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Engineering of Human Corneal Endothelial Grafts

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Abstract

Human corneal endothelial cells (HCEC) play a pivotal role in maintaining corneal transparency. Unlike in other species, HCEC are notorious for their limited proliferative capacity *in vivo* after diseases, injury, aging, and surgery. Persistent HCEC dysfunction leads to sight-threatening bullous keratopathy with either an insufficient cell density or retrocorneal membrane due to endothelial-mesenchymal transition (EMT). Presently, the only solution to restore vision in eyes inflicted with bullous keratopathy or retrocorneal membrane relies upon transplantation of a cadaver human donor cornea containing a healthy corneal endothelium. Due to a severe global shortage of donor corneas, in conjunction with an increasing trend toward endothelial keratoplasty, it is opportune to develop a tissue engineering strategy to produce HCEC grafts. Prior attempts of producing these grafts by unlocking the contact inhibition-mediated mitotic

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent

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Conflict of Interest Statement

The authors declare that they have no conflicts of interests

block using trypsin-EDTA and culturing of single HCEC in a bFGF-containing medium run the risk of losing the normal phenotype to EMT by activating canonical Wnt signaling and TGF- β signaling. Herein, we summarize our novel approach in engineering HCEC grafts based on selective activation of p120-Kaiso signaling that is coordinated with activation of Rho-ROCK-canonical BMP signaling to reprogram HCEC into neural crest progenitors. Successful commercialization of this engineering technology will not only fulfill the global unmet need but also encourage the scientific community to re-think how cell-cell junctions can be safely perturbed to uncover novel therapeutic potentials in other model systems.

Keywords

human corneal endothelial cells (HCEC); endothelial-mesenchymal transition (EMT); Cornea; Corneal Transparency; Vision Restoration

1. Introduction

The cornea is a unique model system that consists of three different tissues, i.e., the stratified corneal epithelium, the avascular stroma containing keratocytes, and the corneal endothelium. The basement membrane-containing Bowman's membrane and Descemet's membrane separate the corneal epithelium and the corneal endothelium from the stroma, respectively (Fig. 1).

Embryologically, human corneal endothelial cells (HCEC) are derived from cranial neural crest cells and form a single monolayer of hexagonal cells lining on the Descemet's membrane of the posterior cornea[1]. By exerting effective barrier and pump functions, HCEC play a pivotal role in regulating proper corneal stromal hydration ensuring transparency and hence vision (reviewed in [2,3]). Unlike other species, HCEC are notorious for having a limited proliferative capacity *in vivo*[4] because of the mitotic block at the G1 phase of the cell cycle due to "contact-inhibition"[5–7]. Consequently, following diseases (such as Fuchs' endothelial dystrophy), aging, injury or surgeries (e.g., post-cataract or corneal graft failure), corneal blindness frequently occurs as a result of dysfunctional HCEC, which present with either insufficient numbers/density in "bullous keratopathy" (reviewed in [8]) or fibroblast metaplasia in "retrocorneal fibrous membrane" due to endothelial-mesenchymal transition (EMT) [9]. Currently, there is no medical treatment for corneal blindness caused by bullous keratopathy or retrocorneal membrane resulting from different causes of dysfunction of HCEC. In the past decade, the surgical treatment has evolved from traditional corneal transplantation (also termed penetrating keratoplasty) to several new surgical procedures, collectively termed "Endothelial Keratoplasties (EKs)" to replace patient's dysfunctional HCEC with a healthy one that contains varying amounts of the posterior corneal stroma or no corneal stroma from a cadaveric donor. This advance in surgical technique has prevailed because EKs facilitate quick postoperative visual recovery, provide the best visual quality, offers the lowest endothelial rejection rate (compared to other forms of corneal transplantations), and is associated with relatively small corneal incision thus contributes to the least amount of induced corneal astigmatism. Eye Bank Association of America statistics showed that the number of EK cases in the US increased from 1,429 in 2005 to 18,221 in 2009 and 24,277 in 2012. That is, EKs represented only 3%

in 2005 but rose to more than 30% in 2009 and 35% in 2012. In 2012, Fuchs' dystrophy was the leading indication for EKs (46.8%), comprising nearly half of all procedures, while post-cataract surgery bullous keratopathy was the second leading indication (19.1%).

As one may predict, the increasing success of EKs generates an increase in demand for donor corneas. However, even under the most ideal scenario, the resultant donor to recipient ratio remains 1:2. Although the current supply of cadaveric donor corneas meets the current domestic demand in the United States, there has been a severe global shortage of donor corneas[10]. Because there is an inverse relationship between age and cornea endothelial density, an increasing aging population worldwide also means a limited donor supply in the future. The older population represented 1 in every 8 Americans in 2005, and this number is expected to more than double by 2030. Hence, successful engineering of HCEC surgical grafts will have a significant impact on solving worldwide corneal blindness, of which the global population was estimated in 2012 by the World Health Organization to be 39 million people[11].

Although the idea of using cultured HCEC as an alternative to full-thickness keratoplasty in the replacement of defective corneal endothelial layer was conceptualized more than 3 decades ago[13,14] (also reviewed in [12]), the successful use of such tissue-engineered corneal endothelium substitutes in EKs has not been reported. In this review, we summarize the literature regarding prior attempts and our recent advances in achieving such a goal by circumventing the drawbacks of the conventional approaches.

II. Isolation of Human Corneal Endothelial Cells

A number of studies indicate that HCEC's limited proliferative capacity is caused by the mitotic block due to an arrest in the G1 phase of the cell cycle (reviewed in[5]). Their proliferative capacity resumes if HCEC are dissociated from the *in vivo* environment and cultured in a medium supplemented with mitogens such as bFGF[15]. Nevertheless, a similar mitotic block reappears in confluent HCEC when intercellular junctions are restored, suggesting that the *in vivo* mitotic block may be linked to *in vitro* "contact inhibition". Mechanical scraping was first used to unlock mitotic block in culturing HCEC^{16–18}. However, scraping not only stimulates proliferation but also leads to EMT when cultured in a medium containing bFGF and/or TGF β in cat corneal endothelial explants [18]. EDTA/trypsin and dispase was subsequently used to unlock the mitotic block [19,20] and aid in isolation of HCEC in bFGF- and serum-containing media for cultured HCEC[15,19–26]. However, these conventional approaches can also potentially trigger EMT, leading to the loss of the HCEC phenotype (reviewed in [9]). Our studies have confirmed that these conventional methods, while successfully unlocking the mitotic block as reported[27], indeed trigger EMT with the loss of the normal HCEC phenotype[28]. We subsequently learned that this pathological state is initiated by proliferation as a result of canonical Wnt signaling in the presence of EGF and/or bFGF and becomes irreversible with cessation of proliferation when TGF- β 1 is added to trigger canonical TGF- β signaling with nuclear translocation of pSmad2/3 and Zeb1/2[29]. Our finding is consistent with several prior studies showing that TGF- β 1/2 suppress proliferation promoted by bFGF in EDTA-treated HCEC[30], rat CEC[31], and bovine CEC[32], and that the use of SB431542, a selective

inhibitor of the TGF- β R, prevents EMT in monkey and HCEC[33]. Because the outcome is EMT with the loss of the normal HCEC phenotype, one strategy of engineering HCEC should be directed to avoiding activation of canonical Wnt-Smad2/3-Zeb1/2 signaling.

To circumvent such a mishap that usually ensues when single HCEC are isolated based on EDTA[19] or EDTA plus bFGF[9], we digested stripped Descemet's membrane with collagenase alone in a serum-containing medium termed supplemented hormonal epithelial medium (SHEM) which consists of DMEM/F-12 (1:1) supplemented with 5% FBS, 0.5% dimethylsulfoxide, 2 ng/ml hEGF, 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml selenium, 0.5 mg/ml hydrocortisone, and 1 nM cholera toxinin^[20]. Because collagenase digestion selectively removes interstitial but not the basement membrane collagens, our method does not disrupt intercellular junctions nor interaction with the basement membrane. Indeed, collagenase digestion results in compact aggregates of HCEC, which retain intercellular junctions mediated by ZO-1 and connexin-43, and maintain their adhesion to such basement membrane components as collagen IV, laminin 5, and perlecan[20]. Presumably because of the preservation of intercellular junctions and cell adhesion to the basement membrane, isolated HCEC aggregates remain viable in a serum-free high calcium medium for at least 3 weeks[20]. The aggregate forms a monolayer of hexagonal HCEC expressing ZO-1 and connexin 43 after being re-seeded in SHEM on collagen IV-coated plastic[20]. The resultant HCEC monolayer maintains hexagonal shape, expresses all adherent junction (AJ) components, and adopt mitotic block due to contact inhibition after three weeks of culturing in SHEM (Fig. 2)[34].

III. Prior Attempts of Engineering HCEC Grafts

Following isolation of single HCEC obtained by trypsin/EDTA, several laboratories have attempted to engineer HCEC grafts in the last decades by culturing them on such substrates as collagen I and fibronectin[35], collagen IV[20,34], chondroitin sulfate and laminin[15], laminin-5[36], matrigel[37] and FNC coating mix[38]. Although HCEC monolayers can be detached from the plastic surface treated with temperature-responsive polymer, poly(N-isopropylacrylamide) (PIPAAm) [25], the engineered graft is easier for surgical manipulation if seeded on carriers such as gelatin hydrogel discs[39], amniotic membrane[21], decellularized thin-layer human corneal stroma[40], a chitosan-based membrane consisting of hydroxyethyl chitosan, gelatin, chondroitin sulfate [41], gelatin hydrogels [42], and atelocollagen I carrier[43,44]. Using our model system, we have screened different substrates such as collagen IV, matrigel, laminin and fibronectin coated on an atelocollagen carrier and noted that collagen IV is essential to be used as the substrate on the atelocollagen carrier for engineering of HCEC grafts (Fig. 3A).

A number of culture media have also been used for cultivating HCEC, including DMEM, DMEM/F12, Opti-MEM-I, and Ham's F12/M199 (reviewed in[45]). Engelmann et al[46] suggested that the Ham's F12 Nutrient Mixture is most suitable to establish a long-term cultivation system for HCEC. Zhu et al[38] noted that the Opti-MEM-1 based medium with various growth factors promotes HCEC growth. Mimura et al[45] reported that DMEM- and DMEM/F12-based media are less effective than Opti-MEM-I- and F12/M199-based medium for expansion of HCEC. Jackel et al[47] evaluated five culture media on the

expansion of HCEC, including F99-based medium, MEM-based medium with 2% fetal calf serum (FCS), MEM-based medium with 5% FCS, and endothelial serum-free medium (SFM) with and without antibiotics and noted that the number of apoptotic cells is much higher when cultured in MEM compared that in F99 and SFM. In our model system, we did not find any significant difference in three media[20,38,46] we chose to compare three media (Fig. 3B) and noted that there was no significant benefit to remove and add different supplements in SHEM (Fig. 3C).

A number of growth factors have been reported to promote growth of HCEC, including bFGF[15,19,48–51], LIF[52,53], EGF[19,20,28,34,49,51,54], NGF[19], endothelial cell growth supplement[49,55], and L-ascorbic acid 2-phosphate (Asc-2P)[56]. It has been reported that Asc-2P, along with basic bFGF, facilitates growth and maintain the polygonal morphology of HCEC on atelocollagen by reducing intracellular oxidative stress (reviewed in[45]). As stated above, we did not find any benefit of adding Asc-2P in our model system (Fig. 3C). However we have recently reported that LIF uniquely effectively promotes *ex vivo* expansion of HCEC growth without any change of the phenotype by delaying contact-inhibition [52].

A major recent advance has been made by the laboratory of Kinoshita by adding the ROCK inhibitor Y27632 in the culture medium. Addition of this small molecule inhibitor preserves proliferation[57] and maintains a normal endothelial phenotype without EMT[58] *in vivo* rabbit[58] or monkey[59] corneas after wounding by mechanical scraping or transcorneal freezing. Such a unique therapeutic efficacy is likely mediated by inhibiting EMT while preserving the activation of Wnt signaling as we have reported in contact-inhibited HCEC monolayers[28] and post-confluent ARPE-19 cells[60]. Our data suggest that the use of small molecule RhoA and ROCK (Y27632) inhibitors may inhibit EMT that is developed after intercellular junctions are disrupted by wounding as trivial as EDTA ± trypsin. Because RhoA activation is suppressed during contact inhibition, their use in our model system of contact inhibited HCEC monolayers might be detrimental[60]. In our model system, where contact inhibition still prevails because of the preservation of intercellular junctions[20], continuous inhibition of RhoA-ROCK signaling does not bode well with the reprogramming of HCEC monolayers because activation of RhoA is a necessary step to trigger either “physiological” non-canonical BMP signaling[28,61] or canonical BMP signaling[53] (see below).

VI. Progenitors of Human Corneal Endothelium

Although there is a *bona fide* mitotic block of HCEC *in vivo*, new evidence has emerged suggesting the presence of HCEC progenitors in the corneal periphery or the junction between the corneal endothelium and the trabecular meshwork. This evidence remains *tangential* as it is based on appearance of BrdU labeling [62], expression of stem cell markers such as Oct3/4 and Wnt1[63] following wounding, and formation of neurospheres with expression of nestin by EDTA-dissociated HCEC[23,64–66]. These distinct subpopulations of cultured CEC show colony-like structures with a small size [67]. Further evidence indicates that these cells are heterogeneous, with tendency of characteristic sphere growth and plasticity to change to other type of cells[23,67] (also reviewed in[64]). These

endothelial progenitors highly expressed p75 neurotrophin receptor (p75NTR), SOX9 and FOXC2, and partially retained the properties of neural crest and periocular mesenchyme with a high proliferative potency[68], suggesting their identity of neural crest progenitors. Our results indicate that peripheral, not central, corneal endothelial aggregates do contain small corneal progenitors with characteristic growth of colony-like structures when isolated by collagenase without disruption of cell-cell junctions and cultured in MESCM, which consists of DMEM/F-12 (1:1) but supplemented with 10% knockout serum, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 4 ng/ml bFGF, 10 ng/ml hLIF[53]. As shown below, we have characterized human corneal endothelial progenitors as a group of cells expressing a number of ESC markers, such as cMyc, KLF4, Nanog, Nestin, Oct4, Rex1, Sox2, SSEA4 and NC markers such as AP2α, AP2β, FOXD3, HNK1, MSX1, p75NTR and Sox9[53], similar to the reports from a number of groups suggesting that these progenitors are a group of small endothelial cells expressing p75NTR, SOX9, FOXC2, Twist, Snail and Slug with higher proliferative potential[23,62,63,66,68–70]. We have gathered additional evidence supporting that the reprogrammed HCEC progenitors also process the plasticity to differentiate into other type of cells such as keratocyte-like cells (unpublished data).

V. Novel Engineering Strategy by Reprogramming into HCEC Progenitors

As stated above, perturbation of contact inhibition to unlock the mitotic block of HCEC through the use of EDTA ± trypsin to disrupt intercellular (adherent) junctions[27,71] runs the risk of EMT into fibroblasts/myofibroblasts leading to retrocorneal membrane formation. [9] In our model system, we have also verified that even a brief treatment of trypsin/EDTA for 5 min also induces a “fibroblastic” appearance that fails to fully recover the hexagonal shape 28 days later in SHEM (Fig. 4A).[53] This morphological alteration is accompanied by activating canonical Wnt signaling as evidenced by nuclear localization of β-catenin and LEF1 (Fig. 4A), increase of the TCF/LEF promoter activity (Fig. 4B), increase of mRNA expression of β-catenin and LEF1 (not shown), and increase of nuclear protein of β-catenin and LEF1 (Fig. 4C). This finding strongly suggests that the demise caused by trypsin/EDTA can be more detrimental than reported (see above).

To search for a new promising strategy, we used the interference RNA technology to target various genes that might be involved in the mitotic block mediated by contact inhibition in our model system of HCEC monolayers.[20,34] We discovered that knockdown of p120 catenin (hereafter p120), but not β-catenin, N-cadherin or ZO-1, uniquely unlocks the mitotic block of contact-inhibited HCEC monolayers[28] and post-confluent ARPE-19 monolayers[60] without disrupting intercellular adherent junctions or activating canonical Wnt signaling (Fig. 4). This novel approach selectively activates p120-Kaiso signaling, which is highlighted by an increase of nuclear BrdU labeling, colocalized with nuclear translocation of p120 that also elicits nuclear release of a transcription suppressor Kaiso[28,53]. We subsequently reported that such knockdown regimen can be augmented by a combination of knockdown with p120 and Kaiso siRNAs (hereafter termed p120 +Kaiso siRNAs) without disrupting the intercellular junction and without causing EMT (Fig. 4) [28,53]. In SHEM, the above signaling activates RhoA-ROCK-non-canonical BMP signaling highlighted by nuclear translocation of pNFκB.[61] Because we believe that peripheral corneas contain HCEC progenitors and we have extensive experience of

expansion of limbal niche progenitors in a serum-free medium termed MESCM[52,53], we thus switched the medium from SHEM to MESCM and interestingly noted dramatic expansion of HCEC monolayers from the size 1.4 ± 0.2 mm of HCEC monolayers in diameter in SHEM to 4.4 ± 0.3 mm in diameter in MESCM after 6 weeks of culture (Fig. 5A)[53] from Descemet's membrane stripped from 1/8 of the corneoscleral rim normally discarded after conventional corneal transplantation[53]. This beneficial effect is mainly due to the delay of contact inhibition by LIF-JAK-STAT3 signaling in the absence of p120-Kaiso knockdown. [52] Nonetheless, additional p120-Kaiso knockdown for 5 weeks results in further expansion of HCEC monolayers to 11.0 ± 0.6 mm in diameter (Fig. 5B).[53] Compared to the *in vivo* stripped Descemet's membrane, the resultant cell shape remained hexagonal during the entire knockdown period (5 weeks of weekly treatment after the first week of culture followed by withdrawal for one week) for both scrambled(sc)RNA and p120-Kaiso siRNAs (Fig. 5C). Additionally, the resultant cell density was maintained at the *in vivo* level by p120-Kaiso siRNAs in MESCM however decreased by scRNA after 3 weeks of culture (Fig. 5D). One week after withdrawal, the resultant HCEC monolayer retained the centric expression of acetylate- α -catenin (a marker of cilium), uniform cytoplasmic expression of γ -tubulin and p75NTR (of which the latter is considered a marker of neural crest cells), junctional expression of p120, N-cadherin, α -catenin, β -catenin, ZO-1, Na-K-ATPase, and F-actin (all are markers of HCEC), without expression of LEF1 and S100A4 1 (both are markers of EMT) (Fig. 5E). This expression of HCEC markers is consistent with the *in vivo* expression pattern previously reported[34]. Further analysis suggests that such a successful expansion of HCEC monolayers is mediated by activating RhoA-ROCK-canonical BMP signaling highlighted by nuclear translocation of pSMAD1/5/8 [53]. The resultant expanded HCEC monolayers carry the phenotype suggestive of reprogramming into neural crest progenitors because we have noted that reprogramming by canonical BMP signaling was correlated with nuclear staining of Oct4, Sox2, and Nanog and up-regulation of miR302b more than miR302c, which suggests that the positive autoregulatory feedback loop between miRNA302b and the Oct4–Sox2–Nanog complex might operate in the reprogramming of HCEC into neural crest progenitors[53].

VI. Transplantation of Engineered HCEC Grafts

Pre-clinical animal studies are the most effective way to study the pathobiology of ocular diseases as well as examine the safety and efficacy of treatment regimens before human testing may begin. Although prior experiments have shown engineered corneal endothelium grafts can be transplanted into animals such as rabbits, cats, monkeys and rats[13,14,21,22,39,44,72–78], the clinical relevance of the animal model must be taken into account. For example, after destruction of 50% of the endothelium by transcorneal freezing, the rabbit corneal endothelium heals in 10 days by extensive cellular division at the margin of the wound, while in cats the corneal endothelium eventually covers the deficit after 1 month but only by enlargement and migration of cells at the margin of the wound[79]. With this in-mind, we have recently begun preliminary studies with our engineered HCEC grafts in mini pigs, which are fully compliant with anesthetic and postoperative experimental procedures following NIH regulations. These animals have become increasingly popular as preclinical disease models[80–82] and can serve as a valuable model for ocular conditions

due to the similarity in corneal diameter, thickness, and refractive power to human corneas. We have established corneal endothelial dysfunction in mini pigs by scoring an area of corneal endothelium and then removing it from the anterior chamber (Fig. 6).

In order to transplant these endothelial grafts, surgical manipulation must be performed in the operating room. As noted above, over the past decade, the paradigm of the surgical approach has shifted from penetrating keratoplasty to EK. EKs have evolved from deep lamellar endothelial keratoplasty (DLEK) to Descemet stripping automated endothelial keratoplasty (DSAEK) and now to Descemet membrane endothelial keratoplasty (DMEK), of which the latter transplants only a single layer of HCEC and the basement membrane. We have tested the feasibility of performing DMEK in our mini pig model by inserting an atelocollagen sheet that has been stained with Vision Blue to aid in visualization and orientation. Using this model system, we are currently testing the safety and efficacy of the aforementioned engineered HCEC grafts.

VII. Future Directions

In summary, the traditional strategy based on EDTA/trypsin to obtain single HCEC perturbs the contact inhibition by activating canonical Wnt signaling (under the influence of bFGF/EGF) and Smad2/3 signaling (Fig. 7). This strategy runs the risk of losing the normal HCEC phenotype to EMT. In contrast, our novel strategy based on transient knockdown of p120 and Kaiso selectively activates p120-Kaiso signaling but not Wnt-Smad2/3 signaling (Fig. 7) to perturb the contact inhibition without altering the normal HCEC phenotype. From one donor corneal rim, this engineering technology can successfully generate a minimum of 8 HCEC grafts with a transplantable size compatible with an *in vivo* density. This technology also differs from all other prior attempts by reprogramming of HCEC into neural crest progenitors. Future studies are needed to further advance our mechanistic understanding of the signaling pathways involved in the reprogramming of HCEC to neural crest progenitors. We speculate that such reprogramming might require a number of mechanisms, of which one is to overcome the senescence barrier mediated by the cyclin-dependent kinase inhibitors (CKIs) such as p16^{INK4a} and p21^{CIP1} or activation of p53.[83,83] Such studies will also help us understand why the central and peripheral corneal endothelium differs *in vivo* regarding the capability of responding to the said reprogramming. The other mechanism might involve the activation of the autoregulatory network between Oct4-Sox2-Nanog and the miR-302 cluster that has been known in maintaining self-renewal and pluripotency. [84,85] This is based on our report showing that activation of RhoA-ROCK-canonical BMP signaling achieve such reprogramming highlighted by BrdU labeling, overexpression of ESC and NC markers, miR 302b/c and nuclear translocation of Oct4, Sox2 and Nanog.⁶¹. Further investigations into the action mechanism whether the nuclear miR 302b/c-Oct4-Sox2-Nanog reprogramming network also operates through the relief of p16^{INK4a}-mediated senescence barrier will help us uncover new therapeutic strategies and design a novel engineering technology to treat corneal blindness caused by dysfunctional HCEC. Successful commercialization of this technology will stimulate the scientific community to re-think how “contact inhibition” can be safely perturbed to our benefit without pathological consequences and whether this new regenerative approach can circumvent the need of using

embryonic stem cells or reprogramming somatic cells all the way to induced pluripotent stem cells.

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Fig. 1.
Diagram of Human Cornea and its Cellular Distribution.

Fig. 2. HCEC Monolayer Model System

(A) Isolation by Collagenase and Expansion in SHEM (A and B) on Collagen IV-coated Dishes leading to restoration of contact inhibition in 3 weeks and a normal phenotype at Day 21 (C, Bar, 25 μ m).

Fig. 3. Growth of HCEC on Different Substrates, in 3 Different Media, and in SHEM with Different Compositions

HCEC attach and expand well on collagen IV-, but not on matrigel-, laminin- or fibronectin-coated atelocollagen sheet at Day 10 (A). HCEC grow similarly in three different media (B) and SHEM with addition or reduction of certain components (C) in six weeks of culture.

Bar, 25 μ m

Fig. 4. Activation of Canonical Wnt Signaling by Trypsin/EDTA but not by p120-Kaiso Knockdown

Contact-inhibited HCEC monolayers cultured at 14 days in SHEM were treated with trypsin-EDTA for 5 min or knockdown with 100 nM p120 siRNA or p120-Kaiso siRNA weekly for 4 weeks. The resultant cells were investigated by phase contrast microscopy, immunofluorescence staining of β -catenin and LEF1 (both green), qRT-PCR of β -catenin and LEF1 transcripts (48 h later), Western blot of nuclear extracts (48 h later, using histone as the loading control), and the TCF/LEF promoter activity (24 h later) (* $p < 0.05$, ** $p < 0.01$, $n = 3$). Scale bar, 25 μ m.

Fig. 5. Dramatic Expansion of HCEC Monolayers without Phenotype Change in MESCM with or without p120-Kaiso siRNAs

The size of HCEC monolayers in diameter was measured when cultured in SHEM and MESCM without (A, * $P < 0.05$, $n = 3$, when compared to SHEM) or with knockdown by 100 nM of scRNA or p120-Kaiso siRNAs (B, * $P < 0.05$, ** $P < 0.01$ when compared to their corresponding scRNA controls; # $P < 0.05$ and ## $P < 0.01$ when compared to SHEM, $n = 3$). The morphology (C) and the density (D, * $P < 0.05$ compared to the control treated with 100 nM scRNA; # $P < 0.05$ compared to the *in vivo* density; $n = 3$) of HCEC monolayers were monitored throughout the entire period. Immunofluorescence staining to acetyl- α -tubulin (red) and other markers (all green) was performed in HCEC monolayers one week after withdrawal (E). Scale bars: 25 μ m.

Fig. 6. Transplantation of HCEC graft in the mini pig model of corneal endothelial dysfunction Continual descemetorhexis was performed using a John dextome spatula (Bausch + Lomb Inc.) to strip the Descemet membrane (A–B). Atelocollagen sheet (C) was stained with Vision Blue (D) and trephined (E) for better orientation to be transplanted as in DMEK (F).

Fig. 7. Activation of Wnt Signaling and p120-Kaiso Signaling to Unlocks the HCEC Mitotic Block with or without EMT.