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Proliferative capacity of corneal endothelial cells

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ABSTRACT

The corneal endothelial monolayer helps maintain corneal transparency through its barrier and ionic "pump" functions. This transparency function can become compromised, resulting in a critical loss in endothelial cell density (ECD), corneal edema, bullous keratopathy, and loss of visual acuity. Although penetrating keratoplasty and various forms of endothelial keratoplasty are capable of restoring corneal clarity, they can also have complications requiring re-grafting or other treatments. With the increasing worldwide shortage of donor corneas to be used for keratoplasty, there is a greater need to find new therapies to restore corneal clarity that is lost due to endothelial dysfunction. As a result, researchers have been exploring alternative approaches that could result in the *in vivo* induction of transient corneal endothelial cell division or the *in vitro* expansion of healthy endothelial cells for corneal bioengineering as treatments to increase ECD and restore visual acuity. This review presents current information regarding the ability of human corneal endothelial cells (HCEC) to divide as a basis for the development of new therapies. Information will be presented on the positive and negative regulation of the cell cycle as background for the studies to be discussed. Results of studies exploring the proliferative capacity of HCEC will be presented and specific conditions that affect the ability of HCEC to divide will be discussed. Methods that have been tested to induce transient proliferation of HCEC will also be presented. This review will discuss the effect of donor age and endothelial topography on relative proliferative capacity of HCEC, as well as explore the role of nuclear oxidative DNA damage in decreasing the relative proliferative capacity of HCEC. Finally, potential new research directions will be discussed that could take advantage of and/or improve the proliferative capacity of these physiologically important cells in order to develop new treatments to restore corneal clarity.

1. Introduction

The corneal endothelium helps maintain corneal transparency via its barrier and ionic "pump" functions. To maintain transparency, endothelial cell density (ECD) must remain above a critical number—usually 400–500 cells/mm^2. Morphometric analyses of ECD in fetal and adult endothelium (Murphy et al., 1984; Bourne et al., 1997; Hollingsworth et al., 2001) indicate that, following formation of the endothelial monolayer during corneal development, human corneal endothelial cells (HCEC) do not normally divide *in vivo* at a rate sufficient to replace dead or injured cells. This results in an average cell loss of 0.3–0.6% per year. The response of the endothelium to this gradual cell loss, as well as to larger wounds, normally involves spreading and/or migration of neighboring cells to cover the wound area (Laing et al., 1976; Honda et al., 1982; Matsuda et al., 1985). The result of this form of wound healing is an increase in overall cell size and an alteration from a hexagonal to a pleomorphic shape. Unfortunately, ECD can be significantly decreased as the result of accidental or surgical trauma, refractive surgery, previous penetrating or endothelial keratoplasty, stress caused by certain diseases such as diabetes or glaucoma, or endothelial dystrophies. If the density of endothelial cells is too low, barrier function is lost and more fluid enters the cornea than can be removed through the activity of the ionic "pumps". Loss of endothelial barrier function results in corneal edema, development of bullous keratopathy, and loss of visual acuity. Current treatments, such as penetrating or endothelial keratoplasty to restore visual acuity generally work well, but can have complications requiring re-grafting or other treatments (Rahman et al., 2010; Lass et al., 2010; Terry et al., 2008; Clements et al., 2011; Shulman et al., 2009). In addition, there is an increasing worldwide shortage of donor corneas that are considered acceptable for transplant purposes and the aging of the "baby boomer" generation will bring a greater need to find new therapies to restore corneal clarity that is lost due to endothelial dysfunction. One approach to develop new therapies to prevent or treat excessive corneal endothelial cell loss

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is to explore the relative ability of HCEC to divide. This review will present information regarding the positive and negative regulation of the cell cycle and discuss results of studies exploring the proliferative capacity of HCEC.

2. The cell cycle

Fig. 1 presents a simplified diagram of the positive and negative regulation of the cell cycle. Additional information related to the cell cycle, but not emphasized here, can be found in recent reviews (Ozaki and Nakagawara, 2011; Kim et al., 2011; Ma and Poon, 2011; and Rieder, 2011). Non-dividing cells normally exist in a "resting" (G_0) state in which DNA is present in an unduplicated (2N) form. Mitogenic stimulation induces G_1-phase entry, which prepares cells for DNA duplication in S-phase. Movement of cells from G_1- into S-phase is highly regulated and involves control of the activity of the E2F transcription factor, which activates genes required for DNA synthesis (Leone et al., 1999). In quiescent cells, E2F is tightly associated with the retinoblastoma tumor suppressor, pRb, which prevents its activation. To inhibit E2F activity, pRb must be in a hypo-phosphorylated state. Hypo-phosphorylation of pRb is maintained, in part, by the activity of the cyclin-dependent kinase inhibitors (CKIs), p27Kip1, p21Cip1, and p16INK4a. Mitogenic stimulation induces a reduction in the protein level of these CKIs due to transcriptional inhibition and/or to increased degradation by the ubiquitin–proteasome pathway. Mitogenic stimulation also induces synthesis of the positive G_1-phase regulatory protein, cyclin D (Sherr, 1993), which binds to cyclin-dependent kinase (CDK)-4, forming an active kinase complex that specifically phosphorylates pRb. This hyper-phosphorylation alters the interaction of pRb with E2F, promoting activation of E2F and leading to S-phase entry. Cyclin E is synthesized late in G_1-phase upon E2F activation. Cyclin E binding to CDK2 helps activate this kinase complex and, in part, promotes continued hyper-phosphorylation of pRb and movement into S-phase. In S-phase, DNA is duplicated under highly controlled conditions, moving DNA from the 2N to a 4N state. Cyclin A synthesis begins in late G_1-phase. Cyclin A binds to CDK2 and the activity of this complex down-regulates E2F activity by facilitating its degradation, thus promoting forward progression from S- to G_2-phase. Cyclin B synthesis is activated at the end of S-phase. In G_2-phase, cyclin B binds to and activates the kinase activity of CDK1, which prepares the cell for M-phase (mitosis), in which cells divide, forming daughter cells, each of which contains 2N DNA.

Negative regulation of G_1-phase involves inhibition of the kinase activity of the G_1-phase cyclin/CDK complexes by CKIs. There are two CKI families. The "INK" family includes p16INK4a, which specifically binds to free CDK4 and prevents its association with cyclin D to form an active complex (Serrano et al., 1993). p16INK4a also competes with cyclin D for binding to CDK4 in existing complexes, thus dissociating the complex. Inhibition of cyclin D/CDK4 kinase activity by p16INK4a prevents the initial downstream hyper-phosphorylation of pRb that is required for E2F activation and S-phase entry. The "Cip/Kip" family includes p21Cip1 and p27Kip1 (Harper et al., 1993; Polyak et al., 1994). Both these inhibitors bind G_1-phase cyclin/CDK complexes, inhibiting their kinase activity. In the presence of p16INK4a, the "Cip/Kip" proteins mainly bind and inhibit the activity of cyclin E/CDK2 complexes. Synthesis of p21Cip1 is induced by the transcription factor, p53, which can be activated by a number of factors, including oxidative DNA damage (Helton and Chen, 2007). Transforming growth factor-β (TGF-β) and formation of mature cell–cell contacts (Polyak et al., 1994) increase the cellular level of p27Kip1. The inhibitory function of all the G_1-phase CKIs is extremely important, because it prevents unscheduled entry into S-phase and inappropriate DNA synthesis.

3. Cell cycle status of HCEC *in vivo*

To understand why HCEC do not proliferate *in vivo*, this laboratory conducted studies to determine the cell cycle status of endothelial cells in *ex vivo* donor human corneas (Joyce et al., 1996a, 1996b). This was accomplished by observing the relative staining intensity and subcellular localization of a battery of key cell cycle

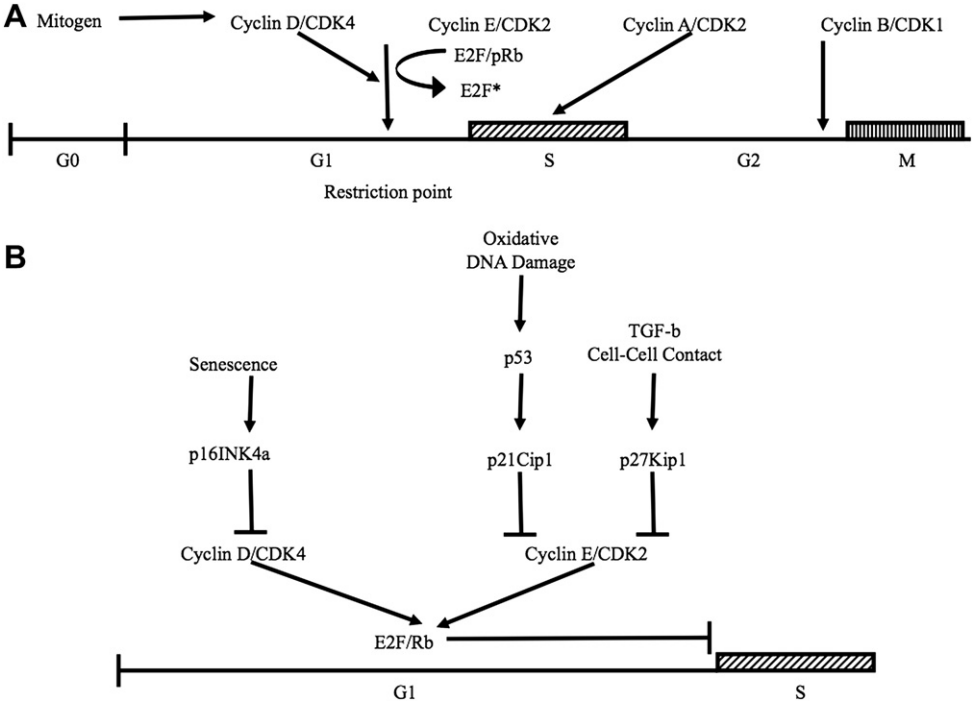

Fig. 1. Diagrams illustrating the positive (A) and negative (B) regulation of the cell cycle. Springer-Verlag is the original copyright holder.

proteins in transverse corneal sections using immunofluorescence microscopy. Together, results strongly suggest that HCEC *in vivo* are arrested in G_1-phase of the cell cycle. Several mechanisms have been identified that contribute to the maintenance of G_1-phase arrest in HCEC *in vivo*. These mechanisms will be discussed below.

3.1. Cell–cell contact-dependent inhibition

Studies (Wulle and Lerche, 1969; Wulle, 1972; Hay, 1980) indicate that corneal endothelium is formed from neural crest-derived mesenchymal cells located at the periphery of the presumptive cornea. During eye development, these cells both proliferate and migrate centrally to form the endothelial monolayer (Nuttall, 1976). Analysis of transmission electron micrographs indicates that proliferation of the presumptive endothelial cells ceases upon formation of cell–cell contacts (Wulle and Lerche, 1969; Wulle, 1972). This same mechanism also plays a role in inhibition of proliferation in the mature endothelium. Studies of the role of cell–cell contacts in the inhibition of proliferation were conducted in this laboratory using corneas obtained from neonatal rats (Joyce et al., 1998), since maturation of rat corneal endothelial cells (RCEC) does not take place until after birth. Results indicated a correlation between the time at which endothelial cell division ceased and the time that mature cell–cell contacts were formed. This timing also correlated with an increase in the expression of p27Kip1 protein, implicating a role for this CKI in mediating contact inhibition in corneal endothelium. Western blot studies showed that the protein level of p27Kip1 in confluent, contact-inhibited cultures of RCEC was 20-fold higher than in subconfluent cultures. When confluent cultures were treated with ethylenediamine tetraacetic acid (EDTA) to release cell–cell contacts, the level of p27Kip1 was greatly reduced, providing additional evidence that p27Kip1 plays a role in mediating contact inhibition in these cells (Joyce et al., 2002). Similar results have been obtained in studies of developing mouse corneal endothelium (Yoshida et al., 2004) and in HCEC. In *ex vivo* human corneas, release of endothelial cell–cell contacts by mechanical wounding (Senoo and Joyce, 2000) or by treatment with EDTA (Senoo et al., 2000) promoted cell division upon exposure to mitogens.

3.2. Lack of effective growth factor stimulation

In vivo, cell division does not appear to occur in HCEC following wounding, even though cell–cell contacts would have been disrupted. This suggests that there may not be sufficient paracrine or autocrine growth factor stimulation to induce cells to divide. Low levels of positive growth factors have been detected in aqueous humor from normal eyes. These include acidic and basic fibroblast growth factor (FGF) (Schulz et al., 1993), insulin-like growth factor-I and –II (Arnold et al., 1993), and hepatocyte growth factor (HGF) (Araki-Sasaki et al., 1997). Descemet's membrane, the thick extracellular matrix secreted by corneal endothelial cells, appears to bind a number of growth factors (Gospodarowicz et al., 1980; Blake et al., 1997). In addition, corneal endothelial cells themselves appear to synthesize a number of growth factors and their receptors, including epidermal growth factor (EGF) and its receptor, acidic and basic FGF and their receptors, TGF-β1, TGF-α, HGF and its receptor, keratinocyte growth factor and its receptor (Wilson and Lloyd, 1991; Wilson et al., 1993a, 1993b, 1994), and platelet-derived growth factor receptor (Hoppenreijs et al., 1993). Although endothelial cells may have access to growth factors, it is possible that they are not present in sufficient concentration, are in inactive forms, or do not bind effectively enough to HCEC receptors to induce and/or sustain a positive mitogenic signal in injured endothelium.

3.3. TGF-β2 suppression of S-phase entry

TGF-β2 is present mainly in latent form in aqueous humor; however, HCEC express proteins, such as thrombospondin-1 (Hiscott et al., 1997), which activate latent TGF-β2 (Schultz-Cherry et al., 1994). HCEC also express mRNA and protein for TGF-β receptors-I, II, and III (Joyce and Zieske, 1997)—all three of which are needed for optimal TGF-β-induced signal transduction. Studies in cultured rabbit (Harris and Joyce, 1999) and rat corneal endothelial cells (Chen et al., 1999) indicate that exogenous active TGF-β2 and activated TGF-β2 from aqueous humor suppress S-phase entry. In rabbit corneal endothelial cells, TGF-β2 down-regulates the expression of CDK4 and prevents nuclear export of p27Kip1 for degradation, thus maintaining a high level of this G_1-phase inhibitory protein (Kim et al., 2001). Evidence also suggests that the TGF-β2 suppressive effect may be due to its stimulation of the synthesis and secretion of prostaglandin E2, which is capable of inhibiting rabbit corneal endothelial cell proliferation in a dose-dependent manner (Chen et al., 2003).

4. Efforts to stimulate corneal endothelial proliferation

A number of methods have been tested to take advantage of the existing proliferative capacity of HCEC as a means of increasing ECD. These will be discussed in more detail below.

4.1. Release of cell–cell contacts and growth factor stimulation

HCEC in *ex vivo* corneas and in culture are able to enter and complete the cell cycle upon release of cell–cell contacts in the presence of mitogens. Fig. 2A shows that nuclei of HCEC in the wounded area of *ex vivo* corneas stain positively for Ki67, a marker of actively cycling cells (Gerdes et al., 1983), whereas there is no positive staining in cells more peripheral to the wound. HCEC can also be isolated from donor corneas, successfully grown in culture, and passaged a limited number of times (Baum et al., 1979; Engelmann and Friedl, 1989; Chen et al., 2001; Li et al., 2007). Fig. 2B shows a subconfluent culture of HCEC with nuclei stained positively for Ki67, indicating that cells are actively proliferating. This data not only provides evidence that HCEC retain proliferative capacity, but also suggests potential methods by which donor corneas with low ECD could be treated to increase cell numbers prior to transplantation.

4.2. Sustaining growth factor-induced stimulation of proliferation

Stimulation of proliferation by EGF involves binding of EGF to its receptor and the subsequent autophosphorylation of specific tyrosine residues in the receptor intracellular domain (Wells, 1999). This autophosphorylation initiates a signaling cascade that leads to cell cycle entry (Marshall, 1995). The protein tyrosine phosphatase, PTP1B, interacts with and dephosphorylates a number of growth factor receptors, including EGF, thereby attenuating EGF-induced downstream signaling (Haj et al., 2003). Studies conducted in both RCEC (Harris and Joyce, 2007) and HCEC (Ishino et al., 2008) have demonstrated that specific inhibition of PTP1B activity was able to sustain EGF-induced tyrosine autophosphorylation of the EGF receptor and increase the number of cells entering the cell cycle, indicating the possibility that the number of proliferating HCEC could be increased by suppressing the down-regulation of growth factor signaling.

4.3. Overcoming G_1-phase inhibition

Studies have been conducted to test whether HCEC can be induced to divide by overcoming G_1-phase inhibition. Ectopic

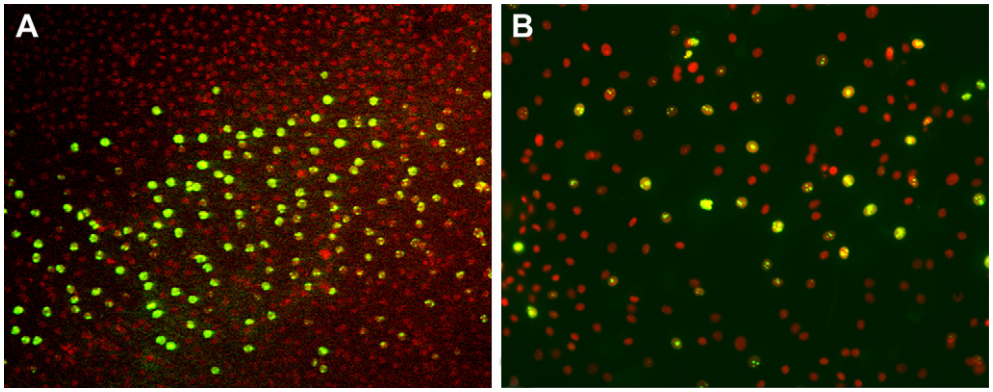

Fig. 2. Evidence that HCEC in the wound area of *ex vivo* corneas (A) and in culture (B) can actively proliferate. Endothelium in the donor cornea in (A) was scrape-wounded, incubated in culture medium, immunostained for Ki67 (green) and counterstained with DAPI (red) to detect nuclei. Nuclei within the wound area are positively stained for Ki67, whereas nuclei in cells in the unwounded part of the endothelial monolayer are not stained for Ki67. HCEC cultured from a 67-year old donor show positive Ki67 staining. Orig. mag. = 16X (A) and 20X (B). Lippincott Williams & Wilkins is the original copyright holder of the image in (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of either the SV40 large-T antigen (Wilson et al., 1993; Aboalchamat et al., 1999) or the human papilloma virus type 16 oncoproteins, E6/E7 (Wilson et al., 1995) in cultured HCEC results in multiple rounds of cell division. Although these treatments have been successful in inducing proliferation of HCEC, questions remain regarding their long-term safety as a treatment to increase ECD. Another possible method to overcome G_1-phase inhibition is to lower the level of one or more cyclin-dependent kinase inhibitors, thereby removing the constraint to S-phase entry. Studies were conducted to determine whether treatment of HCEC with p27Kip1 siRNA would promote proliferation (Kikuchi et al., 2006). Interestingly, siRNA-induced reduction of p27Kip1 protein levels only promoted a significant increase in cell numbers in HCEC cultured from young donors. SiRNA-induced reduction of the protein levels of both p21Cip1 and p16INK4a increased the number of cells entering the cell cycle, as well as total cell numbers in HCEC cultured from both young (<30 years old) and older donors (>50 years old) (Joyce and Harris, 2010). Together, results of these studies indicate that it is possible to overcome G_1-phase inhibition and promote HCEC division by reducing the expression of CKIs. The results also suggest that there is a difference in the relative response of HCEC to these treatments depending on donor age. (See further discussion below.)

4.4. Bypassing G_1-phase inhibition

Studies have tested the effect of ectopically expressing E2F2 in rabbit corneal endothelium (Joyce et al., 2004) and in HCEC in *ex vivo* corneas (McAlister et al., 2005). E2F2 is one of the isoforms of E2F that is responsible for activating genes necessary for S-phase entry (DeGregori et al., 1997). For studies in HCEC, the endothelium of *ex vivo* corneas from both young and older donors was transfected with an adenoviral vector containing the full-length gene for E2F2. Ectopic expression of E2F2 was able to induce S-phase entry, as determined by immunostaining for bromodeoxyuridine, a recognized marker of DNA synthesis. The effect of E2F2 expression on cell division was demonstrated by a significant increase in ECD compared with vector controls. Together, these studies indicate that it is possible to stimulate division in HCEC by bypassing G_1-phase inhibition.

5. Effect of donor age on HCEC proliferative capacity

Of importance is the fact that, although HCEC retain an ability to divide, their relative proliferative capacity is affected by donor age. Baum et al. (1979) first described age-related differences in the proliferation of cultured HCEC. In their studies, cells from donors less than 20 years old grew well in culture, whereas, cells from older donors were either difficult to grow or did not grow at all. Results of those early studies have been confirmed and expanded using *ex vivo* cornea and tissue culture models. The kinetics of cell cycle traverse in HCEC from young (<30 years old) and older donors (>50 years old) were first compared using an *ex vivo* corneal endothelial scrape wound (Senoo and Joyce, 2000). Semi-quantitative analysis of immunostaining for Ki67 in the wound area revealed a significant decrease in the rate of cell cycle entry and in the relative number of dividing HCEC in corneas from older compared with younger donors (Fig. 3A). Direct counting of

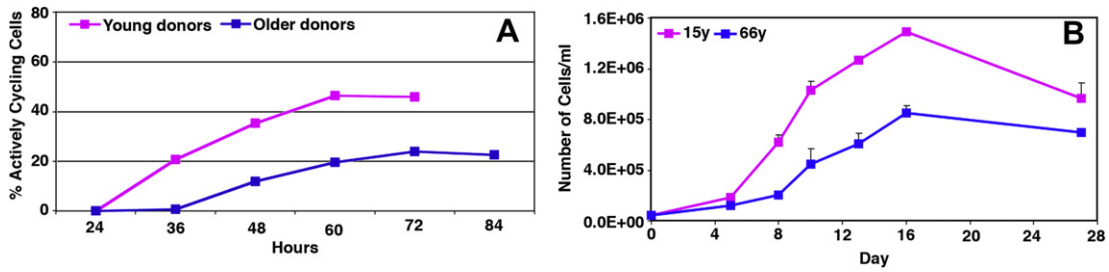

Fig. 3. Growth curves demonstrating age-related differences in relative proliferative capacity in HCEC in an *ex vivo* cornea scrape wound (A) and in culture (B). Graph in (A) presents the average percent of actively cycling cells in the wound area from at least 3 corneas per age-group. Graph in (B) presents direct cell counts at various times after initiation of the culture. Adapted from Joyce (2005). Exp. Eye Res. 81: 629–638. Springer-Verlag is the original copyright holder of this image.

cultured HCEC (Joyce, 2005; Zhu and Joyce, 2004) showed very similar age-related changes in proliferative capacity to those observed using the *ex vivo* cornea wound model (Fig. 3B). Calculations from the log phase of growth indicate that the average doubling time for HCEC cultured from older donors was 90.25 h compared with 46.25 h for cells cultured from young donors (Joyce and Zhu, 2004).

5.1. Age-related changes in the positive regulation of G_1-phase of the cell cycle

As indicated above, mitogenic stimulation leads to the hyper-phosphorylation of pRb by specific G_1-phase cyclin/CDK complexes, resulting in the activation of E2F and the subsequent increased expression of proteins required for S-phase entry. Semi-quantitative analysis (Enomoto et al., 2006) of Western blots of HCEC cultured from young (<30 years old) and older donors (>50 years old) indicated that the overall expression of pRb is very similar in HCEC, regardless of donor age. The same blots indicated that the kinetics of pRb hyper-phosphorylation were much slower and the relative amount of hyper-phosphorylated pRb was significantly lower in HCEC cultured from older compared with younger donors.

Formation of origin-recognition complexes on DNA is required for S-phase entry. These complexes associate with DNA during G_1-phase and are present at sites on chromatin that are at or near future sites of initiation of DNA replication. Binding of these complexes to DNA makes chromatin competent for replication (Stoeber et al., 2001). Minichromosome maintenance-2 (MCM2) protein is a member of the origin-recognition complex and its expression is a reliable marker to identify replication-competent cells (Wharton et al., 2001). Studies using the *ex vivo* cornea wound model compared the relative number of replication-competent HCEC in corneas obtained from young (<30 years old) and older donors (>50 years old) (Mimura and Joyce, 2006) by counting cells immunostained for MCM2. The percentage of MCM2-positive cells in the endothelium of corneas from older donors was significantly less than in the endothelium of younger corneas, indicating that more HCEC from young donors were competent to replicate. In addition, the relative time at which cells became competent to replicate occurred sooner after mitogenic stimulation in HCEC from young compared with older donors.

5.2. Age-related differences in the expression of G_1-Phase inhibitors

The above data provide strong evidence that HCEC from older donors are slower to respond to mitogenic stimulation and exhibit an overall reduced proliferative capacity compared with HCEC from young donors. Since p27Kip1, p21Cip1, and p16INK4a are important negative regulators of G_1-phase of the cell cycle, Western blot studies were conducted to compare the relative expression of these proteins in primary cultures of HCEC from young (<30 years old) and older donors (>50 years old) (Enomoto et al., 2006). Results of the semi-quantitative analysis indicated that the relative expression of p27Kip1 does not differ significantly between age-groups; however, a statistically significant increase in the expression of both p16INK4a and p21Cip1 was detected in HCEC cultured from older donors. Together, results from these studies strongly suggest that p27Kip1, which plays an important role in mediating contact inhibition, does not significantly contribute to the age-related difference in relative proliferative capacity observed in HCEC. On the other hand, the significant increase in p21Cip1 and p16INK4a protein levels in HCEC from older donors provides evidence that both these CKIs help mediate the observed age-related decrease in proliferative capacity.

6. Effect of endothelial topography on HCEC proliferative capacity

The proliferative capacity of HCEC also differs with the position of cells within the endothelial monolayer. Bednarz et al. (1998) found that HCEC cultured from the central 6.5 mm diameter of the endothelium had little-to-no mitogenic activity, whereas, cells cultured from the 6.5–9.0 mm peripheral rim exhibited greater mitogenic activity. Studies from this laboratory further explored the effect of endothelial topography on the replicative competence of HCEC using an *ex vivo* cornea wound model (Mimura and Joyce, 2006). The endothelium was divided into two topographic areas: a 6.0 mm diameter central area and a 6.0 mm–9.5 mm peripheral rim. A mechanical scrape wound was made across the endothelium, including both the center and the peripheral rim. Corneas were incubated in mitogen-containing medium, immunostained for MCM2, and the number of MCM2-positive cells counted. Results showed that the number of MCM2-positive cells, and therefore the number of cells competent to replicate, was consistently lower in central endothelium compared with the peripheral rim in both age-groups. Interestingly, the relative percent of replication-competent cells was significantly lower in the central area of older donors compared with young donors. Results from other laboratories have confirmed the existence of topographical differences related to the relative ability of HCEC to divide (Paull and Whikehart, 2005; Yamagami et al., 2007; Patel and Bourne, 2009).

General characteristics of senescent cells include decreased saturation density, slower cell cycle kinetics, stable arrest with a 2N DNA content, and increased expression of p21Cip1 and p16INK4a protein (Cristofalo, 1988; Campisi, 1996). Staining for beta-galactosidase (SA-β-Gal) at pH 6.0 is a marker of cellular senescence (Dimri et al., 1995). Since HCEC from older donors exhibit a number of senescence-like characteristics, studies were conducted to identify senescent cells in *ex vivo* corneas by staining for SA-β-Gal (Mimura and Joyce, 2006). Scoring of the endothelium for the relative intensity of SA-β-Gal staining showed few-to-no senescent cells in either the central or peripheral area of corneas from young donors. In corneas from older donors, SA-β-Gal staining was detectable at low-to-moderate levels in the periphery, but moderate-to-intense levels were observed in cells within the central area, indicating a greater percentage of senescent cells in central endothelium of older donors. Similar age-related differences in SA-β-Gal staining in HCEC from young and older donors were observed in studies by Song et al. (2008).

Comparative studies using senescence-resistant and senescence-prone strains of mice (Xiao et al., 2009) have indicated that the signaling pathway of the G_1-phase inhibitor, p16INK4a, may play a key role in the early stages of senescence in corneal endothelial cells, while the signaling pathway involving the p53 transcription factor and its downstream activation of p21Cip1 may be active in the later stages of senescence in these cells. These results generally correlate with findings discussed above indicating an up-regulation in the expression of both p21Cip1 and p16INK4a in HCEC cultured from older donors (Enomoto et al., 2006).

7. Molecular basis for decreased proliferative capacity

Together, accumulated data strongly suggest that there is both an age-related and topographical difference in the relative proliferative capacity of HCEC and that this difference is related to cellular senescence. Researchers in the field have identified two forms of cellular senescence: replicative senescence and stress-induced premature senescence. Below is a description of studies conducted to identify the mechanisms responsible for the decreased proliferative capacity observed in HCEC.

7.1. Test for critically short telomeres

Replicative senescence results from the successive shortening of telomeres that occurs during DNA replication (Wright and Shay, 1992). Once telomeres have eroded to a critically short length, the senescence program is activated and cells become irreversibly inhibited from dividing (Ben-Porath and Weinberg, 2005). Egan et al. (1998) measured telomere restriction fragment (TRF) lengths in HCEC isolated from donors 5 weeks to 84 years old and found that the mean TRF length was 12.2 kb, regardless of donor age. This length is sufficient to support several additional rounds of cell division prior to the formation of critically short telomeres. This laboratory confirmed and extended these findings using a fluorescent probe that specifically binds to telomere repeats (Konomi and Joyce, 2007). Semi-quantitative analysis of the intensity of telomere staining in *ex vivo* corneas and in HCEC freshly isolated from donor corneas showed no statistical age-related or topographic difference indicative of a difference in telomere length. Together, these data strongly suggest that HCEC retain the potential to divide based on telomere length and that the observed decrease in proliferative capacity is NOT due to "replicative senescence".

7.2. Role of nuclear oxidative DNA damage

Stress-induced premature senescence is considered to be "premature" because cells lose the ability to proliferate prior to telomere exhaustion. Thus, in this form of senescence, cells retain proliferative potential based on telomere length, but stop dividing due to inhibitory mechanisms activated by stress-induced damage pathways. Corneal endothelium is very metabolically active and lies directly in the light-path, making it potentially vulnerable to oxidative stress and its negative effects on cellular function. This effect on function has been observed in other ocular cells that lie within the light-path, including retinal pigment epithelial cells (Burke and Soref, 1988; Hjelmeland et al., 1999) and lens epithelial cells (Chylack, 1984). Studies were conducted in this laboratory to determine whether there is a relationship between oxidative stress, oxidative nuclear DNA damage, and reduced proliferative capacity in HCEC (Joyce et al., 2009). Oxidized DNA damage was first quantified by a competitive ELISA assay using an antibody directed against 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidized DNA (Beckman and Ames, 1997; Melov, 2000). The average concentration of 8-OHdG per nanogram of DNA was found to be higher in cells isolated from older donors and this difference was mainly contributed by a statistically significant increase in 8-OHdG within central endothelium. Immunostaining for 8-OHdG in the endothelium of *ex vivo* corneas revealed the presence of oxidative DNA damage in mitochondria, regardless of donor age or topographical location. Nuclei were much more intensely stained in central endothelium than in the periphery in corneas from older donors, whereas relatively little nuclear staining was observed in either area of corneas from young donors. Together, these results provide evidence for the existence of nuclear oxidative DNA damage mainly in the central endothelium of older donors. To test whether there is a relationship between oxidative stress and proliferative capacity in HCEC, a study was conducted in which subconfluent HCEC cultured from young donors were exposed to increasing concentrations of hydrogen peroxide (H_2O_2), a known oxidative stressor, and then tested for their ability to divide. The graph in Fig. 4 shows that HCEC not exposed to H_2O_2 and cells exposed to a low concentration (25 μM) of H_2O_2 showed similar robust growth curves. With increasing concentrations of H_2O_2, the growth of HCEC decreased, making the growth kinetics of HCEC from young donors closely resemble those observed in HCEC from older donors (Compare graph in Fig. 4 with graphs in Fig. 2).

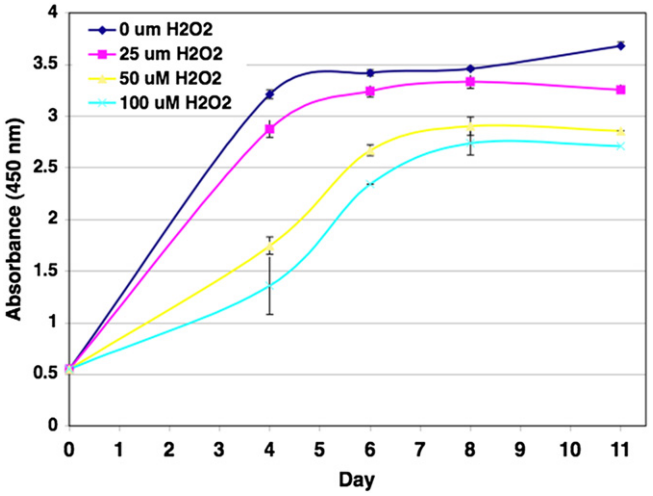

Fig. 4. Effect of mild oxidative stress on the proliferative response of HCEC. Subconfluent HCECs cultured from a 26-year old donor were incubated in the presence of 0, 25, 50, or 100 mM H_2O_2 as the oxidative stressor. Cell numbers were determined using a WST-8 spectrophotometric assay. Results are presented as absorbance at 450 nm. The Association for Research in Vision and Ophthalmology is the original copyright holder.

Together, these results provide strong evidence the reduced proliferative capacity observed in HCEC from older donors is due to oxidative stress-induced premature senescence.

Comparative proteomic analysis of a subset of proteins expressed in HCEC from young (<30 years old) and older donors (>50 years old) (Zhu et al., 2008) found that HCEC from older donors exhibit reduced expression of proteins that support important cellular functions such as metabolism, antioxidant protection, protein folding, and protein degradation. Immunostaining for the phosphorylated histone, H2AX-Ser139, which is associated with DNA damage foci (Paull et al., 2000), revealed significant positive staining in the in the central endothelium of older donors. This result indicates that HCEC are capable of responding to oxidative nuclear DNA damage; however, PCR-based microarray analysis of genes involved in cellular response to oxidative stress and DNA damage showed little change in expression with age, indicating that HCEC do not vigorously defend against or repair oxidative DNA damage by up-regulating the expression of multiple oxidative stress or DNA damage-signaling genes (Joyce et al., 2011).

8. Potential new research directions

It is clear that, although HCECs do not normally proliferate *in vivo*, they do possess proliferative capacity. These studies provide "proof of principle" that it should be possible to take advantage of the existing proliferative capacity of healthy HCECs to increase ECD *in vivo*, in *ex vivo* donor corneas to be used for transplantation, and in cultured cells to be used for corneal bioengineering. Clearly, these methods require further development and testing prior to any effort at clinical application.

The correlation of donor age and endothelial topography with increasing oxidative stress and reduced proliferative capacity is important. These results are consistent with results found in other differentiated cell types (Gaubatz and Tan, 1994; Fortini and Dogliotti, 2010). It is suggested that cells with more extensive DNA damage become arrested in G_1-phase, thereby preventing duplication of damaged DNA and subsequent proliferation. These cells would then be able to concentrate on repair of DNA within the actively transcribed genome, permitting them to survive and

function, but not to divide. Together, this information presents challenges, as well as suggests possible new directions, for basic research to increase ECD. It may be possible to prevent loss of proliferative capacity by treating HCEC with antioxidants to reduce the negative affect of oxidative stress. In addition, it should be possible to ectopically induce expression of specific antioxidant enzymes and/or DNA repair enzymes that could help prevent and/or repair nuclear oxidative DNA damage that would otherwise lead to reduced proliferative capacity. Another approach would be to identify methods to differentiate stem cells to form functional corneal endothelium *in vivo*. This might overcome the chronic need to obtain donor corneal tissue, thereby significantly increasing the number of individuals who could be treated to restore corneal transparency that has been lost due to the dysfunction of these physiologically important cells.

It is important to emphasize that any treatment designed to increase ECD *in vivo*, in *ex vivo* corneas, or for corneal bioengineering needs to be highly controlled. Besides demonstration of the ability of any new treatment to induce cell division, it will be important to demonstrate that the treatment does not negatively affect the karyotype of HCEC, as shown by Miyai et al. (2008). Any treatment would also need to demonstrate that cell division was induced only transiently and could not be re-stimulated. This safeguard is necessary to prevent over-replication of HCEC, which could result in multi-layering or interfere with the aqueous outflow pathway.

Conflict of interest

None.

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