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Expression Of Nucleosomal Protein HMGN1 In The Cycling Mouse Hair Follicle

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Abstract

Here we examine the expression pattern of HMGN1, a nucleosome binding protein that affects chromatin structure and activity, in the hair follicle and test whether loss of HMGN1 affects the development or cycling of the follicle. We find that at the onset of hair follicle development, HMGN1 protein is expressed in the epidermal placode and in aggregated dermal fibroblasts. In the adult hair follicle, HMGN1 is specifically expressed in the basal layer of epidermis, in the outer root sheath, in the hair bulb, but not in the inner root sheath and hair shaft. The expression pattern of HMGN1 is very similar to p63, suggesting a role for HMGN1 in the transiently amplifying cells. We also find HMGN1 expression in some, but not all hair follicle stem cells as detected by its colocalization with Nestin and with BrdU label-retaining cells. The appearance of the skin and hair follicle of *Hmgn1*^{-/-} mice was indistinguishable from that of their *Hmgn1*^{+/+} littermates. We found that in the hair follicle the expression of HMGN2 is very similar to HMGN1 suggesting functional redundancy between these closely related HMGN variants.

Keywords

chromatin; HMGN; p63; hair follicle

1. Results and Discussion

Hair follicle development is initiated by an interaction between ectodermal epithelium and mesenchyme, which is mediated by many growth factors (Schmidt-Ullrich and Paus, 2005). The first appearance of the hair follicle primordium is a hair placode, which is formed by a local thickening of the epithelium that induces aggregation of the underlying mesenchymal cells (dermal condensate). Subsequently, the epithelial cells at the placode proliferate and invaginate into the mesenchyme to form a hair germ, which continues to elongate downward to form a hair peg. At this stage, the dermal condensate is called dermal papilla (DP) and is engulfed by down-growing epithelial cells. The epithelial layer adjacent to the DP is referred to as matrix; these cells grow upward and differentiate into the hair shaft and inner root sheath (IRS). The IRS is surrounded by the outer root sheath (ORS), which is contiguous with skin

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epidermis (Fuchs, 2007; Millar, 2002). The hair follicle stem cells are located in the bulge region of the ORS (Cotsarelis et al., 1990; Taylor et al., 2000). The mature hair follicle undergoes a continuous, cyclic, preprogrammed sequence of events known as growth (anagen), regression (catagen) and rest (telogen) (Alonso and Fuchs, 2006; Muller-Rover et al., 2001).

We previously reported that *Hmgn1* is expressed in the basal layer of skin and cornea epithelium, and that the development and maintenance of the corneal epithelium of *Hmgn1*^{-/-} mice is abnormal (Birger et al., 2006). HMGN (High Mobility Group Nucleosome) is a protein family that specifically binds to nucleosome cores, the building block of the chromatin fiber. The interaction of HMGN with nucleosomes alters the levels of posttranslational modifications in the tail of nucleosomal histones, induces alterations in the local structure of the chromatin fiber and alters transcription (Bustin, 2001; Lim et al., 2004; Lim et al., 2005). Several experiments demonstrated that the expression level of HMGN proteins correlates with tissue differentiation. During embryogenesis the expression of *Hmgn* genes is gradually downregulated throughout the entire embryo except in committed but continuously renewing cells undergoing active differentiation (Furusawa et al., 2006; Hock et al., 2007; Lehtonen et al., 1998). In the corneal epithelium, the expression of HMGN1 coincides with that of p63 (Birger et al., 2006), a protein known to play a role in the epithelial stratification (Mills et al., 1999; Yang et al., 1999). Because both cornea and skin are derived from the ectoderm (Kaufman and Bard, 1999), we examined whether HMGN1 is involved in the formation and maintenance of the hair follicle, which has been extensively used as a model system for studies on development.

1.1. HMGN1 expression during the hair follicle development

To gain insight into the role of HMGN1 in hair follicle development, we examined the distribution of the protein in the follicle and surrounding skin. Whole mount *in situ* hybridization at embryonic day 15.5 (E15.5) reveals that *Hmgn1* expression is observed as a punctuate expression pattern on the skin surface, suggesting that *Hmgn1* transcripts are expressed in the epidermal placode at the onset of hair follicle development (Fig. 1 A,B). Immunofluorescence analyses of newborn skin reveal the presence of HMGN1 protein in the epidermis and in aggregated dermal cells (also known as dermal condensate, DC in Fig. 1C-F). HMGN1 expression persists in the dermal papilla in the later stages (Fig. 1C-N). In the epidermis and in the hair bulb region, HMGN1 expression coincides with that of p63, a major regulator of epidermal morphogenesis and the development of skin appendages, including the hair follicle (Mikkola and Millar, 2006). These results revealed that HMGN1 is expressed both in the epidermis and dermis at the onset of hair follicle morphogenesis.

1.2. HMGN1 expression in the adult hair follicle

In the mature hair follicle, HMGN1 protein is expressed in the basal layer of the skin and throughout the entire growing region of the hair follicle in a pattern similar to that of p63 (Fig. 2). Thus, in the elongated portion of the hair follicle, both HMGN1 and p63 are detected only in the ORS, the outer most region of the hair follicle but not in the IRS or hair shaft (Fig. 2E-2L). In the hair bulb region, HMGN1 is present both in the dermal papilla and in the hair matrix (Fig. 2 M-P). These expression patterns of HMGN1 are especially prominent in the growing region of the hair follicle and are significantly reduced in the more differentiated regions such as IRS and the hair shaft itself. Thus, as the cells differentiate at the matrix, the expression of both p63 and HMGN1 is downregulated. The differentiation-related downregulation of HMGN1 expression during hair follicle development supports our previous observations that HMGN1 is highly expressed in the undifferentiated transiently amplifying cells and downregulated upon differentiation (Birger et al., 2006). Similar to the anagen phase, HMGN1 immunofluorescence signals are observed in the epidermis, ORS, and dermal papilla

of catagen and telogen follicles (supplemental Fig. S1), suggesting that HMGN1 is expressed throughout the hair growth cycle.

1.3. HMGN1 is expressed in hair follicle stem cells

A specialized region of the ORS located just below the sebaceous gland, known as the “bulge”, contains follicle stem cells (Fuchs, 2007). These stem cells are activated at the telogen-to-anagen transition and initiate a new round of hair growth. Since HMGN1 is highly expressed in the ORS, we specifically examined if HMGN1 protein is located in the bulge region. To positively identify the bulge region we counterstained for Nestin, one of several known bulge region markers (Li et al., 2003). We find some of the Nestin positive cells also contain HMGN1 (Fig. 3 A-D).

At the onset of anagen, bulge cells proliferate and incorporate BrdU, but subsequently the label is lost from proliferating cells and retained in the quiescent, non-dividing stem cells (Ito et al., 2004). To further examine whether the Nestin-positive cells in the bulge region which also contain HMGN1 are indeed stem cells, we used an established protocol (Ito et al., 2004) to label cells with BrdU, and visualized the location of the BrdU label and of the HMGN1 protein by immunofluorescence. To this end, the follicles in the back skin of 8 weeks old mice were synchronized into the anagen phase by depilation, the mice injected with BrdU for three days, and the skin collected and examined after a chase period of 18 days. The immunofluorescence analyses revealed a partial colocalization of HMGN1 with the BrdU label (Fig. 3 E-L). Some of the strong BrdU labeled cells had only a weak HMGN1 immunofluorescence signal. In summary, the results indicate that HMGN1 is present in some Nestin- and BrdU-positive cells; however, the HMGN1 signal is not particularly prominent in these stem cells.

1.4. Loss of *Hmgn1* does not affect hair follicle formation

Since we detected HMGN1 expression in the epithelial stem cells, we tested whether loss of HMGN1 affects the expression levels of the hair bulge marker genes. Expression profiling array of skin samples from *Hmgn1*^{+/+} and *Hmgn1*^{-/-} littermates revealed changes in the expression levels of several genes however none of these could be specifically related to the cycling of the hair follicle (not shown). Furthermore, the levels of *Wif1*, *K15*, *CD200*, *CD34*, *Nestin*, *ID2*, *Dkk3*, *Fzd1* and *Fzd2* transcripts, all of which are hair follicle stem cell marker genes (Cotsarelis, 2006; Li et al., 2003) were similar in *Hmgn1*^{-/-} and *Hmgn1*^{+/+} mice (supplemental table S1). This result suggests that the loss of HMGN1 protein does not significantly affect the transcription profile of hair follicle stem cells. Histological analysis also revealed that although HMGN1 is highly expressed in the epidermis, ORS and sebaceous gland (Fig. 2 and Fig. 3), their histological appearance is not affected by the loss of this protein (Fig. 4). Likewise, the expression of p63 in the epidermis and the morphological appearance of the hair shaft in *Hmgn1*^{-/-} mice were not significantly different from that of their *Hmgn1*^{+/+} littermates (Fig. 4 and supplemental Fig. S2). We examined hair growth between *Hmgn1*^{+/+} and *Hmgn1*^{-/-} mice when the hair cycle was synchronized by depilation, but there was no difference (supplemental Fig. S3). Furthermore evaluation of the hair cycle score (Muller-Rover et al, 2001) did not suggest major HMGN1-dependent effects. Taken together, these results indicate that loss of HMGN1 does not significantly alter the formation or cycling of the hair follicle.

The finding that loss of HMGN1 does not affect the hair follicle, although it is prominently expressed in transiently amplifying cells, led us to examine whether a closely related HMGN homologue named HMGN2 is also expressed in the follicle. We found that the expression pattern of the HMGN2 variant is identical to that of HMGN1 except in the matrix (Fig. 5).

In summary, we found that the chromosomal protein HMGN1 is expressed during the hair follicle induction and in the mature hair follicle throughout the hair growth cycle. HMGN1 expression is observed in the ORS, matrix, DP, sebaceous gland, skin epidermis, and in some of the hair follicle stem cells. HMGN1 expression in the ORS, DP and skin epidermis are identical to p63, suggesting that HMGN1 expression is retained in the transient amplifying cells derived from the hair stem cells located in the bulge, and down regulated upon differentiation into IRS and hair shaft in the matrix. Analysis of *Hmgn1*^{-/-} mice reveals that loss of HMGN1 protein did not significantly affect the expression of p63 or the cycling of the hair follicle. We also found that the expression pattern of the HMGN2 variant in the hair follicle is very similar to that of HMGN1, raising the possibility of functional redundancy among these closely related proteins.

2. Experimental Procedures

2.1. Mouse strain

Hmgn1^{-/-} mice, generated as described before (Birger et al., 2003), were backcrossed with C57/BL6 for more than 9 generations. In littermate comparative studies, male and female *Hmgn1*^{+/-} were bred and their litters genotyped. All experiments were performed with the approval of Animal Care and Use Committee (ACUC) of the National Institutes of Health (NIH).

2.2. *In situ* hybridization, Histology, immunofluorescence and BrdU labeling

Whole mount *in situ* hybridization for E15.5 embryo was performed as previously described (Furusawa et al., 2006). Mouse back skin samples from littermate *Hmgn1*^{-/-} and *Hmgn1*^{+/+} C57/BL6 mice were fixed in neutral buffered 10% formalin solution (Sigma-Aldrich, St. Louis, MO) and embedded into Paraplast plus tissue embedding medium (Fisher Scientific, Sewanee, GA). After dehydration, 0.5 mm thick microscope sections were prepared and used for Hematoxylin/Eosin staining. Hair follicle cycle of each specimen was determined according to Muller-Rover et al (2001). For immunofluorescence studies, deparaffinized sections were subjected to antigen retrieval by microwaving for 10 min in 10 mM citric acid buffer (pH 6.0). The treated slides were overlaid with blocking solution (5% goat serum/PBS) for 30 min, washed and incubated with primary antibody at 4 °C for overnight. Primary antibodies used for this study are: rabbit anti mouse HMGN1 polyclonal antibody (dilution rate 1:250) (Birger et al., 2003), rabbit anti HMGN2 polyclonal antibody (1:250) (prepared by Dr. Michael Bustin, against recombinant human HMGN2 protein and which recognize both human and mouse proteins), anti p63 4A4 mouse monoclonal antibody (1:200) (Santa Cruz Biotechnology Inc, Santa Cruz, CA), or anti Nestin mouse monoclonal antibody (1:50) (Developmental Studies Hybridoma Bank, Iowa City, IA). The primary antibodies were detected with secondary antibodies conjugated with either Alexa-fluoro 488 or 568 (Molecular Probes/Invitrogen, Carlsbad, CA) and visualized by fluorescent microscopy. The nuclei in the stained sections were visualized by either embedding the slides with DAPI-containing Vectashield hard set (Vector Laboratories, Burlingame, CA) or Hoechst 33258 (Molecular Probes/Invitrogen, Carlsbad, CA). BrdU labeling and label detection in the hair follicle bulge cells was performed with the BrdU Labeling and Detection Kit I (Roche Diagnostics, Indianapolis, IN). Comparison of phenotypic analysis was examined by three pairs of *Hmgn1*^{+/+} and *Hmgn1*^{-/-} male littermates around twelve weeks old and representative data were presented.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

3. Acknowledgement

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Fig. 1. HMGN1 expression during hair follicle morphogenesis

(A) and (B) Whole mount *in situ* hybridization of *Hmgn1* expression in 15.5 day embryos using antisense (A) and sense (B) probes. The antisense probe generates a spotted expression pattern on the skin surface, representing the developing hair placode. (C) to (N) immunofluorescence analyses of HMGN1 (green) and p63 (red) protein expression in the early stages of the developing hair follicle of the newborn mouse skin. The developing hair follicle is staged as was described (Paus et al., 1999). (C) to (F) In the stage 2 hair follicle, HMGN1 is expressed in the epidermal placode and dermal condensate (DC). (G) to (J) At stage 3, HMGN1 is continuously expressed in the dermal papilla (DP), but not in the hair germ (HG). (K) to (N) At Stage 4, HMGN1 is expressed in the DP and hair matrix. Scale bar: 20 μm.

Fig. 2. Expression of HMGN1 in the anagen phase of adult mouse hair follicle

HMGN1 (green) and p63 (red) protein were detected by immunofluorescence. Nuclei are counter stained with DAPI (blue). Note that HMGN1 and p63 colocalize in most of the hair follicle except the dermal papilla. Scale bar: 50 μ m. **bl**: basal layer; **ors**: outer root sheet; **irs**: inner root sheet; **hs**: hair shaft; **hb**: hair bulb; **dp**: dermal papilla; **mc**: matrix cells.

Fig. 3. HMGN1 is Expressed in the Stem Cells of the Hair Follicle

(A) to (D): Immunofluorescence analysis of Nestin (red) and HMGN1 (green) in the bulge region (BG) of the hair follicle. Cell nuclei are counterstained blue with DAPI. Scale bar: 25 μm. Images are from the sagittal sections of hair follicle. HMGN1 is expressed in the outer root sheath (ORS) and sebaceous gland (SG), but not in the inner root sheath (IRS). Nestin positive cell nuclei are indicated by arrow heads. Note that some Nestin positive cells contain HMGN1. (E) to (L): HMGN1 is detected in BrdU retaining cells. Mice were depilated at the age of 8 weeks and then injected with BrdU twice daily for 3 days. After an 18 day long chase period the skin was collected and the location of BrdU retaining cells (red) and HMGN1

containing cells (green) detected by immunofluorescence. Nuclei are counter stained with DAPI (blue). BrdU positive cell nuclei are indicated by arrow heads. Scale bar: 25 μ m.

Epidermis

Sebaceous gland

Fig. 4. Comparison of epidermis and sebaceous gland between *Hmgn1*^{+/+} and *Hmgn1*^{-/-} hair follicles (A) to (F) The localization of p63 (red) and HMGN1 (green) was detected by immunofluorescence and compared with *Hmgn1*^{+/+} and *Hmgn1*^{-/-} skin. Morphology is compared by phase contrast (Bright field). Note that although HMGN1 is strongly expressed in epidermis and outer root sheath (ORS) and colocalizes with p63, the epidermis and ORS are properly formed both in the wild type (A) to (C) and knock out (D) to (F) mice, and the expression levels of p63 protein (red) in these regions are not affected. Scale bar: 50 μ m. (G) to (L) Comparison of *Hmgn1*^{+/+} and *Hmgn1*^{-/-} skin. HMGN1 (green) was detected by immunofluorescence and Nuclei are counter stained with Hoechst (blue). Morphology is

compared by phase contrast (Bright field). Also no abnormalities are observed in sebaceous gland (SG) formation (G to L). Scale bar: 50 μm .

Fig. 5. Expression of HMGN2 protein in the mouse hair follicle

The localization of HMGN2 (green) was detected by immunofluorescence and compared with p63 protein (red), which is expressed in the basal layer of epidermis, outer root sheath and matrix cells in the hair bulb. Nuclei are counter stained with Hoechst (blue). (A) to (C) Analysis of sections through the surface of back skin show that HMGN2 protein is detected in the epidermis and outer root sheath (ORS). (D) to (I) Sagittal (D to F) and horizontal (G to I) sections of hair follicle at the dermis level. HMGN2 protein is detected in the ORS but not in the inner root sheath (IRS) nor in the hair shaft (HS). (J to L) Analysis of sections through the hair bulb show that HMGN2 protein is detected in the dermal papilla. Scale bar: 50 μ m.