Published in final edited form as:
Differentiation. 2006 February ; 74(1): 19–29. doi:10.1111/j.1432-0436.2006.00054.x.

A role for chromosomal protein HMGN1 in corneal maturation

Yehudit Birger[1],
Protein Section, Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20892, USA, Tel: +1-301-496-5234

Janine Davis[1],
Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892, USA, Tel: +1-301-402-4343

Takashi Furusawa,
Protein Section, Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20892, USA, Tel: +1-301-496-5234

Eyal Rand,
Protein Section, Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20892, USA, Tel: +1-301-496-5234

Joram Piatigorsky, and
Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892, USA, Tel: +1-301-402-4343

Michael Bustin
Protein Section, Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20892, USA, Tel: +1-301-496-5234

Joram Piatigorsky: joramp@nei.nih.gov; Michael Bustin: bustinm@mail.nih.gov

Abstract

Corneal differentiation and maturation are associated with major changes in the expression levels of numerous genes, including those coding for the chromatin-binding high-mobility group (HMG) proteins. Here we report that HMGN1, a nucleosome-binding protein that alters the structure and activity of chromatin, affects the development of the corneal epithelium in mice. The corneal epithelium of *Hmgn1*$^{-/-}$ mice is thin, has a reduced number of cells, is poorly stratified, is depleted of supra-basal wing cells, and its most superficial cell layer blisters. In mature *Hmgn1*$^{-/-}$ mice, the basal cells retain the ovoid shape of immature cells, and rest directly on the basal membrane which is disorganized. Gene expression was modified in *Hmgn1*$^{-/-}$ corneas: glutathione-S-transferase (GST)α 4and GST ω 1, epithelial layer-specific markers, were selectively reduced while E-cadherin and α-, β-, and γ-catenin, components of adherens junctions, were increased. Immunofluorescence analysis reveals a complete co-localization of HMGN1 and p63 in small clusters of basal corneal epithelial cells of wild-type mice, and an absence of p63 expressing cells in the central region of the *Hmgn1*$^{-/-}$ cornea. We suggest that interaction of HMGN1 with chromatin modulates the fidelity of gene expression and affects corneal development and maturation.

Correspondence to: Joram Piatigorsky, joramp@nei.nih.gov.

[1]Y. B. and J. D. made equal contributions.

Keywords

HMG proteins; eye differentiation; corneal epithelium; chromatin

Introduction

Cellular differentiation pathways, including the generation of the mature cornea, involve multiple changes in gene expression, a process known to involve structural and chemical changes in the chromatin fiber (Felsenfeld and Groudine, 2003; Jaskelioff and Peterson, 2003; Hatakeyama et al., 2004; Sarmento et al., 2004; Margueron et al., 2005). Proper development of the transparent cornea is essential to normal vision, as together, the cornea and lens refract light onto the retina (Albert and Jakobiec, 1994). Cornea development commences early in mouse embryos and continues after birth. At birth, the mouse corneal epithelium, derived from head surface ectoderm (Pei and Rhodin, 1970), is one to two cell layers thick (Maurice, 1969), and is separated by a basement membrane from the underlying stromal and endothelial cell layers, derived in part from the neural crest (Haustein, 1983; Trainor and Tam, 1995).

A tightly regulated interplay between proliferative, differentiative, and cell death processes is required to maintain the steady rate of corneal epithelial cell renewal during adult life (Cotsarelis et al., 1989; Lavker et al., 1991; Kruse, 1994). Undifferentiated stem cells, with a high proliferative potential (capacity to divide), move from the limbus towards the central part of the cornea, into the cuboidal basal cell layer (Nagasaki and Zhao, 2003), and transiently become amplifying cells. From this layer, cells committed to terminal differentiation migrate outwards into the wing-shaped, supraba-sal cell layer, which gives rise to several layers of flattened, squamous cells that eventually are sloughed (Klyce and Beuerman, 1988; Chung et al., 1992; Sun et al., 2000). By 3 weeks after birth, the epithelium is six to seven cell layers thick and by 8 weeks of age it reaches its mature size of eight to 10 cell layers (Hay, 1979; Chung et al., 1992; Zieske and Wasson, 1993; Collinson et al., 2002).

The proliferative expansion and differentiation of the corneal epithelium is accompanied by numerous changes in gene expression (Stepp et al., 1995; Francesconi et al., 2000; Sun et al., 2000; Yoshida et al., 2000; Argueso and Gipson, 2001; Wang et al., 2001; Fukushima et al., 2003; West-Mays et al., 2003; Norman et al., 2004). In some cases the expression of specific genes is localized, or highly enriched, in specific layers. For example, p63, a member of the p53 transcription factor family involved in proliferative and differentiative processes in epithelia (Dai and Segre, 2004; Koster et al., 2004; Westfall and Pietenpol, 2004), is expressed exclusively in the basal cell layer (Dua and Azuara-Blanco, 2000; Pellegrini et al., 2001; Moore et al., 2002; Hsueh et al., 2004), glutathione-S-transferase (GST) α 4 (GSTα4) expression is observed mainly in the supraba-sal region, while another GST isoform, GST ω 1(GSTω1), is enriched in the superficial cells of the epithelium (Norman et al., 2004).

It is well documented that differentiation-related changes in transcription levels of specific genes are associated with changes in their chromatin structure (Felsenfeld and Groudine, 2003; Jaskelioff and Peterson, 2003; Hatakeyama et al., 2004; Sarmento et al., 2004; Margueron et al., 2005). Recent SAGE analysis revealed massive changes in gene expression during postnatal maturation of the mouse cornea (Norman et al., 2004). Some of the most affected genes code for chromatin modifying proteins, including members of the high-mobility group (HMG) protein superfamily. HMGs are ubiquitous and relatively abundant nuclear proteins, devoid of enzymatic activity, that bind to the chromatin fiber and

induce changes in both the local and the higher-order chromatin structure (Bustin, 1999, 2001; Reeves, 2001; Agresti and Bianchi, 2003). HMG-mediated changes in chromatin affect a wide range of DNA-dependent activities, including transcription; therefore, alterations in their expression levels may lead to global changes in transcription profile, such as seen during corneal development.

Here we present evidence that the high-mobility group protein N1 (HMGN1) affects the development of the corneal epithelium. HMGN1 is known to bind specifically to nucleosome cores, the building block of the chromatin fiber, to reduce the compaction of the chromatin fiber, to modulate the levels of posttranslational modifications in histones (Lim et al., 2004), and to affect the rate of transcription (Bustin, 2001). We find that mice lacking HMGN1 have multiple abnormalities in their corneas, some of which were not previously described. In the corneas of *Hmgn1*$^{-/-}$ mice, loss of HMGN1 alters the expression levels of cellular adhesion molecules, GSTs, and of p63, a major regulator of epithelial cell differentiation (Koster et al., 2004; Koster and Roop, 2004a, b). In the absence of HMGN1, the development and maintenance of the corneal epithelium is abnormal and characterized by loss of suprabasal cells, a reduction in the epithelial thickness, and tendency to blister. Our studies identify HMGN1 as a new regulator of corneal development.

Materials and methods

RNA blot analysis

A mouse RNA master dot blot (CLONTECH, Palo Alto, CA) was probed with ^{32}P-labeled 3'UTR of the *Hmgn1* cDNA as recommended by the manufacturer. The autoradiograms were scanned using a Molecular Dynamics densitometer and analyzed with Im-ageQuant software (Molecular Dynamics).

Hmgn1$^{-/-}$ *mice* were previously described (Birger et al., 2003).

In situ hybridization

For hybridization of embryo sections, riboprobes were synthesized from a linearized templates containing a 1.2kb cDNA of mouse *Hmgn1* using T3 and T7 RNA polymerases (Stratagene, La Jolla, CA) and digoxigenin-11-UTP (Roche Diagnostics, Indianapolis, IN). Embryos were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and 5 μ*m* sections were collected. Sections were dewaxed, rinsed with phosphate-buffered saline (PBS), and then subjected to protease digestion (1 μ*g* proteinase K/ml PBS). Sections were refixed (4% PFA, 0.2% glutalaldehyde/PBS) and acetylated (0.25% acetic anhydrate, 0.1 M triethanolamine, 0.1% HCl). Hybridization was performed overnight in 50% formamide:5 × SSC:1% SDS at 70°C and washed with 5 × and 1 × SSC. Detection of digoxigenin-labeled hybridization was achieved using an alkaline phosphate (AP)-conju-gated anti-digoxigenin Ab (Roche Diagnostics) at a 1:500 dilution followed by the addition of AP substrates, nitroblue tetrazolium (NBT) and bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) (Roche Diagnostics).

Fresh, frozen, 10μm eye sections from 25-day old mice were fixed, treated with proteinase K (0.2μg/ml PBS) for 8 min, and processed for *in situ* hybridization as described previously (Davis et al., 2003). Riboprobes were synthesized using a DIG RNA Labeling Kit (Sp6/T7) (Roche Molecular Biochemicals, Indianapolis, IN) with linearized, proteinase K-treated, plasmid cDNA templates encoding GST α4 (NM_010357; bases 22–437) and GSTω1 (U80819; bases 62–548). Hybridizations were carried out at 55°C using 200 ng sense or antisense riboprobe/ml hybridization buffer. Hybridization was visualized as described for embryonic sections. The reaction was allowed to proceed until purple color was visible

(approximately 90min), at which time reactions for both the sense and antisense riboprobes were terminated.

Real-time PCR

RNA was isolated from mouse corneas using TriZol Reagent (In-vitrogen, Carlsbad, CA) according to the manufacturer's instructions. Six hundred nanograms of total RNA was reverse transcribed using 1.25U/μl MultiScribe Reverse Transcriptase in 30 μ l of 1 × TaqMan RT buffer containing 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM random hexamers, and 0.4U/μl RNase inhibitor (Applied Biosystems, Foster City, CA). After incubation at 25°C for 10min, mixtures were reverse transcribed at 48°C for 30min followed by heat inactivation at 95°C for 5min. Transcripts were quantified by real-time PCR using reverse transcribed, first-strand cDNA, and SYBR Green PCR Mix (Applied Biosystems). A total of 12.5 m l of 2 × SYBR Green PCR mix was mixed with 0.5 μ l of reverse transcription product and specific primers (final concentration: 0.5 mM) and analyzed using an ABI PRISM 7900HT. The primer sets used in the quantitative real-time PCR were: GST α4: 5'-aaaacccgttacttcccagtgtt-3'/5'-ggatgtctgcccaactgagc-3', 5'-ctcctccagatg-gcccctat-3'/ 5'-ggtgacactgcaattggaacc-3', 5'-actgtcaagagctaaaacccg-ttact-3' /5' -gtctgcccaactgagctggt-3', 5' -gacatccagctcctagaagcca-3'/ 5' -ttgtcttaaatgcctgcagcag-3', and 5' -tgaagttctagtgcagcgtgct-3' /5' -ct-ttgcttctggaatgctctg-3'; p63: 5'-ttgatgccctctctccatcc-3'/5'-gtgcttgac-tgctggaaggac-3', and 5'-aggtcgtgagacgtacgagatgt-3'/5'-gcctgtacgt-ttcgatcgtgt-3'; a-actin: 5'-aaatcagtgcgtgacatcaaa-3'/5'-tctccagggag-gaagaggat-3', and 5'-tcctcctgagcgcaagtactct-3'/5'-gctgatccacatctgct-ggaa-3'; E-cadherin: 5'-ctgtggacgtggtagacg tg-3'/5'-cctgacccacac-caaagtct-3', and 5'-agactttggtgtgggtcagg-3'/5'-tgtccctccaaatccgatac-3'.

Histology and DAPI staining

Whole eyes were immersion-fixed in 4% PFA in PBS overnight at 4°C, washed in PBS and then in saline for 30 min each, dehydrated through a series of ETOH washes, cleared in xylene, and embedded in paraffin. Prior to staining, 10 mm sections were deparaffinized, rehydrated using a graded series of ETOH washes, and rinsed in distilled water for 15 min. Eye sections were stained in Gill's hematoxylin and eosin as described (Luna, 1968). Slides were counter-stained with DAPI as described (Davis and Reed, 1996), coverslipped using Aqua-Poly/Mount (Polysciences Inc., Warrington, PA), and photographed using a Zeiss Axioplan2 microscope with Spot camera.

Electron microscopy

Whole eyes were removed and fixed 24 h at room temperature in a solution of 2.5% glutaraldehyde and 6% sucrose buffered to pH 7.2 with 50mM sodium cacodylate. Small (0.5 ×1 mm) portions of the central cornea were processed for electron microscopy by dehydration in an ethanol series and embedding in epoxy resin. Ultra thin sections were stained with uranyl acetate and lead citrate and photographed using a JEM-100CX electron microscope (JEOL, Pea-body, MA).

BrdU and TUNEL labeling

Four hours prior to sacrifice, *Hmgn1*$^{-/-}$ and *Hmgn1*$^{+/+}$ sibling mice were given an intra-peritoneal injection of BrdU (10mM stock solution used at 1 ml/100 g body weight). Fresh, frozen eye sections were prepared, fixed, and washed in PBS. The sections (10 μm) were incubated in 2N HCl in 0.5% Triton X-100 for 10 min followed by a second incubation of fresh 2N HCl in 0.5% Triton X-100 containing 1 mg/ml pepsin (Sigma P7012, St. Louis, MO) for 30 s. The slides were washed with PBS three times for 10 min each, blocked in 10% normal rabbit serum in a humidified chamber, and then incubated with a rat

monoclonal anti-BrdU antibody (1:100; Accurate Chemical and Scientific Corp., Westbury, NY) overnight at 4°C. Complete processing of the sections was achieved using a rat Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine. TUNEL labeling on parrafin-fixed section was done with the ApopTag kit obtained from Chemicon, according to the manufacturer's instructions.

Immunohistochemistry and immunofluorescence—The following antibodies were used: *anti-Hmgn1* peptide 6, anti-N-terminal keratin 12 (K12) (1 μg/ml; a gift from W. Kao, University of Cincinnati, Cincinnati, OH), anti-aldehyde dehydrogenase 3 (ALDH3) (gift from R. Lindahl), anti-transketolase (TKT) Ab (Sax et al., 1996), anti-p63 (4A4): sc-8431, mouse monoclonal IgG2a, 1–205 a.a. of ΔNp63 of human origin (Santa Cruz Biotechnology, Santa Cruz, CA).

Fresh frozen eye sections (10μm) were collected on Superfrost/ Plus slides (Fisher, Pittsburgh, PA) and stored at −80°C until further use. Skin analyses were performed with PFA-fixed, paraffin-embedded whole embryos. Immunohistochemistry was performed by VectorStain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) with modifications. Briefly, slides were de-paraffinized by immersing in CitriSolv solution (Fisher Scientific). De-paraffinized slides were hydrated by incubating 10min for serial diluted ethanol solutions (100% −>95%–>70%–>50%). Slides were washed with 1× PBS for 10min, permeabilized by 1 × PBS with 0.1% Triton X-100 for 10 min, and then washed with 1 × PBS for 10min. Slides were immersed in 10 mM sodium phosphate (pH6.0) solution and heated by microwave. Sections were blocked with 10% goat normal serum and 3% BSA in PBS at 37°C for 1 h and incubated with primary antibodies. Slides were washed with 1 × PBS containing 0.1% Tween 20 for 5 min and incubated with secondary antibodies at 37°C for 1 h. After incubation, slides were treated as recommended by the manufacture, and then mounted with Aqua Poly/Mount (Polysciences Inc.). For fluorescent microscopy, the sections were incubated with secondary antibodies (1:500 goat anti-mouse 594-Alexa fluor red and goat anti-rabbit 488 Alexa fluor green) at RT for 45min then with 0.5 μg/ml Hoechst 33258 in PBS for 10min. The sections were washed twice with PBS and mounted with Aqua Poly/Mount (Polysciences Inc.). The location of *Hmgn1* and p63 proteins was visualized under a fluorescent microscope.

Western blot analysis

Mice (five $Hmgn1^{-/-}$ and five $Hmgn1^{+/+}$) were euthanized and whole eyes were enucleated and placed in chilled PBS on ice. Using a dissection microscope, corneas (total 20 corneas) were isolated from the rest of the eye by introducing a small opening at the limbus with fine forceps, and then separating the cornea from the conjunctiva by pulling on either side of the opening with fine forceps. The corneas were trimmed free of any remaining non-corneal tissues with a scalpel blade, frozen on dry ice, and stored at −80°C until further use. Tissues were solubilized in 150 mM NaCl, 50 mM Tris, pH 7.4%, 0.5% NP40, 0.5% Na deoxycholate, 5mM EDTA, 0.25% SDS, pepstatin, leupeptin, PMSF, and aprotinin. Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide gel electrophoresis was performed using pre-cast 10% Bis-Tris gels followed by transfer to PVDF membrane in NuPage transfer buffer according to the manufacturer's recommendation (Novex: Invitrogen). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (Blotto) for 1 h at room temperature and incubated with either anti-E-cadherin (Zymed Laboratories Inc., San Francisco, CA), anti α-,β-, or γ-catenin (Transduction Laboratories, Lexington, KY) or anti-actin (Santa Cruz Biotechnology) in Blotto at 4°C overnight. The membrane was washed and incubated with an anti-goat horseradish peroxidase-conjugated secondary antibody (1:10,000 in Blotto,

Amersham Pharmacia Biotech, Piscat-away, NJ) for 30 min. After washing, the immunoreactive complex was visualized using ECL Plus (Amersham Pharmacia Biotech).

Results

Hmgn1 expression in the embryonic and adult mouse eye

A survey of the *Hmgn1* expression in various tissues revealed that the level of *Hmgn1* mRNA was especially high in the eye, as compared with the other tissues examined (Fig. 1A). In the dot blots used, the amounts of RNA in each spot is normalized to the transcription levels of eight housekeeping genes; therefore, the intensity of the spot is indicative of the relative mRNA abundance in a tissue. The relative abundance of *Hmgn1* mRNA in the adult eye was more than twice that of any other tissue tested, including the kidney and uterus, which also had a relatively high expression level. *Hmgn1* mRNA was also detected in lesser amounts in the epididymus, ovary, prostate gland, and liver.

Several experiments indicated that the expression of *Hmgn1* mRNA is related to differentiation (Begum et al., 1990; Crippa et al., 1991; Lehtonen and Leh-tonen, 2001; Korner et al., 2003; Hatakeyama et al., 2004); therefore, we first examined its expression pattern in the eye of 14.5-day-old embryos (E14.5), a developmental stage in which the major structures of the eye have been formed. By *in situ* hybridization, *Hmgn1* transcripts were detected in the neural retina, lens, and cornea of eyes (Fig. 1B). In a magnified view of the cornea, *Hmgn1* mRNA was detected specifically in the epithelial, but not the stromal or endothelial regions of the cornea (Fig. 1B), suggesting lineage-dependent specificity in the timing of *Hmgn1* expression.

Consistent with the *in situ* results for E14.5 eyes, immunohistological analysis revealed that HMGN1 protein was localized specifically to the nuclei of the epithelial, but not stromal or endothelial cornea, cells of the 25-day-old $Hmgn1^{+/+}$ mice (Fig. 1C). Higher magnifications revealed that the HMGN1 protein is present exclusively in the basal cell layer of the stratified cornea epithelium. The presence of HMGN1 in the basal layer of the epithelium is not unique to the eye; the same pattern was observed in analysis of skin from E16.5 day or older embryos: HMGN1 is preferentially expressed in the basal skin layer (Fig. 1D).

Interestingly, not all of the cells in the basal layer of the corneal epithelium were immunoreactive for HMGN1. Instead, a subset of basal cells, appearing as clusters across the central and peripheral cornea, expressed relatively high levels of the protein (Fig. 1C). The basal layer of the corneal epithelium is poised for further differentiation and is the progenitor of the corneal epithelial cells necessary for the constant renewal of the cornea during adult life (Cotsarelis et al., 1989; Lavker et al., 1991; Kruse, 1994). The confinement of HMGN1 protein to the progenitor cells raises the possibility for a role of this protein in cornea epithelium maturation.

Abnormal corneal epithelium morphology in $Hmgn1^{-/-}$ mice

To test the possible involvement of HMGN1 in cornea maturation we first examined the corneal epithelium of $Hmgn1^{-/-}$ and $Hmgn1^{+/+}$ mice that were 25-day old, an age at which the cornea is not fully mature. Histological analysis, by hematoxylin and eosin (H&E) staining, showed a reduction in the thickness of the epithelium in $Hmgn1^{-/-}$ compared with $Hmgn1^{+/+}$ mice (Fig. 2A). The thinner epithelium corresponded to a 25% reduction in cell number in $Hmgn1^{-/-}$ versus $Hmgn1^{+/+}$ mice as determined by counting DAPI-labe-led nuclei (Figs. 2B, 2C). Detailed analysis of the epithelial layers suggested a specific reduction in the number of wing-shaped cells occupying the suprabasal layer of the epithelium in the *Hmgn1−/−* corneas (Fig. 2A—Sb).

The development of the stratified corneal epithelium is not completed until 8 weeks after birth (Collinson et al., 2002). To determine if the lack of HMGN1 also affected the maintenance of the mature corneal epithelium, we examined corneal histology at 4 months of age. Like the 25-day-old corneas, the 4-month-old *Hmgn1*$^{-/-}$ epithelium was thinner than age-matched *Hmgn1*$^{+/+}$ littermates (Fig. 2D) with a few, if any, suprabasal wing cells (Fig. 2D, magnifications). Moreover, the basal cells of the *Hmgn1*$^{-/-}$ corneal epithelium do not acquire the cuboidal-shape characteristic of the mature basal cells in the corneal epithelium of wild-type mice (Fig. 2D). In 4-month-old *Hmgn1*$^{-/-}$ mice the basal cells still have an ovoid shape, characteristic of immature basal cells, which in wild-type mice can be seen prior to, or just immediately after, eyelid opening (Zieske, 2004). Additionally, patches of basal cells in the *Hmgn1*$^{-/-}$ cornea appeared to reside directly on the stroma, rather than on the basement membrane that normally separates the epithelium from the extracellular stroma (Fig. 2D, arrows). In the control *Hmgn1*$^{+/+}$ corneas, the basal cells resided on the basement membrane, as expected. These results indicate that loss of HMGN1 disrupts the maturation of the corneal epithelium.

We examined the possibility that the decrease in corneal cell number in the *Hmgn1*$^{-/-}$ mice is because of either an increase in cells undergoing apoptosis or a decrease in cell proliferation rate. In the TUNEL assay, a small number (0–) of TUNEL-positive cells were detected per corneal section from either 25-day-old *Hmgn1*$^{+/+}$ or *Hmgn1*$^{-/-}$ mice, indicating that loss of HMGN1 does not lead to a significant change in the rate of apoptosis (not shown). To examine whether changes in cell proliferation account for the reduction in corneal cell number in the *Hmgn1*$^{-/-}$ mice, 25-day-old mice were sacrificed 4h after an injection of BrdU and the eyes were processed for immunohistochemistry using an anti-BrdU antibody. Surprisingly, there were more cells incorporating BrdU in S phase in the corneas of *Hmgn1*$^{-/-}$ compared with *Hmgn1*$^{+/+}$ mice. On average, there were 58, as compared with 46, BrdU-labe-led cells per corneal section from HMGN1$^{-/-}$ ($n = 12$) and HMGN1$^{+/+}$ ($n = 10$) mice, respectively. By *t-test* analysis the differences were significant with a $p<0.02$. Thus, loss of HMGN1 altered corneal morphology and increased the frequency of cells in S phase.

Altered cellular adhesion in the *Hmgn1 –/– cornea*

Abnormal corneal morphogenesis, including a reduction in the number of epithelial cell layers have been previously observed in *Sey*$^{+/-}$ mice, and could be indicative of abnormalities in the adhesion properties of the corneal cells (Davis et al., 2003). Electron microscopy studies revealed that the size and number of des-mosomes in the corneal epithelium were indistinguishable between *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ mice (data not shown). However, the superficial cell layer of the epithelium was separated from the underlying layers in the *Hmgn1*$^{-/-}$ mice as compared with *Hmgn1*$^{+/+}$ corneas (Fig. 3). The blistering of the epithelium in *Hmgn1*$^{-/-}$ mice is reminiscent of bullae formation which is characteristic of various ocular diseases (O'Connor, 1983), and suggests adhesion abnormalities in these corneas.

To determine whether adherens junctions, a second type of adhesive complex present in the epithelium, might be altered in the *Hmgn1*$^{-/-}$ cornea, we examined the expression levels of the principal cadherin of epithelial tissue, E-cadherin. Quantitative real time PCR using RNA from 25-day-old corneas showed a 30% increase in the amount of E-cadherin mRNA in corneas from *Hmgn1*$^{-/-}$ mice relative to *Hmgn1*$^{+/+}$ mice (Fig. 4A). Analysis by Western blot showed a twofold increase in the amount of E-cadherin protein in corneal extracts from 25-day-old *Hmgn1*$^{-/-}$ versus *Hmgn1*$^{+/+}$ mice (Fig. 4B). In addition, western blots indicate that three other molecular components of the adherens junction complex, α-, β-, and γ-catenin, were 2.1-fold, 1.7-fold, and 2.3-fold greater, respectively, in corneal extracts from *Hmgn1*$^{-/-}$ than in extracts from *Hmgn1*$^{+/+}$ mice (Fig. 4B).

The distribution of E-cadherin protein, visualized by immunofluorescence, was localized to the plasma membrane of epithelial cells. The pattern of fluorescence was similar in the two mouse backgrounds with highest expression in the most superficial cells in both *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ corneal epithelium (Fig. 4C) suggesting that distribution of E-cadherin protein is not changed in the *Hmgn1* knockout mouse.

Altered differentiation in *Hmgn1*$^{-/-}$ corneal epithelium

The change in cellular morphology and the decreased number of cells in the suprabasal layer of the epithelium suggested that the differentiation pathway may be altered in the *Hmgn1*$^{-/-}$ cornea. To test this possibility we examined the levels of *GSTA4* and *GST*ω 1 transcripts, whose expression is restricted to different layers of the epithelium and correlates with distinct stages of corneal epithelial cell differentiation (Norman et al., 2004). *In situ* hybridization using riboprobes for *GSTA4* or *GSTω1* shows strong hybridization of the antisense (Figs. 5A, 5D), but not the sense (Figs. 5B, 5E) probes, in the middle and superficial epithelial cell layers, respectively, in the *Hmgn1*$^{+/+}$ cornea. In contrast, no *GSTa4* is detectable in the *Hmgn1*$^{-/-}$ cornea (Fig. 5C), while the *GSTω1* signal is clearly reduced in the superficial epithelial layer in the absence *of Hmgn1* (Fig. 5F). Quantitative RT-PCR using RNA isolated from 25-day-old corneas confirmed that *GST α4* RNA was decreased fourfold in the *Hmgn1*$^{-/-}$ cornea relative to wild-type cornea (Fig. 5G), a finding compatible with the decreased number of cells in the suprabasal layer of the epithelium (see Fig. 2). We have previously demonstrated that *GSTα4* is most prominently expressed in these cells (Norman et al., 2004).

The expression of keratin 12 (K12), aldehyde dehydrogenase 3 (ALDH3), and transketolase (TKT), which constitute abundant, corneal enriched proteins (Liu et al., 1993; Sax et al., 1996; Kays and Piatigorsky, 1997), was examined by immunohistochemistry using specific antibodies for each protein. As expected, K12, ALDH3, and TKT, were detected in all layers of the epithelium in the *Hmgn1*$^{+/+}$ cornea (Fig. 5H). The expression levels and localization of K12, ALDH3, and TKT were similar in the *Hmgn1*$^{-/-}$ 25-day-old mice corneas (Fig. 5H) when compared with their *Hmgn1*$^{+/+}$ counterparts. We conclude that loss of HMGN1 altered selectively the levels of proteins in the cornea affecting mostly differentiation related proteins such as E-cad-herin, catenins, GSTα4, and GSTω1.

Altered p63 localization and expression in the cornea epithelium of *Hmgn1*$^{-/-}$ mice

We noted that the pattern of HMGN1 expression in the corneal epithelium (Fig. 1) is similar to that of the transcription factor p63, a major regulator of epidermal morphogenesis, which may play an analogous role in the corneal epithelium (Pellegrini et al., 2001). Both HMGN1 (Fig. 1) and p63 (Collinson et al., 2002; Dua et al., 2003; Hsueh et al., 2004) are expressed in intermittent clusters of cells in the basal layer of the cornea epithelium. Given this similar pattern of expression and the role of p63 in epidermal morphogenesis we examined the possibility that the phenotypic changes observed in the corneal epithelium of *Hmgn1*$^{-/-}$ mice is linked to altered p63 expression.

Double-immunofluorescence on 25-day-old wild-type cornea revealed that HMGN1 and p63 colocalize within clusters of basal epithelial cells (Fig. 6A). HMGN1 protein, visualized using goat anti-mouse alexa fluor red, shows nuclear expression in the small clusters of basal cells. A similar staining pattern is observed for p63, visualized using goat anti-rabbit alexa fluor green (Fig. 6A). An overlay of the green and red images showed a perfect correspondence of the expression pattern, 100% of the time, indicating that HMGN1 and p63 are expressed in exactly the same subset of basal cells in the corneal epithelium (Fig. 6A). The overlap in expression pattern of the two proteins is appreciated further by comparing the Hoechst staining of all nuclei in the same section (Fig. 6A). While most of

the cells in the corneal epithelium were negative for both protein, invariably, cells that were positive for HMGN1 were also positive for p63.

Immunohistochemical analysis of the cornea of 25-day-old mice revealed that the presence of HMGN1 affects the distribution of p63 along the corneal epithelium. Whereas in *Hmgn1*$^{+/+}$ cornea p63 positive clusters of basal cells can be detected across both the peripheral and central cornea (Fig. 6B, +/+), in the corneal epithelium of their *Hmgn1*$^{-/-}$ littermates clusters of p63-positive basal cells are confined to the peripheral region of the corneal epithelium. No p63 protein is observed in the central region of the *Hmgn1*$^{-/-}$ epithelium (Fig. 6B, −/−). Interestingly, the intensity of the p63 immuno stain in the peripheral region of the *Hmgn1*$^{-/-}$ corneas seems to be higher than in the same region of the *Hmgn1*$^{+/+}$ *corneas*, suggesting that the lack of p63-postive cells in the central region is not necessarily because of the absence of sufficient p63 protein. Indeed, by quantitative PCR analysis of the levels of p63 transcripts in the cornea of the *Hmgn1*$^{-/-}$ mice is higher than in the cornea of their *Hmgn1*$^{+/+}$ *littermates* (Fig. 6C). Given the known function of p63 in epithelial development, it is possible that changes in p63 levels and in the distribution of p63-positive cells leads to developmental abnormalities in the corneal epithelium of *Hmgn1*$^{-/-}$ mice.

Discussion

Our main finding is that HMGN1, a chromatin-binding protein that interacts with nucleosomes, plays a role in the development and maintenance of the corneal epithelium. The corneal epithelium of *Hmgn1*$^{-/-}$ mice is thinner and blisters more readily than that of *Hmgn1*$^{+/+}$ mice, an indication that loss of HMGN1 disturbs the normal stratification program in the epithelium. These phenotypic changes are linked to changes in the expression levels of GSTs, of structural components of adherent junctions, and to alterations in the pattern of expression of p63, a key regulator of epidermal development and maintenance (Koster et al., 2004; Koster and Roop, 2004a, b; Westfall and Pieten-pol, 2004).

The thinning of the corneal epithelium and increased tendency to blister in *Hmgn1*$^{-/-}$ mice may be because of a change in its adhesive properties. This finding is consistent with our earlier studies using Pax 6 mutant mice, where a reduction in the thickness of the corneal epithelium was correlated with changes in adhesion (Davis et al., 2003). In *Hmgn1*$^{-/-}$ mice, the superficial epithelial layers appeared loosely adhered to the rest of the cornea, reminiscent of bullae or blister formation, a condition present in various bullous oculocutaneous diseases (O'Connor, 1983; Kaufman, 2000). Blisters forming because of a loss in adhesion (Smolin and Thoft, 1994; Allen et al., 1996; Koch et al., 1997) may represent a premature sloughing of the superficial cells and account for, wholly or in part, the observed reduction in the thickness of the epithelium. Interestingly, while in most pathologies associated with blistering the expression of adhesion molecules is decreased, in the *Hmgn1*$^{-/-}$ corneal epithelium the blistering is associated with an increase in the levels of the adhesion molecules. The changes in adhesion within the *Hmgn1*$^{-/-}$ corneal epithelium may be because of misexpression of E-cadherin, and α-, β-, and γ-catenin, all structural components of adherens junctions, one of several types of adhesion complexes (Angst et al., 2001). We also observed altered expression of N-cadherin, and changes in cell adhesion and motility, in embryo fibroblasts from *Hmgn1*$^{-/-}$ mice (Rubinstein et al., in press).

We find that the absence of HMGN1 impacts the differentiation program of the corneal epithelium. In the corneal epithelium of mature *Hmgn1*$^{-/-}$ mice the basal cells are ovoid rather than cuboid or columnar. Ovoid shaped basal cells are characteristic of corneal epithelium of very young mice; within 1 week of eyelid opening these cells first become

cuboidal and subsequently acquire the columnar-shape characteristic of basal cells of mature corneas (Zieske, 2004). The basal membrane layer of *Hmgn1*$^{-/-}$ corneal epithelium is disorganized and the basal cells penetrate into the stroma (Fig. 2). The suprabasal cell layer is mostly absent in *Hmgn1*$^{-/-}$ mice, and the up-regulation of GSTα4 and GSTω1, in the middle and superficial corneal layers, respectively, is not detected in the *Hmgn1*$^{-/-}$ epithelium; a finding consistent with the absence of the cells expressing these proteins. The results suggest that loss of HMGN1 impacts the movement of cells from the mitotic, basal layer to the non-mitotic, suprabasal layers. In contrast to the GSTs, the expression of several other corneal-enriched proteins, such as K12, ALDH3, and TKT, is not affected by the loss of HMGN1. These proteins are expressed throughout the corneal epithelium (Liu et al., 1993; Sax et al., 1996; Kays and Piatigorsky, 1997) while GST expression is confined to distinct epithelial layers (Davis et al., 2003). The results link HMGN1 expression with specific stages of corneal epithelial cell stratification and differentiation.

Two key findings of this study are that HMGN1 is expressed in the same subset of corneal basal cells as p63, and that the distribution of p63-expressing cells is altered in the *Hmgn1*$^{-/-}$ cornea. The importance of p63 during development of ectodermally derived tissue is well documented. p63 null mice have defects in several epithelial tissues (Mills et al., 1999; Yang et al., 1999), while mutations in p63 are associated with a large number of human syndromes including ectodermal dysplasia (ED) (Brunner et al., 2002). Patients with ED syndromes often display ocular anomalies including recurrent corneal epithelial defects from birth and blindness (Apple, 1998; Donahue et al., 1999; Ca-scallana et al., 2003). Interestingly, p63 is expressed throughout the basal layer of corneal epithelium in mice (Moore et al., 2002; Dua et al., 2003; Ramaesh et al., 2005) and rats (Hsueh et al., 2004) under the age of three months, but is absent in the central portion of the basal epithelial layer of the cornea by 6 months of age, similar to what we observe in the 25 day old *Hmgn1*$^{-/-}$ cornea. The distribution pattern of p63 is altered in the *Hmgn1*$^{-/-}$ cornea and is similar to that of older animals. However the overall levels of p63 are increased rather than decreased. In view of recent findings of uneven distribution of p63 isoforms in limbal keratin-ocytes (Wang et al., 2005) it is conceivable that HMGN1 protein affects p63 isotype synthesis. These data warrant further experiments to test whether the altered localization of p63 positive cells in the cornea of Hmgn1$^{-/-}$ mice is causally connected to the abnormalities in the stratification and differentiation of the corneal epithelium reported here. We find that the expression of *Hmgn1*, as well as that of the closely related *Hmgn2* and *Hmgn3* genes, is down regulated during corneal maturation (Norman et al., 2004). Our present studies indicate that HMGN1 protein plays a regulatory role during corneal maturation. Our finding may be relevant to regulatory mechanisms in other tissues as we find that in the skin the expression of HMGN1 is also localized to the basal cell layers (Fig. 1) and very similar to that of p63 (T. Furusawa and M. Bustin, unpublished). Misexpression of HMGN proteins in C2C12 myoblasts (Pash et al., 1993), in one cell mouse embryos (Mohamed et al., 2001), and in *Xenopus* zygotes (Korner et al., 2003) leads to developmental abnormalities. These, and additional studies (Bustin, 2001) suggest that proper execution of predetermined differentiation programs requires regulated expression of HMGN proteins. Loss of HMGN1 does not result in major phenotypic alterations perhaps because of homeostatic mechanisms involving other HMGNs or/and additional chromatin-binding structural proteins (Agresti and Bianchi, 2003; Catez et al., 2004; Bustin et al., 2005). Indeed, the expression of HMGB1, a highly abundant chromatin structural protein whose function partially overlaps with that of HMGNs is also down-regulated during corneal maturation (Norman et al., 2004). The emerging picture suggests that structural proteins that affect the conformation and accessibility of the chro-matin fiber play regulatory roles in developmental processes including corneal maturation.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, Center for Cancer Research, National Cancer Institute, and National Eye Institute.

References

Agresti A, Bianchi ME. HMGB proteins and gene expression. Curr Opin Genet Dev. 2003; 13:170–178. [PubMed: 12672494]

Albert, D.; Jakobiec, F. Principles and practice of ophthalmology. Philadelphia: W.B. Saunders Company; 1994.

Allen E, Yu QC, Fuchs E. Mice expressing a mutant desmosomal cadherin exhibit abnormalities in desmosomes, proliferation, and epidermal differentiation. J Cell Biol. 1996; 133:1367–1382. [PubMed: 8682871]

Angst BD, Marcozzi C, Magee AI. The cadherin superfamily: diversity in form and function. J Cell Sci. 2001; 114:629–641. [PubMed: 11171368]

Apple, MRaD. Ocular pathology. St. Louis, MI, USA: Mosby Inc; 1998.

Argueso P, Gipson IK. Epithelial mucins of the ocular surface: structure, biosynthesis and function. Exp Eye Res. 2001; 73:281–289. [PubMed: 11520103]

Begum N, Pash JM, Bhorjee JS. Expression and synthesis of high mobility group chromosomal proteins in different rat skeletal cell lines during myogenesis. J Biol Chem. 1990; 265:11936–11941. [PubMed: 1694850]

Birger Y, West KL, Postnikov YV, Lim JH, Furusawa T, Wagner JP, Laufer CS, Kraemer KH, Bustin M. Chromosomal protein HMGN1 enhances the rate of DNA repair in chromatin. Embo J. 2003; 22:1665–1675. [PubMed: 12660172]

Brunner HG, Hamel BC, Bokhoven Hv H. P63 gene mutations and human developmental syndromes. Am J Med Genet. 2002; 112:284–290. [PubMed: 12357472]

Bustin M. Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. Mol Cell Biol. 1999; 19:5237–5246. [PubMed: 10409715]

Bustin M. Chromatin unfolding and activation by HMGN(*) chromosomal proteins. Trends Biochem Sci. 2001; 26:431–437. [PubMed: 11440855]

Bustin M, Catez F, Lim JH. The dynamics of histone H1 function in chromatin. Mol Cell. 2005; 17:617–620. [PubMed: 15749012]

Cascallana JL, Bravo A, Page A, Budunova I, Slaga TJ, Jorcano JL, Perez P. Disruption of eyelid and cornea development by targeted overexpression of the glucocorticoid receptor. Int J Dev Biol. 2003; 47:59–64. [PubMed: 12653252]

Catez F, Yang H, Tracey KJ, Reeves R, Misteli T, Bus-tin M. Network of dynamic interactions between histone H1 and high-mobility-group proteins in chromatin. Mol Cell Biol. 2004; 24:4321–4328. [PubMed: 15121851]

Chung EH, Bukusoglu G, Zieske JD. Localization of corneal epithelial stem cells in the developing rat. Invest Op-hthalmol Vis Sci. 1992; 33:2199–2206.

Collinson JM, Morris L, Reid AI, Ramaesh T, Keighren MA, Flockhart JH, Hill RE, Tan SS, Ramaesh K, Dhillon B, et al. Clonal analysis of patterns of growth, stem cell activity, and cell movement during the development and maintenance of the murine corneal epithelium. Dev Dyn. 2002; 224:432–440. [PubMed: 12203735]

Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell. 1989; 57:201–209. [PubMed: 2702690]

Crippa MP, Nickol JM, Bustin M. Differentiation-dependent alteration in the chromatin structure of chromosomal protein HMG-17 gene during erythropoiesis. J Mol Biol. 1991; 217:75–84. [PubMed: 1988681]

Dai X, Segre JA. Transcriptional control of epidermal specification and differentiation. Curr Opin Genet Dev. 2004; 14:485–491. [PubMed: 15380238]

Davis J, Duncan MK, Robison WG Jr, Piatigorsky J. Requirement for Pax6 in corneal morphogenesis: a role in adhesion. J Cell Sci. 2003; 116:2157–2167. [PubMed: 12692153]

Davis JA, Reed RR. Role of Olf-1 and Pax-6 transcription factors in neurodevelopment. J Neurosci. 1996; 16:5082–5094. [PubMed: 8756438]

Donahue JP, Shea CJ, Taravella MJ. Hidrotic ectodermal dysplasia with corneal involvement. J Aapos. 1999; 3:372–375. [PubMed: 10613583]

Dua HS, Azuara-Blanco A. Limbal stem cells of the corneal epithelium. Surv Ophthalmol. 2000; 44:415–425. [PubMed: 10734241]

Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. Eye. 2003; 17:877–885. [PubMed: 14631392]

Felsenfeld G, Groudine M. Controlling the double helix. Nature. 2003; 421:448–453. [PubMed: 12540921]

Francesconi CM, Hutcheon AE, Chung EH, Dalbone AC, Joyce NC, Zieske JD. Expression patterns of re-tinoblastoma and E2F family proteins during corneal development. Invest Ophthalmol Vis Sci. 2000; 41:1054–1062. [PubMed: 10752941]

Fukushima A, Funaki H, Yaoeda K, Tanaka T, Shirakashi M, Yoshida Y, Yaoita E, Abe H, Yamamoto T. Localization and expression of chondromodulin-I in the rat cornea. Arch Histol Cytol. 2003; 66:445–452. [PubMed: 15018147]

Hatakeyama Y, Tuan RS, Shum L. Distinct functions of BMP4 and GDF5 in the regulation of chondrogenesis. J Cell Biochem. 2004; 91:1204–1217. [PubMed: 15048875]

Haustein J. On the ultrastructure of the developing and adult mouse corneal stroma. Anat Embryol. 1983; 168:291–305. [PubMed: 6660567]

Hay ED. Development of the vertebrate cornea. Int Rev Cytol. 1979; 63:263–322. [PubMed: 395131]

Hsueh YJ, Wang DY, Cheng CC, Chen JK. Age-related expressions of p63 and other keratinocyte stem cell markers in rat cornea. J Biomed Sci. 2004; 11:641–651. [PubMed: 15316140]

Jaskelioff M, Peterson CL. Chromatin and transcription: histones continue to make their marks. Nat Cell Biol. 2003; 5:395–399. [PubMed: 12724776]

Kaufman HE. Treatment of viral diseases of the cornea and external eye. Prog Retin Eye Res. 2000; 19:69–85. [PubMed: 10614681]

Kays WT, Piatigorsky J. Aldehyde dehydrogenase class 3 expression: identification of a cornea-preferred gene promoter in transgenic mice. Proc Natl Acad Sci USA. 1997; 94:13594–13599. [PubMed: 9391071]

Klyce, SD.; Beuerman, RW. Structure and function of the cornea. Kaufman, BBHE.; McDonald, MB.; Waltman, SR., editors. New York: The Cornea. Churchill Livingstone; 1988.

Koch PJ, Mahoney MG, Ishikawa H, Pulkkinen L, Uitto J, Shultz L, Murphy GF, Whitaker-Menezes D, Stanley JR. Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. J Cell Biol. 1997; 137:1091–1102. [PubMed: 9166409]

Korner U, Bustin M, Scheer U, Hock R. Developmental role of HMGN proteins in Xenopus laevis. Mech Dev. 2003; 120:1177–1192. [PubMed: 14568106]

Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR. p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev. 2004; 18:126–131. [PubMed: 14729569]

Koster MI, Roop DR. p63 and epithelial appendage development. Differentiation. 2004a; 72:364–370. [PubMed: 15606495]

Koster MI, Roop DR. The role of p63 in development and differentiation of the epidermis. J Dermatol Sci. 2004b; 34:3–9. [PubMed: 14757276]

Kruse FE. Stem cells and corneal epithelial regeneration. Eye. 1994; 8(Pt 2):170–183. [PubMed: 7958018]

Lavker RM, Dong G, Cheng SZ, Kudoh K, Cotsarelis G, Sun TT. Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. Invest Ophthalmol Vis Sci. 1991; 32:1864–1875. [PubMed: 2032808]

Lehtonen S, Lehtonen E. HMG-17 is an early marker of inductive interactions in the developing mouse kidney. Differentiation. 2001; 67:154–163. [PubMed: 11683498]

Lim JH, Catez F, Birger Y, West KL, Prymakowska-Bosak M, Postnikov YV, Bustin M. Chromosomal protein HMGN1 modulates histone H3 phosphorylation. Mol Cell. 2004; 15:573–584. [PubMed: 15327773]

Liu CY, Zhu G, Westerhausen-Larson A, Converse R, Kao CW, Sun TT, Kao WW. Cornea-specific expression of K12 keratin during mouse development. Curr Eye Res. 1993; 12:963–974. [PubMed: 7508359]

Luna, LG. Manual of histologic staining methods of the armed forces institute of pathology. New York: McGraw-Hill; 1968.

Margueron R, Trojer P, Reinberg D. The key to development: interpreting the histone code? Curr Opin Genet Dev. 2005; 15:163–176. [PubMed: 15797199]

Maurice, DM. The eye. New York: Academic Press; 1969.

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature. 1999; 398:708–713. [PubMed: 10227293]

Mohamed AO, Bustin M, Clarke HJ. High-mobility group proteins 14 and 17 maintain the timing of early embryonic development in the mouse. Dev Biol. 2001; 229:237–249. [PubMed: 11133167]

Moore JE, McMullen CB, Mahon G, Adamis AP. The corneal epithelial stem cell. DNA Cell Biol. 2002; 21:443–451. [PubMed: 12167247]

Nagasaki T, Zhao J. Centripetal movement of corneal epithelial cells in the normal adult mouse. Invest Ophthalmol Vis Sci. 2003; 44:558–566. [PubMed: 12556383]

Norman B, Davis J, Piatigorsky J. Postnatal gene expression in the normal mouse cornea by SAGE. Invest Op-hthalmol Vis Sci. 2004; 45:429–440.

O'Connor, GR. The cornea. Boston: Little, Brown and Company; 1983.

Pash JM, Alfonso PJ, Bustin M. Aberrant expression of high mobility group chromosomal protein 14 affects cellular differentiation. J Biol Chem. 1993; 268:13632–13638. [PubMed: 8514795]

Pei YF, Rhodin JA. The prenatal development of the mouse eye. Anat Rec. 1970; 168:105–125. [PubMed: 5469558]

Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fan-tozzi I, Bondanza S, Ponzin D, McKeon F, DeLuca M. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci USA. 2001; 98:3156–3161. [PubMed: 11248048]

Ramaesh T, Ramaesh K, Martin Collinson J, Chanas SA, Dhillon B, West JD. Developmental and cellular factors underlying corneal epithelial dysgenesis in the Pax6+/− mouse model of aniridia. Exp Eye Res. 2005; 81:224–235. [PubMed: 16080917]

Reeves R. Molecular biology of HMGA proteins: hubs of nuclear function. Gene. 2001; 277:63–81. [PubMed: 11602345]

Rubinstein YR, Furusawa T, Lim JH, Postnikov YV, West KL, Birger Y, Lee S, Nguyen P, Trepel BJ, Bustin M. Chromosomal protein HMGN1 modulates the expression of N-cadherin. FEBS J. 2005; 272:5853–5863. [PubMed: 16279949]

Sarmento OF, Digilio LC, Wang Y, Perlin J, Herr JC, Allis CD, Coonrod SA. Dynamic alterations of specific histone modifications during early murine development. J Cell Sci. 2004; 117:4449–4459. [PubMed: 15316069]

Sax CM, Salamon C, Kays WT, Guo J, Yu FX, Cuthbert-son RA, Piatigorsky J. Transketolase is a major protein in the mouse cornea. J Biol Chem. 1996; 271:33568–33574. [PubMed: 8969223]

Smolin, G.; Thoft, R. The cornea. New York: Little, Brown and Company; 1994.

Stepp MA, Zhu L, Sheppard D, Cranfill RL. Localized distribution of alpha 9 integrin in the cornea and changes in expression during corneal epithelial cell differentiation. J His-tochem Cytochem. 1995; 43:353–562.

Sun L, Sun TT, Lavker RM. CLED: a calcium-linked protein associated with early epithelial differentiation. Exp Cell Res. 2000; 259:96–106. [PubMed: 10942582]

Trainor PA, Tam PP. Cranial paraxial mesoderm and neural crest cells of the mouse embryo: co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. Development. 1995; 121:2569–2582. [PubMed: 7671820]

Wang DY, Cheng CC, Kao MH, Hsueh YJ, Ma DH, Chen JK. Regulation of limbal keratinocyte proliferation and differentiation by TAp63 and {Delta}Np63 transcription factors. Invest Ophthalmol Vis Sci. 2005; 46:3102–3108. [PubMed: 16123408]

Wang I, Kao CW, Liu C, Saika S, Nishina PM, Sundberg JP, Smith RS, Kao WW. Characterization of Corn1 mice: alteration of epithelial and stromal cell gene expression. Mol Vis. 2001; 7:20–26. [PubMed: 11182022]

West-Mays JA, Sivak JM, Papagiotas SS, Kim J, Nottoli T, Williams T, Fini ME. Positive influence of AP-2alpha transcription factor on cadherin gene expression and differentiation of the ocular surface. Differentiation. 2003; 71:206–216. [PubMed: 12694203]

Westfall MD, Pietenpol JA. p63: Molecular complexity in development and cancer. Carcinogenesis. 2004; 25:857–864. [PubMed: 15033906]

Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C, McKeon D. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature. 1999; 398:714–718. [PubMed: 10227294]

Yoshida N, Yoshida S, Araie M, Handa H, Nabeshima Y. Ets family transcription factor ESE-1 is expressed in corneal epithelial cells and is involved in their differentiation. Mech Dev. 2000; 97:27–34. [PubMed: 11025204]

Zieske JD. Corneal development associated with eyelid opening. Int J Dev Biol. 2004; 48:903–911. [PubMed: 15558481]

Zieske JD, Wasson M. Regional variation in distribution of EGF receptor in developing and adult corneal epithelium. J Cell Sci. 1993; 106:145–152. [PubMed: 8270620]

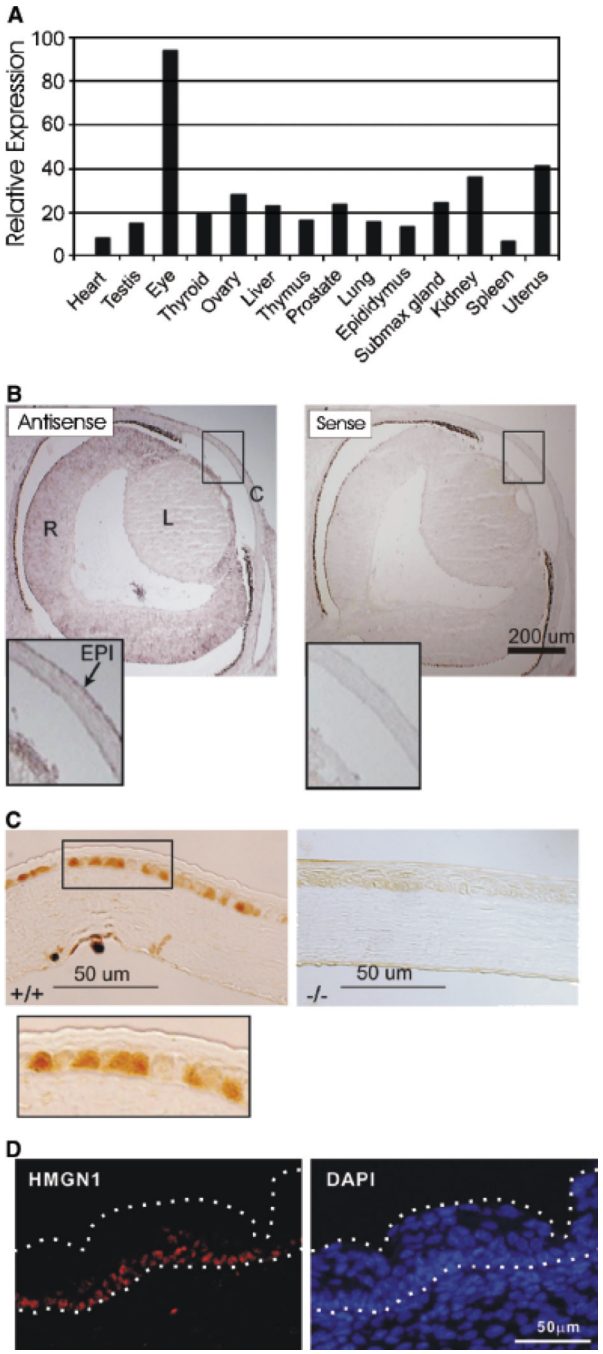

Fig. 1.

Hmgn1 expression in the eye. (A) Quantitative analysis of RNA master dot blot (CLONTECH) demonstrating high levels of *Hmgn1* expression in the eye. (B) *In situ* hybridization of *Hmgn1*$^{+/+}$ embryonic eyes. The arrow in the enlarged box points to the specific expression detected in the corneal epithelium (EPI). C, cornea; R, retina; L, lens. (C) Immunohistochemistry of 25-day-old *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ mice corneas using a mouse high-mobility group protein N1 (HMGN1) specific antibody. Note the expression of the protein in a subset of basal cells in the corneal epithelium of *Hmgn1*$^{+/+}$(left panel) mice. (D) High HMGN1 expression in the basal layer of the epidermis, shown are

immunofluorescence and corresponding DAPI stain. For orientation, the basal and outer layers of the epidermis are outlined.

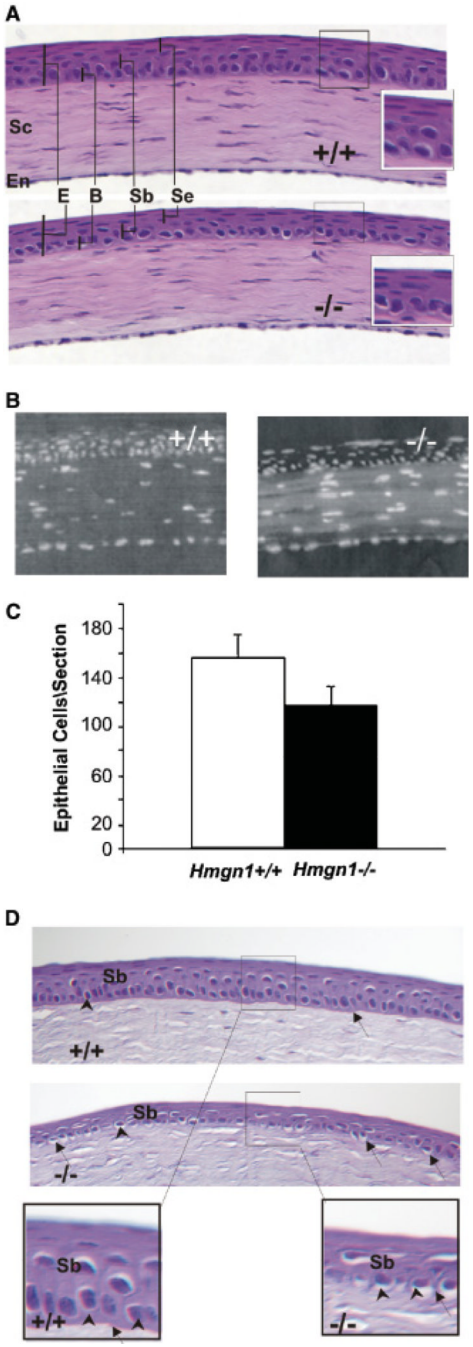

Fig. 2.

Altered properties of *Hmgn1*$^{-/-}$ cornea. (A) Thinning of *Hmgn1*$^{-/-}$ cornea epithelium. Hematoxylin and eosin (H&E) stained sections from the eye of 25-day-old *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ mice reveal a reduced second layer of large nucleated cells (suprabasal cells) in the *Hmgn1*$^{-/-}$ samples. E, epithelium; B, basal layer; Sb, suprabasal cells; Se, surface epithelium; Sc, stroma; En, endothelium. (B) Reduced number of epithelial cells in *Hmgn1*$^{-/-}$ corneas. DAPI staining of 25-day-old *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ eye corneas. (C) Decreased cell number in 25-day-old mice *Hmgn1*$^{-/-}$ cornea epithelium. The bar graph represents the average number of cells counted in six animals. (D) Thinning and stratification changes in H&E-stained 4-month-old *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ mice. Note that

in the *Hmgn1*$^{-/-}$ corneal epithelium there are fewer suprabasal wing cells (Sb), the basal membrane (arrows) is disorganized, and the basal cells are oval rather than columnar (arrowheads).

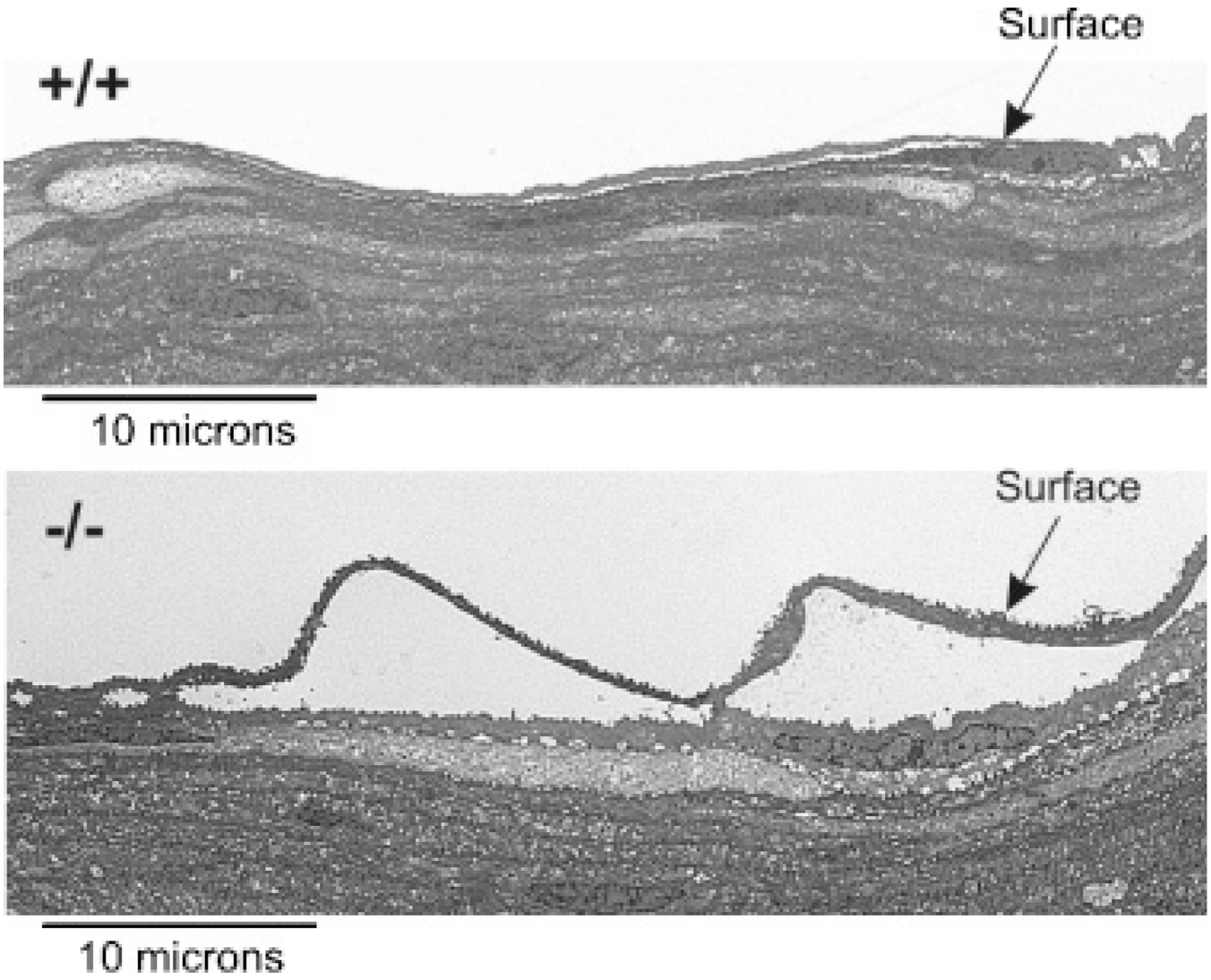

Fig. 3.

Blistering of the epithelial surface in Hmgn1$^{-/-}$ cornea. Electron micrographs of corneal epithelium demonstrating bullous formation in the superficial epithelial layer of corneas of *Hmgn1*$^{-/-}$ mice, but not in their *Hmgn1*$^{+/+}$ littermates.

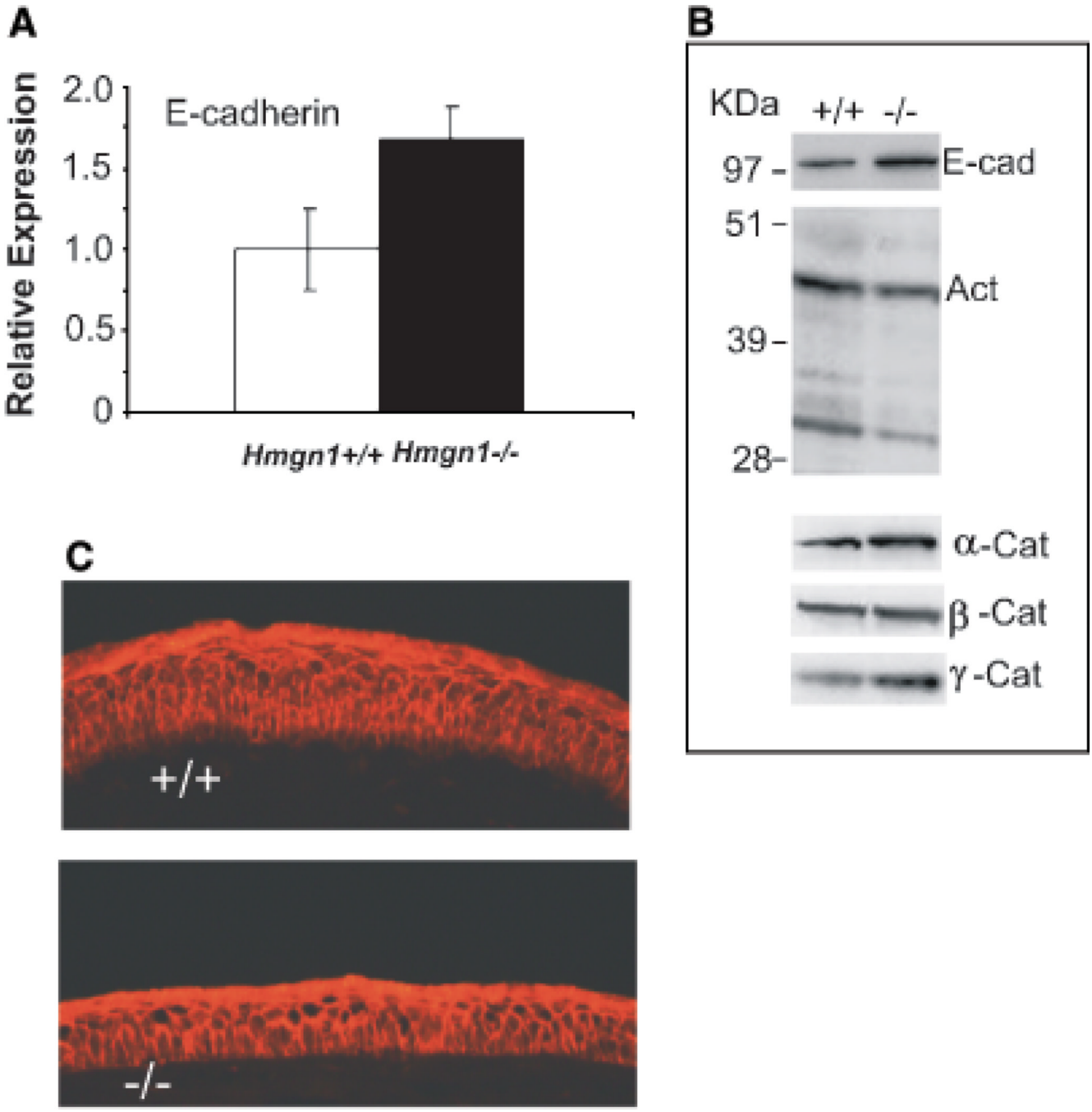

Fig. 4.

Expression of adhesion molecules in the 25-day-old Hmgn1$^{-/-}$ and Hmgn1$^{+/+}$ corneal epithelium. (A) Increased mRNA expression level of E-cadherin (E-cad) in *Hmgn1*$^{-/-}$ cornea. Bars represent quantitative real time PCR of E-cad using RNA isolated from *Hmgn1*$^{-/-}$ and *Hmgn1*$^{+/+}$ corneas. Two sets of primers were used for the detection of the mRNA product in two separate experiments. The results presented in the graph are an average of two RT-PCRs for each experiment. The level of expression is normalized to β actin. (B) Altered expression of E-cad, and of α-, β-, γ-catenin (-Cat) in *Hmgn1*$^{-/-}$ cornea. Shown are western blots of protein extracts from *Hmgn1*$^{-/-}$ and *Hmgn1*$^{+/+}$ cornea probed with the antibodies indicated to the right of each panel. Protein loading normalized to actin.

(C) Immunofluorescence staining of E-cad in *Hmgn1*$^{-/-}$ (−/−) and *Hmgn1*$^{+/+}$(+/+) cornea indicates a similar distribution of this protein, to the plasma membrane.

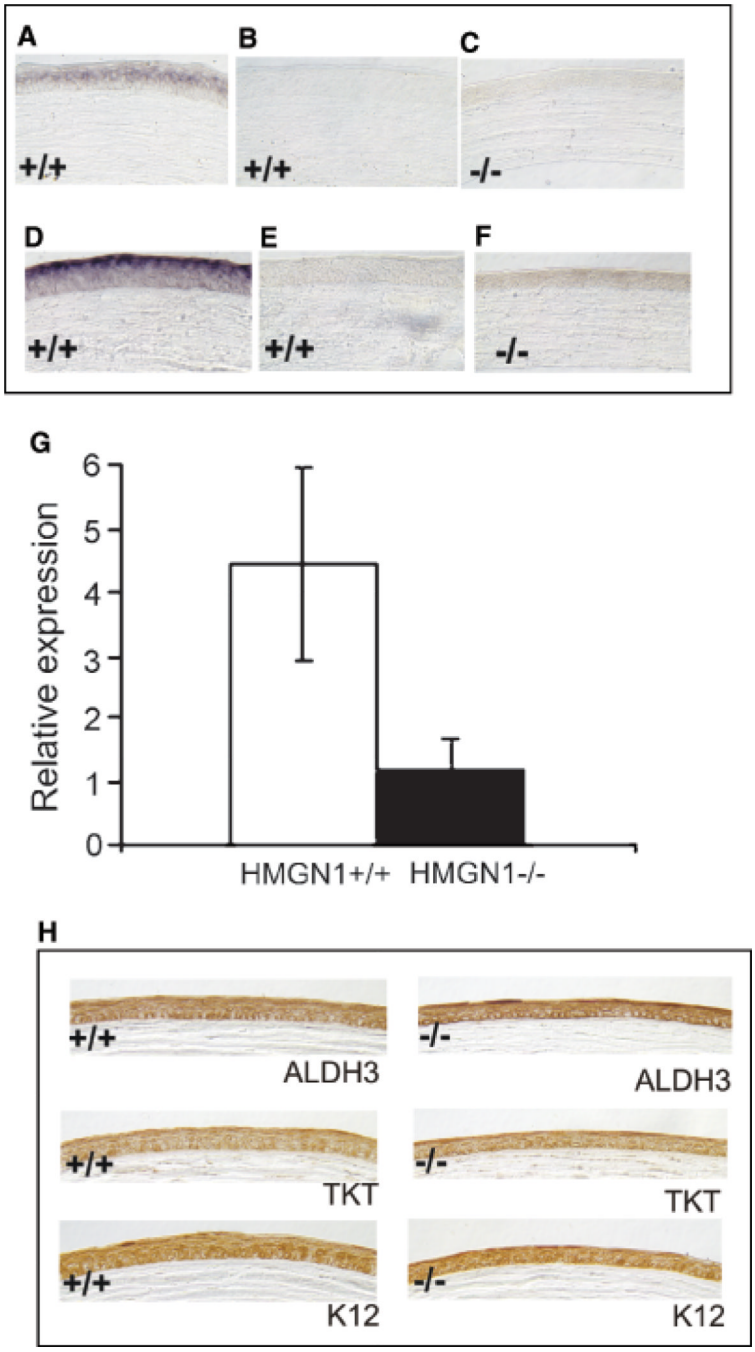

Fig. 5.

Reduced expression of glutathione-S-transferase a 4 (GSTa4) and GST ω 1 (GSTω 1) in Hmgn1$^{-/-}$ cornea epithelium. *In situ* hybridization with riboprobes for GSTa4 (A–C) and GSTω1 (D–E) genes on *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ cornea sections (B, E, controls with sense riboprobes). Note the strong hybridization of the antisense, but not the sense, only in the wild-type (A, D) samples. Note loss of GSTa4 (C) and of GSTo 1 (F), in *Hmgn1*$^{-/-}$ corneas. (G) Real time RT-PCR for GSTa4 gene using RNA isolated from *Hmgn1*$^{-/-}$ and *Hmgn1*$^{+/+}$ corneas. Five sets of primers were used for the detection of the RNA product in two separate experiments. The results presented in the graph are an average of two RT-PCRs for each experiment. The level of expression is relative to actin. (H) Similar expression of aldehyde

dehydrogenase 3, transketolase, and keratin 12, in *Hmgn1*$^{+/+}$ and *Hmgn1*$^{-/-}$ corneas. Immunohistochemistry of cornea from 25-day-old mice.

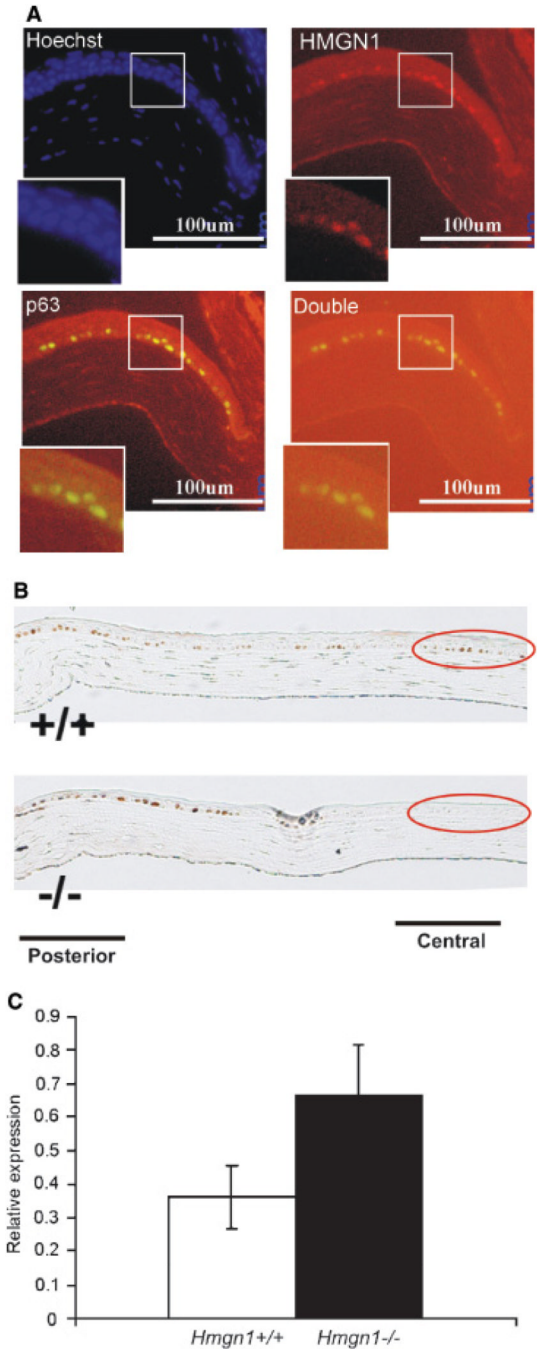

Fig. 6.

p63 expression in the 25-day-old Hmgn1 $^{-/-}$ and Hmgn1$^{+/+}$ corneal epithelium. (A) Double immunofluorescence of high-mobility group protein N1 (HMGN1) and p63. The white boxes in each panel mark a specific area that was enlarged (bottom left). Panels from left to right: Hoechst DNA staining, HMGN1 staining with goat anti mouse 594-Alexa fluor red, p63 staining with goat anti rabbit 488 Alexa fluor green, double staining of HMGN1 and p63, the double expressed cells have yellow staining. Note that HMGN1 and p63 colocalize to the same clusters of basal epithelial cells. (B). Altered distribution pattern of p63-containing cells in the basal layer of *Hmgn1*$^{-/-}$ cornea. Immunohistochemistry of 25-day-old wild-type (+/+) and *Hmgn1*$^{-/-}$ (−/−) cornea using p63 antibody indicates depletion of

the p63-positive cells in the central region of the *Hmgn1*$^{-/-}$ corneal epithelium (red circle). (C) Increased p63 transcript in the corneal *Hmgn1*$^{-/-}$ epithelium. Quantitative real time PCR of p63 using RNA isolated from *Hmgn1*$^{-/-}$ and *Hmgn1*$^{+/+}$ corneas. Two sets of primers specific for the common exons of all p63 isoforms were used for the detection of the RNA product in two separate experiments. The results presented in the bar graph are an average of two PCRs for each experiment.