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K14 + Compound Niches are Present on the Mouse Cornea Early After Birth and Expand After Debridement Wounds

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Background: We previously identified compound niches (CNs) at the limbal:corneal border of the mouse cornea that contain corneal epithelial progenitor cells, express Keratin 8 (K8), and goblet cell mucin Muc5AC. During re-epithelialization after 2.5 mm epithelial debridement wounds, CNs migrate onto the cornea and expand in number mimicking conjunctivalization. When CNs form during development and whether they express corneal epithelial progenitor cell enriched K14 was not known. **Results:** To provide insight into corneal epithelial homeostasis, we quantify changes in expression of simple (K8, K18, K19) and stratified squamous epithelial keratins (K5, K12, K14, and K15) during postnatal development and in response to 2.5 mm wounds using quantitative polymerase chain reaction (Q-PCR), confocal imaging and immunoblots. K14 + CNs are present 7 days after birth. By 21 days, when the eyelids are open, K8, K19, and Muc5AC are also expressed in CNs. By 28 days after wounding, the corneal epithelium shows enhanced mRNA and protein expression for K14 and retains mRNA and protein for corneal epithelial specific K12. **Conclusions:** The keratin phenotype observed in corneal epithelial cells before eyelid opening is similar to that seen during wound healing. Data show K14 + corneal epithelial progenitor cells expand in number after 2.5 mm wounds. *Developmental Dynamics* 245:132–143, 2016. © 2015 The Authors. *Developmental Dynamics* published by Wiley Periodicals, Inc.

Key words: cornea; development; keratins; wound healing; epithelial cells; mouse

Submitted 14 August 2015; First Decision 22 October 2015; Accepted 23 October 2015; Published online 29 October 2015

Introduction

We previously identified compound niches (CNs) at the limbal:corneal border of the BALB/c mouse eye based on the expression of Keratin 8 (K8) and Brdu within morphologically distinct clusters of 4–20 cells (Pajoohesh-Ganji et al., 2012). The CNs on the BALB/c mouse cornea house some of the slow cycling corneal epithelial progenitor cells (Pajoohesh-Ganji et al., 2006). While all of the cells in the CNs express K8, a subpopulation also express K12 and Muc5AC and are morphologically equivalent to goblet cells (Pajoohesh-Ganji et al., 2012).

K8 is also expressed at low levels in the limbal and conjunctival epithelium and within Muc5AC+ and Muc5AC- conjunctival goblet cell clusters located ~300–500 μm from the limbus near the fornix. Like the mouse ocular surface, the human conjunctiva and limbal epithelia also express several simple epithelial keratins including K8, K18, and K19 and stratified squamous epithelial keratins including K5, K14, and K15 (Merjava et al., 2011a,b). The major keratins expressed by corneal epithelial cells are K3, K5, and K12 in the human cornea and K5 and K12 in the mouse cornea. The keratin intermediate fila-

ments play important roles in regulating cell shape and migration. Because keratin expression varies in different epithelial cell types and during epithelial differentiation, changes in keratin expression are often used to characterize changes in epithelial differentiation due to pathology including cancer (Moll et al., 2008; Loschke et al., 2015; Toivola et al., 2015).

Limbal stem cells have recently been shown to express high levels of K14 (Meller et al., 2002; Higa et al., 2009; Echevarria and Di Girolamo, 2011) and lineage tracing experiments performed by two groups in Brainbow mice using K14 promoters (Di Girolamo, et al., 2015; Amitai-Lange et al., 2015) independently confirm that K14 + progenitor cells for the corneal epithelium are found primarily at the limbus. Amitai-Lange and colleagues report “clusters” of K14 + stem cells at the limbus that, unlike most of the K14 + limbal stem cells, did not contribute to homeostasis unless the cornea was injured significantly (Amitai-Lange et al., 2015). After wounds smaller than 2.0 mm, the round nonmotile morphology and expression of K8, ki67, and

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Grant sponsor: NIH; Grant number: EY08512, EY021784, EY023106.

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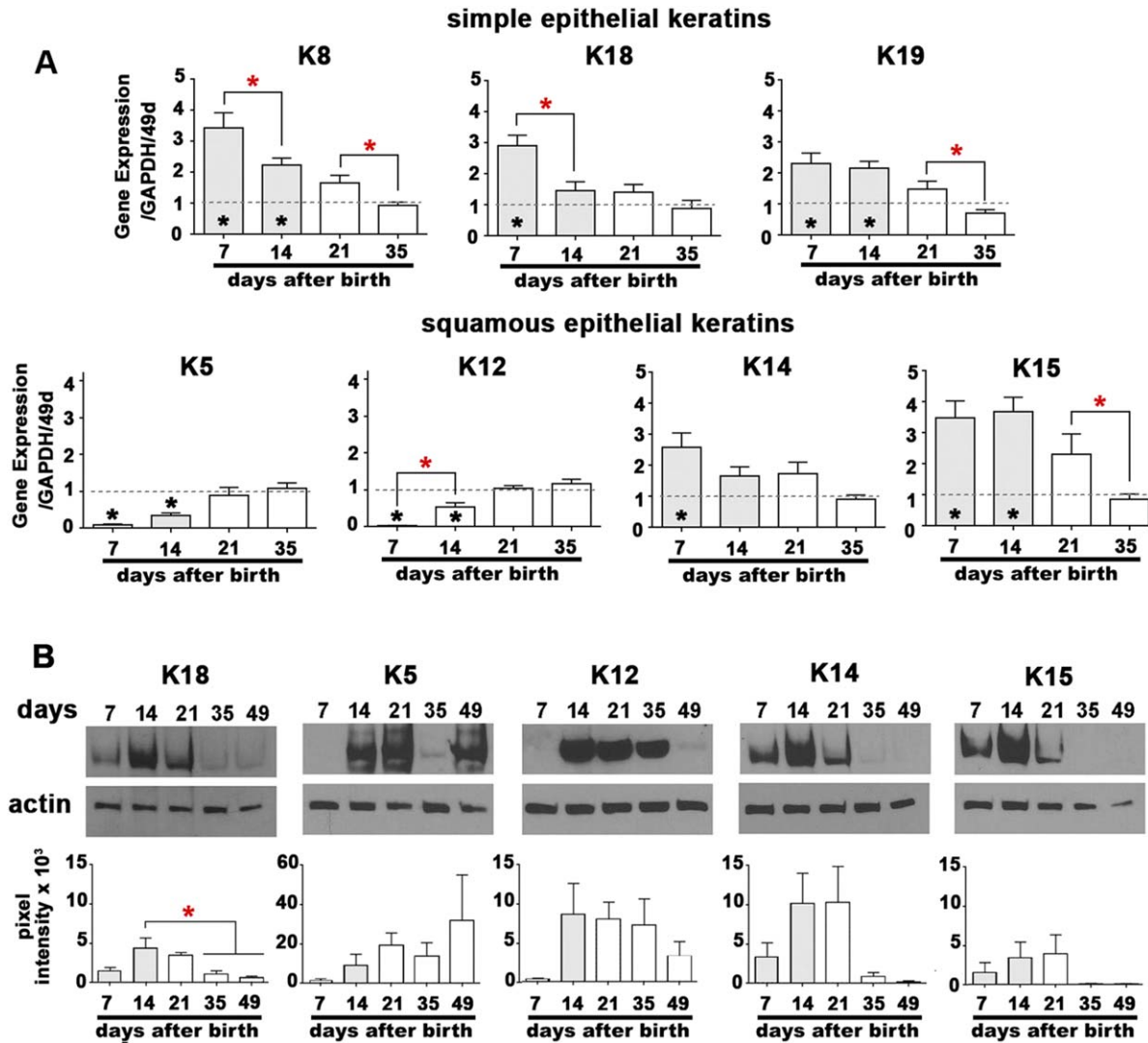


Fig. 1. Corneal epithelial cell differentiation after birth is marked by dynamic changes in keratin mRNA and protein expression. **A:** Q-PCR analyses of mouse corneal buttons at 7, 14, 21, 35, and 49 days after birth was normalized to GAPDH and the 49-day time point as indicated by dotted line. Error bars indicate SEM. The black asterisk indicates a significant difference in expression compared with control and red asterisks indicate a significant difference between time points. Data show that mRNA expression of simple epithelial (K8, K18, and K19) and two stratified squamous epithelial (K14 and K15) keratins is down-regulated after eyelid opening when differentiation of the corneal epithelium takes place, whereas, mRNA expression of the stratified squamous epithelial keratins (K5 and K12) is up-regulated as the ocular surface matures. By 21 days, levels for all the keratins assessed were similar to 49 days controls. **B:** Immunoblot analyses were conducted to assess keratin (K18, K14, K15, K5, K12) protein expression using extracts obtained from corneal buttons at 7, 14, 21, 35, and 49 days after birth. Representative immunoblots are presented. Expression of each keratin relative to actin was determined for three blots and the means obtained are presented; error bars indicate SEM. The red asterisk indicates a significant difference between time points.

Muc5AC of CNs does not change suggesting a minimal role in homeostasis (Pajoohesh-Ganji et al., 2012). By contrast, after larger 2.5 mm corneal epithelial debridement wounds, CNs migrate away from the limbus along with the rest of the migrating epithelial sheet toward the central cornea (Pajoohesh-Ganji et al., 2012). They either expand in size and number or their K8+ cells dissociate from one another and proliferate (Pajoohesh-Ganji et al., 2012; Stepp et al., 2014).

Our goal here is to use a combination of Q-PCR, confocal imaging, and immunoblots to temporally characterize expression of simple and stratified squamous epithelial keratins within the developing and wounded corneal epithelium and CNs with a focus on K14 because of its key role in limbal stem cell specification (Di Girolamo, 2015).

Results

Dynamic Changes in Keratin Expression Accompany Corneal Epithelial Cell Differentiation After Birth

To investigate the temporal changes in expression in the simple (K8, K18, K19) and stratified squamous (K5, K12, K14, K15) epithelial keratins, RNA or protein were extracted from intact corneal buttons before eyelid opening at 7 and 14 days after birth and after eyelid opening at 21 and 35 days after birth; expression was compared with corneal buttons obtained 49 days after birth (adult mice, 7 weeks of age). Q-PCR data are presented in Figure 1A. The dotted line on each graph indicates the mean for each mRNA evaluated in adult mouse tissues. The black asterisks

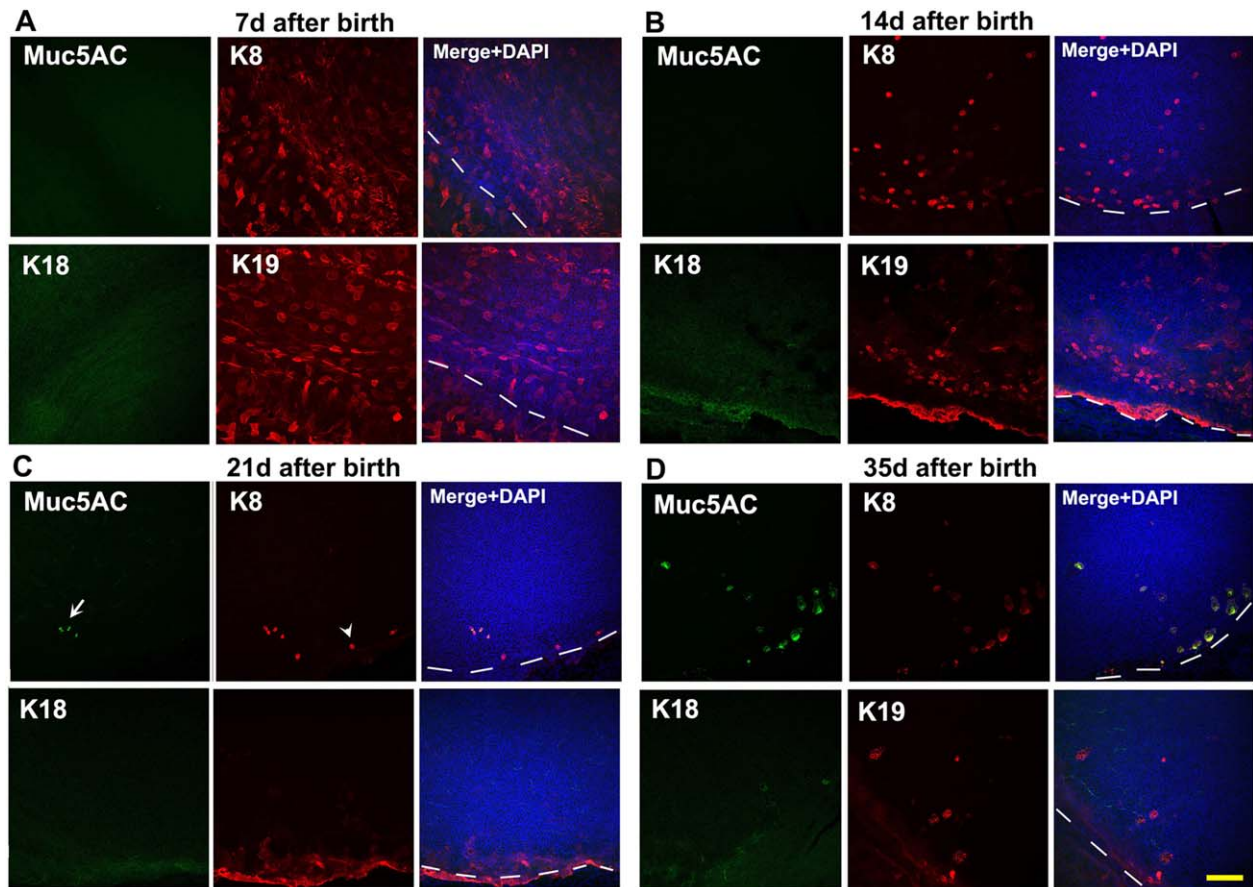


Fig. 2. Changes in simple epithelial keratin protein localization after birth correlate with mRNA expression. Eyes were stained with Muc5AC (green) or K8 (red) or with K18 (green) and K19 (red) and representative images are presented in A–D showing localization at 7 (A), 14 (B), 21 (C), and 35 (D) days after birth. All images are at $10\times$ magnification and oriented with the limbus located at the bottom and the central cornea at the top. The dotted lines indicate position of the limbus. The expression of Set as Muc5AC⁻ increases and K8 and K19 become localized to the limbus over time. K18 expression increases at the limbus at 14 days only. In C, the arrowhead indicates clusters that are K8⁺ and Muc5AC⁻, whereas the arrow shows the cells that are K8 and Muc5AC⁺. Scale bar = 200 μ m.

indicate significant differences compared with adult and the red asterisks indicate a significant difference between developmental time points.

Among simple epithelial keratins, K8 and K19 are expressed at significantly higher levels compared with adults before eyelid opening at 7 and 14 days whereas K18 is expressed at higher levels only at 7 days. K8 and K18 levels both drop significantly between 7 and 14 days and a significant decrease is also seen in K19 levels at 35 days compared with 21 days. In contrast to the simple epithelial keratins, the stratified squamous epithelial keratins K5 and K12 are expressed at significantly lower levels compared with adults before eyelid opening. K12 shows a significant increase between 7 and 14 days. The changes seen in K14 and K15 expression during corneal differentiation are similar to those seen for the simple epithelial keratins. Both are significantly elevated before eyelid opening at 7 days compared with adults and K15 expression remains elevated at 14 days. After eyelid opening, between 21 and 35 days, K15 levels significantly decrease. For K5, K12, and K14, as early as 21 days after birth, the mRNA levels assessed are similar to adults.

We next performed immunoblot analyses over time after birth by harvesting corneal buttons and extracting proteins from three sets of pooled samples (Fig. 1B). K8 and K19 antibodies did not

work for immunoblots but K18 showed a significant decrease beginning 14 days after birth. While K14 and K15 were easily detected at 14 and 21 days after birth, they were barely detected in corneal extracts at 35 and 49 days after birth. Immunoblots for K5 and K12 revealed variability between triplicate sets of pooled corneal epithelial extracts rendering the data not statistically significant. Yet, there is a trend for K5 to increase over time after birth; K12 also increases between 7 and 14 days and plateaus through 35 days. While a decrease in K12 protein is observed between 35 and 49 days in three pooled extracts, its significance is not clear because it does not correspond with changes in K12 protein localization determined by immunofluorescence (IF) shown below.

The changes seen in keratins by immunoblot are consistent with transcriptional regulation of keratin expression during early development for K5, K12, and K15. The increase in K12 expression during development was expected because K12 is considered a marker for the differentiated corneal epithelium (Tanifuji-Terai et al., 2006). K14 protein levels are similar between 14 and 21 days before decreasing at 35 and 49 days. Although K14 mRNA is significantly higher than adults before eyelid opening, it drops to adult levels by 14 days suggesting translational regulation of K14 protein expression between 14 and 21 days after birth.

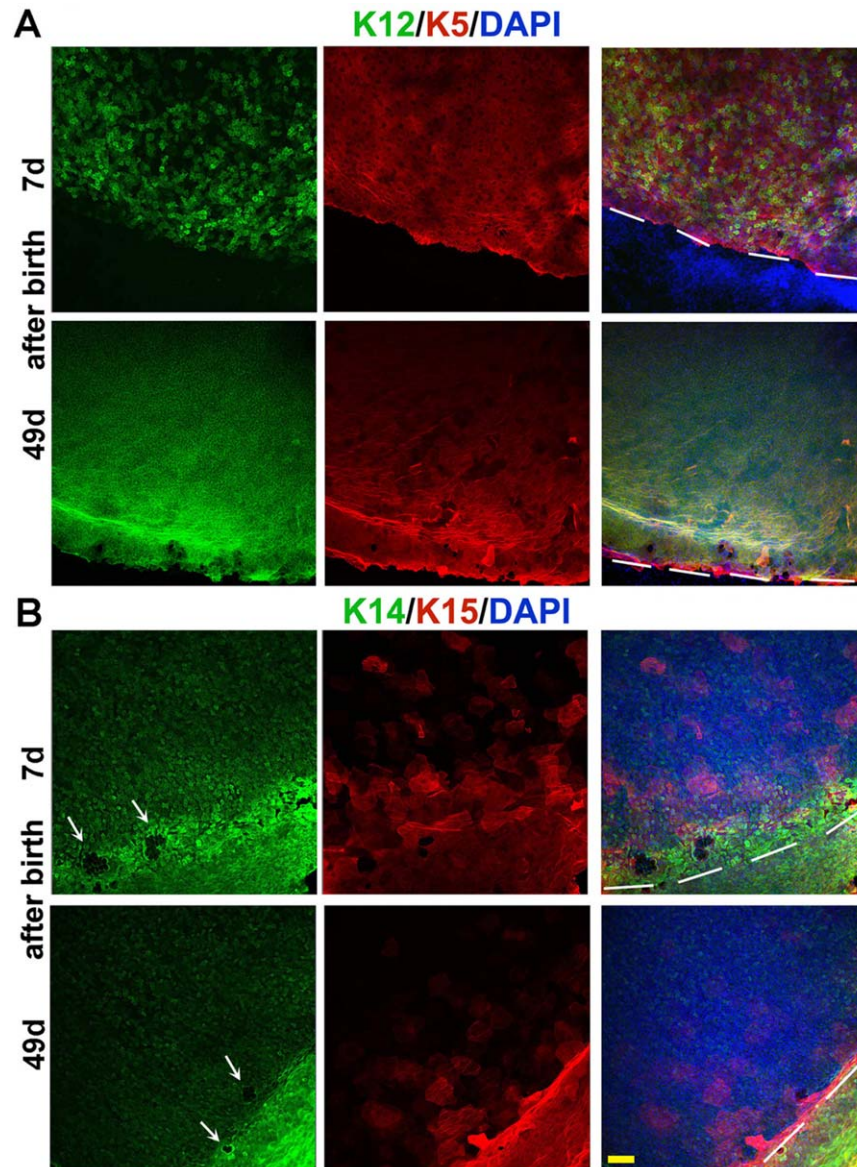


Fig. 3. The localization of stratified squamous epithelial keratins also change during postnatal corneal development. Eyes were stained with K12 (green) and K5 (red) in **A** or with K14 (green) and K15 (red) in **B**; DAPI (blue) is used to show nuclei. Each panel shows a representative image taken from a cornea obtained either 7 or 49 days (7 weeks/adult) after birth. Each image shown was obtained at $10\times$ magnification and is oriented with the limbus located at the bottom and the central cornea at the top. The dotted lines indicate the position of the limbus. **A:** K12 localization is detected in a subpopulation of corneal epithelial cells before eyelid opening at 7 days whereas it is expressed in most cells in adult corneas. By contrast, K5 is expressed at high levels within corneal epithelial cells before and after eyelid opening. **B:** K14 is expressed within cells in the central cornea before eyelid opening 7 days after birth but not in adults. Before eyelid opening, K15 localization is limited to squames and basal cells and squames at the periphery and in adults is primarily restricted to the limbus. Clusters of K14+ cells with morphologies similar to CNs are evident as early as 7 days and are also seen at in adult corneas as indicated by arrows in Figure 3B. Scale bar = $100\ \mu\text{m}$.

Changes in Keratin Protein Localization After Birth Confirm the Changes in mRNA Expression

To confirm the gene expression data in the postnatal cornea and to determine when CNs first become detectable during corneal epithelial differentiation, we performed IF studies. Figure 2 shows data for 7, 14, 21, and 35 days in panels A, B, C, and D, respectively. All images presented are at $10\times$ magnification and oriented with the limbus located at the bottom and the central cornea at the top. K8 and K19 have similar patterns of localization at all time points assessed. At 7 days after birth, K8 and

K19 are present in basal and suprabasal cells throughout the corneal epithelium and limbus (as indicated by the dashed line), as well as in the conjunctiva (Fig. 2A). K18 and Muc5AC are absent at this time point. At 14 days after birth around the time of eyelid opening, K8 and K19 localize to fewer cells on the central cornea and are more restricted toward the peripheral and limbal region. K8+K19+ CNs are not observed until after eyelid opening at 21 and 35 days. K18 is barely detected at all time points studied and Muc5AC is absent until after eyelid opening. By 21 days, although some K8+ CNs are Muc5AC+, others are not; by 35 days, most K8+ CNs are Muc5AC+. Unwounded

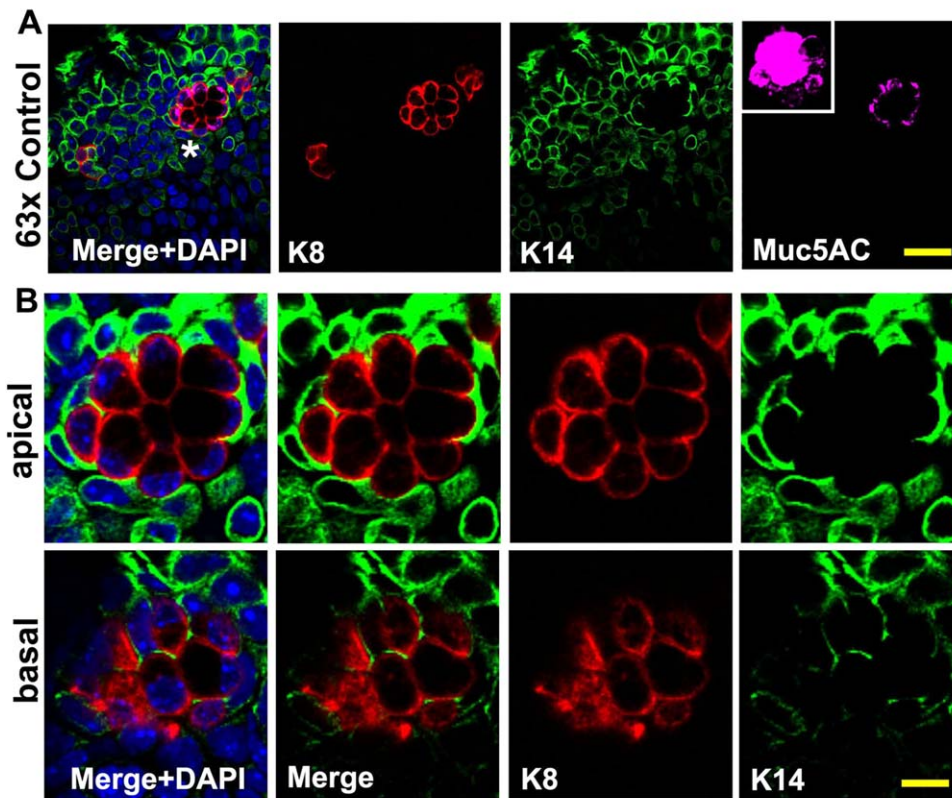


Fig. 4. While K8 and K14 localize within the same cells of the compound niche, they rarely colocalize. Flat mounted corneas from adult mice were used for immunofluorescence to visualize K8, K14, and Muc5AC. **A:** Shows a merged 63 × image showing K8 (red), K14 (green), Muc5AC (magenta), and DAPI (blue) as well as images showing K8, K14, or Muc5AC alone. The insert shows Muc5AC staining in the apical layers where it is preferentially localized. Scale bar = 20 μm. **B:** The CN highlighted by the asterisk was magnified 3.5-fold and is shown. The magenta color (Muc5AC) has been eliminated in these images. The first row in B shows an image projected through an apical focal plane whereas the second row shows a basal focal plane. K8 uniformly labels all the cells of the CN. K14 stains numerous surrounding cells as well as cells of the CN as shown by the basal focal plane image that show K14 within cells at the center of the cluster. K14 is less abundant in the CN than K8 and more K14 seen in the basal most images compared with the apical images. Although cells that express K14 also express K8, there is little evidence of colocalization; note that very little yellow is visible. Scale bar = 5 μm.

adult corneas have fewer K8+Muc5AC + CNs (Pajooesh-Ganji et al., 2012) compared with corneas obtained 21 and 35 days after birth,

Shown in Figure 3A, K5 and K12 are localized within the basal and apical cells of the central and peripheral cornea and limbus as early as 7 days after birth; neither extend past the limbus onto the conjunctiva. K12 expression within basal cells increases over time compared with 7 days, whereas K5 expression remains constant through 49 days after birth. Both K5 and K12 become more enriched within squames over time after birth. K14 staining is present before eyelid opening at 7 days in basal cells over the entire mouse ocular surface with higher expression at the limbal region and conjunctiva compared with the central cornea as shown in Figure 3B. At 7 days, K15 is expressed uniformly in limbal and conjunctival cells but in the corneal periphery and center localization is restricted to well spread apical cells. After eyelid opening, K14 and K15 localization within the central corneal epithelium decreases over time but remains strong at the limbus and conjunctiva as shown at 49 days. K14 + cells with morphologies similar to CNs are evident at 7 and 49 days as indicated by the arrows in Figure 3B. The 7-day time point is before CNs can be detected by staining with K8 or K19 antibodies (see Fig. 2A,B).

To clarify whether K14 + clusters of cells are also K8+, we next stained adult corneas to reveal the localization of K14 and K8 along with Muc5AC using high resolution confocal imaging and data are presented in Figure 4 where a representative 63 × image shows the localization of K8 (red), K14 (green), Muc5AC (magenta), and DAPI (blue) in Figure 4A. The 3D confocal stack is presented as a projection image through the corneal epithelium. When the K8 + CN highlighted by the asterisk in Figure 4A is magnified 3.5-fold and shown (without the Muc5AC staining) as an apical or basal focal plane in Figure 4B, it is clear that K8 and K14 are not colocalizing as indicated by the small amount of yellow. The apical focal plane suggests that K14 is expressed only on cells that surround the CN. Yet, when a focal plane closer to the basement membrane is presented, it becomes clearer that cells within the center of the clusters express K14 and K8.

To summarize, CNs defined by morphology, K8, and Muc5AC expression, do not appear until 21 days. However, if defined by morphology and K14 expression, CNs are seen 7 days after birth. We extend here our characterization of keratin expression in the quiescent adult mouse CN to include K19 and K14 in addition to K8 and K12. While we cannot exclude K5, K15, and K18 as components of the CN intermediate filament cytoskeleton, their low expression levels make them difficult to verify.

Removal of 2.5 mm of Corneal Epithelium at the Corneal Center Induces Alterations in mRNA Expression of Several Epithelial Keratins Over Time After Wounding

To determine whether corneal regeneration after 2.5 mm debridement wounds induces changes in expression and localization of keratin mRNAs, mice were wounded and sacrificed at 1, 2, 7, 14, 28, and 42 days after injury; an uninjured cohort was used as control. For this size wound, re-epithelialization is complete between 2 and 3 days with 100% of the wounded corneas showing sealed wound margins by 3 days after wounding (Pal-Ghosh et al., 2004). As cell proliferation, re-stratification, and reassembly of adhesion structures take place after wound closure, the K8 + CNs that migrate onto the cornea from the limbal periphery expand in number (Pal-Ghosh et al., 2004). Between 2 and 4 weeks after wounding 80% of the corneas develop erosions. Because erosions that form after small wounds do not induce expansion of CNs (Pal-Ghosh et al., 2004), erosion formation is independent of CN expansion.

Q-PCR analyses were performed and results shown in Figure 5A; keratin mRNA expression has been normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control values were set at 1 and all other values expressed relative to controls. The dashed lines represent the normalized mean of each mRNA in control corneal epithelia. Data are presented before (1 and 2 days, gray bars) and after (7, 14, 28, and 42 days) re-epithelialization is completed. Black asterisks indicate a significant difference in expression compared with control; red asterisks indicate a significant difference between time points. Q-PCR data for the simple epithelial keratins K8, K18, and K19 show that all three are up-regulated at 1 day compared with control but K8 and K18 drop dramatically to control levels whereas K19 remained elevated at 2 days. After migration is complete, there are no significant differences in K8, K18, or K19 expression compared with control. By contrast, K5 and K12 are down-regulated significantly at 1 and 2 days after injury. At later time points, K5 expression is not significantly different from controls but K12 expression is significantly decreased at 28 days compared with control. By contrast, K14 expression at 1 day is significantly up-regulated compared with control and 2 days. At later time points, K14 remains elevated compared with control. K15 expression increases during re-epithelialization and the difference is significant by 2 days; K15 drops to levels similar to controls after closure. Taken together mRNA data indicate that during active cell migration, K8, K18, K19, and K14, are up-regulated whereas K5 and K12 are down-regulated. After re-epithelialization is complete, K14 mRNA expression remains increased several fold over controls at 28 and 42 days after wounding.

We also performed immunoblot analyses to assess keratin protein expression during wound healing (Fig. 5B). Pooled corneal buttons were used for immunoblots and experiments performed in triplicate. Because there were few epithelial cells on the corneal buttons 1 day after wounding, that time point was not included. Several of the keratin antibodies used did not yield reliable immunoblots (K8, K15, and K19); data are presented for K5, K12, and K14. There are no significant differences relative to unwounded controls for K5 and K12; the decrease seen by Q-PCR in K5 and K12 at 2 days is not observed. K14 protein shows a dramatic increase of 20-fold 2 days after wounding during active re-epithelialization compared with control. Although expression of K14 protein remains six- to seven-fold higher at 7, 14, and 28

days, one-way analysis of variance (ANOVA) comparing all time points including 2 days shows no significant differences compared with controls. However, when comparing time points after wounding when sheet movement is no longer taking place (control, 7, 14, and 28 days), K14 expression is significantly elevated compared with controls.

Changes in Keratin Protein Localization Are Seen After 2.5 mm Wounds

To evaluate changes in localization and expression of keratin mRNAs differentially expressed by the corneal epithelium during wound repair, IF staining was performed in controls and 1, 7, and 28 days after wounding using whole mount IF. Near the limbus of the 49 days control cornea, clusters of cells morphologically similar to CNs show localization of K19 and low K18 within structures similar to CNs consistent with data presented in Figure 2 from mouse corneas 35 days after birth. Figure 6A shows that by 1 day after wounding, the K19 + clusters develop a migratory phenotype similar to that reported previously for K8 + CNs (Pajoohesh-Ganji et al., 2012); K18 staining is slightly increased. At 7 days after wounding, K19 is localized to cells at the edges of large clusters whereas K18 is localized within cells at the center. The dashed line in Figure 6B shows the limbus 28 days after wounding; the area in the peripheral cornea indicated by the asterisk is shown at higher magnification on the right. By 28 days after wounding, the localization of K19 within CNs is similar to that seen for K8 + CNs near the limbal region and on the peripheral cornea (Pajoohesh-Ganji et al., 2012). K18 localization within CNs appears similar to that seen in unwounded controls.

In Figure 7, K12 and K5 keratins are localized adjacent to the limbal region (dashed lines) in control corneas (Fig. 7A) and 28 days after wounding (Fig. 7B). Beneath 10 × images, the regions highlighted by asterisks are shown at higher magnification. K5 and K12 colocalization are observed throughout all the layers of the corneal epithelium in unwounded corneas with both keratins enriched within squamous cells compared with suprabasal and basal cells. CNs are located among the K12 and K5 positive cells at the limbal border where they are visible as pores (Pajoohesh-Ganji et al., 2012). At 28 days after wounding (Fig. 7B), K12 staining is reduced within patches of K5 + cells adjacent to the limbus (asterisks); this correlates with a transient reduction in K12 expression at this time point.

Figure 8 shows K14 and K15 localization within adult unwounded corneas (Fig. 8A) as well as corneas stained 1 day (Fig. 8B) and 28 days (Fig. 8C) after 2.5 mm debridement wounds. The unwounded central cornea does not express K14 or K15; expression of these keratins is restricted to the limbus and conjunctiva. During active re-epithelialization 1 day after wounding (Fig. 8B), K14 is up-regulated within corneal epithelial cells at and behind the leading edge (LE); some K15+ cells are also observed extending toward the LE (asterisk); at the limbus, K14 and K15 localization appears similar to that seen in controls. At 7 days after wounding (data not shown) the number of cells expressing K14 and K15 remains elevated in the corneal center. By 28 days after wounding (Fig. 8C), K14 and K15 localize within cells adjacent to an erosion at the corneal center (indicated by ^); at the limbus, both keratins are present around goblet cell clusters indicated by the arrows. For K14, Q-PCR and protein data presented in Figure 5 correlate with the changes in localization shown for the protein in Figure 8C. For K15, there is no

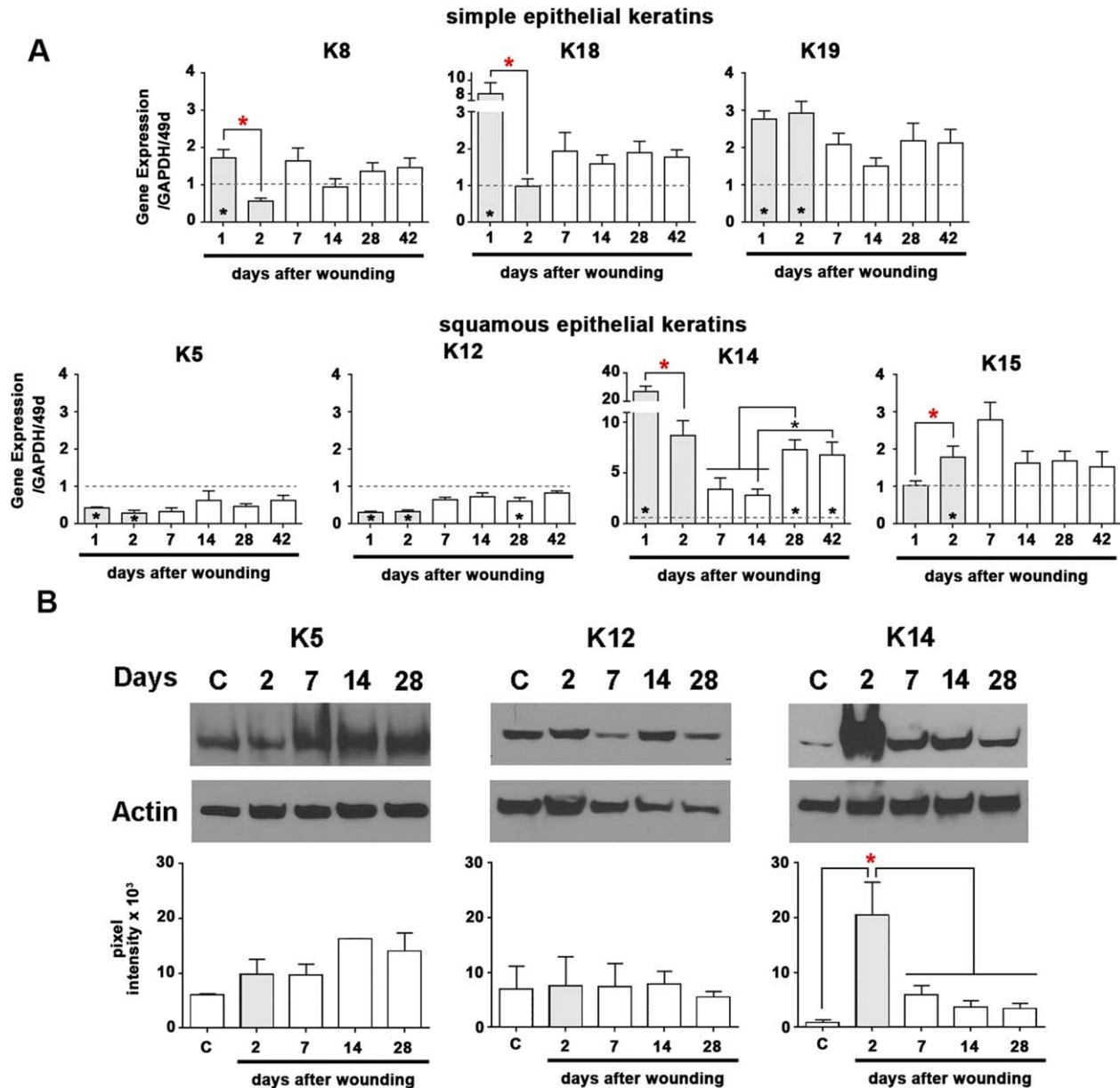


Fig. 5. Q-PCR and immunoblot analyses of corneal buttons isolated at various time points after 2.5mm wounds show retention of K12 mRNA and protein and elevation of K14 mRNA and protein after wounding. **A:** Q-PCR analyses of mouse corneal buttons 1, 2, 7, 14, 28, and 42 days after 2.5mm wounds were normalized to GAPDH and to unwounded controls as indicated by dotted line. Error bars indicate SEM. The black asterisks indicate significant differences in expression compared with unwounded controls while red asterisks indicate significant differences between time points. mRNA data indicate that during active cell migration 1 day after wounding, the simple epithelial keratins K8, K18, and K19 are all up-regulated compared with controls as is the stratified squamous epithelial keratin K14. The other two the stratified squamous epithelial keratins assessed, K5 and K12, are, by contrast, down-regulated at 1 day whereas K15 expression is transiently up-regulated at 2 days. After re-epithelialization is complete at 42 days, only K14 expression is increased several fold higher than controls. K12 is down-regulated significantly at 28 days but expressed at control levels at 14 and 42 days. **B:** Immunoblot analyses were performed on 3 independent blots for wounded samples at 2, 7, 14, and 28 days after wounding with K5, K12, and K14. Representative images for each blot are shown. The red asterisk indicates a significant difference between time points after keratin expression has been quantitated and normalized to actin in triplicate blots. The gray bars indicate time points when reepithelialization is in progress and error bars indicate SEM. The up-regulation of K14 expression at 2 days was high enough to eliminate the differences seen between control and 7–28 days for K14.

significant increase in K15 mRNA despite the increase in the number of K15+ cells.

Discussion

Centrally placed 2.5 mm wounds heal by means of a mechanism that involves migration and expansion of corneal epithelial pro-

genitor cells within CNs giving rise to both corneal epithelial cells and goblet cells on the peripheral and central cornea; wounds smaller than 2.0 mm heal without inducing CN migration (Pajoo-hesh-Ganji et al., 2012). Because the adult mouse cornea is approximately 3 mm in diameter, 2.5 mm wounds remove epithelial tissue from an area approximately 0.5 mm from the limbus; corneal epithelial progenitor cells and CNs are not removed. Due

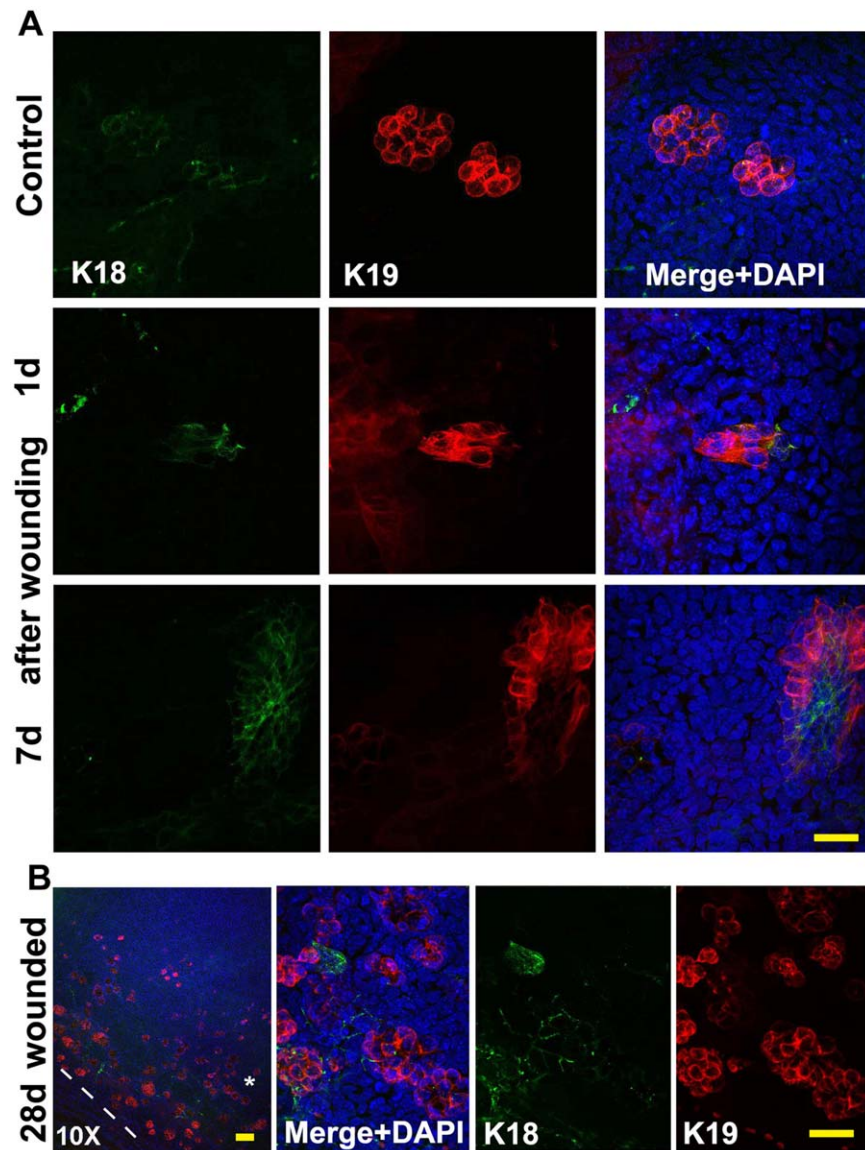


Fig. 6. K19+ CNs migrate from the limbus onto the corneal periphery after 2.5 mm wounds and expand in number and K18 localization increases transiently. Unwounded control (7–8 weeks) and wounded eyes were stained with K18 (green) and K19 (red) and DAPI (blue). Shown are control, 1 and 7 days after wounding in A and 28 days after wounding in B. All images are oriented with the limbus located at the bottom and the central cornea at the top. **A:** In unwounded control tissues, K19 localizes within in cells of the CNs where very faint K18 staining can be observed. One day after wounding, K19+ clusters are migrating away from the limbus and reveal more K18. At 7 days after wounding, clusters of K19+ and K18+ cells have expanded in size with K19 localized at the edges and K18 localized toward the center of the clusters. **B:** By 28 days after wounding, expression of K18 goes down and the localization of K19 appears similar to K8+ CNs near the limbal region. The dotted line in the 10 × image indicates the location of the limbus and the asterisk shows the area where the 40 × image was captured. Scale bar = 50 μm.

to their morphology and expression of K8 and Muc5AC, CN migration can be monitored by confocal imaging. CNs contain a small fraction of the corneal epithelial progenitor cells located at the limbus (Pajoohesh-Ganji et al., 2012).

The term “compound niche” was chosen for K8+ cell clusters because they appear to function as niches for progenitor cells that give rise to corneal epithelial and goblet cells. Evidence for multi-potential stem cells in CNs was two-fold (Pajoohesh-Ganji et al., 2012). First, slow cycling limbal stem cells within CNs are adjacent to K8+Muc5AC+ goblet cells that are K12+ indicating they likely are derived from corneal epithelial progenitors. Second, after 2.5 mm wounds, goblet cell numbers on the peripheral and central cornea increase over time, while conjunctival goblet

cells retain stationary morphologies and remain localized several hundred μm from the limbus near the fornix.

Corneal epithelial stem cell deficiency (CESCD) is characterized by several different clinical signs and symptoms (Baylis et al., 2011; Echevarria and Di Girolamo, 2011; O’Callaghan and Daniels, 2011; Deng et al., 2012; Menzel-Severing et al., 2013) but the most prominent diagnostic criteria are conjunctival epithelial and goblet cells on the cornea. Some patients diagnosed with CESCD have been successfully treated medically suggesting that they may not lack limbal stem cells but have a pathology impacting the differentiation of progenitor cells at the limbus (Kim et al., 2014). In the mouse studies presented here, expression of simple epithelial keratins by corneal epithelial and goblet cells

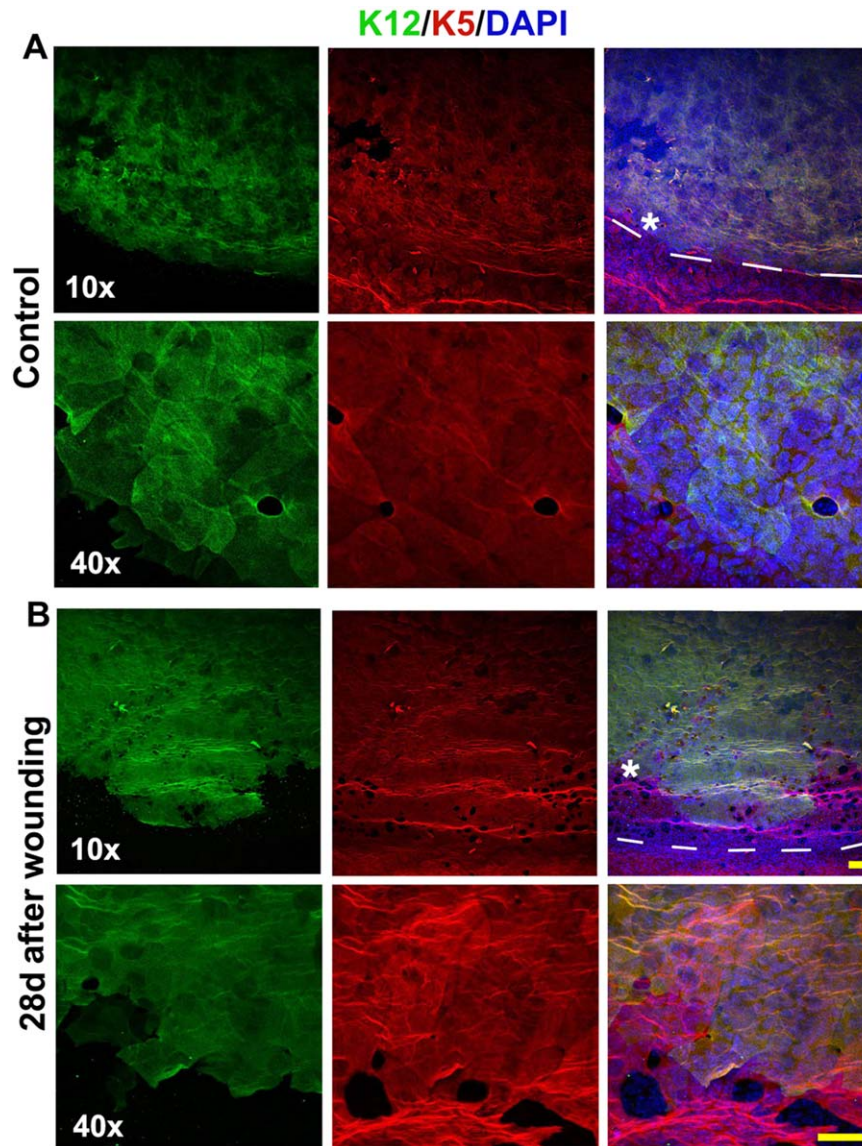


Fig. 7. K12 localization is retained by 2.5 mm wounds but K5 expression appears to increase. Eyes from unwounded control (7 weeks) are shown in A and corneas 28 days after wounding are shown in B after staining with K12 (green), K5 (red), and DAPI (blue). All images are oriented with the limbus located at the bottom. The dotted lines in the 10 × images indicate the location of the limbus and the asterisks indicate the regions shown below at 40 ×. **A:** K12 staining stops at the limbus while K5 staining continues on the conjunctiva. Both basal and apical cells express K12 and K5. In the 40 × images, CNs are visible as dark pores. **B:** At 28 days, while K12 staining is lost within discrete patches of K5 + squames at the corneal periphery adjacent to the limbus, overall, K12 localization is maintained with K5 appearing slightly increased. Scale bar = 50 μm.

after wounds near the limbus suggests CESC; however, the epithelium also expresses the stratified squamous epithelial cell keratins K12 and K14. Elevated levels of the corneal epithelial progenitor cell keratin K14 indicate increased numbers of progenitor cells.

As the ocular surface matures during development, progenitor cells derived from the surface ectoderm generate conjunctival, limbal, corneal epithelium, and goblet cells. Several mouse studies have shown that corneal epithelium converts into epidermis when key transcription factors are deleted, mutated, or expressed at reduced levels including *Dkk2* (Mukhopadhyay et al., 2006), *Notch1* (Vauclair et al., 2007; Ouyang et al., 2014), and *PAX6* (Ramaesh et al., 2005; Ouyang et al., 2014; Li et al., 2015). These data and others (Pearson et al., 2005) indicate that a default dif-

ferentiation pathway generating epidermal keratinocytes exists for corneal epithelial progenitor cells and confirm that corneal epithelial progenitor cells are multipotential progenitor cells. A report appeared recently demonstrating that conditional deletion of *TGFβR2* in K14 expressing cells leads to an expansion of the number of goblet cells in the conjunctiva accompanied by overexpression of SPDEF (McCauley et al., 2014). When mice overexpressing SPDEF in K14 + cells were studied, goblet cell numbers increase in the conjunctiva, limbus, and peripheral cornea. These data are consistent with progenitor cells at the limbus giving rise to both corneal epithelial cells and goblet cells.

To further our understanding of the events that occur during expansion and migration of CNs after debridement wounds near the limbus, we assess expression and localization of K5, K12,

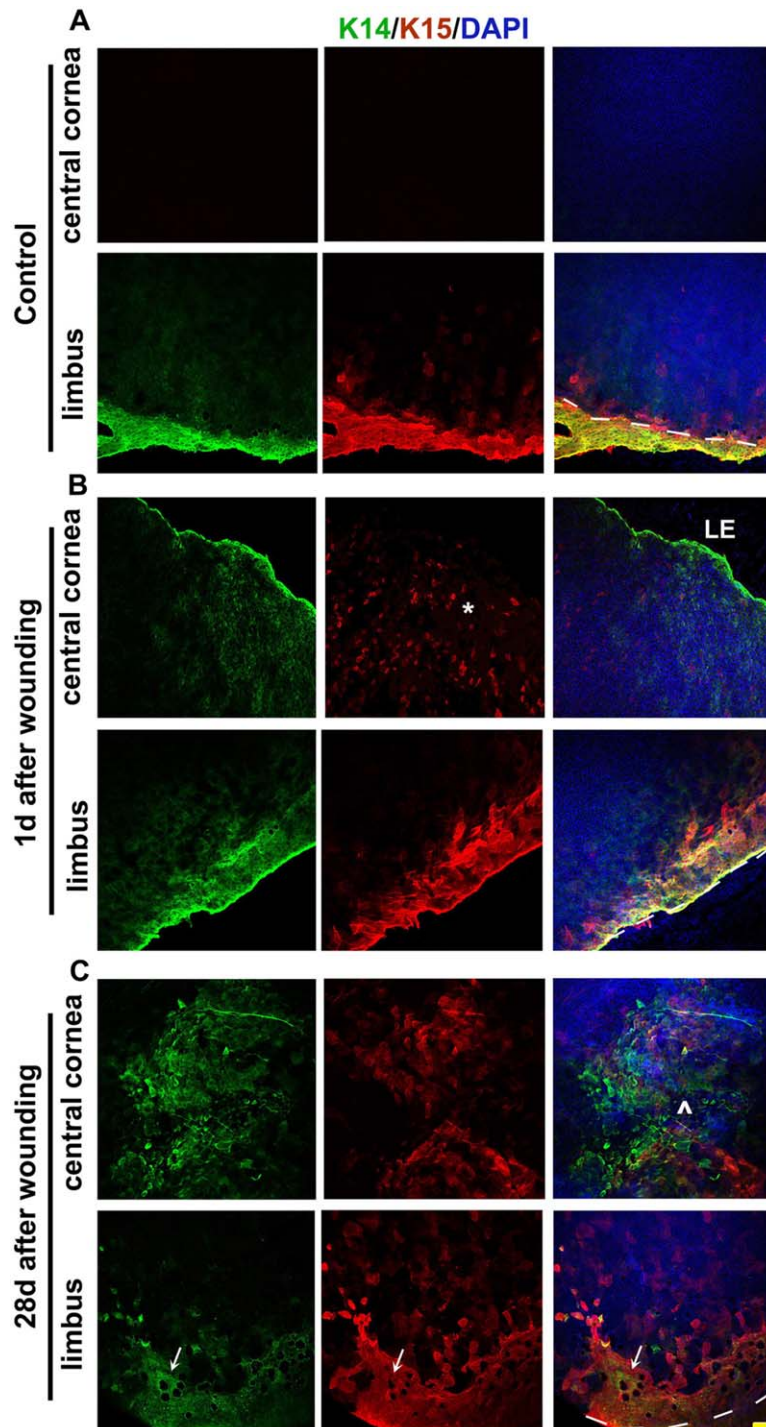


Fig. 8. K14 and K15 increase in localization in the central corneal epithelium over time after 2.5 mm wounds. Unwounded control and corneas 1 or 28 days after 2.5 mm wounds were stained to reveal the localization of K14 (green), K15 (red), and DAPI (blue) are shown in panels A (control), B (1 day after wounding), and C (28 days after wounding). The dashed lines indicate the location of the limbus. **A:** The unwounded central cornea shows no staining for either keratin; both are restricted in expression to the limbus. **B:** During active re-epithelialization at 1 day, K14 is up-regulated within the corneal epithelial cells at and behind the leading edge (LE); a subpopulation of K15+ cells also are present toward the LE (asterisk). **C:** By 28 days after wounding, K14 and K15 localize within cells of an erosion site that developed near the corneal center (indicated by \blacktriangle); at the limbus, both keratins are present around goblet cell clusters as indicated by the white arrows. Scale bar = 50 μ m.

K14, K15, K18, and K19 in the adult mouse cornea during homeostasis, at different stages of postnatal mouse corneal development and at different time points after wounding using Q-PCR, confocal imaging, and immunoblots. The current studies advance

our understanding of ocular surface homeostasis by showing that CNs contain K14+ cells. In addition, in response to wounds near the limbus, (1) corneal epithelial cells increase their expression of K14, (2) K12 expression is maintained, and (3) the keratin gene

expression profile is similar to that seen before eyelid opening when the ocular surface epithelium consists primarily of progenitor cells. Together, these data support the conclusion that the corneal epithelial progenitor cells increase in number on the central cornea after debridement injuries near the limbus.

Injury to the ocular surface near the limbus due to infection, trauma, or surgery triggers activation and proliferation of the progenitor cells. Changes in the microenvironment surrounding stem cells impact the fate acquired by their progeny. Whether proliferation of the progenitor cells generate limbal, goblet, corneal, or epidermal cells will be determined by the microenvironment around the stem cells. A significant expansion or reduction in the relative number of limbal, corneal, and goblet cells will lead to corneal opacification and blindness. Understanding how corneal epithelial and goblet cells are generated and maintained is critical for treating and curing dry eye diseases and CESCD. Now that we appreciate the plasticity of the ocular surface epithelia, we need to determine how this plasticity is regulated in health and disease.

Experimental Procedures

All studies performed comply with the George Washington University Medical Center Institutional Animal Care and Use Committee guidelines and with the ARVO Statement for the Use of Animals in Vision Research. Male BALB/c mice (NCI, Frederick, MD), 7–8 weeks old, were used for all wounding experiments described. Mice were anesthetized with ketamine/xylazine and a topical anesthetic applied to their ocular surface. A 2.5 mm trephine was used to demarcate the wound area and the epithelial tissues within the trephine area were removed using a dulled blade. After wounding, erythromycin ophthalmic ointment was applied to the injured cornea and allowed to heal for 1, 2, 7, 14, 28, and 42 days after which they were sacrificed. For Q-PCR and Western blots (WB) studies, epithelial buttons were frozen in liquid Nitrogen. For IF studies, tissues were fixed immediately (1 × phosphate buffered saline [PBS], 1% formaldehyde, 2 mM MgCl₂, 5 mM EGTA, 0.02% NP-40, Water) for 1 hr and 15 min at 4 degC, followed by 2 washes for 10 min each in 1 × PBS with 0.02%NP40 at room temperature. Tissues were placed in 4:1 Methanol: DMSO at -20 degC for 2 hr and stored in 100% Methanol. For the developmental studies, pups were sacrificed at 7, 14, 21, 35, and 49 days after birth and corneal buttons were rapidly frozen for Q-PCR and WB or whole eyes fixed for IF studies, respectively. A minimum of eight samples were analyzed for Q-PCR studies and each sample contained two corneas, except for 1 day and 2 days after wounding and 7 days after birth samples which contained four corneas. WB samples were run in triplicate and each time point had four samples. Each sample contained 6 corneas except for the 1 day time point after wounding and 7 days after birth which contained 10 corneas per sample. A total of six to eight corneas were stained for each antibody and each time point.

Antibodies

Corneas were stained with the following antibodies: K5 (Covance, #09HC1505), K8 (Developmental Studies Hybridoma Bank, Troma I), K12 (Santa Cruz, #SC-17101), K14 (Covance, #PRB-155p and RDI, #CBL197c for WB), K15 (Covance, #RBP-153p), K18 (RDI, CBL236c), K19 (Developmental Studies Hybridoma Bank, Troma

III), Muc5AC (ThermoScientific #MS-145-P), and actin for immunoblots (Millipore, #MAB1501R). Appropriate secondary DyLite 488, 594, or 647 antibodies from Jackson Immunobiologicals were used for immunolabeling. Corneas were stained with DAPI (Thermo fisher; #46190) before flat mounting to visualize nuclei. To achieve the best flattening, the corneas were placed epithelial side-up with mounting media (Fluoromount G: Electron Microscopy Sciences; #17984-25) and coverslipped.

Microscopy

Confocal microscopy was performed at the Center for Microscopy and Image Analysis (CMIA) at the George Washington University Medical Center. A confocal laser-scanning microscope (Zeiss 710) equipped with a krypton-argon laser was used to image the localization of Alexa Fluor 488 (490 excitation max; 525 emission max), Alexa Fluor 594 (590 nm excitation max; 617 emission max), or Alexa Fluor 647 (650 excitation max; 665 emission max). Optical sections ($z = 0.5 \mu\text{m}$ or $1 \mu\text{m}$) were acquired sequentially with a 10×, 40×, or 63× objective lens. Images presented were generated using Volocity software (Version 6.3, Perkin Elmer).

Q-PCR

Six corneas per sample and at least 8 samples per time point were used for the Q-PCR studies. RNA was extracted from corneal buttons using Arcturus Picopure RNA isolation kit (Applied Biosystems; #12204-01) according to the manufacturer's instructions. Q-PCR was performed using a Bio-Rad CFX384 real-time PCR detection system. The primers used were ordered from Qiagen, unless otherwise specified: K5 (#QT02262169), K8 (#QT00172228), K12 (#QT00172879), K14 (#QT00114422), K15 (#QT00122185), K18 (#QT00248997), K19 (#QT00156667), and GAPDH (Invitrogen, 267553E06). Q-PCR data are normalized against GAPDH.

Statistical Analyses

Quantitative data are presented as mean \pm standard error of the mean. All data were analyzed using one-way ANOVA. All statistical tests were performed using the GraphPad Prism Program, Version 6 (GraphPad Software, Inc. San Diego, CA). A p value < 0.05 was considered statistically significant. Q-PCR data were first normalized against GAPDH and then to either 49 days after birth (in the developmental studies) or control (in the wounding studies). For developmental studies, ANOVA was used to compare RNAs extracted from corneal buttons before eyelid opening (7 and 14 days) and after eyelid opening (21 and 28 days) to adult (49 days). This approach was taken because the dramatic changes seen in protein and mRNA expression before eyelid opening (Stepp et al., 1995; Nakamura et al., 2005) mask the differences occurring after eyelid opening. Likewise, for wound healing studies, one way ANOVA was used to compare RNA expression during active re-epithelialization (1 and 2 days) and the regeneration phase of wound healing (7, 14, 28, and 42 days) to controls. This approach was taken because dramatic increases in protein expression during the migration phase of wound healing (Takahashi et al., 1996) may mask statistical differences at later time points.

Acknowledgments

We thank Dr. Anastas Popratiloff, the director of Center for microscopy and Image Analysis (CMIA) at the George Washington University, for his help with confocal microscopy. We acknowledge and appreciate the valuable advice of Beverly Oakley, Thomas Maynard, and Christophe Cataisson on Q-PCR data analysis.

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