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Quantitative pigment extraction analysis for human pluripotent stem cell derived retinal pigment epithelial cells

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Abstract—**Pigmentation is a typical feature for retinal pigment epithelial cells (RPE), and each RPE cell represents individual characteristics. Although, type and intensity of pigmentation may vary, still most cells are pigmented to some extent.**

In this study, we set-up a standardized quantitative melanin content analysis for human embryonic stem cell (hESC) derived RPEs (hESC-RPE) and compared this method to an image based pigment quantification technique. The optical quantification of pigmentation was done from micrographs of hESC-RPE. The extracted pigment was quantitated with spectral analysis, with UV-Vis spectroscopy, and the size-distribution with dynamic light scattering.

The results revealed that the measured particle sizes of extracted melanin corresponded to known sizes extracted melanosomes. In addition, the optical pigmentation and absolute melanin concentration were clearly correlating.

Our data suggests that image analysis and quantitative melanin content analysis can be interchangeably utilized in pigmentation quantitation: if the relative pigmentation of hESC-RPE cells is desired to be estimated with a non-invasive method then image analysis is the choice of method, but if the exact amount of melanin needs to be evaluated, then the new melanin extraction method presented here, should be chosen.

Keywords—hESC-RPE, Melanin, Pigmentation quantification

I INTRODUCTION

The retinal pigment epithelium (RPE) is a highly melanized cell monolayer, situated between neural retina and choroid in the back of the eye. The RPE fulfills essential tasks for the neighboring neural retina providing the long-term preservation of retinal integrity and visual functions by absorbing stray light, recycling of retinoid, supplying nutrients, secreting growth factors and phagocytosing of shed photoreceptor outer segments (1,2). The RPE cells differentiated from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are proposed to be suitable cell source for transplantation therapy in retinal diseases (3). The current differentiation protocols for the production of RPE cells mainly rely on spontaneous (4), or more directed differentiation methods (5). The characteristics of these generated RPEs should be thoroughly analyzed for down-stream applications.

The mature RPE exhibits three major types of pigmentation: melanin containing melanosomes, lipofuscin and melanolipofuscin. While melanosomes are formed during embryogenesis the accumulation of lipofuscin and melanolipofuscin are a symptom of aging. In cultured hESC-RPE, pigment density initially decreases in the course of cell divisions, which results in a gradual dilution of pigment among the daughter cells, but as the cells become confluent *de novo* synthesis of pigment can be observed (6,7). By day 30, the pigment density is comparable to that in native human RPE (6). Among other features, hESC-RPE are expected to exhibit the same type of pigmentation as native RPE cells.

The pigmentation density at a distinct time point can serve both as a marker for the efficiency of a differentiation protocol and as a guideline for the stage of maturation. Therefore, in this study we introduce a protocol for a standardized quantitative melanin content analysis for RPE cells, comparing this method to an image based pigment quantification technique in respect to sensitivity and reproducibility.

II MATERIALS AND METHODS

Each set of cultured hESC-RPE cells (*A*) was processed as follows: in the end of culture (42d or 135d) cells were photographed (*B*), and the pigment was chemically extracted (*C*). Thereafter the pigment concentration (*C*), spectral properties (*D*) and particle size composition (*D*) of the extracts were determined.

A. hESC and RPE differentiation

Two hESC lines derived at our laboratory were used in this study: Regea08/017 (46,XX) and Regea08/023 (46,XY) (8). The hESCs were differentiated to RPE as floating cell aggregates as described previously (4,9). For the expansion of RPE cells, pigmented areas were manually isolated, dissociated and replated as previously described (4,9). For the experiments the cells were seeded on Collagen IV -coated 24 well plates (Sigma-Aldrich, St. Louis, USA) and cultured for 42 d until light pigmentation was visible; or up to 136 d.

B. Optical pigmentation quantification

The optical pigmentation analysis from bright field micrographs was done as in (9), from five randomly selected areas.

C. Chemical melanin extraction

Cells were dissociated with TrypLE™ Select (Life Technologies, Carlsbad, USA) and counted. The cells were washed twice with 0.5 ml PBS, pelleted at 16.000 g, 5 min at 4 °C. The floating cell-pellets were sonicated in an ice-cooled Bioruptor Standard device (Diagenode, Seraing, Belgium) with the highest intensity, 7 minutes, 30 sec intervals. Afterwards, the cell debris was pelleted, 16.000 g, 5 min at 4 C, and pellet was washed with 500 µl cool wash-solution (0, 25 % Triton X-100, 2 % SDS in PBS) and re-pelleted (16.000 g, 5 minutes at 8 °C), the supernatant discarded and replaced with 500 µl cool PBS. This centrifugation step was repeated and the supernatant was then replaced with 50 µl 1N NaOH (supplemented with 10 % DMSO), followed by an incubation-step at 80° C; until the pellet was completely dissociated i.e. the initially clear NaOH had become brown. Finally, the melanin contents of all extracts were determined with an EnVision 2104 Multilabel reader (Perkin Elmer, Waltham, USA) by measuring the absorbance between 330 and 340 nm (theoretical absorbance maximum of melanin, (10)), using a low auto-fluorescence DELFIA 8x12 strip micro plate (Perkin Elmer, Waltham, USA). Synthetic melanin [100 µg/ml] (Sigma-Aldrich, St. Louis, USA) served as a standard.

D. Spectral analysis and size distribution analysis with dynamic light scattering

The extracted pigment was identified with UV-Vis-spectra (290-890 nm) of both hESC-RPE extracts and synthetic melanin [100 µg/ml] (Sigma-Aldrich, St. Louis, USA) using a LAMBDA 35 UV/Vis spectrometer (Perkin Elmer, Waltham, USA) with a 50 µl quartz-cuvette. Finally the pigment extracts from heavily pigmented and lightly pigmented hESC-RPE samples and synthetic melanin were analyzed with a Zetasizer Nano device (Malvern Instruments, Malvern, UK), using a 50 µl low fluorescence cuvette (Analysis-settings: Dispersant RI: 1.330, Viscosity: 1.5678 mPa·s).

E. Statistical analysis

The statistical analysis was performed using SPSS analyzing software (IBM, Armonk, USA), and statistical significance was determined with two tailed Mann-Whitney U test.

III RESULTS

A. Optical pigmentation quantification

The image analysis of bright field micrographs (Figure 1) of heavily and lightly pigmented hESC-RPE revealed that after 42 d the optical pigmentation values ranged from 4.89 to 7.15. After 136 d of culture with heavy pigmentation, the optical pigmentation ranged from 1.49 to 1.62.

B. Chemical melanin extraction quantification

The photometric concentration determination from the same samples resulted in absolute pigment concentrations; ranging from 89 to 390 µg/ml. Subsequently, these values were normalized by dividing the measured pigment concentrations by the cell count (Figure 1).

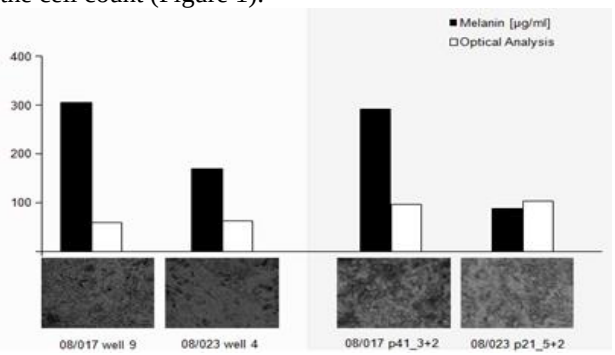

Figure 1: Representative images of examined lightly (left) and heavily pigmented hESC-RPE with corresponding melanin and relative (optical) pigmentation values (without background subtraction).

C. Analysis of pigmentation

The visual comparison of the pigmentation from cells with the same culture time and similar cell count, the Regea08/017 hESC-RPEs displayed a denser and darker pigmentation than Regea08/023 hESC-RPEs. The normalized melanin values of the light-pigmented cells differed significantly from those, obtained from the heavily pigmented ones ($p=0.03$). There was a significant difference between melanin values of lightly pigmented samples from different cell lines: The Regea08/017 hESC-RPEs higher than Regea08/023 hESC-RPEs ($p<0.001$). The lightly pigmented (Regea08/017) samples ($n=12$) yielded optical pigmentation values, ranging from 5.8 to 6.3 (median 6.08) and melanin concentrations, ranging from 266 to 390 µg/ml (median 335 µg/ml. The lightly pigmented Regea08/023 hESC-RPEs ($n=12$), yielded optical pigmentation values, ranging from 4.7 to 5.9 (median 5.3) and melanin concentrations, ranging from 145 to 209 µg/ml (median 179 µg/ml). The heavily pigmented samples

(Regea08/017 and Regea08/023, n=4) had optical pigmentation values ranging from 1.49189 to 1.62916 (median 1.56600), and melanin values, ranging from 89 to 291 µg/ml (median 174 µg/ml) (Figure 2).

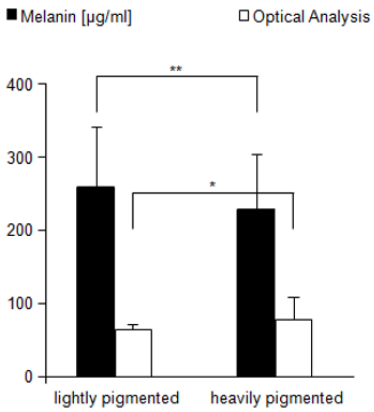

Figure 2: Comparison of absolute melanin-values and relative optical pigmentation-values (without background-subtraction). The heavily pigmented samples n= 4, lightly pigmented samples n= 12 form both (Regea08/017 and 2x Regea08/023) cell lines (*$p \leq 0.05$, **$p \leq 0.01$).

D. Comparison of the methods

The statistical correlation analysis revealed that optical pigmentation values and melanin values are clearly correlating. When all samples were analysed together, the correlation between the optical values and melanin concentration values was 0.63 ($p<0.001$). The correlation was even higher when analysing only lightly pigmented samples ($r=0.76$, $p<0.001$), and further the correlation of the Regea08/017 samples was more obvious, compared to the Regea08/023 samples. In contrast, a correlation of the values resulting from the heavily pigmented samples could not be confirmed possibly due to small sample number and variation between the heavily pigmented Regea08/017 and Regea08/023 samples. The pigmentation values of the lightly pigmented samples differed statistically significantly from those of the heavily pigmented samples.

E. Spectral analysis

The spectral analysis of synthetic melanin and two randomly selected extracts from both cell lines resulted in a complex spectra (Figure 3.), but there was a high similarity between the spectrum from synthetic melanin and the spectra from hESC-RPE extracts (Figure 3).

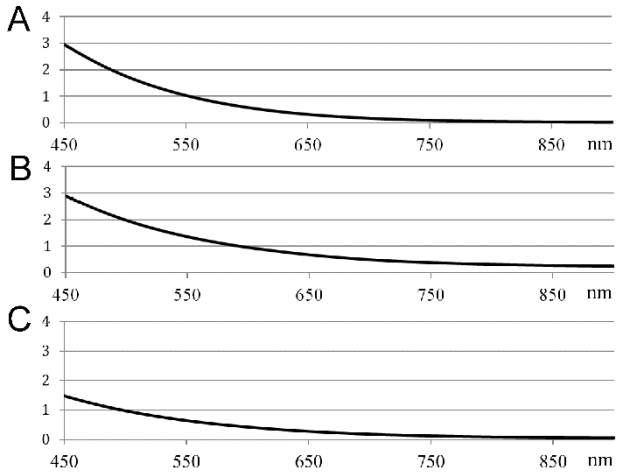

Figure 3: Spectral analysis (450-900nm) of synthetic melanin (A), Regea08/017 (B) and Regea08/023 (C) hESC-RPEs.

F. Dynamic light scattering analysis

The size distribution analysis revealed a particle size between 100 and 800 nm for synthetic melanin (Figure 4A). The diameters of particles detected in the pigment extracts varied from 80 to 8000 nm in the case of Regea08/017 cells (Figure 4B) and from 800 to 8000 nm in extracts of Regea08/023 cells (Figure 4C).

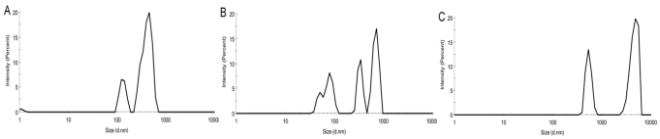

Figure 4: Particle size-distribution recorded from synthetic melanin (A), Regea08/017 (B) and Regea08/023 (C) hESC-RPEs

IV DISCUSSION

In this study, we aimed to develop new melanin extraction method for hESC-RPE cells compared to the previous one (11,12). The UV-Vis-spectra recorded here from synthetic melanin and from hESC-RPE extracts illustrates the complexity and the polymeric nature of the melanin-extracts. This result is in accordance with the published literature (10, 13, 14). Melanosomes of the RPE are non-transparent and appear in two shapes: elliptical melanosomes (0.5 to 1.0 µm in width, 1.0 to 2.5 µm in diameter), spherical or oval melanosomes (smaller one 0.5 to 1.0 µm, larger ones 1.5 to 2.0 µm) (15, 16). In *Sepia*, eumelanin sizes range from 3 to 286 µm in the long dimension and other more densely packed particles, that vary in their lateral dimension from 45 to 230 nm (17,18). This, relatively wide size distribution, was also

documented in this study. In addition the high variation in size (80-8000 nm), detected in here can be explained by the tendency of melanin to form aggregates.

The other aim was to evaluate if the pigment-extraction protocol could verify pigmentation values gained with the optical pigmentation analysis. The pigmentation analysis from the hESC-RPEs has previously been done with image analysis from the bright field micrographs (9,19,20) or by quantitating the extracted melanin (3) but comparison has not been previously done. Here our results demonstrated that optical pigmentation values and melanin values were clearly correlating. Our conclusion is that the pigmentation values obtained with image analysis method can be used to evaluate non-invasively the relative amount of pigmentation in hESC-RPEs. However, in order to acquire absolute melanin concentration then the invasive, "end-point analysis", of quantitative pigment extraction should be used.

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Conflict Of Interest

The authors declare that they have no conflict of interest.

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