Downregulation of Foxe1 by HR suppresses Msx1 expression in the hair follicles of HrHp mice

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Hairless (HR), a transcriptional cofactor, is highly expressed in the skin and brain. To characterize the effects of HR expression in the skin, we examined its capacity for transcriptional regulation of its target genes in mouse skin and keratinocytes. We found that Foxe1 mRNA expression was suppressed in HR-overexpressing skin, as well as in HR-expressing keratinocytes. In turn, Msx1 expression was downregulated contingent on Foxe1 downregulation in skin and keratinocytes. We also found that expression of Sfrp1 was also correlated with that of Foxe1. Further investigation of the mechanisms involved in the transcriptional regulation of these genes will facilitate our understanding of the relationship among genes involved in hair follicle morphogenesis and cycling.

INTRODUCTION

The hair follicle (HF) constitutes a distinct compartment within the skin that is formed prior to growth of a hair and maintained during the hair growth-cycle: This cycle comprises three phases, including anagen (growth), catagen (transition), and telogen (rest) (1, 2).

The HF forms through close crosstalk between epithelial and mesenchymal cells throughout its developmental stages, with development progressing via the participation of numerous genes whose expression is tightly regulated in time and space. Four major signaling pathways are involved in HF development: the Wnt, TGFβ/BMP, Sonic hedgehog, and FGF (3-5).

Many genes involved in HF development and cycling have been identified in studies of spontaneous or induced mutant mouse models. Most recently, the Hairpoor mouse has been identified as a mouse model for Marie Unna hereditary hypotrichosis (MUHH, OMIM; 146550) (7). Autosomal dominant MUHH is characterized by sparse or coarse hair during childhood and progression to gradual baldness in adulthood. Similar to MUHH, Hairpoor mice also display short and sparse hair at birth that is gradually lost by the 40th week after birth in heterozygotes. Hairpoor homozygote mice are naked at birth. MUHH patients and Hairpoor mice exhibit similar mutations: in each case a single-nucleotide substitution in the second upstream open reading frame of the Hairless (Hr) gene results in a gain-of-function mutation that leads to overexpression of HR in skin during HF development (8).

In skin, the Hr mRNA is expressed in the suprabasal cell layers of the interfollicular epidermis, bulge, outer root sheath (ORS), and bulb cells, but not in the dermal papilla of the HF. Hr is expressed in the lower portion of the HF and is induced phasically within the hair growth-cycle; transcription commences in catagen and endures until early anagen of the next cycle (9). HR is a transcriptional corepressor that interacts with transcription factors such as the Vitamin D Receptor, Thyroid Hormone Receptor, and Retinoic-Acid Related Orphan Receptor to downregulate expression of their respective target genes (10-12). In particular, HR stimulates Wnt signaling via the suppression of its inhibitors, such as Secreted Frizzled-related Protein 2 (Sfrp2) and Sclerostin-Domain Containing 1 (Sostdc1) (8, 9).

To identify HR’s target genes during early stages of HF development, we performed microarray analysis using the skin of HrHp/HrHp homozygote mice and identified genes that were differentially expressed compared with wild-type skin (13). Among these genes, we found that Foxe1, a Forkhead family member, was significantly downregulated in HrHp/HrHp mice compared with wild-type mice. The Forkhead family, which is characterized by a winged helix DNA-binding domain, is involved in embryogenesis, cell differentiation and development, and hormone-responsiveness (14). Another member of the Forkhead family, the Forkhead Box N1, effects the terminal differentiation of epithelial cells in the epidermis and HF, and its inactivation causes nude skin in mice (15, 16).

FOX1 is a transcription factor that was initially identified in the thyroid and regulates the expression of thyroid-specific genes, such as Thyroglobulin, Thyroid Peroxidase, NK2 Homeobox 1, and Paired-Box Gene 8, thus contributing to thyroid morphogenesis (17). Recently, Foxe1 mutant mice were shown

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to exhibit thyroid agenesis, spiky hair, and cleft palate, suggesting that FOXE1 is critical for developmental processes in many tissues, including the vertebrate HF. The FOXE1 gene is also a direct target of the GLI Family Zinc Finger 2 (GLI2), which is a key mediator of Sonic hedgehog (Shh) signaling during HF morphogenesis and cycling in humans (18).

In the present study, we aimed to delineate the relationship between Hr and Foxe1 and identified potential crosstalk between Wnt and Shh signaling during HF development.

RESULTS

Foxe1 transcription is downregulated in the skin of Hr/Hr mice and keratinocytes

In a previous study using microarray analysis to identify HR target genes, we found that expression of the Foxe1 mRNA was decreased to 0.27-fold in the skin of Hr/Hr mice compared with wild-type skin at postnatal day 0 (P0). This suppression of Foxe1 mRNA expression was validated using real-time quantitative PCR (RQ-PCR) with mRNAs originally used in the microarray analysis as templates. We observed comparable reduction in Foxe1 mRNA expression in the present study (0.41 ± 0.03 vs. 0.27-fold) at P0 (Fig. 1A).

We additionally found that expression of Foxe1 was significantly reduced during HF development in the skin of Hr/Hr mice. Foxe1 expression in the skin of Hr/Hr mice was decreased to 0.14 ± 0.04, 0.1 ± 0.04, 0.13 ± 0.01, and 0.25 ± 0.14 fold compared with that observed in wild-type mice at P10, P14, P17, and P35, respectively (Fig. 1B).

![Fig. 1. Foxe1 expression is reduced in the skin of Hr/Hr mice.](http://bmbreports.org)

We probed Foxe1 mRNA expression in Hr/Hr mice via *in situ* hybridization analysis on sections of P7 mouse skin using a DIG-labeled probe specific for the mouse Foxe1 mRNA. As previously reported (18, 19), Foxe1 transcripts were detected at the ORS of the HF compartment in wild-type mice, as shown by Foxe1 colocalization with the ORS marker Keratin 14 (K14). In contrast, mRNA was almost undetectable in the ORS of Hr/Hr mice (Fig. 2A), although low but detectable amplification was apparent through RQ-PCR using RNA samples from the same stage (P7; Fig. 2B). The results of the *in situ* hybridization and RQ-PCR analyses, which revealed reduced expression of the Foxe1 mRNA in the skin of Hr/Hr mice, suggest that enhanced HR expression suppresses that of Foxe1.

To investigate whether Foxe1 transcription was directly regulated by HR, expression levels of Foxe1 were determined in a mouse keratinocyte cell line (PAM212) transfected with an HR overexpression construct. Foxe1 mRNA expression was significantly reduced to 0.34 ± 0.03 fold in HR-overexpressing cells compared with mock-transfected cells (Fig. 2C); the consistency of these findings with our *in vivo* results suggests that HR serves to downregulate Foxe1 expression.

Foxe1 downregulation suppresses Msx1 and Sfrp1 expression

Previously, we identified Msx1 (homeobox, msh-like 1) as one of the genes downregulated in the skin of Hr/Hr mice (0.64-fold expression level compared with that of wild-type mice at P0) (13). In addition, Msx1 was recently classified as a potential target in the developing epithelial cells of the palatal shelves (20). We therefore investigated Msx1 expression in Hr/Hr mice using *in situ* hybridization and RQ-PCR to elucidate the relationship between Foxe1 and Msx1 in HF. In the anagenic HF of P10 wild-type mice, we found widespread expression of Msx1 mRNA, including in the matrix and ORS as previously reported (21). In contrast to the pronounced expression of Msx1 in the wild-type HF, Msx1 mRNA staining in Hr/Hr mice was very weak and limited to the HF matrix (Fig. 3A). We also detected abnormal HF formation in Hr/Hr mice compared with wild-type mice at P10, with underdeveloped hair bulbs and DP and shorter hair shafts. The weak staining was in agreement with results obtained with RQ-PCR showing significantly reduced expression of Msx1 mRNA (0.6 ± 0.14 fold in the skin of Hr/Hr mice compared with wild-type mice at P10; Fig. 3B).

We further analyzed the relationship between expression of Foxe1 and Msx1 in cultured keratinocytes. RQ-PCR analysis revealed that Msx1 mRNA levels increased 1.86 ± 0.28 fold in Foxe1-overexpressing cells compared with a mock transfection (Fig. 3C). Conversely, Msx1 mRNA levels were reduced to 0.47 ± 0.07 fold in HaCaT cells in which Foxe1 expression was knocked down by 35.8% relative to control cells by FOXE1-specific siRNA. These results demonstrate that the expression of Msx1 is regulated by FOXE1 (Fig. 3D).

The Secreted Frizzled-related Protein 1 (Sfrp1), which is an upstream inhibitor of WNT, was previously documented as be-
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Fig. 2. Downregulation of Foxe1 expression by HR. (A) RNA in situ hybridization of 3-μm-thick sections of mouse skin at P7 using a DIG-labeled antisense probe for mouse Foxe1 (purple). The Foxe1 mRNA was detected in the ORS of the HF (arrowhead). Scale bars, 20 μm. Immunostaining of ORS markers (K14; green) in mouse skin section at P10. Nuclei were detected by DAPI staining (blue). Scale bars, 20 μm. (B) Relative expression of the Foxe1 mRNA in the skin of HrHp/HrHp mice was diminished to 0.48 ± 0.07 at P7 compared with the skin of wild-type mice, as shown by RQ-PCR analysis. Western blot analysis showed overexpression of HR in the dorsal skin of HrHp/HrHp mice. (C) Relative expression of the Foxe1 mRNA in PAM212 cells. Foxe1 mRNA expression was reduced in PAM212 cells transfected with an HR expression construct, as analyzed by RQ-PCR (left panel). HR expression was detected in transfected cells by western blot analysis using a polyclonal α-HR antibody developed in-house. β-Actin served as a loading control (right panel). Values are expressed as the SEM of two replicates per experiment from three independent experiments. P < 0.05 using Student’s t test.

DISCUSSION

Crosstalk between major signaling pathways regulates intercellular interactions and modulates development of numerous tissues, including the HF, lung, teeth, and taste papilla (23-25). Many signaling pathways are involved in HF development, such as the Wnt, Shh, TGFβ/BMP, and FGF pathways (2, 26). Crosstalk between these signaling pathways has been suggested during the development of the HF (27).

Foxe1, a direct target of GLI2, is regulated by Shh signaling and plays important roles in vertebrate development, including in epithelial-mesenchymal interactions in the HF through the regulation of cell differentiation and proliferation (18, 19, 28, 29). In addition to decreased expression of Foxe1 in HrHp/HrHp mice, the expression of patched homolog 1 (Path1), another Shh-related gene, was increased to 1.43-fold compared with wild-type mice (13), suggesting that HR may affect the Shh signaling pathway. HR also transcriptionally regulates several Wnt signaling associated genes, especially those such as Sostdc1, Sfrp1, Sfrp2, and Wnt Inhibitory Factor 1 (Wif1), which repress Wnt signaling (8, 9, 13). Moreover, the downregulation of these genes by HR causes Wnt pathway induction, which is critical for the proliferation and differentiation of HF cells and cyclic regeneration of the HF (8, 9).

In the present study, we showed that HR suppressed the expression of the Foxe1 mRNA, a downstream effector of Shh signaling. In turn, the FOXE1 transcription factor regulated expression of Msx1, a member of the Hox family; MSX1 appeared to modulate expression of Sfrp1. Although the current results do not exclude the possibility of direct regulation of Sfrp1 by HR in partnership with a nuclear receptor, our study clearly suggests that Sfrp1 is regulated by a cascade of transcriptional regulation initiated by HR. Thus, HR seems to function as a bridge between the Shh and Wnt signaling pathways, via the regulation of Foxe1.

It is noteworthy that the ORS is the common compartment in which all these molecules are expressed, suggesting that their interplay is critical for the proliferation and/or differ-
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Fig. 3. Msx1 expression in HrHp/HrHp mice. (A) In situ hybridization of β-μm-thick mouse skin sections at P10 using a DIG-labeled antisense probe for mouse Msx1. The Msx1 mRNA was broadly expressed in the HF including Matrix (arrow) and ORS (arrowhead). High magnification of the HF of the Wild-type mice (a) and HrHp/HrHp mice (b). (B) RQ-PCR was performed using the dorsal skin of P10 mice. (C) Regulation of Msx1 expression by FOXE1. Western blot analysis showed the expression of FOXE1 in PAM212 cells transfected with an HA-FOX1 expression vector (left panel). RQ-PCR analysis revealed increased expression of the Msx1 mRNA in FOXE1-transfected PAM212 cells. (D) RQ-PCR measurement of FOXE1 and MSX1 mRNA levels in FOXE1 siRNA-transfected HaCaT cells (+). Scrambled siRNA was used as control (−). (E) Expression of Sfrp1 in cells overexpressing FOXE1. (F) RT-PCR analysis of Foxe1, Msx1, Sfrp1, and Gapdh mRNA levels in FOXE1 siRNA-transfected HaCaT cells (+). β-galactosidase activity was used to normalize transfection efficiency for all transfection experiments.

Cell culture and transient transfection assays

Mouse keratinocyte (PAM212) and human keratinocyte (HaCaT) cell lines were cultured in Dulbecco’s modified Eagle medium (high glucose, Gibco) with 10% fetal bovine serum and 1% streptomycin/penicillin at 37°C and 5% CO2.

PAM212 cells were transfected with 3 μg vector DNA (empty vector or expression construct). Transient transfection experiments were performed using GeneExpresso (Excellgen) according to manufacturer’s instructions; cells were harvested 48 hrs after transfection.

FOXE1 siRNA (5'-CUCAACGACUGCUCUCUAA-3' and 5'-UGAGGAAGCAGAGCUU-3') and a scrambled control siRNA (5'-ACGTGACACGTTCGGAGAAUU-3' and 5'-UUCUCCGACUGUCAGCUUU-3') were custom-designed (Genolution). HaCaT cells were transfected with siRNAs separately at final concentration of 20 nM using G-lectin according to manufacturer’s instructions (Genolution). At 48 hr after the initial transfection, cells were re-transfected via the same protocol; total RNAs were extracted from transfected cells after 48 hrs’ additional incubation. β-galactosidase activity was used to normalize transfection efficiency for all transfection experiments.

Plasmids

The Hr expression construct was purchased from Invitrogen (BC049182). The Foxe1 expression plasmid was constructed by PCR-amplifying 1,116 bp (Expand High Fidelity PCR System, Roche) with the following primers: forward 5'-ATGACGGCCAGAGAGCGCGCC-3' and reverse 5'-TCACATGCAGACACGA-3'. PCR products were introduced into the pcDNA3-HA HA-tagging vector (kindly provided by Dr. H Rhim, the Catholic University of Korea) and for FOXE1 expression in transfection experiments. The in situ hybridization probe plasmid was also constructed by PCR using the following primers: forward 5'-ATGACGGCCAGAGAGCGCGCC-3' and reverse 5'-TCACATGCAGACACGA-3'. PCR products were introduced into the pcDNA3-HA HA-tagging vector (kindly provided by Dr. H Rhim, the Catholic University of Korea) and for FOXE1 expression in transfection experiments. The in situ hybridization probe plasmid was also constructed by PCR using the following primers: forward 5'-ATGACGGCCAGAGAGCGCGCC-3' and reverse 5'-TCACATGCAGACACGA-3'. PCR products were introduced into the pcDNA3-HA HA-tagging vector (kindly provided by Dr. H Rhim, the Catholic University of Korea) and for FOXE1 expression in transfection experiments. The in situ hybridization probe plasmid was also constructed by PCR using the following primers: forward 5'-ATGACGGCCAGAGAGCGCGCC-3' and reverse 5'-TCACATGCAGACACGA-3'. PCR products were introduced into the pcDNA3-HA HA-tagging vector (kindly provided by Dr. H Rhim, the Catholic University of Korea) and for FOXE1 expression in transfection experiments.

RNA isolation and analysis

Total RNA was extracted from the skins of HrHp/HrHp and
Table 1. List of gene-specific primers

<table>
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<th>Gene</th>
<th>Accession No.</th>
<th>Gene name</th>
<th>Primer</th>
<th>Ref.</th>
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<td><em>Foxe1</em></td>
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<td></td>
<td></td>
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<tr>
<td><em>Msx1</em></td>
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<td></td>
<td></td>
<td>R: 5’-AGTGGAGCTGTTGGAAGG-3’</td>
<td>(32)</td>
</tr>
<tr>
<td><em>Sfrp1</em></td>
<td>NM_013834</td>
<td>Secreted frizzled-related protein 1</td>
<td>F: 5’-TACAGCTGCAAGGATGTC-3’</td>
<td>(13)</td>
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<tr>
<td><em>Gapdh</em></td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>F: 5’-AACACCGGACTTGGAAAAGG-3’</td>
<td>(8)</td>
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<tr>
<td><em>GAPDH</em>†</td>
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<td></td>
<td></td>
<td></td>
<td>R: 5’-TGAGTTGGAGGATCG-3’</td>
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</tr>
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Primer design for *mouse and †human.

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Western blot analysis

Protein extracts of mouse dorsal skin tissue and cells were prepared in RIPA buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)) following standard protocols (31). Protein concentrations were determined by the Bradford assay (Bio-Rad); Home-made rabbit polyclonal α-HR antibody (8), α-HA (Santa Cruz), β-actin antibody (Ahn), and α-tubulin antibody (Santa Cruz) were used for western blotting at dilutions of 1 : 500, 1 : 2,500, 1 : 10,000, and 1 : 2,500, respectively. Protein signal was detected via enhanced chemiluminescence (ECL) following manufacturer’s instructions and exposure to X-ray film (Kodak).

In situ hybridization and immunohistochemistry

Mouse skin was fixed in Accustain (Sigma) overnight at 4°C, embedded in paraffin, and sectioned. Tissue sections were treated with proteinase-K at 10 μg/ml at 37°C for 20 min and prehybridized in a solution of 50% Formamide and 5XSSC then incubated at 55°C for 30 min. Hybridization was carried out in hybridization solution (50% Formamide, 5XSSC, Heparin 5 μg/ml, Yeast tRNA 500 μg/ml, EDTA 1 mM, CHAPS 0.1%) containing 1 μg DIG-labeled *Foxe1* Riboprobe at 60°C overnight. After washing, sections were blocked with blocking solution (10% fetal bovine serum (FBS) in TBS buffer) and incubated overnight with α-DIG-alkaline phosphatase conjugated antibody (Roche) diluted 1 : 1,000 in blocking solution. Probes were visualized in NBT/BCIP (Promega) solution. Developed slides were dehydrated through a graded ethanol series followed by xylene and mounted in Shandon Synthetic Mountant solution (Thermo) before examination under a light microscope (Olympus). Antisense and sense probes were transcribed using a SP6 RNA polymerase (ambion) and DIG labeling kit (Roche) following manufacturer’s instructions. Sense probe was used as a control in in situ hybridization experiments.

Immunohistochemistry analysis was performed as described previously (8). The primary antibody against K14 was used (1 : 1,000; Covance) and counterstained with DAPI.

Acknowledgements

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REFERENCES