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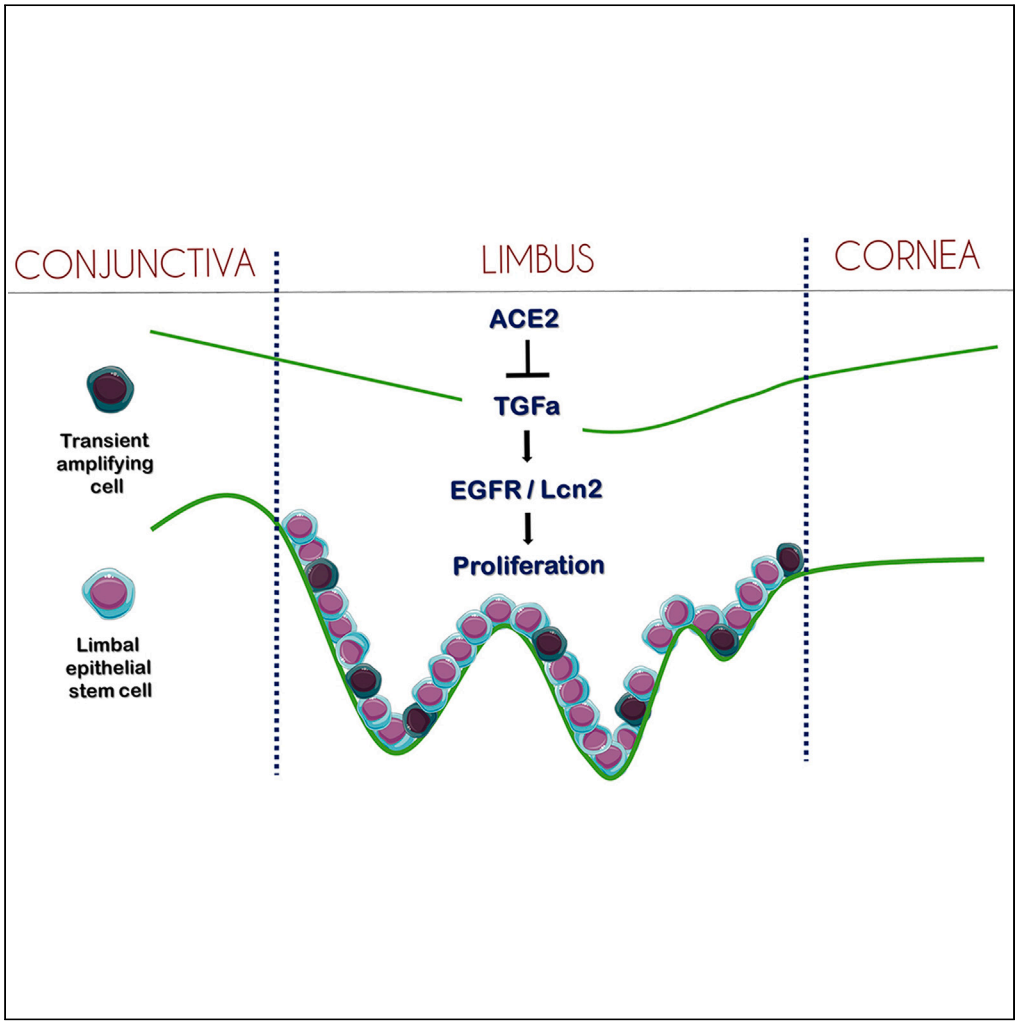

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Highlights

Following corneal injury, reduced ACE2 contributes to limbal epithelial activation

The effect of ACE2 in proliferation is independent of the renin-angiotensin system

ACE2 positively regulates limbal epithelial activation via TGFA/EGFR/LCN2 pathway

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Activation of limbal epithelial proliferation is partly controlled by the ACE2-LCN2 pathway

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SUMMARY

In response to corneal injury, an activation of corneal epithelial stem cells and their direct progeny the early transit amplifying (eTA) cells to rapidly proliferate is critical for proper re-epithelialization. Thus, it is important to understand how such stem/eTA cell activation is regulated. Angiotensin-converting enzyme 2 (ACE2) is predominantly expressed in the stem/eTA-enriched limbal epithelium but its role in the limbal epithelium was unclear. Single cell RNA sequencing (scRNA-seq) suggested that Ace2 involved the proliferation of the stem/eTA cells. Ace2 was reduced following corneal injury. Such reduction enhanced limbal epithelial proliferation and downregulated LCN2, a negative regulator of proliferation in a variety of tissues, via upregulating TGFA and consequently activating epidermal growth factor receptor (EGFR). Inhibition of EGFR or overexpression of LCN2 reversed the increased proliferation in limbal epithelial cells lacking ACE2. Our findings demonstrate that after corneal injury, ACE2 is downregulated, which activates limbal epithelial cell proliferation via a TGFA/EGFR/LCN2 pathway.

INTRODUCTION

Proper corneal wound healing requires activation of quiescent limbal epithelial stem cells (LESCs) and their progeny the early transit amplifying (eTA) cells to rapidly proliferate.[1,2] As the LESCs and eTA cells reside in the basal layer of the limbal epithelium, such activation results in a marked increase in proliferation in the basal cells of the limbal epithelium[2] and initiates corneal epithelial regeneration.[2,3] A defect in limbal stem cell/eTA cell activation can result in delayed corneal wound healing.[4] Thus, it is essential to understand the mechanisms underlying how limbal epithelial cell proliferation is regulated during wound healing.

Angiotensin-converting enzyme 2 (ACE2) is a protein known for its involvement in the renin-angiotensin system (RAS), which regulates blood pressure and fluid balance.[5–14] An important function of ACE2 is to convert angiotensin II (AngII) into angiotensin-(1–7) (Ang-[1–7]).[5–11,13] AngII is the major effector peptide of the RAS which positively regulates blood pressure, the inflammatory response, and cell proliferation.[5–10] Ang-(1–7) counteracts the effects of AngII by promoting blood vessel dilation, decreasing inflammation, and reducing cell proliferation.[5–9] We have shown that ACE2 is prominently expressed in limbal basal epithelial cells, where the corneal epithelial stem cells reside.[15] This specific spatial expression of ACE2 in the ocular anterior segment, suggests that ACE2 may have a regulatory role in the stem cell-enriched limbal epithelial basal cells.

ACE2 has been detected in stem cells in variety of tissues including neural[16] and hematopoietic stem cells.[17] Treatment with Ang II, a main substrate of ACE2, induces proliferation of hematopoietic stem cells.[18] In addition to regulation of proliferation of hematopoietic stem cells, ACE2/Ang-(1–7) signaling has been shown to have an inhibitory role in proliferation in many other cell types.[7,8] For example, ACE2 suppresses vascular smooth muscle cell proliferation, which may be due to inhibition of Akt/ERK phosphorylation.[7] Interestingly, ACE2 interacts with epidermal growth factor receptor (EGFR) signaling,[19–22] which is a key regulator of cell proliferation via modulating Akt and ERK activation. Although the role of ACE2 in proliferation has been studied in other tissues, it remains to be investigated whether ACE2 regulates proliferation in the limbal/corneal epithelia. Herein, we report that single cell RNA sequencing (scRNA-seq) and proliferation assays reveal that loss of *ACE2* enhances proliferation in LESC-enriched epithelial basal cells via alteration of *LCN2* (lipocalin-2) through EGFR signaling. Importantly, corneal injury results in downregulation of *ACE2* and such downregulation has a positive role in the activation of limbal epithelial cells to rapidly proliferate in response to wounding.

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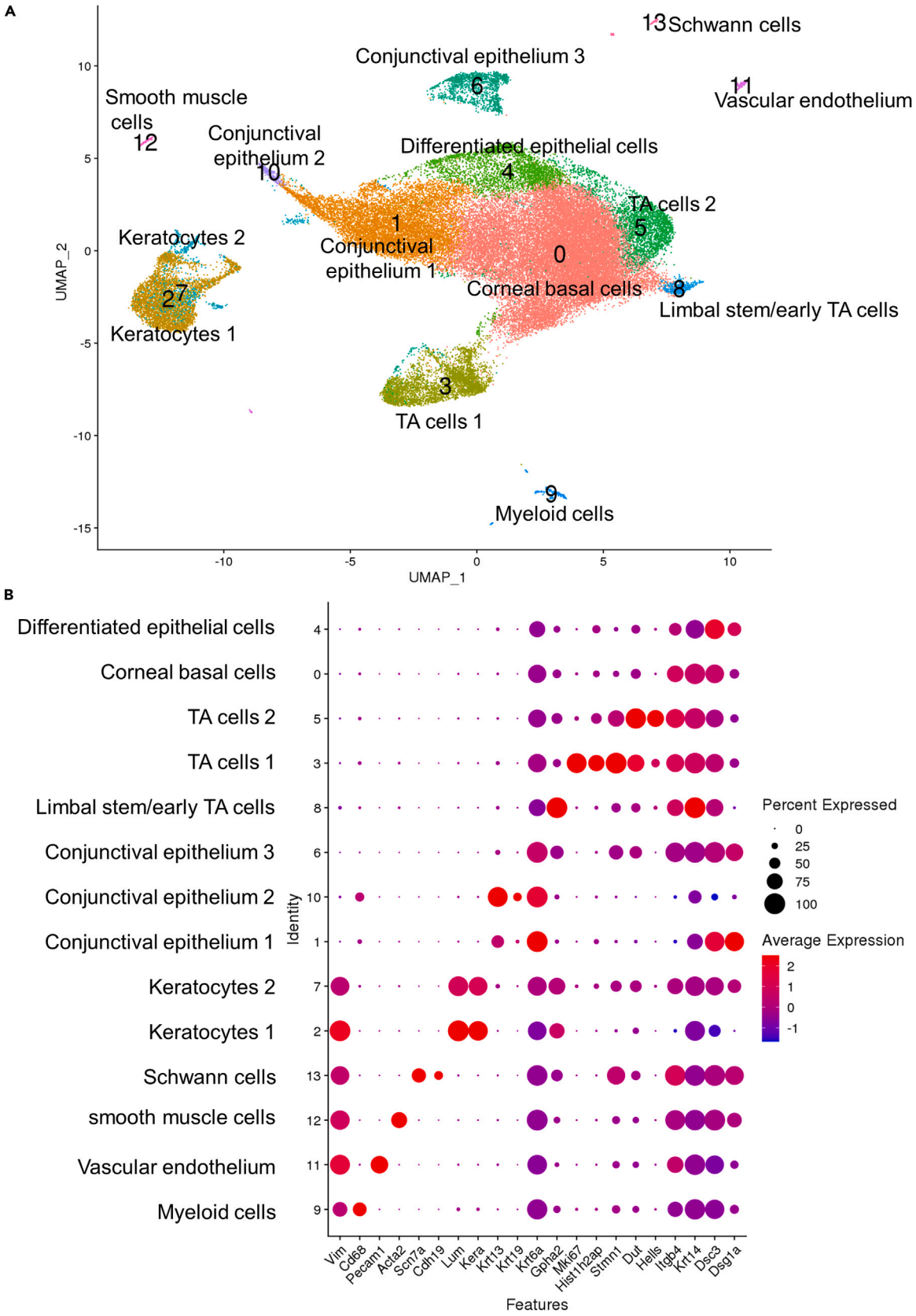
A
13 Schwann cells
Conjunctival epithelium 3
6
11
Vascular endothelium
Smooth muscle cells
12
Conjunctival epithelium 2
10
Differentiated epithelial cells
4
TA cells 2
5
1
Conjunctival epithelium 1
0
Corneal basal cells
8
Limbal stem/early TA cells
Keratocytes 2
27
Keratocytes 1
3
TA cells 1
9
Myeloid cells
UMAP_2
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B
Differentiated epithelial cells
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TA cells 2
TA cells 1
Limbal stem/early TA cells
Conjunctival epithelium 3
Conjunctival epithelium 2
Conjunctival epithelium 1
Keratocytes 2
Keratocytes 1
Schwann cells
smooth muscle cells
Vascular endothelium
Myeloid cells
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Cd68
Pecam1
Acta2
Scn7a
Cdh19
Lum
Kera
Krt13
Krt19
Krt6a
Gpha2
Mki67
Hist1h2ap
Stmn1
Dut
Hells
Itgb4
Krt14
Dsc3
Dsg1a
Features
Percent Expressed
0
25
50
75
100
Average Expression
2
1
0
-1

Figure 1. scRNA-seq demonstrated 14 clusters in cells isolated from limbal/corneal tissues of WT and *Ace2* KO mice

(A)UMAP visualized 14 differential clusters of cells.

(B) Dot plot was used to visualize conserved cell type markers and show both the expression level and the percentage of cells in a cluster expressing those marker genes.

RESULTS

scRNA-seq analyses of *Ace2* KO mouse corneas reveal that *Ace2* is associated with the pathways regulating proliferation in limbal epithelium

To understand the roles of *ACE2* in corneal and limbal epithelia, we conducted scRNA-seq analysis using cells isolated from the limbal and corneal tissues of both WT ($n = 3$) and *Ace2* KO ($n = 3$) mice utilizing the 10x Genomics platform.[23] In total, scRNA-seq yielded 8090, 7276, and 8234 cells from WT1, WT2, and WT3 mice respectively, as well as 8029, 10119, and 7949 cells from *Ace2* KO 1–3 mice, respectively. Analysis of the scRNA-seq data using Seurat demonstrated 14 distinct clusters in a UMAP plot (Figure 1A). UMAP plot showed that cell proportions of each cluster had no significant difference between WT and *Ace2* KO mice and the cells from WT and *Ace2* KO mice were evenly integrated (Figure S1A). The expression of marker genes in 14 clusters was visualized using a dot plot (Figure 1B). Among the 14 clusters, 6 cell clusters were mesenchymal cells, which expressed the mesenchymal marker vimentin (*Vim*). These 6 clusters included keratocytes (lumican [*Lum*]+ and keratocan [*Kera*]+), corneal Schwann cells, smooth muscle cells, vascular endothelial cells, and myeloid cells.

The remaining eight cell clusters were identified as epithelial in nature. Clusters 1, 6, and 10 were identified as conjunctival epithelial cells based on the high expression of conjunctival markers such as Keratin 13 (*Krt13*), Keratin 19 (*Krt19*), and Keratin 6a (*Krt6a*)[24–26] (Figures 1B and S1B). Among the other epithelial clusters, cluster 8 highly expressed glycoprotein hormone subunit alpha 2(*Gpha2*), a limbal epithelial stem/eTA cell marker,[26,27] as well as Keratin 14 (*Krt14*),[28] a basal cell marker. In addition, cluster 8 expressed low levels of differentiation markers such as desmocollin-3 (*Dsc3*) and desmoglein 1alpha (*Dsg1a*),[26,29] indicative of a less differentiated more primitive cell. This suggests that cluster 8 is predominately limbal epithelial stem/eTA cells. Clusters 3 and 5 were identified as more mature TA cell populations since TA cell markers including the marker of proliferation *Mki67*, *Histone cluster 1 H2ap* (*Hist1h2ap*), *Stathmin 1* (*Stmn1*), *Deoxyuridine triphosphatase* (*Dut*), and *Helicase, lymphoid specific* (*Hells*)[30–33] were enriched in these two clusters. Cluster 0 highly expressed basal cell markers (e.g., *Integrin beta 4* [*Itgb4*], *Krt14*)[28,34]; while cluster 4 highly expressed differentiation markers (e.g., *Dsc3*, *Dsg1a*).[26,29] Thus, cluster 0 was designated as corneal epithelial basal cells and cluster 4 as differentiated corneal epithelial cells.

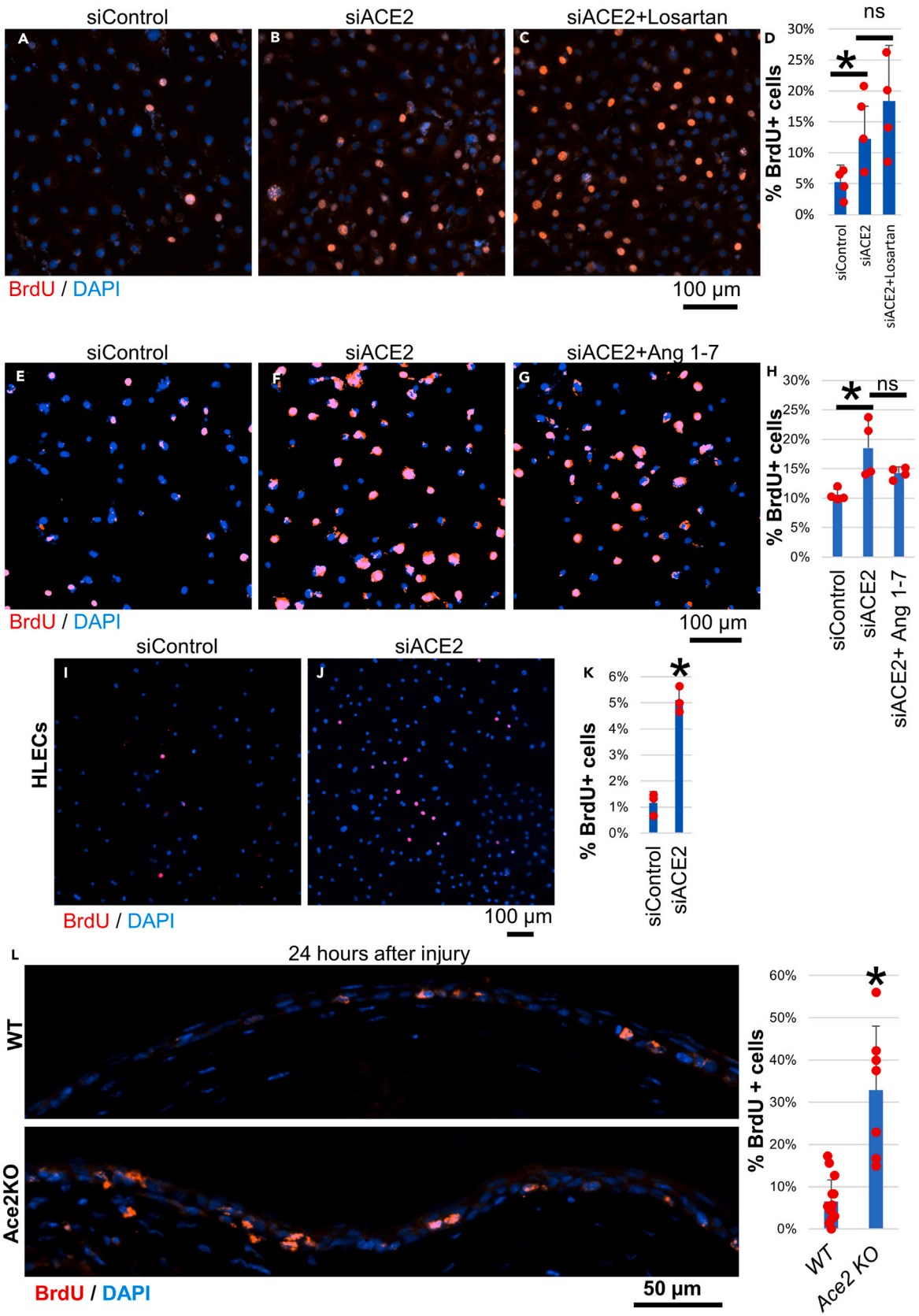
To explore the role of ACE2 in limbal epithelial stem/eTA cells, the differentially expressed genes (DEGs) in cluster 8 between WT and *Ace2* KO mice were subjected to two gene ontology (GO) programs: database for annotation, visualization and integrated discovery (DAVID) as well as clusterProfiler (Figures S1C and S1D). The top significant GO terms included "cell cycle" (Figure S1C), "epithelial cell proliferation", and "regulation of epithelial cell proliferation" (Figure S1D). Interestingly, in human and wild type mouse limbal epithelial basal layers, ACE2 proteins were detected by immunostaining in the cells expressing p63 and Keratin 15 (two putative LESC markers[35]; Figure S2A). These observations suggest that ACE2 may play a role in regulating proliferation of limbal epithelial stem/eTA cells, which reside in limbal epithelial basal layer.

ACE2 negatively regulates proliferation in limbal epithelial cells *in vitro* and *in vivo*

To investigate the role of ACE2 in the proliferation of limbal epithelial cells, we utilized hTCEpi cells, a human limbal epithelial cell line.[36] These cells were transfected with siRNA pools against *ACE2* (si*ACE2*) and a significant reduction of *ACE2* in hTCEpi cells was detected (Figure S2B). To assess cell proliferation, a BrdU labeling assay was conducted, which detects cells in the S phase of DNA synthesis. After a 72-h transfection period, we observed a significant increase in the percentage of BrdU positive cells in siACE2 transfected cells (Figures 2B, 2D, 2F, and 2H) when compared to the scrambled control siRNA (siControl) transfected cells (Figures 2A, 2D, 2E, and 2H). The alteration of the proliferative status of the siACE2 transfected cells was also evidenced by a significant increase in the number of hTCEpi cells in the S phase of the cell cycle detected by flow cytometry assays (Figure S2C). Consistently, in primary human limbal epithelial cells (HLECs), knockdown of *ACE2* also markedly enhanced BrdU labeling (Figures 2I-2K). Furthermore, we examined the role of ACE2 in mouse limbal epithelial cell proliferation *in vivo* during wound healing. We conducted BrdU labeling assays in young adult *Ace2* KO mouse corneas that were injured by removal of the central corneal epithelium. Immunostaining for BrdU showed that after injury, *Ace2* KO mice had enhanced limbal epithelial proliferation, as evidenced by more BrdU+ cells, compared with WT mice (Figure 2L). This suggests that a decrease in ACE2 promotes proliferation of the mouse limbal epithelium in the context of corneal injury.

ACE2 negatively regulates limbal epithelial cell proliferation in an Ang II/AT1 receptor and Ang-(1–7) signaling independent way

ACE2 is a key component in RAS and counteracts AngII/AT1 receptor signaling via converting AngII into Ang-(1–7). Thus, we explored whether the increased proliferation induced by loss of *ACE2* was due to enhanced AngII/AT1 receptor signaling. To test this, we utilized losartan (50μM),[37] an angiotensin II receptor type 1 (AT1) antagonist. Interestingly, treatment with losartan failed to rescue the increased BrdU+ cells in those cells transfected with siACE2 (Figures 2A–2D). To examine whether the increased proliferation induced by loss of *ACE2* was due to reduction of Ang-(1–7), cells were treated with Ang-(1–7) peptide (1 μM).[38] Such treatment with Ang-(1–7) also failed to reverse the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Ace2 KO
BrdU / DAPI
50 µm

Figure 2. ACE2 negatively regulates proliferation *in vitro*, which is independent of RAS signaling

(A–H) A limbal epithelial cell line hTCEpi cells were transfected with siControl or siACE2 for 72 h. AT1R antagonist losartan (50μM) and Ang-(1–7) peptide (1 μM) were added for 24 h.

(A–C, E-H) Immunofluorescent images visualized the BrdU+ cells at 72 h after transfection.

(D and H) The percentage of BrdU+ cells were quantified by ImageJ and the results showed an increased percentage of BrdU+ cells after siACE2 transfection. This increased proliferation was not reversed by the treatment of losartan (N = 4 of independent experiments) or Ang-(1–7) peptides (N = 4 of independent experiments). One-way ANOVA was conducted, *$p < 0.05$.

(I-K) Primary human limbal epithelial cells (HLECs) were transfected with siControl or siACE2 for 72 h. Immunofluorescent images visualized the BrdU+ cells at 72h after transfection (I and J). The percentage of BrdU+ cells were quantified by ImageJ and the results showed an increased percentage of BrdU+ cells after siACE2 transfection (K). N = 3 of independent. *$p < 0.05$. As BrdU is utilized for nuclear labeling, only signals within nuclei were quantified, ensuring colocalization with DAPI. The percentages of BrdU+ cells = numbers of BrdU+ cells/(numbers of BrdU+ cells + numbers of BrdU- cells).

(L) Immunofluorescent images showed that after injury, BrdU+ cells in the limbal epithelium of *Ace2* KO mice (N = 7) were increased compared to WT (N = 15). The percentage of BrdU+ cells was quantified using ImageJ. *$p < 0.05$.

increased BrdU+ cells in those cells lacking *ACE2* (Figures 2E-2H). These observations indicate that the role of *ACE2* in limbal epithelial cell proliferation is not mediated by counteracting AngII/AT1 receptor signaling or producing Ang-(1–7).

Bioinformatics analysis suggests that EGFR, LCN2, and GSN form a downstream signaling network in regulating cell proliferation

To understand how ACE2 regulates proliferation in limbal epithelial cells, we explored the DEGs that are involved in the cell cycle and epithelial proliferation in cluster 8 (stem/eTA cell cluster) and interrogated the top 5 DEGs using violin plots (Figure 3A). Peptidylprolyl isomerase A (*Ppia*) is a housekeeping gene[39] and plays a critical role in the development of many human cancers by regulating cell growth.[40] Additionally, it also has a function in promoting inflammation and vascular smooth muscle cell proliferation.[41] Mesoderm induction early response 1 (*Mier1*) is reported to have an influence on cell cycle gene expression and regeneration.[42] Gelsolin (*Gsn*) has been identified as a multifunctional regulator involved in various physiological and pathological cellular processes, including the regulation of cell migration, cell morphology, proliferation, and apoptosis.[43] Leucyl-TRNA synthetase 2 (*Lars2*) is correlated with proliferation in tumor cells.[44,45] *Lcn2* has been associated with cell proliferation, angiogenesis, cell invasion, and metastasis.[46]

To validate whether *ACE2* regulates the expression of these 5 candidate genes, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was conducted using RNAs from cells transfected with siACE2 or siControl. RT-qPCR indicated that knockdown of *ACE2* significantly downregulated *GSN* and *LCN2* expression (Figure 3B), suggesting that *ACE2* positively regulates *GSN* and *LCN2*. To explore how *ACE2* regulates *GSN* and *LCN2*, we conducted a network analysis using GeneMANIA,[47] which predicted a regulatory network that connects *Ace2* with *Gsn* and *Lcn2*. Interestingly, this network indicated that *Egfr* is a central node in connecting *Ace2* with *Gsn* and *Lcn2* (Figure 4A).

ACE2 regulates proliferation via EGFR signaling

Since ACE2/EGFR signaling crosstalk has been reported in various tissues,[19–22] we investigated whether ACE2 regulated limbal epithelial cell proliferation via EGFR signaling. Western blotting showed that knockdown of ACE2 markedly increased phosphorylated EGFR (*p*-EGFR; active form) as well as phosphorylated Akt (*p*-Akt, a downstream effector of EGFR signaling) in hTCEpi cells (Figure 4B). This suggests that loss of ACE2 activates EGFR signaling. The binding of a ligand to EGFR causes phosphorylation of EGFR and activation of downstream signaling pathways.[48] Thus, we explored the ligand/receptor interaction of the EGFR receptor family in our scRNA seq data using CellChat in R. Interestingly, *transforming growth factor α* (*Tgfa*)/Egfr interaction was detected in *Ace2* KO mice but not in WT mice (Figure 4C). ScRNA-seq data showed that *Tgfa*, one of the ligands of Egfr, was upregulated in the stem/eTA cell cluster (cluster 8) of *Ace2* KO mice compared to WT (Figure 4D). RT-qPCR confirmed that in hTCEpi cells, knockdown of *ACE2* increased the expression of *TGFA* (Figure 4E), indicating that *ACE2* negatively regulates *TGFA/EGFR* signaling. To explore whether ACE2 regulates limbal epithelial cell proliferation via EGFR signaling, we utilized two EGFR inhibitors: AG1478 (1μM) and erlotinib (1μM).[49,50] BrdU labeling assays revealed that knockdown of *ACE2* increased BrdU labeling, indicating enhanced proliferation (Figures 5B and 5G). Such increased BrdU labeling was reversed by inhibition of EGFR signaling (Figures 5D, 5F, and 5G), demonstrating that *ACE2* affects limbal epithelial cell proliferation via negatively regulating TGFA/EGFR signaling.

ACE2 attenuates cell proliferation via positively regulating LCN2

To investigate whether *ACE2* regulates proliferation via *GSN* and/or *LCN2*, we tested the effects of knockdown of *GSN* and *LCN2* on cell proliferation. Transfection with an siRNA against *LCN2* (siLCN2) reduced *LCN2* expression (Figure 6G) and increased the percentage of BrdU+ cells compared to siControl (Figures 6E and 6F). However, BrdU labeling assays revealed that hTCEpi cells transfected with an siRNA against *GSN* (siGSN; Figure S3A) didn't show a significant effect on the percentage of BrdU+ cells compared to the siControl. This suggests that *ACE2* and *LCN2* represent a regulatory axis, which inhibits limbal epithelial cell proliferation.

To further validate this finding, we conducted a rescue experiment by overexpressing LCN2 in hTCEpi cells lacking ACE2. RT-qPCR results confirmed a significant increase of *LCN2* expression (Figure 6H) after cells were transfected with a lentivirus encoding LCN2 (LV-LCN2). We

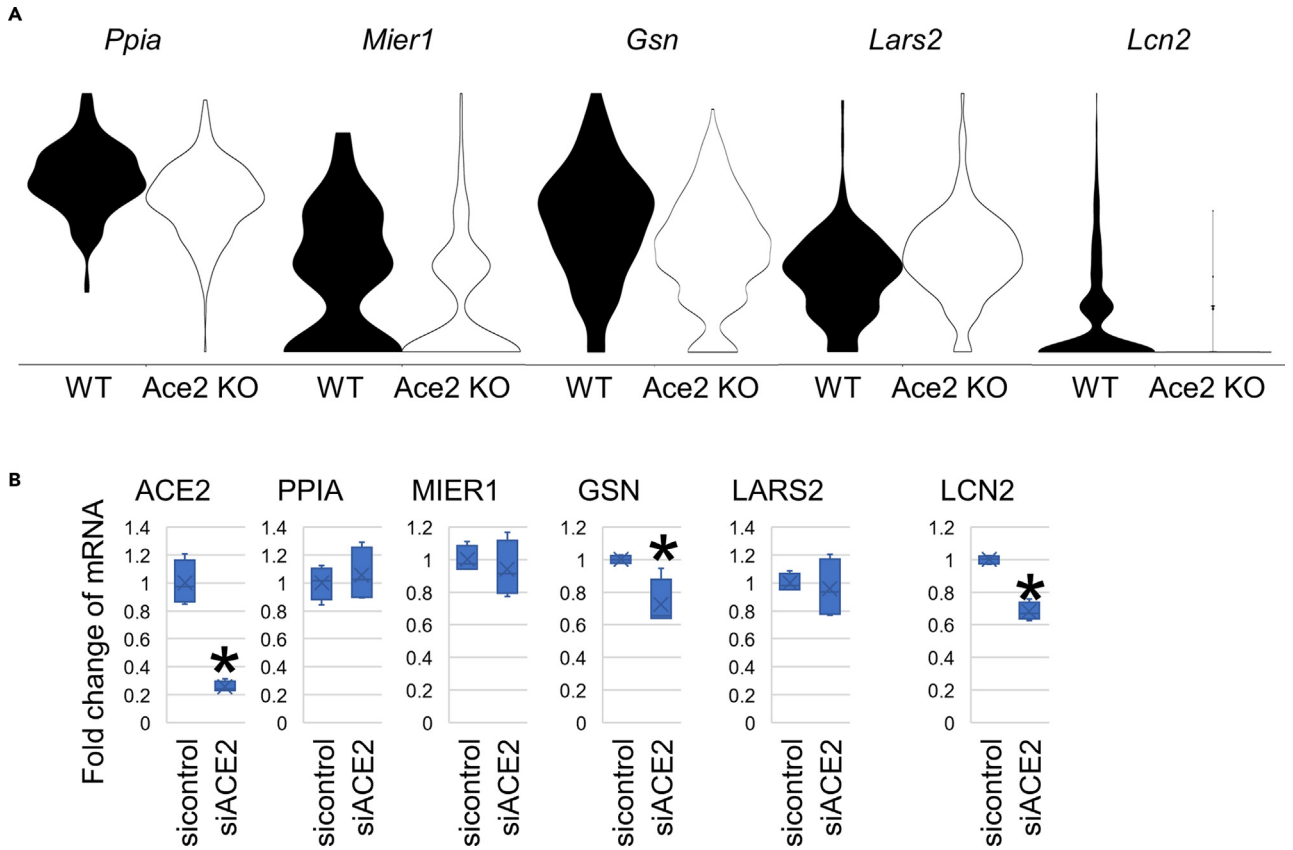


Figure 3. scRNA-seq identifies ACE2 downstream genes that involve cell proliferation

(A) In cluster 8 (stem/eTA cell cluster), violin plots show the expression of the top differentially expressed genes (DEGs) that are related to cell proliferation.

(B) hTCEpi transfected with siACE2 and siControl. After 72 h, total RNAs were isolated from these cells for RT-qPCR to examine the expression of the top DEGs in cluster 8. A markedly decreased expression of GSN and LCN2 was detected in cells transfected with siACE2 compared to cells transfected with siControl. Graph showed the fold changes. $N = 4$ of independent experiments. *$p < 0.05$. *Ppia*: peptidylprolyl isomerase A, *Mier1*: mesoderm induction early response 1, *Gsn*: gelsolin, *Lars2*: Leucyl-TRNA synthetase 2, *Lcn2*: lipocalin-2.

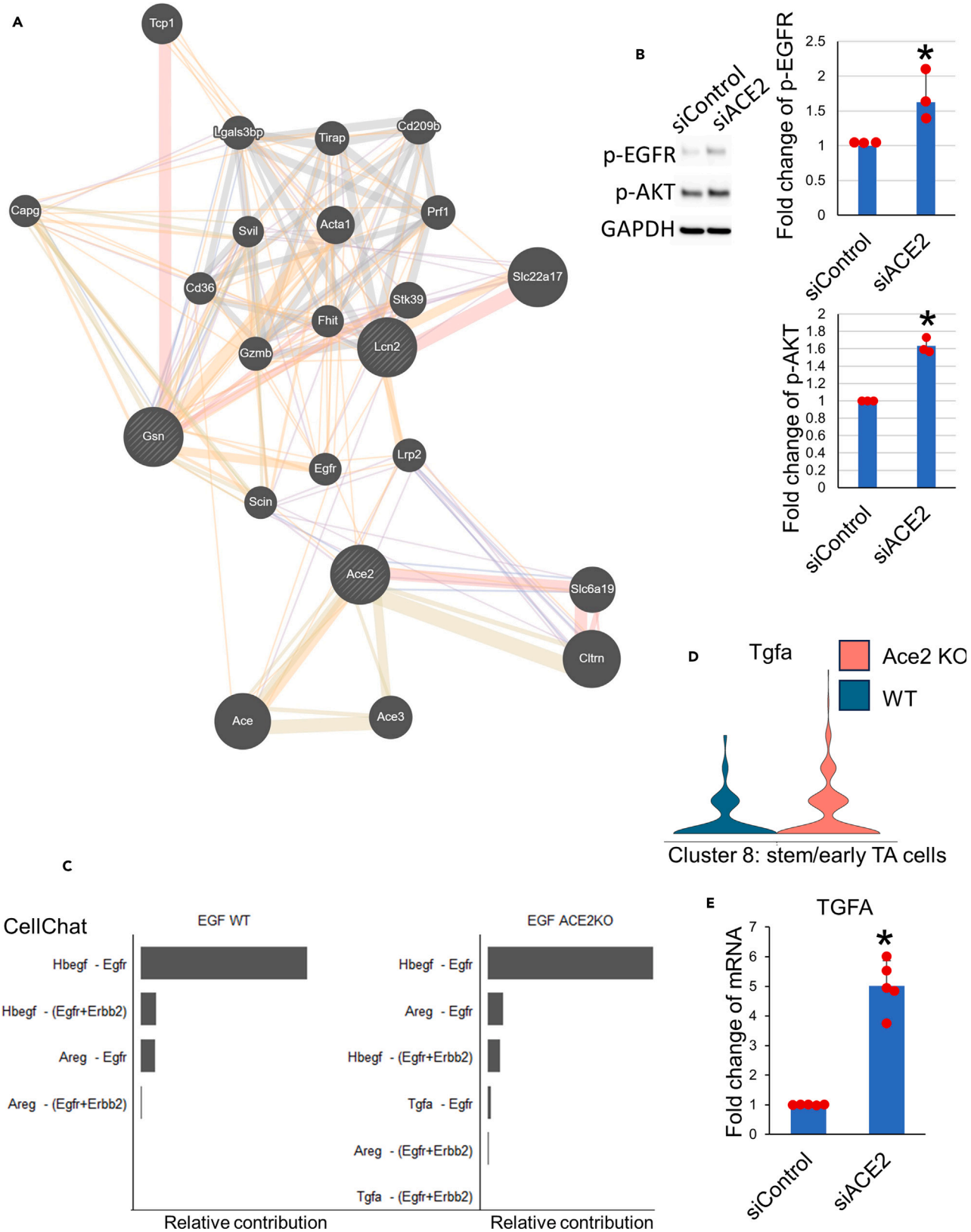
observed that overexpression of *LCN2* could rescue the increased BrdU+ cells in cells transfected with siACE2 (Figures 6A–6D and 6F). This strongly indicates that *ACE2* negatively regulates cell proliferation via upregulation of *LCN2*.

LCN2 expression is regulated by ACE2/TGFA/EGFR signaling

It has been reported that the activation of EGFR induces the expression of LCN2 in a renal tubular cell line.[51] To investigate the possible regulatory role of *ACE2* in *LCN2* expression through TGFA/EGFR signaling pathway, RT-qPCR revealed an increase in *LCN2* expression in hTCEpi cells treated with AG1478(1μM), an EGFR inhibitor, while a decrease in cells treated with TGFA (50 ng/ml) (Figure 6I).[52] However, overexpression of *LCN2* failed to alter *TGFA* expression (Figure 6H). These observations suggest that *LCN2* is a downstream effector of TGFA/EGFR signaling.

Interestingly, LCN2 plays a positive role in EGFR recycling to the plasma membrane in a mouse epithelial kidney cell line.[53] Such EGFR recycling contributes to sustained activation of EGFR signaling.[53] We conducted surface biotinylation assays to determine the level of EGFR proteins on the plasma membrane. Interestingly, in hTCEpi cells, knockdown of *LCN2* increased the level of EGFR proteins on the plasma membrane (Figure S3B). This suggests that in limbal epithelial cells, downregulation of LCN2 promotes EGFR recycling, which can result in sustained activation of EGFR.[53] Since EGFR signaling negatively regulates LCN2 expression (Figure 6I), this forms a double-negative feedback loop between EGFR and LCN2, which could facilitate switch-like behavior to turn on/off EGFR/LCN2 signaling.

Furthermore, we explored whether ACE2 regulates LCN2 in limbal epithelium *in vivo*. We conducted immunofluorescence staining with an LCN2 antibody in WT and *Ace2* KO mouse eyes. LCN2 was predominantly expressed in the basal layer of the limbal epithelium in WT mice (Figure S3C). In wild type mouse limbal epithelial basal layers, LCN2 proteins were detected by immunostaining in the cells expressing Keratin 15 (a putative LESC markers[35]; Figure S3C). These observations suggest that LCN2 may regulate proliferation of limbal epithelial stem/eTA cells in limbal epithelial basal layer. Quantification of LCN2 relative expression levels (Figure S3C) revealed that, in comparison to WT mice, *Ace2* KO mice exhibited a marked reduction in LCN2 expression levels.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Gzmb
Gsn
Lrp2
Egfr
Scin
Ace2
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Cltrn
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siControl
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GAPDH
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Ace2 KO
WT
Cluster 8: stem/early TA cells
C
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EGF WT
EGF ACE2KO
Hbegf - Egfr
Hbegf - (Egfr+Erbb2)
Areg - Egfr
Areg - (Egfr+Erbb2)
Tgfa - Egfr
Tgfa - (Egfr+Erbb2)
Relative contribution
Relative contribution
E
TGFA
Fold change of mRNA
siControl
siACE2

Figure 4. ACE2 negatively modulates TGFA/EGFR signaling

(A) Network analysis of scRNA-seq data predicts the connection between ACE2 and its downstream gene using Genemania. This indicates that ACE2 connects with *GSN* and *LCN2* through EGFR.

(B) Western blotting showed that knockdown of ACE2 increased *p*-EGFR, an active form of EGFR, as well as *p*-Akt, a downstream effector of EGFR signaling. Densitometry was performed using ImageStudio Lite. Bar graph showed the fold changes. $N = 3$ of independent experiments. $^*p < 0.05$.

(C) Cell-cell communication network and relative contribution of each ligand-receptor pair within the EGF signaling were analyzed by CellChat package. Tgfa interaction was only observed in *Ace2* KO mice.

(D) Violin plots showed that in cluster 8 of our scRNA-seq data, *Tgfa*, a ligand of EGFR, was increased in Ace2 KO mice compared to WT.

(E) RT-qPCR showed that knockdown of *ACE2* in hTCEpi cells increased *TGFA* expression. Bar graph showed the fold changes. $N = 5$ of independent experiments. $^*p < 0.05$.

Decrease in ACE2 contributes to corneal wound healing

Two decades ago, it was demonstrated that activation of proliferation in the limbal epithelium is critical for wound healing after corneal injury.[2,3] To explore whether such activation is attributed to a decrease of ACE2 levels *in vivo*, we examined ACE2 expression following mechanically or chemically induced corneal injury. To induce a mechanical wound in the central cornea, mouse central corneal epithelium (1mm) was removed using a diamond burr (debridement wounding), which induced a wound with only minimum impact on the stroma and basement membrane.[54] Immunostaining indicated that after wounding, ACE2 expression was reduced in the limbal epithelium (Figure 7A). To induce chemical injury, mouse corneas were also injured by exposure to 0.5% nitrogen mustard (NM) solution for 1 min or 1M NaOH solution for 30 s. NM is an analog of sulfur mustard (SM) and can cause severe corneal inflammation and epithelial damage in exposed eyes, which mimics perturbations caused by SM.[55] RT-qPCR demonstrated that NM induced corneal injury also resulted in a decrease in *Ace2* expression in mouse corneas (Figure S4A). Immunostaining showed those 3 days after exposure to NaOH, ACE2 expression was reduced in limbal epithelium (Figure S4B). Collectively, these observations show a decrease in epithelial ACE2 during the course of corneal wound healing.

DISCUSSION

Until recently, little if any attention has been focused on how a corneal injury activates limbal epithelial cells to proliferate. We demonstrate that in response to either a mechanical or chemical injury to the cornea, ACE2 expression is reduced *in vivo*. ScRNA seq combined with bioinformatic analysis reveal that a novel ACE2/TGFA/EGFR/LCN2 signaling pathway is involved in activating limbal epithelial proliferation. Reduction of ACE2 causes the initiation of the TGFA/EGFR signaling pathway along with the downregulation of LCN2, which results in the induction of limbal basal epithelial cell proliferation both *in vitro* and *in vivo* (Figure 7B). Importantly, this is the first demonstration that LCN2 has a role in ocular surface physiology. The Ang II/Ang-(1–7)-independent function of ACE2 has recently attracted attention because the role of ACE2 in SARS-CoV2 invasion does not require its substrate AngII. An Ang II/Ang-(1–7) independent role of *ACE2* in proliferation via regulation *LCN2* could expand our knowledge of non-canonical ACE2 signaling. It should be noted, however, that ACE2 has a substrate spectrum that goes beyond the RAS. Although Ang II and Ang-(1–7) are the main known ACE2 substrates, ACE2 can hydrolytically target several other biologically active peptides from bradykinin, apelin, and other cascade peptide systems.[56,57] We have suggested that the *Ace2* KO mouse is an ideal model for a cytokine storm-driven inflammation.[15] ACE2 has been identified as a receptor for SARS-CoV-2, which enters cells via binding to ACE2. Such infection results in the downregulation of ACE2.[58–61] It has been suggested that SARS-CoV-2-induced downregulation of ACE2 results in the cytokine storm induced inflammation.[62–65] Interestingly, in the area where severe infection of SARS-CoV-2 occurs in lungs of human patients, proliferation in epithelial basal cells is enhanced.[66] These observations support our hypothesis that downregulation of ACE2 contributes to enhanced inflammation and increased proliferation, two key processes in wound healing.

Corneal wound healing involves multiple processes including inflammation and epithelial proliferation too. Here, we demonstrated that corneal injury downregulated Ace2 expression. Such downregulation is associated with induction of corneal inflammation[15] and activation of limbal epithelial cell proliferation (Figure 7B). However, when ACE2 is completely depleted in the cornea (*Ace2* KO mice), severe corneal inflammation occurs, which can be accompanied with corneal epithelial hyperplasia.[15] The most plausible explanation for downregulation of *ACE2* expression after corneal injury might be related to disintegration of corneal areas where *ACE2* usually resides causing its loss. It is possible that this time-tuned downregulation of *ACE2* expression observed after corneal injury might have desirable consequences, i.e., for initiation in inflammation and activation of limbal epithelial proliferation, two necessary factors in wound healing. On other hand, as our previous studies suggest, a complete or prolonged Ace2 deficiency could lead to chronic inflammation and keratinization of corneal epithelium.[15] We therefore propose that a precise and balanced regulation of ACE2 expression in the cornea is critical for tissue homeostasis.

In many tissues, the expression of ACE2 and EGFR is associated with each other. For example, in human patients, expression of ACE2 and EGFR are negatively associated in ovarian tumors.[22] Knockdown of ACE2 in an ovarian cancer cell line upregulated EGFR protein.[22] More interestingly, it has been suggested that SARS-CoV-2 binds to EGFR with high affinity.[67] Infection of SARS-CoV-2, which downregulates ACE2,[58–61] increased EGFR signaling via activation of EGFR phosphorylation in Caco-2 and A549 cells expressing ACE2 (A549-ACE2 cells).[21,68,69] We have shown that knockdown of ACE2 induces phosphorylation of EGFR (Figure 4B), and inhibition of EGFR reverses the effect of reduced ACE2 on limbal epithelial cell proliferation (Figure 5). These observations indicate that ACE2 negatively regulates EGFR signaling, which has a role in cell proliferation. We have shown that chemical and mechanical injuries to the cornea, induces limbal/corneal epithelial proliferation,[2,70,71] and downregulates the expression of *Ace2* (Figures 7A and S4). It has been demonstrated that upregulation of TGFA and activation of EGFR signaling are early responses following corneal epithelial cell wounding.[72–76] These observations suggest that

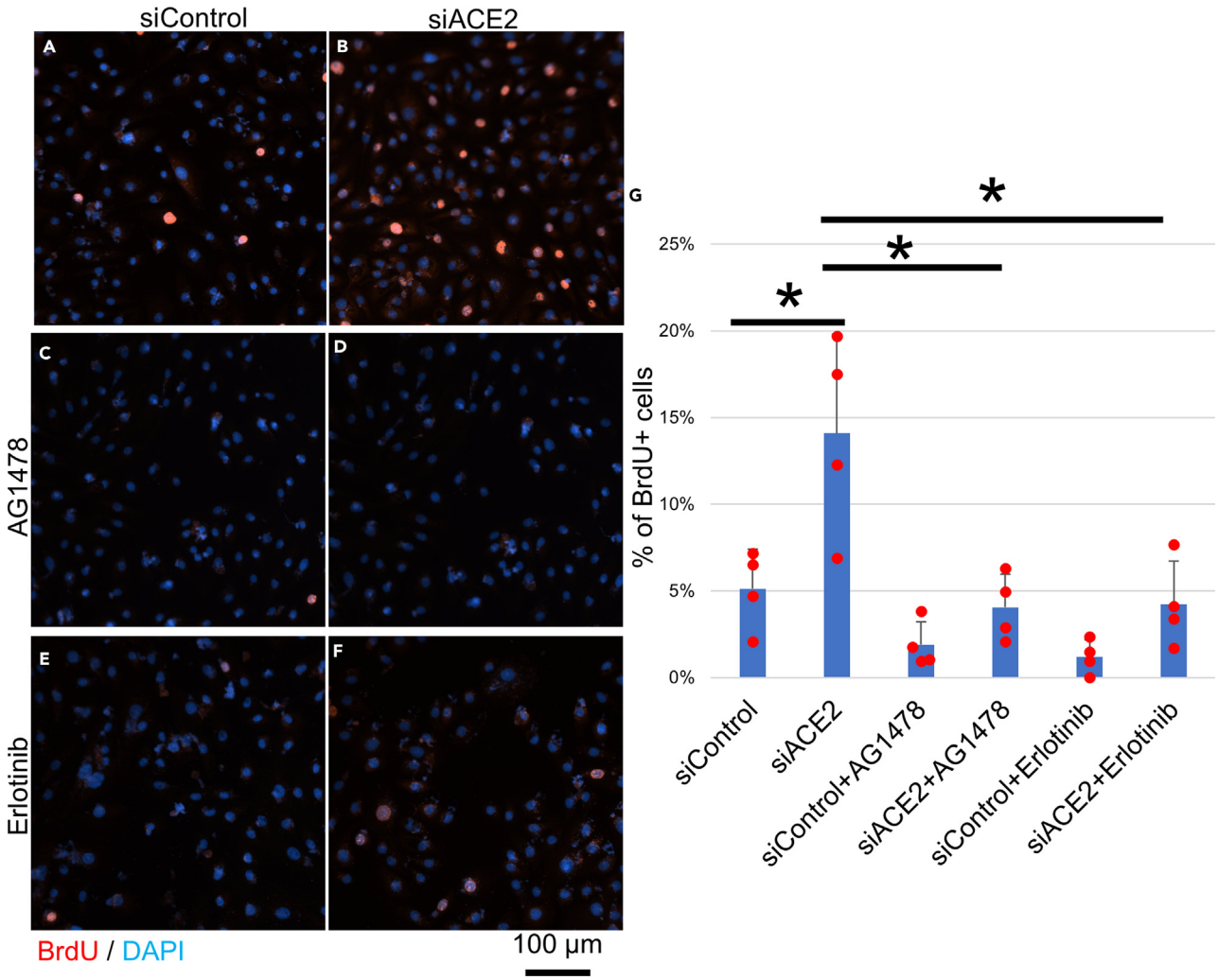

Figure 5. ACE2 negatively regulates proliferation via negatively modulates EGFR/LCN2 signaling

(A–F) Immunofluorescent microscopy visualized the BrdU+ hTCEpi cells. Cells were transfected with siControl or siACE2 for 72 h. EGFR inhibitor Erlotinib (1μM) and AG1478 (1μM) were added for 24 h before immunostaining.

(G) The percentage of BrdU+ cells were quantified by ImageJ. $N = 4$ of independent experiments. Welch ANOVA was conducted since the data had unequal variances. *$p < 0.05$.

downregulation of ACE2 and subsequent activation of TGFA/EGFR signaling play an important role in corneal wound healing. It also has been demonstrated that in human lung cancer cell lines, EGF, a ligand of EGFR, induces the expression of ACE2,[20] indicating that EGFR signaling positively regulates ACE2 expression in this cancer cell line. This suggests that there may be a feedback loop between EGFR and ACE2. It has been demonstrated that increasing the magnitude and duration of EGFR activation elicits adverse effects.[77] Thus, maintaining a balanced and controlled EGFR signaling in cornea is crucial. We posit that the feedback loop between ACE2 and EGFR plays a pivotal role in keeping EGFR signaling in check since activation of EGFR increases ACE2, which curbs EGFR ligand expression.

Previously, we have shown that ACE2 negatively regulates inflammation via Ang II/AT1R pathway.[15] Interestingly, here we demonstrate that enhanced proliferation in cells lacking ACE2 is independent of Ang II/Ang-1-7 signaling pathways, the two key branches of RAS signaling. Thus, scRNA seq assay combined with bioinformatic analysis revealed that loss of ACE2 upregulates proliferation by activating EGFR via increasing TGFA. It has been reported that the activation of EGFR in a mouse model of chronic kidney disease induces the expression of LCN2.[51,53] Such increased LCN2 facilitates recycling of EGFR and maintains EGFR activation.[51,53] Interestingly, in human limbal epithelial cells, EGFR negatively regulates LCN2 expression (Figure 6I). On the other hand, LCN2 negatively regulates EGFR recycling (Figure S3B) and thus may have an inhibitory role in EGFR sustained activation. These observations suggest a double-negative feedback loop between EGFR and LCN2 in human limbal epithelial cells. Double-negative feedback loops behave as a bistable switch and functions in controlling cell status such as cell cycle control.[78,79] Therefore, the double-negative feedback loop of EGFR/LCN2 may function as a switch to turn on/off the limbal epithelial cell proliferation.

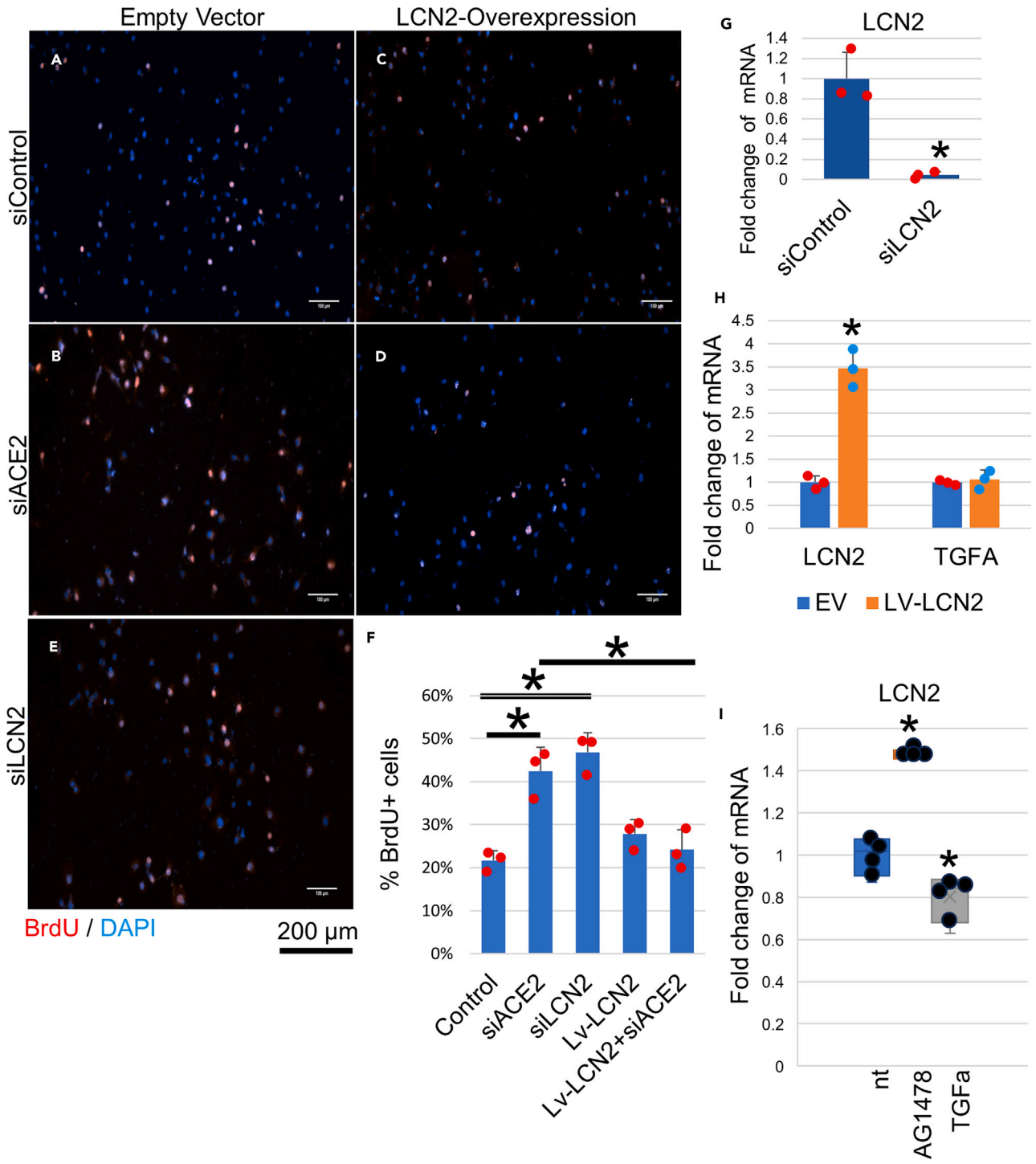

Figure 6. The limbal epithelial cell proliferation induced by knockdown of ACE2 can be rescued by overexpression of LCN2

(A–E) hTCEpi cells transduced with lentiviral-LCN2 (LV-LCN2) or lentiviral—empty vector (EV) were transfected with siControl, siACE2, or siLCN2. Immunofluorescent microscopy visualized the BrdU+ cells 72 h after transfection.

(F) The percentage of BrdU+ cells was quantified by ImageJ. *N* = 3 of independent experiments. One-way ANOVA was conducted, *$p < 0.05$.

(G) RT-qPCR results showed a significant reduction of LCN2 gene expression after siLCN2 transfection compared with siControl. Bar graph showed the fold changes. *N* = 3 of independent experiments. *$p < 0.05$. (H) hTCEpi cells were transduced with lentiviral-LCN2 (LV-LCN2) or lentiviral—empty vector (EV).

Figure 6. *Continued*

LV-LCN2 transduction resulted in a significant increase of *LCN2* while had no significant effect on the expression level of *TGFA*. Bar graph showed the fold changes. $N = 3$ of independent experiments. $^{*}p < 0.05$.

(l) RT-qPCR showed that *LCN2* expression level in hTCEpi cells was increased when treated with AG1478 (1μM) and decreased when treated with TGFA (50 ng/ml), compared with untreated cells (nt). Graph showed the fold changes. $N = 4$ of independent experiments. $^{*}p < 0.05$.

Interestingly, bulk RNA and scRNA-seq suggest that increased *Lcn2* is detected in diabetic corneas,[33,80] in which EGFR signaling is down-regulated[81] and limbal epithelial proliferation is inhibited even under injured conditions.[4] Therefore, our data provide a rationale to develop a novel therapy for decreased proliferation and delayed wound healing in diabetic corneas as well as other corneal diseases with defective proliferation by inhibition of LCN2.

Limitations of the study

We found a significant correlation between ACE2 and the activation of TGFA/EGFR signaling. However, the specific mechanism by which ACE2 regulates TGFA is still unclear, which is the drawback of this study. We demonstrated the role of ACE2/TGFA/EGFR/LCN2 pathway in limbal epithelial cell proliferation using ACE2 knockout mouse, a limbal epithelial cell line as well as primary human limbal epithelial cells. However, this study could have benefited from generating conditional knockout mice for ACE2 and LCN2, which is a logic extension of future investigations.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHODS DETAILS
  - Animal experiments
  - Single-cell isolation and scRNA-seq analysis
  - Cell transfection
  - Immunostaining
  - Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)
  - Western blotting
  - Cell cycle analysis
  - Cell surface biotinylation
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110534.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.B., S.D.M., K.L., and H.P.; methodology, H.J., M.L., W.Y., Y-K.H., D.X., E.K.N., E.D.C., P.F., N.K., J.W., and H.P.; data curation, H.J., M.L., W.Y., Y-K.H., D.X., E.K.N., E.D.C., and P.F.; investigation, H.J., M.L., W.Y., Y-K. H., D.X., E.K.N., E.D.C, P.F., H.P.; formal analysis, H.J., M.L., W.Y., Y-K.H., D.X., E.K.N., E.D.C., P.F., H.P.; validation, H.J., M.L., and H.P. ; resources, D.B., S.D.M., and K.L.; writing – original draft, H.J. and H.P.; review and editing, D.B., S.D.M., K.L., and H.P.; supervision, H.P.; project administration, H.P.; funding acquisition, H.P. All authors have read, edited, and approved the final manuscript.

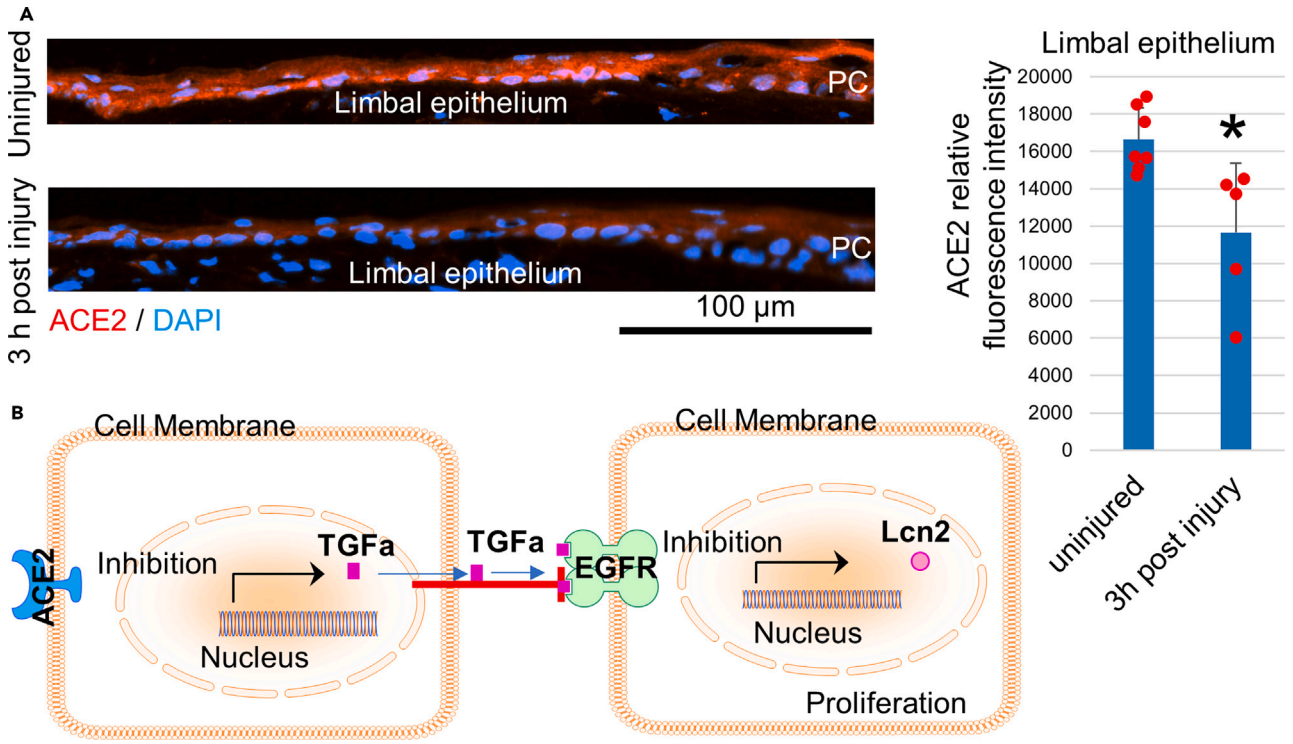

Figure 7. ACE2 expression is reduced in response to corneal injury

(A) WT mouse central corneas were subjected to debridement wounding. Three hours post injury, immunofluorescent staining showed that ACE2 expression was reduced in limbal epithelium ($N = 7$) compared to uninjured controls ($N = 5$). ACE2 relative fluorescence intensity was quantified using ImageJ. *$p < 0.05$. PC: peripheral cornea.

(B) A diagram summarizing how ACE2 negatively regulates limbal epithelial proliferation via inhibiting TGFA/EGFR signaling, which consequently modulates LCN2 expression.

DECLARATION OF INTERESTS

D Batlle and J Wysocki are coinventors of patents entitled "Active Low Molecular Weight Variants of ACE2," and "Soluble ACE2 Variants and Uses therefor." D Batlle is founder of Angiotensin Therapeutics Inc. D Batlle has received consulting fees from Advicenne unrelated to this work and received unrelated research support from a grant from AstraZeneca; J Wysocki reports scientific advisor capacity for Angiotensin Therapeutics Inc. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Krt15	Invitrogen	Cat:# MA5-11344; RRID: AB_10999819
Rabbit anti-Lcn2	Invitrogen	Cat:# PA5-116987; RRID: AB_2901617
Rabbit anti-Ace2	Proteintech	Cat:# 21115-1-AP; RRID: AB_10732845
Rabbit anti-p63	Cell Signaling Technologies	Cat:# 13109S; RRID: AB_2637091
Mouse anti-BrdU	Developmental Studies Hybridoma Bank	Cat:#G3G4; RRID: AB_1157913
Anti-Phospho-Akt	Cell Signaling Technologies	Cat:# 4060S; RRID: AB_2315049
Anti-Phospho-EGFR	Cell Signaling Technologies	Cat:# 3777S; RRID: AB_2096270
Bacterial and virus strains		
Lentiviral pseudo particles	Northwestern University Skin Disease Research Center GET IN Core Facility)	N/A
Biological samples		
Human cornea tissue	Eversight Eye Bank	N/A
Chemicals, peptides, and recombinant proteins		
Lipofectamine RNAiMAX	Thermo Fisher Scientific	Cat:# 13778150
Losartan	Sigma	Cat:# SML3317
Ang-(1–7)	Sigma	Cat:# A9202-1MG
Nitrogen mustard	Sigma	Cat:# 122564
BrdU	Sigma	Cat:#B5002
AG1478	Sigma	Cat:#T4182
Erlotinib	Sigma	Cat:# SML3621
TGFA peptide	Cell Signaling Technologies	Cat:# 44909S
Critical commercial assays		
BCA Protein Assay Kit	Thermo Fisher Scientific	Cat:# 23227
miRNeasy kit	Qiagen	Cat:# 217004
Roche FastStart Essential DNA Green Master	Roche	Cat:# 06402712001
Deposited data		
Single-cell RNA sequencing data	GEO	GSE263994
Experimental models: Cell lines		
hTCEpi cells	A gift from Dr. Robertson	N/A
Experimental models: Organisms/strains		
WT C57Bl/6 mice	The Jackson Laboratory	N/A
ACE2-deficient ($Ace2^{-/-}$) on C57Bl/6 mice	This paper	N/A
Oligonucleotides		
ACE2 siRNA	Invitrogen	CAT:# 4392420
LCN2 siRNA	Invitrogen	CAT:# AM51331
GSN siRNA	Invitrogen	CAT:# AM16708
Primers for RT-PCR: ACE2 Forward	IDT	5′- AAT TCC ATG CTA ACG GAC CCA
Primers for RT-PCR: ACE2 Reverse	IDT	5′- CTG TCA GGA AGT CGT CCA TTG T

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primers for RT-PCR: LCN2 Forward	IDT	5′- GGA GCT GAC TTC GGA ACT AAA G
Primers for RT-PCR: LCN2 Reverse	IDT	5′- CGT CGA TAC ACT GGT CGA TTG
Primers for RT-PCR: GSN Forward	IDT	5′- GCC AGA AAC AGA TCT GGA GAA T
Primers for RT-PCR: GSN Reverse	IDT	5′- TAG CTG TCG CCT CCA TAG AA
Primers for RT-PCR: PPIA Forward	IDT	5′- CAA GAC TGA GTG GTT GGA TGG
Primers for RT-PCR: PPIA Reverse	IDT	5′- GGT GAT CTT CTT GCT GGT CTT
Primers for RT-PCR: MIER1 Forward	IDT	5′- GTT TGG ACA GAG GAA GAG TGT AG
Primers for RT-PCR: MIER1 Reverse	IDT	5′- ACT GAC CTT GTT CGG ACT TTA T
Primers for RT-PCR: LARS2 Forward	IDT	5′- GCT GCT GAG TAA CAA GGA GAA
Primers for RT-PCR: LARS2 Reverse	IDT	5′- GAA ATG GGT GGT CAC CTG AG
Primers for RT-PCR: TGFA Forward	IDT	5′- CTC AGA AAC AGT GGT CTG AAG AG
Primers for RT-PCR: TGFA Reverse	IDT	5′- CAG GTG ATT ACA GGC CAA GTA G
Software and algorithms		
Cell Ranger	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest
R 4.2.1	http://www.rstudios.co	N/A
Seurat v4.3.0	https://satijalab.org/seurat/	N/A
Cell Ranger	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest
DAVID	NIH	https://david.ncifcrf.gov/
Cluster Profiler	https://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html	N/A
Genemania	https://genemania.org/	N/A
ImageJ	https://imagej.nih.gov/ij/	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Han Peng (han-peng@northwestern.edu).

Materials availability

This study did not generate new unique reagents and material.

Data and code availability

- scRNA-seq data have been deposited at NCBI GEO repository and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All the data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal procedures were approved by the Northwestern University Animal Care and Use Committee (IS00006868, IS00018311, IS00001798) and adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. w/w mice were purchased from The Jackson Laboratory and *Ace2* KO mouse was previously provided to us as a gift by Susan Gurley.[15] All mice were housed in a specific pathogen-free (SPF) environment with free access to food and water. Both male and female mice at 6–8 weeks old were used. hTCEpi cells, which were immortalized from the epithelial cells isolated from the limbal region,[36] were cultured in keratinocyte serum-free media with supplements (Thermo Fisher Scientific, Waltham, MA, USA) and 0.15 mM $CaCl_2$.[15]

Primary human limbal epithelial cells (HLECs) were isolated from cadaver donor corneas provided by Eversight Eye Bank (Ann Arbor, MI) and cultured in CnT-20 media with supplements (CellnTech; Bern, Switzerland) on collagen IV-coated plates (Corning; Tewksbury, MA).

METHODS DETAILS

Animal experiments

To generate debridement wounds (1mm) in the central corneal epithelium, a rotating diamond burr was gently applied to the surface of the central cornea to remove the corneal epithelium while the peripheral corneal and limbal epithelia remained intact. For nitrogen mustard (NM) injury, mouse corneas were exposed (1 min) to 0.5% NM in PBS. Three days post injury, mouse corneas were isolated as previously described.[30] For NaOH perturbation, mouse corneas were exposed (30 s) to 1M NaOH solution. Three days post injury, mouse corneas were isolated as previously described.[30] For the BrdU pulse labeling assay, mice were injected with BrdU (50 μg/kg) intraperitoneally 1 h prior to euthanasia.[82]

Single-cell isolation and scRNA-seq analysis

Corneal and limbal tissues were harvested from 3 healthy *Ace2* KO female and 3 healthy C57/BL6 wild-type littermate control female mice. Single-cell isolation and scRNA-seq analysis were conducted as previously described.[30] Transcripts were mapped to the mm10 reference genome (GRCm38.91) using Cell Ranger. Data normalization by R Package Seurat was used to account for the differences in the cell number between samples during integration and clustering as previously described.[30,83] For data alignment, we selected 2,000 highly variable genes in each data matrix and performed 'FindIntegrationAnchors' and 'IntegrateData' functions. Next, we performed the clustering using 'FindClusters' in Seurat to identify cell type clusters and yielded 14 clusters. UMAP of each dataset were visualized. The Seurat FindConservedMarkers() function was used to obtain a list of marker genes conserved across conditions. These marker genes for each cluster were used to annotate these 14 clusters as specific cell types. Dot Plot was used to visualize conserved cell type markers and show both the expression level and the percentage of cells in a cluster expressing those marker genes. 'FindMarkers' was used to identify the differentially expressed genes (DEG) between WT vs. *Ace2* KO in each cluster. Cell-cell communication network and relative contribution of EGF pathway were analyzed by CellChat package.[84] Functional Annotation Clustering of DEGs was performed in DAVID Functional Annotation Bioinformatics Resources. The clusterProfiler package was also utilized to conduct functional enrichment analysis of Gene Ontology (GO) terms on DEGs.[85] Genemania program in Cytoscape was used to predict a network connecting between *Ace2, Lcn2* and *Gsn*. The scRNA seq data that support the findings of this study are submitted to NCBI GEO database (GSE263994).

Cell transfection

For siRNA studies, cells were transfected with a 5 nM siRNA pool against ACE2, LCN2, GSN (Invitrogen, Carlsbad, CA, USA) or nontarget control (Invitrogen, Carlsbad, CA, USA) as previously described.[82] For lentiviral transduction, cells were incubated within lentiviral supernatants (produced by the Northwestern University Skin Disease Research Center GET IN Core Facility) for 6 h and then switched to fresh growth medium.[71] Cells were treated with losartan (50 μM), Ang-(1–7) (1 μM), AG1478 (1μM), Erlotinib (1μM), and TGFA peptide (50 ng/mL) for 24 h. Cells incubated with BrdU (10μM) for 1h were used for the BrdU pulse labeling assay.[82] Total RNA was extracted for RT-qPCR as described below.

Immunostaining

Eyes were embedded for optimal cutting temperature compound (OCT) sections. OCT sections were fixed with 4% PFA at room temperature for 10min. After blocking in 10% goat serum, sections were incubated overnight at 4°C with primary antibodies: Krt15 mouse monoclonal antibody, LCN2 rabbit polyclonal antibody (Invitrogen, Carlsbad, CA, USA), ACE2 rabbit polyclonal antibody (Proteintech, Rosemont, IL, USA) at 1:50 dilution, or p63 rabbit monoclonal antibody at 1:50 dilution (Cell Signaling Technologies, Massachusetts, USA). For BrdU labeling, sections with mouse eye tissues or coverslips with cells were fixed in cold methanol at −20°C for 10min. Following antigen retrieval with 2 N HCL for 30 min at room temperature, sections were incubated overnight at 4°C with BrdU mouse monoclonal antibody (G3G4, Developmental Studies Hybridoma Bank) at 1:10 dilution. Appropriate secondary antibodies conjugated to Alexa 555 or Alexa 488 (Invitrogen, Carlsbad, CA, USA) at 1:300 dilution were used to detect primary antibodies. DAPI (4′,6-diamidino-2-phenylindole) was used to label nuclei and mounted using Gelvatol. Slides were visualized with a Nikon Ti-2 (Nikon, Tokyo, Japan) microscope (Northwestern CAM core).[30] ImageJ was used for cell counting and relative fluorescence analysis. When counting positive cells, 4 random locations in each slide were analyzed. For analysis of BrdU+ cells, as BrdU is utilized for nuclear labeling, only signals within nuclei were quantified, ensuring colocalization with DAPI. Any signal not overlapping with DAPI indicated background noise originating from the cytosol. The percentages of BrdU+ cells = numbers of BrdU+ cells/(numbers of BrdU+ cells + numbers of BrdU- cells).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNAs were isolated from cells and mouse corneas and purified using a miRNeasy kit (Qiagen, Hilden, Germany). RT-qPCR was performed with a Roche LightCycler 96 System using the Roche FastStart Essential DNA Green Master (Roche, Branchburg, NJ, USA) according to the manufacturer's instructions. The primer pairs for RT-qPCR were designed using the IDT PrimerQuest Primer Design Tool (IDT, Coralville, IA, USA).

Western blotting

Proteins from total cell lysates were resolved with a precast TrisHCl gradient gel (Bio-Rad Laboratories, Hercules, CA, USA), transferred to NC membranes, blocked in 5% nonfat milk in TBS/Tween 20, and blotted with the following antibodies: GAPDH (Santa Cruz Biotechnology, Texas, USA), *p*-Akt, *p*-EGFR (Cell Signaling Technologies, Massachusetts, USA). Blots were visualized using an AZURE chemidoc system (Azure Biosystems, Dublin, CA, USA) according to the manufacturer's instructions.[30] Full Western blot images were presented as Figure S5.

Cell cycle analysis

Cells were fixed in 1 mL 80% cold ethanol and then were incubated in 500 μL propidium iodine (50 μg/mL) containing 200 μg/mL RNase A (Sigma; St. Louis, MO) at 37°C for 20 min. Cell cycle distribution was calculated from >10,000 cells using FACSCalibur Flow Cytometer (BD Biosciences; San Jose, CA).[30,86]

Cell surface biotinylation

Cells were incubated with 0.5 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (ThermoFisher Scientific) for 1 h at 4°C. To quench the reaction, 100 mM Glycine was added and incubated for 10 min at 4°C. After washing once with wash buffer, cells were harvested with lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS with protease and protein phosphatase inhibitors tablets). After centrifuged at 16 000 g for 15 min at 4°C, an equal amount of proteins was incubated with Pierce Streptavidin Magnetic Beads on a roller for 2 h. Beads were washed three times with lysis buffer priori to elution in 2X Laemmli buffer and reduction with β-mercapthoethanol for 5 min at 95°C. Full Western blot images were presented as Figure S5.

QUANTIFICATION AND STATISTICAL ANALYSIS

Unpaired t test, paired t test,one-way ANOVA and Welch ANOVA were performed to determine statistical significance. The data are shown as means ± standard deviation (SD). Statistical significance was defined as $*p < 0.05$. All experiments were replicated at least three times.