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Perspective

On the Misuse of Virus-Transformed Human Corneal Epithelial Cells as Surrogates for Normal Cells

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Research in corneal biology and pathology including disease modeling and drug testing largely depends on the availability of human ex vivo cultures. Because normal corneal epithelium can be cultured only for several passages, stable epithelial cell lines transformed by different agents or spontaneously have been developed. Telomerase immortalized diploid cell lines introduced more recently were shown to recapitulate traits of normal cells including the expression of some markers and stratification ability. At the same time, there are frequently used cell lines generated by transformation with viruses. The most popular lines were shown to be unstable, tetraploid, lacking important epithelial markers, having aberrant differentiation and deviating from normal cells by gene expression patterns. This perspective reviews properties of various corneal epithelial cell lines and cautions against the use of virally transformed, especially tumorigenic cell lines, as seriously deviating form normal cells. We also provide commercial sources of normal corneal cells and briefly discuss possible future use of limbal organoids.

Keywords: corneal epithelium, cultured cells, viral transduction, telomerase immortalization, primary corneal cells

Corneal epithelium, the outermost part of the eye, is fully exposed to the environment and constantly renews itself. This transparent multilayered structure is a robust biobarrier controlling and restricting passage of pathogens and many drugs to the inner layers of the cornea and to the posterior eye. The expansion of the topically applied drug armamentarium triggered a significant interest in testing drug permeability, toxicity, and efficacy using corneal epithelium.[1–3] For this purpose and for research in corneal stem cell biology, a number of human corneal epithelial culture models were developed, including primary cells from donor eyes, telomerase or spontaneously immortalized cell lines, and virally transformed lines. As the fully differentiated central corneal epithelium can be reproducibly maintained in vitro only as part of organ cultures, monolayer cultures originating from isolated or explanted limbal-like epithelium with retained proliferative capacity were developed.[4] Primary cultures can be maintained only for a limited number of passages,[4] heightening the interest in developing virus-transformed and telomerase-immortalized cell lines with increased lifespan, together with occasional spontaneously immortalized cell lines. For the purpose of this paper, we critically review each of these major human corneal epithelial cell lines. Rodent immortalized cell lines are not very much in use, apparently due to the availability of primary cells.

Virus-Transformed Immortalized Cell Lines

The first successful rabbit corneal epithelial cell lines were obtained using transduction into primary cells of the simian vacuolating virus 40 (SV40)–adenovirus (Ad) vector where the Ad early region was substituted by the SV40 early region.[5] The transduced and cloned cells grew up to 400 passages and maintained the epithelial appearance and expression of keratins and SV40 large T antigen (Tag). Tag is known to interfere with tumor suppressor retinoblastoma and p53 pathways. Importantly, these cells could grow as a tumor in immunodeficient mice. A similar rabbit cell line was obtained independently by large T-antigen gene transfection.[6] A human equivalent of these cells (line 50.B1) was generated by transduction of primary explant cultures from a male donor with Ad12–SV40 hybrid virus or plasmid Rous sarcoma virus (RSV)-T.[4] This cell line is known as human

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corneal epithelial-2 (HCE-2) and is manufactured by American Type Culture Collection (Manassas, VA, USA). Another similar and still popular human cell line was made in 1995 and is known as HCE-T.[7] This cell line also expresses several keratins and paired box 6 (PAX6), although at a low level.[8] Subsequently, other virus-transformed cell lines based on the SV40 large T antigen gene were made using retroviral vectors.[9,10] These transformed cells are still being frequently used in corneal research.[11–17] Other virally transformed cell lines were generated with retroviral vector containing human papilloma virus *HPV-16-E6/E7* viral genes, with[18] or without[19–21] a tetracycline on (Tet-On) inducible system. These cells express some keratins (K19 and K12; weak K3) along with progenitor cell marker ATP-binding cassette subfamily G member 2 (ABCG2). These systems are more rarely used.

Telomerase-Immortalized Cell Lines

Transduction of the human telomerase gene (*hTERT*) into corneal epithelial cells was also shown to render them immortalized. Initially, a complex transduction protocol was used whereby primary cultures of human corneal limbal epithelium were sequentially transduced retrovirally with pBABE (cdk4R)hygro, which expresses a p16INK4A-resistant point mutant (R24C) of CDK4, and pL(p53DD)SN, which expresses a dominant-negative fragment of p53. The cells became insensitive to CDK4 and p53 and were finally transduced with retroviral pBABE(hTERT)puro to expresses the catalytic subunit of human telomerase.[22,23] These cells resembled normal corneal epithelium based on stratification, keratin K3/K12, and membrane-associated mucin expression. Other lines of immortalized cells were obtained by direct transduction of the *hTERT* gene using a retroviral vector. The cells expressed K5/K14 and even K3 when air-lifted,[24] as well as normal isoforms of the putative stem cell marker ΔNp63.[25,26] They could be successfully transplanted onto the epithelium-denuded corneas of nude mice where they formed stratified epithelium expressing K3.[27] They are diploid[24] as opposed to virally transformed cells. However, their stability upon subculture and degree of plasticity for differentiation have not been systemically studied and they may have to be authenticated with several known corneal epithelial markers.

Spontaneously Immortalized Corneal Epithelial Cells

HCE-S spontaneously immortalized cell line expressed markers of primary corneal epithelial cells K3 and PAX6 as well as progenitor cell marker ABCG2.[28] Another cell line, TKE2, was generated from sparse cultures of mouse corneal epithelium. It could stratify in culture and express K14, involucrin, connexin 43, and p63 but not K12 or PAX6.[29] It is commercially available from several sources.

Known Problems With Virally Transformed Cells

Special studies have examined molecular and functional markers and biological behavior of virally transformed, immortalized versus primary human corneal epithelial cells. A human papillomavirus (HPV)-transformed cell line[19] failed to establish epithelial barrier in culture, whereas a SV40-transformed HCE-T cell line showed markedly higher permeability to fluorescein than normal corneal cells in culture.[30] In HCE-T cells, epithelial efflux transporters were also different from cultured primary corneal epithelial cells.[31] HCE-T cells expressed several simple epithelial keratins not found in the ex vivo corneal tissue,[32] and the expression of several differentiation markers, including PAX6, at the mRNA and protein level was significantly decreased.[8,33] Additionally, miR-204-5p upregulated *PAX6* expression levels in HCE-T cells but not in human primary or telomerase-immortalized cultured cells.[34]

Gene expression studies also revealed significant changes in HCE-T cells compared to normal ones. Agilent 244K (Agilent Technologies, Santa Clara, CA, USA) microarray analysis showed aberrant genomic content in HCE-T cells, with gained and lost regions found on several chromosomes.[35] Many corneal epithelial genes were not expressed in different clones, although the respective proteins were identified in the initial report, suggesting significant heterogeneity in this tetraploid line.[35,36] Similar results on gene expression differences from normal cells were obtained in stratified HCE-T cells cultured at a liquid–air interface. These cells had deviation from normal with 22% overexpressed genes and 14% underexpressed genes identified on Affymetrix HG-U133A (Applied Biosystems, Waltham, MA, USA) microarrays. Notably, underexpressed genes included those associated with corneal phenotype and stratified epithelium, such as *KRT12* (keratin 12), *GJA1* (connexin 43), *PAX6*, and *ALDH3A1* and *ALDH1A1* (aldehyde dehydrogenases).[37] At the same time, telomerase-immortalized cells were found to be closer to normal cells in cell-cycle gene expression analysis by RNA sequencing than HCE-T.[36]

HCE-2 is another popular transformed cell line used as a surrogate of normal corneal epithelial cells; however, these cells are mildly tumorigenic and significantly aneuploid.[4] They reportedly have abnormal gene expression profiles and require prolonged air–liquid interface cultures (over 1 month) to achieve physiological protein expression.[19,38] A study of HCE-2 ultraviolet B (UV-B) exposure after priming with lipopolysaccharide or TNF-α revealed a dramatically smaller response of IL-1β compared to primary corneal epithelial cells. Additionally, intracellular NLR family pyrin domain containing 3 (NLRP3) inflammasome levels were increased in TNF-α–primed primary cells after the UV-B exposure, contrary to HCE-2 cells.[39] Analysis of endoplasmic reticulum stress also identified some differences in hyperosmotic medium–induced changes in the levels of key mediators in HCE-2 versus primary cells.[40] These data on HCE-2 differences from primary cells are less abundant than for the HCE-T line but still raise concerns about their applicability in corneal studies.

Conclusions

The popular virally transformed human corneal epithelial cell lines appear to be significantly different from normal corneal epithelial cells. In our opinion, these cell lines should not be recommended for in vitro studies of corneal epithelium. Telomerase-immortalized human cells, on the other hand, are closer to normal corneolimbal cells, are readily available, and could be used more frequently with the understanding that they still may be in some metabolic aspects different from primary cells. This may necessitate additional comparison with normal cells for the studied parameters and authentication of the cell lines

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using several markers in line with a previous perspective.[41] The marker-validated cultured human primary corneal cells[38,42,43] remain the gold standard for corneal cell culture work. Although in many countries there is shortage of human corneas for research, primary corneal epithelial cells are commercially available from many sources (e.g., PCS-700-010 from American Type Culture Collection; CSC-C9224J from Creative Bioarray, Shirley, NY, USA; FC-0029 from Lifeline Cell Technology, San Diego, CA, USA; P10871 from Innoprot, Bizkaia, Spain; 6510 from ScienCell Research Laboratories, Carlsbad, CA, USA; HCEP from CELLnTEC, Bern, Switzerland; H-6048 from Cell Biologics, Chicago, IL, USA; DBFF-1123-HX955 from Creative Biolabs, Shirley, NY, USA).

Another potential source of normal human corneal epithelial cells developed more recently is represented by limbal organoids.[44] Such organoids have been derived from human embryonic stem cells, induced pluripotent stem cells and small pieces of limbal tissues.[45–48] They can be suitable for drug screening and disease modeling,[44] and some organoids provided epithelial cell supply when transplanted onto rabbit corneas with limbal stem cell deficiency.[47,48] The organoid epithelial cells expressed standard normal corneal markers. However, epithelial cell culture from these organoids has not been attempted.

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