·Review·

Research progress on the negative factors of corneal endothelial cells proliferation

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Abstract

• The human corneal endothelium forms a boundary layer between anterior chamber and corneal stoma. The corneal endothelial cells are responsible for maintaining cornea transparency, which is very vital for our visual acuity, via its pump and barrier functions. The adult corneal endothelial cells *in vivo* lack proliferation in response to the cell loss caused by outer damages and diseases. As a result, in order to compensate for cell loss, corneal endothelial cells migrate and enlarge while not via dividing to increase the endothelial cell density. Therefore, it is not capable for corneal endothelium to restore the corneal clarity. Some researches have proved that *in vitro* the corneal endothelial maintained proliferation ability. This review describes the current research progress regarding the negative factors that inhibit proliferation of the corneal endothelial cells. This review will mainly present several genes and proteins that inhibit the proliferation of the corneal endothelial cells, of course including some other factors like enzymes and position.

• KEYWORDS: corneal endothelium; proliferation; age; PLZF; telomerase; PTP1B; cAMP; position
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INTRODUCTION

Corneal endothelium located in the posterior part of the cornea. Corneal endothelium consists of an important monolayer of cells whose primary function is to maintain transparency of the cornea via the pump function. HCECs (human corneal endothelial cells) *in vivo* usually do not divide sufficiently to replace the dead and injured cells caused by various reasons. In response to the cell loss, the neighboring cells normally cover the loss area through enlargement or migration [1]. Hence, this causes the inability of corneal endothelial cells to pump the fluid out of the stroma, resulting in the loss of visual acuity and corneal clarity [2]. Recent therapies based on the penetrating or endothelial keratoplasty to rebuild pump function work well, while there is a severely shortage of donor corneas all around the world and serious complications so that it is necessary to explore new treatments to restore corneal clarity [3,4]. Study on the cell cycle-associated proteins indicates that human corneal endothelial cells *in vivo* do not exit cell cycle while remain arrested in the G1 phase of the cell cycle [5]. They retain proliferative capacity and can divide both in culture and in *ex vivo* corneas if cell-cell contacts are disrupted and cells are exposed to positive growth factors [6,7]. However, there are many anti-proliferative factors that more or less inhibit the division of the corneal endothelial cells. This review mainly presents current information concerning the negative factors that affect proliferation of the corneal endothelial cells.

NEGATIVE EFFECT OF INNATE FACTORS

To understand the influence of innate factors towards the proliferation, the growth and anatomical positions should not be neglected. The proliferation capacity of the old and the young is different. And physiological process of the cell senescence needs to be taken into consideration.

Age and Anatomical Factors: Different Proliferative Capacity in Different Positions A result demonstrated that corneal endothelial cells from both the central and peripheral areas retained potential proliferative capacity, regardless of donor age. However, the percentage of hCEC that retained replicative competence from older donors was lower than that of younger donors [8]. And there was a tendency that age-related factors affected the proliferation ability, the outcome was that the cells from older donors need a much longer doubling time and cells from the younger divided more readily, besides, endothelium from the young grow more robustly and be passaged more times than the older donors [9,10]. Some research indicated that human central endothelial cell density decreased at an rate of approximately 0.6% on average per year in normal corneas throughout adult life[11]. Older age, male sex, higher

intraocular pressure and history of outdoor work were negatively related with lower endothelial cell density[12]. Cell density in the peripheral cornea is greater than that of central cornea. Studies also indicated that human cornea had an increased endothelial cell density in the paracentral and peripheral regions of cornea compared with the central region [13]. Several findings demonstrated that peripheral rabbit endothelial had a stronger capacity to renew than central endothelial[14]. However, the endothelial proliferative response noted in the human central cornea was greater than in the peripheral area. Cells from the old were competent to proliferate but responded slowly than from the young [15]. As the above description, there are regional differences in proliferation-ability within the endothelial population *in vivo*. While *in vitro* corneas, hCECs from the peripheral area retain higher replication competence, regardless of donor age. The relative percentage of central area of human corneal endothelial cells from older donors that are competent to replicate is significantly lower than in the periphery or in the central area of corneas from younger donors. Recent study showed that hCECs cultured from either central or peripheral cornea retained proliferative competence, and there was a tendency for central endothelial cells to exhibit longer population-doubling time, although there was no significant difference [8]. Some researchesshowed that decreased proliferation was noted in the peripheral or mid cornea compared with the central corneal region. Unexpectedly, the decreased proliferation in the peripheral or mid region corresponded to a trend of higher endothelial cell density in the peripheral or mid region compared with the central region. There was a clear trend in their study that when cell density was greater than 2000 cells/mm^2, corneal endothelial cells tended to no proliferation[16].

Role of Cell Senescence Cell senescence is a process that excessive cell division is limited and early neoplastic progression is halted. Cultured hCECs enter senescence after relatively short proliferative lifespan (typically 20-30 population doublings). The cellular senescence is an irreversible proliferation arrest, and celluar senescence secretome contributes to a mainstay of proliferation arrest[17]. Researchers demonstrated that there were two forms of cellular senescence: replicative senescence, caused by the shortening of telomeres that occured during division, and stress-induced premature senescence, resulted from certain environmental stresses [18, 19]. Many primary cell types are prone to senescence to many factors, including oxidative stress, tumor suppressor, oncogene-signaling, and DNA damage signaling [20]. Previous studies showed reduction of telomere was observed with cell senescence, and its length was altered by cellular senescence[21]. The telomere length of hCEC retain sufficient to permit cell division[22]. HCECs *in vivo* have a long telomere to permit the cell division, regardless of age. The result was interpreted as indicating that senescence perhaps was induced by telomere-independent mechanisms in corneal endothelial cells *in vivo*[23]. Besides, there was finding revealed that the shortening of the telomere was not the senescence-related mechanism in hCECs *in vivo*. Senescence in human endothelium is not driven by telomere or stress signaling but regulated by extrinsic factors that impact upon oxidative stress. The combination of telomerase expression with the knockdown of p53 or ectopic expression will immortalize hCEC [24]. Age-dependent decreases in proliferative capacity observed in hCECs resulted, at least partly, from nuclear oxidative DNA damage, which was more serious in the cells of older donors and highest in the central endothelium area of older donors [25]. p16INK4a, p21WAF/CIP1 p53 and p27Kip1 are all senescence-related genes. They can inhibit cell cycle progression and maintain the G1 phase arrest of cells [26]. They all express in hCECs regardless of donor ages. Recent study showed that an age-related increase in p16INK4a expression was observed, while p27KIP1 and p21WAF/CIP expression tended no significant difference between the young and old donors. It was concluded that p16INK4a signaling pathway might play an important role in the early stages of senescence in rat CECs, while the p53 and p21WAF/CIP1 signaling pathway might exert its principle effect in the late stages of senescence in CECs [27]. Further study first indicated that cellular senescence was associated with high expression of p16INK4a and low expression of polycomb ring finger oncogene Bmi1 in human cornea [28]. The reduction that the relative percentage of hCECs from older donors that were competent to replicate was significantly lower than the cells from younger donors negatively correlated with the observed increase in the population of central hCECs exhibiting senescence-like characteristics[23].

EFFECTS OF CELL CYCLE INHIBITION FACTORS TO CEC PROLIFERATION

Various cell cycle inhibition factors have been shown to be responsible for the arrest of corneal endothelial cells. Many studies showed that they played an important role during the cell cycle. Decreased expression might bring cell division. These will be discussed in more detail below.

Age And Cyclin Kinase Inhibition Protein: HCECS Express Different Amount Of Cyclin Kinase Inhibition Proteins A research demonstrated the first proteomic comparison of proteins expressed in hCECs cultured from the young and old donors. Results indicated that hCECs from older donors exhibited reduced expression of proteins that support important cellular functions such as metabolism, antioxidant protection, cellular regulation [29]. Human corneal endothelial cells *in vivo* are arrested in the

G1-phase but have not exited the cell cycle [30]. It is suggested that cell contact, TGFβ , and p53 are all responsible for negative regulation of the cell cycle through related CKIs [31]. CKI proteins play vital roles in keeping G1-phase arrest and include two families, INK and CIP/KIP family [32]. Previous studies have shown that INK family member p16INK4a (p16 cyclin-dependent kinase inhibitor 4a) and CIP/KIP family members p21WAF/CIP (p21 cyclin-dependent kinase-interacting protein 1) and p27Kip1 (p27 kinase inhibitor protein1) are expressed in corneal endothelial cells from several species[31, 33, 34]. The population of hCECs exhibiting senescence-like characteristics increases with age, while p16INK4a, p21WAF1/Cip1, and p27Kip1 are expressed in hCECs despite donor ages. The G1-phase inhibitors p21Cip1 and p16INK4a from the older donors expressed at a higher level than the younger donors. The molecular basis for this age-related difference in proliferative capacity appears to involve an age-dependent increase in the expression of the cyclin-dependent kinase inhibitors, p21Cip1 and p16INK4a, which reduce the ability of mitogens to stimulate cell-cycle progression. Age-related differences in the relative expression of p16INK4a and p21WAF1/Cip1, except for the inhibition activity of p27Kip1, led to the conclusion that, there was an age-dependent increase in negative regulation of the cell cycle. This additional molecular mechanism may be responsible, partly, for the reduced proliferative response observed in hCECs from older donors[32]. The CKI, p21Cip1 is an important transcriptional target of the tumor suppressor, p53, and mediates both G1 and G2-phase checkpoint arrest in response to stresses, such as oxidative DNA damage [35, 36]. A study recently showed that the proliferation capacity of hCECs *in vitro* correlated with the amount of oxidative DNA damage received by the cells *in vivo* prior to explants [37]. Molecular studies have also demonstrated an important role of p16INK4a in the development of cellular senescence, p16INK4a strongly inhibited DNA synthesis and imposed a durable block to cell proliferation [38]. The population of hCECs exhibiting senescence-like characteristics increases with age. The p16 (INK4a) signal pathway might play a key role in the process of senescence in hCECs[39]. Both p21Cip1 and p16INK4a are involved in down-regulation of the cell cycle in hCECs and, thereby, provide an effective barrier to cell division. The small inference RNA-induced increase in expression of these proteins reduces the number of cells entering the cell cycle, of course the total cell numbers. Thereby, reduction of levels of p21Cip1 and p16INK4a would be effective in the treatment progress to induce the cell entering cell cycle, and then increase the total number of corneal endothelial cells[40].

Though p27Kip1 was related with negative regulation of proliferation, and expressed regardless of donor age, of interest was the fact that there was no statistically significant difference in the relative expression of p27Kip1 in primary cultures of hCECs from the young and old donors, and the expression of p27Kip1 appeared to decrease in an age-related manner in hCECs at passage-4 [41]. RNA interference (RNAi) was an effective means to down-regulate the p27Kip1 expression and then perhaps promoted the proliferation of CECs [42]. Small hairpin RNA-p27Kip1 could also negatively and effectively regulate the expression of p27Kip1 and increase the growth of bovine CECs. Thereby, shRNA-p27Kip1 RNA interference perhaps was an effective method to promote the proliferation of CECs [43]. Previous research showed that p27Kip1 antisense oligonucleotides brought a lower p27Kip1 protein levels and positively regulated the proliferation in confluent cultures of rat CECs [44]. Interestingly, research indicated that transfection of p27kip1 siRNA was sufficient to promote proliferation in confluent cultures of hCECs from young donors, but not older donors. These results indicated that inhibition of proliferation in older donors was regulated by other mechanisms in addition to p27 (kip1) [41]. Connective tissue growth factor (CTGF) -specific siRNA could inhibit the expression of CTGF mRNA and negatively regulate the expression of p27 in bovine CECs. We need more researches to prove the same effect in hCECs [45]. There was research demonstrated that p27(Kip1) was involved in the regulation of the proliferation in the developing corneal endothelium [46]. Besides, some researchers demonstrated that p27 had a closely negative relationship with the proliferation of hCECs. FGF-2 stimulates cells' proliferation via a linear signal transduction, PI-3 kinase and the downstream target extracellular signal-regulated kinase 1/2. Human corneal endothelium employs phosphorylation of p27 (Kip1) at two sites to accomplish the proliferation mediated by FGF-2[47]. One site is at Ser10 through pathway KIS (kinase-interacting stathmin) during early G1 phase, which is the major mechanism for G1/S transition, the other is at Thr187 through pathway Cdc25A (cell division cycle 25A/Cdk2 during late G1 phase[48].

c AMP (Cyclic Adenosine Monophpsphate) FGF-2 (fibroblast growth factor) markedly stimulates corneal endothelial proliferation [49], in response to FGF-2 stimulation, the mitogenic signaling pathway uses PI (phosphatidylinositol)3-kinase as one of the key downstream pathways [50]. Previous study showed that CDK4 (cyclin dependent kinase) and p27Kip1 involved in FGF-2-stimulated mitogenesis [51]. PI-3 kinase stimulates cell proliferation by up-regulating expression of CDK4, facilitating the nuclear import of CDK4, and sequestering CDK4 in the nuclei as it simultaneously down-regulates

expression of p27 and facilitates the proteolysis of the molecule by phosphorylation [52]. Some data support the hypothesis that cAMP inhibits the proliferation of CECs, preventing them from entering the S phase by negatively regulating PI -3kinase [53]. In corneal epithelial and endothelial cells, β-adrenergic stimulation leads to activation of PKA (protein kinase A) via stimulation of adenylyl cyclase, and α-adrenoceptor stimulation inhibits PKA activity via inhibition of adenylyl cyclase. Stimulation and inhibition of corneal cAMP-PKA pathway may play a role in important functions. Long-term therapy with α-agonists or β-antagonists perhaps influence these functions in a currently unknown way[54]. Findings indicated that in bovine corneal epithelial and endothelial cells, m4 muscarinic cholinoceptor inhibited the cAMP-PKA pathway, which resulted decreased PKA activity. Further work will be necessary to clarify the physiological function of this pathway in CEC [55]. There was study that demonstrated that native endothelial and epithelial cells expressed a functional 5-HT receptor positively correlated to adenylyl cyclase and PKA formation. However, currently it was still a matter of speculation of the physiological function of 5-HT receptors and the cAMP-PKA pathway in the cornea [56]. Dopamine includes two receptor subgroups, D1-like and D2-like receptors, the bovine corneal endothelium and epithelium showed D1-like receptor positive staining but negative for D2-like receptors. The stimulation of dopamine to D1-like receptors revealed a dose-dependent increase of the intracellular cAMP concentration. While presently the physiological function of the receptor was still our speculation[57].

PLZF (Promyelocytic Leukemia Zinc Finger) PLZF, a transcriptional repressor and negative regulator of cell cycling, is able to suppress the transcription of genes such as cyclin A2 and c-myc. Besides, continued PLZF expression induces cell cycle arrest in phase G1 and eventual apoptosis[58,59]. Cyclin A2 protein is essential for two critical points that are phase transitions during cell proliferation progression [60]. Hence, PLZF plays a negative role of cell proliferation. Study demonstrated that cell-cell contact inhibition was an important mechanism of growth arrest during corneal endothelial development[61]. There were findings that showed expression of PLZF in hCECs was closely related to the formation of cell-cell contacts, study showed that expression of PLZF mRNA varied according to the cell-cell contact state in hCECs *in vivo*, interestingly, the expression of mRNA returned to a normal level as cell-cell contact reformed, so it was suggested that PLZF played an important role in the expression of proliferation of hCECs [62].

PTP (Protein Tyrosine Phosphatase) PTPs can mediate dephosphorylation events, and protein tyrosine phosphorylation and dephosphorylation are common mechanisms that control cell replication [63]. EGF(epithelial growth factor) has been shown that it is competent to induce proliferation in corneal endothelium from several species[64,65]. A few PTPs associate with EGF-receptor, and may be active in regulating cell cycle signaling. Tyrosine-phosphorylated EGF receptor peptide, modeled on tyrosine 992 and 1148, inhibited the binding of PTP1B. It was revealed that clear sequence specificity in the binding of proteins involved in the regulation of intracellular signaling by receptor tyrosine kinases [66]. PTP1B can interact with proteins that contain adhesion junctions, in confluent rat corneal endothelium, phosphatase inhibition induced by SOV (sodium orthovanadate), a general phosphatase inhibitor, may disrupt the integrity of cell-cell junctions and promote cell cycle entry [67]. PTP1B interacts with and is tyrosine-phosphorylated by EGFR (epidermal growth factor receptor), thereby attenuating ligand-induced downstream signaling. Comparison of PTP1B mRNA and protein levels shows that PTP1B expression is modulated primarily at the protein level in the cultured rat corneal endothelial cells. The fact that EGFR is internalized in response to EGF stimulation indicates that it could interact with and regulated by PTP1B. The ability of PTP1B inhibitor resulted in a more rapid response to EGF stimulation and increases the time to sustain EGFR Tyr992 phosphorylation, then increase the number of rat corneal endothelial cells which express Ki67, a recognized marker of actively cycling cells. These findings indicated that PTP1B played a role in the negative regulation of EGF-induced signaling and helped suppress cell cycle entry [68]. PTP1B expression was significantly higher in hCECs from older people than young people, while there was no significant age-related difference in the expression of EGFR, suggesting that the decreased activity of proliferation in response to EGF is partly due to PTP1B activity. Study also indicated that inhibition of PTP1B increased the relative number of cells entering S-phase strongly suggested that PTP1B help negatively regulate EGF-stimulated cell cycle entry in hCEC. These results suggest that perhaps it is possible to increase the proliferative competence of hCEC, particularly in cells from older donor s, by inhibiting the activity of this important PTPs[69].

In conclusion, it is clear that hCECs do possess proliferative capacity. Many studies provide the proof that the hCECs remain arrested in G1 phase, thereby preventing subsequent proliferation causing by different negative factors. Many negative factors are responsible to interpret the arrest of cell cycle. It is possible to increase the density of endothelial cells to maintain the pump function to restore corneal transparency via inhibiting these factors. The correlation of people ages and hCEC senescence with reduced proliferative capacity is important, while unchangeable. Cell

cycle negative regulators involved in the proliferation of hCECs present challenges, as well as lead possible new directions to induce cell division. It may be possible to promote proliferative capacity by treating hCEC with anti-negative cycle factors to weaken the negative affect. This might overcome the serious shortage of cornea donors, and increase the number of individuals who could maintain the corneal transparency via sufficient cell division.

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