Lack of Collagen VI Promotes Wound-Induced Hair Growth

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Collagen VI is an extracellular matrix molecule that is abundantly expressed in the skin. However, the role of collagen VI in hair follicle growth is unknown. Here, we show that collagen VI is strongly deposited in hair follicles, and is markedly upregulated by skin wounding. Lack of collagen VI in *Col6a1*$^{-/-}$ mice delays hair cycling and growth under physiological conditions, but promotes wound-induced hair regrowth without affecting skin regeneration. Conversely, addition of purified collagen VI rescues the abnormal wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice. Mechanistic studies revealed that the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is triggered by activation of the Wnt/β-catenin signaling pathway, and is abolished by inhibition of this pathway. These findings highlight the essential relationships between extracellular matrix (ECM) and hair follicle regeneration, and suggest that collagen VI could be a potential therapeutic target for hair loss and other skin-related diseases.

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INTRODUCTION

Mammalian skin is a complex organ that contains three epithelial compartments, including the interfollicular epidermis, sebaceous glands, and hair follicles (Stenn and Paus, 2001). Each hair follicle in adult mammals goes through cycles of anagen (growth), catagen (regression), and telogen (quiescence) phases, a process that relies on a group of stem cells, including bulge cells and secondary hair germ (Greco *et al.*, 2009; Myung and Ito, 2012). In addition, hair follicles have an important role in early epidermal repair following skin wounding (Ito *et al.*, 2005), where they regenerate *de novo* in adult mice in a manner similar to embryonic hair follicle development (Ito *et al.*, 2007). Remarkably, the expression pattern of epithelial stem cells in hair follicles around wound regions, and the signals coordinating the growth and activation of follicular epithelial cells, are similar to that in embryonic hair development (Ito and Kizawa, 2001; Millar, 2002). These findings suggest that characterization of the molecular signals governing the wound-induced hair regrowth may reveal the general understanding of hair growth. The central pathway that mediates wound-induced hair follicle regeneration and growth is Wnt/β-catenin (Ito *et al.*, 2007; Gay *et al.*, 2013; Myung *et al.*, 2013). However, it is currently unclear how Wnt/β-catenin signaling is regulated during hair follicle growth after skin wounding.

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Abbreviations: DKK-1, dickkopf-related protein 1; ECM, extracellular matrix; K79, keratin 79

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It has been shown that the onset of epithelial stem cell development is triggered by environmental signals, defined as niche (Fuchs *et al.*, 2004). Extracellular matrix (ECM) is an important hair follicle stem cell niche that regulates bulge cell behavior and hair development (Gattazzo *et al.*, 2014). Collagen VI is abundantly deposited in a variety of tissues, including skin (Chen *et al.*, 2013, 2014a, b). The most abundant form of collagen VI is made of three α1(VI), α2(VI), and α3(VI) chains encoded by distinct genes (*Col6a1*, *Col6a2*, *Col6a3*), although recent studies identified further genes (*Col6a4*, *Col6a5*, *Col6a6*) coding for three chains with a high degree of similarity to α3(VI) (Fitzgerald *et al.*, 2008; Gara *et al.*, 2008). Our recent work demonstrated that collagen VI is a key component of adult muscle stem cell niche required for proper muscle regeneration after injury (Urciuolo *et al.*, 2013). These findings suggest that collagen VI may have critical functions in stem cell niches in tissues, thus affecting tissue homeostasis and regeneration. Microarray studies showed that the expression levels of *Col6a1* and *Col6a2* genes in mouse bulge cells are higher than in differentiated keratinocytes (Fujiwara *et al.*, 2011). However, it is still completely unknown whether collagen VI contributes to hair follicle growth and regeneration.

Here we show that collagen VI is abundantly deposited in hair follicles and upregulated by skin wounding. By using collagen VI-null (*Col6a1*$^{-/-}$) mice, where a targeted inactivation of the *Col6a1* gene prevents the assembly and secretion of collagen VI in the ECM (Bonaldo *et al.*, 1998; Irwin *et al.*, 2003; Chen *et al.*, 2014b), we demonstrate that lack of collagen VI delays hair cycling and growth under physiological conditions, but promotes wound-induced hair regrowth by regulating the Wnt/β-catenin signaling pathway.

RESULTS

Collagen VI is expressed in hair follicles and regulated by skin wounding

To analyze the deposition of collagen VI in hair follicles, we labeled murine skin with different antibodies against collagen VI chains. Immunofluorescence analysis showed that collagen VI is abundantly deposited in different regions of the hair follicle, including bulge, sebaceous gland, hair germ, and dermal papilla (Figure 1a). Double immunofluorescence showed that α1(VI) and α3(VI) chains were adjacent to the keratin 15 (K15)-positive bulge stem cells, and partially colocalized with K15, suggesting that collagen VI is an extracellular component of bulge stem cell niche (Figure 1b–d). Together with previous microarray data showing that the expression levels of *Col6a1* and *Col6a2* genes in bulge stem cells are higher than in differentiated keratinocytes (Fujiwara *et al.*, 2011), these findings suggest a potential role for collagen VI in hair follicle.

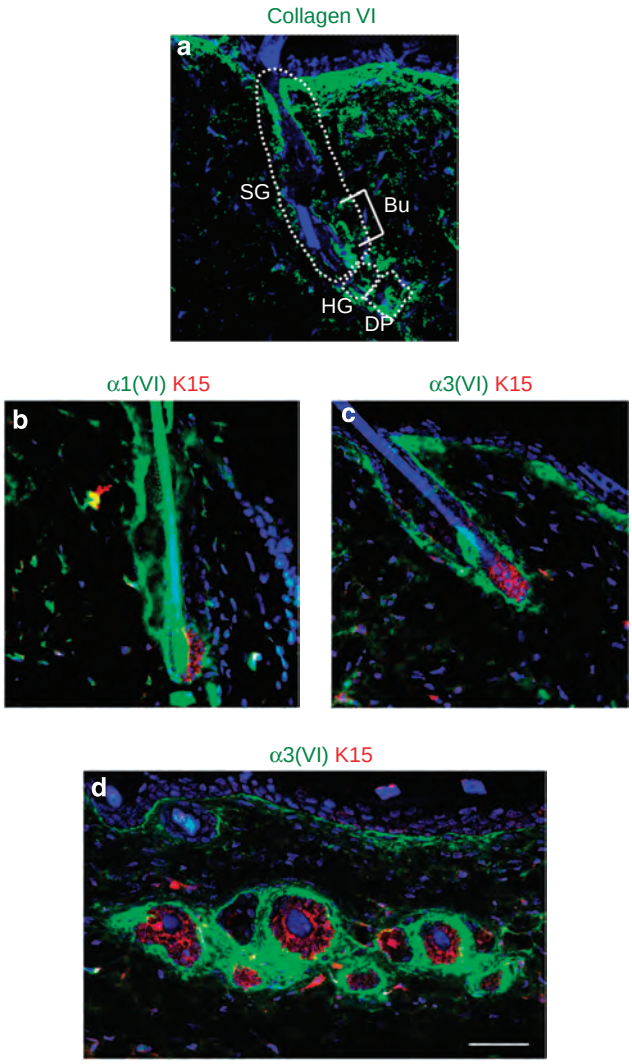

Figure 1. Collagen VI is deposited in hair follicles. (**a**) Immunofluorescence for collagen VI in hair follicles of wild-type adult mice. (**b**–**d**) Coimmunofluorescence labeling of keratin 15 (K15; red) with α1(VI) (green, **b**) and α3(VI) (green, **c** and **d**) in hair follicles of wild-type mouse. The dotted areas mark dermal papilla (DP), hair germ (HG), and sebaceous gland (SG) of hair follicle, as indicated. Nuclei were stained with Hoechst (blue). Scale bar = 50 μm. Bu, bulge.

In addition, we examined whether skin wounding impinges on collagen VI expression in adult mice. Real-time reverse-transcriptase–PCR showed that the levels of *Col6a1*, *Col6a2*, and *Col6a3* transcripts were increased at days 4 and 8 after wound (Figure 2a–c). Western blot analysis showed that the protein levels of α1(VI) were first decreased at days 2 and 4 after wound and then increased at day 8 after wound, whereas α3(VI) levels were increased as early as at day 2 after wound (Figure 2d and e). Immunofluorescence showed that the level of collagen VI was increased after skin wounding (Figure 2f). Taken together, these data indicate that the expression of collagen VI is strongly regulated upon skin wounding, pointing at a potential role for this molecule in skin pathology.

Lack of collagen VI promotes wound-induced hair regrowth

On the basis of our previous findings that collagen VI is required for muscle and peripheral nerve regeneration (Urciuolo *et al.*, 2013; Chen *et al.*, 2015), we hypothesized that collagen VI may be involved in skin wound healing. To test this hypothesis, we made full-thickness excisional wounds (1 cm^2 square) on the shaved dorsal skin of wild-type and *Col6a1*$^{-/-}$ mice and monitored the wound areas for 2 weeks. Surprisingly and unexpectedly, we found that wound healing was similar in two genotypes, but the wound-induced hair regrowth was markedly accelerated in *Col6a1*$^{-/-}$ mice (Figure 3a and b). To further confirm these results, we performed another skin injury model in which mice were subjected to three 6 mm incisional wounds. The results demonstrated that the wound-induced hair regrowth in this model was also significantly promoted in *Col6a1*$^{-/-}$ mice when compared with wild-type mice (Figure 3c), but hair growth was similar in both genotypes when mice received hair shaving alone (Supplementary Figure S1 online). Hematoxylin and eosin staining showed that the wound-induced initiation of hair follicle anagen was higher in *Col6a1*$^{-/-}$ mice, and that hair follicles of *Col6a1*$^{-/-}$, but not wild-type mice, were in the anagen phase even at day 14 after wound (Figure 3d). Altogether, these findings indicate that lack of collagen VI facilitates wound-induced hair regrowth.

To confirm that the promoted hair growth of *Col6a1*$^{-/-}$ mice is induced by wounding, we analyzed hair cycles in mice of both genotypes under physiological conditions. Hematoxylin and eosin staining showed that the hair cycling was delayed in *Col6a1*$^{-/-}$ mice when compared with wild-type mice during the physiological postnatal hair cycle (Supplementary Figure S2a online). Similar results were displayed by depilation-induced adult hair cycling (Supplementary Figure S2b online). Moreover, the hair growth in wild-type mice was faster than that in *Col6a1*$^{-/-}$ mice after depilation (Supplementary Figure S2c online). These findings suggest that lack of collagen VI delays hair cycling and growth under physiological conditions.

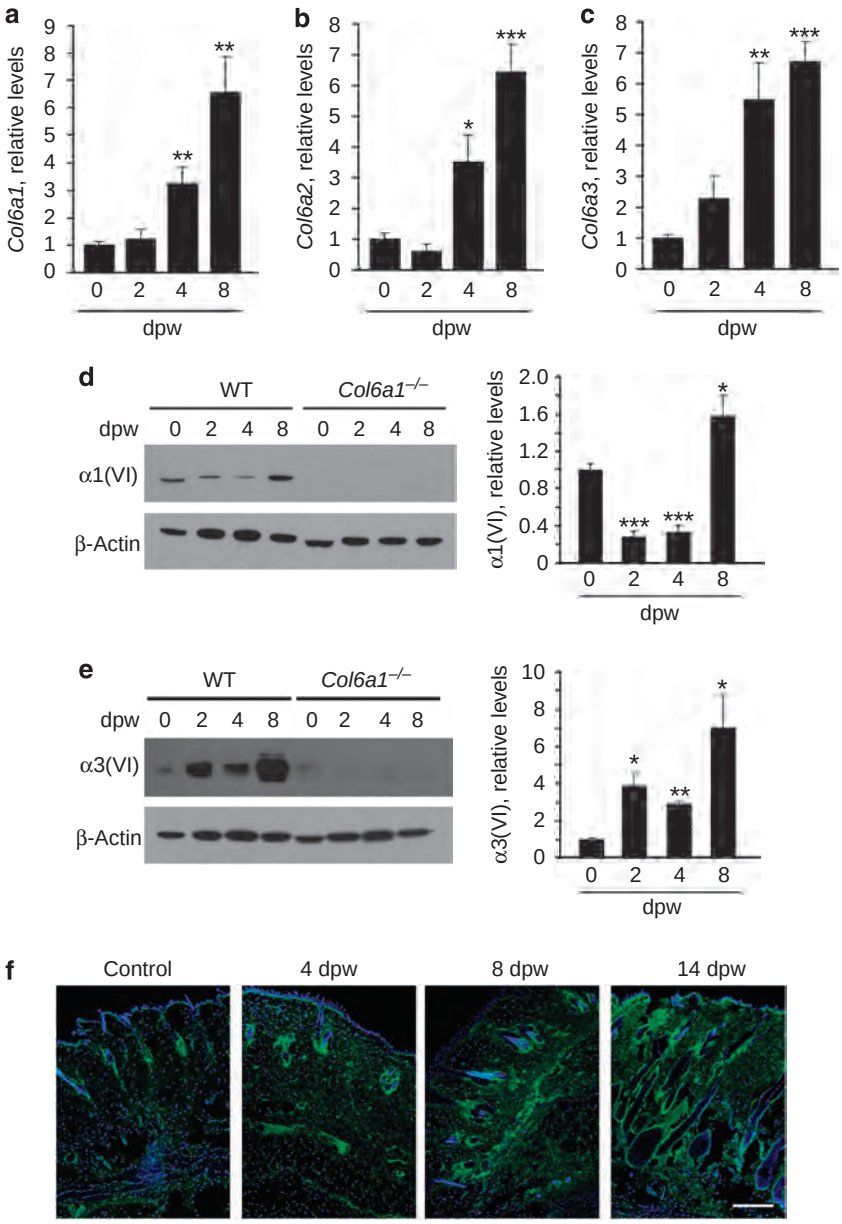

Figure 2. Skin injury regulates collagen VI expression. (**a**–**c**) Real-time reverse-transcriptase–PCR (RT-PCR) for (**a**) *Col6a1*, (**b**) *Col6a2*, and (**c**) *Col6a3* transcripts in wild-type (WT) skin under uninjured conditions and at the indicated times after wound ($n = 5$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (**d** and **e**, left panels) Western blot for α1(VI) and α3(VI) in WT and *Col6a1*$^{-/-}$ skin under uninjured conditions and at the indicated times after wound. (**d** and **e**, right panels) Densitometric quantification of α1 or α3(VI) versus actin ($n = 4$–5; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (**f**) Immunofluorescence labeling of collagen VI (green) in WT skin under uninjured conditions and at the indicated times after wound. Analysis of wounded skin was performed close to the wound edge. Nuclei were stained with Hoechst (blue). Scale bar = 200 μm. dpw, days post wound.

The enhanced would-induced hair regrowth of *Col6a1*$^{-/-}$ mice involves upregulation of K79, increased number of hair follicle stem cells, and activation of Wnt/β-catenin signaling

Keratin-79 (K79)–positive cells are a population of migratory epithelial cells that initiate hair regeneration (Veniaminova *et al.*, 2013). We thus investigated whether the facilitated wound-induced hair regrowth of *Col6a1*$^{-/-}$ animals is paralleled by the upregulation of K79. Western blot analysis showed that although ablation of collagen VI attenuated K79 expression under physiological conditions, K79 protein levels in wounded skin of *Col6a1*$^{-/-}$ mice were higher than those of wild-type mice at day 8 after wound (Figure 4a). As discussed above, collagen VI is a component of bulge stem cell niche; we therefore investigated whether stem cells contribute to the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice. Toward this aim, we analyzed CD39 and α6 integrin (CD49f) double-positive cells, a distinct population of hair follicle keratinocyte stem cells (Trempus *et al.*, 2003; Morris *et al.*, 2004; Jensen *et al.*, 2008). Flow cytometric analysis showed that although CD34-/CD49f-positive stem cells were mildly

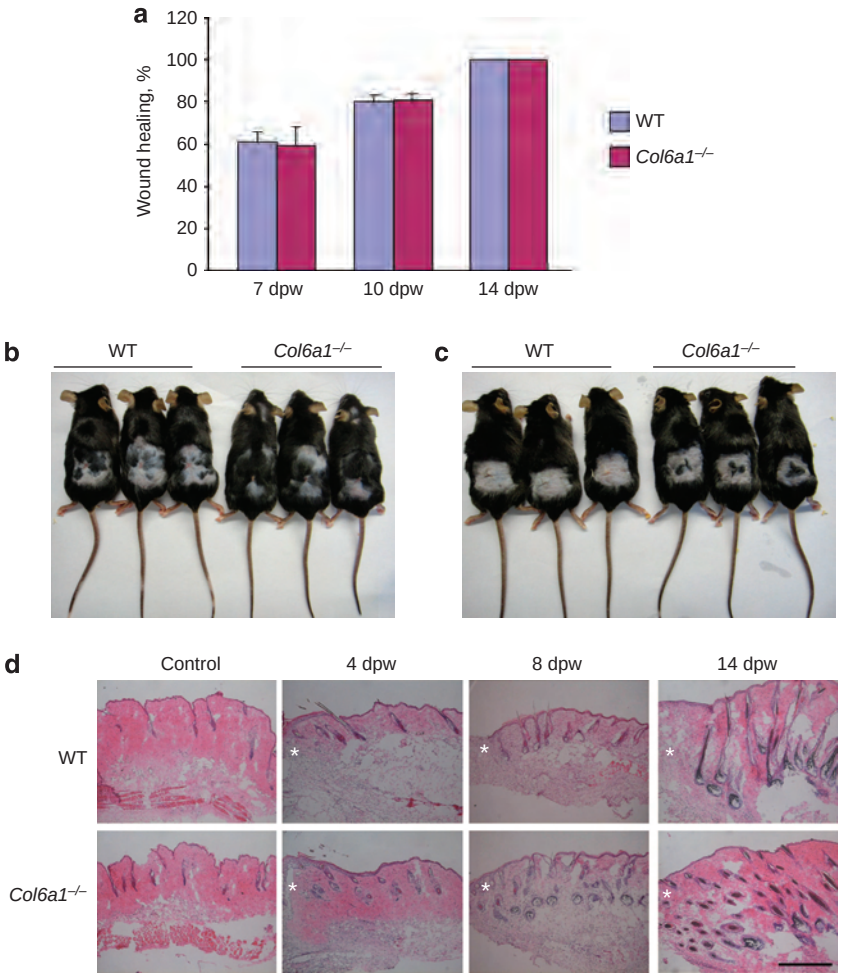

Figure 3. Lack of collagen VI does not affect wound healing, but promotes wound-induced hair regrowth. (**a**) Quantification of the percentage of initial wound areas in wild-type (WT) and *Col6a1*$^{-/-}$ mice after excising a 1 cm^2 square of full-thickness dorsal skin ($n = 3$). (**b** and **c**) Wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice is faster than in WT mice in the injury models obtained by excising a 1 cm^2 square of full-thickness dorsal skin (**b**) or by performing three 6 mm incisional wounds (**c**). Photographs were taken at day 14 after wound. (**d**) Hematoxylin and eosin (H&E) staining in wounded skin from WT and *Col6a1*$^{-/-}$ mice under uninjured conditions and at the indicated times after wound ($n = 3$–4). The asterisks label the wound edge. Scale bar = 200 μm. dpw, days post wound.

reduced (although not significantly) in *Col6a1*$^{-/-}$ mice with respect to controls under physiological conditions, the percentage of CD34-/CD49f-positive cells in wounded skin of *Col6a1*$^{-/-}$ mice was significantly higher than those of wild-type mice at day 8 after wound (Figure 4b). Altogether, these findings suggest that K79-positive cells and CD34-/CD49f-positive stem cells are involved in the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice.

Wnt/β-catenin signaling is a key signaling pathway in wound-induced hair regrowth (Ito *et al.*, 2007; Gay *et al.*, 2013). To determine whether Wnt/β-catenin signaling contributes to the enhanced wound-induced hair regrowth of collagen VI–deficient mice, we performed a thorough investigation of this pathway in wounded skin of wild-type and *Col6a1*$^{-/-}$ mice. Western blotting showed that although β-catenin levels were decreased upon skin wounding in both genotypes, β-catenin levels in wounded skin of *Col6a1*$^{-/-}$ mice were significantly higher than those of wild-type mice at day 8 after wound (Figure 5a). Immunofluorescence analysis confirmed that β-catenin labeling in hair follicles was much stronger in *Col6a1*$^{-/-}$ mice than in wild-type mice at day 8 after wound (Figure 5b). In addition, we examined β-catenin levels in nuclear fraction extracts, and found that they were significantly increased in *Col6a1*$^{-/-}$ mice when compared with wild-type mice at day 8 after wound (Figure 5c). Furthermore, immunofluorescence for the Wnt inhibitor dickkopf-related protein 1 (DKK-1) at day 8 after wound showed lower labeling in the hair follicles of *Col6a1*$^{-/-}$ mice than in wild-type mice (Figure 5d). These findings indicate that Wnt/β-catenin signaling is involved in the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice.

Notch signaling is involved in embryonic and postnatal hair homeostasis. Inactivation of Notch results in hair loss (Vauclair *et al.*, 2005). Lhx2 is a Lim-homeodomain transcription factor that has an important role in controlling the switch between stem cell quiescence and activation in hair follicles, and Lhx2 deficiency is associated with the acceleration of wound-induced hair growth (Mardaryev *et al.*, 2011).

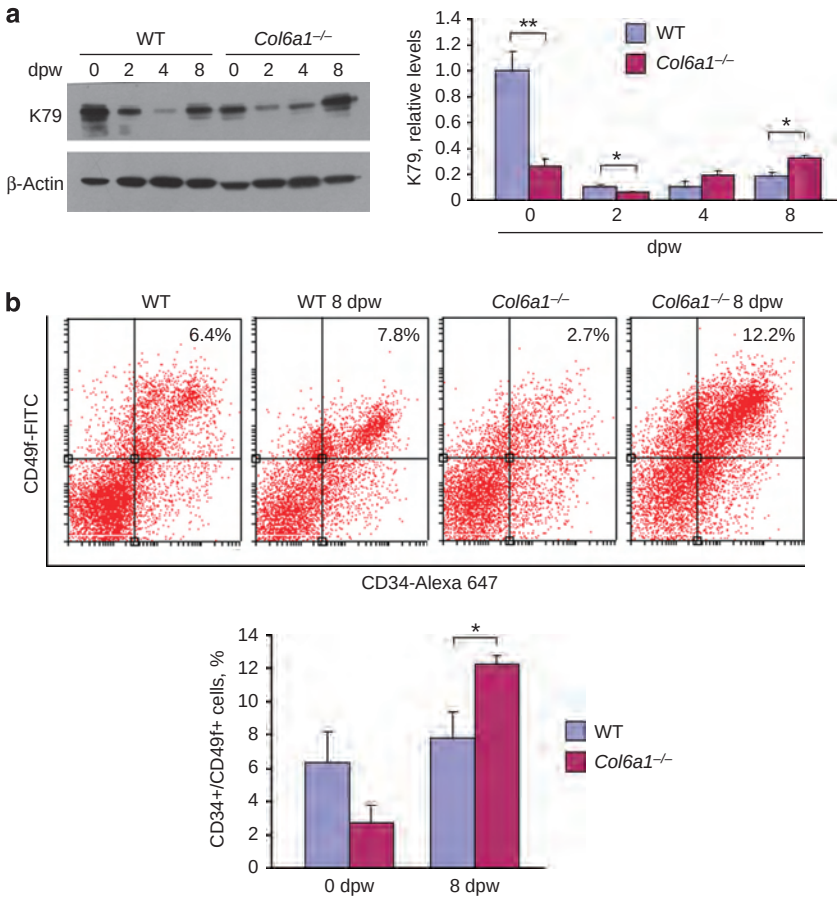

Figure 4. Ablation of collagen VI increases keratin 79 (K79) expression and CD34-/CD49f-positive cells after skin wounding. (**a**, left panel) Western blot for K79 in skin from wild-type (WT) and *Col6a1*$^{-/-}$ mice under uninjured conditions and at the indicated times after wound. (**a**, right panel) Densitometric quantification of K79 versus actin ($n=4$; *$P<0.05$; **$P<0.01$). (**b**, top panel) Flow cytometry analysis with stem cell markers CD34 and CD49f in dorsal skin keratinocytes isolated from WT and *Col6a1*$^{-/-}$ mice under uninjured conditions and at day 8 after wound. (**b**, bottom panel) Quantification of flow cytometric data ($n=3$–4). dpw, days post wound.

Thus, we investigated whether Notch and Lhx2 are involved in mediating the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice. Western blot and/or immunofluorescence analysis at day 8 after wound showed that the levels of Notch1 and Lhx2 in wounded skin of *Col6a1*$^{-/-}$ mice were similar to those of wild-type mice (Supplementary Figure S3a–c online). Taken together, these data indicate that upregulation of K79 and activation of the Wnt/β-catenin pathway may contribute to the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice.

The increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is abolished by addition of collagen VI or by inhibition of Wnt/β-catenin pathway

To confirm the role of collagen VI in wound-induced hair regrowth and the underlying molecular mechanism involving modulation of the Wnt/β-catenin signaling pathway, we treated *Col6a1*$^{-/-}$ mice with purified native collagen VI or with the Wnt/β-catenin inhibitor ICG-001 after skin wounding. Notably, the enhanced hair regrowth of *Col6a1*$^{-/-}$ mice was rescued by the addition of purified collagen VI protein (Figure 6a) and by treatment with ICG-001 (Figure 6b), suggesting that the inhibitory effect of collagen VI in wound-induced hair regrowth is regulated by the Wnt/β-catenin pathway. Hematoxylin and eosin staining demonstrated that treatment with purified collagen VI protein or with ICG-001 inhibited wound-induced hair follicle anagen initiation in *Col6a1*$^{-/-}$ mice (Figure 6c). Furthermore, western blotting showed that addition of purified collagen VI or of ICG-001 blocked the wound-induced upregulation of K79 and β-catenin in *Col6a1*$^{-/-}$ mice (Figure 6d). Altogether, these data indicate that the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is regulated by the activation of the Wnt/β-catenin signaling pathway.

DISCUSSION

Previous microarray studies showed that the expression levels of *Col6a1* and *Col6a2* in bulge stem cells are higher than in differentiated keratinocytes, suggesting that collagen VI may contribute to bulge function and hair development. However, a direct evidence for such a role was still lacking (Fujiwara *et al.*, 2011). In this study, we showed that collagen VI has a

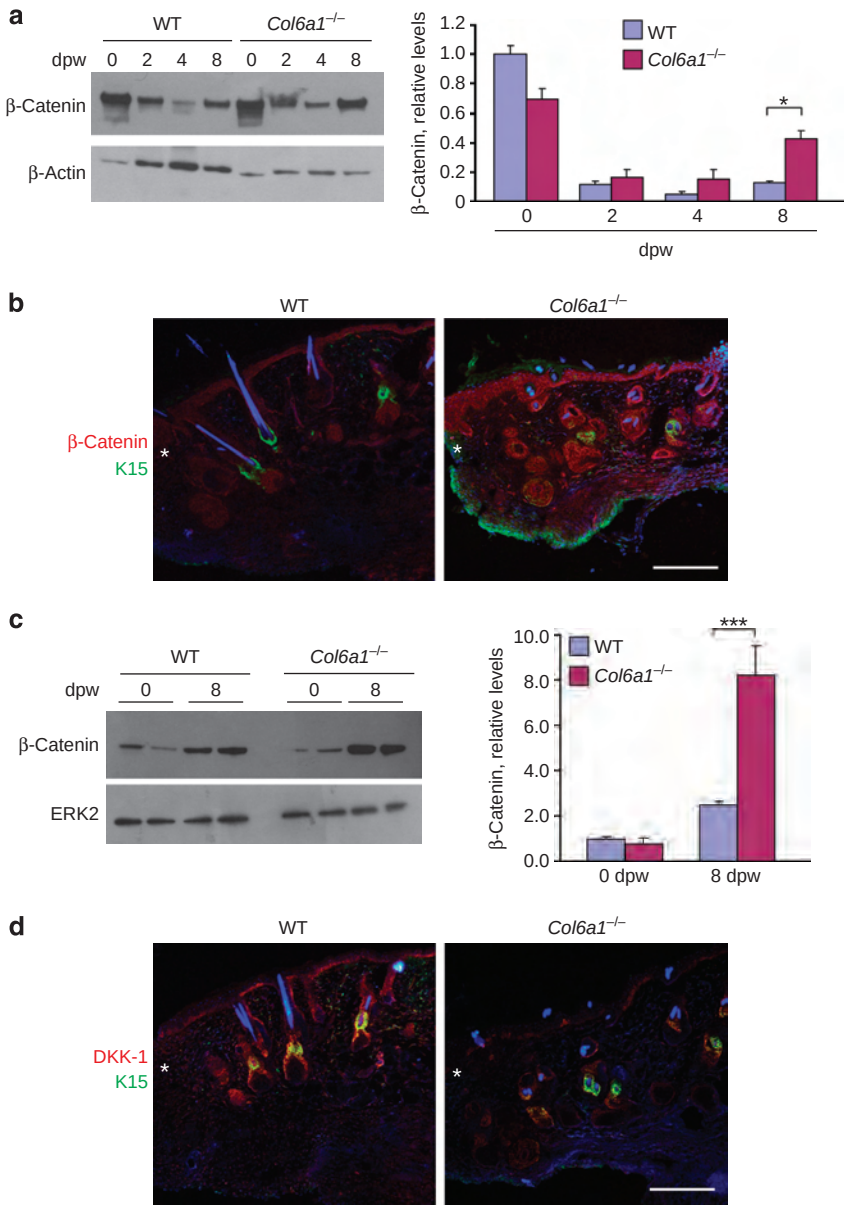

Figure 5. Lack of collagen VI activates Wnt/β-catenin signaling after skin wounding. (**a** and **c**, left panels) Western blot for (**a**) cytoplasmic and (**c**) nuclear β-catenin in wild-type (WT) and *Col6a1*$^{-/-}$ skin under uninjured conditions and at the indicated times after wound. (**a** and **c**, right panels) Densitometric quantification of (**a**) β-catenin versus actin or (**c**) β-catenin versus ERK2 ($n = 4–5$; $^{*}P < 0.05$; $^{***}P < 0.001$). (**b** and **d**) Coimmunofluorescence labeling of K15 (green) with β-catenin (red, **b**) or DKK-1 (red, **d**) in skin from wild-type and *Col6a1*$^{-/-}$ mice at day 8 after wound. The asterisks label the wound edge. Nuclei were stained with Hoechst (blue). Scale bar = 200 μm. DKK-1, dickkopf-related protein 1; dpw, days post wound; ERK2, extracellular signal–regulated kinase 2; K15, keratin 15.

distinct distribution in hair follicles and responds to skin wounding, and demonstrated that lack of collagen VI in *Col6a1*$^{-/-}$ mice alters the hair follicle cycle and has a striking impact on wound-induced hair regrowth.

Skin is normally able to regenerate hair follicles during wound healing in rodents (Breedis, 1954; Ito *et al.*, 2007). However, wound healing sometimes results in inadequate tissue regeneration by fibrosis or scarring, especially for the cutaneous wounds in adult humans (Gay *et al.*, 2013). The underlying mechanisms controlling wound scarring and tissue regeneration are not yet fully understood (Nelson *et al.*, 2013). It has been well established that ECM molecules have an important role in skin wound healing (Olczyk *et al.*, 2014). Given our previous studies showing that collagen VI is essential for muscle regeneration by modulating satellite cell activities (Urciuolo *et al.*, 2013), and for peripheral nerve regeneration by modulating macrophage function (Chen *et al.*, 2015), we originally predicted that the *Col6a1*$^{-/-}$ mice may have deficits in skin regeneration. However, we found that lack of collagen VI in *Col6a1*$^{-/-}$ mice does not affect skin wound healing (Lettmann *et al.*, 2014). Interestingly, a previous study demonstrated that lack of

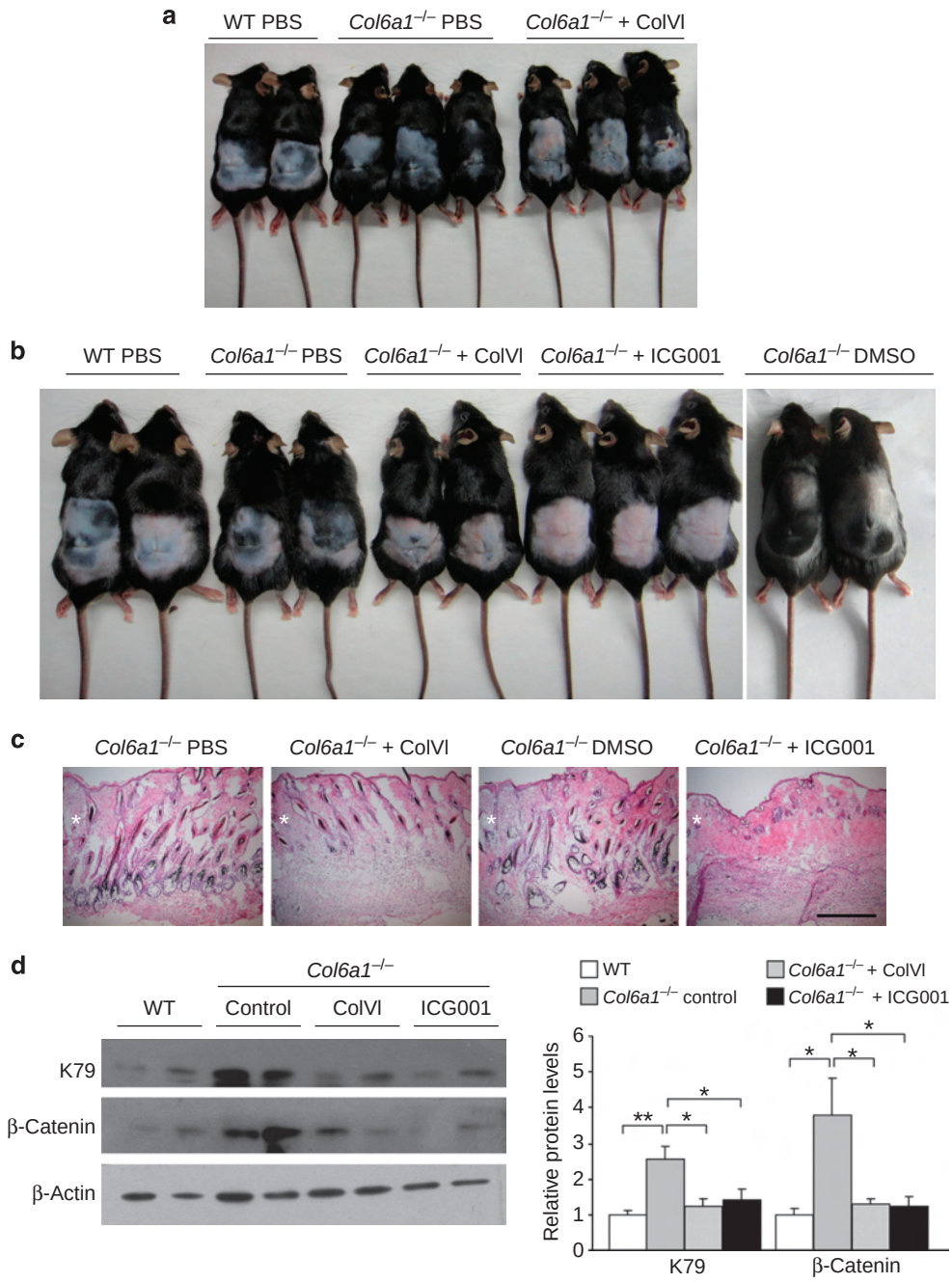

Figure 6. Purified collagen VI or Wnt/β-catenin inhibitor abolishes the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice. (**a** and **b**) Representative images showing the effect of indicated treatments on wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice at day 14 after wound ($n = 4$–6). (**c**) Hematoxylin and eosin (H&E) staining in wounded skin of *Col6a1*$^{-/-}$ mice under indicated conditions at day 14 after wound ($n = 3$–4). The asterisks label the wound edge. Scale bar = 200 μm. (**d**, left panel) Western blot for keratin 79 (K79) and β-catenin in wounded skin of wild-type (WT) and *Col6a1*$^{-/-}$ mice at day 8 after wound under indicated conditions. (**d**, right panel) Densitometric quantification of K79 or β-catenin versus actin ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$). Collagen VI in PBS (3 mg kg^{-1} per day) or ICG-001 in 1% DMSO (5 mg kg^{-1} per day) was subcutaneously administered at 0.5 cm away from the wound edge starting at day 4 after wound. ColVI, purified collagen VI; PBS, phosphate-buffered saline.

collagen VI in *Col6a1*$^{-/-}$ mice improves cardiac function, structure, and remodeling after myocardial infarction (Luther *et al.*, 2012). These findings suggest that this ECM molecule has different roles in distinct tissues during regeneration that may be owing to the specific context of each tissue. Further studies will be needed to understand the detailed underlying mechanisms involved in the specific functions of collagen VI during regeneration in different organs.

Similar to the previous unexpected finding that *Col6a1*$^{-/-}$ mice have improved cardiac function after myocardial infarction (Luther *et al.*, 2012), in this study we found that lack of collagen VI unexpectedly promotes wound-induced

hair regrowth. To further confirm that the promoted hair regrowth of *Col6a1*$^{-/-}$ mice is triggered by wounding, we investigated the hair follicle cycle in both genotypes under physiological conditions and found that the hair follicle cycle is delayed in *Col6a1*$^{-/-}$ mice when compared with wild-type mice. These findings suggest that the wound-induced hair regrowth elicited by lack of collagen VI should be regulated by wounding-related cell components and/or signals.

K79-positive cells are a recently identified population of epithelial cells that are essential for initiating hair canal morphogenesis and regeneration independently of the Notch signaling pathway (Veniaminova *et al.*, 2013). Interestingly, our data show higher immunolabeling for K79, but not for Notch1, in wounded skin of *Col6a1*$^{-/-}$ mice than those of wild-type mice. These data suggest that regulation of K79-positive cells is correlated with the increased hair regrowth in *Col6a1*$^{-/-}$ mice independently of Notch signaling.

The Wnt/β-catenin pathway has a central role in regulating embryonic and adult hair follicle growth under both physiological and pathological conditions. For example, it has been shown that activation of Wnt/β-catenin signaling is essential for the initiation of embryonic hair follicle development (Huelsken *et al.*, 2001; Andl *et al.*, 2002; Zhang *et al.*, 2009). Wnt/β-catenin signaling is also required for wound-induced hair neogenesis, in which overexpression of Wnt7a in the mouse epidermis enhances wound-induced hair follicle growth, whereas development of new hair follicles after wounding is blocked by overexpression of DKK-1 or by ablation of β-catenin (Ito *et al.*, 2007). Interestingly, in this study we found that the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice was associated with higher β-catenin levels at 8 days after wound, whereas under physiological conditions β-catenin levels were slightly (although not significantly) lower in the skin of *Col6a1*$^{-/-}$ mice with respect to wild-type mice. These findings are consistent with previous studies that showed that collagen VI is able to stabilize and activate β-catenin in cancer cells (Iyengar *et al.*, 2005), pointing at a potential positive regulation between collagen VI and Wnt/β-catenin signaling. In this context, it must be underlined that Wnt/β-catenin signaling is dynamically regulated and dependent on distinct microenvironments. For example, during the initiation of hair follicle development, Wnt/β-catenin signaling is first enhanced uniformly in the upper dermis and then focally in both the underlying dermal condensate and the epithelial hair follicle placode (Zhang *et al.*, 2009; Chen *et al.*, 2012; Myung *et al.*, 2013). In agreement with this concept, previous studies showed that lack of collagen VI inhibits tumor growth by destabilizing and inactivating β-catenin signals (Iyengar *et al.*, 2005), whereas here we show that ablation of collagen VI promotes wound-induced hair regrowth by activation of the Wnt/β-catenin pathway. The contribution of Wnt/β-catenin signals in wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is also supported by the pharmacological treatment with the Wnt/β-catenin inhibitor ICG-001. Our results show that ICG-001 blocks the increased wound-induced hair regrowth and enhanced K79 expression in *Col6a1*$^{-/-}$ mice, thus providing further evidence that the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice relies upon activation of Wnt/β-catenin signals. Although further studies aimed at elucidating in detail the link between collagen VI, skin wounding, and Wnt/β-catenin signaling are needed to obtain a thorough understanding of the mechanisms governing hair follicle development and wound-induced hair regrowth, the present findings reveal an unanticipated role for collagen VI in wound-induced hair regrowth.

The reasons why hair growth is induced by wounding are not yet fully understood (Stenn and Paus, 2001). In this study, we demonstrate that collagen VI is mainly deposited in hair follicles and is regulated by skin wounding that in turn regulates hair regrowth by modulation of Wnt/β-catenin signaling. Interestingly, we found that the enhanced wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice is abolished by treatment with purified collagen VI. Altogether, this study provides evidence on the role of specific ECM molecules in wound-induced hair regrowth, and sheds light on the potential therapeutic benefit in accelerating impaired hair growth by targeting collagen VI.

MATERIALS AND METHODS

Animals

Col6a1$^{+/+}$ (wild type) and *Col6a1*$^{-/-}$ mice in the C57BL/6 background (Bonaldo *et al.*, 1998; Irwin *et al.*, 2003) were used in this study. The *in vivo* experiments were performed in 2- to 3-month-old mice, except for the studies investigating hair follicle development in which mice were used at the indicated ages. Native collagen VI protein was purified from newborn mice, as previously described (Irwin *et al.*, 2003). Animal procedures were authorized by the Ethics Committee of the University of Padova and by the Italian Ministry of Health.

Tissue preparation and histology

Dorsal skin was obtained from mice at postnatal days 1, 4, 7, 14, 21, and 28 for analyzing the physiological postnatal hair cycle. The adult hair cycle was induced by wax depilation, as described previously (Ishimatsu-Tsuji *et al.*, 2005), and dorsal skin was obtained at days 1, 3, 5, 13, 20, and 23 after depilation, encompassing hair follicle development from anagen to telogen. For the studies of wound-induced hair follicle growth, the dorsal skin of healthy and wounded wild-type and *Col6a1*$^{-/-}$ mice was harvested and frozen in liquid nitrogen. After cutting at 10 μm using a cryostat (Leica, Wetzlar, Germany), samples were stained with hematoxylin and eosin to analyze the morphology of hair follicles.

Surgical procedure

Mice were anesthetized with xylazine (8 mg kg^{-1} body weight) and ketamine (100 mg kg^{-1} body weight), and the dorsal hair was shaved. Skin wounds were made by excising a 1 cm^2 square of full-thickness dorsal skin (used for investigating wound healing and related hair regrowth as indicated) or by cutting three 6 mm wounds (1 midline and 2 on each side of the midline).

RNA isolation and real-time reverse-transcriptase–PCR

Total RNA from mouse dorsal skin of healthy and wounded wild-type mice was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. A measure of 200 ng of total RNA was used to make complementary DNA using

the Superscript III kit (Invitrogen, Carlsbad, CA). Quantitative PCR was carried out using the LightCycler 480 system (Roche, Basel, Switzerland). The expression level of each gene was calculated by comparing with the *Gapdh* housekeeping gene. Primers used in this study are shown in Supplementary Table S1 online.

Immunofluorescence

Immunofluorescence was performed on frozen skin sections of healthy and wounded wild-type and *Col6a1*$^{-/-}$ mice. After blocking with 10% goat serum for 1 hour, sections were incubated with primary antibodies (1:200) overnight at 4 °C. Primary antibodies against the following proteins were used: β-catenin, Notch1 (rabbit monoclonal; Abcam, Cambridge, MA); collagen VI (rabbit polyclonal antibody raised against α1, α2, and α3(VI) collagen; (Fitzgerald, Acton, MA); DKK-1, α1(VI) collagen (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA); Lhx2 (goat polyclonal; Santa Cruz Biotechnology); α3(VI) collagen (guinea pig polyclonal, a gift of Raimund Wagener, Cologne, Germany; Lettmann *et al.*, 2014); and K15 (mouse monoclonal; Thermo Scientific, Rockford, IL). The samples were then transferred to secondary antibodies (1:200) and Hoechst 33258 (Sigma, St. Louis, MO) for 1 hour at room temperature. The following secondary antibodies were used: anti-goat CY3, anti-rabbit CY2 and CY3, anti-guinea-pig CY2, and anti-mouse CY2 and CY3 (Jackson Immunoresearch, West Grove, PA). After washing three times in phosphate-buffered saline, slides were mounted using 80% glycerol.

Western blotting

Mice were killed by cervical dislocation, and dorsal skin of healthy and wounded (including the wound edge and 5 mm of the surrounding skin) wild-type and *Col6a1*$^{-/-}$ mice was removed and frozen in nitrogen immediately. The tissues were homogenized in lysis buffer (Millipore, Billerica, MA) with protease inhibitors (Roche, Basel, Switzerland) and phosphatase inhibitors (Sigma). Preparation of nuclear protein extracts was carried out with a commercially available Nuclear Extraction Kit (Abcam), according to the manufacturer's instructions. Protein concentration was determined using the BCA assay (Thermo Scientific). Samples of 20 μg of protein were applied to SDS-PAGE gels (Invitrogen) and blotted onto polyvinylidene fluoride membrane (Millipore). Membranes were incubated with primary antibodies (1:1,000) overnight at 4 °C. Primary antibodies against the following proteins were used for western blotting: α1 and α3 (VI) collagens; ERK2 (rabbit polyclonal; Santa Cruz Biotechnology); K79 (goat polyclonal, Santa Cruz Biotechnology); β-actin (mouse monoclonal; Sigma); β-catenin; and Notch1. After washing three times with Tris-buffered saline and Tween-20, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (1:1,000; Amersham Bioscience, Dübendorf, Switzerland) for 1 hour at room temperature. Detection was conducted using chemiluminescence (Pierce, Rockford, IL). Densitometric quantification was performed by the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). Each assay was repeated at least 3 times.

Flow cytometric analysis

Single-cell suspensions from mouse dorsal skin were prepared as described previously (Bohr *et al.*, 2013). Briefly, dissected skin was floated on 0.25% trypsin (Invitrogen) at 37 °C for 2 hours followed by separation of dermis from epidermis. Separated dermis was cut into small pieces, digested with 0.15% type IV collagenase (Sigma) at 37 °C for 40 minutes, and then filtered through 70-μm strainers (BD Biosciences, San Jose, CA). The isolated cells were washed with phosphate-buffered saline and then incubated for 30 minutes at 4 °C with FITC-conjugated antibody to CD49f and Alexa 647–conjugated antibody to CD34 (BD Biosciences). The stainings were analyzed with a flow cytometer (BD Biosciences).

Statistical analysis

Data are represented as mean ± SEM. Statistical analysis of data was carried out using Student's *t*-test. A *P*-value of <0.05 was considered as a significant difference.

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 http://www.nature.com/jid

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