Nuclear p120-catenin Unlocks Mitotic Block of Contact-inhibited Human Corneal Endothelial Monolayers without Disrupting Adherent Junction

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Summary

Contact-inhibition ubiquitously exists in non-transformed normal cells when neighboring cells are in contact. This phenomenon explains poor regenerative capacity of in vivo human corneal endothelial cells during aging, injury, and surgery. This study demonstrated that the conventional approach of expanding human corneal endothelial cells by disrupting contact inhibition with EDTA followed by bFGF actually activated the canonical Wnt signaling and lost the normal phenotype to endothelial-mesenchymal transition, especially if TGF-β1 was added. In contrast, p120 siRNA also uniquely promoted proliferation of the endothelial cells by activating p120-catenin trafficking to the nucleus, thus relieving repression by nuclear Kaiso. This nuclear p120-catenin/Kaiso signaling is associated with activation of RhoA-ROCK signaling, destabilization of microtubules, and inhibition of Hippo signaling, but not with activation of Wnt/β-catenin signaling. Consequently, proliferating human corneal endothelial cells maintained a hexagonal shape with junctional expression of N-cadherin, ZO-1, and Na-K-ATPase. Further expansion of human corneal endothelial monolayers with a normal phenotype and a higher density became feasible by prolonging treatment of p120-catenin siRNA followed by withdrawal. This new strategy of perturbing contact inhibition by selective activation of p120-catenin/Kaiso signaling without disrupting adherent junction may be deployed to engineer surgical grafts containing normal human corneal endothelial cells to meet global corneal shortage and for endothelial keratoplasties.
Introduction

The corneal endothelium forms a single monolayer of hexagonal cells lining on the basement membrane-containing Descemet membrane of the posterior cornea and facing the TGF-β2-containing aqueous humor (Chen et al., 1999). Through expression of ZO-1, a tight junction component, and Na-K-ATPase to exert barrier and pump functions, respectively, human corneal endothelial cells (HCEC) play a pivotal role in regulating corneal stromal hydration and hence transparency (reviewed in (Bonanno, 2003)). Unlike other species such as murine, rabbit, and bovine, HCEC are notorious for their limited proliferative capacity in vivo after injury, aging, and surgery (Laing et al., 1984). HCEC’s limited proliferative capacity is caused by the mitotic arrest at the G1 phase of the cell cycle (reviewed in (Joyce, 2005)). A similar mitotic block is also reported in cat (Petroll et al., 1998) and human (Senoo et al., 2000) corneal explants, as well as confluent rat corneal endothelial cultures (Joyce et al., 2002) because of “contact inhibition.” The conventional approach of expanding HCEC in vitro is to disrupt cell junctions by EDTA followed by culturing them in a medium supplemented with mitogens, such as bFGF (Engelmann et al., 1988). Nevertheless, such a culturing method casts the concern of activating “endothelial-mesenchymal transformation” (EMT), a pathologic process that may generate “retrocorneal fibrous membrane,” leading to corneal blindness (reviewed in (Lee and Kay, 2006)).

Intercellular junctions include gap junction, adherent junction (AJ), and tight junction, among which AJ plays an important role in controlling many cellular behaviors, including proliferation, differentiation, and survival (reviewed in (Perez-Moreno et al., 2003)). Although not fully elucidated, the mechanism governing contact inhibition-mediated mitotic block likely involves signaling transmitted from AJ to the nucleus (reviewed in (Matter et al., 2005; Perez-Moreno et al., 2003)). Conventionally, two signaling pathways could be elicited via β-catenin and p120-catenin (hereafter p120), respectively, when the AJ junction is disrupted. The former is known as the canonical Wnt pathway, in which β-catenin, if accumulated in the cytoplasm without prompt degradation via binding with β-TrCP, can be translocated into the nucleus, where it acts as a transcriptional coactivator through binding with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (reviewed in (Nelson and Nusse, 2004)). The
latter may trigger the p120/Kaiso pathway, in which nuclear translocated p120 relieves the repressor activity of Kaiso, a member of BTB/POZ-ZF transcription factor family ([Kelly et al., 2004], also reviewed in [Daniel, 2007]). It has been known that p120 negatively regulates RhoA ([Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001]). However, it is unclear whether and how activation of RhoA following the release of p120 inhibition can be linked to p120 nuclear trafficking and subsequent signaling. Most recently, the Hippo pathway, an evolutionarily conserved protein kinase cascade, has also been identified to control in vivo organ size and in vitro contact inhibition by governing cell proliferation and apoptosis ([Zeng and Hong, 2008]). Cytoplasmic phosphorylated and nuclear non-phosphorylated Yes associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) have been proposed as the prime mediators of the Hippo pathway ([Zhao et al., 2010]). The Hippo signaling can intercede Wnt/β-catenin signaling via an interaction between phosphorylated TAZ and CK1δ/3-mediated phosphorylation of Disheveled in the cytoplasm ([Varelas et al., 2010]) and between non-phosphorylated YAP and β-catenin in the nucleus ([Heallen et al., 2011]). It is unclear whether and how any of the aforementioned three signaling pathways might be linked to contact inhibition of HCEC monolayers.

To resolve these questions, we have isolated HCEC by collagenase instead of trypsin/EDTA or dispase to avoid disrupting intercellular junctions and cell matrix (basement membrane) interaction ([Li et al., 2007]), and we have demonstrated that such cultured HCEC monolayers also exhibit contact inhibition when AJ matures to an in vivo pattern ([Zhu et al., 2008]). Herein, we demonstrate that the aforementioned mitotic block, unlocked by EDTA with bFGF, is mediated by the canonical Wnt signaling and induces EMT, especially if TGF-β1 is added. In contrast, the mitotic block unlocked by p120 siRNA is causatively related to activation of p120 trafficking to the nucleus, relief of repression by nuclear Kaiso, activation of RhoA-ROCK signaling, inhibition of Hippo signaling, but not activation of β-catenin/Wnt signaling. Consequently, HCEC retain a normal phenotype without EMT despite addition of bFGF and TGF-β1. These results highlight the feasibility of deploying a novel strategy of tissue engineering of HCEC and other similar tissues by selective activation of p120/Kaiso signaling without the use of single cells by disrupting adherent junction.
Results

Mitotic block unlocked by EDTA-bFGF induces EMT, especially if TGF-β1 is added.

As a first step of exploring the signaling involved in controlling contact inhibition, we isolated HCEC by collagenase, but not trypsin or dispase, to retain cell junctions and cell-basement membrane interaction (Li et al., 2007), and we demonstrated that in vitro HCEC monolayers exhibit contact inhibition when their AJ matures to an in vivo pattern (Zhu et al., 2008). Similar contact inhibition can be unlocked by scraping, followed by culturing with bFGF or bFGF plus TGFβ in cat corneal buttons (Petroll et al., 1998), or by disrupting cell junctions with EDTA in human corneal buttons pre-incubated in a medium with EGF and FGF (Senoo et al., 2000). Exposure of mitotically quiescent HCEC monolayers to 5 mM EDTA for 1 h alone led to notable disruption of intercellular junction (Fig. 1A). Without bFGF, cells restored their pre-treatment monolayer morphology in two days (not shown). However, immediately following EDTA treatment, when 20 ng/ml bFGF was added for 2 days, 10 ng/ml TGFβ1 was added for 3 days, or 20 ng/ml bFGF was added for 2 days followed by 10 ng/ml TGFβ1 for 3 days, HCEC turned into fibroblastic-like cells with relocation of N-cadherin from the cell junction to the cytoplasm (Fig. 1A). Nuclear BrdU staining was negative when the cells were treated with PBS or EDTA alone (Fig. 1A). However, the BrdU labeling index was promoted to ~19% by bFGF, but not TGFβ1 alone or bFGF plus TGFβ1 (Fig. 1A), suggesting that the mitotic block could only be unlocked by bFGF when EDTA was used to disrupt cell junctions. Immunostaining to S100A4, an early marker of epithelial mesenchymal transition (Basile et al., 2011), showed the S100A4 staining was negative in cells with or without EDTA treatment, but became positive in the cytoplasm and the nucleus after addition of bFGF or TGF-β1, and exclusively in the nucleus when both bFGF and TGFβ1 were added (Fig. 1A). In addition, α-smooth muscle actin (α-SMA), a marker indicative of myofibroblast differentiation (Gabbiani, 2003), became strongly positive only when TGF-β was added (Fig. 1A). The EMT caused by EDTA-bFGF was accompanied by the loss of the normal HCEC phenotype because junctional staining of N-cadherin, ZO-1, and Na-K-ATPase turned into the cytoplasmic staining pattern (Fig. 1B). Collectively, these results indicated that the contact inhibition unlocked by EDTA-
bFGF initiated EMT with proliferation, while full-blown EMT without proliferation developed after additional TGF-β1.

Mitotic block unlocked by EDTA-bFGF is causatively mediated by the canonical Wnt signaling.

Because S100A4 is a transcriptional target of the canonical β-catenin/Wnt signaling (Sack and Stein, 2009), we wondered whether the Wnt signaling was activated to unlock the mitotic block of HCEC monolayers. Compared to the PBS control, EDTA alone did not change the expression of β-catenin and LEF1 mRNAs and proteins (not shown). However, EDTA-bFGF elevated 2- and 3-fold of the level of β-catenin and LEF1 mRNAs, respectively (Fig. 2A, n=3). Immunostaining confirmed the junctional localization of β-catenin and the absence of nuclear LEF1 in cells treated with PBS (Fig. 2B) or EDTA alone (not shown). In contrast, EDTA-bFGF reduced β-catenin in the cell junction, increased its accumulation in the cytoplasm and the nucleus, and notably enhanced the LEF1 nuclear staining (Fig. 2B). The aforementioned change of immunostaining was confirmed by semi-quantitative protein dot blotting using membranous, cytosolic, and nuclear extracts. Based on the loading control, i.e., CN43, α-tubulin, and histone, respectively, the β-catenin protein level was decreased by 5-fold in the membranous compartment, but increased 3- and 6-fold in cytosolic and nuclear compartments, respectively. At the same time, EDTA-bFGF increased 3-fold of the LEF1 level in the nuclear compartment (Fig. 2C). These results collectively supported the notion that the mitotic block unlocked by EDTA-bFGF correlated with activation of the canonical Wnt signaling. Such an activation of Wnt signaling was not observed when TGF-β1 was added (not shown).

To confirm that the above correlation was causative, we transfected HCEC monolayers with a plasmid containing TCF/LEF promoter construct and simultaneously treated the cells with XAV939, an inhibitor of β-catenin-mediated signaling through inhibiting the poly-ADP-ribosylating enzymes tankyrase 1 and tankyrase 2 (Huang et al., 2009). As expected, the promoter activity was low in PBS or EDTA treated groups alone, but was elevated 6-fold in EDTA-bFGF treated group (Fig. 2D). Such an elevated
promoter activity was completely suppressed by XAV939 (Fig. 2D). As a result, the BrdU labeling reverted to the baseline quiescence, while β-catenin remained in the junction and S-100A4 was negative without EMT (Fig. 2E). In fact, the junctional staining of N-cadherin, ZO-1, and Na-K-ATPase was retained (Fig. 2E). In addition, the notion that activation of Wnt signaling induced proliferation and EMT was further confirmed by overexpression of stable S33Y β-catenin in contact-inhibited HCEC monolayers treated with EDTA, but without bFGF. In such cases, Wnt signaling was activated (Fig. 2G) while nuclear BrdU labeling, β-catenin, LEF-1 and S100A4 as well as cytoplasmic α-SMA and N-cadherin were significantly increased (Fig. 2F). In fact, overexpression of stable S33Y β-catenin also rescued proliferation and EMT inhibited by XAV939 (not shown).

**HCEC mitotic block is uniquely unlocked by p120 siRNA without EMT.**

Because disruption of AJ by EDTA induced EMT by activating the Wnt signaling (Fig. 1 and 2), we took a different approach by perturbing intercellular junction components with siRNAs to downregulate p120, β-catenin, N-cadherin, or ZO-1. The knockdown efficiency of these siRNAs was verified by both real-time PCR and Western blotting (Supplemental Fig. 1A and 1B). To our surprise, the BrdU labeling index was also promoted to ~18% in HCEC monolayers when treated with p120 siRNA, but not with siRNA of β-catenin, N-cadherin, or ZO-1 (Fig. 3A). Unlike EDTA treatment (Fig. 1A), addition of bFGF did not further increase BrdU labeling that was already promoted by p120 siRNA (Fig. 3B). The above result was confirmed by using 3 other independent p120 siRNAs with target sequences 5’GCCAGAGGTGGTTCGGATA3’ (Wildenberg et al., 2006), 5’AACGAGGTTATCGCTGAGAAC3’ (Davis et al., 2003) and 5’GCGATTGCTTCGAAAGGCTCGTGAT3’ (designed by us, not shown). Most importantly, HCEC treated with p120 siRNA remained hexagonal and did not express cytoplasmic or nuclear S100A4 and cytoplasmic α-SMA, even with additional bFGF and TGFβ (Fig. 3B). These results collectively indicated that the mitotic block unlocked by p120 siRNA retained the normal HCEC phenotype without inducing EMT. In contrast, p120 siRNA decreased HCEC proliferation by 7-fold when the cells were dissociated into single cells by EDTA/trypsin (Fig. 3C).
p120 siRNA activates p120/Kaiso but not β-catenin/Wnt signaling

Although p120 is usually found at the cell junction and undergoes nucleocytoplasmic shuttling (Kelly et al., 2004; Rocznik-Ferguson and Reynolds, 2003; van et al., 1999), the controversy of nuclear p120 in normal or tumor cells exists (Daniel, 2007). We suspected that such a controversy might stem from the difference in the fixative used for immunostaining. In fact, the nuclear p120 staining was not apparent when HCEC monolayers were fixed with 4% paraformaldehyde as practiced in prior studies (Davis et al., 2003; Rocznik-Ferguson and Reynolds, 2003; Wildenberg et al., 2006). In contrast, nuclear p120 staining was apparent when cells were fixed by 25% acetic acid plus 75% methanol (Supplemental Fig. 2). To determine whether nuclear accumulation of p120 triggered by p120 siRNA might cause the release of nuclear Kaiso’s transcriptional repression as suggested (reviewed in (Daniel, 2007)), we first examined the expression of p120 and Kaiso transcripts by real-time PCR. Compared to the control treated with scRNA, HCEC treated with p120 siRNA expressed 20- and 3-fold decrease of p120 and Kaiso transcripts, respectively (Fig. 4A). As a contrast, p120 siRNA did not alter the levels of β-catenin and LEF1 transcripts (Fig. 4A). Compared to scRNA, p120 siRNA markedly reduced p120 staining in the cell junction, but increased that in the nucleus, where it was colocalized with increased BrdU nuclear staining (Fig. 4B). At the same time, nuclear staining of Kaiso was markedly reduced (Fig. 4B). As a comparison, the junctional β-catenin staining and the nuclear LEF1 staining were not changed by p120 siRNA when compared to the controls (Fig. 4B). The aforementioned immunostaining differences were confirmed by semi-quantitation of these protein levels by dot blotting using membranous, cytosolic, and nuclear lysates. p120 siRNA indeed reduced 4-fold of the membranous p120 level, but increased 3-fold of the nuclear p120 level, while reduced 4-fold of the nuclear Kaiso level (Fig. 4C). As a comparison, the dot blotting showed that p120 siRNA did not change β-catenin and LEF1 levels in these 3 compartments (Fig. 4C). EDTA-bFGF, which activated the Wnt signaling (Fig. 2), neither affected junctional p120 and nuclear Kaiso staining, (Fig. 4D) nor reduced p120 and Kaiso protein levels in these 3 compartments (Fig. 4E). Collectively, these results
showed that the mitotic block unlocked by p120 siRNA led to nuclear translocation of p120, which correlated well with the decrease of nuclear Kaiso, and that such an unlocking effect was not accompanied by changes of cytolocalization of β-catenin and LEF1 and the TCF/LEF promoter activity (not shown).

**p120 nuclear translocation is directly responsible for releasing nuclear Kaiso to unlock HCEC mitotic block.**

Although p120 siRNA led to nuclear translocation of p120 and nuclear release of Kaiso (Fig. 4B and 4C), it also reduced the level of Kaiso transcript by 70% (Fig. 4A). To discern whether Kaiso nuclear release was directly influenced by p120 nuclear translocation or indirectly via downregulation of Kaiso transcription, we tested the effect of Kaiso siRNA knockdown, of which the efficiency was confirmed by reducing 85% of Kaiso transcripts (Supplemental Fig. 1). When HCEC monolayers were treated with Kaiso siRNA alone, the protein dot assay showed that nuclear Kaiso level (Fig. 5A) and Kaiso staining (Fig. 5B) were not altered when compared to the control treated with scRNA. Double immunostaining confirmed that junctional staining of p120 was not reduced, and nuclear BrdU labeling was not promoted (Fig. 5B). These results indicated that reduction of Kaiso transcription alone by Kaiso siRNA knockdown could not alter the nuclear Kaiso level and hence was not sufficient to unlock the mitotic block. Nonetheless, the nuclear Kaiso level was reduced 4-fold when cells were treated with p120 siRNA alone, and reduced 6-fold when cells were treated with both p120 and Kaiso siRNAs (Fig. 5A). Consequently, the BrdU labeling index was increased 18% by treatment of p120 siRNA alone, and increased 26% by treatment of both p120 and Kaiso siRNAs (Fig. 5B). These results further suggested that the release of nuclear Kaiso was crucial for unlocking the mitotic block of HCEC monolayers, and such an effect could not be achieved by Kaiso siRNA alone, unless p120 siRNA was instituted to trigger p120 nuclear translocation.

To further prove that nuclear translocation of p120 was crucial for Kaiso nuclear release to unlock the mitotic block, we examined the effect of nocodazole or taxol (Rocznik-Ferguson and Reynolds, 2003), which is known to depolymerize or stabilize the microtubule network, respectively, to affect the cytoplasmic p120 pool, thus
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indirectly affecting p120 nuclear translocation (Yanagisawa et al., 2004). Compared to the control treated with scRNA, addition of nocodazole increased the nuclear p120 level from 3- to 5-fold when HCEC monolayers were treated with p120 siRNA (Fig. 5C). In contrast, addition of taxol reduced the p120 nuclear level to that of the control (Fig. 5C and 5E). Consequently, the nuclear Kaiso level was decreased 2-fold by nocodazole, but increased by taxol to the level of the scRNA control (Fig. 5D). The aforementioned changes of nuclear p120 and Kaiso levels were also reflected by the extent of nuclear p120 and Kaiso staining (Fig. 5E). Consequently, the BrdU labeling index was enhanced from 18% to 24% by nocodazole, but decreased to that of control level by taxol (Fig. 5E). Collectively, these results indicated that the extent of p120 nuclear translocalization played a dominant role in reducing the nuclear level of Kaiso to unlock the mitotic block of HCEC monolayers.

Nuclear p120-mediated proliferation is controlled by RhoA-ROCK signaling

p120 is known to interact and stabilize microtubules (Franz and Ridley, 2004) and to inhibit RhoA (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001). Activation of RhoA signaling can destabilize microtubules (Takesono et al., 2010). Because destabilization of microtubules by nocodazole facilitated p120/Kaiso signaling (Fig. 5), we wondered whether RhoA was activated by p120 siRNA in a similar manner to nocodazole, resulting in proliferation mediated by p120 nuclear translocation. Indeed we noted that the level of active RhoA was increased 3-fold by p120 siRNA and 4-fold with additional nocodazole (Fig. 6A). In contrast, the level of active RhoA promoted by p120 siRNA or p120 siRNA and nocodazole was suppressed by Taxol to the baseline level. Under this scenario, nuclear p120 staining and BrdU labeling promoted by p120 siRNA and nocodazole was abolished by taxol, CT-04 (a RhoA inhibitor), or Y27632 (a ROCK inhibitor) (Fig. 6B). These results suggested that activation of RhoA-ROCK signaling was correlated with destabilization of microtubules and p120 nuclear translocation and proliferation.

Hippo signaling is inhibited by p120 siRNA, but not EDTA-bFGF
The Hippo pathway is a key protein kinase cascade involved in regulating in vivo organ size and in vitro contact inhibition by governing cell proliferation and apoptosis (Zeng and Hong, 2008). To determine whether Hippo signaling was also affected, we examined and quantified the cytolocalization of phosphorylated and non-phosphorylated YAP and TAZ, which are the prime mediators of the Hippo pathway (Zhao et al., 2010). Both immunostaining and Western blotting showed that non-phosphorylated YAP and TAZ were accumulated in the nucleus after treatment of p120 siRNA, while cytoplasmic phosphorylated YAP and TAZ was dramatically reduced in the cytoplasm as compared to the control treated with scRNA (Fig 7A and 7B). As a comparison, EDTA-bFGF failed to elicit any change (Fig 7C and 7D). Because the pYAP (S127) antibody detected both pYAP and pTAZ (Habbig et al., 2011; Lei et al., 2008), we performed Western blotting to confirm the presence of cytoplasmic pTAZ and pYAP and nuclear TAZ and YAP. These results indicated that p120 siRNA, but not EDTA-bFGF, inhibited Hippo signaling by decreasing cytoplasmic pTAZ and pYAP, but increasing nuclear TAZ and YAP, further suggesting that selective activation of p120/Kaiso signaling also uniquely inhibited the Hippo signaling.

**Prolonged treatment of p120 siRNA followed by withdrawal leads to further expansion of HCEC monolayers without EMT**

The above experiments based on a short pulse of 100 nM p120 siRNA knockdown for 48 h was capable of unlocking the HCEC mitotic block without inducing EMT by selective activation of p120/Kaiso and inhibition of Hippo signaling. It remained unclear whether proliferation could be sustained without EMT by prolonging treatment of p120 siRNA. A total of 18 HCEC monolayers derived from three separate donors with ages of 55 (Fig. 8A, d1 and D1), 58 (d2 and D1), and 76 (d3 and D3) were treated with 40 nM of either p120 siRNA or scRNA (n=3 for each for each donor) weekly from Day 14 for two weeks followed by withdrawal on Day 28 for 10 days. The HCEC monolayers treated with scRNA virtually turned quiescent after 18 days of culturing (Fig 8A, d1-d3). In contrast, the HCEC monolayers treated with p120 siRNA continued growth during the entire 27 days of culturing (Fig 8A, D1-D3). At Day 27, the monolayer size of the
control treated with scRNA rapidly declined to an average of $1.7 \pm 0.4$ mm$^2$ (n=9), while that of the p120 siRNA-treated counterpart continued to expand to $3.7 \pm 0.7$ mm$^2$ (n=9).

To ensure that the aforementioned change of monolayer size was resulted from cell proliferation but not cell enlargement, we measured the HCEC cell density. HCEC of the Descemet membrane stripped from the donor peripheral cornea showed a characteristic in vivo hexagonal pattern with the average density of $2241 \pm 104$ /mm$^2$ (Fig 8B, a, n=5 donors). HCEC monolayers cultured in SHEM up to Day 14 also maintained a similar hexagonal pattern with a significant increase of cell density to $2548 \pm 93$ /mm$^2$ (Fig 8B, b, n=5). For the control treated with scRNA, although the hexagonal pattern was maintained, the cell density was $2083 \pm 86$ /mm$^2$ at Day 28 (after two weeks of treatment) (Fig 8B, c, n=5), and dropped to $1764 \pm 96$ /mm$^2$ on Day 38 (10 days after the withdrawal), indicative of cell enlargement (Fig 8B, d, n=5). In contrast, prolonged treatment of p120 siRNA maintained not only the hexagonal pattern but also the cell density at $2316 \pm 79$ /mm$^2$ at Day 28 and $2289 \pm 113$ /mm$^2$ at Day 38 (Fig 8B, e and f, respectively, both n=5).

Immunostaining confirmed that the normal HCEC phenotype, judged by junctional staining of p120, N-cadherin, ZO-1 and Na-K-ATPase as well as by perimembranous staining of F-actin, was maintained in the control treated with scRNA (Fig 8C, the top row). Prolonged treatment of p120 siRNA for two weeks resulted in significant reduction of p120 junctional staining and an increase of p120 nuclear staining (Fig 8C, the middle row) similar to what was shown in Fig 4. Furthermore, it also reduced peri-junctional staining of F-actin, but only mildly reduced the junctional staining of N-cadherin, ZO-1, and Na-K-ATPase respectively (Fig 8C, the middle row). Ten days after withdrawal of p120 siRNA, the immunostaining pattern was completely reverted to that of the control for all these 5 markers (Fig 8C, the bottom row). These results collectively indicated that prolonged treatment of p120 siRNA further expanded HCEC monolayer sizes while maintaining the normal hexagonal morphology at the same cell density without EMT (not shown). Following withdrawal of p120 siRNA, the normal HCEC phenotype was completely restored in 10 days.
Discussion

Disruption of cell junctions by EDTA (Senoo et al., 2000), scraping (Petroll et al., 1998), or freezing (Petroll et al., 1997) is a pre-requisite to unlock the mitotic block mediated by contact inhibition in corneal endothelial cells. In cat corneal endothelial explants (Petroll et al., 1998), scraping not only stimulates proliferation but also leads to EMT when cultured in a medium containing bFGF and/or TGF-β (Petroll et al., 1998). In cultured HCEC monolayers, contact inhibition disrupted by EDTA did not unlock the mitotic block even in EGF-containing SHEM until bFGF was added (Fig. 1). By that time HCEC had lost their normal phenotype expressing the junctional staining of N-cadherin, ZO-1, and Na-K-ATPase, changed to a slender shape, and begun to express cytoplasmic and nuclear staining of S100A4 (Fig. 1), a marker of epithelial-mesenchymal transition (Zeisberg et al., 2007). Such a phenotypic transition reminisced an early phase of EMT elicited in confluent rabbit corneal endothelial cells when bFGF was added to promote proliferation in a serum-containing medium (Kay et al., 1993). Upon addition of TGFβ1 to EDTA-bFGF treated cells, full blown EMT ensued as evidenced by cytoplasmic α-SMA and heralded by cessation of BrdU labeling (Fig. 1). These results collectively let us conclude that conventional methods of expanding single EDTA or EDTA/trypsin-treated HCEC in a medium containing such growth factors as EGF, bFGF, and TGFβ1 are potentially at risk of losing HCEC’s important barrier and pump functions to EMT, which is a pathologic process leading to corneal blindness by forming a retrocorneal membrane (reviewed in (Lee and Kay, 2006)).

We further demonstrated that the mitotic block of HCEC monolayers unlocked by EDTA-bFGF was caused by selective activation of the canonical Wnt signaling. EDTA-bFGF led to nuclear translocation of β-catenin, a significant increase of nuclear β-catenin and LEF1 protein levels, and a significant increase of the TCF/LEF promoter activity (Fig. 2). Furthermore, addition of a specific Wnt inhibitor XAV939 (Huang et al., 2009) abolished BrdU labeling, cytoplasmic and nuclear staining of S100A4, cytoplasmic α-SMA, and retained the junctional staining of N-cadherin, ZO-1 and Na-K-ATPase in EDTA-bFGF treated HCEC even if TGFβ1 was added (Fig. 2). Such a change can be completely reverted by overexpression of stable S33Y β-catenin (Fig. 2). To our best
knowledge, the above finding is the first demonstrating a clear causative role played by
the canonical Wnt signaling in governing the transition from contact inhibition to EMT
with proliferation in HCEC monolayers. This finding remisces the known role of the Wnt
signaling in epithelial-mesenchymal transition (Polette et al., 2007), where the canonical
Wnt signaling cooperates with TGF-\(\beta\)-mediated Smad signaling in mouse epithelial cells
(Eger et al., 2004) or proximal tubular epithelial cell lines (Masszi et al., 2004).

Realizing the aforementioned drawback of activating the Wnt signaling by
EDTA-bFGF in HCEC, herein we discovered that contact inhibition of HCEC
monolayers could also be unlocked by p120 siRNA. Similar to EDTA-bFGF treatment, a
high BrdU labeling index was uniquely promoted by knockdown of p120, but not \(\beta\)-
catenin, N-cadherin, or ZO-1 (Fig. 3). Unlike EDTA-bFGF, p120 siRNA, which
downregulated p120 transcript and protein levels, actually perturbed neither transcription
and junctional staining of \(\beta\)-catenin and N-cadherin (not shown) nor transcription and
nuclear protein level of LEF1 (Fig. 4). The above finding was surprising, given the fact
that p120 is known to play a key role in stabilizing cadherins at the membrane when AJ
matures in many types of cells (Anastasiadis and Reynolds, 2001; Rossman et al., 2005).
In fact, p120 siRNA reportedly decreases the level of \(\beta\)-catenin (Davis et al., 2003) and
E-cadherin in tumor epithelial cells (Davis et al., 2003; Ireton et al., 2002), VE-cadherin
in vascular endothelial cells (Davis et al., 2003; Ferreri et al., 2008), and N-cadherin in
cardiac myocytes (Davis et al., 2003) and vascular endothelial cells (Ferreri et al., 2008).
But none of these studies showed increased proliferation after treatment of p120 siRNA.
We attributed such a discrepancy to their use of EDTA/trypsin dissociated cells, because
p120 siRNA actually reduced cell proliferation when HCEC were dissociated into single
cells by EDTA/trypsin (Fig. 3). These data highlights a unique role of p120 in governing
contact inhibition in post-confluent cultures.

The mitotic block of contact-inhibited HCEC monolayers unlocked by p120
siRNA was mediated by selective activation of the p120/Kaiso signaling. Previous
studies have shown that intrinsic nucleocytoplasmic shuttling activity of p120 can be
modulated by extrinsic factors, such as cadherin binding (Rocznik-Ferguson and
Reynolds, 2003; van et al., 1999) and interactions with the microtubule network
(Rocznik-Ferguson and Reynolds, 2003; Yanagisawa et al., 2004). However, the importance of nuclear p120 in eliciting downstream signaling has not been appropriately addressed, presumably because of the controversy surrounding nuclear p120 staining when cells were fixed by paraformaldehyde. We discovered that p120 nuclear staining was apparent in HCEC monolayers only when fixed by methanol and acetic acid (Supplemental Fig. 2). Accompanied by downregulation of p120 transcript and protein, p120 siRNA dramatically shifts p120 cytolocalization from the junction/membrane to the nucleus, where it was colocalized with BrdU-labeling and correlated with the release of nuclear Kaiso (Fig. 4). Consistent with what has been noted in a number of cell lines (Rocznik-Ferguson and Reynolds, 2003), the extent of nuclear p120 level elicited by p120 siRNA could be augmented by nocodazole, a microtubule-disrupting agent, but diminished by taxol, a microtubule-stabilizing agent (Fig. 5), confirming that p120 shuttling from the cytoplasm to the nucleus could be facilitated by perturbing the microtubular network. Consequently, the nuclear p120 level manipulated by nocodazole and taxol, respectively, was inversely correlated with the nuclear level of Kaiso, strongly suggesting that the former dictates the latter. Similar to porcine pulmonary artery endothelial cells (Zhang et al., 2010), p120 knockdown in HCEC monolayers also downregulated Kaiso transcription (Fig. 4). Herein, for the first time, we showed that Kaiso siRNA per se, which downregulated Kaiso transcript and protein (Supplemental Fig. 1), was incapable of reducing the nuclear Kaiso level unless nuclear p120 was elicited (Fig. 5). Such a finding suggests that the homeostasis of nuclear Kaiso is tightly regulated and cannot be influenced by its own transcription and translation in a short term. Nonetheless, Kaiso siRNA was synergistic with p120 siRNA in further lowering the nuclear Kaiso level (Fig. 5), a finding resembling what has been shown in H. pylori mediated up-regulation of MMP-7 in MKN28 cells (Ogden et al., 2008). These data collectively support the notion that nuclear translocalization of p120 is a prerequisite to relieve nuclear Kaiso in agreement with the data that the Kaiso’s binding domain with p120 is the same as that with DNA (Kelly et al., 2004).

The notion that p120/Kaiso signaling activated by p120 siRNA was facilitated by destabilization of microtubules is supported by the finding that p120 is capable of regulating microtubule dynamics in a cadherin-independent manner (Ichii and Takeichi,
2007). Consistent with the known action of p120 in downregulating RhoA (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001), p120 siRNA activated RhoA-ROCK signaling (Fig. 6), which was associated with destabilization of microtubules. We discovered that p120 nuclear translocation induced by p120 siRNA was dependent on RhoA-ROCK signaling and destabilization of microtubules because inhibition of RhoA by CT-4 or ROCK by Y-27632 or stabilization of microtubules by Taxol abolished p120 nuclear translocation and BrdU labeling (Fig. 6). Although p120 signaling may modulate canonical and non-canonical Wnt signaling (Kim et al., 2004; Ruzov et al., 2009; Spring et al., 2005), p120 knockdown did not activate canonical Wnt signaling leading to EMT in HCEC monolayers. The finding that p120 signaling did not activate Wnt signaling might partly be explained by our finding that p120 siRNA, but not EGTA-bFGF, elicited dramatic decrease of cytoplasmic p-YAP and p-TAZ, but increase of nuclear non-phosphorylated YAP and TAZ (Fig 7). This was because Hippo signaling intercedes with Wnt signaling through the binding between p-TAZ and CK1δ/3 to block phosphorylation of Disheveled in the cytoplasm (Varelas et al., 2010) and interaction between non-phosphorylated YAP and β-catenin in the nucleus (Heallen et al., 2011) and disruption of cadherin-catenin complex (Kim et al., 2011). Such a contrast between p120 and β-catenin when the adherent junction is perturbed by EDTA and p120 knockdown, respectively, paves the way for future investigation of how Hippo signaling might be controlled by contact inhibition.

p120 is the prototypic member of the subfamily of p120 armadillo-related proteins (Hatzfeld, 2005) associated with cadherin-mediated adherent junctions (Davis et al., 2003; Ireton et al., 2002; Xiao et al., 2003). Several authors have reported that the cell proliferation was promoted in a p120 siRNA stable-transfected NIH3T3 cell line (Wildenberg et al., 2006), in the keratinocytes of p120 conditional knockout mouse (Perez-Moreno et al., 2006; Perez-Moreno et al., 2008) by activation of RhoA signaling. Such effect of p120 may be reversely linked to E-cadherin or isoform 1A of p120 at the cell membrane (Slorach et al., 2011; Soto et al., 2008). However, others have demonstrated that the growth of MDA-MB-231 cells was promoted by p120 (Soto et al., 2008) or the growth of single MDCK cells was inhibited by p120 knockdown (Dohn et al., 2009). Our results clearly indicate that relief of inhibition of RhoA activity by p120...
siRNA may play a major role in promoting proliferation of contact-inhibited HCEC monolayers. Such an activation of RhoA may be due to the fact that p120 acts as a guanine nucleotide dissociation inhibitor (GDI) by binding to and preventing RhoA activity (Anastasiadis et al., 2000; Rossman et al., 2005). Interestingly, downregulation of p120 was not associated with change of junctional N-cadherin. Such a finding is not in line with the finding that p120 siRNA resulted in downregulation of N-cadherin in bovine pulmonary artery endothelial cells (Ferreri et al., 2008). We attribute such a discrepancy to a different type of endothelial cells and different culture conditions.

To rule out the concern of carcinogenesis based on the finding that enhanced nuclear p120 levels are noted in several tumor cell lines (reviewed in (Daniel, 2007)), we prolonged p120 siRNA treatment for two weeks. Our results showed a wider spread of BrdU labeling from the periphery to the center of HCEC monolayers (not shown). Consequently, the mitotically arrested HCEC monolayers continued to expand its size from an average of 1.6 mm² to 3.7 mm² while maintaining the same cell density. Importantly, prolonged p120 knockdown did not alter the characteristic hexagonal shape and junctional expression of N-cadherin, ZO-1, and Na-K-ATPase, suggesting their barrier and pump functions were preserved (Fig 8). Both p120 nuclear translocation and dissolution of peri-membranous F-actin cables were transient and reversed to the normal pattern following the withdrawal of p120 siRNA. Hence, it is plausible to deploy this novel strategy of selectively activating the p120/Kaiso signaling to expand HCEC monolayers using the donor scleral rim left after corneal transplantation. This new engineering strategy will not only obviate the use of enzymatic digestion to produce single HCEC but also avoid the loss of their normal phenotype to EMT during ex vivo expansion of HCEC. If accomplished, this new technology will not only help solve the global shortage of human donor corneas but also facilitate the current popular trend of transplanting only the corneal endothelium in procedures collectively termed “endothelial keratoplasty” (reviewed in (Terry, 2006)). Our on-going experiments showed that this technology can also be applied to achieve the same goal in the human retinal pigment epithelium.
Materials and Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM), Ham’s/F12 medium, human epidermal growth factor (hEGF), HEPES buffer, Hanks’ balanced salt solution (HBSS), phosphate-buffered saline (PBS), gentamicin, fetal bovine serum (FBS), Texas Red®-X phalloidin and Alexa Fluor-conjugated secondary IgG were purchased from Invitrogen (Carlsbad, CA, USA). Collagenase A was obtained from Roche Applied Science (Indianapolis, IN, USA). Hydrocortisone, dimethyl sulfoxide, cholera toxin, insulin-transferrin-sodium selenite media supplement, bovine serum albumin, agarose, PCR marker, paraformaldehyde, methanol, Triton X-100, Hoechst 33342 dye, Y-27632, CT-04, XAV 939, nocodazole and taxol were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Cytoskeleton (Denvor, CO) or Calbiochem (La Jolla, CA, USA). Specific monoclonal antibodies against α-tubulin, β-catenin, BrdU, Kaiso, Na-K-ATPase, N-cadherin as well as polyclonal antibodies against α-SMA, Histone, N-cadherin (type I), p120-catenin, RhoA, S100A4, ZO-1, YAP, TAZ and pYAP (S127) were purchased from Abcam (La Jolla, CA, USA), Cell Signaling (Boston, MA, USA), Chemicon (Billerica, MA, USA), Upstate (Billerica, MA), Cytoskeleton, Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sigmal and Zymed (Carlsbad, CA, USA) (Supplemental Table 1). RNeasy Mini Kit was purchased from Qiagen (Valencia, CA, USA). Dual luciferase assay system was purchased from Promega (Madison, WI, USA). High Capacity Reverse Transcription Kit and TaqMan Universal PCR Master Mix were obtained from Applied Biosystems (Foster City, CA, USA). Control scRNA and HP validated siRNAs to β-catenin, N-cadherin, ZO-1, and Kaiso (Qiagen, Catalog Number SI02662478, SI02663927, SI02655149 and S104165924, respectively), HiPerFect® siRNA and SuperFect® plasmid transfection reagents were obtained from Qiagen. p120 siRNA was designed by us and obtained from Invitrogen with the target sequence of 5’CAGAGGTGATCGCCATGCTTGGATT3’. The TCF/LEF reporter plasmid kit was from SABiosciences (Valencia, CA, USA). Both control and stable S33Y β-catenin plasmids were a gift from Dr. Jan-Kan Chen (The Chang Gung University, Taiwan).(Chen et al., 1994)

HCEC Isolation and Culture
A total of 336 human corneas with ages of 20 to 81 years and maintained at 4°C in Optisol (Chiron Vision, Irvine, CA) for less than 5 days were obtained from the Florida Lions Eye Bank (Miami, FL, USA) and handled according to the declaration of Helsinki. The isolation and culture of HCEC followed what we have reported (Li et al., 2007; Zhu et al., 2008). In short, after central corneal buttons had been used for corneal transplantation, the remaining corneoscleral tissues were rinsed three times with DMEM containing 50 mg/mL gentamicin and 1.25 mg/mL amphotericin B. Under a dissecting microscope, the trabecular meshwork was cleaned, the rim was trephined within Schwalbe’s line, and Descemet’s membranes containing HCEC were stripped. After digestion at 37°C for 16 h with 1 mg/mL collagenase A in SHEM, which was made of an equal volume of HEPES-buffered DMEM and Ham’s F12 supplemented with 5% FBS, 0.5% dimethyl sulfoxide, 2 ng/mL hEGF, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 0.5 μg/mL hydrocortisone, 1 nM cholera toxin, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B, HCEC aggregates were collected by centrifugation at 2,000 rpm for 3 min to remove the digestion solution, and they were cultured in 24-well dishes coated with Collagen IV in SHEM. Cultures were monitored by phase contrast micrography, and the size of monolayer was determined by digitizing the surface area using J Imaging.

**siRNA Transfection and Other Treatments**

HCEC monolayers were cultured to Day 14-19, when they exhibited contact inhibition coinciding with the maturatation of AJ and ZO-1 resembling the in vivo pattern (Zhu et al., 2008). Such HCEC monolayers were treated with PBS with or without 5 mM EDTA at 37°C for 1 h, followed by culturing in SHEM with or without addition of 20 ng/ml bFGF for 2 days, which was then followed with or without 10 ng/ml TGFβ1 for 3 additional days or TGFβ1 for 3 days. For the short pulse siRNA knockdown, parallel HCEC monolayers were subjected to scRNA or siRNA transfection by mixing 50 μl of serum-free, antibiotic-free SHEM with 1 μl of HiPerFect® siRNA transfection reagent (final dilution, 1:300) and 3 μl of 20 μM of scRNA or siRNA (final concentration, 100 nM) to p120, β-catenin, N-cadherin, ZO-1, or Kaiso dropwise followed by culturing in 250 μl of fresh SHEM at 37°C for 2 days. For prolonged p120 siRNA knockdown, HCEC monolayers were treated with 40 nM of scRNA or p120 siRNA added once a
week. on Day 14 for two weeks before switching to siRNA-free fresh SHEM for 10 days. BrdU was added at a final concentration of 10 μM in the culture medium for 24 h before termination. Some cultures were treated with 1 μM XAV 939 in the culture medium for 48 h immediately following 5 mM EDTA treatment (Senoo et al., 2000) or with 5 μg/ml nocodazole or 10 μM taxol or 5 μg/ml CT-04 or 20 μM Y27632 in the culture medium (Rocznia-Ferguson and Reynolds, 2003; Yanagisawa et al., 2004) during the entire period of p120 siRNA transfection. For cultures receiving transfection of the control plasmid or the S33Y β-catenin plasmid, 0.2 μg of the control plasmid or the S33Y β-catenin plasmid was mixed with 3 μl of SuperFect transfection reagent (Qiagen, Valencia, CA) and 50 μl of a serum-free DMEM/F12 medium for 30 min before being dropwise added to the culture and incubated for 1 day.

**TCF/LEF Promoter Assay**

HCEC monolayers in 24-well dishes were cotransfected with 0.4% of the TCF/LEF construct that harbors TCF/LEF-binding sites and 0.01% of pRL-TK internal control plasmids with 1% SuperFect® plasmid transfection reagent in SHEM. Twenty-four hours after transfection, the transfection medium was removed. HCEC monolayers were then treated with PBS or 5 mM EDTA for 1 h and cultured in SHEM with or without bFGF or TGFβ1 or both or with or without addition of XAV 939 for another 48 h. The samples collected were assayed for firefly luciferase and Renilla luciferase activities using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA, USA). The ratio of firefly luciferase and Renilla luciferase activities was used to determine whether the promoters are activated.

**RNA Extraction, Reverse Transcription and Real-time PCR**

Total RNAs were extracted using RNeasy Mini Kit (Qiagen) and were reverse-transcribed using High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA of each cell junction component was amplified by real-time RT-PCR using specific primer-probe mixtures and DNA polymerase in 7000 Real-time PCR System (Applied Biosystems). Real-time RT-PCR profile consisted of 10 min of initial activation at 95 °C, followed by 40 cycles of 15 sec denaturation at 95 °C, and 1 min annealing and extension at 60 °C. The genuine identity of each PCR product was confirmed by the size
determination using 2% agarose gels followed by ethidium bromide staining together with PCR marker according to EC3 Imaging System (BioImaging System, Upland, CA, USA).

**Immunostaining**

HCEC monolayer cultures were air-dried and fixed in 4% formaldehyde, pH 7.0, for 15 min at room temperature, rehydrated in PBS, incubated with 0.2% Triton X-100 for 15 min, and rinsed three times with PBS for 5 min each. For double immunostaining to both BrdU and p120 or nuclear S100A4 or Kaiso, samples were fixed with 75% methanol plus 25% acetic acid for 15 min, denatured by 2 M HCl for 30 min at 37 °C and neutralized by 0.1 M borate buffer, pH 8.5 for 5 min three times. After incubation with 2% BSA to block non-specific staining for 30 min, they were incubated with the desired first antibody (all at 1:50 dilution) for 16 h at 4 °C. After three washes with PBS, they were incubated with corresponding Alexa Fluor-conjugated secondary IgG for 60 min. The samples were then counterstained with Hoechst 33342 and analyzed with Zeiss LSM 700 confocal microscope (Thornhood, NY, USA). Corresponding mouse and rabbit sera were used as negative controls for primary monoclonal and polyclonal antibodies, respectively.

**RhoA Activity Assay**

The assay of Rho activation was performed in 10-50 μg of protein of cell lysates using RhoA Activation Assay Biochem Kit (Cytoskeleton) to pull down the GTP-bound form of RhoA by a GST fusion protein containing rhotekin (7-89 residues) and RBD protein using brightly colored glutathione affinity beads. The amount of activated RhoA pulled down was quantitatively determined by Western blot using anti-RhoA antibody.

**Dot or Western Protein Blotting**

Cell lysates were prepared in RIPA buffer and electrophoresized on 4-15 % (w/v) gradient acrylamide ready gels under denaturing and reducing conditions for Western blotting. To prepare protein extracts from the membrane, the cytosol, and the nucleus, we followed Qproteome Cell Compartment protocol (Qiagen). Briefly, cells were first added with Extraction Buffer CE1, which selectively disrupts, but without solubilizing, the plasma membrane, followed by centrifugation at 100 x g for 10 min to pellet plasma membranes and compartmentalized organelles, such as nuclei, mitochondria, and the
endoplasmic reticulum. The pellet was then resuspended in Extraction Buffer CE2, which solubilizes the plasma membrane as well as all organelle membranes except the nuclear membrane, followed by centrifugation at 6000 x g for 10 min to pellet nuclei. The supernatant contains membrane proteins and proteins from the endoplasmic reticulum and mitochondria. Finally, the pellet containing nuclei was solubilized using Extraction Buffer CE3 and pelleted by centrifugation at 6800 x g for 10 min. The protein extracts from the above three compartments were transferred to a nitrocellulose membrane, which was then blocked with 5% (w/v) fat-free milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20), followed by sequential incubation with specific primary antibodies against β-catenin, LEF1, p120, Kaiso, RhoA, and their respective secondary antibodies using connexin (CN) 43, α-tublin, and histone as the loading control for the membranous, cytosolic, and nuclear compartments, respectively. Immunoreactive proteins were detected with Western Lighting™ Chemiluminesence Reagent.

Statistics

All summary data were reported as means ± s.d. calculated for each group and compared using Student's unpaired t-test by Microsoft Excel™ (Microsoft, Redmont, WA). Test results were reported as two-tailed p values, where p < 0.05 was considered statistically significant.
Acknowledgements

This study has been supported in part by R43 EY 022502 (to Ying-Ting Zhu and Scheffer C. G. Tseng) from National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA, and in part by TissueTech, Inc, Miami, Florida, USA. The aforementioned innovations, covering methods for promoting cellular proliferation that is limited by contact inhibition because of adherent junctions in cells including HCEC, and for generating surgical grafts and tissues, were filed in International PCT Patent Application (PCT/US07/79757) on September 27, 2007.
References


Figure Legends

Fig. 1. EMT with or without proliferation is caused by EDTA with or without bFGF and TGFβ1. (A) HCEC changed to a fibroblastic shape after additional bFGF and/or TGFβ. BrdU labeling was increased by 19% only by EDTA-bFGF (n=3, * p<0.05) but not by EDTA-TGFβ1 or EDTA-bFGF-TGFβ1. S100A4 staining was increased by EGTA-bFGF or TGFβ1, but became exclusively nuclear by EDTA-bFGF-TGFβ1. Cytoplasmic α-SMA staining was apparent in EDTA-TGFβ1 and increased in EDTA-bFGF-TGFβ1 treated cells. In addition, N-cadherin switch from the membrane to the cytoplasm was apparent by EDTA-bFGF, EDTA-TGFβ1 or EDTA-bFGF-TGFβ1 (B) Membrane staining of N-cadherin, ZO-1 and Na-K-ATPase was disrupted in EDTA-bFGF treated cells. All Bar=100 μM.

Fig. 2. EMT induced by EDTA-bFGF is mediated by the Wnt signaling. (A) Real-time PCR showed 2 and 3 fold upregulation of β-catenin and LEF1 transcripts by EDTA-bFGF, respectively (n=3, both * p<0.05). (B) EDTA-bFGF promoted translocalization of β-catenin from the membrane to the nucleus and nuclear accumulation of LEF1. (C) Dot protein blotting further confirmed that EDTA-bFGF caused a shift of β-catenin from the membrane to the nucleus and promoted nuclear accumulation of LEF1 (n=3, * p<0.05; CN43, α-tubulin, and histone were used as the loading control for membranous, cytosolic and nuclear compartments, respectively). (D) EDTA-bFGF causitively activated Wnt signaling by activating the TCF/LEF promoter activity by 6-fold (n=3, p<0.05). Addition of a specific Wnt inhibitor, XAV 939, completely abolished the activity of TCF/LEF promoter and BrdU labeling. (E) EMT caused by EDTA-bFGF was prevented by XAV 939. After addition of XAV 939, the staining of β-catenin, S100A4, N-cadherin, ZO-1 and Na-K-ATPase was similar to that from normal HCEC monolayers (c.f., Fig. 1B and 2B). (F) Overexpression of S33Y β-catenin in HCEC monolayers treated with EGTA without growth factors increased BrdU labeling (by 21-fold, n=3, p<0.05), nuclear β-catenin, LEF-1, S100A4, and cytoplasmic α-SMA, N-cadherin. (G) The activation of Wnt signaling by S33Y β-catenin was confirmed by Westen blotting, indicating nuclear β-catenin and LEF-1 were activated. All Bar=100 μM.
**Fig. 3. Mitotic block is uniquely unlocked by p120 siRNA without EMT.** (A) HCEC monolayers treated with siRNA to p120 but not β-catenin, N-cadherin, or ZO-1, significantly promoted nuclear BrdU labeling up to 18-fold (n=3, * p<0.05). (B) The proliferation promoted by p120 siRNA was not further promoted by additional bFGF (n=3, p>0.05). Addition of bFGF and/or TGF-β1 did not induce staining of S100A4 or α-SMA. However, addition of TGF-β1 abolished BrdU labeling promoted by p120 siRNA (n=3, * p<0.05). (C) In contrast, the proliferation of HCEC monolayers was decreased 7-fold (n=3, p<0.05) by p120 siRNA when the cells were dissociated into single cells by EDTA/trypsin. All Bar=100 μM.

**Fig. 4. p120 siRNA triggers p120/Kaiso, but not Wnt signaling.** (A) p120 siRNA downregulated expression of both p120 and Kaiso transcripts by 95% and 70% respectively (n=3, both * p<0.05) but not that of β-catenin and LEF1 transcripts. (B) p120 siRNA induced nuclear translocalization of p120 (green) from the junction, colocalized with nuclear BrdU labeling (red) (n=3, * p<0.05), decreased nuclear Kaiso staining, but did not change membrane β-catenin, or activate nuclear LEF1 staining. (C) Dot blotting confirmed that p120 siRNA increased the level of p120 and decreased that of Kaiso in the nuclear compartment, but did not altering that of β-catenin and LEF1. (D) In comparison, EDTA-bFGF did not alter the staining pattern of p120 and Kaiso. All Bar=100 μM. (E) Dot blotting confirmed that EDTA-bFGF did not change quantitation of p120 and Kaiso.

**Fig. 5. p120 nuclear translocation plays an important role in releasing nuclear Kaiso to unlock HCEC mitotic block.** (A) The nuclear Kaiso level was significantly decreased by p120 siRNA and further by combined p120 and Kaiso siRNAs (n=3, both * p<0.05), but not by Kaiso siRNA (n=3, p>0.05). In contrast, the nuclear p120 level was not affected by Kaiso siRNA, single or in combination with p120 siRNA (not shown, n=3, p>0.05). (B) Double immunostaining of p120 and BrdU (left) showed that BrdU labeling (red) correlated with nuclear p120 staining (green) was promoted by p120 siRNA but not Kaiso siRNA. A synergistic effect was noted by combined treatment with both p120 and
Kaiso siRNAs (n=3, all * p<0.05). (C) Dot blotting showed the nuclear Kaiso protein level decreased by p120 siRNA was further decreased by nocodazole, but was increased by taxol to that of the control level (n=3, * p<0.05). (D) Nuclear dot blotting showed the nuclear p120 protein level promoted by p120 siRNA was further enhanced by nocodazole, but was decreased by taxol (n=3, * p<0.05). (E) The extent of nuclear p120 and BrdU labeling was negatively correlated with nuclear Kaiso levels, which was decreased by nocodazole but increased by taxol (n=3, * p<0.05). All Bar=100 μM.

Fig. 6. RhoA-ROCK signaling controls p120 nuclear translocation and its associated proliferation. (A) The level of active RhoA was promoted 3-fold by p120 siRNA and 4-fold with addition of nocodazole. The level of active RhoA promoted by p120 siRNA or p120 siRNA+nocodazole was inhibited by taxol to the baseline. (B) Nuclear BrdU and p120 promoted by p120 siRNA+nocodazole was abolished by Taxol, CT-04 (RhoA inhibitor), or Y27632 (ROCK inhibitor). All Bar=100 μM.

Fig. 7. p120 knockdown but not EDTA-bFGF inhibits Hippo signaling. Immunostaining (A) showed that p120 siRNA led to nuclear accumulation of non-phosphorylated TAZ and YAP and cytoplasmic deletion of phosphorylated TAZ and YAP when compared to that of scRNA. Because the same antibody detects both pYAP and pTAZ, Western instead of dot blotting (B) was used to confirm that both pTAZ and pYAP bands were decreased in the cytoplasm, while TAZ and YAP (detected by different antibodies) were increased in the nuclear extract. In contrast, immunostaining (C) and Western blotting (D) showed that EDTA-bFGF failed to elicit such a change.

Fig. 8. Prolonged treatment of p120 siRNA causes further expansion of HCEC monolayers without EMT. (A) The HCEC monolayer surface area reached a plateau when treated with scRNA (d1, d2 and d3), averaged 1.7 ± 0.4 mm² (n=9, p<0.05), but was continuously promoted by p120 siRNA (D1, D2 and D3) (p<0.05 on day 18 except the D3/d3 pair, and on Day 21, 24 and 27 for all three pairs), averaged 3.7 ± 0.7 mm² (n=9, p<0.05) without cell enlargement in the center. (B) The HCEC density was 2241 ± 104 /mm² when the Descemet membrane was stripped from the peripheral cornea (a). It
was increased to \(2548 \pm 93 \text{ /mm}^2\) for HCEC monolayers cultured on Day 14 (b, \(n=5\), \(p<0.05\)). For the control treated with scRNA, the HCEC density dropped to \(2083 \pm 86 \text{ /mm}^2\) on Day 28 (c, \(n=5\), \(p>0.05\)) and \(1764 \pm 96 \text{ /mm}^2\) on Day 38 (d, \(n=5\), \(p<0.05\)), i.e., 10 days after withdrawal. In contrast, the HCEC density was maintained at \(2316 \pm 79 \text{ /mm}^2\) on Day 28 (i.e., two weeks of p120 siRNA3 treatment) (e, \(n=5\), \(p>0.05\)), and \(2289 \pm 113 \text{ /mm}^2\) on Day 38 (i.e., 10 days after withdrawal, f, \(n=5\), \(p>0.05\)). (C) Prolonged p120 siRNA treatment resulted in nuclear translocation of p120 and dissolution of F-actin without disturbing the junctional staining pattern of N-cadherin, ZO-1, and Na-K-ATPase. Ten days after withdrawal of p120 siRNA, the staining pattern of p120 and F-actin reverted to the normal pattern while that of the rest remained unchanged. All Bar=100 \(\mu\text{M}\).
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A

B

PBS EDTA+bFGF

EDTA +bFGF

β-cat

LEF-1

PBS

EDTA +bFGF

C

PBS EDTA+bFGF

PBS EDTA+bFGF

PBS EDTA+bFGF

Membrane

Cytoplasm

Nucleus

D

E

BrdU

β–cat

S100A4

F

G

α-SMA

S100A4

N-cadherin

92 kDa

46 kDa

17 kDa

anti β-cat

anti LEF-1

anti Histone

Vector

S33Y

Nucleus
A

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- anti RhoA-GTP
- anti total RhoA
- anti α-tublin

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A. scRNA (d1, d2, d3) or p120 siRNA (D1, D2, D3)

B. Images showing different conditions:
   - a, b
   - c, d
   - e, f

C. Images of p1120, F-actin, N-cad, ZO-1, ATPase with different treatments:
   - scRNA
   - p120 siRNA
   - Withdrawal
**Fig S1 Knockdown Efficiency:** Knockdown efficiency of various junctional proteins was verified by real-time PCR and Western blotting. (A) siRNA to p120, β-catenin, N-cadherin, ZO-1 or Kaiso significantly downregulated corresponding mRNA levels (n=3, * p<0.05). (B) siRNA to p120, β-catenin, N-cadherin, ZO-1, but not to Kaiso, significantly downregulated corresponding proteins (n=3, * p<0.05).
**Fig S2 Nuclear p120 Staining:** Nuclear p120 staining was clearly observed when HCEC were fixed with acidic acid and methanol, but not with paraformaldehyde. Bar=100 µM.