Pigmentation Is Associated with Stemness Hierarchy of Progenitor Cells Within Cultured Limbal Epithelial Cells

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ABSTRACT

Ex vivo cultured human limbal epithelial stem/progenitor cells (hLESCs) are the main source for regenerative therapy of limbal stem cell deficiency (LSCD), which is worldwide one of the major causes of corneal blindness. Despite many stemness-associated markers have been identified within the limbal niche, the phenotype of the earliest hLESCs has not been hitherto identified. We sought to confirm or refute the use of tumor protein p63 (p63) and ATP binding cassette subfamily B member 5 (ABCB5) as surrogate markers for hLESCs early within the limbal differentiation hierarchy. Based on a robust fluorescence-activated cell sorting and subsequent RNA isolation protocol, a comprehensive transcriptomic profile was obtained from four subpopulations of cultured hLESCs. The subpopulations were defined by co-expression of two putative stem/progenitor markers, the p63 and ABCB5, and the corneal differentiation marker cytokeratin 3. A comparative transcriptomic analysis yielded novel data that indicated association between pigmentation and differentiation, with the p63 positive populations being the most pigmented and immature of the progenitors. In contrast, ABCB5, either alone or in co-expression patterns, identified more committed progenitor cells with less pigmentation. In conclusion, p63 is superior to ABCB5 as a marker for stemness. Stem Cells 2018;36:1411–1420

SIGNIFICANCE STATEMENT

This study has first conducted a biomarker-oriented comparative transcriptomic analysis of cultured and sorted human limbal epithelial stem/progenitor cells (hLESCs), and found out that the p63 but not the ATP binding cassette subfamily B member 5 predicts the immaturity of niche progenitors. The comparative analysis of the functional gene networks furthermore revealed an association of stemness with pigmentation, which highlights the role of pigmentation in the protection of corneal limbus from radiation damage. These findings have implications for the acceptance and use of p63 as a marker for early hLESCs, and contribute to better understanding of hLESCs differentiation biology.

INTRODUCTION

The human limbal epithelial stem/progenitor cells (hLESCs) are believed to play a central role in renewing and repairing cornea [1]. However, this biological process may be disturbed thus leading to a condition termed limbal stem cell deficiency (LSCD). As a result, the cornea becomes opacified and vascularized, with a concomitant visual impairment that often results in complete blindness [2]. At present, transplantation of ex vivo cultured limbal epithelial cells is considered to be the most efficient treatment [2]. Since the first cultured limbal epithelial transplantation (CLET) was conducted in 1997 [3], thousands of CLET procedures have been reported from around the world [4]. Despite its indisputable success, the overall long-term success rate of this procedure does not surpass 80% [5–7]. Considerable effort is therefore currently being invested into better understanding of the biomolecular and developmental cues that control the limbal stem cell niche, so that the current CLET may be improved.

The hLESCs are usually identified by a combination of markers, which include p63, ABCG2, integrin α9, keratin 15, N-cadherin, NGF/TrkA, integrin α6/CD71, Hes1, p75, nectin 3, importin 13, nucleostemin, CD38/157, Lrig1,
ABCBS, and WNT7A [8]. Of special significance appears the p63 marker, since Pellegrini et al. in 2001 suggested that its deltaNp63α isoform is required to support the normal development of corneal epithelium [9]. Furthermore, the proportion of p63 positive cells in the limbal epithelial cell culture is a key factor influencing the success of CLET [5]. Consequently, it is being used as a surrogate marker for hLESCs in the world’s first commercial stem cell product for the treatment of LSCD, the Holoclar [10, 11]. While this marker has a notable prognostic value, enrichment strategies based on antibody-sorting of cells are hindered by the fact that p63 is an intracellular protein. Alternatively, the ATP binding cassette subfamily B member 5 (ABCB5) surface protein, has been suggested as a putative hLESCs marker. Recent findings have shown that ABCBS is critical for corneal epithelial homeostasis and repair [12] and it is often co-expressed with p63 in hLESCs both in situ [12] and ex situ [13, 14]. However, many concerns were recently raised regarding the ability of p63, as well as of ABCBS to accurately detect hLESCs [11, 15, 16]. Thus, in spite of clear significance, the specific placement of these markers within the limbal differentiation hierarchy remains unresolved.

Earlier attempts to clarify the developmental biology and differentiation hierarchy of hLESCs have been hampered by the use of nondiscovery based methods such as microarray analysis [17–23], poor study material such as nonhuman or whole unfractonated tissue [24–29], or impure hLESC cultures [30, 31]. A few discovery based next-generation sequencing hLESCs transcriptomic studies have been conducted but these were also limited by the use of in situ material [32, 33] or non-human models [34–36].

To overcome these limitations, we have in this study combined our earlier optimized pipeline for high quality transcripts from human limbal epithelial cellular subpopulations sorted by fluorescence-activated cell sorting (FACS) [37] with discovery based next-generation sequencing. By using this strategy, we have as the first conducted a biomarker-oriented comparative transcriptome analysis of cultured and sorted hLESCs, and by this sought to refute or confirm the use of p63 and ABCBS as surrogate markers for hLESCs.

MATERIALS AND METHODS

Cell Culture

For isolation of hLESCs, corneal scleral rings were procured from the Danish Cornea Bank (Aarhus University Hospital, Arhus, Denmark) in accordance with the applicable Danish legislation. For a single isolation procedure, 10 to 12 randomly collected rings (donor age 22–86 years, 64% men, and absence of corneal disease) were used, and, altogether, three independent primary cell lines were established. The protocol for isolation and culture of hLESCs was based on our previous report [38]. In brief, after gross debridement and removal of the endothelium, the rings were incubated with 2.4 U/ml dispase II (Life Technologies, Naerum, Denmark) in sterile phosphate-buffered saline (sPBS; Gibco, Taastrup, Denmark) for 1 hour at 37°C. The limbal epithelial cell layer was then scraped and further digested with TrypLE (Gibco) for 15 minutes at 37°C. The obtained cell suspension was filtered through a 70 μm mesh (BD Biosciences, San Jose, CA), seeded into T25 culture flasks (Corning CellBIND, Sigma–Aldrich, Copenhagen, Denmark), and cultured in complete keratinocyte-SFM (Life Technologies). At 80%–90% confluency, the cells were detached using TrypLE for subsequent procedures.

In Situ Direct Immunofluorescence Assay

Live cell cultures at P2 to P3 were used to reveal the surface ABCBS epitope (LifeSpan BioSciences, Seattle, WA), whereas cells fixed and permeabilized with 4% formaldehyde and 0.1% Triton X-100 (both from Sigma–Aldrich), respectively, were used to target the intracellular markers p63 and CK3 (both from US Biological, Salem, MA). Specifications of the used conjugates and their preimmune controls are listed in Supporting Information Table S1. The antibodies were diluted as recommended by manufacturers in sterile phosphate buffered saline (sPBS) supplemented with 10% fetal bovine serum (FBS) and 0.1% sodium azide in the case of unfixed cells, and incubated with the cells for 1 hour at 4°C. After a brief washing with PBS, the nuclei were stained with 0.1 μg/ml Hoechst 33342 for 10 minutes at 4°C, which was followed by the final washing and mounting in fluorescent mounting medium (DAKO, Glostrup, Denmark). The signal was visualized and recorded with Axio Observer.Z1 microscope (Carl Zeiss, Göttingen, Germany) equipped with Orca Flash 4.0 camera (Hamamatsu, Ballerup, Denmark). The images were processed using Zen (blue edition) software from Carl Zeiss. In additional experiments, the ABCBS antibody was validated against previously established ABCBS monoclonal (clone 5H3C6) [14] (data not shown).

Immunofluorescent Labeling for Cell Sorting

The experimental set up was previously optimized to reveal markers pertinent to this study using a set of directly labeled antibodies and to determine the sorting thresholds using matching isotype controls for p63 and CK3 and fluorescence minus one (FMO) for ABCBS [39]. All buffers used in staining and subsequent FACS sorting were sPBS based, supplied with 50% Accumax (Sigma-Aldrich) and 25 mM HEPES (Life Technologies) to prevent cell clumping and to maintain a proper pH range. The cell suspensions were first stained for surface antigen ABCBS at a working dilution of 1:50 for 30 minutes at 4°C, followed by washing, and then fixation and permeabilization with 70% ethanol (VWR, Herley, Denmark) for 10 minutes at 4°C. After permeabilization, an Rnase inhibitor (Rnasin plus; Promega, Roskilde, Denmark) was utilized with each step. The intracellular antigens p63 and CK3 were targeted after additional washing with p63 (1:200) and CK3 (1:100), or with isotype controls for 30 minutes at 4°C. Finally, the labeled cells were transferred into a 5 ml round-bottom polystyrene tube (BD Falcon, Albertslund, Denmark) for the flow cytometric sorting and analysis. Using the previously established primary cell lines, three independent staining and sorting experiments were carried out.

FACS Cell Sorting

MoFlo Astrios cell sorter and Summit Software v4.3 (both from Beckman Coulter, Brea, CA) were used for both FACS and flow cytometric analysis. Gate strategies were set with reference to isotype and FMO controls, and discrimination limit for positive events was set at a fluorescence intensity higher than the top 2.5 percentile from the control samples. Before sorting, the
instrument was sequentially decontaminated with RNase ZAP (Sigma-Aldrich), 70% ethanol, and milli-Q water. To minimize mechanical stress during sorting, system pressure was set at 20 psi, and a 100 μm sorting nozzle was used. FACS sorting was performed at 4°C, and it typically took 2 to 3 hours to complete. Four hLESCs phenotypic subpopulations, including p63+, ABCB5+, p63 + ABCB5+, and the differentiation control p63 + ABCB5+ CK3+ were obtained. After each sorting, aliquots for total RNA quality control were withdrawn, and the cell remainders were kept at −80°C as frozen pellets until RNA extraction, at which point subpopulations from the three different sorting runs were pooled.

**Total RNA Extraction and Next-Generation Sequencing**

The RNA extraction and next-generation sequencing was carried out on a commercial basis by the AROS Applied Biotechnology (Aarhus, Denmark). The technologies used were based on the QiAsymphony RNA Kit (QiAGEN, Copenhagen, Denmark) for RNA isolation and the SMART-Seq v4 Ultra Low Input RNA Kit and Low Input Library Prep Kit V2 (both from Takara Bio, Otsu, Japan) for cDNA and sequence library preparation, respectively. The RNA-seq was done using an Illumina HiSeq 2000 instrument (Illumina Inc., San Diego, CA). Quality of obtained raw sequencing data was assessed with the aid of Quailmap v2.2 [40]. The sequences were submitted to Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under accession number PRJNA387095.

**Transcript Assembly and Differential Expression Analysis**

After trimming, the sequenced transcripts were imported as paired-ends reads into Cufflinks v2.21 for transcriptome assembly and differential expression analysis [41, 42]. Assembly of transcripts was performed against an annotated Homo Sapiens reference genome (Human genome 19). Six distinct RNA-seq experiments were created for each pair combination from the four hLESC phenotypic variants. These included, ABCB5+ versus p63+, p63 + ABCB5+ versus p63+, p63 + ABCB5 + CK3+ versus p63+, p63 + ABCB5+ versus ABCB5+, p63 + ABCB5+ CK3+ versus ABCB5+, and p63 + ABCB5+ CK3+ versus p63 + ABCB5+. Significance of differential gene expression was assessed at Benjamini & Hochberg false discovery rate (FDR)-adjusted p-values (q-values) <.05 [43]. InteractiVenn was used to render 4-way interactions among the independent libraries according to selected criteria [44]. CummeRbund v2.0.0 was invoked to produce heat maps of hierarchical clustering of genes and samples based on fragments per kilobase of transcript per million mapped reads (FPKM) values [45].

**Gene Ontology Analysis**

Significantly differentially expressed genes were annotated for over-represented gene ontology (GO) terms in biological process using Database for annotation, visualization and integrated discovery (DAVID) [46, 47]. To graphically render the relationships between significantly enriched (q < .05) GO terms, hypergeometric tests were performed and the resulting GO categories were visualized in a network fashion using BiNGO [48] in the Cytoscape environment [49].

**Statistics**

Data represent the mean (± standard deviation, SD) of three independent FACS procedures. For the data that is shown in differential gene expression as well as gene ontology analyses, an FDR adjusted p-value (q-value) was applied for multiple hypothesis testing based on Benjamini-Hochberg procedure [43].

**RESULTS**

**Immunophenotype Analysis and RNA Isolation and Sequencing**

The staining patterns of the selected antibodies were first confirmed by in situ immunofluorescence microscopy. The ABCB5 antibody produced a cell surface signal, whereas the p63 and CK3 antibodies reacted with intranuclear and cytoplasmic epitopes, respectively (Fig. 1A). The sorting was carried out from three independent cultures of hLESCs, and after averaging values from the sorted populations, the frequency of individual markers was, 31.5% ± 1.5% for p63, 27.7% ± 5.1% for ABCB5, and 43% ± 2.5% for CK3 (mean ± SD). The flow cytometric traces, which were obtained in one of the sorting experiments are shown in Figure 1B as representative data. The analysis of co-expression patterns further revealed that most of the cells did not bear any of the studied markers (40.4% ± 4.4%), and of the sorted subpopulations, the most prevalent phenotype was p63 + ABCB5 + CK3+ (red; 15.1% ± 2.9%), followed by ABCB5+ (green; 7.8% ± 3.0%), p63+ (blue; 3.9% ± 1.3%), and p63 + ABCB5+ (orange; 2.6% ± 1.3%) (Fig. 1B).

Before sequencing, the RNA quality was analyzed using parameters described previously [39], and the values pertinent to individual populations are shown in Supporting Information Table S2. Although the RNA yield varied broadly, up to 2.8-fold between the highest and lowest values, the RIN appeared consistent and sufficiently high (7.7 ± 0.4, n = 4) to meet the requirements of the protocol.

The analysis of the sequencing process by invoking the PHRED quality score confirmed high reliability of the obtained data, since more than 80% of all base calls scored higher than 30 (Fig. 1C). Importantly, a satisfactory depth of sequencing was achieved to perform differential expression profiling, as on average 1.14E + 08 reads were acquired per sample. More than 80% of these reads mapped in pairs, which translates into a total average of 94.27 x10⁶ pair-end reads (Table 1). Additional quality parameters were explored, and they are included in Figure 1B and Table 1. In particular, the average GC content for the mapped reads was 49.25% and the GC content per sample displayed a normal distribution. All four sequence libraries exhibited expected, nearly identical log-normal profile of coverage per mapped position (FPKM), and, similarly, appeared to cover the whole genome, where on average 86.96% of the pair-end reads mapped to exonic regions of the reference human genome 19.

**Differential Gene Expression Between the Sorted Cultured hLESC Subpopulations**

Transcripts from the sorted subpopulations were analyzed for differential expression. Global comparison revealed that the transcriptomes were overall quite similar in terms of genes identified,
quality of sequences and gene coverage. However, pairwise comparisons showed significant differential gene expression between the individual subpopulations (Fig. 2A,B). The actual number of significantly expressed genes (q < .05) regulated in either direction varied from 24 to 81, and both the up- and downregulation appeared to be in balance (Fig. 2C). The largest amount of differential regulation was observed when comparing p63+ ABCB5+ CK3+ vs. p63+ (159 genes), and the least when

Table 1. Parameters of RNA sequencing from fluorescence-activated cell sorting purified cultured human limbal epithelial stem cells subpopulations

| Subpopulation | Reads count | Reads mapped in pairs (%) | Mapped reads GC content (%) | Reads mapped to exonic regions (%) |
|---------------|-------------|---------------------------|----------------------------|----------------------------------|
| p63+          | 1.23E+08    | 82.67                     | 49.12                      | 88.13                            |
| ABCB5+        | 1.09E+08    | 81.18                     | 48.91                      | 85.61                            |
| p63+ABCB5+    | 1.13E+08    | 82.54                     | 49.22                      | 85.33                            |
| p63+ABCB5+CK3+ | 1.11E+08   | 85.87                     | 49.76                      | 88.78                            |

*Fraction out of pair-end reads mapped to human genome 19.
Abbreviation: GC, guanine-cytosine.
comparing p63 + ABCB5+ versus p63 (53 genes) subpopulations. These differences appeared sufficient to provide for a clear discrimination of the four subpopulations by principal component analysis (Fig. 2D). The co-expression patterns were further analyzed in by four-way Venn diagram plots to visualize the amount of shared and uniquely expressed genes of significance (q < .05) (Fig. 2E). The largest group is represented by genes that are shared by all four subpopulations (21.5%). Genes that are shared by two or three subpopulations in various combinations represent 38.4% and 21.8%, respectively, and the uniquely expressed genes represent 18.3% (Supporting Information Table S3). Since all the analyses above demonstrated that the isolated subpopulations displayed unique although closely related transcriptional activation, the degree of relatedness was explored by K-means-20 cluster analysis and hierarchical alignment (Fig. 2F). The dendrogram revealed a surprising pattern, where the ABCB5+ transcriptional signature was closer to the CK3 subpopulation than to the p63+ or p63 + ABCB5+ subpopulations. Significant differentially expressed genes (q < .05) were subsequently annotated for functional biological processes in the GO hierarchy.

**GO Analysis**

To evaluate the differentially expressed genes in terms of biological significance for a given subpopulation, functional annotations were applied using the DAVID tool. Significantly overrepresented (q < .05) GO terms are listed for each
subpopulation in Table 2. The GO terms pertinent to stem or progenitor cells, such as “positive regulation of exit from mitosis,” “regulation of chromosome segregation,” or “spindle organization” were markedly enriched in the p63+ subpopulation. Another noticeable finding in this group was that the pigmentation-related processes, such as “melanin biosynthetic process” or “developmental pigmentation,” scored among the highest overrepresented terms. As expected, the terms relevant to cell differentiation, such as keratinocyte differentiation or keratinization were enriched in p63 + ABCB5+ CK3+ subpopulation. Surprisingly, however, they were found enriched practically to the same degree also in ABCB5+ subpopulation. As for the double marker-expressing p63 + ABCB5+ subpopulation, the most notable processes were those that were found in the single marker p63+ and the ABCB5+ associated with extracellular matrix and collagen metabolism.

Further understanding of the implications of above described biological processes for the relationship between the subpopulations was provided by constructing GO networks with the aid of BiNGO tool (Fig. 3). A pairwise comparison clearly demonstrates that the p63+ subpopulation is set apart by a predominance of pigmentation-associated processes,

### Table 2. Enrichment of functional annotation terms using DAVID\(^a\) for genes differentially expressed between the cultured human limbal epithelial stem cells subpopulations, with values <.05 after Benjamini multiple testing correction

| BP_direct GO terms | Fold enrichment | p value | Benjamini |
|--------------------|----------------|---------|-----------|
| GO:0051301~cell division | 8.27 | 2.78E-12 | 1.43E-09 |
| GO:0008283~cell proliferation | 5.14 | 7.89E-06 | 1.01E-03 |
| GO:0008284~positive regulation of cell proliferation | 4.04 | 8.41E-05 | 6.17E-03 |
| GO:0031536~positive regulation of exit from mitosis | 72.38 | 6.85E-04 | 3.02E-02 |
| GO:0007067~mitotic nuclear division | 10.51 | 1.05E-12 | 1.08E-09 |
| GO:0051439~regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle | 31.47 | 1.67E-05 | 1.72E-03 |
| GO:0051430~negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle | 10.19 | 1.43E-03 | 4.94E-02 |
| GO:0051427~positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition | 11.43 | 1.73E-04 | 9.86E-03 |
| GO:0000086~G2/M transition of mitotic cell cycle | 8.45 | 4.34E-05 | 3.72E-03 |
| GO:0000070~mitotic sister chromatid segregation | 28.95 | 2.36E-05 | 2.21E-03 |
| GO:0007062~sister chromatid cohesion | 14.05 | 3.54E-08 | 9.11E-06 |
| GO:0051983~regulation of chromosome segregation | 54.28 | 1.27E-03 | 4.56E-02 |
| GO:0007050~chromosome segregation | 10.64 | 1.21E-03 | 4.52E-02 |
| GO:0007094~mitotic spindle assembly checkpoint | 28.95 | 3.28E-04 | 1.67E-02 |
| GO:0007052~mitotic spindle organization | 19.30 | 1.11E-03 | 4.30E-02 |
| GO:0007051~spindle organization | 45.24 | 3.57E-06 | 5.24E-04 |
| GO:0051310~metaphase plate congression | 48.25 | 6.58E-05 | 5.20E-03 |
| GO:0007080~mitotic metaphase plate congression | 19.56 | 1.16E-04 | 7.43E-03 |
| GO:0031145~anaphase-promoting complex-dependent catabolic process | 14.66 | 1.13E-06 | 1.95E-04 |
| GO:0009910~cytokinesis | 15.08 | 3.22E-04 | 1.73E-02 |
| GO:0000281~mitotic cytokinesis | 19.97 | 1.00E-03 | 4.05E-02 |
| GO:0042438~melanin biosynthetic process | 77.95 | 1.49E-10 | 5.12E-08 |
| GO:0030318~melanocyte differentiation | 36.19 | 9.29E-06 | 1.06E-03 |
| GO:0048066~developmental pigmentation | 62.04 | 9.55E-04 | 4.02E-02 |
| GO:0042787~protein ubiquitination involved in ubiquitin-dependent protein catabolic process | 7.57 | 8.75E-05 | 9.99E-03 |
| GO:1901215~negative regulation of neuron death | 18.09 | 1.58E-04 | 9.51E-03 |
| GO:0007517~muscle organ development | 9.76 | 3.62E-04 | 1.76E-02 |
| GO:0030574~collagen catabolic process | 18.09 | 2.65E-07 | 5.45E-05 |
| GO:0030198~extracellular matrix organization | 5.91 | 4.01E-04 | 1.86E-02 |
| ABCB5+ | | | |
| GO:0030216~keratinocyte differentiation | 18.94 | 2.19E-08 | 1.47E-05 |
| GO:0031424~keratinization | 23.32 | 4.85E-07 | 8.12E-05 |
| GO:0008544~epidermis development | 16.93 | 5.35E-08 | 1.19E-05 |
| GO:0018149~peptide crosslinking | 25.59 | 2.29E-08 | 7.66E-06 |
| p63+ABCB5+ | | | |
| GO:0030199~collagen fibril organization | 31.31 | 2.65E-04 | 2.27E-02 |
| GO:0003057~collagen catabolic process | 42.93 | 2.57E-11 | 1.33E-08 |
| GO:0070208~protein heterotrimerization | 87.23 | 1.12E-05 | 1.93E-03 |
| GO:0030198~extracellular matrix organization | 12.46 | 2.95E-06 | 7.67E-04 |
| GO:0071230~cellular response to amino acid stimulus | 32.48 | 1.54E-05 | 2.00E-03 |
| GO:0007155~cell adhesion | 5.99 | 1.01E-04 | 1.05E-02 |
| GO:0001649~osteoblast differentiation | 14.68 | 3.46E-04 | 2.54E-02 |
| p63+ABCB5+CK3+ | | | |
| GO:0030216~keratinocyte differentiation | 18.04 | 2.72E-07 | 2.04E-04 |
| GO:0031424~keratinization | 17.85 | 1.68E-04 | 2.48E-02 |
| GO:0008544~epidermis development | 12.10 | 1.32E-04 | 2.44E-02 |
| GO:0018149~peptide crosslinking | 20.56 | 1.00E-05 | 3.74E-03 |
| GO:00010951~negative regulation of endopeptidase activity | 9.91 | 7.16E-05 | 1.77E-02 |

\(^a\)Database for annotation, visualization, and integrated discovery. Subset of biological process gene ontology terms.

Abbreviations: ABCB5, ATP binding cassette subfamily B member 5; CK3, cytokeratin 3; p63, protein p63.
whereas all the other subpopulations are subject to epithelial-specific differentiation. This indicates that the p63+ variant is the common progenitor phenotype. On the other hand, as expected due to the expression of cytokeratin, the p63+ ABCB5+ CK3+ variant represents the most differentiated phenotype. Looking at the remaining two variants, the ABCB5+ and p63+ ABCB5+, intriguingly we found that the former phenotype appears developmentally downstream from the latter one. These relationships thus provided for a framework, upon which we based our proposal for a developmental hierarchy within the limbal niche (Fig. 4). We hypothesize that within the scope of studied phenotype variants, the p63+ represents the most immature progenitor, and the ABCB5+ marker either alone or in co-expression pattern identifies progressively more committed precursors.

**DISCUSSION**

The gene ontology analysis revealed that pigmentation and epithelial differentiation were mutually exclusive processes, which enabled us to determine a developmental hierarchy within the sample of isolated cultured hLESC phenotypical variants. Intriguingly, the most immature phenotype was found associated with p63 as a single marker, whereas ABCB5 alone or in co-expression was found on the descendant variants. Relationship between melanin pigmentation and limbal stemness has previously been investigated, and interestingly, the initial indication of the presence of hLESCs was inspired by observation of pigment movement from the limbus toward epithelial defect in wounded corneas [50]. In situ, the limbal palisades of Vogt, which contain pigment granules that are aligned with the microvilli of the corneal epithelium, are believed to be the source of hLESCs [51].
attributed to the protection from ultraviolet radiation and oxidative damage [52, 53]. Melanocytes that can be found scattered in the basal limbus epithelium have been highlighted as a major site of pigment production [54–57], nevertheless, our current investigation, in line with some previous studies, demonstrates that the limbal corneal progenitors are by themselves involved in melanin turnover [19, 32, 58]. Importantly, a direct communication between both cell lineages has as well as been documented [52–54]. This observation thus provides structural basis for realization that a cellular network, possibly also including players from additional compartments, that entails a comprehensive crosstalk is essential in order to properly maintain the limbal niche.

Although the molecular basis for the relationship between pigmentation and the hLESC maintenance or p63 is not fully understood, there is a plethora of evidence that implicates the SERPINF1, which encodes the pigment epithelium-derived factor (PEDF). PEDF was first identified as a 50 kDa secreted protein in conditioned medium from cultured fetal human retinal pigment epithelium (RPE) cells [59], and was recognized as a potent inhibitor of vascular endothelial growth factor (VEGF) [60]. Recently, PEDF was proposed to regulate the proliferation and differentiation of human embryonic stem cells [61] as well as multiple tissue-specific stem cells [62], and was also found in developing and mature human cornea [58]. With regard to hLESCs, it has been reported that the PEDF has the capacity to promote self-renewal [17] and that such effect may be associated with the p63 expression [63]. PEDF’s effect on regeneration of a functional limbus was further confirmed by Yeh et al. in 2016 in the rabbit model of LSCD [64, 65]. Interestingly, a possible regulatory mechanism was unveiled, when the PEDF was reported to be a direct target gene for p63 [66]. The results from our current investigation provide additional support for the PEDF role by demonstrating its exclusive association with earliest developmental phenotype, which is marked by single p63 expression.

Based on our model of differentiation hierarchy within the cultured limbal epithelial cells, the ABCB5 designates a lineage that is still of a precursor type, but is clearly distinct from the pigmented lineage that is associated with p63. Previously, based on the morphological criteria, differentially pigmented precursor types have been identified in the palisades of Vogt and in the transition zone closer to the peripheral cornea [67, 68]. It is highly likely, that the phenotypical variants analyzed in our study correspond to the in situ progenitors from the above studies, nevertheless, only direct identification of the place of residence within the limbus of the four studied phenotypes can give a definitive answer. Such study would undoubtedly shed more light on the developmental relationship between p63 and ABCB5 as well as the role of pigmentation in the maintenance of the limbal niche.

**CONCLUSION**

Both p63 and ABCB5 have been well established as markers associated with limbal stemness, nevertheless, their placement within the differentiation hierarchy has not been known until now. Although we were not able to confirm that the variant bearing single p63 corresponds to the true limbal stem cell, we have demonstrated that it is a more immature progenitor than those featuring ABCB5 alone or in co-expression patterns. Building on our approach, and invoking phenotypes with complex marker repertoires, it may be possible to infer in high detail the developmental hierarchy relevant for the limbal niche. Such knowledge will in turn have practical implications, so that the perspective treatments would be based on the rational selection of the earliest progenitors available.

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**AUTHOR CONTRIBUTIONS**

L.L.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; F.M.N.: collection of data, final approval of manuscript; J.E., S.R., T.F., and C.P.P.: data analysis and interpretation, manuscript writing, final approval of manuscript; C.B.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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