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ER71/ETV2 Promotes Hair Regeneration from Chemotherapeutic Drug-Induced Hair Loss by Enhancing Angiogenesis

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Abstract

Chemotherapy-induced alopecia and hair loss can be stressful in patients with cancer. The hair grows back, but sometimes the hair tends to stay thin. Therefore, understanding mechanisms regulating hair regeneration may improve the management of chemotherapy-induced alopecia. Previous studies have revealed that chemotherapeutic agents induce a hair follicle vascular injury. As hair growth is associated with micro-vessel regeneration, we postulated that the stimulation of angiogenesis might enhance hair regeneration. In particular, mice treated with 5-fluorouracil (5-FU) showed delayed anagen initiation and reduced capillary density when compared with untreated controls, suggesting that the retardation of anagen initiation by 5-FU treatment may be attributed to the loss of perifollicular micro-vessels. We investigated whether the ETS transcription factor ETV2 (aka ER71), critical for vascular development and regeneration, can promote angiogenesis and hair regrowth in a 5-FU-induced alopecia mouse model. *Tie2-Cre; Etv2* conditional knockout (CKO) mice, which lack *Etv2* in endothelial cells, presented similar hair regrowth rates as the control mice after depilation. Following 5-FU treatment, *Tie2-Cre; Etv2* CKO mice revealed a significant reduction in capillary density, anagen induction, and hair restoration when compared with controls. Mice receiving lentiviral *Etv2* injection after 5-FU treatment showed significantly improved anagen induction and hair regrowth. Two-photon laser scanning microscopy revealed that enforced *Etv2* expression restored normal vessel morphology after 5-FU mediated vessel injury. Our data suggest that vessel regeneration strategies may improve hair regrowth after chemotherapeutic treatment.

Key Words: Angiogenesis, Chemotherapy-induced alopecia, ETS transcription factor, ETV2/ER71, Hair regeneration, 5-FU

INTRODUCTION

Hair follicles continuously cycle through anagen (growth), catagen (regression), and telogen (resting) stages (Schneider *et al*., 2009). These cyclic changes involve rapid remodeling of both epithelial and dermal components (Paus and Cotsarelis, 1999). Hair growth is associated with perifollicular angiogenesis, which is highly active during the anagen phase (Mecklenburg *et al*., 2000; Yano *et al*., 2001). The interaction of vascular endothelial growth factor (VEGF) with its receptors plays a critical role in angiogenesis. VEGF receptor 2 (VEGFR2; FLK1), the primary receptor for VEGF, mediates most of the mitogenic, survival, and vascular permeability effects of VEGF. Consistently, VEGF delivery into the dorsal subcutaneous tissue of mice significantly promotes angiogenesis and hair follicle growth (Ozeki and Tabata, 2002). Notably, the perivascular niche in the upper bulge maintains the hair follicle stem cells (Xiao *et al*., 2013).

Chemotherapy or radiation therapy, which targets rapidly dividing cancer cells, frequently leads to visibly distressing

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side effects such as hair loss. Chemotherapy-induced alopecia, ranging from mild to severe, often causes patients to refuse chemotherapy (Yeager and Olsen, 2011; Dunnill *et al*., 2018). Hair loss can occur from a few days to weeks after chemotherapy, and distinct hair loss patterns can be observed based on severity (Rossi *et al*., 2020). Chemotherapy-induced alopecia most prominently disturbs the highly proliferative activity of matrix keratinocytes in anagen hair follicles (Trüeb, 2010; Paus *et al*., 2013). Telogen hair follicles are less sensitive to chemotherapy than anagen hair follicles, presumably because of their low proliferative activity. In certain instances, hair-follicle stem cells are also injured, leading to permanent hair loss (Trüeb, 2010; Paus *et al*., 2013). Several drugs or devices have been used to prevent or treat chemotherapy-induced alopecia; however, the results have been unsatisfactory in several cases (Rubio-Gonzalez *et al*., 2018).

5-Fluorouracil (5-FU) is an antimetabolite anticancer drug widely used to treat gastrointestinal, breast, gynecological, and head and neck cancers (Grem, 1997). However, patients receiving 5-FU treatment commonly display endovascular injury in various organs (Jensen and Sørensen, 2012; Focaccetti *et al*., 2015). As chemotherapeutic agents induce vascular damage to the hair follicle (Amoh *et al*., 2007), we hypothesized that the stimulation of vascular regeneration might enhance hair regrowth. The ETS transcription factor ETV2 (ER71) is essential for hematopoietic and vessel development (Lee *et al*., 2008). *Etv2* is reactivated in endothelial cells and upregulates *Flk1* expression in an ischemic hindlimb vascular regeneration model (Park *et al*., 2016). In the present study, we investigated whether Etv2/Er71 can promote perifollicular angiogenesis and hair regrowth in a mouse model of 5-FU induced alopecia. We revealed that endothelial *Etv2* enhanced hair regeneration by promoting vessel regeneration after 5-FU mediated vascular injury and hair loss.

MATERIALS AND METHODS

Generation of CKO mice

Tie2-Cre; Etv2 CKO (*Tie2-Cre; Etv2*$^{flox/flox}$) mice were generated from *Tie2-Cre; Etv2*$^{flox/+}$×*Etv2*$^{flox/flox}$ mating, as previously reported (Park *et al*., 2016). *Tie2-Cre* mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA); the generation of *Etv2*$^{flox/flox}$ mice has been previously described (Lee *et al*., 2011). Animal husbandry, generation, handling, and experimentation were performed in accordance with protocols (20170245) approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine in St. Louis (MO, USA).

Depilation induced hair growth measurement

Synchronized anagen was induced in the dorsal skin of approximately 7-week-old (P50-53) C57BL/6 mice by depilation, as previously described (Paus *et al*., 1990; Yano *et al*., 2001). The dorsal skin surface was photographed at days 12 and 18 after depilation, followed by quantitative assessment of anagen induction by dotmatrix planimetry. Areas that were in different stages (pink, telogen; anagen, black) were marked on a mouse photo, and the percentage of anagen induction was calculated using the equation [(black skin/total skin)×100]. Data are presented as mean ± standard deviation (SD) (n=4-8).

5-FU treatment

P50-53 wild-type C57BL/6 mice and Tie2cre; *Etv2* CKO mice were intraperitoneally injected with 200 mg/kg or 250 mg/kg of 5-FU or phosphate-buffered saline (PBS) at day 0 after depilation.

Lentiviral particle production

Lentivirus was produced as previously reported (Park *et al*., 2016). Briefly, 293T cells were transfected with pCSII-EF1α-ETV2-IRES-VENUS (pCSII-EF1α-IRES-VENUS or pCSII-EF1α-FLK1), pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev (4:3:1) using the calcium phosphate method. Sixteen hours after transfection, the medium was changed, and cells were grown for an additional 48 h. Subsequently, the supernatant was harvested and concentrated using a Lenti-X-Concentrator (Clontech, Mountain View, CA, USA). The virus titer was determined using a Lenti-X™ p24 Rapid Titer Kit (Clontech). For the injection study, the IFU was approximately 3×10^7/mL.

Lentiviral Etv2 injection

P50-53 C57BL/6 mice were depilated and intraperitoneally injected with 250 mg/kg 5-FU or PBS. Lentiviral *Etv2* (or control) (100 μL) was intradermally injected the following day.

Immunohistochemistry

In brief, the dorsal skin was excised at day 13 (late anagen) after depilation and fixed using 4% paraformaldehyde in PBS. The skins were then frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Finetk, Torrance, CA, USA) at −20°C, and cut into 10-μm thick sections using a Cryostat Cryocut Microtome (CM1850, Leica, Nussloch, Germany). For CD31 staining, tissue sections were incubated overnight at 4°C with rat anti-platelet endothelial cell adhesion molecule (CD31, PECAM-1) (1:25) antibody (BD Bioscience, San Jose, CA, USA), followed by staining with Alexa Fluor 594-labeled goat anti-rat (1:200) for 1 h at room temperature (Molecular Probes, Grand Island, NY, USA). Nuclei were counterstained with 4,6 diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) and examined using a fluorescence microscope (Axiovert 200M, ZEISS, Göttingen, Germany). At least eight images were randomly selected from each slide, and fluorescent vessels were counted to quantify CD31 positive vessels in the skin.

Histology

The dorsal skins were excised on day 13 after depilation, fixed in 4% paraformaldehyde in PBS, dehydrated in a graded ethanol series, and embedded in paraffin. Tissue sections (4-μm thick) were stained with hematoxylin and eosin. Five images were randomly selected from each slide (n=1). The skin thickness was measured in at least five different regions and averaged per image using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Two-photon imaging analysis

Mice were injected intravenously with 70 kDa rhodamine-dextran (50 μL) on day 13 after depilation. A two-photon laser tuned to 890 nm was used to excite dextran. In each experiment, a total of 50 two-dimensional (2D) slices were obtained at 2.5 μm steps and extrapolated into a three-dimensional (3D) volume using the Imaris software. Blood vessel diameters and volumes were measured from 3D rendering using

Imaris's FilamentTracer module (OXFORD Instruments, Zurich, Switzerland). The fractal dimension of the vasculature was calculated in MATLAB using an iterative box-counting algorithm on the maximum intensity projection of the 3D volume. Data are presented as the mean ± standard error (SE) (n=7-8, z-stack images).

Statistical analysis

Statistical analysis of blood vessel density measurements and quantification of anagen induction was performed using analysis of variance (ANOVA). For 2P image quantification, repeated measures analysis of covariance (ANCOVA) was performed using SAS statistical software (Campus Drive, Cary, NC, USA) with proc mixed. Multiple images per mouse were obtained, and model-fitting statistics, such as AIC and log-likelihood, suggested that a diagonal variance component matrix best fits the repeated measures effect. Outcome variables were adjusted for covariance from naturally occurring predictor variables (e.g., longer vessels have higher curvature regardless of treatment). Statistical contrasts were performed to assess within-group differences, and a *p*-value of <0.05 was considered significant.

RESULTS

5-FU treatment delays anagen induction

Previous studies have revealed that depilation (shaving) induces anagen in telogenic mice (Yano *et al*., 2001). Consistently, on inducing the hair cycle by depilation in 7-week-old C57BL/6 mice, which were almost exclusively in the telogenic hair follicle phase, anagen was initiated in 77.3 ± 15.4% of the depilated dorsal skin on day 12. The coat was almost fully restored by the end of anagen (day 18, 98.1 ± 2.2%, Fig. 1). In contrast, mice treated with 5-FU (200 mg/kg) after depilation showed delayed anagen initiation when compared with untreated controls, as indicated by the decreased percentage of black skin observed at day 12 (42.9 ± 10.3%) and day 18 (77.5 ± 26.1%). In a cohort of mice administered a higher dose of 5-FU dosage (250 mg/kg), anagen induction and hair regeneration were decreased further to 31.4 ± 12.5% on day 12 and 52.1 ± 27.8% on day 18, indicating that 5-FU treatment resulted in retarded anagen induction (Fig. 2A, 2B). Importantly, 5-FU treatment resulted in reduced capillary density (Fig. 2C, 2D, Supplementary Fig. 1).

Endothelial Etv2 deletion leads to delayed hair regeneration after 5-FU treatment

As *Etv2* is required for vascular regeneration after ischemic injuries (Park *et al*., 2016), we determined whether *Etv2* is also necessary for hair regeneration. First, we assessed hair regrowth after depilation in *Tie2-Cre; Etv2* conditional knockout (CKO) mice, which lack *Etv2* in endothelial cells. *Tie2-Cre; Etv2* CKO mice displayed similar hair regrowth rates as compared with control mice after depilation, as shown by the similar anagen initiation (day 12, C57BL/6, 77.3 ± 15.4%; *Tie2-Cre; Etv2* CKO, 65.8 ± 10.1%) and the fully restored hair by the end of anagen (day 18, C57BL/6, 98.1 ± 2.2%; *Tie2-Cre; Etv2* CKO, 98.6 ± 0.6%) (Fig. 1). These results indicate that in addition to being dispensable for maintaining steady-state blood vessels (Park *et al*., 2016), *Etv2* is dispensable for normal hair regrowth by steady-state blood vessels. However, when wild-type and *Tie2-Cre; Etv2* CKO mice underwent depilation and treatment with 5-FU at 200 mg/kg, a dosage that allows substantial hair regrowth in wild-type mice by day 18 (Fig. 1), *Tie2-Cre; Etv2* CKO mice showed diminished anagen (day 12, 26.6 ± 11.1% *vs.* 42.9 ± 10.3%, respectively), as well as reduced capillary density (Supplementary Fig. 1). Although hair regrowth in control mice reached 77.5 ± 26.1% by day 18, 5-FU-treated *Tie2-Cre; Etv2* CKO mice showed significantly delayed hair regrowth (63.5 ± 27.0%).

Lentiviral Etv2 gene delivery enhances vessel regeneration and hair regrowth

In a mouse hindlimb ischemic injury model, lentiviral *Etv2* gene delivery robustly enhances vessel angiogenesis and tissue repair (Park *et al*., 2016). Thus, we determined whether *Etv2* gene delivery could augment vessel regeneration and enhance hair regrowth. Telogen C57BL/6 mice were depilated and administered 5-FU at 250 mg/kg, a dosage that severely impairs hair regrowth in wild-type mice; this was followed by intradermal lentiviral *Etv2* injection. We confirmed the lentiviral

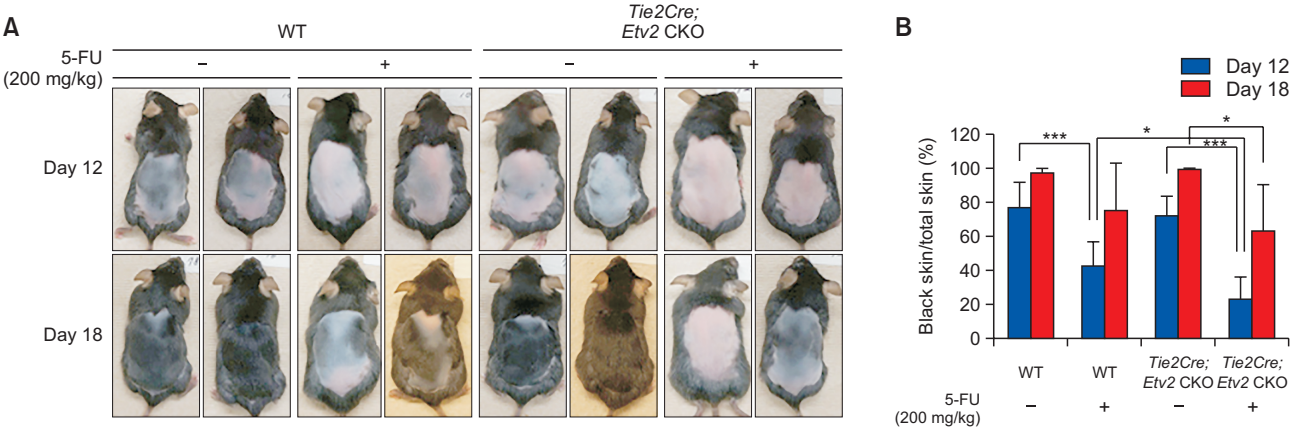

Fig. 1. *Etv2* is required for hair follicle anagen induction after 5-FU injury. (A) Representative images of the depilated dorsal skin of wild-type and *Tie2-Cre; Etv2* CKO mice, with or without 5-FU treatment, at days 12 and 18. (B) Quantification of anagen induction in (A). **p*<0.05; ****p*<0.001. 5-FU, 5-fluorouracil.

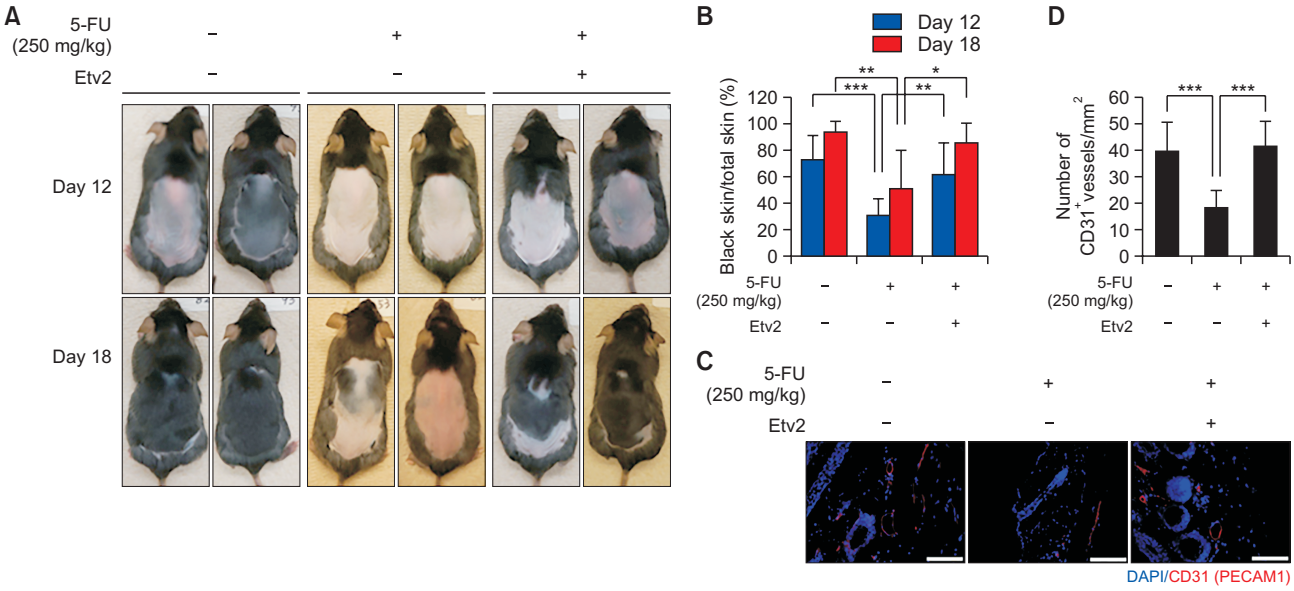

Fig. 2. *Etv2* enhances hair follicle anagen induction by angiogenesis. (A) Representative images of the depilated dorsal skin surface in 5-FU treated mice ± *Etv2* at days 12 and 18. (B) Quantification of anagen induction in (A). Mean values ± standard error (SE) (n≥4); **p*<0.05; ***p*<0.01; ****p*<0.001. (C) Representative images of the CD31 immunohistochemistry in the 5-FU-treated perifollicular region after lentiviral *Etv2* Injection. The nuclei were stained with DAPI (blue). Scale bars: 100 μm. (D) Quantitative analysis of CD31-positive vessels in (C). Mean values ± standard deviation (SD); ****p*<0.001. At least 8 fields generated from n≥2 mice were examined. 5-FU, 5-fluorouracil.

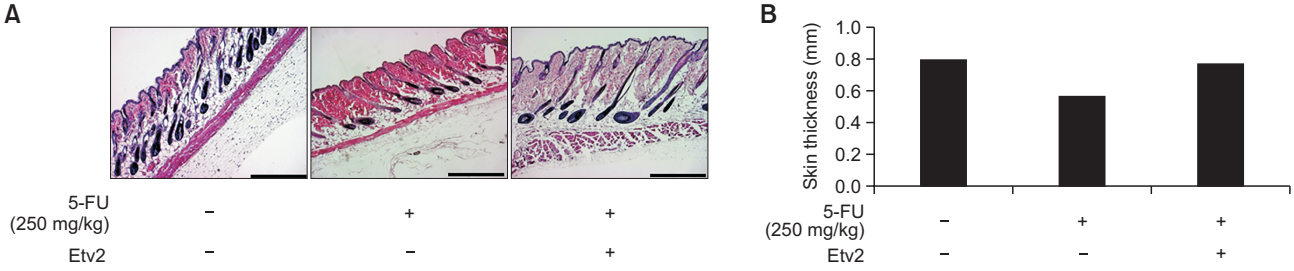

Fig. 3. *Etv2* increases dorsal skin thickness after 5-FU injury. (A) Hematoxylin and eosin staining of histological dorsal skin samples at day 13 after depilation. Scale bars: 500 μm. (B) Quantification of skin thickness in (A) (n=1). 5-FU, 5-fluorouracil.

infection of endothelial cells by assessing the co-expression of CD31 and lentiviral-encoded GFP (Supplementary Fig. 2). Mice receiving lentiviral *Etv2* injection revealed significantly improved anagen induction and hair regrowth (62.6 ± 23.2% at day 12 and 86.6 ± 14.2% at day 18) (Fig. 2A, 2B), while the lentiviral vector control group showed no improvement when compared with controls (not shown). Notably, hair-follicle-associated micro-vessel density, decreased in the 5-FU treatment group, was increased to control levels following the *Etv2* injection (control, 40.0 ± 10.7; 5-FU, 18.9 ± 6.2; 5-FU and *Etv2*, 41.2 ± 9.1; Fig. 2C, 2D).

Moreover, the 5-FU treatment decreased the thickness of the dermis and subcutaneous tissue of the dorsal skin, and *Etv2* injected mice showed a similar thickness of the whole dermis and subcutaneous tissue to that of normal dorsal skin (Fig. 3). We employed *in vivo* two-photon laser scanning microscopy to assess the impact of *Etv2* on the morphology and density of vessels in depilated dorsal skin (Fig. 4). Vessels in 5-FU treated mice appeared discontinuous and fragmented (Fig. 4A). Additionally, vessels in 5-FU-treated mouse skin revealed decreased vascular volume when compared with those in the control mouse skin. However, lentiviral expression of *Etv2* rescued 5-FU mediated vessel defects and restored normal vessel morphology in terms of tortuosity and branching, as measured by the fractal dimension (Fig. 4B-4D).

DISCUSSION

Approximately 80% of patients with cancer have reported that chemotherapy-induced alopecia is a severe problem associated with cancer treatment after nausea and vomiting (Yeager and Olsen, 2011). Accordingly, several patients refuse to undergo cancer chemotherapy (Yeager and Olsen, 2011). Understanding hair loss mechanisms and hair regeneration after chemotherapy can improve the quality of life in patients undergoing cancer treatment.

Chemotherapeutic agents reportedly cause vascular dam-

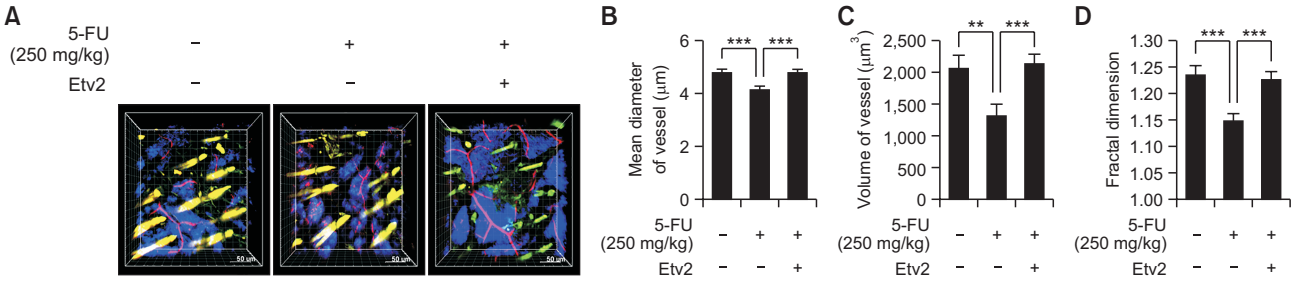

Fig. 4. *Etv2* enhances perifollicular angiogenesis after 5-FU injury. (A) Two-photon microscopy images of dorsal skin at day 13 after depilation. Red colors indicate vascular signal. Hair shafts display auto-fluorescence. Scale bars: 50 μm. (B) Vessel diameter from Z-stacks of two-photon images of dorsal skin at day 13 after depilation. Data are presented as the mean ± standard error (SE) (n=7-8 z-stack images). ***p<0.001. (C) Vessel volume from Z-stacks of two-photon images of dorsal skin at day 13 after depilation. Data are presented as the mean ± SE (n=7-8 Z-stack images). **p<0.01; ***p<0.001. (D) Fractal dimension of vessels from the Z-stacks of two-photon images at day 13 after depilation. ***p<0.001. 5-FU, 5-fluorouracil.

age to hair follicles (Amoh *et al*., 2007). Angiogenesis is essential for regulating the hair follicle cycle and hair regrowth (Yano *et al*., 2001). During the anagen phase, the vascular network is highly developed (Mecklenburg *et al*., 2000). Hair follicle dermal papilla and outer root sheath keratinocytes are known to secrete VEGF at higher levels, thereby controlling hair growth (Man *et al*., 2009). Moreover, VEGF can induce hair follicle growth by promoting angiogenesis (Ozeki and Tabata, 2002). These studies support the notion that angiogenic stimulation may promote hair regeneration. In the present study, we examined whether hair regeneration could be promoted by enhancing angiogenesis. We specifically tested whether *Etv2* deficiency affected chemotherapy-induced alopecia. Our data indicate that 5-FU treated *Tie2-Cre; Etv2* CKO mice showed delayed anagen initiation when compared with control mice after 5-FU treatment (Fig. 1). Additionally, lentiviral *Etv2* injected mice demonstrated significantly improved anagen induction and hair regrowth (Fig. 2, 4). We confirmed lentiviral Etv2 gene delivery to endothelial cells (Supplementary Fig. 2). These results indicate that hair regeneration after 5-FU treatment can be facilitated by enhancing perifollicular capillary angiogenesis and anagen initiation.

Etv2 is critical for vascular development and regeneration (Lee *et al*., 2008; Park *et al*., 2016). Previously, we showed that endothelial Etv2/Er71 reactivation was essential for angiogenesis in hindlimb ischemia and a tumor model (Park *et al*., 2016; Kabir *et al*., 2018). Etv2 was reactivated in endothelial cells at the site of hindlimb ischemic injury in wild-type mice, while Etv2 expression and angiogenesis decreased in Etv2 CKO mice, such as *Tie2-Cre; Etv2* CKO mice (Park *et al*., 2016). Additionally, *Etv2* gene delivery to endothelial cells by lentiviral *Etv2* injection enhanced angiogenesis and reduced the fibrosis area in a mouse hindlimb ischemic injury model (Park *et al*., 2016). In contrast, Etv2 shRNA inhibited angiogenesis and tumor growth when Etv2 shRNA was delivered to the tumor (Kabir *et al*., 2018). In the present study, 5-FU treatment in *Tie2-Cre; Etv2* CKO mice delayed anagen induction and reduced capillary density. Lentiviral *Etv2* gene delivery rescued impaired angiogenesis at the anagen stage, as well as hair regeneration in 5-FU treated wild-type mice. Therefore, Etv2 reactivation in endothelial cells induced angiogenesis, which was followed by hair regrowth. Hair growth is associated with perifollicular angiogenesis (Mecklenburg *et al*., 2000; Yano *et al*., 2001). Several studies have shown that angiogenesis promotes hair growth. VEGF delivery and platelet-rich plasma (Ozeki and Tabata, 2002; Cheng *et al*., 2017) successfully enhanced angiogenesis and hair growth. Based on previous reports, the difference in anagen induction in 5-FU treated *Tie2-Cre; Etv2* CKO mice and 5-FU treated, lentiviral Etv2 gene-delivered mice is dependent on angiogenesis. Therefore, Etv2 can regulate hair regeneration via angiogenesis in hair loss induced by vascular injury.

In the present study, we were unable to confirm the effect of Etv2 on the regulation of the normal hair cycle; however, Etv2 activation might also enhance hair regeneration under normal conditions. ER71 promotes FLK1+ mesoderm formation by directly activating *Flk1* gene expression (Lee *et al*., 2008). Furthermore, *Er71* deficient mice demonstrated considerably reduced FLK1 expression, died early during gestation, and displayed severe blood and vessel defects (Lee *et al*., 2008). Reportedly, Etv2 regulates FLK1 expression in endothelial cells (Park *et al*., 2016; Kabir *et al*., 2018). Endothelial cells derived from Etv2 deletion mice revealed reduced FLK1 expression when compared with endothelial cells derived from normal mice (Park *et al*., 2016; Kabir *et al*., 2018). Endothelial cells derived from Etv2 deficient mice showed reduced VEGF response, while an increase in Etv2 expression enhanced FLK1 expression (Kabir *et al*., 2018). It has been reported that VEGF is produced at higher levels during the anagen phase by dermal papilla and outer root sheath keratinocytes, which control hair growth (Kozlowska *et al*., 1998; Man *et al*., 2009). Therefore, the reactivation of Etv2 in endothelial cells increased the VEGF response secreted from dermal papilla and outer root sheath keratinocytes and could enhance angiogenesis, which can accelerate anagen induction and improve hair growth.

In conclusion, our data demonstrated that Etv2 deficiency in endothelial cells reduces hair regeneration. Conversely, enforced Etv2 expression led to improved angiogenesis and hair regeneration after 5-FU treatment. Our data support the notion that Etv2-mediated enhanced vascularization may offer a novel approach for hair regeneration after chemotherapy. Our studies further support the idea that vessel regeneration strategies may be integrated into future tissue regeneration therapies.

CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

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