# Cryopreservation and Lentiviral-Mediated Genetic Modification of Human Primary Cultured Corneal Endothelial Cells

*Leejee H. Sub*,<sup>1</sup> *Cheng Zhang*,<sup>1</sup> *Roy S. Chuck*,<sup>1</sup> *Walter J. Stark*,<sup>1</sup> *Stuart Naylor*,<sup>2</sup> *Katie Binley*,<sup>2</sup> *Shukti Chakravarti*,<sup>1,3</sup> *and Albert S. Jun*<sup>1</sup>

**PURPOSE.** To determine the viability and potential usefulness of cryopreserved human primary cultured corneal endothelial cells by characterizing their morphology, gene expression, and ability for genetic modification by the lentiviral vector equine infectious anemia virus (EIAV).

**METHODS.** Primary cultured endothelial cells were dissociated from human corneas and grown in organ culture medium. Corneal endothelial cell origin was confirmed by morphology and immunostaining with polyclonal anti- collagen VIII antibodies. Cells of different passages were cryopreserved in medium containing dimethyl sulfoxide and were assessed after thawing for morphology, proliferative capacity, gene expression, and ability to form cell-cell junctions. EIAV encoding enhanced green fluorescent protein (eGFP) was used to transduce cryopreserved human corneal endothelial cells. Transduced cells were then sorted by fluorescence-activated cell sorting (FACS) and imaged with fluorescence microscopy.

**R**ESULTS. Cryopreserved, primary, cultured human corneal endothelial cells are viable and retain their ability to proliferate, produce collagen VIII, and express ZO-1, a tight-junction protein. EIAV-based gene transfer of eGFP is highly efficient and nontoxic to cryopreserved human primary cultured corneal endothelial cells. These genetically modified cells can be selected to nearly pure populations with FACS sorting.

Conclusions. Human primary cultured corneal endothelial cells retain their phenotypic properties after cryopreservation. The ability to store, genetically modify, and sort these cells through FACS to pure populations has the potential to greatly expand their future therapeutic application to treat corneal endothelial disorders. (*Invest Ophthalmol Vis Sci.* 2007;48:3056–3061) DOI:10.1167/iovs.06-0771

Disclosure: L.H. Suh, None; C. Zhang, None; R.S. Chuck, None; W.J. Stark, None; S. Naylor, Oxford Biomedica (E); K. Binley, Oxford Biomedica (E); S. Chakravarti, None; A.S. Jun, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertise-ment*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Albert S. Jun, Wilmer Eye Institute/Woods 474, The Johns Hopkins Medical Institutions, 600 N. Wolfe Street, Baltimore, MD 21287-9238; aljun@jhmi.edu.

s the "barrier and pump" of the cornea, the endothelium Aplays an essential role in maintaining corneal transparency. In patients with endothelial injury such as pseudophakic bullous keratopathy and primary endotheliopathy such as Fuchs endothelial corneal dystrophy (FECD), human corneal endothelial cells (CECs) do not significantly proliferate because they are arrested in the G<sub>1</sub> phase of the cell cycle.<sup>1-4</sup> However, several groups have demonstrated that under the appropriate in vitro culture conditions, human CECs do have limited proliferative capacity<sup>5-7</sup> and can be passaged a relatively small number of times before they exhibit characteristics of senescence.<sup>1</sup> Although immortalization of primary endothelial cells has been established,<sup>8</sup> such approaches have potential disadvantages for clinical use. Thus, the ability to store primary cultures for subsequent research or disease therapy is desirable. Reports of successful transplantation of CECs seeded onto substrates, such as amniotic membranes,<sup>9</sup> collagen sheets,<sup>10</sup> and denuded Descemet membrane,<sup>7,11</sup> further underscore the potential usefulness of expanding populations and storing endothelial cells for later clinical use. Several groups have demonstrated the viability of endothelial cells after the cryopreservation of ex vivo corneas.12-14 However, successful cryopreservation of human primary cultured CECs has not been reported.

Important markers for corneal endothelial cell identity and function include collagen VIII and zonula occludens-1 (ZO-1). Collagen VIII is a trimer composed of various combinations of two subunits, the  $\alpha$ -1 and the a-2 chains.<sup>15-18</sup> Previous immunologic studies have demonstrated that collagen VIII is present in corneal endothelial cells and Descemet membrane but not in corneal stroma or keratocytes,<sup>19,20</sup> and mutations in the  $\alpha$ 2 chain of collagen VIII are associated with Fuchs endothelial corneal dystrophy.<sup>21,22</sup> ZO-1 is a protein component of tight junctions at the apical endothelial cell borders.<sup>6,7,23</sup> Correct localization of ZO-1 is an important functional marker for CECs, which indicates intact intracellular protein trafficking to apical cell membranes, formation of a polarized, contact-inhibited monolayer, and establishment of a physiological endothelial barrier.<sup>6,7</sup>

Long-term genetic modification of CECs could have great therapeutic potential for endothelial disorders such as FECD, pseudophakic bullous keratopathy, and graft failure. Lentiviruses are attractive vectors for gene transfer because they can stably transduce nondividing or slowly dividing cells and can be stripped of proinflammatory viral components.<sup>24</sup> Recombinant human immunodeficiency virus (HIV)-based lentiviral vectors have allowed stable and nontoxic gene transduction in mouse endothelium.<sup>25</sup> Potential safety issues surrounding the clinical use of HIV-based vectors have prompted investigation into non-HIV retroviral vectors. Equine infectious anemia virus (EIAV) is a promising vector system that is more efficient than HIV in transducing mouse and rabbit endothelial cell lines and ex vivo human corneas.<sup>26</sup>

In this study, we sought to establish methods to successfully cryopreserve human primary cultured CECs and to character-

From the <sup>1</sup>Wilmer Eye Institute and the <sup>3</sup>Department of Medicine, The Johns Hopkins Medical Institutions, Baltimore, Maryland; and <sup>2</sup>Oxford BioMedica (UK) Ltd., Oxford Science Park, Oxford, United Kingdom.

Supported by a Research to Prevent Blindness Career Development Award (ASJ), a research grant from the Medical Illness Counseling Center (ASJ, Silver Spring, MD) and the Walter J. Stark Research Grant Award funded in part by an unrestricted educational grant from Alcon, Inc. (LHS).

Submitted for publication July 7, 2006; revised December 22, 2006, and February 2, 2007; accepted May 3, 2007.

ize these cells after cryopreservation with respect to morphology, proliferative capacity, and expression of collagen VIII and ZO-1. Furthermore, we sought to characterize the ability of EIAV to genetically modify these cells after cryopreservation.

# **MATERIALS AND METHODS**

#### Materials

Materials were obtained from the following sources: serum-free medium (OptiMEM-I), Dulbecco phosphate-buffered saline (PBS), gentamicin, and trypsin-EDTA (Gibco BRL/Life Technologies, Rockville, MD); mouse submaxillary gland nerve growth factor and bovine pituitary extract (Biomedical Technologies, Stoughton, MA); mouse submaxillary gland epidermal growth factor (Upstate Biotechnologies, Lake Placid, NY); fetal bovine serum (FBS; Hyclone, Logan, UT); ascorbic acid, chondroitin sulfate, calcium chloride, 0.02% ethylene diamine tetraacetic acid (EDTA) solution, dimethyl sulfoxide (DMSO), and antibiotic/antimycotic solution (Sigma, St. Louis, MO); cell attachment reagent (FNC Coating Mix; Biological Research Faculty and Facility, Ijamsville, MD); mouse monoclonal antibody to the  $\alpha$ 1 chain of bovine collagen VIII (Seikagaku America, East Falmouth, MA); rabbit polyclonal antibody to the  $\alpha$ 2 chain of bovine collagen VIII (gift of Paul F. Davis,<sup>18</sup> Wellington, New Zealand); rabbit polyclonal antibody to ZO-1 (Zymed Laboratories, Inc., South San Francisco, CA); Cy-3- conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA); Hoechst nuclear staining (Molecular Probes, Eugene, OR); and eight-well glass chamber slides (Laboratory-Tek, Nunc, Rochester, NY). High-titer (5  $\times$  10<sup>8</sup> to 1  $\times$  10<sup>9</sup> transducing (T) U/mL) preparations of EIAV pseudotyped with a vesicular stomatitis virus (EIAV-VSVG) or with rabies virus (EIAV-rabies) envelope protein with the cytomegalovirus (CMV) promoter driving marker gene enhanced green fluorescent protein (eGFP) expression were obtained from Oxford BioMedica (Oxford, UK).26

#### Human Corneal Endothelial and Keratocyte Cell Culture

Corneas from donors aged 19 and 3 years were obtained from the Washington Eye Bank and the Lions Eye Institute for Transplant and Research and were stored in corneal preservation media (Optisol-GS; Bausch & Lomb, Rochester, NY) at 4°C. Standard eye bank protocol for informed consent and for protection of donor confidentiality were used. Both donors' corneas were unsuitable for transplantation because of defects in the stroma within the optical zone. The 19-year-old donor died of a stroke, and the death-to-preservation time was 12 hours. The 3-year-old donor died of respiratory arrest from pneumonia, and the death-to-preservation time was 14 hours. Standard serologies for infectious agents were negative for both tissues. Primary cultures were initiated within 1 to 3 days of preservation in corneal preservation media (Optisol-GS; Bausch & Lomb).

Human corneal endothelial cell culture lines were established according to published methods.<sup>6</sup> Corneas were removed from corneal preservation media (corneal preservation media [Optisol-GS; Bausch & Lomb]) and were washed with culture medium consisting of serumfree medium (OptiMEM-I, Gibco BRL/Life Technologies), 8% FBS, 5 ng/mL epithelial growth factor (EGF), 20 ng/mL NGF, 100 µg/mL pituitary extract, 20 µg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, 50 µg/mL gentamicin, and antibiotic/antimycotic solution diluted 1/100. Descemet membrane with intact endothelium was dissected in small strips from corneal buttons. The strips were incubated in culture medium overnight at 37°C under 5% CO2 and were then centrifuged at 3000 rpm for 6 minutes and incubated in 0.02% EDTA for 1 hour at 37°C to loosen cell-cell junctions. The tissue was manually disrupted by passage through a glass pipette. The dissociated cells were centrifuged again at 3000 rpm for 6 minutes, and the pellet was gently resuspended in culture medium. Isolated cells and pieces of Descemet membrane were plated in a six-well tissue culture plate precoated with undiluted cell attachment reagent (FNC Coating Mix; Biological Research Faculty and Facility). Cultures were incubated at  $37^{\circ}$ C in a 5% CO<sub>2</sub>, humidified incubator, and the medium was changed every other day. Once the cells approached approximately 80% to 90% confluence, the cells were trypsinized with trypsin-EDTA and passaged at 1:2 ratios.

Stromal fibroblasts were dissociated as follows: the epithelium and endothelium were removed from the corneal button of 19-year-old donor. The corneal button was left in culture medium (same as that used for growing endothelial cells) for 1 to 2 weeks until a monolayer of spindle-shaped cells (keratocytes) was established along the bottom of the tissue culture plate.

#### Immunostaining of Human Corneal Endothelial Cells and Stromal Fibroblasts

At the time of passaging cells, small aliquots of resuspended cells were plated on eight-well chamber slides precoated with cell attachment reagent (FNC Coating Mix; Biological Research Faculty and Facility) and were allowed to grow to confluence in the endothelial cell culture medium. The cells were then fixed in 2% paraformaldehyde for 20 minutes and treated with 0.1% Triton X-100 for 15 minutes at room temperature. Nonspecific antibody binding was blocked by incubating cells in 5% donkey serum for 1 hour before incubation with the following primary antibodies overnight at 4°C: mouse monoclonal antibody to the  $\alpha 1$  chain of bovine collagen VIII (1:50; Seikagaku America, East Falmouth, MA) or rabbit polyclonal antibody to the  $\alpha 2$ chain of bovine collagen VIII (1:1500; kindly provided by Paul F. Davis, Wellington, New Zealand). The cells were further incubated in Cy-3 conjugated donkey anti-mouse or anti-rabbit secondary antibodies (1:100; Jackson ImmunoResearch) for 1 hour at room temperature and were counterstained with Hoechst nuclear staining (1:2000; Molecular Probes) according to the manufacturer's recommendations.

Cell-cell junction formation was assessed by immunostaining with rabbit polyclonal antibodies against ZO-1, a junctional complex protein expressed in corneal endothelium. Cells plated for ZO-1 staining were grown in the same culture medium used to grow endothelial cells but with a lower serum concentration of 6% FBS to optimize the formation of tight junctions (N. Joyce, personal communication, 2006). Cells were incubated with primary rabbit polyclonal antibodies against ZO-1 (1:100; Zymed) overnight at 4°C. Secondary staining was performed with Cy-3- conjugated donkey anti-rabbit antibodies (1:100) for 1 hour at room temperature, and cells were counterstained with Hoechst nuclear staining (1:2000).

## Cryopreservation of Human Corneal Endothelial Cells

Confluent cells were trypsinized and resuspended in culture medium. They were then gently centrifuged and resuspended in freezing medium consisting of 90% heat-inactivated fetal bovine serum and 10% dimethyl sulfoxide. The cells were then slowly frozen overnight in an isopropanol bath freezing container (Nalgene; Nunc, Rochester, NY) at -80°C and transferred to liquid nitrogen. For recovery from cryopreservation, all cells were thawed at room temperature after 2 months of cryopreservation and promptly were resuspended drop by drop with prewarmed culture medium. The cells were then centrifuged, washed, and resuspended in culture medium to remove residual dimethyl sulfoxide and plated on precoated standard tissue culture plates. The medium was changed the next day, and the cells were passaged when confluence was reached. Aliquots of cells were grown on chamber slides and processed for immunochemistry as described.

# Retroviral Transduction of Human Corneal Endothelial Cells

Human CECs from a 3-year-old donor were passaged, and aliquots were cryopreserved at passage 3. Cells were thawed after 2 months of cryopreservation and were grown to approximately 80% confluence in 24-well plates (approximately  $1.5 \times 10^5$  cells/well). The medium was



FIGURE 1. Phase-contrast microscopy of primary human corneal endothelial cells at passage 5 (A) and stromal fibroblasts at passage 2 (B) from the same 19-year-old donor. At confluence (C), corneal endothelial cells exhibit a polygonal shape similar to the in vivo hexagonal morphology.

changed 3 to 4 hours before transduction. High-titer (5  $\times$  10<sup>8</sup> to 1  $\times$  10<sup>9</sup> TU/mL) viral particles, pseudotyped with either vesicular stomatitis virus (EIAV-VSVG) or rabies virus (EIAV-rabies) with the CMV promoter driving eGFP expression, were used to obtain a multiplicity of infection of approximately 100 in a final volume of 200  $\mu$ L. After 12 hours of initial exposure to virus, fresh medium was added to each well containing the viral particles. Two to 3 days after transduction, the cells were imaged with fluorescence microscopy to assess the percentage of cells expressing eGFP. Five to 12 days after transduction, FACS and additional imaging were performed.

#### Imaging and Fluorescence-Activated Cell Sorting

Immunostained cells were viewed and photographed with an inverted fluorescent digital microscopy/photography system (Eclipse TE2000-U; Nikon, Melville, NY). FACS for eGFP expression was performed (MoFlo High-Performance Cell Sorter; Cytomation, Fort Collins, CO) approximately 7 days after transduction.

### RESULTS

#### Morphology and Collagen VIII Staining of Human Cultured Corneal Endothelial Cells

Pure cultures of CECs were obtained from both corneas. By phase-contrast microscopy, these subconfluent cells grew in a monolayer and showed the characteristic medium-sized, polygonal morphology with elongated processes (Fig. 1A) which was distinct from the smaller, spindle-shaped stromal fibroblasts cultured from the same donor (Fig. 1B). At confluence, CECs exhibited a more compact polygonal morphology similar to their in vivo appearance (Fig. 1C). At progressively higher passage numbers, endothelial cell size and heterogeneity of shape increased as the cells exhibited reduction in and eventual loss of proliferative capacity.

To confirm the endothelial origin of our CEC lines, we performed immunofluorescence staining with anti-collagen VIII ( $\alpha$ 1) and ( $\alpha$ 2) antibodies of our cultured cell lines. Keratocytes showed no staining for either antibody (Fig. 2A). In contrast, endothelial cells at passage 3 showed consistently high levels of diffuse staining in the cytoplasm for both antibodies (Fig. 2B). Secondary antibody alone and mouse antihuman Thy-1 IgG (BD PharMingen, San Diego, CA) as negative controls showed no staining of endothelial cells (data not shown).

## Cryopreservation of Human Corneal Endothelial Cells

CEC lines of various passages were cryopreserved, as described in Materials and Methods. CECs from a 19-year-old donor cryopreserved at passage 5 were thawed and characterized after four passages to ensure that morphology and collagen VIII expression were stable and resulted from postthaw metabolic activity. Each vial of thawed cells showed a relatively limited number of nonviable cells, consistent with other commonly used cell culture lines after thawing.

Cells recovered from cryopreservation showed characteristic endothelial cell morphology and were able to proliferate after thawing for an additional four passages. CECs before (Fig. 2B), two passages after (Fig. 2C), and four passages after (Fig. 2D) cryopreservation have a polygonal appearance similar to that of the light microscopy images of the cells at confluence



FIGURE 2. Immunofluorescence microscopy of human primary cultured corneal cells from a 19-year-old donor. (A) Anti-collagen VIII( $\alpha 1$ ) antibodies were used to stain keratocytes, (B) corneal endothelial cells at passage 3 before cryopreservation, and (C) endothelial cells at passage 7 (two passages after cryopreservation). (**D**) Anti-collagen VIII( $\alpha 2$ ) antibodies were used to stain corneal endothelial cells at passage 9 (four passages after cryopreservation). Hoechst dye was used as nuclear stain. All panels were taken at the same magnification. Scale bar, 30 μm (A).

FIGURE 3. (A) Immunofluorescence microscopy using anti-ZO-1 antibodies of human primary cultured corneal endothelial cells from a 19-yearold donor at passage 9 (four passages after cryopreservation). (B) Negative control staining of identical cells with no primary antibody. Comparable levels of nonspecific, perinuclear fluorescence  $(\mathbf{A}, \mathbf{B})$  indicate that the cell border staining in (A) is specific and not related to differences in brightness between the two panels. Hoechst dye was used as nuclear stain. Both panels were taken at the same magnification. Scale bar, 30 μm (A).



before cryopreservation (Fig. 1C). In addition, the amount of collagen VIII ( $\alpha$ 2) immunofluorescence is consistent in CECs before and up to four generations after cryopreservation (Figs. 2B-D). With successive passages after thawing, the CECs showed a tendency to enlarge (Figs. 2B-D), which has been noted previously to correspond with senescence and gradual loss of replicative capacity.<sup>1</sup>

Immunofluorescence with antibodies to the tight junction protein ZO-1 was performed on cryopreserved cells at passage 9 (postthaw passage 4; Fig. 3A). The positive staining pattern of the cell borders indicated that cryopreserved CECs were able to form tight junctions after thawing.

#### EIAV Transduction of Human Corneal Endothelial Cells

Primary cultures of human corneal endothelial cells obtained from a 3-year-old donor and thawed at passage 3 after 2 months' cryopreservation were used for EIAV-eGFP transduction with vesicular stomatitis virus- or rabies virus-pseudotyped retroviral particles. Two to 3 days after transduction, approximately 30% of cells showed eGFP expression (Fig. 4A). No toxic effect of transduction was observed for the VSVG or the rabies-pseudotyped retroviral particles. EIAV-transduced cells showed normal cell morphology and continued to express collagen VIII ( $\alpha$ 1) and ( $\alpha$ 2) by immunofluorescence (Fig. 4B). These cells were resuspended in culture medium and FACS sorted at passage 7 (four passages after cryopreservation and transduction). With the use of a high eGFP fluorescence threshold to reduce the number of falsely positive cells, we found a higher percentage of eGFP-positive cells with the EIAV-rabies vector (16.12%) over the EIAV-VSVG vector (4.92%). FACS-sorted cells were plated and imaged by fluorescence microscopy, which showed near homogeneity of eGFPexpressing cells (Fig. 4C). Transduced cells had normal cell morphology without evidence of toxicity, continued to express collagen VIII, proliferated for four more passages after cryopreservation similar to untransduced cells, and were able to maintain a high level of eGFP expression at passage 7 (Fig. 4C), 12 days and four passages after transduction.

# DISCUSSION

Loss of corneal endothelial cells from trauma, inflammation, genetic disorders, and graft failure is a common cause of vision loss and accounts for approximately half the full-thickness corneal transplantations performed annually in the United States.<sup>27</sup> In vivo, corneal endothelial cells are arrested in the  $G_1$  phase of the cell cycle and do not replicate.<sup>1-4</sup> The discovery that human CECs can be cultured in vitro<sup>5-7</sup> has created substantial interest in using these cells either alone or placed on various substrates as an alternative to penetrating keratoplasty for the treatment of endothelial cell loss. One potential shortcoming of this approach is the finite proliferative capacity of nontransformed primary CEC cultures,<sup>1</sup> which would limit the amount of time a potential donor cell line could be used therapeutically. Thus, the ability to extend the length of time primary CECs are capable of proliferating could increase the usefulness of these cells for treatment or research.

A similar rationale has been proposed for studies of cryopreservation of whole ex vivo corneas from human and rabbit for keratoplasty using reagents, such as dextran,<sup>12</sup> propane-1,2diol (PROH),<sup>13</sup> and dimethyl sulfoxide.<sup>14</sup> One study found the longest viability of a transplanted, cryopreserved cornea in a human subject to be 16 years.<sup>14</sup> However, although a single cryopreserved whole cornea can still only be used for a single recipient patient, an expanded, cryopreserved primary endothelial cell line could theoretically be used as a donor to multiple patients if a practical, cell-based method of transplantation is developed. One limitation in this study is that we used human CECs from only two corneas. However, we specifically wanted to study human CECs from younger corneas because

FIGURE 4. (A) Fluorescence microscopy of human primary cultured corneal endothelial cells from a 3-year-old donor transduced with EIAV-eGFP pseudotyped with rabies virus at passage 3 (first passage after cryopreservation). (B) Merged eGFP fluorescence and anti-collagen VIII( $\alpha$ 1) staining of the same field shown in (A). (C) EGFP fluo-



rescence of cells from the same culture (**A**, **B**) after FACS at passage 7 (four passages after cryopreservation). Hoechst dye was used as nuclear stain. All panels were taken at the same magnification. Scale bar, 30  $\mu$ m (**A**).

they have been shown to have greater proliferative capacity.<sup>6</sup> Procuring and storing younger human CECs would have greater clinical usefulness with their greater proliferative response. Another limitation in this study is the assessment of endothelial cell functionality. In this study, we interpret the cells' ability to form cell-cell junctions by expression of ZO-1 as a possible method of evaluating cell function. Functional studies, such as measuring the endothelial pump function, are needed.

In addition to cryopreservation, the ability to genetically manipulate human primary cultured CECs could greatly increase their usefulness for cell-based transplantation approaches. Possible modifications could include the introduction of trophic factors or inducible replication control molecules because transplanted endothelial cells undergo accelerated attrition even years after surgery.<sup>28,29</sup> Several studies demonstrate that genetic modification of growth phase-arrested CECs with the transcription factor E2F2 or an antisense oligonucleotide to p27(kip1) induces proliferation.<sup>30-32</sup> Genetic modification of CECs with immunomodulatory proteins may also increase the success of cell-based transplantation methods because allografts of cultured CECs would still be susceptible to immunologic rejection. Recent reports by Beutelspacher et al.<sup>33,34</sup> support the important role of indoleamine 2,3-dioxygenase in regulating corneal allograft rejection and demonstrate that overexpression of this protein through EIAVmediated transduction prolongs corneal graft survival in a mouse transplant model.

Our results provide additional support for EIAV as a vector for genetic modification of human CECs. EIAV has been shown to achieve efficient and long-term transduction of dividing and nondividing cells in a multitude of tissues.<sup>35-37</sup> Beutelspacher et al.<sup>26</sup> reported that EIAV was more efficient than HIV-1 based vectors for transduction of a murine corneal endothelial cell line and human ex vivo corneas. Furthermore, intracameral administration of EIAV-VSV-G has been demonstrated to produce efficient and prolonged (10-month) expression of GFP in mouse corneal endothelium with no evidence of cellular dysfunction such as corneal edema.38 Interestingly, the EIAV rabies-pseudotyped virus had a higher transduction efficiency than the EIAV-VSV-G virus. This is in contrast to findings shown by Balaggan et al.<sup>38</sup> in which either EIAV-rabies or EIAV-VSV-G virus was administered intracamerally into mice. They report a higher transduction efficiency of endothelial cells and trabecular meshwork with the EIAV-VSV-G virus. Mazerakis et al.<sup>36</sup> report that EIAV pseudotyped with rabies virus glycoprotein enabled retrograde axonal transport of a marker gene that was not observed with VSV-G-pseudotyped virus. Thus, it is possible that differences in pseudotyping may result in target cell type-specific phenotypes for EIAV vectors. Such differences may explain the variation in endothelial cell transduction efficiencies and warrant further study.

Our studies show that cryopreserved human primary cultured endothelial cells can be efficiently transduced by EIAV without phenotypic alteration. Furthermore, these cells can be sorted to nearly pure populations by FACS techniques. Thus, a repository of cryopreserved human CECs capable of genetic modification to increase survival or reduce immunogenicity and sorted to homogeneity is now possible and could significantly improve the success of future endothelial cell transplantation approaches.

Our findings represent, to our knowledge, the first report that primary human CEC cultures can be cryopreserved and that these cells retain their morphology, proliferative capacity, and collagen VIII and ZO-1 expression after thawing. Furthermore, these cells are able to be genetically modified by EIAV and sorted to a nearly pure population. These results underscore the potential usefulness of this technique in expanding the supply of these cells for research and therapeutic applications, including gene therapy to prolong survival or to prevent immunologic rejection. Additional characterization of cryopreserved human CECs, particularly an assessment of pump function and their ability to be transplanted successfully, deserves additional study.

#### Acknowledgments

The authors thank Lindsey Savino and Luke Roberts for their technical assistance.

#### References

- Joyce NC. Proliferative capacity of the corneal endothelium. Prog Retinal Eye Res. 2003;22:359-389.
- 2. Joyce NC. Cell cycle status in human corneal endothelium. *Exp Eye Res.* 2005;81:629-638.
- Joyce NC, Meklir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. *Invest Ophthalmol Vis Sci.* 1996;37:645–655.
- 4. Joyce NC, Navon SE, Roy S, Zieske JD. Expression of cell cycle associated proteins in human and rabbit corneal endothelium in situ. *Invest Ophthalmol Vis Sci.* 1996;37:1566–1575.
- Baum JL, Niedra R, Davis C, Yue BY. Mass culture of human corneal endothelial cells. Arch Ophthalmol. 1984;97:1136–1140.
- Zhu C, Joyce NC. Proliferative response of corneal endothelial cells from young and older donors. *Invest Ophthalmol Vis Sci.* 2004; 45:1743-1751.
- 7. Chen KH, Azar D, Joyce NC. Transplantation of adult human corneal endothelium ex vivo. *Cornea*. 2001;20:731-737.
- Bednarz J, Teifel M, Friedl P, Engelmann K. Immortalization of human endothelial cells using electroporation protocol optimized for human corneal endothelial and human retinal pigment epithelial cells. *Acta Ophthalmol Scand*. 2000;78:130–136.
- Ishino Y, Sano Y, Nakamura T, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophtbalmol Vis Sci.* 2004;45:800-806.
- Mimura T, Yamagami S, Yokoo S, et al. Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. *Invest Ophthalmol Vis Sci.* 2004;45:2992–2997.
- 11. Mimura T, Amano S, Usui T, et al. Transplantation of corneas reconstructed with cultured adult human corneal endothelial cells in nude rats. *Exp Eye Res.* 2004;79:231–237.
- Halberstadt M, Bohnke M, Athmann S, Hagenah M. Cryopreservation of human donor corneas with dextran. *Invest Ophthalmol Vis Sci.* 2003;44:5110–5115.
- 13. Routledge C, Armitage WJ. Cryopreservation of cornea: a low cooling rate improves functional survival of endothelium after freezing and thawing. *Cryobiology*. 2003;46:277-283.
- Brunette I, Le Francois M, Tremblay M, Guertin MC. Corneal transplant tolerance of cryopreservation. *Cornea*. 2001;20:590– 596.
- Benya PD, Padilla SR. Isolation and characterization of type VIII collagen synthesized by cultured rabbit corneal endothelial cells: a conventional structure replaces the interrupted-helix model. *J Biol Chem.* 1986;261:4160–4169.
- Tamura K, Konomi H, Sawada H, Takashima S, Nakajima A. Tissue distribution of type VIII collagen in human adult and fetal eyes. *Invest Ophthalmol Vis Sci.* 1991;32:2636–2644.
- 17. Sawada H, Konomi H, Hirosawa K. Characterization of the collagen in the hexagonal lattice of Descemet's membrane: its relation to type VIII collagen. *J Cell Biol*. 1990;110:219–227.
- Greenhill NS, Ruger BM, Hasan Q, Davis PF. The alpha1 (VIII) and alpha2 (VIII) collagen chains form two distinct homotrimeric proteins in vivo. *Matrix Biol.* 2000;19:19–28.
- Gottsch JD, Zhang C, Sundin OH, Bell WR, Stark WJ, Green WR. Fuchs corneal dystrophy: aberrant collagen distribution in an L450W mutant of the COL8A2 gene. *Invest Ophthalmol Vis Sci.* 2005;46:4504-4511.

- Leung EW, Rife L, Smith RE, Kay EP. Extracellular matrix components in retrocorneal fibrous membrane in comparison to corneal endothelium and Descemet's membrane. *Mol Vis.* 2000;6:15–23.
- 21. Biswas S, Munier FL, Yardley J, et al. Missense mutations in COL8A2, the gene encoding the  $\alpha$ 2 chain of type VIII collagen, cause two forms of corneal endothelial dystrophy. *Hum Mol Genet.* 2001;10:2415-2423.
- 22. Gottsch JD, Sundin OH, Liu SH, et al. Inheritance of a novel COL8A2 mutation defines a distinct early-onset subtype of Fuchs corneal dystrophy. *Invest Ophthalmol Vis Sci.* 2005;46:1934– 1939.
- 23. Mandell KJ, Holley GP, Parkos CA, Edelhauser HF. Antibody blockade of junctional adhesion molecule-A in rabbit corneal endothelial tight junctions produces corneal swelling. *Invest Ophthalmol Vis Sci.* 2006;47:2408–2416.
- Naldini L, Blomer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996;5259:263–267.
- 25. Bainbridge JWB, Stephens C, Parsley K, et al. In vivo gene transfer to the mouse eye using an HIV-based lentiviral vector; efficient long-term transduction of corneal endothelium and retinal pigment epithelium. *Gene Ther.* 2001;8:1665-1668.
- Beutelspacher SC, Ardjomand N, Tan PH, et al. Comparison of HIV-1 and EIAV-based lentiviral vectors in corneal transduction. *Exp Eye Res.* 2005;80:787-794.
- 27. Maeno A, Naor J, Lee HM, et al. Three decades of corneal transplantation: indications and patient characteristics. *Cornea*. 2000;19:7-11.
- Armitage WJ, Dick AD, Bourne WM. Predicting endothelial cell loss and long-term corneal graft survival. *Invest Ophthalmol Vis Sci.* 2003;44:3326-3331.
- 29. Bourne WM. Cellular changes in transplanted human corneas. *Cornea*. 2001;20:560-569.

- Kikuchi M, Harris DL, Obara Y, Senoo T, Joyce NC. P27kip1 antisense-induced proliferative activity of rat corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 2004;45:1763–1770.
- Joyce NC, Harris DL, McAlister JC, Ali RR, Larkin DF. Effect of overexpressing the transcription factor E2F2 on cell cycle progression in rabbit corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 2004;45:1340–1348.
- McAlister JC, Joyce NC, Harris DL, Ali RR, Larkin DFP. Induction of replication in human corneal endothelial cells by EF2 transcription factor cDNA transfer. *Invest Ophthalmol Vis Sci.* 2005;46:3597– 3603.
- Beutelspacher SC, Tan PH, McClure MO, Larkin DF, Lechler RI, George AJ. Expression of indoleamine 2,3-dioxygenase (IDO) by endothelial cells: implications for control of alloresponses. *Am J Transplant*. 2006;6:1320-1330.
- 34. Beutelspacher SC, Pillai R, Watson MP, et al. Function of indolamine 2,3-dioxygenase in corneal allograft rejection and prolongation of allograft survival by over-expression. *Eur J Immunol.* 2006;36:690–700.
- Azzouz M, Ralph S, Wong LF, et al. Neuroprotection in a rat Parkinson model by GDNF gene therapy using EIAV vector. *Neuroreport*. 2004;15:985–990.
- 36. Mazarakis JD, Azzouz M, Rohll JB, et al. Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. *Hum Mol Genet*. 2001;10:2109–2121.
- 37. O'Rourke JP, Hiraragi H, Urban K, et al. Analysis of gene transfer and expression in skeletal muscle using enhanced EIAV lentivirus vectors. *Mol Ther*. 2003;7:632–639.
- Balaggan KS, Binley K, Esapa M, et al. Stable and efficient intraocular gene transfer using pseudotyped EIAV lentiviral vectors. *J Gene Med.* 2006;8:275–285.