of metabolomic profiles of the culture media identified subsets of metabolites that correlated with the presence of CST. The clusters were divided into at least four metabolite subsets: (1) increased metabolites in all chCECs (Nos. 66, 72, and 55); (2) increased metabolites mainly in No. 58 (donor age = 66 years, donor ECD = 5127); (3) metabolites most profoundly decreased in No. 66;
FIGURE 3. Alteration of EMT, cell senescence, and fibrosis-related genes by glucose starvation. Upregulation was assigned to MMP1, MMP2, BMP2, and TGFβ1, while TGFβ2 showed decrease (A). The genes related to EMT and fibrosis were uniformly downregulated. The downregulated genes included SPARC, TGFβ2, IL-1β, CD44, CD24, CDH2, VIM, SNAIL1, SNAIL2, and IGFBP 3, 4, and 7 (B). (A) Cultured HCECs for No. 55 (passage 5, donor age = 49 years, donor ECD = 2699) were either glucose starved, or not. The qRT-PCR experiments were performed in duplicate. *P < 0.05, **P < 0.01. (B) Cultured HCECs for No. 72, as in Figure 2. The qRT-PCR experiments were performed for pre- and poststarvation chCECs in triplicate. *P < 0.05, **P < 0.01.
FIGURE 4. Intracellular metabolomic profile clustering among heterogeneous cHCEC SPs. Unsupervised HCA showed clear separation among the three lots of cHCECs (A). Despite the superficially similar microscopic features, the HCA differed greatly, especially between C16, C164, and C21 (B). Cultured HCECs subjected to analysis were C16 (P6, 19Y, 3062), C21 (P3, 23Y, 3884), and No. 164 (P1, 15Y, 3226). (A) Hierarchical clustering of the metabolites among cHCECs, C16, C21, and No. 164. (B) Cellular features of cHCECs subjected to analysis and the composites of SPs revealed by FACS. (C) Illustration of the relative amount of representative metabolites among cHCECs, C16, C21, and No. 164.
FIGURE 5. Metabolomic profile clustering of secreted metabolites in the supernatants of cHCECs. The clusters were divided into at least four metabolite subsets: (1) increased metabolites in all cHCECs (Nos. 66, 72, and 55); (2) increased metabolites in No. 72 and No. 55, but not in No. 66; (3) metabolites most profoundly decreased in No. 66; and (4) subtypes appearing, more or less, in all three groups (A). The lactate to pyruvate ratios were higher in No. 72 and No. 55 than in No. 66 (B). Three lots exhibited a nice morphology under microscopic examination (C). Cultured HCECs subjected to the analysis were No. 66 (P4, 23Y, 3504), No. 72 (as in Fig. 2), and No. 55 (as in Fig. 3).
and (i) subtypes appearing, more or less, in all three groups (Fig. 5A). Several intermediates in glycolysis were lower in No. 72 and No. 55 than in No. 66, consistent with the “Warburg effect” of increased glycolysis rate. The Warburg effect also includes increased lactate production. Indeed, the lactate to pyruvate ratios were higher in No. 72 and No. 55 than in No. 66 (Fig. 5B). It is of note that these three lots exhibited nice morphology under microscopic inspection (Fig. 5C).

Distinction of Secreted Metabolites Among Quality-Controlled cHCECs Without CD44+, CD24+, and/or CD26+ SPs

The culture medium of cHCECs produced under good manufacturing practice conditions and devoid of CD44++, CD24+, or CD26+ cells was analyzed. The four investigated cHCECs differed in their composite SPs in the context of CD44++ versus CD44++ SPs. The quality can be monitored by the ratio of CD44+ to CD44++ SPs, or CD26+ and CD26++ SPs. The four investigated cHCECs had similar metabolite profiles; however, C23 exhibited a strong disposition for mitochondria-dependent OXPHOS instead of anaerobic glycolysis, as verified via the clustering described above, HCA identified four metabolite subsets as described above (Figs. 6B, 6C). The four lots exhibited similar metabolite profiles; however, C23 exhibited a strong disposition for mitochondria-dependent OXPHOS instead of anaerobic glycolysis, as verified via the lowest production of lactate, the lowest lactate to pyruvate ratio, and the highest production of tricarboxylic acid cycle intermediates such as citrate/isocitrate and cis-aconitate. Interestingly, glucose consumption was highest in C24, whereas C21 showed the lowest consumption, thus indicating a possible difference in gluconeogenesis in these two. No big differences were distinct in amino acid metabolism and redox status. To confirm the reduction of glycolysis and the disposition for mitochondria-dependent OXPHOS in the most ideal cHCECs, C23, we propose a simple monitoring method for metabolites in culture media via the ratio of citrate to lactate. Figure 7 illustrates the difference in the citrate to lactate ratios among No. 66 and No. 55 versus No. 72, different in the content of CD44++ SPs, and those among A21, A22, A23, and A24, which differed only in the content of CD44− to CD44+ versus CD44++ SPs. The quality can be monitored by the ratio of citrate over lactate in cHCEC culture supernatants.

Discussion

In this current study, we found a way to discriminate SPs in cHCECs by their secretory metabolites, and found that the CST SPs exhibited disposition for anaerobic glycolysis instead of mitochondria-dependent OXPHOS. To the best of our knowledge, these findings are the first to open a pathway for monitoring the disposition of cHCECs in regard to their energy metabolism.

Since HCECs can be grown in culture, cell-based therapy of using cHCECs to treat corneal endothelial dysfunction has been extensively explored.11,41−45 One of the most notable obstacles to the application of cHCECs for cell-based therapy is the lack of an established reliable method for verifying the quality of cHCECs. In this study, we presented a noninvasive method for monitoring cell quality, as opposed to the invasive method that involves following surface CD markers. The comprehensive survey in this study of the secretory metabolites in culture medium precisely assigned cHCEC SPs distinct in the expression levels of surface CD44 antigen.

It is known that the metabolic state influences the cell state, and the Warburg effect in cancer cells destined to aerobic glycolysis is one representative example.27 Nonetheless, the requirement for specific metabolic reprogramming in the maturation and differentiation of HCECs remains to be explored.46 To the best of our knowledge, our findings in this present study are the first describing the profound change of energy metabolism states among distinct SPs in cHCECs. Cell-state transition-cHCECs switched to a glycolytic metabolotype, whereas differentiated cHCECs became more oxidative. Low lactate production and elevated activating phosphorylation levels in cHCEC mature SPs suggest a possible application of metabolomics in cell quality control and/or their usefulness as biomarkers for diagnosis, prognosis, and therapeutic efficacy for corneal endothelial dysfunctions, such as Fuchs’ endothelial corneal dystrophy (FECD). Our data were not sufficient to deduce a statistical correlation between the variation of cell area and the percentage of hexagonal cells. Hence, we were unable to further detail the correlation between the morphometric characteristics of cultured cells, phenotype characteristics, and the metabolites.

C-Myc reportedly regulates lactate production through transcriptional regulation and is an important regulator for cell cycle and glycolysis in transformed cells.28,47−49 Enzymes involved in glucose uptake, lactate excretion, and glutamine catabolism are important targets of c-Myc regulation, and it has been suggested that c-Myc can drive cells to specific metabolic substrates.49 The reduction of glycolysis in mature differentiated cHCEC SPs might be triggered by downregulation of the c-Myc pathway. In this context, it is noteworthy that c-Myc is also regulating CD44 expression, which is key in distinguishing differentiated cHCECs from either nondifferentiated cHCECs or cHCECs with CST. Reportedly, CD44 plays a critical role as a major adhesion molecule of the extracellular matrix and in TGFβ-mediated mesenchymal phenotype induction. Loss of CD44 reportedly abrogates those changes.50 In that study, CD44 ablation by RNA interference increases metabolic flux to mitochondrial respiration and concomitantly inhibits entry into glycolysis.

Apart from the pathway relating to c-Myc/CD44 regulation, it is noteworthy that mutant p53 promotes aerobic glycolysis by inducing GLUT1 translocation to the plasma membrane, which is mediated by activated Ras homolog gene family, member A (RhoA) and its downstream effector Rho-associated protein kinase (ROCK).51 In our culture system, the presence of ROCK inhibitor Y27632 dramatically induced the differentiation of cHCECs to a mature state devoid of OXPHOS with reduced expression of CD44 and GLUT1. The transition between regulated GLUT1 translocation and RhoA/ROCK pathway in cHCECs might be elusive. It should be noted that CD44 might be repressed by wild-type p53.52

Our findings also provide novel insights into the origin of guttae in FECD, with cardinal features such as abnormalities of cells, coalescence of multiple guttae, and contour reported in previous studies.53,54 Considering the necessity of matching metabolic output with the demands of cellular function for tissue integrity under homeostatic and stress conditions, future elucidation of the origins of guttae in regard to the metabolic changes will provide new insights into pathogenesis of FECD.

In summary, the findings in this study provide a pathway for monitoring the disposition of cHCECs via their energy metabolism, thus leading to safe and stable regenerative medicine by metabolically defined cHCECs in the form of a cell suspension. Simultaneously, our novel findings, albeit very preliminary, provide evidence of a possible link between metabolic regulation in the corneal endothelium and the development of more effective targeted therapies to treat patients with bullous keratopathy, including FECD.
FIGURE 6. Fine distinction of secreted metabolites in the culture supernatants of cHCECs. Hierarchical cluster analysis identified four metabolite subsets, as described in Figure 5B and 5C. C23 exhibited a strong disposition for mitochondria-dependent OXPHOS instead of anaerobic glycolysis. Cultured HCECs subjected to the analysis were C21 (P2, 23Y, 3884), C22 (P2, 29Y, 3309), C23 (P2, 18Y, 3280), and C24 (P3, 29Y, 3412). (A) The composites of cHCECs, C21, C22, C23, and C24 revealed by FACS. (B) Hierarchical clustering of the metabolites among cHCECs, C21, C22, C23, and C24.
C23, and C24, which differed only in the content of CD44/C0+
+ + + in the citrate to lactate ratios among No. 66 and No. 55 versus No. 72,
supernatants of distinct cHCECs. The figure illustrates the difference
between these supernatants.

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