Perspective

# Cell Line Authentication in Vision Research and Beyond: A Tale Retold

Jeremy Peavey[1] and Goldis Malek[1,2]

[1]Duke Eye Center, Department of Ophthalmology, Duke University School of Medicine, Durham, North Carolina, United States
[2]Department of Pathology, Duke University School of Medicine, Durham, North Carolina, United States

Correspondence: Goldis Malek, Duke University School of Medicine, Department of Ophthalmology, 2351 Erwin Road, PO Box 3802, Room 4006, Durham, NC 27710, USA; gmalek@duke.edu.

**Received:** January 31, 2020
**Accepted:** May 11, 2020
**Published:** June 9, 2020

Citation: Peavey J, Malek G. Cell line authentication in vision research and beyond: a tale retold. *Invest Ophthalmol Vis Sci.* 2020;61(6):19. https://doi.org/10.1167/iovs.61.6.19

We live in an age where new technologies, and organizations involved in the distribution of biological materials, such as cell culture lines, have eased accessibility to a variety of in vitro models, developed, and/or harvested from different sources. In translational and basic ophthalmology research, in vitro assays are an essential component to discovery and preclinical studies. It is, therefore, of utmost importance for vision researchers to be cognizant of the risks surrounding the use of newly developed cell culture models and how scientific integrity could be impacted when standard operating procedures are not followed for cell line validation and identification. Herein, we discuss authentication challenges we faced when we obtained a newly marketed human choroidal endothelial cell line for vision research, and outline our process of validating and characterizing primary human choroidal endothelial cell lines in the laboratory.

Keywords: choroidal endothelial cells, cell culture authentication, tissue culture, retinal disease, rigor, reproducibility

Misidentification and cross-contamination of cell lines were originally recognized by Rothfels et al. in 1958, and have since become well-documented phenomena in the basic sciences.[1–14] To date, the International Cell Line Authentication Committee has identified a growing list of 486 misidentified cell lines[15] that have, in turn, negatively impacted more than 32,000 published articles.[16] In a 2007 press release, the National Institutes of Health (NIH) responded to these concerns by adopting cell line authentication guidelines. Subsequently, in early 2016, all proposals submitted to the NIH were required to include a dedicated section reporting protocols utilized to "authenticate key biological resources," and, of relevance here, to provide information on how the identity, purity, and fidelity of cell lines used in the grant will be verified. Other grant-awarding institutions have since followed suit, yet strict authentication practices across disciplines remain inconsistent.

The field of vision research is not exempt from the woes of misidentified cell lines.[17–20] The human eye is a complex organ structure where specialized niches of cells reside. The retinal pigment epithelium and choriocapillaris endothelium are two examples of cell types that are frequently isolated for the study of blind eye diseases of the posterior pole of the eye, such as age-related macular degeneration, proliferative vitreoretinopathy, and diabetic retinopathy, to name a few. The challenge of culturing these specialized cell types can be exacerbated by cell heterogeneity. To ensure culture purity, laboratories are expected to consider implementing additional isolation methods, such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS; Miltenyi Biotech Inc., Somerville, MA, USA), both of which may be cumbersome and costly. Another important factor affecting in vitro models is that most adult ocular cell types are non-proliferative in vivo, and cell sorting of ocular tissues, an expensive endeavor, does not guarantee the survival of the cells unless placed in a specific "proliferation-friendly" environment. Determining said "proper" environment in and of itself requires supplementary trial-and-error assays and further expenses. Critically, when the culture environment is unfavorable, cells can rapidly dedifferentiate and/or reach phases of senescence and/or apoptosis. To circumvent issues of cell senescence in vitro, researchers have turned to exploiting a variety of stem cell technologies,[21–24] immortalization techniques,[25–27] and cell line transformations.[28] These modern approaches have again proven to be costly and untimely, and have ultimately resulted in the emergence of biotechnology organizations who fill the void with the promise of providing "authenticated" primary cell lines, the acquisition of which often needs to be accompanied by purchasing proprietary reagents to propagate and culture at a high cost. Here, we present a narrative of our experience with a commercially obtained, purported human choroidal endothelial cell line and outline methods associated with authentication in support of the principles of rigor and reproducibility.

## Methodology and Biological Resources

Commercially obtained "human choroidal endothelial cells" (Celprogen; cat. 36052-03) were cultured on Celprogen T75 pre-coated human choroidal endothelial primary cell culture complete extra-cellular matrix flasks (E36052-03-T75) in Celprogen human choroidal endothelial cell complete growth media with serum (M36052-03S) as described in the

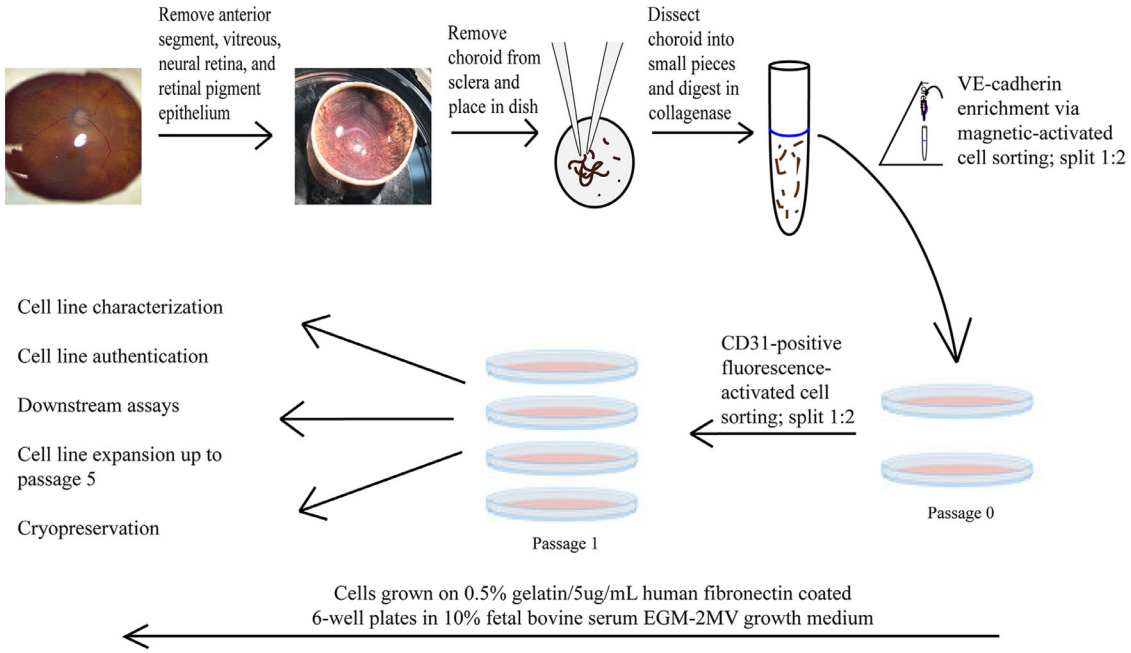



**Figure 1.** Schematic diagram of primary human choroidal endothelial cell (hCEC) isolation and expansion.

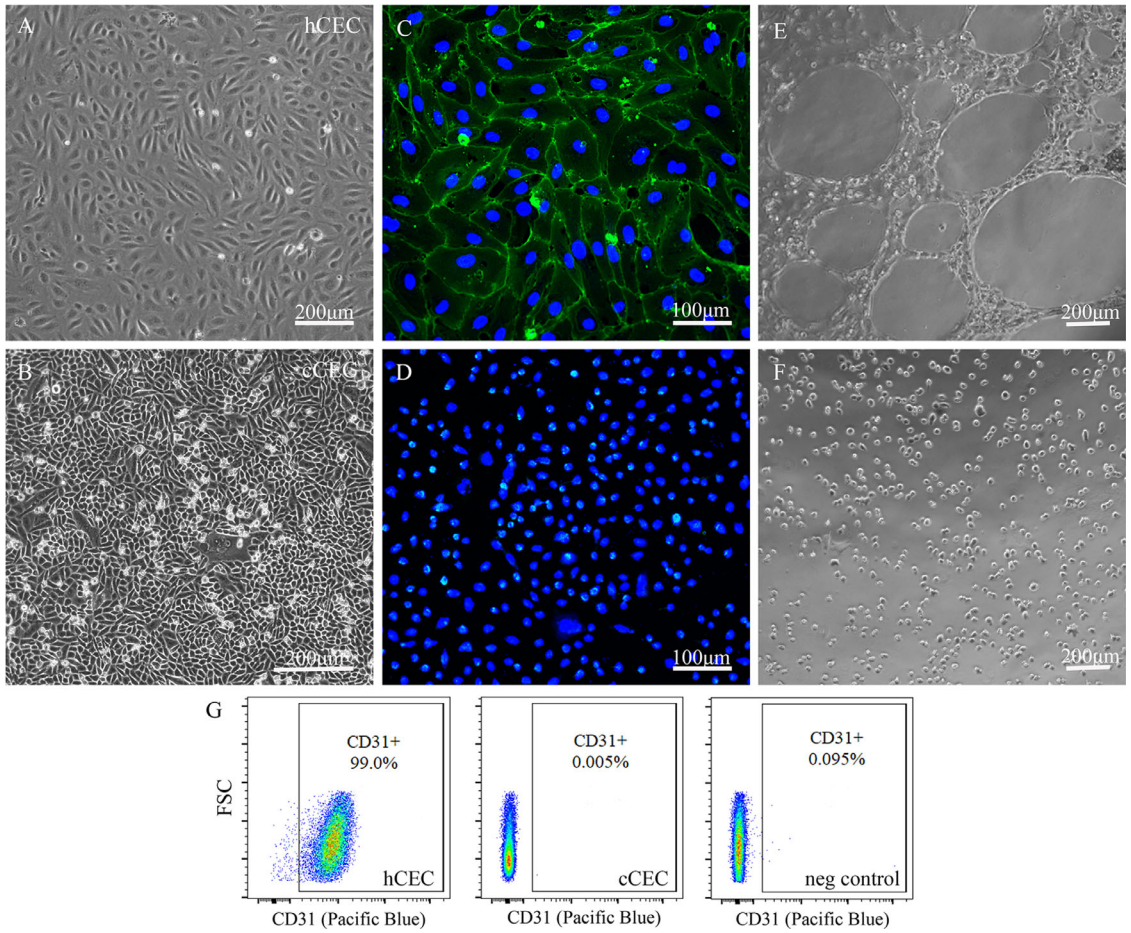



**Figure 2.** Commercial human choroidal endothelial cells (cCECs) diverge significantly from primary human choroidal endothelial cells (hCECs) isolated from donor tissues. (**A**) hCEC and (**B**) cCEC morphology 24 hours post-confluence, plated at 150,000 cells/6-well, at passage 2; (**C**) hCEC and (**D**) cCEC cellular immunofluorescent staining for CD31 surface expression with Alexa Fluor 488 secondary antibody (green) and the nuclear stain Hoechst (blue); degree of tube-formation by (**E**) hCECs and (**F**) cCECs cultured on Geltrex coated plates, (**G**) flow cytometry analysis for CD31. FSC = forward scatter area; negative control = no antibody hCEC samples; aquablue = live-dead stain for live cell gating.

Investigative Ophthalmology & Visual Science

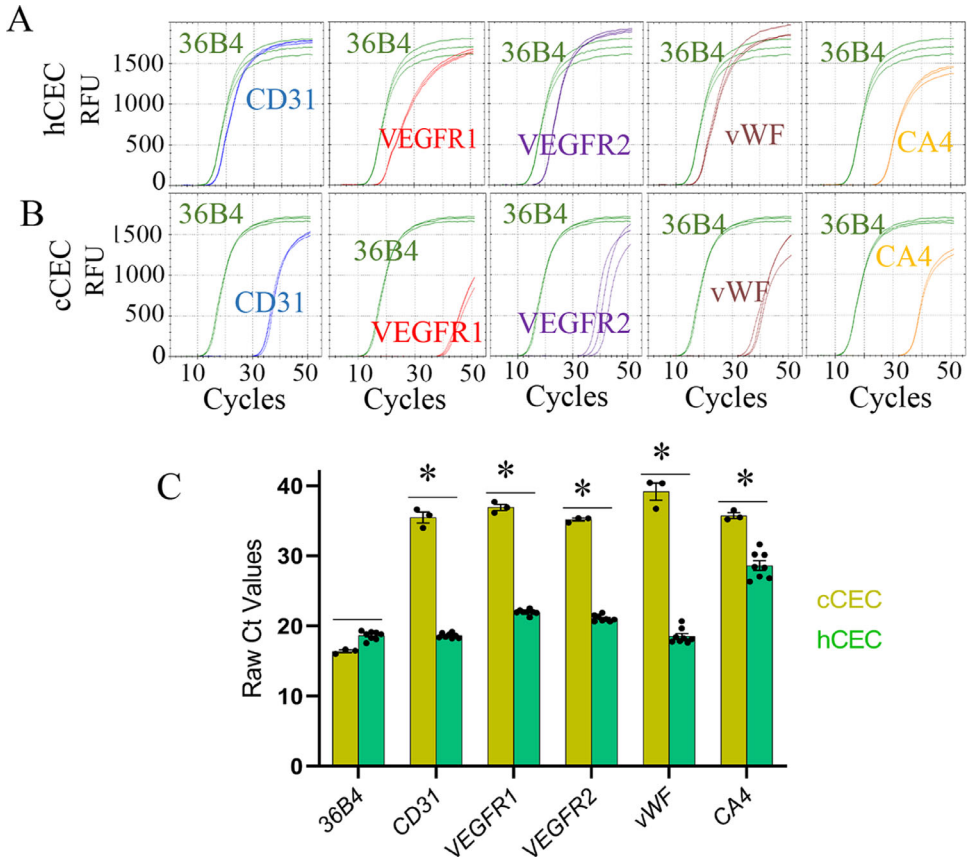


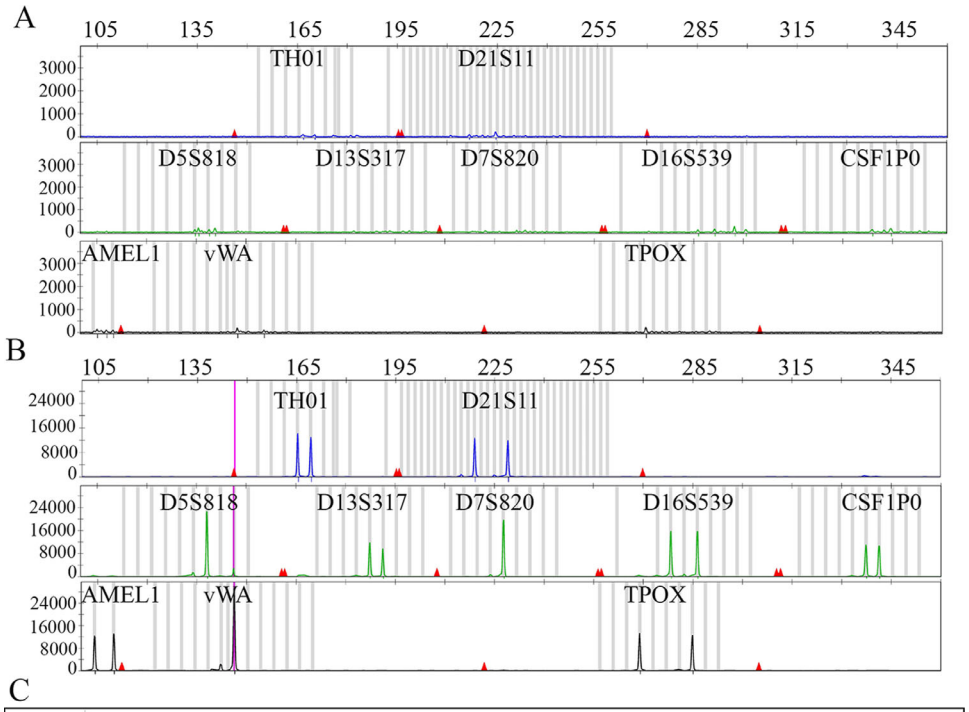

**Figure 3.** The qRT-PCR analysis for RNA pan-endothelial markers (CD31, VEGFR1, VEGFR2, and vWF) and a choriocapillaris-specific marker expression (CA4) relative to the housekeeping reference gene 36B4. (**A**) The hCEC threshold cycle curves from passage 2 cells. (**B**) The cCEC threshold cycle curves from passage 2 cells. (**C**) Comparison of raw CT values for cCEC (3 technical replicates) and hCEC (3 technical replicates from 8 biological replicates) at 5 ng cDNA per qRT-PCR (statistical significance; two-sample *t*-test; $^*P < 0.05$).

product data sheet. These commercial choroidal endothelial cells (cCECs) were compared to primary choroidal endothelial cells (hCECs) isolated in the laboratory from human eye donor tissue within 6 hours from the time of death (BioSight, NC; $n = 6$ male donors, 2 female donors; ages 47 to 90 with normal ocular history). Microdissected choroids were digested with collagenase (Sigma-Aldrich; C2674), blocked with FCR-blocking reagent (Miltenyi; 130-059-901), and labeled with VE-cadherin magnetic beads (Miltenyi; 130-097-857). Labeled cells were enriched with MACS on LS columns (Miltenyi; 130-042-301) and plated on 5 ug/mL human fibronectin/0.5% gelatin (Sigma-Aldrich; F0895, Sigma Aldrich; G1393) coated 6-well plates in 10% FBS EGM-2MV media (Lonza; CC-3202) supplemented with 1× nonessential amino acids (Corning; 25-025-CI), 1× penstrep (Sigma-Aldrich; P0781), and 2.5 ug/mL amphotericin B (Thermo Fisher; 15290018). At 90% confluency, hCECs were dissociated with Accumax (Invitrogen; 00-4666-56) into a single-cell solution and labeled with CD31-Pacific Blue conjugated antibody (Biolegend; 303114) and Aquablue live-dead Stain (Invitrogen; 43110). The hCECs were then sorted with an eSony SH800 cell sorter gated against Aquablue live-dead and CD31-Pacific Blue. Sorted hCECs were next expanded in coated six-well plates as previously described, and antibiotics were removed prior to dissociating cells at 90% confluence with Accumax. The quantitative real-time polymerase chain reaction (qRT-PCR),[29–31] immunocytochemistry,[32] and tube formation assays[29,33,34] were performed as previously described. Short-tandem repeat (STR) profiling was carried out by co-amplification of nine human-specific polymorphic STR markers plus amelogenin for gender-specification with the Geneprint 10 kit (Promega; B9510). Allele size and identity were determined on an ABI 3130xl automated capillary DNA sequencer.

## The Need for Authentic Human Primary Choroidal Cell Lines: We Won't Know if We Don't Test

Age-related macular degeneration (AMD) is the leading cause of blinding eye disease in elderly populations of the developed world.[35] Traditionally, much of AMD-related research carried out with in vitro assays have focused on utilizing culture models of retinal pigment epithelial (RPE) cells. However, there has been a steady demand for cell culture models of "AMD-vulnerable" cells other than RPE cells, importantly, choroidal endothelial cells, which have been shown to be compromised not only in wet or neovascular AMD, but also in the early and late dry forms of the disease.[36–39]

Given the paucity of protocols available for isolating, purifying, and expanding primary human choroidal endothelial cell lines, we ordered commercially available "human choroidal endothelial cells" (cCECs) from a biotechnology company founded in 2002 under the premise of stem




C

| | Short-Tandem Repeat Markers | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | THO1 | D21S11 | D5S818 | D13S317 | D7S820 | D16S539 | CSF1P0 | AMEL | vWA | TPOX |
| cCEC | np | np | np | np | np | np | np | np | np | np |
| hCEC | 7, 8 | 29, 31.2 | 13, 13 | 11, 12 | 10, 10 | 9, 11 | 11, 12 | x, y | 16, 16 | 8, 12 |

**Figure 4.** Short-tandem repeat profiling (STR) of cells. (**A**) Commercial human choroidal endothelial cells (cCECs) lack human-specific STR allelic peaks as compared to (**B**) human primary choroidal endothelial cells (hCECs). (**C**) Human-specific STR profiles establish allelic identities for hCEC and cCEC by co-amplification and capillary DNA sequencing analysis. np = not present; STR markers: THO1, D21S11, D5S818, D13S317, D7S820, D16S539, CSF1P0, AMEL, vWA, and TPOX.

cell research and 3D technology.[40,41] To our knowledge, this is the only company currently offering primary human choroidal endothelial cells. Per rigor and reproducibility protocol, we characterized the cells prior to use as outlined in our research proposals. We settled on the need for the "choroidal endothelial" cell line to meet at least five benchmarks: (1) morphological evaluation: visible "cobblestone-like" appearance characteristic of choroidal endothelial cells, (2) cell-specific gene expression: confirmed expression of choroidal endothelial associated gene markers by qRT-PCR analysis, (3) evidence of cell-specific protein marker expression: positive staining for morphological marker(s) through flow analysis and/or immunofluorescent staining, (4) functional evaluation: evidence of ability to form tubes in vitro, and (5) genomic analysis: human cell line authentication. The commercial cell line purchased was compared to human primary choroidal endothelial cells isolated from donor eyes in the laboratory per schema presented in Figure 1.

## Morphological Evaluation

Abnormal morphology was the first indication that the commercial "choroidal endothelial" cell line (cCEC) was not of endothelial origin. Human primary choroidal endothelial cells harvested from donor tissues (hCEC) typically maintain a "dark-centered, cobblestone-like" monolayer at confluence[42–44] (Fig. 2A), yet cCEC did not present with these hallmarks (Fig. 2B). Furthermore, the cCECs retained visibly distinct cell borders at confluence, whereas the hCEC borders were largely unnoticeable. Finally, when plated at the same density, cCECs reached confluence in less than half the time required by hCECs, and were approximately one-fourth the measurable size of hCECs.

## Cell-Specific Protein and Gene Expression

In cCECs, low to absent levels of CD31, an established endothelial cell surface marker, was confirmed qualitatively by immunofluorescent staining (Fig. 2D) and quantitatively by flow cytometry analysis (Fig. 2G). Conversely, hCEC were positive for CD31 cell surface staining (Fig. 2C) with a measured purity of 99.0% CD31-positive cells (Fig. 2G). Similarly, characterization of the commercial cell line, cCEC, in comparison to hCEC, by qRT-PCR, revealed significantly lower RNA levels of pan-endothelial markers (CD31, VEGFR1, VEGFR2, and vWF) and lower levels of the choriocapillaris-specific marker (CA4; Figs. 3A–C).

## Functional Evaluation

Functionally, whereas hCEC formed capillary-like structures typical of endothelial cells (Fig. 2E), cCECs were unable to form tubes when grown on Geltrex-coated plates (Fig. 2F).

### Genomic Analysis

The STR profiling, which has become a requirement for publication in many journals, revealed cCEC lack 10 human-specific STR loci (THO1, TPOX, VWA, CSF1PO, D16S539, D75820, D13S317, D21S11, D75820, D13S317, D5S818, and amelogenin). All peaks were appropriately identified in hCEC. Furthermore, amelogenin, a gender-specific STR, confirmed the gender of the human donor profiled, male in this case (Fig. 4).

## Final Thoughts

Here, we provide one example of the imperative need to authenticate biological materials used in the laboratory regardless of the originating source. We found "human choroidal endothelial cells" obtained from a life sciences and biotechnology company failed to meet all five benchmarks used to authenticate the nature of the cells. They lacked typical choroidal endothelial morphology, did not express pan-endothelial or cell-specific markers, were functionally dissimilar to primary human choroidal cells, and lacked human DNA markers. Although the initial reaction to the commercial availability of a highly sought after cell line may be to operate on a sense of "trust" and begin utilizing the cells without further in-house characterization, these findings strongly reinforce the need for vision research laboratories to use robust standard operating procedures for authentication when receiving new cell lines regardless of origin. By creating a baseline, cell lines may be periodically monitored for genetic drift and cross contamination. When possible, comparison across culture models is preferable. In-house derived and characterized primary cell lines are particularly useful for confirming new cell culture models. Without such due diligence, laboratories will likely fall victim to the risks associated with the steady flow of new technological advances in cellular biology that are either created in-house or enter through the market.

### *Acknowledgments*

The authors thank the donors and donor families for their generosity and BioSight for their assistance and timely procurement of donor eyes. Furthermore, the authors appreciate the assistances of Scott Langdon, Manager of the Duke DNA analysis facility; J. Michael Cook, Director of the Duke Flow Cytometry Core; and Joan Kalnitsky for support with flow cytometry.

Supported by the National Eye Institute grants R01 EY027802 (to GM), R01 EY028160 (to GM), P30 EY005722 (to the Duke Eye Center), and the Research to Prevent Blindness, Inc. (RPB) Core grant (to the Duke Eye Center).

Disclosure: **J. Peavey**, None; **G. Malek**, None

### *References*

1. Gartler SM. Apparent HeLa cell contamination of human heteroploid cell lines. *Nature*. 1968;217:750–751.
2. MacLeod RA, Drexler HG. Cell banks detect false cell lines: journals must act too. *Lancet Oncol*. 2001;2:467–468.
3. Stacey GN. Cell contamination leads to inaccurate data: we must take action now. *Nature*. 2000;403:356–356.
4. Markovic O, Markovic N. Cell cross-contamination in cell cultures: the silent and neglected danger. *In Vitro Cell Dev Biol Anim*. 1998;34:1–8.
5. Satoh M, Takeuchi M. Cross-contamination of cell lines as revealed by DNA fingerprinting in the IFO animal cell bank. *Res Commun Inst Ferment*. 1993;:18–23.
6. Nelson-Rees WA, Daniels DW, Flandermeyer RR. Cross-contamination of cells in culture. *Science*. 1981;212:446–452.
7. Lavappa KS, Macy ML, Shannon JE. Examination of ATCC stocks for HeLa marker chromosomes in human cell lines. *Nature*. 1976;259:211–213.
8. Masters J. False cell lines. *Int J Cancer*. 2002;99:154.
9. Drexler HG, Dirks WG, Matsuo Y, MacLeod RAF. False leukemia-lymphoma cell lines: an update on over 500 cell lines. *Leukemia*. 2003;17:416–426.
10. Masters JR. Human cancer cell lines: fact and fantasy. *Nat Rev Mol Cell Biol*. 2000;1:233–236.
11. Azari S, Ahmadi N, Tehrani MJ, Shokri F. Profiling and authentication of human cell lines using short tandem repeat (STR) loci: report from the National Cell Bank of Iran. *Biologicals*. 2007;35:195–202.
12. Nelson-Rees WA. Responsibility for truth in research. *Philos Trans R Soc Lond B Biol Sci*. 2001;356:849–851.
13. Arlett CF. The use of dubious cell lines in research: is trust enough? *Lancet Oncol*. 2001;2:467.
14. MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, Drexler HG. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int J Cancer*. 1999;83:555–563.
15. Capes-Davis A, Theodosopoulos G, Atkin I, et al. Check your cultures! A list of cross-contaminated or misidentified cell lines. *Int J Cancer*. 2010;127:1–8.
16. Horbach SPJM, Halffman W. The ghosts of HeLa: how cell line misidentification contaminates the scientific literature. *PLoS One*. 2017;12:e0186281.
17. Krishnamoorthy RR, Clark AF, Daudt D, Vishwanatha JK, Yorio T. A forensic path to RGC-5 cell line identification: lessons learned. *Invest Ophthalmol Vis Sci*. 2013;54:5712–5719.
18. Folberg R, Kadkol SS, Frenkel S, et al. Authenticating cell lines in ophthalmic research laboratories. *Invest Ophthalmol Vis Sci*. 2008;49:4697–4701.
19. Di Girolamo N, Chow S, Richardson A, Wakefield D. Contamination of primary human corneal epithelial cells with an sv40-transformed human corneal epithelial cell line: a lesson for cell biologists in good laboratory practice. *Invest Ophthalmol Vis Sci*. 2016;57:611–616.
20. Makin RD, Apicella I, Nagasaka Y, et al. RF/6A chorioretinal cells do not display key endothelial phenotypes. *Invest Ophthalmol Vis Sci*. 2018;59:5795.
21. Smith EN, D'Antonio-Chronowska A, Greenwald WW, et al. Human iPSC-derived retinal pigment epithelium: a model system for prioritizing and functionally characterizing causal variants at AMD risk loci. *Stem Cell Reports*. 2019;12:1342–1353.
22. Songstad AE, Worthington KS, Chirco KR, et al. Connective tissue growth factor promotes efficient generation of human induced pluripotent stem cell-derived choroidal endothelium. *Stem Cells Transl Med*. 2017;6:1533–1546.
23. Deng F, Hu H, Chen M, et al. Generation of induced pluripotent stem cells from human Tenon's capsule fibroblasts. *Mol Vis*. 2012;18:2871–2881.
24. Ji S-L, Tang S-B. Differentiation of retinal ganglion cells from induced pluripotent stem cells: a review. *Int J Ophthalmol*. 2019;12:152–160.
25. Giacalone JC, Miller MJ, Workalemahu G, et al. Generation of an immortalized human choroid endothelial cell

line (iChEC-1) using an endothelial cell specific promoter. *Microvasc Res*. 2019;123:50–57.
26. Rambhatla L, Chiu C-P, Glickman RD, Rowe-Rendleman C. In vitro differentiation capacity of telomerase immortalized human RPE cells. *Invest Ophthalmol Vis Sci*. 2002;43:1622–1630.
27. Ding QJ, Zhu W, Cook AC, Anfinson KR, Tucker BA, Kuehn MH. Induction of trabecular meshwork cells from induced pluripotent stem cells. *Invest Ophthalmol Vis Sci*. 2014;55):7065–7072.
28. Kim C-W, Park G-T, Bae O-N, Noh M, Choi K-C. Application of SV40 T-transformed human corneal epithelial cells to evaluate potential irritant chemicals for in vitro alternative eye toxicity. *J Pharmacol Toxicol Methods*. 2016;80:82–89.
29. Choudhary M, Kazmin D, Hu P, Thomas RS, McDonnell DP, Malek G. Aryl hydrocarbon receptor knock-out exacerbates choroidal neovascularization via multiple pathogenic pathways. *J Pathol*. 2015;235:101–112.
30. Hu P, Herrmann R, Bednar A, et al. Aryl hydrocarbon receptor deficiency causes dysregulated cellular matrix metabolism and age-related macular degeneration-like pathology. *Proc Natl Acad Sci USA*. 2013;110:E4069–E4078.
31. Dwyer MA, Kazmin D, Hu P, McDonnell DP, Malek G. Research resource: nuclear receptor atlas of human retinal pigment epithelial cells: potential relevance to age-related macular degeneration. *Mol Endocrinol*. 2011;25:360–372.
32. Yao P-L, Chen L, Hess RA, Müller R, Gonzalez FJ, Peters JM. Peroxisome proliferator-activated receptor-D (PPARD) coordinates mouse spermatogenesis by modulating extracellular signal-regulated kinase (ERK)-dependent signaling. *J Biol Chem*. 2015;290:23416–23431.
33. Choudhary M, Ding J-D, Qi X, et al. PPAR$\beta/\delta$ selectively regulates phenotypic features of age-related macular degeneration. *Aging (Albany NY)*. 2016;8:1952–1978.
34. Choudhary M, Safe S, Malek G. Suppression of aberrant choroidal neovascularization through activation of the aryl hydrocarbon receptor. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864:1583–1595.
35. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health*. 2014;2:e106–116.
36. Chirco KR, Sohn EH, Stone EM, Tucker BA, Mullins RF. Structural and molecular changes in the aging choroid: implications for age-related macular degeneration. *Eye (Lond)*. 2017;31:10–25.
37. Mullins RF, Johnson MN, Faidley EA, Skeie JM, Huang J. Choriocapillaris vascular dropout related to density of drusen in human eyes with early age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2011;52:1606–1612.
38. Bhutto I, Lutty G. Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex. *Mol Aspects Med*. 2012;33:295–317.
39. McLeod DS, Taomoto M, Otsuji T, Green WR, Sunness JS, Lutty GA. Quantifying changes in RPE and choroidal vasculature in eyes with age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2002;43:1986–1993.
40. Celprogen, Inc: Biotechnology, https://www.linkedin.com/company/celprogen-inc. Accessed January 24, 2020.
41. Celprogen: Stem Cell Research & Therapeutics, https://www.celprogen.com/. Accessed January 24, 2020.
42. Skeie JM. *Choroidal endothelial cell activation in age-related macular degeneration*. Spring 2010, https://ir.uiowa.edu/cgi/viewcontent.cgi?article=1787&context=etd.
43. Browning AC, Gray T, Amoaku WM. Isolation, culture, and characterisation of human macular inner choroidal microvascular endothelial cells. *Br J Ophthalmol*. 2005;89:1343–1347.
44. Skeie JM, Mullins RF. Elastin-mediated choroidal endothelial cell migration: possible role in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2008;49:5574–5580.