TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Activation of the OVOL1-OVOL2 Axis in the Hair Bulb and in Pilomatricoma

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OVOL1 and *OVOL2*, ubiquitously conserved genes encoding C2H2 zinc finger transcription factors in mammals, control epithelial cell proliferation, and differentiation, including those in skin. *OVOL1* and *OVOL2* expression is coordinately mediated via the Wnt signaling pathway, and *OVOL1* negatively regulates *OVOL2* expression in a transcriptional manner. Our previous study of *OVOL1* expression in human skin revealed that *OVOL1* is preferentially expressed in the inner root sheath of the hair follicle. Therefore, we hypothesized that the *OVOL1*-*OVOL2* axis is involved in normal and neoplastic follicular differentiation. Immunohistochemical analysis showed that *OVOL1* and *OVOL2* were strongly expressed in a mutually exclusive manner in the cytoplasm of inner root sheath cells and matrix cells, respectively, in normal follicles. *OVOL2* was also expressed in pilomatricoma, with only partial expression of *OVOL1*. Cultured human keratinocytes expressed *OVOL1* and *OVOL2* on both the mRNA and protein levels. The expression of *OVOL2* was higher in keratinocytes transfected with siRNA of *OVOL1*. Ketoconazole, a hair growth stimulant, up-regulated the expression of *OVOL1* but did not affect *OVOL2* expression. These results indicated that the *OVOL1*-*OVOL2* axis may actively contribute to cell differentiation and proliferation in the hair bulb, suggesting that the *OVOL1* and *OVOL2* may be therapeutic targets of hair disorders, including alopecia, and play important roles in the tumorigenesis of pilomatricoma. (*Am J Pathol* 2016; 186: 1036–1043; <http://dx.doi.org/10.1016/j.ajpath.2015.12.013>)

OVOL1 and *OVOL2* are ubiquitously conserved genes encoding C2H2 zinc finger transcription factors in mammals. Functional studies in *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus* have suggested that these genes play key roles in the development of epithelial tissues arising from germ cells.^{1–3}

OVOL1 is expressed in multiple somatic epithelial tissues, including skin.¹ Recent studies have shown that the activation of *OVOL1* redirects cell proliferation to cell differentiation.³ *OVOL1* expression is transcriptionally regulated via signal transduction of the lymphoid enhancer factor/β-catenin complex in the Wnt signaling pathway, which governs homeostasis, including the development, maintenance, and differentiation of epithelial and hair follicle stem cells.^{4,5}

OVOL2 expression has been also observed in murine and human epithelial tissues, such as skin and the germinal

epithelium of the testes.⁶ A molecular study of *OVOL2* expression demonstrated that *OVOL1* represses the activity of the *OVOL2* promoter in a DNA-binding–dependent manner.⁷ Furthermore, genetic deletion of *OVOL1* up-regulates *OVOL2* expression, leading to functional compensation by *OVOL2*, which suggests that *OVOL1* negatively regulates *OVOL2* expression.⁷

The *OVOL1*-*OVOL2* axis is reportedly crucial for the normal development and differentiation of hair follicles in a murine model; *OVOL1*-deficient mice manifest ruffled hair coat and hair abnormalities.¹ Our previous study showed that human *OVOL1* is expressed in the inner root sheath of hair

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Figure 1 Immunohistochemical staining of OVOL1 (brown staining) and OVOL2 (brown staining) with counter hematoxylin staining in the normal skin and hair follicle. Expression of OVOL1 in basal and suprabasal keratinocytes in the epidermis (A) and the follicular isthmus (arrows; B). The staining patterns are nuclear dominant. Similar staining pattern of OVOL2 in the epidermis (C) and in the follicular isthmus (D). Arrows in A, C, and D indicate keratinocytes expressing OVOL. Scale bars: 100 μ m (A–D).

follicles,⁸ which is consistent with the confirmed expression of OVOL1 in the hair follicles and suprabasal layer of the epidermis in mice.¹ Although localization of OVOL2 has not been observed in human hairs, the OVOL1-OVOL2 axis may be involved in differentiation and tumorigenesis, including pilomatricoma in human hair bulbs.

Herein, we found that OVOL1 and OVOL2 are differentially expressed in the normal hair bulb and in pilomatricoma, a common skin tumor derived from the hair matrix. Our results showed that cultured keratinocytes expressed mRNA and proteins of OVOL1 and OVOL2, with up-regulated transcription of *OVOL2* under *OVOL1* knockdown conditions. Moreover, the hair growth stimulant ketoconazole increased the expression of OVOL1, but not that of OVOL2, implying that ketoconazole might lead to hair growth via OVOL1 expression.

Materials and Methods

Tissue Samples

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Kyushu University Hospital (Fukuoka, Japan). We examined 12 pilomatricoma, 1 pilomatrix carcinoma, and 5 normal skin samples. All formalin-fixed, paraffin-embedded tissues were obtained from the archives of the Department of Dermatology of Kyushu University Hospital and the Division of Dermatology of Tottori University Hospital (Tottori, Japan). Clinical and demographic data were retrieved from patient files. The diagnosis of pilomatrix carcinoma was confirmed through comprehensive assessment with immunohistochemistry and electron microscopy.

Immunohistochemical Analysis

All samples were fixed with 10% buffered formalin. The archival paraffin-embedded tissue blocks were cut into sections

(4 μ m thick). The sections were deparaffinized with xylene for 10 minutes and rehydrated with a graded ethanol series. Antigen retrieval was performed with Heat Processor Solution pH6 (Nichirei Biosciences Inc., Tokyo, Japan) at 100°C for 40 minutes, and endogenous peroxidase was blocked by incubating the sections with 3% H₂O₂ (Nichirei Biosciences Inc.). The sections were then incubated with rabbit antibody against human OVOL1 (1:200; LifeSpan BioSciences, Inc., Seattle, WA), rabbit antibody against human OVOL2 (1:50; Novus Biologicals, Inc., Littleton, CO), mouse antibody against human cytokeratin 5 (CK5; 1:50; Abcam, Inc., Cambridge, UK), or mouse antibody against human trichohyalin (AE15; 1:50; Abcam, Inc.) at 4°C overnight, followed by incubation with secondary antibody, N-Histofine Simple Stain MAX-PO MULTI (Nichirei Biosciences Inc.). Immunodetection was conducted with 3,3-diaminobenzidine as a chromogen, followed by light counterstaining with hematoxylin. Sections stained without primary antibody served as the negative control. For immuno-double staining, in addition to the immunodetection using the horseradish peroxidase method mentioned above, N-Histofine Simple Stain AP (Nichirei Biosciences Inc.) and PermaBlue/AP (Diagnostic BioSystems, Inc., Pleasanton, CA) were used as the secondary antibody and the chromogen, respectively.

Cell Culture of NHEKs

Normal human epidermal keratinocytes (NHEKs) obtained from Lonza Ltd (Basel, Switzerland) were grown in culture dishes at 37°C in 5% CO₂. NHEKs were then cultured in serum-free keratinocyte growth medium (Lonza, Walkersville,

Figure 2 Immunohistochemical staining of OVOL1 (brown staining) and OVOL2 (brown staining) with counter hematoxylin staining in the normal hair bulb. A and B: OVOL1 expression in the normal hair bulb. Positive staining for OVOL1 is observed in cells of the inner root sheath (cytoplasmic), hair germ (nuclear), matrix (cytoplasmic and nuclear), cortex (nuclear), and outer root sheath (nuclear). C and D: OVOL2 expression in the normal hair bulb. Positive staining for OVOL2 is observed in the cytoplasm of cells in the matrix, medulla, and cortex and in the nuclei of hair germ cells and outer root sheath cells. Inner root sheath cells show both cytoplasmic and nuclear staining. Scale bars: 200 μ m (A and C); 100 μ m (B and D).

Table 1 Expression Patterns of OVOL1 and OVOL2 in the Hair Bulb

Part of hair bulb	OVOL1	OVOL2
Hair germ cells	Nuclear (++++)	Nuclear (++++)
Medulla	(–)	Cytoplasmic (++++)
Matrix	Nuclear (+), cytoplasmic (+)	Cytoplasmic (++++)
Cortex	Nuclear (+)	Cytoplasmic (++++)
Inner root sheath	Cytoplasmic (++++)	Nuclear (+), cytoplasmic (+)
Outer root sheath	Nuclear (+)	Nuclear (+)

+, Expression present; +++, strong expression; –, no expression.

MD) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine. The culture medium was replaced every 2 days. When the cells were near confluence (70% to 90%), they were disaggregated with 0.25 mg/mL trypsin/0.01% EDTA and subcultured. Second- to fourth-passage NHEKs were used in all experiments.

NHEKs (1×10^5) were seeded in 24-well culture plates, allowed to attach for 24 hours, and treated with ketoconazole or dimethyl sulfoxide. Various concentrations of ketoconazole (up to 10 μ M/L) were prepared with cell culture medium. Control cultures used media containing comparable amounts of dimethyl sulfoxide (up to 0.02%). Fresh medium containing ketoconazole (Sigma-Aldrich, St Louis, MO) or dimethyl sulfoxide (Sigma-Aldrich) was added as indicated in the figure legends.

Immunofluorescence and Confocal Laser-Scanning Microscopic Analysis

NHEKs cultured on slides were washed in phosphate-buffered saline (PBS), fixed with acetone for 10 minutes, and blocked with 10% bovine serum albumin in PBS for 30 minutes. Samples were incubated with primary rabbit anti-OVOL1 (1:50), anti-OVOL2 (1:50), or control rabbit IgG (1:100) in PBS overnight at 4°C. Slides were washed in PBS before incubation with anti-rabbit (Alexa Fluor 546 or 488; Molecular Probes, Eugene, OR) secondary antibody for 1 hour at room temperature. After nuclear staining with DAPI, the slides were mounted with Ultra-Cruz mounting medium (Santa Cruz Biotechnology, Santa Cruz, CA). All samples were analyzed with a D-Eclipse confocal laser-scanning microscope (Nikon, Tokyo, Japan).

RT-PCR and Quantitative RT-PCR Analysis

Total RNA was extracted with an RNeasy Mini kit (Qiagen, Courtaboeuf, France). Reverse transcription was performed with a PrimeScript RT-reagent kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR was performed on a Mx3000p real-time system (Stratagene, La Jolla, CA) with SYBR Premix Ex Taq

(Takara Bio). Amplification was started at 95°C for 30 seconds as the first step, followed by 40 cycles of quantitative RT-PCR at 95°C for 5 seconds and 60°C for 20 seconds. mRNA expression was measured in triplicate and normalized to β -actin expression levels. The primers, from Takara Bio and SABiosciences (Frederick, MD), were as follows: OVOL1 (forward, 5'-ACGATGCCCCATCCACTACCTG-3'; reverse, 5'-TTTCTGAGGTGCTGGTCATCATTG-3'), OVOL2 (forward, 5'-GGCAAGGGCTTCAACGACA-3'; reverse, 5'-CTTCAGGTGGGACTCCAGAGA-3'), and β -actin (forward, 5'-ATTGCCGACAGGATGCAGA-3'; reverse, 5'-GAGTACTTGCGCTCAGGAGGA-3'). The CYP1A1 primers were PPH01271E (SABiosciences).

Western Blot Analysis

NHEKs were incubated with lysis buffer (Complete Lysis-M; Roche Applied Science, Indianapolis, IN). The lysate protein concentration was measured with a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of protein (20 μ g) were dissolved in NuPage LDS Sample Buffer (Invitrogen, Carlsbad, CA) and 10% NuPage Sample Reducing Agent (Invitrogen). Lysates were boiled at 70°C for 10 minutes and loaded and run on 4% to 12% NuPage Bis-Tris Gels (Invitrogen) at 200 V for 40 minutes. The proteins were transferred to polyvinylidene fluoride membranes (Invitrogen) and blocked in 2% bovine serum albumin in 0.1% Tween-20 (Sigma-Aldrich) and Tris-buffered saline. Membranes were probed with anti-OVOL1, anti-OVOL2, or anti- β -actin antibodies overnight at 4°C. The secondary antibody was anti-rabbit horseradish peroxidase-conjugated IgG. Protein bands were detected with a Western Breeze kit (Invitrogen). Densitometric analysis of

Figure 3 **A:** Immuno-double staining of OVOL1 (brown staining) and cytokeratin 5 (CK5; blue staining) with counter hematoxylin staining. CK5 stains in cytoplasm of the outer root sheath, which highlights the cytoplasmic staining of OVOL1 in the inner root sheath. **B:** Immuno-double staining of OVOL2 (brown staining) and trichohyalin (AE15; blue staining). AE15 stains in the cytoplasm of the inner root sheath, which highlights the cytoplasmic staining of OVOL2 in the matrix. **C:** Double immunostaining of OVOL1 (blue staining) and OVOL2 (brown staining). Cells in the outer root sheath showed nuclear staining of OVOL1, OVOL2, or both. Scale bars: 100 μ m (A–C).

Figure 4 Immunohistochemical staining of OVOL1 (brown staining) and OVOL2 (brown staining) with counter hematoxylin staining in pilomatricoma. Only small OVOL1-positive cell nests are observed. **C and D:** OVOL2 expression in pilomatricoma. Most of the basaloid cells show strong cytoplasmic expression that gradually fades during the transition into shadow cells. Tumor cells at the periphery of the nests show nuclear staining. The **boxed areas** in **A** and **C** are magnified in **B** and **D**, respectively. Scale bars: 500 μ m (**A** and **C**); 100 μ m (**B** and **D**).

protein bands was performed with ImageJ software version 1.49 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

Results

Immunohistological Localization of OVOL1 and OVOL2 in the Hair Bulb and in Pilomatricoma

In agreement with results reported previously,⁸ we detected faint expression of OVOL1 in the basal and suprabasal keratinocytes of normal epidermis with a nuclear-dominant pattern (Figure 1A). Positive signals for OVOL2 were also faintly detected in the basal keratinocytes and in suprabasal cells, mainly with a nuclear pattern (Figure 1C). Similar localization of OVOL1 and OVOL2 was detected in cells of the follicular isthmus (Figure 1, B and D) and sebaceous glands.

In the hair bulb, germ cell nuclei were strongly positive for OVOL1, which maintained moderate expression during transit to the matrix and cortex. Cells in the outer root sheath also stained positively for OVOL1. Abundant cytoplasmic staining was evident in inner root sheath cells (Figure 2, A and B). OVOL2 was also strongly expressed in the nuclei of hair germ cells and cells in the outer root sheath. Notably, strong cytoplasmic staining of OVOL2 (Figure 2C) was observed in cells in the matrix, cortex, and medulla, which was a stark contrast to the strong cytoplasmic staining of OVOL1 in inner root sheath cells (Figure 2A). Weak staining of OVOL2 in the inner root sheath cells was observed in both nuclei and cytoplasm (Figure 2D). The expression patterns of OVOL1 and OVOL2 in the hair bulb are summarized in Table 1.

To further examine whether the OVOL1-OVOL2 axis is involved in the cell differentiation toward outer root sheath and inner root sheath in the hair bulb, we used double

immunostaining using OVOL1, OVOL2, CK5, and AE15. Double immunostaining highlighted the localization of OVOL1 and OVOL2 (Figure 3, A and B). AE15 and CK5 strongly stain in the cytoplasm of inner root sheath cells and outer root sheath cells, respectively. Strong cytoplasmic expression levels of OVOL1 in inner root sheath and OVOL2 in matrix were observed (Table 1). Co-expression of OVOL1 and OVOL2 was also observed in the nuclei of outer root sheath cells and germ cells (Figure 3C).

OVOL1 and OVOL2 expression in the pilomatricoma is shown in Figures 4 and 5. Notably, moderate to strong OVOL2 expression was observed in all (12 of 12) of the pilomatricoma samples (Figure 4, C and D). The OVOL2 was expressed in the nuclei of the tumor cell at the periphery of the nests and in the cytoplasm of most of the basaloid cells. Cells in the transitional zone showed weak to mild OVOL2 expression in the cytoplasm, which gradually faded during the transition into shadow cells (Figure 4, C and D). The nuclear OVOL2-positive cells were only focally positive for CK5, suggesting the differentiation toward outer root sheath in part (Figure 5A). Most of the pilomatricoma cells were negative or only faintly positive (nuclear) for OVOL1; small

Figure 5 **A:** Double immunostaining of OVOL2 (brown staining) and cytokeratin 5 (CK5; blue staining) in pilomatricoma with faint counterstaining with hematoxylin. The nuclear OVOL2-positive cells are only focally positive for CK5, suggesting the focal differentiation toward the outer root sheath. **B:** Double immunostaining of OVOL1 (brown staining) and trichohyalin (AE15; blue staining) in pilomatricoma with faint counterstaining with hematoxylin. OVOL1-positive cells are also positive for AE15, suggesting the differentiation toward the inner root sheath. Scale bars: 200 μ m (**A**); 100 μ m (**B**).

Table 2 Expression Pattern of OVOL1 and OVOL2 in Pilomatrixcoma

Pilomatrixcoma	OVOL1	OVOL2
Periphery of the nest	(-)/Nuclear (+)*	Nuclear (+++)
Transitional zone	(-)/Nuclear (+)*	Nuclear (+/+++), cytoplasmic (+/+++) [†]
Shadow cells	(-)	(-)

*Focally strong cytoplasmic expression.
[†]Expression gradually fades during the transition into shadow cells in a regular manner.
+, Expression present; +++, strong expression; -, no expression.

OVOL1-positive (cytoplasmic) cell nests were occasionally detected (Figure 4, A and B). The cytoplasmic OVOL1-positive cells were also positive for AE15, suggesting the differentiation toward inner root sheath (Figure 5B). These OVOL1- and OVOL2-positive cells were regularly arranged. The expression patterns of OVOL1 and OVOL2 in pilomatrixcoma are summarized in Table 2.

To further examine how the OVOL1-OVOL2 axis is regulated in the malignant neoplasm of the hair bulb, we evaluated the distribution of OVOL1 and OVOL2 expression in pilomatrix carcinoma. We found that OVOL1 and OVOL2 were expressed in the nucleus (Supplemental Figure S1). However, further studies are necessary to conclude the expression of OVOL1 and OVOL2 axis in pilomatrix carcinoma, because we could examine a single case of pilomatrix carcinoma, a rare malignant neoplasm.

Expression of OVOL1 and OVOL2 in Cultured Human Keratinocytes

We next examined the expression of OVOL1 and OVOL2 in cultured human keratinocytes. Human keratinocytes expressed the mRNA and proteins of OVOL1 and OVOL2 (Figure 6, A and B). The immunofluorescence study demonstrated nuclear and cytoplasmic distribution of OVOL1 and nuclear distribution of OVOL2 (Figure 6C). A previous murine study showed that the genetic deletion of *OVOL1* up-regulates *OVOL2* expression.⁷ Because the expression of OVOL1 and OVOL2 was differentially regulated in human hair bulbs, we next examined the mutual regulatory capability of *OVOL1* and *OVOL2* in human keratinocytes. Compared with those observed after transfection with control siRNA, mRNA levels of OVOL1 and OVOL2 were 28.5% ± 2.4% and 35.8% ± 2.8%, respectively, lower in keratinocytes transfected with *OVOL1* siRNA or *OVOL2* siRNA (Supplemental Figure S2). Notably, the expression of *OVOL2* was up-regulated in the *OVOL1* knock-down keratinocytes (Figure 7A). However, the expression of *OVOL1* was not significantly affected in *OVOL2* knockdown keratinocytes (Figure 7B).

Ketoconazole Up-Regulation of OVOL1 But Not OVOL2 Expression

To examine further the machinery of OVOL1-OVOL2 axis regulation in keratinocytes, we treated the keratinocytes with various concentrations (≤10 μmol/L) of ketoconazole, a potent

Figure 6 RT-PCR analysis (A) and Western blot analysis (B) of cultured human keratinocytes. Cultured human keratinocytes express the mRNA and proteins (arrows) of OVOL1 and OVOL2. C: The results of the immunofluorescence study demonstrate the nuclear and cytoplasmic distribution of OVOL1 (green) and the nuclear distribution of OVOL2 (green). Blue staining (DAPI) indicates nuclei. Original magnification: ×400 (C, insets).

Figure 7 Quantitative RT-PCR analyses of cultured human keratinocytes. **A:** OVOL2 expression is up-regulated in OVOL1 knockdown keratinocytes. **B:** OVOL1 expression is slightly decreased in OVOL2 knockdown keratinocytes. **C and D:** Ketoconazole (KCZ) up-regulates OVOL1 expression in a dose-dependent manner, whereas it has no effect on OVOL2 expression. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

hair growth stimulant.^{9,10} Ketoconazole up-regulated OVOL1 expression in a dose-dependent manner (Figure 7C), whereas it did not affect the expression of OVOL2 (Figure 7D). The ketoconazole-induced up-regulation of OVOL1 was also evident in protein levels determined with Western blot analysis (Figure 8A). The protein level of OVOL2 was unaffected by ketoconazole. The effects of ketoconazole were also confirmed with immunofluorescence staining. The staining intensity of OVOL1, but not OVOL2, in keratinocytes was again significantly up-regulated by ketoconazole treatment (Figure 8, B and C).

Discussion

Our results included several key findings about OVOL1 and OVOL2 expression in the hair bulb and in

pilomatricoma. OVOL1 and OVOL2 were expressed in normal human skin, especially in the hair bulb. As demonstrated in Figures 2 and 3, the expression of the proteins was highly site specific, with a cytoplasmic pattern, nuclear pattern, or both. Both proteins were expressed in the nuclei of hair germ cells, and expression was maintained during the differentiation toward hair follicles. OVOL1 was specifically observed in the nuclei of cortex and outer root sheath cells, and OVOL2 was present in the nuclei of outer root sheath cells. It is known that OVOL1 is recruited to epidermal differentiation complex genes, leading to promoting keratinocyte differentiation,¹¹ and OVOL2 suppresses terminal differentiation of keratinocytes by directly repressing *c-myc* and Notch-1. The nuclear staining of OVOL1 and OVOL2 in ORS and germ (in addition to above mentioned facts) suggests that OVOL1 and OVOL2 are important transcriptional factors

Figure 8 Western blot analysis of cultured human keratinocytes. **A:** An increased protein level of OVOL1 in keratinocytes treated with ketoconazole (KCZ) is evident compared with that in control. KCZ statistically increases the protein level of OVOL1 (**B**), but does not affect the OVOL2 protein level (**C**) in keratinocytes. * $P < 0.05$, *** $P < 0.001$.

mediating epidermal differentiation complex gene expression and transcriptional activity of *c-myc* and Notch-1 in the hair bulb.¹² Interestingly, the strong cytoplasmic expression of OVOL1 in the inner root sheath cells contrasted starkly with that of OVOL2 in the cells of the matrix and cortex. The cytoplasmic staining of OVOL1 and OVOL2 was reciprocally exclusive in hair follicle cells (Figures 2 and 3). Because *OVOL1*-deficient mice produce aberrant hair¹ and OVOL1 inhibits OVOL2 function,⁷ OVOL1 and OVOL2 may coordinately regulate hair differentiation and growth in the development of the hair shaft structure.

Another important observation was that OVOL1 and OVOL2 proteins were expressed in pilomatricoma. Staining for OVOL2 was strongly positive in the cytoplasm of basaloid cells (matrix germinative cells) in pilomatricoma, a hair matrix–derived tumor. This expression faded as cells transitioned into shadow cells. Some basaloid cells at the periphery of the nests showed nuclear staining. The expression pattern closely resembled that of normal hair germ cells and hair matrix cells, which suggests that OVOL proteins may be useful markers for determining the origin of tumors with follicular differentiation. OVOL1, by contrast, showed partial, but strong, cytoplasmic staining in pilomatricoma. These OVOL1-positive cells might differentiate toward inner root sheath cells.

Furthermore, the expression of OVOL1 and OVOL2 with nuclear and cytoplasmic localization is evident in cultured human keratinocytes. Although the cell system using NHEKs might not entirely correspond to keratinocytes of the hair sheath, it has been reported that NHEKs, cultured keratinocytes of the basal layer, and keratinocytes in the lower part of the outer root sheath have similar features that they are positive for keratin 15 and negative for CD34.¹³ A previous murine study demonstrated that *Ovol2* is a downstream target of *Ovol1* by showing that the expression of *Ovol2* is up-regulated in *Ovol1*-deficient epidermis and that *Ovol1* represses the activity of the *Ovol2* promoter in a DNA-binding–dependent manner.⁷ Consistent with this finding, significant up-regulation of *OVOL2* was observed in *OVOL1*-knockdown human keratinocytes in the present study, whereas the expression of *OVOL1* was unaffected under the *OVOL2* knockdown condition. The clear down-regulation of OVOL2 in OVOL1-abundant inner root sheath cells parallels the *OVOL1*-induced repression of *OVOL2*. In addition, we found that ketoconazole, a potent hair growth stimulator,^{9,10} up-regulated *OVOL1*, but not *OVOL2*, expression. Our recent study demonstrated that ketoconazole activates aryl hydrocarbon receptors in keratinocytes,¹⁴ which implies that OVOL1 acts in concert with the activation of aryl hydrocarbon receptors (AhRs) and leads to hair growth; however, the molecular mechanisms by which AhR activation by ketoconazole up-regulates OVOL1 expression remain unclear. Because AhR-dependent gene expression requires the nuclear translocation of AhR from cytoplasm to nuclei,¹⁵ AhR nuclear transporter (ARNT), a binding partner of the AhR, has a pivotal role in the AhR activation. Considering the facts that the AhR-ARNT

system regulates keratinocyte differentiation¹⁵ and ARNT expression is involved in the hair development,¹⁶ there is a possibility that OVOL1, in conjunction with the AhR-ARNT system, might play a major role during hair production and development.

These findings, along with those of the present study, suggest that the OVOL1-OVOL2 axis is an active regulator of normal hair development and differentiation. OVOL1 and OVOL2 may be therapeutic targets in the treatment of hair disorders, including alopecia, especially androgenetic alopecia. Furthermore, pilomatricoma conserves the expression of OVOL proteins, and the distribution pattern of this protein expression may be useful in the differentiation of the tumor.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.12.013>.

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