The basal layer of limbal and central corneal epithelium is enriched in stem cells and transient amplifying cells, respectively. This physical separation of stem and transient amplifying cells makes the limbal/corneal epithelium an exceptionally suitable system for isolating basal cells enriched in these two proliferative populations. Prior attempts to isolate epithelial stem cells used methods such as proteolytic tissue dissociation and cell sorting that could potentially alter their gene expression profile. Using laser capture microdissection, we were able to isolate resting limbal and corneal basal cells from frozen sections with minimal tissue processing, thereby improving the yield and quality of RNA. Analyses of RNA isolated from 300 limbal and corneal basal cells from eight mice revealed a set of ~100 genes that are differentially expressed in limbal cells versus corneal epithelial basal cells. Semiquantitative reverse transcription-PCR confirmed the up-regulation of three limbal and three corneal genes. LacZ identification of epiregulin from epiregulin-null mice and immunohistochemical staining of wild type mice confirmed that epiregulin, one of the limbal epithelial-enriched genes, was associated with the limbal epithelial basal cells. Within the limbal and corneal basal cells, we detected previously unknown genes that were differentially expressed in these two regions that contribute further to our understanding of the unique heterogeneity of these two closely related basal cell populations. Our findings indicate that we can obtain accurate gene expression profiles of the stem cell-enriched limbal basal cell population in their “natural” quiescent state.

The ability to identify, purify, and characterize epithelial stem cells is a critically important issue in epithelial stem cell biology as it will further our understanding on how stem cells are regulated. Of the various epithelial tissues studied, the limbal/corneal epithelium has yielded a great deal of information about stem cells (1). Based on a combination of (i) keratin expression data (2); (ii) in vivo and in vitro cell kinetic data (3–7); (iii) centripetal migration studies (8–13); and (iv) corneal regeneration and reconstitution studies (14–20), it is well accepted that the corneal epithelial stem cells are preferentially located in the limbal epithelial basal layer (for reviews, see Refs. 1 and 21–27). Furthermore, since the limbal stem cells can be long-term cultured, we can use them to study the behavior of epithelial stem cells in vivo.

A lack of stem cell-specific markers has been an impediment for the isolation and subsequent biological and biochemical characterization of epithelial stem cells. Recently, transcriptional profiling of enriched populations of putative epithelial stem cells has been used to search for epithelial stem cell-specific markers. Methods used to isolate the putative stem cells prior to analyses include (i) rapid adherence of trypsinized epithelial cells to collagen-coated plates (i.e. epithelial cells that adhere within 20 min are thought to be putative stem cells, whereas slowly adhering cells are defined as the TA or terminally differentiated cells); (ii) the active efflux of the DNA binding dye Hoechst 33342 by the ABCG2/BCRP1 transporter, yielding a cohort of cells known as the side population (32–34); (iii) the engineering of transgenic mice to express histone H2B-green fluorescent protein in a manner that specifically tags the label-retaining cells followed by FACS sorting of single cell preparations of these label-retaining cells (35); and (iv) the engineering of transgenic mice to express green fluorescent protein under the keratin 15 promoter, which preferentially tags cells in the hair follicle bulge (site of the hair follicle stem cells), followed by FACS sorting of this green fluorescent protein cell population (36). Although all of these methods yield cells for subsequent transcriptional profiling, these cells are subjected to a variety of manipulations prior to analysis, including proteolytic tissue dissociation, cell culturing, and FACS sorting. These manipulations can potentially perturb the observed genetic profiles.

In an effort to obtain epithelial stem cells and their progeny, the TA cells, in their more “natural” quiescent state, we used laser capture microdissection (LCM) to isolate limbal epithelial basal cells (enriched in corneal epithelial stem cells) and corneal epithelial basal cells (enriched in TA cells). The LCM system that we used enabled us to isolate these cell populations directly...
from frozen sections with a minimum of tissue processing, thereby improving the yield and quality of RNA. In the current study, as few as 50–300 limbal and corneal epithelial basal cells were sufficient to generate an accurate representation of the expression profiles of the “normal” *in vivo* limbal and corneal epithelial basal cell populations. Comparison of these profiles identified many previously unreported limbal- and corneal-enriched genes. The expression of some of these was validated using semiquantitative RT-PCR. The expression profile data were further validated by the preferential LacZ and immunohistochemical detection of one of the limbal-enriched genes, epiregulin, in a subset of limbal epithelial basal cells. Analysis of the gene expression profiles from the limbal basal cells revealed the elevated expression of certain genes that are up-regulated in the hair follicular bulge stem cells, suggesting the existence of a common “signature set” of epithelial stem cell genes.

**EXPERIMENTAL PROCEDURES**

**Animal and Tissue Preparation**—All animal protocols were approved by Northwestern University Animal Care and Use Committee. Eight-to-ten-week-old male Balb/c mice (Charles River) were killed by CO2 asphyxiation and cervical dislocation. Whole globes with surrounding tissues were excised, immediately embedded in OCT compound, and stored at −80 °C until sectioning. Eyes from epiregulin null mice (37) were kindly provided by Dr. D.W. Threadgill. (Department of Genetics, University of North Carolina, Chapel Hill, NC).

**Isolation of Limbal and Corneal Epithelial Basal Cells by LCM**—The LCM system that we used (P.A.L.M. MicroBeam System, Zeiss Instruments, Berneid, Germany) obviated the need for tissue dehydration prior to microdissection. This enabled us to isolate quiescent limbal and corneal epithelial basal cells directly from 6–μM frozen sections of mouse eyes (Fig. 1), thereby increasing the yield and quality of RNA (see below). Frozen sections, mounted on special membrane-coated slides (P.A.L.M. Microbeam), which facilitated the capture of cells, were briefly (1 min) stained with hematoxylin (HistogeneTM; Arcturus, Mountain View, CA). In addition to visualizing the limbal and corneal epithelial basal cells, this staining procedure also removed the OCT mounting medium. Stained sections were viewed with a Zeiss Axiovert 200/200 M inverted light microscope (Zeiss Instruments) using a ×40 objective. Basal cells to be isolated were outlined with a “light” pen or cursor on a monitor screen using PALM RoboSoftware (Fig. 1, A and B). Such an outline defined the area that would be cut (Fig. 1, C and D) and catapulted (Fig. 1, E and F) intact into a microtube cap containing adhesive material for RNA isolation. In this manner, 300 basal epithelial cells from the limbus and from the cornea were isolated from each of eight mice. The mouse limbal epithelium, located at the edge of the corneal epithelium, is ~50 cells wide and 2–4 layers thick and overlies a loosely organized stroma containing a network of capillaries. The basal cells of the limbal epithelium are smaller and more electron-dense than those of the corneal epithelium. The central corneal epithelium is 5–9 cell layers thick and overlies a compact “plywood-like” stroma free of vasculature.

**Total RNA Isolation and cRNA Amplification**—Total cellular RNA from LCM-captured cells was isolated and purified (PicoPureTM; Arcturus). Samples of the total starting RNA were analyzed by capillary electrophoresis (Agilent Technologies, Palo Alto, CA) to assess the degree of purification. Approximately 3 ng of total cellular RNA could be extracted from 300 limbal and corneal basal epithelial cells that were isolated by LCM. When this RNA was contrasted with commercially prepared total RNA from mouse liver using picogram chips and a Bioanalyzer (Agilent Technologies), sharp bands corresponding to the 18S and 28S RNA were observed for all samples (Fig. 2, A–C). Although we could detect signals from as few as 50 limbal and 50 corneal basal cells (Fig. 2, lanes 6 and 7), we routinely collected 300 basal cells from each region to ensure that the data were representative and reproducible. RNA quality was further assessed by calculating the RNA integrity number, which is based on a proprietary Agilent Technologies algorithm (38). Total RNA from the isolated limbal and corneal epithelial basal cells was subjected to cRNA amplification. Briefly, two rounds of cRNA amplification were accomplished using a RibonAmp® HS RNA amplification protocol (Arcturus). First strand cDNA was generated by reverse transcription using the total RNA. After the second strand cDNA was synthesized, a T7 RNA polymerase-driven cRNA synthesis was performed to obtain the first round of cRNA amplification (see Fig. 3). A second double strand cDNA synthesis was performed followed by a second round of cRNA amplification. A BioArray™ HighYield™ RNA transcript labeling protocol (T7) (Enzo Life Sciences, Famingdale, NY) was employed for the second round of amplification to biotinylate the cRNAs. To examine the reaction quality, an aliquot from the first stand cDNA synthesis in the first round cRNA amplification and another from the second strand cDNA synthesis in the second round amplification sample were removed for real-time PCR analysis.

**Real-time PCR**—To determine the quality of cRNA amplification, β-actin mRNA from corneal and limbal epithelia were examined from separate synthesized cDNA samples from both first and second round synthesis using real-time PCR (Applied Biosystems, Foster City, CA). The cycle time was calculated,

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**Gene Profiles of Limbal and Corneal Basal Cells**

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**FIGURE 1. Isolation of mouse limbal and corneal epithelial basal cells using laser capture microdissection.** A–F, micrographs of frozen sections of cornea (A, C, and E) and limbal (B, D, and F) epithelia, lightly stained with hematoxylin. Basal cells to be isolated are outlined with a “light” pen (A and B; blue line), which demarcates the zone cut by the laser. After cutting (C and D), cells are catapulted (E and F; captured) by the laser into a capsule, which allows the isolated cells to be processed.
and the signal amplification thresholds between the first round and second round amplification were compared (see Fig. 3). Prior to biotinylation, we compared the results of the quantitative RT-PCR, and those samples with a large variation (=10-fold) were eliminated.

**Microarray Hybridization Functional Genomics**—cRNA fragmentation and hybridizations were performed at the University of Chicago. Briefly, 30 μg of cRNA was incubated in the fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) for 35 min at 94 °C, and then 12 μg of fragmented cRNA was hybridized to an Affymetrix mouse 430 2.0 chip (Affymetrix, Santa Clara, CA) for 16 h at 45 °C and 60 rpm in an Affymetrix hybridization oven 640. The arrays were washed and stained with streptavidin phycoerythrin in an Affymetrix fluids station 450 using an Affymetrix GeneChip protocol and then scanned using an Affymetrix GeneChip scanner 3000 (Affymetrix).

**Data Acquisition and Analysis**—The acquisition and initial quantification of array images was performed using the GCOS software (Affymetrix). Subsequent data analysis was performed using DNA-Chip Analyzer 1.3 with the *.CEL files obtained from GCOS (39). We used a PM-only model to estimate gene expression level (any array with a percentage of outliers >10% was eliminated from analyses). We chose the perfect match-only model due to its superior reproducibility as compared with the perfect match/mismatch model. The invariant set approach was used for normalization. For comparative analyses, thresholds for selecting significant genes were set at a relative difference of >3-fold, an absolute difference of >100 signal intensity, and a statistical difference at p < 0.05. Five hundred permutations were performed to estimate the false discovery rate. In this study, all false discovery rates were <10%. Gene ontology analysis was performed using classify gene function in dCHIP (39).

**Semiquantitative RT-PCR**—We used the same method to isolate basal cells for RT-PCR analysis as we did for the microarray experiments. Therefore we were only able to collect ~3 ng of total RNA/sample, an amount far below what is needed for qRT-PCR; therefore we used semiquantitative RT-PCR. For semiquantitative RT-PCR, limbal basal (300 cells) and central corneal basal (300 cells) epithelial cells were isolated, and total RNA was extracted as described above. The RNA samples were purified and subjected to one round of amplification. The second round double strand cDNA was synthesized using amplified cRNA as the template. To perform semiquantitative PCR for selected genes from microarray analysis, glyceraldehyde 3-phosphate dehydrogenase and 18 S ribosomal RNA were
used as quantitative controls. PCR primers were designed using the data provided by GenBank™ (National Center for Biotechnology Information (NCBI)) as templates. PCR products were run electrophoretically on 2% agarose gels and the density of the bands were measured using a densitometer and one-dimensional image analysis software (Eastman Kodak Co.).

β-Galactosidase Staining and Immunohistochemistry—β-Galactosidase staining of limbal and corneal epithelia from epiregulin-null mice was performed as described (37). We generated anti-epiregulin polyclonal antibodies in rabbit against a peptide of 12 (TALVQTENDNPR) amino acids (Invitrogen). We preincubated cryostat sections of mouse eyes with blocking buffer (phosphate-buffered saline supplemented with 0.2% normal goat serum, 0.1% Triton, and 0.1% NaN₃) for 30 min. Sections were incubated for 2 h at room temperature in anti-epiregulin antibody (diluted 1:100 in blocking buffer), washed with phosphate-buffered saline, and incubated with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (1:1000) as the second antibody at room temperature for 1 h. Slides were washed, mounted, and viewed by fluorescence microscopy (Zeiss Axiophot 2). We performed control experiments using either preimmune serum or normal rabbit IgG, as well as blocking the binding by co-incubating the antibody with the peptide.

RESULTS AND DISCUSSION

Previous studies have used microarray technology to investigate gene expression in normal human corneas (40, 41). In these global studies, the entire cornea was used as the starting material, and no attempt was made to separate the epithelial compartment from the underlying stroma. cDNA microarray has also been used to assess the gene expression profile of the corneal epithelium under pathological conditions (e.g., keratoconus (42, 43), pterygium (44), diabetic corneas (45), hypoxia (46)) and after exposure to various cytokines (e.g., epidermal
growth factor (47), interleukin-1 (48), and keratinocyte growth factor (47)). In these studies, the entire corneal epithelium was used to obtain RNA for the microarray analysis. Thus far, no attempts have been made to examine the gene profiles of the various regions of the corneal epithelium (e.g. basal, wing, and superficial cells) in their natural state and/or compare the basal cells of the limbal versus corneal epithelium. In the present study, we obtained reproducible gene profiles from relatively small subpopulations (50–300 cells) of stem cell-(limbal) and TA cell-(corneal) enriched epithelial basal cells using LCM, RNA amplification, and cDNA microarray technologies. These relatively pure populations of basal cells were isolated with a minimum of perturbation, and the gene profiles that we observed represent the natural in vivo state of these cells. Within the limbal and corneal epithelial basal cells, we detected previously unreported genes that were differentially expressed in these two regions that contribute further to our understanding of the unique heterogeneity of these two closely related basal cell populations.

**Basal Cells Isolated by LCM Yield High Quality RNA**—We performed three separate gene profiling experiments on Balb/c mice using groups of two, three, and three mice for a total of eight mice. In each experiment, 300 limbal and corneal basal epithelial cells isolated by LCM yielded ~3-ng of total cellular RNA, which could be amplified to yield ~30 μg of high quality cRNA (Fig. 3). A second round of cRNA amplification was performed, and the biotinylated cRNA products were fragmented. Comparison of the bioanalyzer plots and agarose gels of the biotinylated cRNA products with commercially prepared samples (Arcturus) indicated that the molecular size distribution of the amplified and biotinylated cRNA was in the required range of the commercially prepared samples (Fig. 4). Furthermore, the intensity of our biotinylated products was more consistent than the commercial samples. Examination of the fragmented, biotinylated products using agarose gels indicated that all products were within 200 bp, which is required for successful hybridization to microarray chips (Fig. 4E).
TABLE 1
The top 50 differentially expressed genes in limbal versus corneal epithelial basal cells

| Prob Set          | Gene                                                                 | Accession       | Fold change | p value |
|-------------------|----------------------------------------------------------------------|-----------------|-------------|---------|
| Apoptosis/anti-apoptosis | Tumor necrosis factor (ligand) superfamily, member 6 | NM_010177 | 6.76 | 0.04    |
|                   | Baculoviral IAP repeat-containing 5                                  | AF416641         | −4.13       | 0.05    |
|                   | B-cell leukemia/lymphoma 2                                           | BC024780         | −3.63       | 0.03    |
|                   | Diablo homolog (Drosophila)                                          | AW542545         | −3.6        | 0.04    |
| Cell adhesion     | Thrombospondin 2                                                     | BB233297         | 7.77        | 0.03    |
|                   | AE binding protein 1                                                 | NM_006936        | 7.31        | 0.05    |
|                   | Catenin α 2                                                          | NM_009819        | 4.34        | 0.05    |
|                   | Procollagen, type VI, α 2                                            | BI455189         | 4.27        | 0.04    |
|                   | Spondin 1, (f-spondin) extracellular matrix protein                   | BC020531         | 3.81        | 0.01    |
|                   | Armadillo repeat gene deleted in velo-cardio-facial syndrome         | BB336845        | −3          | 0.03    |
|                   | Multiple PDZ domain protein                                          |                 | −3          | 0.05    |
| Cell differentiation | Disabled homolog 2 (Drosophila)                                      | BC006588         | 4.25        | 0.02    |
|                   | Twist homolog 2 (Drosophila)                                         | NM_007855        | 3.15        | 0.01    |
| Cell growth/development | Dachshund 2 (Drosophila)                                             | BB175077         | 4.3         | 0.04    |
|                   | v-Crk sarcoma virus CT10 oncogene homolag (avian)-like               | AW541802         | 4.26        | 0.01    |
|                   | Epiregulin                                                           | NM_007950        | 4.22        | 0.02    |
|                   | Nuclear receptor subfamily 2, group E, member 1                       | BG072053         | 4.3         | 0.01    |
|                   | Suppressor of cytokine signaling 3                                   | BB241535         | 3.8         | 0.04    |
|                   | Cytokine inducible kinase                                             | BM497855         | 3.33        | 0.04    |
|                   | Fibroblast growth factor receptor 1                                  | M33760           | 4.83        | 0.05    |
|                   | Cell division cycle 37 homolog (Saccharomyces cerevisiae)-like       | BE824561         | 3.28        | 0.02    |
|                   | Coiled-coil domain containing 5                                      | NM_018827        | −4.27       | 0.01    |
|                   | Neurotrophic tyrosine kinase, receptor, type 1                       | AIF48260        | −3.77       | 0.04    |
| Cytoskeleton      | Keratin complex 1, acidic, gene 13                                   | AV085233         | 9.46        | 0.03    |
|                   | Keratin complex 2, basic, gene 6                                     | NM_010669        | 4.21        | 0.03    |
|                   | Keratin complex 2, basic, gene 7                                     | BC010357         | 3.02        | 0.03    |
|                   | W/D repeat domain 1                                                  | BB053942         | −3.56       | 0.05    |
|                   | Cortactin                                                            | BB317004         | −3.13       | 0.02    |
| Extracellular matrix protein | Tissue inhibitor of metalloproteinase 2                             | M93954           | 5.62        | 0.01    |
|                   | Platelet derived growth factor receptor, α polypeptide               | AW537708         | 5.2         | 0.03    |
|                   | Tumor necrosis factor receptor superfamily, member 11a              | BC019185         | 3.1         | 0.03    |
| Receptors         | CD86 antigen                                                         | NM_019388        | 5.73        | 0.01    |
|                   | Mus musculus 7 days embryo whole body cDNA                           | BB409477         | 3.98        | 0.01    |
|                   | Opioid growth factor receptor like 1                                 | BE650508         | 3.28        | 0.03    |
|                   | Membrane associated protein 17                                       | AA396586         | 4.11        | 0.04    |
|                   | Neuronal pentraxin receptor                                           | AK010743         | −6.19       | 0.05    |
|                   | Synaptic vesicle glycoprotein 2 b                                    | BB674794         | −3.03       | 0.01    |
| Signal transduction | Receptor (calcitonin) activity modifying protein 1                   | NM_016894        | 3.66        | 0.05    |
|                   | Calcitomin receptor-like                                             | AF209905         | 3.18        | 0.01    |
|                   | Wingless-related MMTV integration site 5A                            | BC018425         | 3.01        | 0.05    |
|                   | Neurpeptide Y                                                       | BB817939         | −6.91       | 0.02    |
|                   | Calcitomin receptor                                                  | AI596632         | −5.83       | 0.01    |
|                   | Chemokine-like receptor 1                                            | BE892944         | −4.71       | 0.05    |
|                   | Phosphodiesterase 1A, calmodulin-dependent                            | BM208103         | −3.44       | 0.04    |
|                   | LanC (bacterial lantibiotic synthetase component C)-like             | BB667274         | −3.26       | 0.03    |
| Transcription regulation | SRY-box containing gene 4                                            | AI428101         | 5.75        | 0.03    |
|                   | Early growth response 3                                              | AV346607         | 4.23        | 0.04    |
|                   | Kruppel-like factor 13                                               | AK002926         | 3.38        | 0.03    |
|                   | Cofactor required for Sp1 transcriptional activation subunit 2       | AF302127         | −4.05       | 0.02    |

The ability to isolate basal cells directly from fresh frozen sections with a minimum of fixation and dehydration yielded good quality RNA from cells that were subjected to little if any perturbation. Consequently, the gene profiles that we generated (see below) were reflective of cells in their natural state. We used the RNA integrity number to assess the RNA quality since this has been shown to be the method of choice when evaluating RNA integrity from LCM samples (49). Using the RNA integrity number scale, which ranges from 10 (ideal intact RNA) to 1 (totally degraded RNA (38)), we routinely obtained values ~7.5 and ~6.6 for limbal and corneal RNAs, respectively. To date, few studies using LCM have examined the quality of the isolated RNA; however, a recent study reported that the RNA isolated from LCM of stained cerebellum had an RNA integrity number of ~5 (49). Although we had some RNA degradation, this was probably due to staining, which has been shown to decrease RNA quality (49). Since staining is essential for cell localization, some RNA degradation is inevitable. Other investigators have used LCM to isolate epithelial cells from stem cell-
Gene Profiles of Limbal and Corneal Basal Cells

enriched regions (e.g. hair follicle bulge, intestinal crypts, basal epidermal keratinocytes); however, the isolation required either fixation and/or dehydration of the samples, which could have affected the quality of the RNA (50–52). In the majority of these investigations, LCM was combined with RT-PCR to check for the presence of specific genes. Only one study employed a similar combination of LCM, RNA amplification, and microarray analysis to define the molecular features of a stem cell-enriched population (53). In that study, a much higher number (>3,500 versus 300 in our case) of epithelial crypt progenitor cells served as the starting material for the extraction of the total cellular RNA.

Limbal and Corneal Microarray Data Are Highly Reproducible—To obtain gene profiles of limbal and corneal epithelial basal cells, we analyzed the biotinylated and fragmented cRNA samples from the corneal and limbal basal cells of the Balb/c mice. Initially, we evaluated the quality of the microarray hybridization by examining the report file, which showed that: (i) most of the housekeeping genes were present including β-actin and glyceraldehyde 3-phosphate dehydrogenase; (ii) the 3’ to 5’ ratio for most of the housekeeping genes was ≤3; (iii) the hybridization background was ≤300; and (iv) the percentage of the present genes ranged from 21 to 33%, which is the typical range for tissues derived from a single cell type. We analyzed the consistency of the gene expression patterns of a given sample of basal cells isolated from different mice (Fig. 5B) or between two different regions of basal cells from the same mouse (Fig. 5A). In both scenarios, the scatter plot analyses revealed an overall similarity in gene expression profiles. Since basal cell populations from two closely related regions of the anterior segmental epithelium were being compared, the overall similarity in genes expressed is to be expected. The lower correlation coefficient observed between limbal and corneal basal cells (Fig. 5A) is consistent with the unique lateral heterogeneity of the limbal/corneal epithelial basal layer (2, 54–56). When microarray data were analyzed with the sample clustering software, the profiles of these two populations were consistently clustered into identical groups (Fig. 6). Gene clustering analysis indicated an overall uniformity between limbal and corneal samples with respect to genes that were either up-regulated or down-regulated (Fig. 6). This further indicates the reliability and feasibility of gene profiling using LCM with small numbers of cells.

To determine the differentially up- and down-regulated genes in both limbal and corneal epithelial basal cells, we analyzed the microarray data generated from limbal and corneal samples from eight mice. We used a 3-fold or greater change and a p value of 0.05 as criteria for gene selection and identified ~100 genes. Following the elimination of unknown genes (designated as RIKEN), we sorted out 50 genes on the basis of their biological function (Table 1). Many of these genes have not been reported to be associated with either limbal and/or corneal epithelial basal cells.

Gene Profiling Reveals the Presence of New Limbal- and Corneal-enriched Genes—To further validate the results of the cDNA microarray data, we randomly picked several genes that were highly expressed in either limbal or corneal epithelial basal cells and performed semiquantitative RT-PCR. Epiregulin, dachshund, and Sry were at least 4-fold higher in limbal basal cells according to the microarray data (Table 1), and the semiquantitative PCR products for these genes were indeed found exclusively in limbal basal cells but not in corneal basal cells (Fig. 7). In a similar fashion, Diablo, cyclin M2, and multiple PDZ domain protein were up-regulated in corneal basal cells according to the microarray data (Table 1), and their PCR products were indeed expressed at significantly higher levels in corneal than limbal basal cells (Fig. 7).

To further validate the up-regulation of epiregulin in limbal basal cells, we obtained eyes from six mice that had a lacZ cassette inserted into the epiregulin locus by homologous recombination in embryonic stem cells (37). In such mice, β-galactosidase staining was restricted to limbal epithelial basal cells (Fig. 8A). β-Galactosidase staining was not detected in limbal stromal cells, corneal endothelium, corneal keratocytes, or corneal endothelium (Fig. 8A). We also found that in wild type mice, epiregulin protein was restricted to the limbal basal cells, with no appreciable staining in corneal basal cells (Fig. 9). Taken together, the β-galactosidase staining and immunohistochemical data provide strong support to our microarray data indicating that epiregulin is restricted to the limbal basal epithelial cells.

The functional significance of epiregulin, a member of the epidermal growth factor family of peptide growth factors (57), in limbal basal cells needs to be explored; however, its roles in wound healing and regulating proliferation are not inconsistent with a limbal location. For example, recent studies revealed that epiregulin was more potent than either epidermal growth factor or transforming growth factor-α in promoting both in vivo and in vitro wound closure in epidermal keratinocytes (58, 59). Since the stem cell-enriched, limbal epithelial basal cell population has been shown to play a central role in wound repair, the preferential distribution of epiregulin in limbal basal cells may affect the increased proliferative response of these cells observed following wounding (4, 6, 28). Even more intriguing is the observation that epiregulin is highly up-regulated in human

FIGURE 7. Semiquantitative RT-PCR results confirm the microarray data. Semiquantitative RT-PCR was performed on the cDNA obtained from amplified RNA extracted from 300 limbal and corneal basal cells isolated as shown in the legend for Fig. 1. The PCR products of Sry (A), epiregulin (B), and Dachs- hund (C) are seen in limbal (l) but not in corneal (c) basal cells and were markedly up-regulated in microarray analysis (Table 1). Conversely the PCR products of Diablo (D), cyclin M2 (E), and multiple PDZ domain protein (F), genes that were corneal-enriched in the microarray analysis, are strongly expressed in corneal basal cells and only weakly expressed in limbal basal cells. The PCR product of 18 S ribosomal RNA serves as a control (G).
Gene Profiles of Limbal and Corneal Basal Cells

FIGURE 8. Epiregulin is expressed in limbal basal epithelial cells. A and B, frozen sections of the limbus (A) and cornea (B) from a mouse that had a lacZ cassette inserted into the epiregulin locus, enabling the detection of the expression of epiregulin. β-Galactosidase staining (arrows) was restricted to a subset of limbal (I) epithelial basal cells. Note the absence of epiregulin expression in the limbal fibroblasts, corneal epithelium (c), and keratocytes. C and D, age-matched wild type controls.

Gene Profiling Studies

The expression of epiregulin in limbal basal epithelial cells was validated using RT-PCR, ACSP2, Diablo, and the multiple PDZ-domain protein (MUPP) genes have not previously been reported in these cells, and their functional significance remains to be determined.

When the gene expression profile data from eight separate mice were pooled and a 3-fold or greater cut-off was applied (Table 1), a number of diverse genes was found to be preferentially up-regulated in either the limbal or the corneal epithelial basal cells. The observed changes in transcript levels could be clustered into groups with specific functions. For example, 42% of all the up-regulated genes were involved in protein metabolism, 24% were associated with transport, and 13% were associated with cell surface receptors. The significant expression of epiregulin and its functional role in the regulation of cell proliferation and survival remain to be determined.

Some of the genes that were preferentially (≥3-fold) expressed in limbal epithelial basal cells were keratin complex (K6), fibroblast growth factor receptor 1 (FGFr1), and the adaptor molecule disabled homolog 2 (Dab2). Interestingly, these molecules have also been reported to be up-regulated in keratinocytes present in the bulge region of the hair follicle (35, 36), a site enriched in epithelial stem cells (67–70). A second set of genes including S100A4, S100A6, Idb2, and Idb4 was up-regulated to a lesser extent.

telomerase reverse transcriptase (hTERT)-immortalized fibroblasts and is an important factor for the maintenance of the infinite proliferation of these fibroblasts (60). Thus it is possible that epiregulin may contribute to the high proliferative capacity of limbal epithelial basal cells (5, 7). As with epiregulin, the functional significance of an enrichment in Dach2 in limbal basal cells is unclear; however, in developing tissues (e.g. the mouse retina), this gene has been shown to act in concert with Six6 to repress the transcriptional activity of p27Kip1. The result of such repression is the expansion of retinal precursor cell populations during development (61). Since the limbal epithelial stem cells are the precursor population of the corneal epithelium, Dach2 may function at times when the limbal stem cell population needs to be expanded (e.g., during wound repair and/or in development). Our gene profiling studies were conducted using male mice, and this accounts, in part, for the detection of a male sex-determining gene Sry (62, 63); however, the functional significance of Sry expression in limbal basal cells is unclear. Sry expression, like epiregulin and Dach2, is associated with an increase in proliferation; however, in the case of Sry, the increased proliferation occurs in Sertoli cell precursors and results in testes formation (64, 65). It would be of interest to see whether limbal basal cells from female mice contain a preferential distribution of Dax1, which is a female-specific gene involved in gonadal development (66). The presence of epiregulin, Dach2, and Sry, all potent growth stimulators, in limbal basal cells at first may seem inconsistent with the slow cycling feature of these cells (4, 28). However, these three genes appear either to act at specific times such as the expansion of precursor cell populations and in wound healing or to maintain proliferative capacity, events known to involve stem cell activation. With respect to the genes enriched in corneal basal cells that were validated using RT-PCR, ACDP2, Diablo, and the multiple PDZ-domain protein (MUPP) genes have not previously been reported in these cells, and their functional significance remains to be determined.

Is There a Signature Set of Epithelial Stem Cell Genes?—When the gene expression profile data from eight separate mice were pooled and a 3-fold or greater cut-off was applied (Table 1), a number of diverse genes was found to be preferentially up-regulated in either the limbal or the corneal epithelial basal cells. The observed changes in transcript levels could be clustered into groups with specific functions. For example, 42% of all the up-regulated genes were involved in protein metabolism, 24% were associated with transport, and 13% were associated with nucleotide regulation, intracellular signaling, and transcription, whereas only 4% were associated with cell surface receptor-linked signal transport and regulation of signal transduction. It was also possible to group limbal and corneal genes into categories that reflected functions that might be relevant to basal cells (Table 1).

Some of the genes that were preferentially (≥3-fold) expressed in limbal epithelial basal cells were keratin complex 2, basic, gene 6g (K6), fibroblast growth factor receptor 1 (FGFr1), and the adaptor molecule disabled homolog 2 (Dab2) (Table 1). Interestingly, these molecules have also been reported to be up-regulated in keratinocytes present in the bulge region of the hair follicle (35, 36), a site enriched in epithelial stem cells (67–70). A second set of genes including S100A4, S100A6, Idb2, and Idb4 was up-regulated to a lesser
degree (>1.5-fold) in limbal epithelial basal cells, yet these genes were also found to be enriched in bulge keratinocytes as well. It should be noted that different methods (e.g. proteolytic digestion, cell sorting, green fluorescent protein transgenic mice) were used to isolate the bulge keratinocytes prior to gene profiling, and this might reflect the differences in the degree of up-regulation of some of the genes. Nevertheless, the detection of common genes that were markedly up-regulated in stem cell-derived populations from distinct epithelia such as the hair follicle and the limbus supports the idea of a signature set of genes that may help define epithelial stem cells, in a manner similar to the subset of genes that have been shown to be present in neural, hematopoietic, and embryonic stem cells (71, 72).

It should be noted that the presence and/or absence of specific markers will not be sufficient to define epithelial stem cells, or for that matter, any stem cell. For a cell to be classified as a stem cell, it must possess a variety of attributes, which still need to be fully delineated (73).

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