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Targeting of CXXC5 by a Competing Peptide Stimulates Hair Regrowth and Wound-Induced Hair Neogenesis

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The Wnt/β-catenin pathway has been implicated in hair follicle development and hair regeneration in adults. We discovered that CXXC-type zinc finger protein 5 (CXXC5) is a negative regulator of the Wnt/β-catenin pathway involved in hair regrowth and wound-induced hair follicle neogenesis via an interaction with Dishevelled. CXXC5 was upregulated in miniaturized hair follicles and arrector pili muscles in human balding scalps. The inhibitory effects of CXXC5 on alkaline phosphatase activity and cell proliferation were demonstrated using human hair follicle dermal papilla cells. Moreover, *CXXC5*$^{-/-}$ mice displayed accelerated hair regrowth, and treatment with valproic acid, a glycogen synthase kinase 3β inhibitor that activates the Wnt/β-catenin pathway, further induced hair regrowth in the *CXXC5*$^{-/-}$ mice. Disrupting the CXXC5-Dishevelled interaction with a competitor peptide activated the Wnt/β-catenin pathway and accelerated hair regrowth and wound-induced hair follicle neogenesis. Overall, these findings suggest that the CXXC5-Dishevelled interaction is a potential target for the treatment of hair loss.

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INTRODUCTION

Androgenetic alopecia (AGA), the most common type of hair loss, is caused by androgens in genetically susceptible women and men (Price, 2003). Hair follicles in AGA have excessive 5α-reductase enzyme activity and increased levels of dihydrotestosterone (DHT) (Sawaya, 1998). DHT binds more firmly to the androgen receptor than testosterone and activates the genes involved in the transformation of terminal follicles to miniaturized follicles in AGA (Kaufman, 1996; Yang et al., 2013). Finasteride, a 5α-reductase inhibitor, and topical minoxidil are the only FDA-approved treatments for AGA (Dhurat et al., 2013), but their utilities are limited by low efficacy and potentially undesirable side effects (Crabtree et al., 2010).

The Wnt/β-catenin pathway plays important roles in hair follicle development (Millar, 2002) and hair regeneration in adults (Collins et al., 2011). Activation of the Wnt/β-catenin pathway rescues DHT-induced suppression of the hair follicle stem cell differentiation that results in the hair follicle miniaturization (Leirós et al., 2012). Moreover, the treatment of glycogen synthase kinase 3β inhibitors, such as 6-bromoindirubin-3′-oxime or valproic acid (VPA), in human dermal papilla cells activates the Wnt/β-catenin pathway and increases the activity and expression of alkaline phosphatase (ALP), a prominent dermal papilla marker (Lee et al., 2012a; Yamauchi et al., 2009). Inducing β-catenin deletion or Dkk1 expression during the anagen phase in mice also inhibits hair follicle matrix proliferation (Choi et al., 2013). In contrast, the Wnt/β-catenin pathway activators, such as VPA or hair stimulating complex, promote human hair follicle cell proliferation (Jo et al., 2013; Zimber et al., 2011).

CXXC5 is a member of a small protein family containing the CXXC-type zinc-finger domain (Ravasi et al., 2003) and is a Wilms Tumor 1 target gene that attenuates the Wnt/β-catenin pathway (Andersson et al., 2009; Kim et al., 2010). CXXC5 interacts with Dishevelled (Dvl) via its C-terminal Dvl-binding motif, and this motif is crucial for CXXC5 function (London et al., 2004). CXXC5 is known as a negative regulator of the Wnt/β-catenin pathway in the neural stem cells and zebrafish (Andersson et al., 2009; Kim et al., 2010). Inhibition of the Wnt/β-catenin pathway by CXXC5 has been previously demonstrated, but the role of CXXC5 as a therapeutic target for AGA is unknown.

Wound-induced hair follicle neogenesis (WIHN) could be potential treatment for hair loss by regenerating follicles through wounding and Wnt/β-catenin pathway activation (Ito et al., 2007). The de novo hair regeneration after wounding was observed in humans, but its efficiency was relatively low,

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Abbreviations: AGA, androgenetic alopecia; ALP, alkaline phosphatase; DBM, Dvl-binding motif; DHT, dihydrotestosterone; Dvl, Dishevelled; HFDPC, human hair follicle dermal papilla cell; PCNA, proliferating cell nuclear antigen; PTD, protein transduction domain; VPA, valproic acid; WIHN, wound-induced hair follicle neogenesis

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suggesting the necessity of activator increasing regeneration efficiency (Gay et al., 2013). However, the small molecule and peptide drugs increasing hair regeneration efficiency after wounding has yet to be developed.

In this study, we monitored the status of CXXC5 on haired and bald scalps of patients with AGA and characterized the effect of competitor peptide disrupting the CXXC5-Dvl interaction on hair regrowth, WIHN, and ALP expression in vitro and in vivo.

RESULTS

CXXC5 is highly expressed in miniaturized hair follicles in balding human scalps and catagen follicles in mice skins

To investigate the clinical implications of Wnt/β-catenin signaling and CXXC5 in AGA, we monitored the levels of β-catenin and CXXC5 in haired and bald scalps from individuals with AGA. High expression and nuclear localization of β-catenin was observed in the hair matrix cells of haired scalps, but its expression was lower in the bald scalps (see Supplementary Figure S1a online). Conversely, the normal hair follicle exhibited little or no CXXC5 expression, whereas its expression was high in the miniaturized follicles of the bald scalps (see Supplementary Figure S1a). CXXC5 was also highly expressed in the dermal papilla as well as follicular keratinocytes in the catagen follicle of bald scalps (Figure 1a). We next characterized the factors influencing human hair growth via immunohistochemistry. Proliferating cell nuclear antigen (PCNA)-positive cells were prominent among the matrix keratinocytes in the haired scalps, but the number of PCNA-positive cells was severely reduced in the bald scalps (see Supplementary Figure S1a). Although ALP was strongly expressed in cells toward the apex of dermal papilla in the haired scalps, its expression was undetectable in the bald scalps (see Supplementary Figure S1a). The hair follicle stem cell marker keratin 15 (Garza et al., 2011) and the progenitor cell markers (Garza et al., 2011), such as CD34 and CD200, were primarily expressed in the outer-root sheath cells in the haired scalps (see Supplementary Figure S1a). However, keratin 15 expression was retained, whereas CD34 and CD200 were undetectable in the follicles of bald scalps (see Supplementary Figure S1a). Quantitative analyses of the immunohistochemical staining also confirmed the inverse expression patterns of β-catenin and CXXC5 in terminal and miniaturized follicles of patients with AGA (Figure 1a). To better understand the role of CXXC5 on balding, we traced CXXC5 expression in the bald scalps via quantitative analyses of the immunohistochemical staining. CXXC5 was highly expressed in the hair follicle keratinocytes of miniaturized follicles and arrector pili muscles compared with its expression in sebaceous glands (Figure 1b, 1c). Moreover, CXXC5 was expressed in the keratin 15-negative cells of follicles in the bald scalps (Figure 1c).

To further evaluate the role of CXXC5 on hair growth in mice, we monitored the levels of β-catenin and CXXC5 during the depilation-induced, synchronized adult hair cycle. Immunohistochemical and western blot analyses showed that β-catenin was highly expressed in the anagen phase, whereas CXXC5 expression was highest during the anagen-catagen transition (Figure 1d, Supplementary Figure S2a online). β-Catenin expression peaked on day 10 after depilation, and subsequently, CXXC5 expression peaked on day 18 after depilation, as measured by quantitative analyses of western blotting (see Supplementary Figure S2b online). Given the inverse relationship between β-catenin and CXXC5 expression, we hypothesized that the subsequent induction of CXXC5 may be a negative feedback mechanism of the Wnt/β-catenin pathway. To verify this hypothesis, we further analyzed the expression patterns of β-catenin and CXXC5 during the physiological first postnatal hair cycle. Similarly, β-catenin expression peaked on postnatal day 6, followed by the peak of CXXC5 on postnatal day 17 (see Supplementary Figure S2c online). At day 18 after depilation, CXXC5 was extensively expressed in keratin 15-negative keratinocytes (see Supplementary Figure S2d online). Overall, CXXC5 is highly expressed in the miniaturized follicles of bald human scalps and catagen follicles in mice skins, suggesting that CXXC5 negatively regulates hair growth in humans and mice.

CXXC5 attenuates ALP activity and proliferation in human hair follicle dermal papilla cells via interaction with Dvl-1

The role of CXXC5 in modulating ALP activity and cell proliferation was investigated using human hair follicle dermal papilla cells (HFDPCs). Transfection with *CXXC5* significantly inhibited expression of β-catenin, ALP, and PCNA (Figure 2a). Analyses using immunocytochemical and ALP staining also showed that the levels of both β-catenin and ALP were diminished by *CXXC5* transfection (Figure 2b). In contrast, CXXC5 knockdown induced the expression of β-catenin, ALP, and PCNA (see Supplementary Figure S3 online). CXXC5 possesses a Dvl-binding motif at its C terminus that is essential for CXXC5 function as a negative regulator of the Wnt/β-catenin pathway (London et al., 2004). Transfection with *CXXC5ΔDBM*, which lacks the DBM (London et al., 2004), did not inhibit the expression of β-catenin and ALP, as well as PCNA in HFDPCs (Figure 2c). Overexpression of CXXC5 markedly reduced the transcriptional activity of a Wnt/β-catenin reporter gene (Figure 2d). Furthermore, *ALPL* promoter activity and ALP activity were significantly lowered by CXXC5 overexpression (Figure 2e, 2f). Cell proliferation was also decreased in HFDPCs by 66% at 72 hours after transfection with *CXXC5* (Figure 2g). To further clarify the role of the Wnt/β-catenin pathway in CXXC5 function, we confirmed the effect of Wnt3a in HFDPCs. Interestingly, β-catenin and CXXC5 were concomitantly increased in human dermal papilla cells treated with Wnt3a as shown by western blot and immunocytochemical analyses (see Supplementary Figure S4 online). On the basis of in vivo results, we expected Wnt3a-dependent induction of CXXC5 to be a negative feedback mechanism. We also found that CXXC5 interacted with Dvl-1 in human hair follicle cells, but only detected this interaction when HFDPCs were treated with Wnt3a (Figure 2h). However, CXXC5ΔDBM failed to interact with Dvl-1 in HFDPCs, even when the cells were treated with Wnt3a (Figure 2h). Endogenous CXXC5 also bound to Dvl-1 in Wnt3a-treated HFDPCs as shown by western blot analyses of immunoprecipitates

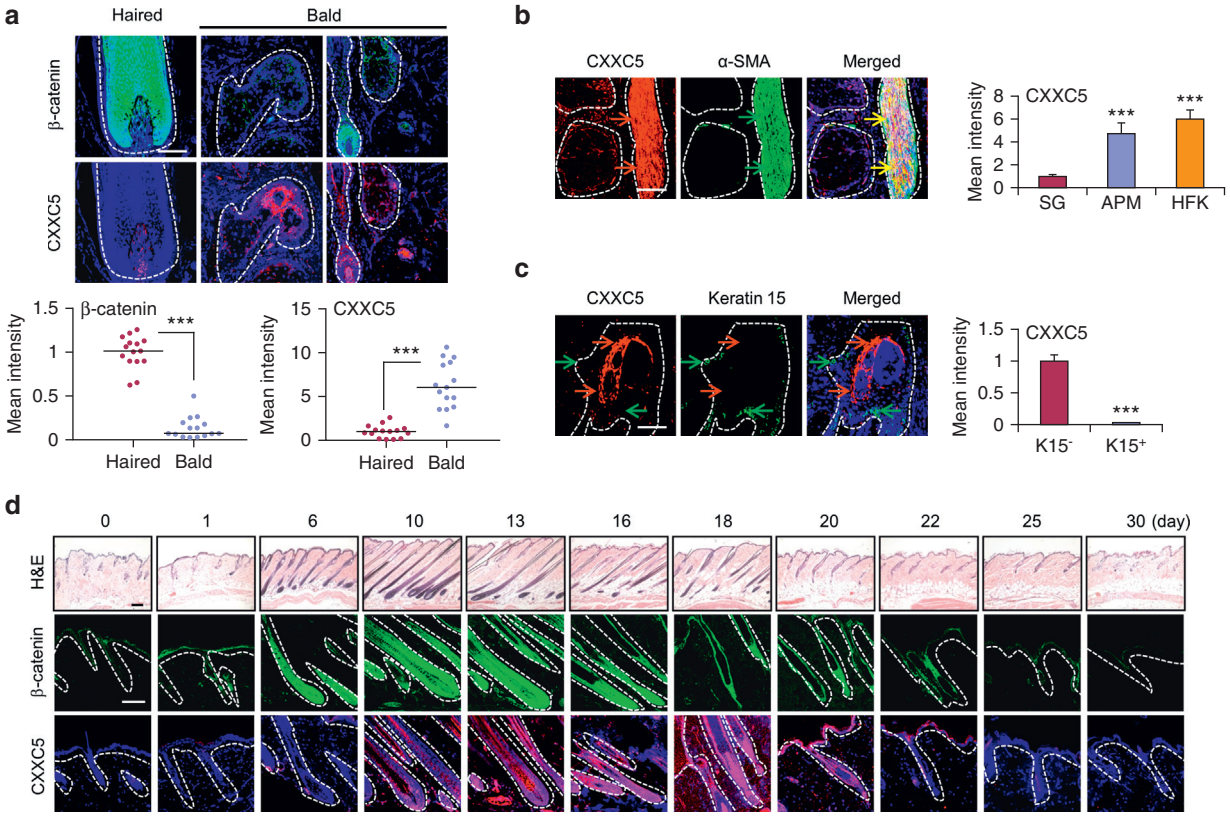

Figure 1. CXXC5 levels are upregulated and inversely correlated with β-catenin levels in balding human scalp. Haired and matching bald scalp tissues (n = 5) were originated from the same individuals of male patients with AGA and subjected to H&E staining and immunohistochemical analyses. (**a**) H&E and immunohistochemical staining for β-catenin, CXXC5, with DAPI nuclear counterstaining (blue) in the human haired and bald scalps. Dashed lines indicate the outlines of hair follicles. (**b**) Immunohistochemical staining performed with antibodies against CXXC5 (red) or α-SMA (green) in bald scalps (left panels) and quantitative analyses (n = 3) of immunohistochemical staining for CXXC5 in sebaceous glands (SG), arrector pili muscles (APM), and hair follicle keratinocytes (HFK) of bald scalps (right graph). Yellow arrows indicate the coexpression of CXXC5 and α-SMA. Dashed lines delineate SG and APM. (**c**) Immunohistochemical staining was performed with antibodies against CXXC5 (red) or keratin 15 (green) in bald scalps (left panels) and quantitative analyses (n = 3) of immunohistochemical staining for CXXC5 in keratin 15-positive or keratin 15-negative regions of bald scalps (right graph). Red and green arrows indicate that CXXC5 is expressed in the keratin 15-negative cells of follicles. Dashed lines outline hair follicles. (**d**) H&E and immunohistochemical staining for β-catenin (green) or CXXC5 (red) with DAPI staining (blue) in mice skin during the depilation-induced hair cycle. Dashed lines outline epidermis and hair follicles. All data are representative of at least three independent experiments. Scale bars = 100 μm for panels (**a**–**d**). Data are presented as means ± SD. ***$P < 0.0005$ for panels (**a**–**c**). AGA, androgenetic alopecia; α-SMA, alpha-smooth muscle actin; H&E, hematoxylin and eosin; SD, standard deviation.

(see Supplementary Figure S5 online). To confirm the possibility of further Wnt/β-catenin pathway activation after disrupting the negative feedback regulation of CXXC5, we cotreated HFDPCs with both CXXC5 small interfering RNA and Wnt3a. Expression of β-catenin, ALP, and PCNA was synergistically increased by cotreatment with CXXC5 small interfering RNA and Wnt3a (Figure 2i, 2j). In summary, our data indicate that CXXC5 interacts with Dvl in a Wnt3a-dependent manner to negatively regulate the Wnt/β-catenin pathway in HFDPCs.

Loss of CXXC5 promotes hair regeneration in the normal and wounded skins and VPA treatment further induces the phenotype of CXXC5-deficient mice

To assess whether Wnt/β-catenin signaling and CXXC5 are involved in anagen induction and hair regrowth, 7-week-old littermate *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice were treated every day for 25 days with either vehicle or VPA, a Wnt/β-catenin pathway activator (Lee et al., 2012b). *CXXC5*$^{-/-}$ mice treated with VPA exhibited markedly accelerated hair regrowth as determined by measurement of hair weight (Figure 3a). Histologic analyses also showed that VPA significantly induced the telogen-anagen transition in *CXXC5*$^{-/-}$ mice, whereas hair follicles from vehicle-treated wild-type mice skin remained in the telogen phase (Figure 3b). Moreover, the number of hair follicles and dermal thickness were significantly increased in VPA-treated *CXXC5*$^{-/-}$ mice skin (Figure 3b). Expression levels of β-catenin and PCNA were significantly increased in the epidermis and hair bulb, including the hair matrix and dermal papilla of VPA-treated *CXXC5*$^{-/-}$ mice as determined by immunohistochemical analyses (Figure 3c, 3d). The mice deleted for CXXC5 and/or treated with VPA displayed normal skin architecture as indicated by proper maintenance of keratin 14 expression (Figure 3c). ALP levels were significantly increased toward the apex of dermal papilla in VPA-treated *CXXC5*$^{-/-}$ mice (Figure 3d). Western blot analyses also showed that expression levels of β-catenin, ALP, and PCNA were synergistically increased in VPA-treated *CXXC5*$^{-/-}$ mice (Figure 3e). Furthermore,

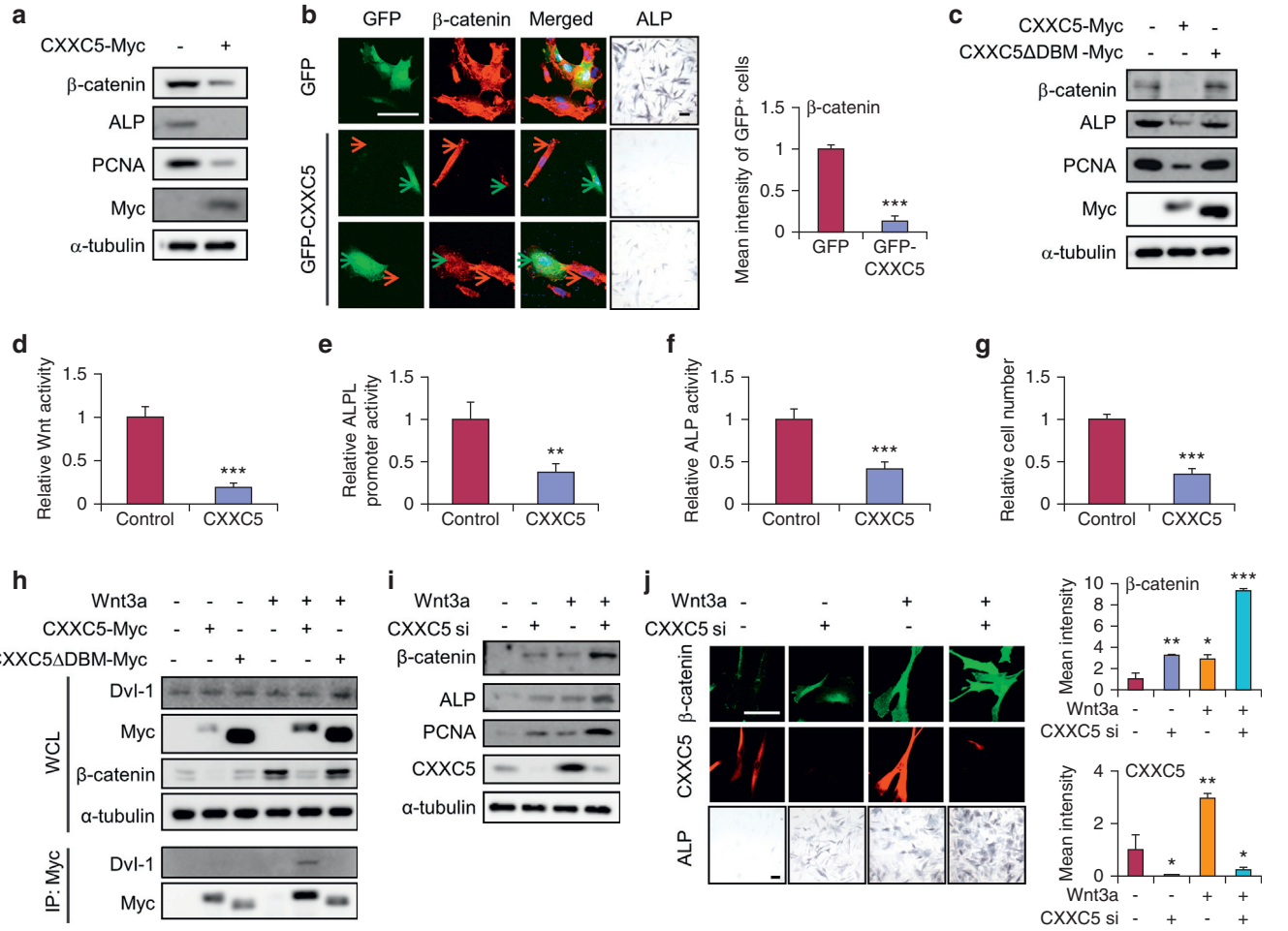

Figure 2. CXXC5 attenuates ALP activity and proliferation in HFDPCs. (**a**) HFDPCs (n = 3) were transfected with pcDNA3.1 or pcDNA3.1-CXXC5-Myc. The cells were further cultured for 3 days before harvesting and whole cell lysates (WCLs) were subjected to western blot analyses to detect the protein levels of β-catenin, ALP, PCNA, Myc, and α-tubulin. (**b**) Immunocytochemical staining for β-catenin (red) with DAPI staining (blue), ALP staining (dark blue) in HFDPCs transfected with pEGFP-c3 or GFP-CXXC5 (green) (left panels) and quantitative analyses (n = 3) of immunocytochemical staining for β-catenin in GFP-positive cells (right graph). Red arrowheads indicate the expression of β-catenin and green arrowheads indicate the expression of GFP. (**c**) HFDPCs were transfected with pcDNA3.1, pcDNA3.1-CXXC5-Myc, or pcDNA3.1-CXXC5ΔDBM-Myc. The cells were further incubated for 3 days and WCLs were subjected to western blot analyses to examine the expression of β-catenin, ALP, PCNA, Myc, and α-tubulin. (**d**–**g**) Wnt reporter assay, *ALPL* reporter assay, ALP activity assay, and MTT assay (n = 3) in HFDPCs transfected with pcDNA3.1 or pcDNA3.1-CXXC5-Myc. (**h**) HFDPCs were transfected with pcDNA3.1, pcDNA3.1-CXXC5-Myc, or pcDNA3.1-CXXC5ΔDBM-Myc. The transfected cells were treated with or without 50 ng/ml recombinant Wnt3a for 72 hours. WCLs or cell lysates immunoprecipitated with anti-Myc were analyzed by western blotting to detect Dvl-1, Myc, β-catenin, or α-tubulin. (**i, j**) HFDPCs were treated with or without 50 ng/ml recombinant Wnt3a for 3 days after transfection with control siRNA or CXXC5 siRNA (CXXC5 si). (**i**) Western blot analyses of WCLs were performed with antibodies against β-catenin, ALP, PCNA, Myc, and α-tubulin. (**j**) Immunocytochemical staining for β-catenin (green) or CXXC5 (red), ALP staining (dark blue) (left panels), and mean intensity quantitation (n = 3) (right graphs) were also performed. All results presented are representative of at least three independent experiments. Scale bars = 100 μm for panels (**b**) and (**j**). Data are expressed as means ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ for panels (**b**), (**d**), (**e**), (**f**), (**g**), and (**j**). ALP, alkaline phosphatase; Dvl, Dishevelled; GFP, green fluorescent protein; HFDPC, human hair follicle dermal papilla cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen; SD, standard deviation; siRNA, small interfering RNA.

quantitative analyses of immunohistochemical staining demonstrated that the levels of β-catenin and PCNA were significantly increased in the epidermis and hair bulb of VPA-treated *CXXC5*$^{-/-}$ mice (Figure 3f). Especially, the hair follicles of VPA-treated *CXXC5*$^{-/-}$ mice were transformed to late anagen as shown by hair cycle analyses (Figure 3f).

To test whether CXXC5 deletion and/or VPA treatment promotes hair regeneration in the wounds, we created full-thickness wounds (diameter = 1 cm) on the backs of *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice and then treated the wounds with either vehicle or VPA. VPA-treated *CXXC5*$^{-/-}$ mice wounds showed marked increases in WIHN and expression levels of β-catenin and Fgf9, a growth factor involved in enhancement of WIHN (Gay et al., 2013) compared with vehicle-treated wild-type mice wounds (see Supplementary Figure S6 online). Overall, deletion of CXXC5 promotes hair regeneration in the normal and wounded skins, and VPA treatment further induces the phenotype in the *CXXC5*$^{-/-}$ mice.

Combination treatment with PTD-DBM and Wnt3a synergistically induces expression of β-catenin, ALP, and PCNA in HFDPCs

To activate the Wnt/β-catenin pathway by disrupting the CXXC5-Dvl interaction, we used a peptide, specifically

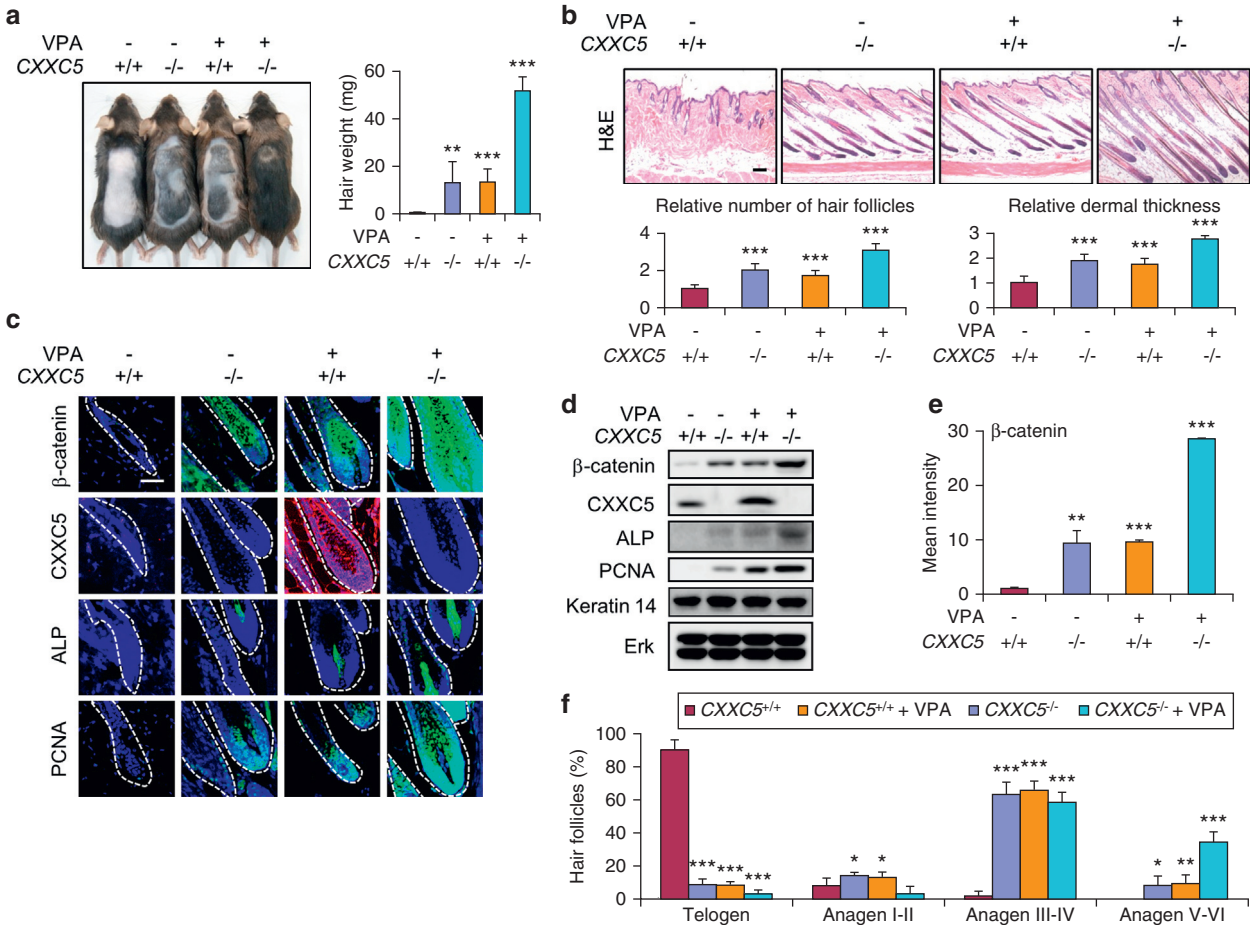

Figure 3. Loss of CXXC5 promotes hair regrowth and VPA treatment further induces the hair regrowth in the CXXC5-deficient mice. Vehicle or 500 mM VPA was topically applied to the shaved back skin of 7-week-old *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice (n = 7 per group) daily for 25 days. (**a**) Representative gross images (left panel) and quantitative estimation (n = 7) of hair regrowth (right graph). Hair regrowth in each group was determined by the measurement of the weight of newly grown hairs. (**b**) H&E staining (top panels) of the skins from *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice treated with or without VPA for 25 days and measurements (n = 21) of hair follicle number and dermal thickness (lower graphs) in H&E-stained skin tissue sections taken randomly in three fields per mouse. (**c**) Immunohistochemical staining for β-catenin, CXXC5, ALP, or PCNA in the hair bulb of *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice skins treated with or without VPA treatment for 25 days. Dashed lines outline hair bulbs. (**d**) Western blot analyses of β-catenin, CXXC5, ALP, PCNA, keratin 14, and Erk in vehicle- or VPA-treated mice skin tissues from *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice. (**e**) Quantitative analyses of immunohistochemical staining shown in (**c**). (**f**) Quantitative hair cycle analyses (n = 5 per group) using at least 50 hair follicles per mouse per group. All results presented are representative of at least three independent experiments. Scale bars = 100 μm for panels (**b**) and (**c**). Data are expressed as means ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ for panels (**a**), (**b**), (**e**), and (**f**). ALP, alkaline phosphatase; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; SD, standard deviation; VPA, valproic acid.

competed for CXXC5 binding to Dvl. This peptide consists of a protein transduction domain (PTD; PolyR) for delivery, a linker for flexibility, the DBM from CXXC5, and a lysine conjugated to fluorescein isothiocyanate for visualization (see Supplementary Figure S7a online). The PTD-DBM peptide induced the expression of β-catenin, ALP, and PCNA in a concentration-dependent manner in HFDPCs (see Supplementary Figure S7b, S7c online). To further confirm the role of CXXC5 as a negative feedback regulator of the Wnt/β-catenin pathway, we cotreated HFDPCs with PTD-DBM and Wnt3a or VPA. PTD-DBM or Wnt3a treatment alone induced β-catenin, ALP, and PCNA expression, and combination treatment synergistically induced the expression of these markers in HFDPCs as shown by western blot and immunocytochemical analyses, as well as ALP staining (Figure 4a, 4b). Cotreatment with PTD-DBM and VPA also synergistically induced the expression of β-catenin and ALP, as well as PCNA (Figure 4c, Supplementary Figure S8 online). We also observed synergistic increases in Wnt reporter activity, *ALPL* reporter activity, and ALP activity in HFDPCs treated with both PTD-DBM and Wnt3a (Figure 4d–f). In addition, cell proliferation was significantly increased at 72 hours after treatment with both PTD-DBM and Wnt3a (Figure 4g). Western blot analyses of immunoprecipitates demonstrated that PTD-DBM significantly interrupted the Wnt3a-induced interaction between CXXC5 and Dvl-1 (Figure 4h). Moreover, transfection with β-catenin small interfering RNA abolished the PTD-DBM-induced increases in ALP and PCNA in HFDPCs (Figure 4i), indicating that PTD-DBM induced the levels of ALP and PCNA in a β-catenin-dependent manner. We also found that PTD-DBM induced the expression of β-catenin and ALP, and PCNA regardless of cotreatment with DHT in HFDPCs (Figure 4j).

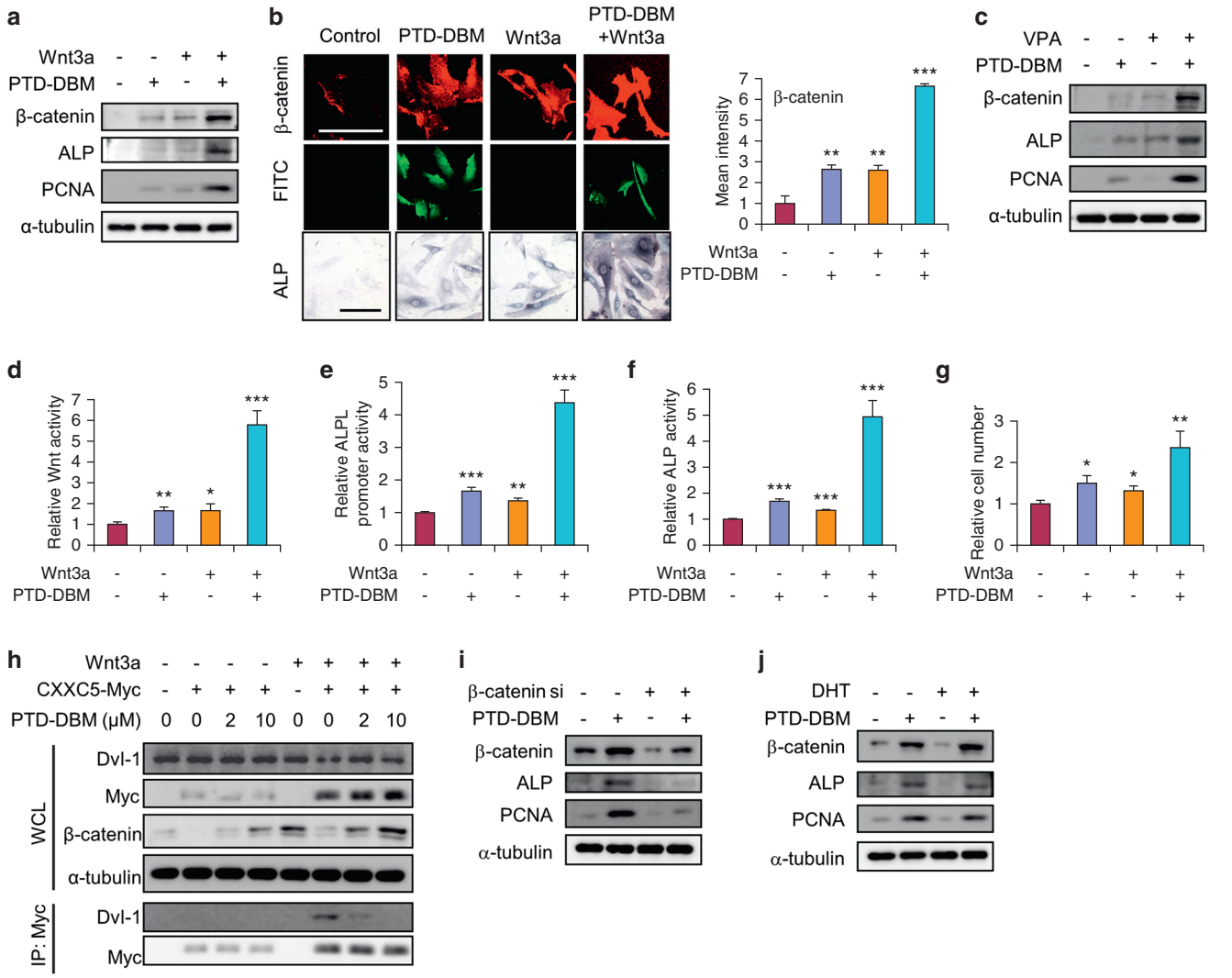

Figure 4. Combination treatment with PTD-DBM and Wnt3a synergistically induces ALP activity and proliferation in HFDPCs. (**a, b**) HFDPCs were treated with 2 μM PTD-DBM and/or 50 ng/ml recombinant Wnt3a for 3 days. (**a**) WCLs were subjected to western blotting to determine the levels of β-catenin, ALP, PCNA, and α-tubulin. (**b**) Immunocytochemical staining for β-catenin (red), FITC (green) for PTD-DBM peptide detection, ALP staining (dark blue) (left panels), and mean intensity quantitation (n = 3) (right graphs) were also performed. (**c**) Western blot analyses of β-catenin, ALP, PCNA, and α-tubulin on WCLs from HFDPCs cotreated with 2 μM PTD-DBM and/or 1 mM VPA for 3 days. (**d**–**g**) Wnt reporter assay, *ALPL* reporter assay, ALP activity assay, and MTT assay (n = 3) in HFDPCs cotreated with 2 μM PTD-DBM and/or 50 ng/ml recombinant Wnt3a for 3 days. (**h**) HFDPCs were treated with 2 μM PTD-DBM and/or 50 ng/ml Wnt3a after transfection with pcDNA3.1 or pcDNA3.1-CXXC5-Myc. The lysates were used for immunoprecipitation with an anti-Myc antibody. (**i**) HFDPCs were treated with 2 μM PTD-DBM for 3 days after transfection with β-catenin siRNA (β-catenin si). Western blot analyses of WCLs were performed with antibody against β-catenin, ALP, PCNA, or α-tubulin. (**j**) HFDPCs treated with 2 μM PTD-DBM and/or 100 nM DHT for 3 days were analyzed by western blotting to detect β-catenin, ALP, PCNA, and α-tubulin. All data are representative of the results of at least three independent experiments. Scale bars = 100 μm for panel (**b**). Results are shown as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$ for panels (**b**) and (**d**–**g**). ALP, alkaline phosphatase; DBM, Dvl-binding motif; DHT, dihydrotestosterone; FITC, fluorescein isothiocyanate; HFDPC, human hair follicle dermal papilla cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen; PTD, protein transduction domain; SD, standard deviation; siRNA, small interfering RNA; VPA, valproic acid; WCL, whole cell lysate.

These results demonstrate that disrupting the CXXC5-Dvl interaction induces ALP activity and proliferation in HFDPCs.

Cotreatment with PTD-DBM and VPA promotes hair regrowth and significantly induces β-catenin, ALP, and PCNA expression in mice

To determine the effect of cotreatment with PTD-DBM and VPA on hair regrowth in vivo, we shaved the dorsal skin of mice and topically treated the shaved area with PTD-DBM and/or VPA for 28 days on a daily basis. When the skin was treated with PTD-DBM, we detected strong fluorescence in the stratum corneum and epidermis 30 minutes after treatment (see Supplementary Figure S9 online). Although there were regional differences in the strength of the fluorescein isothiocyanate signal, PTD-DBM was effectively delivered to both the epidermis and dermis in mice skin 2 hours after treatment (see Supplementary Figure S9). Combination treatment with PTD-DBM and VPA remarkably accelerated hair regrowth and anagen induction in the shaved area (Figure 5a). Cotreatment with PTD-DBM and VPA enhanced hair regrowth much more efficiently than treatment with minoxidil alone as determined by measurements of hair weight and hair follicle number, as well as

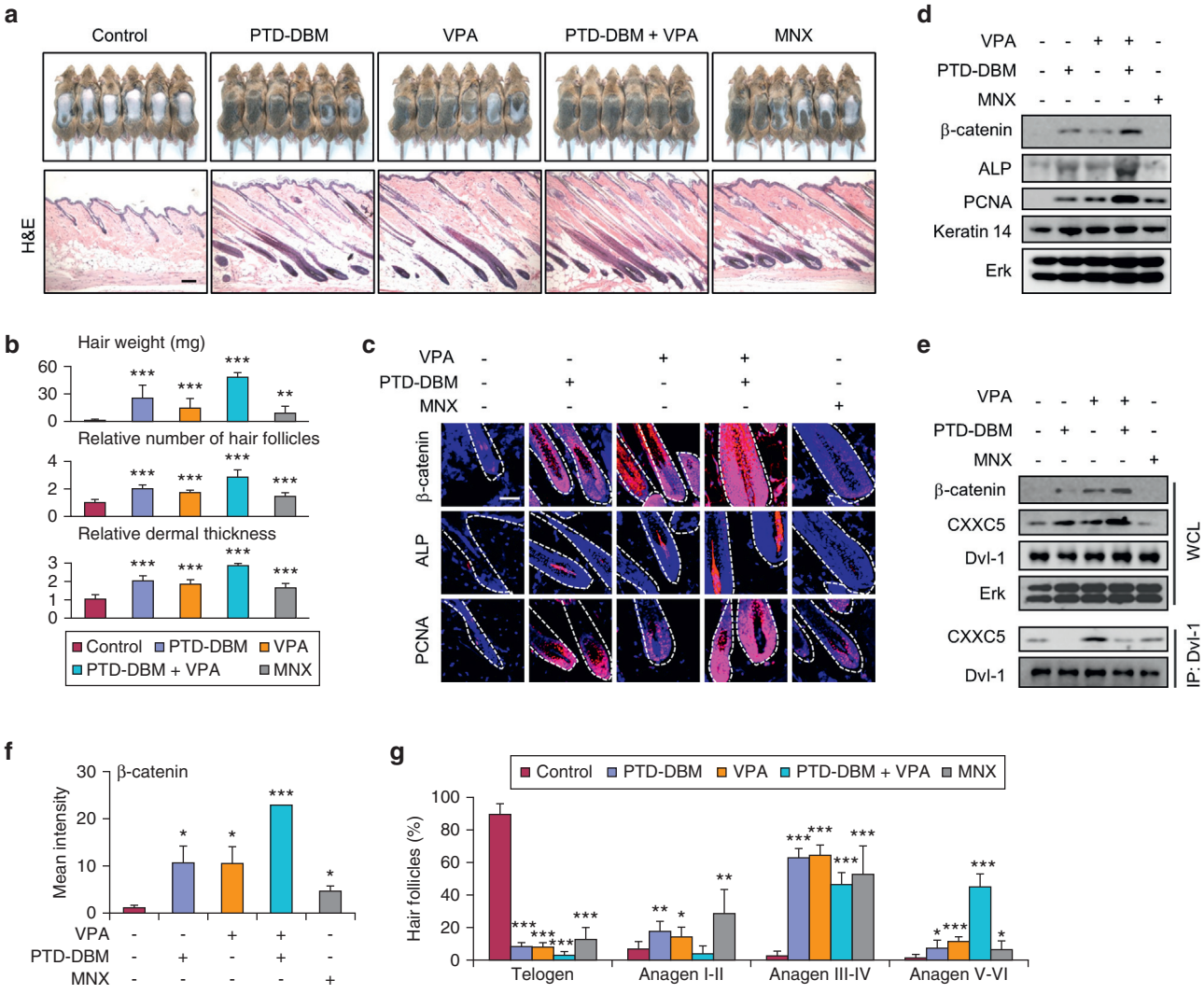

Figure 5. Cotreatment with PTD-DBM and VPA significantly promotes hair regrowth in mice. The back skins of 7-week-old male C3H mice (n = 12 per group) were shaved and treated topically daily with 2 mM PTD-DBM and/or 500 mM VPA or with 100 mM MNX for 28 days. (**a**) Gross images (top panels) showing hair regrowth and H&E staining (lower panels) of skins with the different treatments for 28 days. (**b**) Measurements of hair weight (top graph; n = 12) of regrown dorsal hairs, as well as hair follicle number (middle graph; n = 24) and dermal thickness (lower graph; n = 24) in H&E-stained skin tissue sections in two fields per mouse. (**c**) Immunohistochemical staining for β-catenin, ALP, and PCNA in the hair bulbs of skins from mice treated with PTD-DBM and/or VPA or MNX for 28 days. Dashed lines delineate hair bulbs. (**d**) Western blot analyses of β-catenin, ALP, PCNA, keratin 14, and Erk in skin tissues with the different treatments for 28 days. (**e**) Immunoprecipitation analyses with anti-Dvl-1 antibody using lysates from skin tissues with the different treatments for 28 days. (**f**) Quantitative analyses (n = 3) of immunohistochemical staining shown in (**c**). (**g**) Quantitative hair cycle analyses ($n = 6$ per group) using at least 50 hair follicles per mouse per group. All data are representative of the results of at least three independent experiments. Scale bars = 100 μm for panels (**c**). Results are shown as means ± SD. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$ for panels (**b**), (**f**), and (**g**). ALP, alkaline phosphatase; DBM, Dvl-binding motif; Dvl, Dishevelled; H&E, hematoxylin and eosin; MNX, minoxidil; PCNA, proliferating cell nuclear antigen; PTD, protein transduction domain; SD, standard deviation; VPA, valproic acid; WCL, whole cell lysate.

dermal thickness (Figure 5b). Immunohistochemical analyses also confirmed that β-catenin and PCNA expression levels were significantly increased in epidermis and hair bulb by treatment with a combination of PTD-DBM and VPA (Figure 5c, 5d). However, overall the skin architecture remained the same in all the treatment groups as shown by proper maintenance of keratin 14 expression (Figure 5c). The combination treatment group showed a significantly higher ALP level than the other treatment groups (Figure 5d). The expression levels of β-catenin, ALP, and PCNA were synergistically increased in the combination treatment groups, whereas the keratin 14 protein level was not obviously changed in all of the treatment groups as determined by western blot analyses (Figure 5e). We also confirmed that VPA treatment enhanced the CXXC5-Dvl interaction, whereas PTD-DBM treatment disrupted this interaction in mice (Figure 5f). Moreover, quantitative analyses of immunohistochemical staining indicated that cotreatment with PTD-DBM and VPA induced the levels of β-catenin and PCNA in the epidermis and hair bulb of mice skin (Figure 5g). All treatment groups showed accelerated hair cycle into the anagen phase, and the hair follicles after combination treatment with PTD-DBM and VPA especially entered into the late anagen as shown by hair cycle analyses (Figure 5h).

These combined data show that PTD-DBM, which disrupts the CXXC5-Dvl interaction, together with the Wnt/β-catenin pathway activator VPA stimulates hair regrowth.

Cotreatment with PTD-DBM and VPA enhances WIHN and significantly induces the expression of β-catenin and Fgf9 in mice wounds

To confirm the effect of combination treatment with PTD-DBM and VPA on WIHN, full-thickness wounds (diameter = 1 cm) were created on the backs of mice and then the wounds were treated with PTD-DBM and/or VPA. Cotreatment with PTD-DBM and VPA critically enhanced WIHN in mice wounds (Figure 6a). Significant increases in both β-catenin and Fgf9 expression were observed after combination treatment with PTD-DBM and VPA, causing the enhanced WIHN (Figure 6a, 6b). Immunohistochemical and western blot analyses also revealed that epidermal and dermal cell proliferation was significantly increased in the combination treatment group (Figure 6a, 6b). Moreover, there was a significant increase in keratin 17- or ALP-positive neogenic follicles in the combination treatment group (Figure 6a–c). Cotreatment with PTD-DBM and VPA also significantly induced the expression levels of β-catenin and PCNA in the epidermis and dermis as revealed by quantitative analyses of immunohistochemical staining (Figure 6d). To address the presence of a positive feedback loop between the Wnt and Fgf9 pathways during WIHN, we further monitored levels of β-catenin and Fgf9 in the dermis of wounds. Simultaneous inductions of β-catenin and Fgf9 were observed in the dermis of wounds treated with both PTD-DBM and VPA (Figure 6e). Whole mount ALP staining also showed that cotreatment of PTD-DBM and VPA significantly increased WIHN (Figure 6f). Consistently, combination treatment with PTD-DBM and VPA significantly induced Axin2-LacZ expression in the dermis of *Axin2*$^{LacZ/+}$ mice wounds (Figure 6g). Interestingly, combination treatment with PTD-DBM and VPA significantly induced the formation of new unpigmented hairs at the center of re-epithelialized wounds 25 days after wounding (Figure 6h). These data indicate that blockade of the CXXC5-Dvl interaction is a possible strategy for the development of novel drugs that potentiate hair follicle neogenesis.

DISCUSSION

CXXC5 is induced on Wnt/β-catenin pathway activation, followed by inhibition of this pathway through a negative feedback mechanism in normal mice skin tissues and human dermal papilla cells. We also postulate that the sustained and abnormal expression of CXXC5 in the miniaturized hair follicles could be caused by other factors, despite scarce β-catenin expression. Similarly, Dkk1, a secreted Wnt antagonist (Niida et al., 2004), is upregulated by Wnt3a in human dermal papilla cells (Shin et al., 2010), and it is also induced in balding dermal papilla cells by DHT (Kwack et al., 2008). In addition, Dkk1 is also upregulated in the balding scalp compared with the haired scalp of patients with AGA (Kwack et al., 2008). Our findings suggest CXXC5 as a possible target of treatment for hair loss, but further studies are needed to elucidate the detailed mechanism underlying upregulation of CXXC5 in AGA.

We also found that CXXC5 was highly expressed in the dermal papilla of bald hair follicle, whereas it was slightly expressed in the dermal papilla of normal hair follicle, suggesting a potential role of CXXC5 in dermal papilla. Moreover, CXXC5 was expressed during the anagen-catagen transition of depilation-induced hair cycle and physiological first postnatal hair cycle, and its expression peaked in the catagen phase. Considering that several proteins that were involved in the inhibition of hair growth and elongation, such as prostaglandin D2 synthase and fibroblast growth factor 5, were expressed in the anagen-catagen transition (Garza et al., 2012; Hebert et al., 1994), these data indicate the inhibitory role of CXXC in hair growth in humans and mice.

WIHN could be used as treatment for hair loss (Gay et al., 2013; Ito et al., 2007). Transient induction of Dkk1 after wound closure completely abrogates WIHN, whereas overexpression of Wnt7a in the epidermis increases WIHN (Ito et al., 2007). In addition, Fgf9 from γδ T cells triggers Wnt activation in wound fibroblasts and induces WIHN (Gay et al., 2013). However, because of the low number and sequestered location of γδ T cells, humans lack appreciable hair follicle regeneration after wounding compared with mice (Gay et al., 2013). Hence, testing activators of the Wnt or Fgf pathways during wound healing is essential for enhancing the efficiency of WIHN. VPA-treated *CXXC5*$^{-/-}$ mice and the combination treatment group (PTD-DBM and VPA) at 14 days after wounding had increased β-catenin and Fgf9 expression and keratin 17- and ALP-positive neogenic hair follicle. Furthermore, cotreatment with PTD-DBM and VPA markedly induced the formation of new unpigmented hairs in the wounds 25 days after wounding. Taken together, these results suggest that combination treatment with PTD-DBM and VPA may possess a strong potential for enhancing WIHN that activates Wnt and Fgf pathways.

Multiple signaling pathways including Wnt/β-catenin, stem cell factor/c-kit, and bone morphogenetic protein signaling pathways are involved in the control of melanocyte development, regeneration, and pigment production (Botchkareva et al., 2001, 2003; Rabbani et al., 2011; Sharov et al., 2005). Especially, the delicate balance of the Wnt/β-catenin pathway in skin is required for the differentiation into melanocytes (Lim et al., 2013). Loss of β-catenin in melanocytes results in the formation of unpigmented hair, and ectopic activation of the Wnt/β-catenin pathway in melanocyte stem cells also induces premature hair graying (Rabbani et al., 2011). According to our observation, the hair follicle of mice skins treated with PTD-DBM and/or VPA did not show any abnormalities of hair pigmentation. Ultimately, combination treatment with a Wnt/β-catenin pathway activator and the peptide blocking the CXXC5-Dvl interaction may represent a potential therapeutic strategy to promote hair regeneration and treat hair loss.

MATERIALS AND METHODS

Human tissue samples

To investigate the effect of CXXC5 on baldness development, haired and matching bald scalp specimens were obtained from the same individuals of five male patients with AGA by 5-mm biopsy punch.

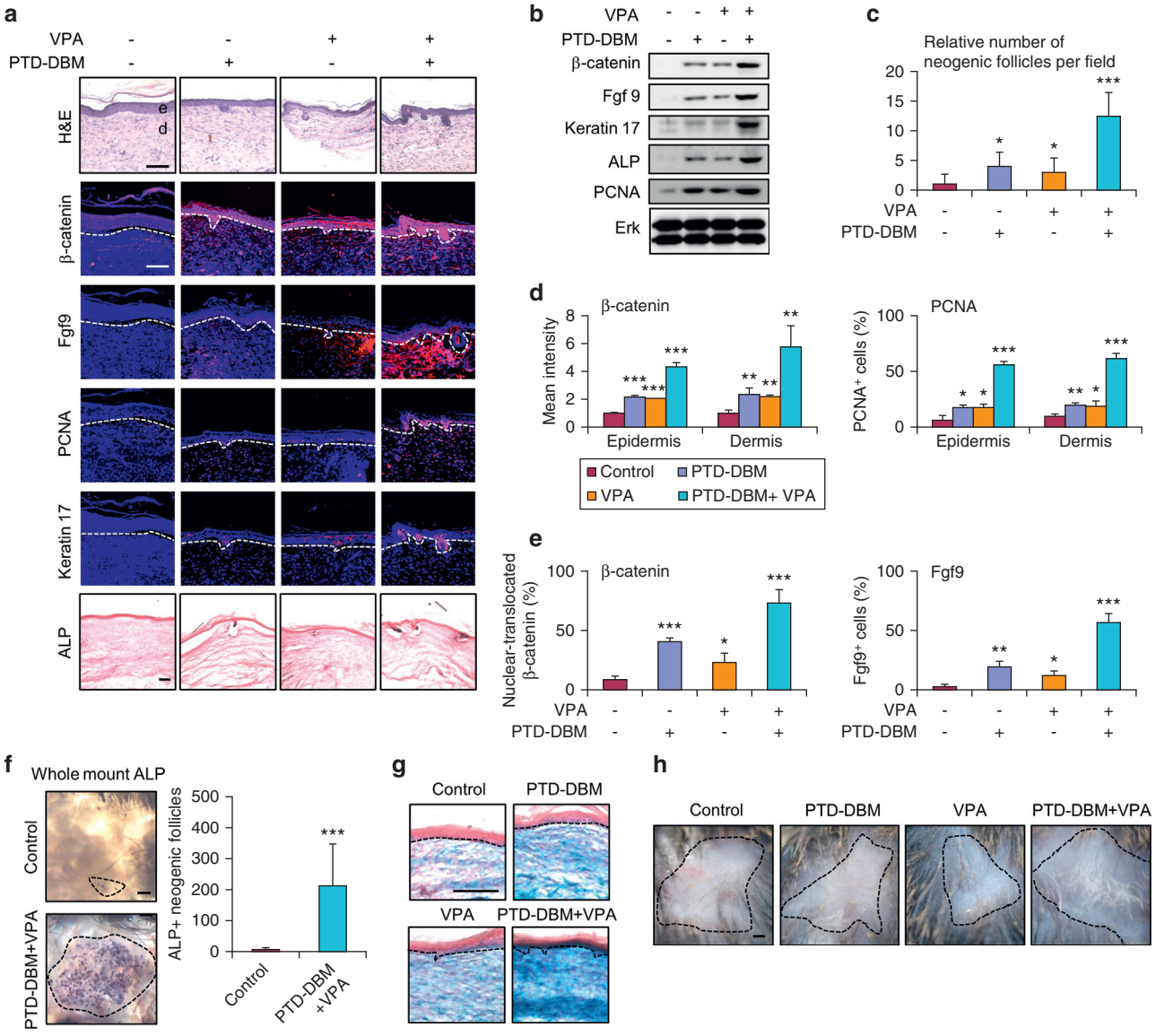

Figure 6. Cotreatment with PTD-DBM and VPA significantly enhances WIHN in mice. The wound (diameter = 1 cm) of 3-week-old male C3H mice (n = 12 per group) was treated daily with 2 mM PTD-DBM and/or 500 mM VPA 14 days after wounding. (**a**) H&E, immunohistochemical staining for β-catenin, Fgf9, PCNA, or keratin 17 with DAPI staining (blue), and ALP staining (dark blue) in the wounds treated with PTD-DBM and/or VPA 14 days after wounding. e: epidermis; d: dermis. The epidermal-dermal junction is indicated by a dashed line in each panel. (**b**) Western blot analyses of β-catenin, Fgf9, keratin 17, ALP, PCNA, and Erk in the wounds 14 days after wounding. (**c**) Measurements (n = 7) of neogenic hair follicle number per field in H&E-stained sections for each group of mice. (**d**) Quantitative analyses (n = 3) of immunohistochemical staining shown in (**a**) for β-catenin (left graph) or PCNA (right graph) in the epidermis and dermis of the wounds treated with PTD-DBM and/or VPA 14 days after wounding. (**e**) Quantitative analyses (n = 3) of immunohistochemical staining shown in (**a**) for nuclear β-catenin (left graph) or Fgf9 (right graph) in the dermis of wounds treated with PTD-DBM and/or VPA 14 days after wounding. (**f**) Whole mount ALP staining and its quantification (n = 15 per group). (**g**) X-gal staining (top panels) of wounded tissues from *Axin2*$^{LacZ/+}$ mice treated with PTD-DBM and/or VPA 14 days after wounding. Dashed lines indicate the epidermis-dermis border. (**h**) Representative gross images of the wounds treated with PTD-DBM and/or VPA 25 days after wounding. Dashed lines outline the wound. All data are representative of the results of at least three independent experiments. Scale bars = 100 μm for panels (**a**), (**f**), (**g**), and (**h**). Results are shown as means ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ for panels (**c**–**f**). ALP, alkaline phosphatase; DBM, Dvl-binding motif; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; PTD, protein transduction domain; SD, standard deviation; VPA, valproic acid; WIHN, wound-induced hair follicle neogenesis.

The Institutional Ethics Committee of Yonsei University Wonju College of Medicine (approval no. YWMR-14-9-022) approved all of the described studies, which were conducted according to the Declaration of Helsinki Principles. All patients provided informed written consent before participating in the study. The tissue specimens were fixed overnight in 10% (v/v) formalin in phosphate buffered saline for immunohistochemical analyses.

Mice

The generation of *CXXC5*$^{-/-}$ mice was previously described (Lee et al., 2015). *CXXC5* heterozygous mice were intercrossed for four generations to obtain littermate wild-type and *CXXC5*$^{-/-}$ mice and were maintained on a C57BL/6 background. *Axin2*$^{LacZ/+}$ mice were obtained from Jackson Laboratory. Wild-type C3H mice were purchased from Orient Bio. All animal protocols were approved by the

Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (09-013).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.04.038.

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