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BALB/c and C57BL6 mouse strains vary in their ability to heal corneal epithelial debridement wounds

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Abstract

Genetically engineered mice are usually produced on a mixed genetic background and can be derived from several mouse strains including 129SvJ, C57BL6, and BALB/c. To determine whether differences in recurrent corneal epithelial erosions (RCEEs), corneal epithelial stem cell deficiency (CESCD), and cell migration rate vary between two different mouse strains (BALB/c and C57BL6), 8 week mice were subjected to 1.5 (small) or 2.8 mm (large) manual debridement wounds and allowed to heal for 4 weeks. Syndecan-1 (sdc-1) null mice backcrossed seven generations onto a BALB/c genetic background were also included in the RCEE and CESCD studies to permit comparisons between genotypes within a single strain. After sacrifice, corneas were assessed for the presence of recurrent erosions; no fewer than 15 corneas were used for each strain or genotype studied. Data show that the frequency of recurrent erosions after small wounds was 81 $\pm$ 9% in the C57BL6 mice, 73 $\pm$ 2% in the BALB/c mice, and 32 $\pm$ 6% in sdc-1 null mice. Neither strain developed CESCD after small wounds. The frequency of erosions after large wounds was greater (88 $\pm$ 8%) in the C57BL6 mice compared to BALB/c (60 $\pm$ 2%), and sdc-1 null mice (32 $\pm$ 5%). 4 weeks after the large wounds, fixed, flat mounted corneas were assessed for evidence of CESCD with antibodies against the conjunctival keratin K8 and the goblet cell marker, the mucin Muc5AC. The frequency of CESCD 4 weeks after the large wounds was significantly greater in the C57BL6 mice than in the BALB/c or sdc-1 null mice. To assess cell migration rates, corneas were subjected to 1.5 mm wounds and allowed to heal for 12, 15, 18, 21, and 24 hours. After sacrifice, corneas were stained with Richardson stain (BALB/c) or propidium iodide (C57BL6) to assess reepithelialization rates. While reepithelialization rates were similar for the early times after wounding, by 24 hours the C57BL6 corneas had healed faster: 16 of 30 corneas from the C57BL6 mice were closed compared to 9 of 30 of the BALB/c wounds. BALB/c corneas appeared larger overall compared to C57BL6 corneas; measurements of the overall mass of the enucleated eyes and diameters of the flat-mounted corneas confirmed that C57BL6 eyes and corneas were 6.8% and 4.4% smaller respectively than those of BALB/c mice even though the masses of the two mouse strains at 8 weeks of age were identical. Using BrdU to label dividing cells, we found that 18 hours after wounding, C57BL6 and BALB/c corneal epithelia showed similar numbers of proliferating cells. To determine if the enhanced corneal epithelial cell migration rate seen in the C57BL6 mice was specific to the cornea,

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we conducted time-lapse studies to assess random cell migration rates in vitro using primary cultures of mouse epidermal keratinocytes. Consistent with the in vivo data, epidermal keratinocytes derived from BALB/c mice migrated 60% slower than C57BL6 cells. These data prove that strain-specific differences in cell migration rate in vivo are present in the cornea and are accompanied by differences in the frequencies of recurrent erosions and corneal epithelial stem cell deficiency.

Introduction

The cornea is the primary refractive surface of the eye and damage or diseases affecting it can lead to blindness. Nearly 10 million people worldwide are blind due to corneal disease or trauma making it the second leading cause of blindness following cataracts (Whitcher, et al., 2001). Due to their exposure to the elements, the epithelial cells of the cornea are frequently injured by scratches, debris, and eye rubbing secondary to allergies. The loss of the epithelial barrier increases the risk of eye infections by bacterial and fungal pathogens. Delayed reepithelialization secondary to various diseases leads to increased risk of corneal infection especially for the elderly and those with diabetes (Schultz, et al., 1981; Chikama, et al., 2007). Refractive surgical techniques make use of the cornea's refractive power to correct visual impairment resulting from myopia and astigmatism. Several million procedures are performed each year worldwide with over one million per year in the United States. In spite of improvements in LASIK, in the UK, studies have shown that LASIK significantly increased the risk of recurrent corneal epithelial erosions (RCEEs; Ramamurthi, et al., 2006). Injuries to the cornea resulting from LASIK or trachoma infection in the developing world increase the frequency of corneal scarring and RCEEs.

The stem cells that give rise to the corneal epithelium are located at the periphery of the cornea within the limbus (Stepp and Zieske, 2005; Pajooheh-Ganji and Stepp, 2005). Corneal Epithelial Stem Cell Deficiency (CESCD) due to loss of a portion or all of the corneal epithelial stem cells (CESCs) at the limbus is most often caused by chemical injury to the eye (Avunduk and Tekelioglu, 2006). Surgical procedures to treat retinal diseases and glaucoma, which involve incisions made through the limbus, can also lead to CESCD (Schwartz and Holland, 2001). Although harder to treat than injuries to the central cornea, progress is being made in the techniques to restore vision of those suffering from CESCD using CESC transplants grown on amniotic membranes (Grueterich, et al., 2003; Kenyon, 2005; Sangwan, et al., 2007).

We have been studying corneal wound healing in rodents and have developed mouse models for the study of RCEEs and CESCD using BALB/c mice (Pal-Ghosh, et al., 2004). The current study was initiated to confirm that these models also apply to C57BL6 mice. BALB/c mice remain a commonly used strain in vision research due to early studies of autoimmunity and to their use in making monoclonal antibodies. Over the past 25 years, mouse genetic engineering has led to the development of many interesting mouse models for ocular diseases which have mixed genetic backgrounds. While backcrossing transgenic and knockout mice into commonly used mouse strains such as BALB/c and C57BL6 can largely eliminate genetic differences, backcrossing is time consuming and it remains to be determined whether it should be routine for studies of corneal wound healing.

We have been maintaining a colony of syndecan-1 (sdc-1) null mice which were backcrossed seven generations onto the BALB/c genetic background and have shown that the corneal and skin wound healing of mice lacking sdc-1 is delayed (Stepp, et al., 2002) in part due to differences in epithelial cell adhesion and altered cell migration (Stepp, et al., 2007). Since the DNA sequence of the C57BL6J mouse strain has been determined (Waterston, et al., 2002), we decided to use C57BL6, BALB/c, and sdc-1 null mice on the BALB/c background to compare healing after 1.5 (small) and 2.8 (large) mm corneal epithelial debridement wounds

to look at the frequency of RCEEs and CESC4 4 weeks after wounding. We also assessed cell migration and proliferation rates *in vivo* after small corneal debridement wounds. Finally, we compared *in vivo* corneal epithelial cell migration with the cell migration *in vitro* using time-lapse studies of mouse epidermal keratinocytes isolated from both strains. Data show strain-dependent differences in erosions, stem cell deficiency, proliferation, and migration rate in response to corneal debridement wounds.

Methods

Materials and chemicals

The majority of chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO), including bovine serum albumin (BSA) and horse serum. Ketamine was purchased from Fort Dodge Veterinary Supplies (Fort Dodge, IA) and xylazine from Vedco (St. Joseph, MO). Proparacaine eye drops and erythromycin ophthalmic ointment were obtained from Baush & Lomb (Tampa, FL). We purchased slides and coverslips from Thermo Fisher Scientific (Somerville, NJ). For antibodies, we used a rat antimouse antibody against keratin-8 (K8; Troma-1 Developmental Hybridoma Data Bank, University of Iowa, Iowa City, IA), Muc5AC Ab-1 (MS 145-P1; NeoMarkers, Fremont, CA).

Manual Corneal Debridement—All experiments described in this paper were conducted in voluntary compliance with the Association for Research in Vision and Ophthalmology Guidelines for the Use of Animals in Research and all procedures were approved by the GWU Animal Care and Use Committee. The technique used has been previously described (Stepp, et al., 1990). In brief, 7–8 week old mice were anesthetized, topical anesthetic applied to their ocular surface, and corneas scraped with a dull scalpel to remove the corneal epithelium. For the small wounds, a 1.5 mm central corneal area was demarcated with a trephine and removed manually by gentle scraping with a dulled blade. For large wounds, a 2.8 mm area of epithelial tissue was removed taking care to avoid limbal epithelium. After wounding, all eyes were treated with erythromycin ophthalmic ointment to minimize inflammation and keep the ocular surface moist while the mice were under anesthesia. This procedure has been determined previously to leave the basement membrane intact and non-denatured. For studies of recurrent corneal erosion and corneal epithelial stem cell deficiency, the mice were allowed to heal *in vivo* for 4 wk. For the recurrent erosion studies, after sacrifice, eyes were enucleated and stained with Richardson stain (1:1 mixture v/v of A: 1% methylene blue and B: 1% sodium borate + 1% azure II) for albino mice or, for C57BL6 mice, eyes were fixed as for whole mounts as described below and stained with a 1:1000 dilution of a 1mg/ml propidium iodide (PI; P1304MP; Invitrogen, Eugene, OR) for 10 min and viewed under the dissecting microscope to assess erosions. For studies of corneal epithelial stem cell deficiency (CESCD), eyes were processed for whole mount staining. To study the rate of reepithelialization, after sacrifice at each time point, corneas were stained with either Richardson stain or PI and photographed using an Olympus SZX7 dissecting microscope equipped with a SZX MGFP-A fluorescent filter and Q color 5 cooled RTV digital camera. Areas of the remaining open wounds were determined using NIH Image.

Whole mounts—Eyes were enucleated and fixed for two hours with pre-chilled 4:1 dilution of 100% methanol and DMSO at -20°C and then stored in 100% methanol. Using the dissecting microscope, the retina, lens, and iris were discarded. Corneas were transferred into PBS (1× made from 10× PBS #17515Q, Lonza, Walkersville, MD) in a graded methanol series (70%, 50%, and 30% methanol and PBS, 30 minutes each). All incubations were performed with gentle shaking at room temperature unless otherwise specified. Following two 30 minute PBS washes, the corneas were incubated with blocking buffer (1% BSA and horse serum in 1× PBS) for 2 hours. The tissues were then incubated with primary antibody overnight at 4°C . The next

day, corneas were washed five times with PBS and 0.02% Tween 20 in (PBST) for 1 hour each. The tissues were blocked for 2 hours and then incubated with secondary antibodies conjugated to Alexa 488 (K8) and/or Alexa 594 (Muc5AC) overnight at 4°C. The eyes were finally washed 3 times with PBST for one hour each and then stained with a 1:2000 dilution of a 5mg/ml stock solution of DAPI (D21490; Invitrogen, Eugene, OR) for 5 min. To achieve the best result in flattening the corneas, four incisions were made in each cornea and placed epithelial side up before adding Fluoromount G mounting media (17984-25, EMS, Hattfield, PA) and cover slipping. The images were captured at 20× with a Nikon Eclipse E600 upright microscope equipped with a RT Slider SPOT camera and SPOT Advanced 4.5 Image acquisition software. Only K8 staining is shown in the images; goblet cells were generally both K8 and Muc5AC positive but the intensity of the K8 staining made it hard to see both markers simultaneously.

Bromodeoxyuridine (BrdU) cell proliferation analyses—Cell proliferation was assessed using the BrdU Labeling and Detection Kit (Roche Diagnostics; Indianapolis, IN), as recommended by the manufacturer. Control and wounded mice were injected with 500 µl of a 0.5 mg/ml solution of BrdU 2 hours prior to sacrifice. Eyes were fixed and processed as for whole mounts. Corneas were rehydrated in PBS and re-fixed in ice-cold 70% methanol in 50 mM glycine buffer, pH-2, for 20 minutes at -20°C. After fixation, corneas were incubated in PBS twice for 10 minutes each, then with 2.5% trypsin in 0.1% CaCl₂ in PBS for 10 minutes at room temperature, and then transferred to a solution containing 4 M HCl for 10 minutes at room temperature. Corneas were washed twice for 5 minutes each in PBS and then transferred to blocking buffer and then processed for immunofluorescence. The number of labeled cells in 4 different fields representing the 4 quadrants from each cornea were counted; regions selected for counting were 0.5–1 mm from the limbus in both control and wounded corneas to minimize any differences that might arise near the leading edges. Data are expressed as the number of cells labeled per unit area and analyzed for significance using the unpaired t-test.

Primary mouse keratinocyte cell culture

BALB/c and C57BL6 neonates were obtained from NCI-Frederick (Frederick, MD). Tissue culture media (S-MEM), stocks, and buffers were obtained from Gibco/Invitrogen (Carlesbad, CA) unless otherwise indicated. Primary mouse keratinocytes were isolated from skin of newborn mice as described (Dlugosz et al., 1995), resuspended in freezing media (S-MEM with 8% FCS fetal calf serum, 1.4 mM calcium, 10% dimethylsulfoxide, 10 mM Hepes, pH=7.3) and stored in liquid nitrogen. For time-lapse experiments, primary keratinocytes were grown in low-calcium media (S-MEM and 8% FCS with calcium concentration 0.05 mM) for 3 days. Tissue culture plates were routinely coated with a mixture of human plasma fibronectin and collagen I (FNCNI; 10 µg/ml human plasma FN (354008; BD Pharmingen, San Jose, CA); 1% Vitrogen (v:v) and 100 µg/ml bovine serum albumin (BSA) in S-MEM) for 15 minutes at 37°C prior to plating keratinocytes.

Time-Lapse Studies

Cells were seeded on 24 well plates and allowed to grow for 3 days before imaging on an Olympus IX81 research microscope (Olympus America, Melville, NY) equipped with a Proscan motorized stage (Prior Scientific Instruments Ltd., Rockland, MA) and placed in a temperature and CO₂ controlled chamber (LiveCell Incubation System, Neue Biosciences, Camp Hill PA). Using relief-contrast optics, 10× images were taken of each well every 10 min for 16 h 40 m (100 images). For each variable, triplicate wells were tracked and images managed using Slidebook 4.0 Digital Microscopy Solutions software (Intelligent Imaging Innovations, Denver CO, 80216). Images were transferred to a workstation equipped with Metamorph image analysis software (Molecular Devices Corporations, Chicago, IL) and velocities of 20 cells were calculated using the track cell module. A visual basic program was

written to allow us to choose the cells to track randomly from each field and to assist in data analysis. A minimum of three different sets of C57BL/6 and BALB/c keratinocytes were used for time-lapse analyses and the cells were tracked in triplicate wells. A minimum of 120 cells were tracked for each mouse strain studied.

Statistics

Data were subjected to statistical analyses using InStat software (Graphpad Software, Inc., San Diego, CA). Unless indicated, differences between groups were determined by two tail unpaired t-tests and data were considered significant for p values of less than or equal to 0.05. When standard deviations between groups were not equal, significance was determined using Welch corrected unpaired t tests and when the distributions of values within a data set were not Gaussian, significance was determined using the Mann-Whitney test. Bar graphs were generated using means and standard error of the mean (SEM) values.

Results

The frequency of recurrent corneal erosions varies by mouse strain and genotype

To begin to look at strain differences between mice, we first used a model for the study of recurrent corneal epithelial erosions (RCEEs). After a single manual 1.5 mm or 2.8 mm manual debridement wound to the cornea, wounds are completely reepithelialized by 30 and 60 hours respectively. However, two weeks after the wounds initially close, they begin to spontaneously re-open (Pal-Ghosh, et al., 2004). We have hypothesized that the hemidesmosomes, which must disassemble to allow reepithelialization, do not get the opportunity to reassemble properly after migration is complete leaving the epithelial sheet at risk of eroding. For these studies, shown schematically in Figure 1A, we made 1.5 or 2.8 mm manual corneal debridement wounds and allowed the corneas to heal for 4 weeks. Mice were then sacrificed and corneas stained with Richardson stain or PI to reveal the presence of erosions as shown in Figure 1B.

As reported previously (Pal-Ghosh, et al. 2004), there is no significant difference in the frequency of erosions in BALB/c mice after small compared to large wounds (Figure 1B). Both wound sizes result in 60–73% recurrent erosions in BALB/c mice, 80–85% in C57BL/6 mice, and 30–32% in *sdcl* null mice after 4 weeks. Thus, *sdcl* null mouse had a significantly reduced frequency of corneal erosions compared to BALB/c and C57BL/6 mice. Further, the C57BL/6 mice had more erosions compared to both the BALB/c and *sdcl* null mice after the large wounds. From these results, we conclude that mouse strain is important to the development of erosions and is a more significant factor after large wounds compared to small wounds. In addition, the *sdcl* null mice showed greater than 50% reduction in erosion frequency regardless of wound size.

The frequency of corneal epithelial stem cell deficiency (CESCD) varies by mouse strain and genotype

To further assess the role of mouse strain on corneal wound healing, we used a model for the study of CESCD (Pal-Ghosh, et al., 2004). 2.8 mm manual debridement wounds to the cornea are reepithelialized by 60 hours. However, beginning two weeks after the wounds initially close, loss of limbal $\alpha9\beta1$ integrin, a transit amplifying cell marker (Pal-Ghosh, et al., 2004), and the presence of goblet cells in the central cornea are observed. CESCD progresses between 2–4 weeks and stabilizes between 6–8 weeks after wounding. To study CESCD, larger debridement wounds were made and allowed to heal and CESCD was assessed as shown schematically in Figure 2A. At 4 weeks after wounding, corneas were fixed for whole mount immunofluorescent analyses and triple stained with the conjunctival keratin K8, the goblet cell marker Muc5AC, and DAPI to visualize the nuclei. The severity of CESCD was assessed in each of the 4 quadrants of the cornea and scored ranging from 0, indicating no evidence of

stem cell deficiency (no goblet cells past the limbus) to 5 (goblet cells over the entire quadrant). For the C57BL6 mice, 72 quadrants, 18 corneas, for BALB/c mice, 112 quadrants, 28 corneas, were assessed, and for the *sd-1* null for, 76 quadrants, 19 corneas, were assessed. Typical immunofluorescence data are shown in Figure 2B and are presented graphically in Figure 2C with the mean scores $\pm$ SEM and the % of quadrants receiving each score from 0–5 shown.

Unwounded BALB/c and C57BL6 corneas show few if any K8 positive goblet cells near the limbus and none present in the central cornea (Figure 2B and C). In the wounded C57BL6 corneas, goblet cells were present all the way to the central cornea (mean score = 2.1) whereas for BALB/c and *sd-1* null mice, fewer goblet cells were seen (mean scores of 1.6 and 1.75 respectively). Further, for C57BL6 mice, only 10% of the quadrants assessed were free of goblet cells past the limbus and received scores of 0 whereas for BALB/c and *sd-1* null mice, over 30% of the quadrants assessed had no goblet cells present. Thus, these data show that mouse strain is important for CESC since the C57BL6 mice were more prone to the development of stem cell deficiency compared to either BALB/c or *sd-1* null mice.

C57BL6 mice heal corneal epithelial debridement wounds faster than BALB/c mice

So far, we have focused on long term wound healing after manual debridement. While performing long terms wounds on mice, we observed that the eyes and corneas of the BALB/c mice appeared larger than those of the C57BL6 mice. To quantify any differences in gross anatomy and the sizes of the eyes and corneas, at sacrifice we weighed the mice and the eyes after enucleation and measured the diameters of the corneas on flat-mounts of the eyes using NIH image. We found that both strains weighed the same at the time of the experiments (8 weeks of age); the mass of the C57BL6 eyes was 6.8% and the diameter of the corneas was 4.4% less than of the BALB/c eyes (Figures 4A). To test whether the initial wound healing responses of C57BL6 and BALB/c mice vary, 1.5 mm manual debridement wounds were performed and the corneas allowed to heal for 12, 15, 18, 21, and 24 hours after wounding. After mice were sacrificed and Richardson and/or PI stain were used to visualize the open wound areas in BALB/c and C57BL6 mice respectively. Preliminary studies were conducted to confirm that similar results were obtained with Richardson stain or PI staining for measuring areas of open wounds (data not shown).

Data in Figure 3B and C show that the initial rate of reepithelialization was identical between genotypes but by later time points, statistically significant differences were seen with the C57BL6 strain corneal wounds closing faster than the BALB/c corneas. Not included in the data presented were results from *sd-1* null mice, which had been shown previously to heal slower than BALB/c mice (Stepp, et al., 2002). The areas presented in Figure 3C include data for closed wounds; by 24 hours, 16 of the 30 C57BL6 corneal wounds had closed whereas for the BALB/c mice, only 9 of 30 were closed. The fact that there was a time lag before the difference in migration became apparent suggests that had we performed large wounds that take longer to heal, differences may have arisen earlier. The estimated surface area of the C57BL6 corneal epithelium calculated from the diameter measurements to be $3.14(3.3/3)^2 = 8.55 \text{ mm}^2$ compared to 9.18 mm^2 in BALB/c; since 1.5 mm wounds remove 1.77 mm^2 of corneal epithelial tissue from the cornea, these wounds remove 20.7 and 19.3% of the total surface epithelium from C57BL6 and BALB/c corneas. This 1.4 % difference in the percentage of the total epithelial tissue removed by 1.5 mm debridement wounding from the two strains is unlikely to impact healing overall.

From these experiments, we do not know whether differences in cell proliferation might underlie the differences in migration rate. To study the role played by cell proliferation, we allowed the small wounds to reepithelialize for 18 hr, a time point at which the percentage of the wound closed for both mouse strains was identical (Figure 3C). We injected control and wounded mice with BrdU 2 hours before sacrifice; no fewer than 5 corneas from a minimum

of 3 mice per variable were assessed. While wounding increased cell proliferation 3–4 fold compared to unwounded control eyes, there were no significant differences between strains. Therefore, cell proliferation differences do not explain the differences in cell migration rates.

Epidermal keratinocytes derived from C57BL6 mice migrate faster in vitro compared to keratinocytes from BALB/c mice

To determine if the difference in migration rate seen at the final stages of corneal reepithelialization in C57BL6 mice was unique to corneal epithelium or an inherent property of epithelial cells derived from this mouse strain, we performed time-lapse studies to assess spontaneous cell migration in vitro using primary mouse epidermal keratinocytes derived from wt C57BL6 and BALB/c neonates. Images were acquired every 10 minutes for 16 hours and 40 minutes (100 images). Shown in Figure 4A are typical data from one such experiment with the tracks in red; the tracks from the C57BL6 keratinocytes are longer than those of the BALB/c. Time-lapse experiments were repeated three times on three different sets of keratinocytes harvested from different neonatal mice. Cell velocities were derived from the images using Metamorph Image Analysis Cell Tracking software. The individual cells tracked were selected randomly and cells that divided during the time-lapse were excluded from the analysis. Data presented in Figure 4B show that C57BL6 keratinocytes migrated 60% faster than BALB/c keratinocytes. Thus, these results show that the enhanced corneal reepithelialization rate in the C57BL6 mouse cornea is an inherent property of stratified squamous epithelial cells derived from the mouse strain and not specific to the corneal epithelium.

Discussion

Mouse strain differences alter disease phenotypes in mice

Use of transgenic and knockout strains of mice provide an important tool for testing and verification of hypotheses regarding the mechanisms regulating the healing of corneal and skin wounds. These mice are often on mixed genetic backgrounds and present challenges to those trying to use the most appropriate controls for their studies. To determine whether corneal wound healing varied among strains, we tested BALB/c and C57BL6 mice for their ability to heal manual corneal epithelial debridement wounds. To make our experimental procedures robust enough to detect differences in wound healing among strains, we included syndecan-1 (sdc-1) null mice which showed delayed reepithelialization compared the BALB/c mice (Stepp, et al., 2002). Sdc-1 null mice had been backcrossed 7 generations onto a BALB/c genetic background to minimize variation among individuals due to differences in mouse strain. Backcrossing mice is time consuming and expensive and may not be necessary for some analyses. However, without backcrossing, strain variations among individuals would contribute additional variability and further undermine attempts to derive statistically significant data from corneal wound healing studies.

BALB/c is an old strain of mice (see :<http://jaxmice.jax.org/jaxnotes/archive/443a.html>) widely used for the production of monoclonal antibodies beginning in the early 1970's and is the strain of choice for the study of autoimmunity and arthritis (Zhang, 2003). Issues related to availability and costs lead to their use for our studies (Stepp, et al., 1990). The C57BL6 mice are also an old strain, first described by Dr. C.C. Little, the founder of Jackson Labs (<http://jaxmice.jax.org/strain/000664.html>). The C57BL6/J substrain housed at the Jackson Labs was the source for DNA sequenced for the mouse genome project and are used in the development of transgenic mice. They have been shown to carry a mutation in their Cadherin 23 gene that leads to spontaneous hearing loss (Erway, et al., 1996). C57BL6 mice did not initially yield stable ES cell lines. Until recently the ES cell lines in common use for making transgenic and knockout mice were made from substrains derived from 129SvJ mice, progeny of strains originating later than those of the BALB/c and C57BL6 mice. As a result of the use

of C57BL6 and 129SvJ mouse strains, genetically engineered mice have mixed genetic backgrounds.

As mouse genetic engineering became a popular tool for biologists, scientists encountered problems arising from variability in experimental results related to differences in mouse strains. In skin cancer studies, mouse strain variation in susceptibility to tumor induction protocols has been a frequent cause for concern and has been addressed in several different studies (Mock, et al., 1998; Angel and DiGiovanni, 1999; Woodworth, et al., 2004). This variability has been used to uncover genetic factors that contribute to tumor formation in humans (reviewed in Hahn and Weinberg, 2002). Mouse strain variations in inflammation and wound healing have also been reported. The affect of cigarette smoke on the healing of skin wounds was tested in BALB/c, C56BL6, and Swiss mice (Cardoso, et al., 2007). Contraction of skin wounds in BALB/c and C57BL6 was inhibited compared to Swiss mice and each strain had its own distinct inflammatory response to injury and to cigarette smoke. Scientists developing transgenic mice have long noted that the penetrance of phenotype was impacted by variations in the strain onto which the mouse was bred. A recent study by Tylzanowski and colleagues (2007) showed that while deletion of *Noggin*, a gene important in bone development, caused lethality at 18.5 pdc in 129 $\times$ 1/SvJ mice, more moderate phenotypes were seen in CD1, DBa1, and C57BL6 mouse strains. Immunologists have often exploited differences in the immune systems of different mouse strains to probe immune cell functions. For example, IL10 deficiency in C57BL6 mice only slightly increases the risk of mice developing inflammatory bowel disease, but the same mutation in BALB/c mice greatly increases the risk and severity of disease (Beckwith, et al., 2005).

Corneal epithelial wound healing varies between BALB/c and C57BL6 mouse strains

After small wounds, the frequency of recurrent erosions was the same for both strains; yet, the *sdcl* null mice had more than 50% fewer erosions compared to either strain of wt mice. Thus, the increased $\alpha 6 \beta 4$ expression and enhanced adhesion of epithelial cells seen in vitro in cells derived from the *sdcl* null mice (Stepp, et al., 2007) appears to offer partial protection to their corneas from recurrent erosions. When we looked at CESCD, we found that stem cell loss was more profound in the C57BL6 mice compared to BALB/c and *sdcl* null mice. Since CESCD studies are analyzed after large wounds, it is possible that the increase in erosion frequency in C57BL6 mice could either cause or be caused by the increased frequency and severity of CESCD. In the *sdcl* null mice, the reduced frequency of erosions in the *sdcl* null genotype does not alter the frequency of CESCD.

$\alpha 9$ integrin positive cells at the limbus are the progeny of the corneal epithelial stem cells (Pajooesh-Ganji, et al., 2006). We know that the distribution of $\alpha 9 \beta 1$ integrin within basal cells at the limbus is the same in the C57BL6 cornea (Singh, et al., 2008, in press) and the large wounds leave these early transit amplifying cells behind (Pal-Ghosh, et al., 2004). Therefore, these data suggest that similar numbers of corneal epithelial stem cells are present in the adult BALB/c and C57BL6 corneas prior to wounding. The increased frequency of CESCD in the C57BL6 mice correlates with increased corneal epithelial cell migration in vivo and epidermal cell migration in vitro. Due to a mutation in C57BL6 mice in the cadherin 23 protein which interacts with the protocadherin 15 to form the cilia of the hair cells in the ear, this mouse strain develops adult onset hearing loss (Erway, et al., 1996; Kazmierczak, et al., 2007). Since we only studied the mice at 8 weeks of age, we are unable to determine whether older mice have more severe corneal wound healing defects. Regardless, our results affirm the importance of cadherins in retention of corneal epithelial stem cells within their niche at the limbus. Studies have shown that N-cadherin expression by osteoblasts mediates adhesion of hematopoietic stem cells within their bone marrow niche (Zhang, et al., 2003); Hayashi and colleagues (2007) have shown that N-cadherin is expressed on both melanocytes and putative corneal

epithelial stem cells at the limbus and suggested that this interaction may prove critical for retention of stem cells at the niche. It will be important to determine whether cadherin 23 plays a direct role in mediating corneal epithelial stem cell retention or whether compensation for its loss of function by another cadherin might impact the stem cell compartment in the limbus.

The rate of reepithelialization varied between the two mouse strains; previous studies had shown that corneal debridement wounds heal slower in *sd-1* null mice on a BALB/c background (Stepp, et al., 2002). While data were not significantly different at 21 hours, by 24 hours, the enhanced reepithelialization rate in C57BL/6 mice was significant. In vivo corneal reepithelialization after debridement is subject to variation due to differences in age, inflammation, innervation, and tear production. Differences in age were controlled in this study; we also rule out differences in cell proliferation rates. While the enhanced corneal reepithelialization rate seen in the C57BL/6 mice could be due to enhanced migration of the epithelial cells, other contributing factors can not be ruled out. We used time-lapse studies in vitro to prove that wt C57BL/6 epidermal keratinocytes migrated faster in culture compared to BALB/c derived cells. Thus, the difference in migration seen during corneal wound healing was due in part to inherent differences in stratified squamous epithelial cells from the two mouse strains.

An important study by Adriouch and colleagues (2002) sought to determine the reasons why mouse P2X7, an ATP-gated channel, forms smaller, less efficient pores than the human ortholog of the protein. That study led to the discovery of a single amino acid change in the P2X7 protein in C57BL/6 and DBA/2 strains of mice compared to the protein in BALB/c and wild mice which blocks its ability to respond to ATP; those data suggested that wt C57BL/6 mice might well serve as a model for humans having the same mutation in their P2X7 protein (Gu, et al., 2001; Wiley, et al., 2002). Recently, Mayo and colleagues (2008) showed that P2X7 null mice have defective reepithelialization and defects in the organization and synthesis of collagen in their corneas. There are likely numerous genetic differences that exist between the various inbred mouse strains that alter protein structure, function, and expression. At present, most of these are unknown and only one substrain of the C57BL/6 strain of mice has been subjected to complete genetic analysis. Additional research is going to be needed to develop a more complete understanding of how such differences impact corneal structure and its ability to heal after injury or in response to disease.

Sdc-1 null mice have fewer recurrent erosions but the same frequency of CESCD as BALB/c mice

We have already shown that in response to 1.5 mm debridement wounds, reepithelialization is slower in *sd-1* null mice. To begin to get at the mechanism underlying the difference in corneal reepithelialization rate we have developed in vitro models using mouse epidermal keratinocytes (Stepp, et al., 2007) and dermal fibroblasts (Jurjus, et al., 2008, in press). We have found that the epithelial cells derived from the *sd-1* null mouse are more adherent via $\alpha 6\beta 4$:LN-332 interactions and that this adhesion is a primary factor underlying their slower migration in vitro (Stepp, et al., 2007). We also know that the *sd-1* null keratinocytes and fibroblasts are more responsive to TGF β 1. TGF β 1 can affect the recruitment of inflammatory cells into the cornea after wounding and is an important modulator of corneal wound healing (Zieske, et al., 2001; Stramer, et al., 2003). Taken together, these data suggest that the wound healing delays in the *sd-1* mice are due to increased adhesion of the epithelial cells to their underlying basement membrane and altered growth factor signaling induced by loss of *sd-1* as an important integrin co-receptor.

In the *sd-1* null mouse, increased epithelial cell adhesion is the likely cause for reduced erosions. The epithelial cells on the quiescent *sd-1* null cornea proliferate faster than those of the BALB/c mouse. The increased adhesion of the *sd-1* epithelial cells via $\alpha 6\beta 4$:LN332 could

allow for increased stem cell retention within the niche and reduce stem cell loss. One of the major differences between the corneal response to the small wounds, which never cause stem cell deficiency, and the large wounds is the enhanced proliferative burden the wounds involve. This leads us to hypothesize that enhanced stem and transit amplifying cell proliferation within the moving epithelial sheet during reepithelialization could result in niche emptying and stem cell deficiency. After small wounds, the proliferative burden is less and stem cells may not proliferate to close wounds. However, in large wounds, the stem cells would be required to divide while the epithelial sheet surrounding them was in motion. In our earlier corneal wound healing study (Stepp, et al., 2002), we showed that after small wounds, the cell proliferation rate does not increase in the *sd-1* null corneas, yet, over time, their corneal epithelium recovers the appropriate numbers of cell layers. It is possible that this reduced proliferative response to injury offers partial protection from stem cell loss after large wounds. However, much more needs to be known about the niche and its composition to sort out the interactions that are most critical for retaining stem cells within their niche.

These studies show that it is important to control for differences in mouse strain when assessing corneal wound healing in mice. Failure to do so will negatively impact the quality of the data generated from wounded mouse corneas. However, if strain-matched controls are used, important new insights into the causes of recurrent corneal erosions, corneal epithelial stem cell deficiency, and delayed reepithelialization can be generated.

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Figure 1. Erosion frequency is less in sdc-1 null mice compared to BALB/c or C57BL/6 mice regardless of wound size

A. After small or large wounds, mice were allowed to heal for 4 weeks. Erosion frequency, determined after staining corneas for open wound area, was determined for each strain of mouse. **B.** The erosion frequency in C57BL/6 corneas is similar after both small and large wounds at 80–85%. In the BALB/c mice, the erosion frequency is 75% and 60% after small and large wounds respectively. The difference between the two strains was significant only after the large wounds. The sdc-1 null mice had similar erosions frequencies after both small and large wounds but the values were significantly less at 25–30%. The n values listed refer to the numbers of corneas assessed per variable. Error bars represent SEM values and asterisks indicate data that are significant by the unpaired t test with p values < 0.05.

Figure 2. C57BL6 mice have greater corneal epithelial stem cell deficiency (CESCD) compared to BALB/c or sdc-1 null mice

A. 4 weeks after large wounds eyes were stained with K8 and Muc5AC to visualize the goblet cells that invaded the central cornea. **B.** Representative results from immunofluorescence microscopy show no goblet cells on the central cornea in unwounded C57BL6 and BALB/c corneas. The inset in the control BALB/c cornea shows K8+goblet cells at the limbus (indicated by the white asterisk) stained with Muc5AC. There are more goblet cells in the central cornea in the of C57BL6 mice compared to BALB/c or sdc-1 null mice. The white arrows in the C57BL6 cornea show goblet cells in the center of the cornea which were K8+Muc5AC+; in the plane of the section shown, they appear negative. **C.** The extent of goblet cell invasion onto

central corneas after the large wounds was assessed by quadrant for C57BL6, BALB/c and sdc-1 null mice. Scores were assigned ranging from 0–5 with 0 indicating no evidence of CESC and 5 indicating the presence of goblet cells in the central cornea. The n values on the bar graphs show the numbers of quadrants assessed with the numbers of corneas assessed indicated in parentheses below. The graph on the left shows the mean scores $\pm$ SEM with the asterisk indicating that C57BL6 mice had significantly higher ($p < 0.05$) scores for CESC than either BALB/c or sdc-1 null mice. The graph on the right shows the % of each quadrant assigned each individual score. Note that while 25% of the quadrants in the BALB/c and sdc-1 mice were assigned scores of 0 indicating that there was no evidence of CESC, only 10% of the C57BL6 quadrants were free of evidence of CESC.

Figure 3. 1.5 mm manual debridement wounds heal faster in C57BL/6 mice

A. The mass of the C57BL/6 and BALB/c eyes and the diameters of their corneas were measured and found to be smaller despite the fact that the mass of both strains of mice are identical. **B.** Corneas were stained with either Richardson stain or propidium iodide (PI). Shown are representative images taken of corneas from mice at 12, 15, 18, 21, and 24 hours after wounding. The n values shown in white are the numbers of corneas assessed per time point. **C.** The areas of the open wound immediately after wounding as well as at the indicated time points were determined and data expressed as the % of the remaining wound; error bars indicate the SEM values derived from the area data. The healing rates for C57BL/6 and BALB/c mice are similar for the first 18 hours. By 21 hours, the open wound areas in C57BL/6 corneas are smaller but the data are not significant. However, by 24 hours, the C57BL/6 wound areas are statistically significantly smaller. **D.** BrdU-labeled cells were counted for 4 separate fields from control and wounded corneas; the numbers of BrdU labeled cells were similar in both strains and increased 3–4 fold after wounding. Asterisks indicate data that are significant by the Mann-Whitney test ($p < 0.05$).

BALB/c **C57BL6**

Figure 4. Time-lapse studies in vitro show that C57BL6 epidermal keratinocytes migrate faster than BALB/c epidermal keratinocytes

A. Epidermal keratinocytes were isolated and used for time-lapse studies over a 16 hour 40 min time period with 100 images taken every 10 min. The tracks of 15 randomly selected C57BL6 and BALB/c keratinocytes during the time-lapse are indicated in red and suggest that the C57BL6 cells had a faster migration rate. **B.** The velocities were calculated for a minimum of 50 cells per variable and confirm that the C57BL6 cells migrate faster than the BALB/c cells. Error bars indicate the SEMs of the velocities. Asterisks indicate data that are significantly different by the Mann-Whitney test ($p < 0.05$).