The co-repressor hairless has a role in epithelial cell differentiation in the skin

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Summary

Although mutations in the mammalian hairless (Hr) gene result in congenital hair loss disorders in both mice and humans, the precise role of Hr in skin biology remains unknown. We have shown that the protein encoded by Hr (HR) functions as a nuclear receptor co-repressor. To address the role of HR in vivo, we generated a loss-of-function (Hr<sup>−/−</sup>) mouse model. The Hr<sup>−/−</sup> phenotype includes both hair loss and severe wrinkling of the skin. Wrinkling is correlated with increased cell proliferation in the epidermis and the presence of dermal cysts. In addition, a normally undifferentiated region, the infundibulum, is transformed into a morphologically distinct structure (utricle) that maintains epidermal function. Analysis of gene expression revealed upregulation of keratinocyte terminal differentiation markers and a novel caspase in Hr<sup>−/−</sup> skin, substantiating HR action as a co-repressor in vivo. Differences in gene expression occur prior to morphological changes in vivo, as well as in cultured keratinocytes, indicating that aberrant transcriptional regulation contributes to the Hr<sup>−/−</sup> phenotype. The properties of the cell types present in Hr<sup>−/−</sup> skin suggest that the normal balance of cell proliferation and differentiation is disrupted, supporting a model in which HR regulates the timing of epithelial cell differentiation in both the epidermis and hair follicle.

Key words: Hair follicle, Alopecia, Nuclear receptor, Repression, Epidermis, hr

Introduction

The skin is a dynamic and complex organ. To serve its function as a barrier to the environment, the skin must maintain its integrity by continuously renewing the epidermis, while also maintaining associated appendages such as hair. The surface-exposed epidermis, a stratified squamous epithelium composed primarily of keratinocytes, is continuously regenerated as cells in the outer cornified layer are sloughed off and replaced by newly differentiated cells. Hair is produced and maintained by the pilosebaceous unit, which includes a hair-producing follicle and a sebaceous gland. Unlike the epidermis, the mature pilosebaceous unit is a topologically complex mosaic of multiple cell types. The hair follicle undergoes a temporarily organized cyclical process of growth (anagen), regression (catagen) and rest (telogen) (Hardy, 1992). Both skin development and its post-maturation homeostasis require interactions between the epithelium and underlying mesenchyme (Hardy, 1992; Oro and Scott, 1998). These interactions help to control the mitotic activity and fate of epithelial stem cells that reside in specialized parts of the outer root sheath (ORS) (Cotsarelis et al., 1990; Ghazizadeh and Taichman, 2001; Oshima et al., 2001; Taylor et al., 2000). These stem cells provide precursors for the cells that ultimately populate the epidermis, hair follicles and sebaceous glands (Alonso and Fuchs, 2003; Niemann and Watt, 2002). The molecular mechanisms underlying these processes are being increasingly well defined, and include signaling pathways such as WNT/β-catenin, sonic hedgehog (SHH) and fibroblast and epidermal growth factors (Dlugosz, 1999; Fuchs and Raghavan, 2002; Millar, 2002; Steen and Paus, 2001). Much remains to be learned, and the many spontaneous and genetically engineered mutations that display phenotypic abnormalities in murine skin provide an invaluable resource for this endeavor.

The mouse mutant hairless (Hr; previously known as hr) was first recognized in 1926 for its characteristic hair loss phenotype, in which initial hair growth is normal but after shedding the hair does not grow back (Brooke, 1926). The Hr gene was identified by mapping the retroviral insertion in the original murine allele (Cachon-Gonzalez et al., 1994; Stoye et al., 1988) and as a thyroid hormone-regulated gene in rat brain (Thompson, 1996). Identification of the human ortholog revealed that mutations in the Hr gene result in congenital hair loss disorders (alopecia universalis, papular atrichia) (Ahmad et al., 1998b; Cichon et al., 1998; Sprecher et al., 1999). Multiple mutant Hr alleles in both mice and humans show phenotypic variations that can include skin wrinkling and papular rash (Panteleyev et al., 1998b). Although mechanisms have been proposed to explain histological changes in Hr mutant skin that include absence of hair follicles, epidermal...
utricles and dermal cysts (Mann, 1971; Montagna et al., 1952; Panteleyev et al., 1999), the precise role of \( Hr \) in hair follicle biology remains unknown.

The \( Hr \) gene encodes a 130 kDa protein (HR) that is highly expressed in skin and brain (Cachon-Gonzalez et al., 1994; Potter et al., 2001a). Although the HR protein lacks sequence identity to proteins of known structure or function, we recently demonstrated that HR functions as a nuclear receptor co-repressor (Potter et al., 2001a). Nuclear receptors are transcription factors that regulate specific changes in gene expression in response to the binding of their cognate ligands (Mangelsdorf et al., 1995; McKenna and O’Malley, 2002). The transcriptional activity of nuclear receptors depends on the association of additional proteins (co-activators and co-repressors), many of which function in chromatin remodeling (Glass and Rosenfeld, 2000; Jepsen and Rosenfeld, 2002). HR interacts with and influences the transcriptional activity of multiple nuclear receptors, including thyroid hormone receptor (TR), retinoic acid receptor-related orphan receptor \( \alpha \) (ROR\( \alpha \)) and vitamin D receptor (VDR) (Hsieh et al., 2003; Moraitis et al., 2002; Potter et al., 2001a; Thompson and Bottcher, 1997). In the context of TR, HR mediates repression in the absence of thyroid hormone, probably through interaction with histone deacetylases (Potter et al., 2001a; Potter et al., 2002). In the case of ROR\( \alpha \), a constitutively active orphan receptor, HR inhibits transcriptional activation via a novel mechanism using co-activator-type binding motifs (LXXLL) (Moraitis et al., 2002). HR function in the context of VDR is distinct, as HR also inhibits transcriptional activation by the ligand-bound receptor (Hsieh et al., 2003).

The nuclear receptors with which HR interacts have been implicated in epithelial development and function. In humans, thyroid hormone (TH) deficiency frequently causes thickening of the skin and hair loss (Bernhard et al., 1996; Freinkel and Freinkel, 1972). Like mutations in \( Hr \), some mutations in the gene encoding VDR result in congenital hair loss in mice and humans (Malloy et al., 1999; Miller et al., 2001). Similarly, conditional inactivation in mouse skin of the genes encoding heterodimeric partners of TR and VDR (RXR\( \alpha \) and RXR\( \beta \)) results in progressive alopecia (Li et al., 2001; Li et al., 2000). The phenotypic similarities of \( Hr \), VDR and RXR (retinoid X receptor) mutant animals suggest that the biochemical interaction of these proteins is functionally relevant in vivo.

As HR is a transcriptional regulator, the phenotype of \( Hr \) mutants likely results from a perturbation of gene expression. To study the role of HR in vivo, we generated a null allele (\( Hr^{-/-} \)) using homologous recombination. In this study, we find specific changes in gene expression in \( Hr^{-/-} \) skin, which includes upregulation of keratinocyte differentiation markers. Based on the properties of the cell types present in \( Hr^{-/-} \) skin, we propose that the role of HR in the skin is to regulate the timing of epithelial progenitor cell differentiation, and that disruption of timing in \( Hr \) mutants leads to changes in cell fate favoring epidermis and sebaceous glands at the expense of the hair follicle.

### Materials and methods

#### In situ hybridization

In situ hybridization was performed using frozen sections (20 \( \mu m \)) (Hsieh et al., 2003). For each gene analyzed, digoxigenin-labeled sense (control) and antisense cRNA probes were transcribed from linearized plasmids. Plasmids used to make probes: mouse \( Hr \) (Hsieh et al., 2003); WNT10B, p3J-WNT10b (G. Shackelford, USC, Los Angeles, CA); filaggrin, ATCC clone 949741; SCD1, ATCC clone MGC-6427; lefl1, pBS-lefl1 was made by subcloning an EcoRI to Xbal fragment from pCDNA6/V5-6-Lefl1 (H. Varmus, Sloan-Kettering Institute, New York, NY) into pBS KS\( + \) (Stratagene, La Jolla, CA); SHH, pBS-shh was made by subcloning an EcoRI/HindIII fragment from RK5mycSHH (P. Beachy, Johns Hopkins University) into pBS KS\( + \); caspase 14, pBS-casp14 was made by subcloning an EcoRI fragment from ATCC clone 3451156 into pBS KS\( + \).

#### RNA and protein analysis

Mouse strains C57BL/6, HRS/J \( Hr \), SKH2/J and RHJ/LeJ \( Hr^{-/-} \) were obtained from The Jackson Laboratory; CBA-Hr\( ^{+/+} \) was obtained from Taconic. For RNA preparation, mouse backskin was pooled from two male and two female mice for each age and genotype, and RNA was isolated using an RNeasy kit (Qiagen). Primary keratinocytes were cultured in EMEM (0.05mM Ca\( ^{2+} \)) with 8% chelex treated fetal bovine serum as described (Filkasof et al., 1994; Hennings et al., 1980; Yuspa et al., 1989). Cultures were treated with 0.12 mM Ca\( ^{2+} \) for 24 hours before harvesting total RNA using Trizol (Sigma).

Microarray analysis was performed using Affymetrix gene array chips (U74Av2) following Affymetrix specifications (Johns Hopkins Medical Institutions Core Facility). First-strand cDNA was synthesized using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primer (SuperScript Choice System, Life Technologies). After synthesis of double-stranded cDNA, biotinylated antisense cRNA was generated by in vitro transcription using the BioArray RNA High Yield Transcript Labeling kit (ENZO Life Sciences). Biotinylated cRNA was fragmented and hybridized to the GeneChip array, followed by two rounds of staining with a streptavidin-phycocerythrin conjugate. Image analysis was carried out using the Agilent GeneArray Scanner and MicroArray Suite 5.0 software (Affymetrix). For comparison between different chips, global scaling was used, scaling all probe sets to a user defined target intensity of 150. Significant changes were defined as greater than twofold with a \( P \) value of less than 7\( \times 10^{-6} \).

Northern analysis was as described (Potter et al., 2001b). Results were quantitated using a Fujifilm BAS-2500 phosphorimager to scan two independent blots for each probe; values were normalized to signal for \( \beta \)-tubulin. DNA fragments used as probes were from the indicated plasmids: rat \( Hr \) cDNA (Thompson, 1996); keratin 10 (pET-K10); loricrin (ATCC clone 1747977); filaggrin (ATCC clone 949741); caspase 14 (ATCC clone 3451156); Kdap (ATCC clone 1477125); calmodulin 4 (ATCC clone 1766225) and \( \beta \)-tubulin (A. Lanahan, Dartmouth University).

For protein preparation, mouse backskin was frozen, crushed and homogenized in 1.5 ml of an isotonic detergent buffer. After centrifugation, 15-20 \( \mu l \) of supernatant was loaded onto a SDS-polyacrylamide gel. Western analysis was performed as described (Potter et al., 2001a).

### Generation of \( Hr^{-/-} \) mice

The targeting vector was constructed from the plasmid PGK-tk by first subcloning the PGK NEO loxP cassette from pKSneo-12 (C.-M. Fan, Carnegie Institution of Washington, Baltimore, MD). PCR amplification of 129SV genomic DNA with specific primers was used to generate fragments spanning exon 6-7 (2 kb) and exon 11-17 (4.5 kb). Fragments were inserted into restriction sites flanking the NEO cassette. The targeting vector was linearized and electroporated into 129/Sv embryonic stem (ES) cells (JHU Transgenic Core Facility), followed by selection with G418 and gancyclovir. Colonies were screened using Southern analysis with probes flanking the recombination site (Fig. 1, data not shown). Four out of 143 colonies showed homologous recombination. Chimeric mice were generated using two independent ES cell lines. C57BL/6J mice were mated with chimeric mice to generate heterozygous animals. Offspring were
genotyped by Southern analysis of EcoRI-digested genomic DNA. Subsequent generations were genotyped using PCR. Heterozygotes were interbred to obtain $Hr^{-/-}$ animals. Studies were carried out with mice of mixed background; preliminary studies with seventh generation backcross to C57BL/6 have yielded comparable results.

**Histology and immunostaining**

For histology, shaved skin was immersed in Bouin’s fixative overnight, transferred to 70% ethanol, then paraffin wax embedded, sectioned at 5 μm and stained with Hematoxylin-Eosin (AML Laboratories, Baltimore, MD). For immunohistochemistry, antibodies were: K17 (McGowan and Coulombe, 1998), K10, PCNA (Sigma), K14, filaggrin, loricrin (Covance), K16 (Bernet et al., 2002) and trichohyalin (AE15) (O’Guin et al., 1992). Signal was detected with biotinylated secondary antibodies followed by ABC Vectastain kit (Vector Laboratories) and visualized using DAB (3,3’-diaminobenzidine) (Sigma). For immunofluorescence, sections were incubated with K17-specific antibody and detected with Cy3-conjugated anti-rabbit antibody, followed by incubation with FITC-coupled K14-specific antibody. Cell nuclei were visualized with DAPI (4’,6-diamidino-2-phenylindole) (Molecular Probes). For Immunohistochemistry, antibodies sectioned at 5 μm were fixed overnight, transferred to 70% ethanol, then paraffin wax embedded, and stained with Hematoxylin-Eosin (AML Laboratories, Baltimore, MD). For immunohistochemistry, antibodies were: K17 (McGowan and Coulombe, 1998), K10, PCNA (Sigma), K14, filaggrin, loricrin (Covance), K16 (Bernet et al., 2002) and trichohyalin (AE15) (O’Guin et al., 1992). Signal was detected with biotinylated secondary antibodies followed by ABC Vectastain kit (Vector Laboratories) and visualized using DAB (3,3’-diaminobenzidine) (Sigma). For immunofluorescence, sections were incubated with K17-specific antibody and detected with Cy3-conjugated anti-rabbit antibody, followed by incubation with FITC-coupled K14-specific antibody. Cell nuclei were visualized with DAPI (4’,6-diamidino-2-phenylindole) (Molecular Probes). For Bromodeoxyuridine (Brdu) incorporation, mice were injected intraperitoneally with 100 μg/g body weight of Brdu (Roche) and sacrificed after 2 hours. Bouin’s fixed sections were digested with Proteinase K, then incubated with antibodies specific for Brdu and stained with Hematoxylin. Digital images were captured using a Zeiss Axiocam and analyzed using Zeiss Axiosvidion and Adobe Photoshop software (Potter et al., 2001b).

**Results**

**Generation of $Hr$ null allele**

To help decipher the role of $Hr$ in skin, we examined $Hr$ mRNA and protein expression in mice with spontaneous mutations in the $Hr$ gene ($Hr^{br}$, hairless allele; $Hr^{rh}$, rhino allele). Using northern analysis, expression of the 6 kb $Hr$ transcript was detected in the skin of homozygous mutant mice of both $Hr^{br}$ and $Hr^{rh}$ alleles (Fig. 1A). Homozygous $Hr^{br}$ mice show a small decrease in $Hr$ mRNA levels and two additional $Hr$-related mRNA species that probably arise from aberrant splicing resulting from the retroviral insertion that causes this mutation (Stoye et al., 1988). The skin of homozygous $Hr^{br}$ mice shows only a small amount of the 6 kb $Hr$ mRNA. A 3 kb mRNA from the $Hr$ gene was detected in other tissues, including brain and pituitary; this message appears to be present in skin at low levels and was unaffected by the mutant alleles (Fig. 1A, data not shown). Using western analysis, HR protein expression was detected in extracts from adult +/+Hr/hr and Hr/hr/Hr/hr mutant skin but not in extracts from Hr/hr/Hr/hr mutant skin (Fig. 1B). Thus, the $Hr$ allele maintains expression of $Hr$ mRNA and protein, while the $Hr^{rh}$ allele retains expression of the 3 kb $Hr$ mRNA.

To generate a defined mouse model that lacks $Hr$ expression, a targeted deletion of the $Hr$ locus was created using homologous recombination in mouse embryonic stem (ES) cells (Fig. 1C). The targeting construct was designed to excise exons 8-10, to both disrupt the 3 kb $Hr$ mRNA (which initiates in exon 8) and remove a functional domain required for interaction with nuclear receptors (Moraitis et al., 2002; Potter et al., 2001b).

![Fig. 1. Generation of $Hr$ null mice.](image)

(A) Expression of $Hr$ mRNA in $Hr$ mutant mice. Northern analysis of mRNA from backskin of adult (10 week old) mice. hr, $Hr^{br}$ allele (HRS/J); hr, $Hr^{rh}$ allele (RHJ/LeJ); +, wild type allele. Asterisks indicate high molecular weight RNA species detected only in $Hr^{br}$ allele. (B) Expression of HR protein in $Hr$ mutant mice. Western analysis of extracts from adult backskin shows protein detected by HR-specific antibody (α-Hr). hr, $Hr$ allele (SKH2/J); hr, $Hr^{rh}$ allele (CBA-hr/h); +, wild type allele; Ponceau, Ponceau Red stained membrane. Molecular weight is shown in kDa. (C) Targeting vector used to create $Hr$ null (−). Exons 8-10 were replaced with a neo cassette (PGKneo) using homologous recombination. Exons are not drawn to scale. RI, EcoRI sites. (D) Southern analysis of mice with targeted gene deletion. Southern blot of EcoRI-digested genomic DNA. Probe is region indicated by black bar in C. Band of 9 kb represents wild-type allele; 6 kb band represents recombinant allele; +, wild type allele; −, recombinant (HR−) allele. (E) $Hr$ mRNA is disrupted in mice carrying targeted gene deletion. Northern analysis of RNA prepared from skin of P15 mice. +/+, wild type; −/−, $Hr^{-/-}$. (F) Mice carrying targeted gene deletion do not express HR protein. Western analysis of extracts from skin with HR-specific antibody (α-Hr). (G) Skin wrinkling in $Hr^{-/-}$ mouse (1 year old). (H) Comparison of 1-year-old $Hr$ mouse mutants. Null, $Hr^{-/-}$ mouse; rhino, $Hr^{br}$/Hr/hr mouse (CBA-Hr/h); Hr, Hr/hr/Hr/hr mouse (SKH2/J).
et al., 2001a). Chimeric mice were generated from ES cells carrying the recombined allele and crossed to C57BL/6 mice to produce heterozygous animals. Heterozygotes were intercrossed to generate mice with two copies of the recombinant allele, which segregated in a Mendelian fashion. Mice homozygous for the Hr targeted gene deletion were identified by the presence of the recombinant allele (6 kb) and absence of the wild-type allele (9 kb) using Southern analysis (Fig. 1D). Although the recombinant allele allows for the potential production of truncated RNA and protein (exons 1-6), no full-length Hr mRNA was detected (Fig. 1E). In addition, no full-length or truncated HR protein was detected in the skin of mice carrying the recombinant allele using an antibody that recognizes epitopes that would be present in the putative truncated protein (Fig. 1F). We refer to the mouse strain homozygous for the targeted allele as Hr–/–.

Examination of Hr–/– mice during postnatal development shows that, as in other Hr alleles, the first hair coat grows normally. Hair loss is observed at ~P18, beginning at the head and proceeding caudally in a scattered pattern. Once the hair is lost, the skin of Hr+/+ mice appears wrinkled, similar to that of rhino alleles. The histology of Hr–/– skin is also initially similar to previously described alleles as the pilary canal widens to form a utricle, hair follicles fail to regenerate and small dermal cysts become visible. However, as the Hr–/– mice age, the skin becomes progressively more wrinkled (Fig. 1G). When compared with Hr+/Hr+/ and Hr+/Hr– mutants, Hr–/– mice exhibit a more prominent wrinkling phenotype (Fig. 1H). This wrinkling phenotype resembles that of the rhino-Yurolo allele, which (like Hr–/+) is caused by an insertion that would disrupt both Hr mRNAs (Ahmad et al., 1998c; Cachon-Gonzalez et al., 1994; Panteleyev et al., 1998a), therefore the severe wrinkling phenotype is probably due to complete loss of Hr expression.

Utricle formation in Hr–/– skin

Utricle formation is the first morphological change observed in Hr–/– skin. Histological analysis pointed to postnatal day 12 as the age at which a change is visible, near the top of the pilary canal (infundibulum) (Fig. 2A). Once formed, the utricle remains a prominent feature as the hair is lost and persists as hair follicles fail to regenerate. The mechanism of utricle formation is unclear. To characterize the cells that comprise the utricle, immunohistochemical detection of keratinocyte differentiation markers was examined at P19 (Fig. 2B). Keratin 10 (K10), which localizes to the suprabasal layer of the epidermis in wild type skin, was detected both in the epidermis and in the utricle walls in Hr–/– skin. Keratin 14 (K14) is normally expressed in basal cells of the epidermis and pilosebaceous unit. In Hr–/– skin, Arrowheads indicate morphological change in the infundibulum of Hr–/– skin. (B) Expression of keratin markers in utricles. Immunohistochemical detection of keratin markers in consecutive serial sections of backskin from P19 Hr+/+ (top panels; –/+) and wild-type (+/+; mice (bottom panels). H&E, Hematoxylin-Eosin; K10, keratin 10; K14, keratin 14; K17, keratin 17. Arrowheads indicate positive signal in utricle; arrows indicate epithelial remnants; thick arrows indicate sebaceous glands. (C) Utricles (arrowheads) do not express hair markers. Immunohistochemical detection of Trichohyalin, a hair marker, in P15 Hr–/– backskin. (D) Immunofluorescent detection of K10 (red) and K17 (green) in sagittal skin sections from Hr+/+ and wild-type mice. Brackets indicate expansion of K10-positive region. Scale bars: 50 µm.
K14 was present in the basal layer of the epidermis as well as the basal layer surrounding the utricle and sebaceous gland. Keratin 17 (K17), which localizes to the outer root sheath (ORS) in wild-type epidermis, was expressed in cells that comprise the utricle walls. Filaggrin, a marker of late stage epithelial differentiation, was detected in the innermost layers of the utricle wall (data not shown, see Fig. 7). In contrast to epidermal markers, the utricle does not express a hair marker (trichohyalin) (Fig. 2C). Both K14 and K17 were detected in cell clusters in the dermis distal to the utricles and sebaceous glands; these cells may represent remnants of the receding hair follicle.

The location of the utricle suggests that it is derived from the infundibulum, normally a small population of epithelial cells at the top of the pilary canal that is characterized by a high rate of proliferation (Ghazizadeh and Taichman, 2001). Although there is no specific marker for the infundibulum, this region can be defined as the junction between K10-expressing epidermal cells and K17-expressing cells of the pilary canal. Double immunostaining with K10 and K17 revealed that in \( Hr^{+/−} \) skin, the region of the pilary canal that is K10 positive is greatly expanded (Fig. 2D). This extended expression of K10, an epidermal marker, suggests that cells in the infundibulum have differentiated into epidermis, or that the interfollicular epidermis has been expanded. Based on its expression of keratinocyte differentiation markers, the utricle exhibits the molecular characteristics of normal epidermis. Consistent with epidermal function, \( Hr^{+/−} \) skin exhibits normal barrier function, as it excludes water permeable dye (data not shown).

The expansion of the utricle led us to examine cell proliferation. Identification of mitotically active cells at P16 (prior to hair loss) showed that the number of proliferating cells in the epidermis is greater in \( Hr^{+/−} \) than in wild-type skin (Fig. 3A). In \( Hr^{+/−} \) skin, proliferation above wild-type levels is observed both in the utricle and the interfollicular epidermis. Cells in the utricle region remain mitotically active as hair follicles undergo telogen (Fig. 3B) and after hair loss (data not shown). Mitotic activity in the infundibulum prior to hair loss may contribute to utricle formation and continuing proliferation may help maintain the utricle. Detection of keratin markers in combination with BrdU incorporation indicated that proliferating cells are K14-positive (Fig. 3B). Keratin 16 (K16) expression remains comparable in wild-type and \( Hr^{+/−} \) skin, suggesting that the infundibulum is not a site of aberrant differentiation (Fig. 3C). Thus, the increase in cell proliferation in \( Hr^{+/−} \) skin is probably a direct consequence of loss of HR function. As the animals age, increased proliferation probably contributes to the increase in skin surface area, and is also necessary to accommodate tissue expansion that results from cyst formation.

**Hair marker expression in \( Hr^{+/−} \) mice**

After utricle formation, the next phase of the \( Hr^{+/−} \) phenotype is failure to initiate the first hair cycle. Part of the mechanism by which \( Hr^{+/−} \) mice fail to re-grow hair may be the inability of cells responsible for re-generating the hair follicle to follow their usual fate. To examine whether cells that normally contribute to regenerating hair follicles are present in \( Hr^{+/−} \) skin, we examined expression of early markers of hair differentiation including sonic hedgehog (\( Shh \)) (Chiang et al., 1999) and \( Lef1 \) (van Genderen et al., 1994) (Fig. 4A). At P9, an age at which both wild-type and \( Hr^{+/−} \) animals have normal hair, \( Shh \) is expressed in a subset of matrix cells, in the anagen bulb. Similarly, at P6, \( Lef1 \) is expressed in matrix cells at the base of the hair bulb in both wild-type and \( Hr^{+/−} \) skin. At P28, when hair follicles are regenerating and expression of SHH and \( Lef1 \) is detected in wild-type skin, no expression of these genes was detected in \( Hr^{+/−} \) skin (Fig. 4B). Although \( Hr^{+/−} \) skin lacks follicles at this age, progenitor cells that would normally be fated to become follicular structures may still be present in the dermis. The absence of these markers suggests that either these cells are absent, or that the dermis of \( Hr^{+/−} \) animals lacks the signals necessary for progenitor cells to differentiate towards a hair follicle fate. These results suggest that \( Hr \) acts upstream of \( Shh \) and \( Lef1 \) function in the genetic pathway that regulates the hair cycle.
Dermal cyst formation in \( Hr^{-/-} \) skin

The severe wrinkling observed in adult \( Hr^{-/-} \) animals appears well after utricle formation and hair loss, and is correlated with the development of dermal cysts. Histological analysis shows that cysts increase in size as the mice age with utricle size remaining relatively constant (Fig. 5A). Previous work on other \( Hr \) mutants has suggested that sebaceous glands contribute to dermal cyst make up (Bernerd et al., 1996), and consistent with this idea we find that some cells at the cyst perimeter express stearoyl-coenzyme A desaturase 1 (\( Scd1 \)) (Fig. 5B) (Zheng et al., 1999). Most cysts also contain lipids, a component of sebum (Fig. 5C). The cellular makeup of the cysts was examined by detection of keratinocyte differentiation markers (Fig. 5D). Cells surrounding the cysts express both K14 and K17 (Fig. 5D), but do not express terminal epidermal keratinocyte differentiation markers such as filaggrin, loricrin (Fig. 5E) and K10 (data not shown) or a hair marker (trichohyalin) (Fig. 5E). Thus, cells in the cysts are not undergoing epidermal differentiation. Simultaneous detection of K14 and K17 shows that the cyst walls are multilayered (Fig. 5D). Cell proliferation may contribute to cyst expansion, as dividing cells are detected in the cyst walls (Fig. 5F). Thus, dermal cyst expansion may result from a combination of sebaceous cells producing sebum into a sealed compartment, and continuing cell proliferation. The formation of abnormal structures (utricles and cysts) in \( Hr^{-/-} \) skin may point to the function of HR in processes that regulate cellular fates.

![Fig. 4.](image1.png) Expression of hair bulb markers in \( Hr^{+/+} \) skin. (A) Normal expression of hair bulb markers during initial hair growth. In situ hybridization of sagittal sections from backskin with cRNA probes specific for \( Lef1 \) (top panels, P6) and sonic hedgehog (\( Shh \)) (bottom panels, P9) in wild-type (+/+), \( Hr^{-/-} \) (-/-), mice. (B) Absence of hair markers during second anagen. In situ hybridization with a sebaceous gland marker (\( SCD1 \)). In situ hybridization of adjacent sagittal sections of backskin from adult (1 year) \( Hr^{-/-} \) mouse with \( Scd1 \)-specific antisense (AS, left panel) or sense (S) probes (right panel). Arrowheads indicate positive signal in subset of cells surrounding cyst. Scale bar: 50 \( \mu m \). (C) Lipids detected in dermal cysts. Oil red \( O \) staining on sagittal sections from backskin of adult (1 year) \( Hr^{-/-} \) mouse. Scale bar: 50 \( \mu m \). (D) Immunohistochemical detection of keratinocyte differentiation markers. Positive staining in the cyst walls for K14 (green) and K17 (red) and both (K14/K17, yellow) in sagittal sections of backskin from adult (1 year) \( Hr^{-/-} \) mouse. Scale bar: 20 \( \mu m \). (E) Cysts do not express epidermal or hair markers. Immunohistochemical detection of loricrin, filaggrin and trichohyalin in adult \( Hr^{-/-} \) backskin. Scale bar: 50 \( \mu m \). (F) Proliferation in cyst wall colocalizes with K14. Immunofluorescent detection of BrdU (red) and K14 (green) in sagittal section of \( Hr^{-/-} \) mouse backskin (P45). DAPI, nuclear stain (blue). Scale bar: 20 \( \mu m \).
Transcriptional changes in Hr<sup>−/−</sup> skin

Although loss of Hr leads to a number of morphological alterations in the skin, little is known about the molecular basis of these changes. As we have previously shown that the HR protein functions as a transcriptional co-repressor (Hsieh et al., 2003; Moraitis et al., 2002; Potter et al., 2001a), we asked whether loss of HR results in specific changes in gene expression. To examine the initial events underlying the Hr<sup>−/−</sup> phenotype, we identified genes whose expression is altered in Hr<sup>−/−</sup> skin using microarray analysis. P12 was analyzed, as there are no morphological changes in Hr<sup>−/−</sup> skin at this age, thus changes in gene expression are likely to be direct rather than misexpression in an altered structure such as a utricle or cyst. Of the 8000 genes screened, expression of only 15 was significantly changed (Table 1). As expected from removing the function of a repressor, expression of 14 of the 15 genes was upregulated in Hr<sup>−/−</sup> skin. We verified that expression of eight genes was higher in the skin of Hr<sup>−/−</sup> mice using northern analysis (Fig. 6A, Table 1). Interestingly, six of the identified genes are associated with epidermal differentiation. Genes that are directly associated with epidermal differentiation include K10, loricrin and filaggrin, while upregulated genes with a putative role in epidermal differentiation are caspase 14, a gene that is induced during terminal differentiation of keratinocytes (Ahmad et al., 1998a; Eckhart et al., 2000a; Van de Craen et al., 1998), keratinocyte differentiation-associated protein (Kdap; Napsa – Mouse Genome Informatics) (Oomizu et al., 2000) and calmodulin 4 (Calm4 – Mouse Genome Informatics) (Koshizuka et al., 2001), also known as skin calmodulin-related factor (Scarf) (Hwang and Morasso, 2003). Thus, we have identified the first known changes in gene expression in Hr mutant mice; altered expression of these genes may underlie phenotypic changes such as the conversion of infundibulum into epidermis.

Table 1. Results of microarray analysis for Hr<sup>−/−</sup> versus wild-type skin

<table>
<thead>
<tr>
<th>Gene name (GenBank Accession Number)</th>
<th>Fold difference (knockout/wild type)</th>
<th>Microarray</th>
<th>Northern</th>
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<td>11.8</td>
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<td>5.7</td>
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<td>Neurone (D30785)</td>
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<td>4.8</td>
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<td>Loricrin (U09189)</td>
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<td>2.5</td>
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<td>Histidine ammonia lyase (L07645)</td>
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Fold difference is expression value for Hr<sup>−/−</sup> divided by value for wild type. Northern analysis was quantitated by phosphorimager and values normalized to β-tubulin expression. P value indicates statistical significance of microarray data.

To analyze the potential role of the upregulated genes, we examined expression during postnatal skin development (Fig. 6B). Measuring expression at various postnatal ages revealed that expression of all six genes was upregulated at least twofold in Hr<sup>−/−</sup> skin by P9 (Fig. 6B, data not shown). Thus, altered expression occurs prior to any morphological changes (see Fig. 2). The difference in expression between Hr<sup>−/−</sup> and wild-type skin peaks at P16 for loricrin (2.6-fold), K10 (2.8-fold),...
calmodulin 4 (P16, 2.9-fold) and KDAP (P12, 2.5-fold) (Fig. 6, data not shown). The change in expression of caspase 14 and filaggrin over the course of development is quite dramatic. Although detectable filaggrin mRNA is typically heterogeneous (Haydock and Dale, 1986; Rothnagel et al., 1987), the difference in filaggrin expression between \(^{Hr^{-/-}}\) and wild-type skin is similar to that of caspase 14. Expression is upregulated at P9 and the difference in expression between \(^{Hr^{-/-}}\) and wild type increases as development proceeds. By P19, expression of filaggrin is 17-fold higher in \(^{Hr^{-/-}}\) skin than in wild type, and expression of caspase 14 is ~30-fold higher in \(^{Hr^{-/-}}\) skin. Increased expression of caspase 14 and filaggrin is maintained in adult animals.

We next assessed which epithelial compartment contributes to the increase in mRNA of the most highly regulated genes, caspase 14 and filaggrin (Fig. 7). At P6, caspase 14 and filaggrin are expressed in the infundibulum and the suprabasal layer of the epidermis in both wild-type and \(^{Hr^{-/-}}\) skin. At this age, caspase-14 and filaggrin expression in \(^{Hr^{-/-}}\) skin is similar in abundance and expression pattern to wild-type skin, consistent with northern analysis. As postnatal development proceeds, expression of both genes is downregulated in the interfollicular epidermis in both wild-type and \(^{Hr^{-/-}}\) mice. As \(^{Hr^{-/-}}\) skin matures (P9-P19), caspase 14 and filaggrin expression in the infundibulum not only persists but increases as the utricle develops. Increased gene expression occurs before the morphologically distinct utricle can be identified, indicating that the upregulation is probably a cause rather than a consequence of utricle formation. Indeed, filaggrin expression in \(^{Hr^{-/-}}\) skin (P9) is detected in the shape of a utricle despite the absence of a histologically detectable utricle structure. Expression of other identified upregulated genes was also detected in utricles (data not shown).

The observed increase in gene expression, prior to utricle formation, suggests that transcription of such genes is normally inhibited by HR. To determine whether expression of the upregulated genes is normally repressed by HR, we examined expression in primary newborn keratinocytes cultured from \(^{Hr^{-/-}}\) and \(^{Hr^{+/+}}\) mice. Using northern analysis, we find that expression of both filaggrin (2.9-fold) and caspase 14 (2.0-fold) is significantly higher in \(^{Hr^{-/-}}\) keratinocytes grown in elevated Ca\(^{2+}\) (0.12 mM) to promote differentiation (Fig. 8) and in proliferating keratinocytes grown in low Ca\(^{2+}\) (data not shown). Thus, the increase in filaggrin and caspase 14 expression in \(^{Hr^{-/-}}\) skin is not simply a result of utricle formation, and probably occurs because HR represses transcription of these genes.

![Fig. 7. Developmental expression of caspase 14 and filaggrin in epidermis. In situ hybridization of sagittal sections from mouse backskin at the indicated ages using caspase 14-specific (left columns) or filaggrin-specific (right columns) cRNA probes. Shown is a comparison between wild type (+/+) and \(^{Hr^{-/-}}\) (-/-). Arrowheads, specific signal. epi, epidermis; inf, infundibulum; u, utricle. Scale bar: 50 \(\mu\)m.](image)
Discussion

Although histological analyses have provided insight into the pathogenesis of the $Hr$ mutant phenotype, the molecular mechanisms that underlie the morphological changes in $Hr$ mutant skin have remained a mystery. An important advance was the cloning of the rodent $Hr$ gene (Cachon-Gonzalez et al., 1994; Thompson, 1996), which allowed the pattern of $Hr$ mRNA expression to be determined, revealing potential sites of $Hr$ action in the skin (Cachon-Gonzalez et al., 1994; Cachon-Gonzalez et al., 1999; Panteleyev et al., 2000). The presence of conserved cysteine residues in the putative HR protein suggested that HR might be a transcription factor (Cachon-Gonzalez et al., 1994). Our work has demonstrated that the HR protein functions as a nuclear receptor co-repressor (Hsieh et al., 2003; Moraitis et al., 2002; Potter et al., 2001a). Thus, though not a typical transcription factor, HR does regulate gene expression. Here, we have identified the first specific changes in gene expression in $Hr$ mutant skin. Evidence that transcriptional changes occur before the phenotype is visible supports the biochemical function of HR as a transcriptional regulatory protein in vivo.

Similarly, work over the past 50 years examining histology, cell proliferation and cell death in spontaneous $Hr$ alleles led to predictions that HR coordinates the balance between cell proliferation, differentiation and/or apoptosis in the epidermis and hair follicle (Mann, 1971; Montagna et al., 1952; Panteleyev et al., 1999). By combining detailed molecular analysis of the cell types present in $Hr^{+/–}$ skin with current models of epithelial cell fate determination, our results provide evidence that HR has a role in regulating the timing of cell differentiation in the skin. HR plays a similar role in both the epidermis and the hair follicle, in both cases influencing cell fate.

Gene expression is altered in $Hr^{+/–}$ skin

Our recent demonstration that the HR protein functions as a nuclear receptor co-repressor suggested that HR regulates gene expression through its interaction with other proteins. We show here that loss of HR function results in specific changes in gene expression. The identified genes showed increased expression in $Hr^{+/–}$ skin relative to wild type, consistent with removing the function of a transcriptional repressor. Temporally, we detect changes in gene expression well before the onset of the $Hr$ mutant phenotype. Spatially, we find that altered gene expression in $Hr^{+/–}$ skin is first detected in the infundibulum and is restricted to epidermal structures (utricule). The change in expression is intrinsic to keratinocytes, as expression is upregulated in keratinocytes isolated from $Hr^{+/–}$ skin, suggesting that HR directly represses transcription of the identified genes. As both phenotypic alterations and significant changes in gene expression in $Hr$ mutant skin occur in the infundibulum, the utricule is probably derived from the infundibulum by a combination of changes in gene expression and altered cell proliferation.

Many of the misregulated genes are involved in keratinocyte terminal differentiation (K10, loricrin, filaggrin), consistent with the role of HR in the epidermis. The roles of other upregulated genes are less obvious, but their expression and putative functions suggest that they too have a role in keratinocyte terminal differentiation. The most highly upregulated gene, caspase 14, is a member of the caspase family of proteins and is the only caspase with tissue-restricted expression (Ahmad et al., 1998a; Eckhart et al., 2000a; Hu et al., 1998; Van de Craen et al., 1998). Although caspase 14 does not cleave classical caspase substrates and is not activated by apoptosis-inducing agents, the protein is processed during epidermal differentiation and processing is associated with terminal keratinocyte differentiation (Chien et al., 2002; Eckhart et al., 2000b; Lippens et al., 2000). Calmodulin 4/Scarf is a Ca$^{2+}$-binding protein expressed exclusively in differentiating keratinocytes, and is proposed to control Ca$^{2+}$-mediated signaling in epidermal differentiation (Hwang and Morasso, 2003; Koshizuka et al., 2001). Kdap was isolated based on its specific expression in developing epidermis, and is expressed in suprabasal cells of epidermis and the infundibulum (Oomizu et al., 2000). Thus, although the functions of caspase 14, calmodulin 4/Scarf and Kdap are unclear, these genes probably have important roles in the skin, and their aberrant expression may promote the conversion of infundibulum to epidermis.

HR action as a co-repressor fits well with the role of transcriptional regulatory proteins in mediating hair and skin development and function. As a co-repressor, HR does not directly regulate transcriptional activity but instead acts in concert with other transcription factors. Both biochemical and physiological evidence supports the idea that in the skin, HR acts at least in part through VDR: RXR heterodimers (Hsieh et al., 2003). Physiologically, the phenotypes of $Hr$, VDR or RXR$\alpha$ mutants are similar as initial hair growth is normal but subsequent hair cycles fail (Li et al., 2001; Li et al., 2000; Li et al., 1997; Miller et al., 2001; Yoshizawa et al., 1997). In addition, VDR mutants show reduced epidermal differentiation, indicating that VDR also has a role in epidermis (Xie et al., 2002). However, the phenotypes are not identical as RXR$\alpha$ mutants show a hyperproliferative response in the epidermis (Li et al., 2001). In addition, hair loss in VDR and RXR mutants is delayed relative to $Hr$, and neither show severe wrinkling. These phenotypic variations indicate that HR may also act through other nuclear receptors in the skin, such as TR, and may influence the activity of other transcription factors as well.

Role of HR in regulating cell differentiation

The $Hr$ mutant phenotype is observed in temporally and spatially distinct compartments, the epidermis and hair follicle. We propose that the role of HR in both compartments can be
understood within the context of epithelial cell differentiation. Continuous regeneration of the epidermis and cyclical regeneration of the hair follicle both rely on a pool of epithelial stem cells that reside in specialized parts of the outer root sheath (ORS) (Braun et al., 2003; Cotsarelis et al., 1990; Fuchs et al., 2001; Oshima et al., 2001). Stem cells give rise to multipotent progenitor cells that migrate in a bidirectional manner (Taylor et al., 2000). Cells that travel upwards ultimately populate the epidermis and sebaceous glands, while cells that migrate downwards normally contribute to the regenerating hair follicle. Progenitor cells differentiate into distinct cell fates by responding to cues provided by multiple signaling molecules, which include secreted factors such as WNTs, SHH and BMP4, and transcription factors such as MYC, LEF1 and TCF3 (Kratochwil et al., 1996; Merrill et al., 2001; Millar et al., 1999; Niemann et al., 2002; St-Jacques et al., 1998; Waikel et al., 2001; Wilson et al., 1994).

Our data support a model in which the multiple phenotypic changes in Hr mutant skin result from the loss of a repressive influence on differentiation. Normally, multipotent progenitors originating from the bulge and/or other locations within the ORS transit through the infundibulum on their way to the epidermis (Ghazizadeh and Taichman, 2001; Kratochwil et al., 1996; Niemann and Watt, 2002). Keratinocytes located in the infundibulum exhibit a relatively high proliferation rate, but do not terminally differentiate (Morris et al., 2000; Schmitt et al., 1996). In the absence of HR, infundibular keratinocytes continue to proliferate; however, differentiation is no longer inhibited. Instead, these cells adopt an epidermal fate in response to local cues. This interpretation is supported by HR mRNA expression in the infundibulum of mature hair follicles, and by the conversion of the infundibulum to utricles in Hr−/− follicles.

The idea that HR regulates entry into differentiation can also account for the phenotypic alterations seen in the lower part of the hair follicle. Multipotent progenitor cells migrating downward from the ORS-associated stem cells should normally give rise to all the lineages required to form a hair bulb capable of producing hair. In the absence of HR, the inductive signal(s) required to trigger the stem cells into forming a hair bulb are missing, and in response to signals from the prevailing microenvironment, they instead attempt to produce sebaceous glands. This results in the absence of hair follicles and development of an abnormal dermal structure, the cyst. Continuing cell proliferation leads to expansion of the cysts and skin, probably contributing to the progressive wrinkling phenotype. Evidence that cysts are related to sebaceous glands includes expression of K14 and SCD1, and lack of filaggrin or K10 expression.

Precedence for the transformation of cell fates in the skin comes from studies in which specific signaling pathways have been disrupted. For example, inhibition of WNT signaling by inhibiting either β-catenin or LEF1 causes transformation of cells fated to become inner root sheath or hair into sebocytes, thereby suppressing differentiation of hair cells and increasing sebocyte and dermal cyst formation (Huelsken et al., 2001; Merrill et al., 2001; Niemann et al., 2002). Conversely, stimulation of WNT signaling by expressing a constitutive β-catenin results in hair follicle morphogenesis and differentiation in cells derived from epidermis and ORS (Gat et al., 1998).

Although HR is expressed early in development, phenotypic manifestations in Hr−/− mutant skin do not appear until the onset of postnatal hair cycling. One possibility is that loss of HR function is effectively compensated for only during development. Alternatively, the influence of HR on terminal differentiation is not crucial during development, possibly because the environmental cues responsible for the adoption of specific lineages are better segregated at spatial and temporal levels. There are many instances in which gene manipulation differentially affects developing skin epithelia and postnatal hair cycling (Gat et al., 1998; Koch et al., 1997; McGowan et al., 2002).

Our results provide a unifying model for the role of HR in epidermis and hair follicle, in which HR acts alongside local environmental cues to regulate the entry of multipotent progenitor keratinocytes into specific programs of terminal differentiation. The role of HR in influencing cell differentiation can probably be extended to other tissues in which HR is expressed, such as the brain. In addition, we provide the first direct evidence that the HR protein is a transcriptional repressor in vivo, supporting a model in which the Hr mutant phenotype arises from changes in the normal pattern of gene expression that regulates the timing of epithelial cell differentiation.

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