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Wound healing protects against chemotherapy-induced alopecia in young rats via up-regulating interleukin-1β-mediated signaling

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Abstract

Wound healing is a complex process regulated by various cell types and a plethora of mediators. While interactions between wounded skin and the hair follicles (HFs)

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could induce HF neogenesis or promote wound healing, it remains unknown whether the wound healing-associated signaling milieu can be manipulated to protect against alopecia, such as chemotherapy-induced alopecia (CIA). Utilizing a well-established neonatal rat model of CIA, we show here that skin wounding protects from alopecia caused by several clinically relevant chemotherapeutic regimens, and that protection is dependent on the time of wounding and hair cycle stage. Gene expression profiling unveiled a significant increase in interleukin-1 beta (IL-1β) mediated signaling by skin wounding. Subsequently, we showed that IL-1β is sufficient and indispensable for mediating the CIA-protective effect. Administration of IL-1β alone to unwounded rats exhibited local CIA protection while IL-1β neutralization abrogated CIA protection by wounding. Mechanistically, IL-1β retarded postnatal HF morphogenesis, making HFs at the wound sites or IL-1β treated areas damage-resistant while the rats developed total alopecia elsewhere. We conclude that wound healing switches the cutaneous cytokine milieu to an IL-1β-dominated state thus retarding HF growth progression and rendering the HFs resistant to chemotherapy agents. In the future, manipulation of HF progression through interfering with the IL-1β signaling milieu may provide therapeutic benefits to a variety of conditions, from prevention of CIA to inhibition of hair growth and treatment of hirsutism.

Keywords: Medicine

1. Introduction

Increasing clinical and experimental evidence has demonstrated intimate interactions between the skin wound healing and the hair follicle (HF) development and/or regeneration processes. On the one hand, epithelial cells from the HFs have been shown to contribute to re-epithelialization during wound healing, some of which remain within the repaired epidermis for many months (Ito et al., 2005; Levy et al., 2007; Snippert et al., 2010). Clinicians have long reported that wound heals faster in hair-bearing areas—such as the scalp—than non-hairy areas such as the palm (Jimenez et al., 2015). Additionally, skin wound heals more rapidly when the HFs are in the growth phase of the hair cycle (Garcin et al., 2016). In mice, wound healing is accelerated during anagen (growth) rather than during telogen (quiescent) phase of the hair cycle (Ansell et al., 2011; Schneider et al., 2009; Stojadinovic et al., 2011). In the clinic, several studies have successfully treated chronic leg ulcers using epidermal equivalents derived from cells of anagen HF outer root sheath, taking advantage of their high proliferation potential (Fox et al., 2016; Jimenez et al., 2015; Limat et al., 1996; Martinez et al., 2016; Ortega-Zilic et al., 2010; Renner et al., 2009; Tausche et al., 2003). On the other hand, it has been known, for decades, that wounding the murine skin can induce the telogen to anagen transition (Hoffmann et al., 1996; Jiang et al., 2010). Additionally and remarkably, wounding the skin can also induce epidermal cells to assume a HF

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stem cell phenotype and produce HFs *de novo*, in a process termed wound induced hair follicle neogenesis (WIHN), in mice (Ito et al., 2007; Seifert et al., 2012) and possibly in humans (Sun et al., 2009). Furthermore, macrophages, one of the key regulators of wound healing in mice and humans, are also important regulators of HF cycling (Castellana et al., 2014). Numerous cytokines, growth factors, and neuropeptides not only regulate HF growth and cycling, but also impact on wound healing (Barrientos et al., 2008; Krause and Foitzik, 2006; Rognoni et al., 2016). Importantly, all major wound healing-associated cytokines are hair growth-inhibitory (Hoffmann et al., 1998; Philpott et al., 1996; Tong and Coulombe, 2006; Yu et al., 2008). These observations raise the possibility for manipulation of the microenvironment shared by the HFs and adjacent skin to promote wound healing and HF regeneration, or to prevent alopecia.

Once HFs are formed through *de novo* morphogenesis, they go through cycles of anagen, apoptosis-driven regression (catagen), and telogen (Geyfman et al., 2015; Lee and Tumbar, 2012; Stenn and Paus, 2001). HF cycling occurs over the lifespan, well beyond the organogenesis of other systems and the cycling lifetime of the ovary or endometrium. This cyclic regeneration requires many of the cellular signals integral to other morphogenetic (e.g., salivary glands, kidney, breast, and tooth) and regenerating systems (e.g., the amphibian limb). The massive cell proliferation that feeds the elongation of the lower HF during late anagen make HFs at this stage (anagen VI) highly susceptible to anti-proliferative chemotherapy agents. Because the majority of scalp HFs are at this stage at any given time, the result of antineoplastic chemotherapy is alopecia—termed chemotherapy-induced alopecia (CIA)—in approximately 65% of patients (Paus et al., 2013).

In a previous study, we made the serendipitous discovery that wounding prevented CIA in a rat model. We noticed several neonatal rat pups had been unintentionally wounded (Fig. 1B) by their mother when she carried them around in between her teeth (Rosenblatt, 1967). These wounded pups and their unwounded littermates (Fig. 1A, B) were then given chemotherapeutic agent etoposide to induce total alopecia, on postnatal day 11–13 (PD11-13) during late stage HF morphogenesis (Wikramanayake et al., 2012). By PD21, while the unwounded pups developed total alopecia of the trunk (Fig. 1C) as expected (Wikramanayake et al., 2012), patches of hair were retained at the wounded sites of their littermates (Fig. 1B, D). This observation suggested that wounding may have induced changes to the HFs to render them less susceptible to CIA.

Considering this observation, the current study set out to examine the effects of wounding on HF morphogenesis and CIA. To do so, we induced incisional wounds in neonatal rats on PD3, before treating the pups with chemotherapeutic agents on PD11-13 to induce alopecia (Wikramanayake et al., 2012). Once we verified that induced wounds protected the HFs from CIA (Fig. 1E), we determined gene

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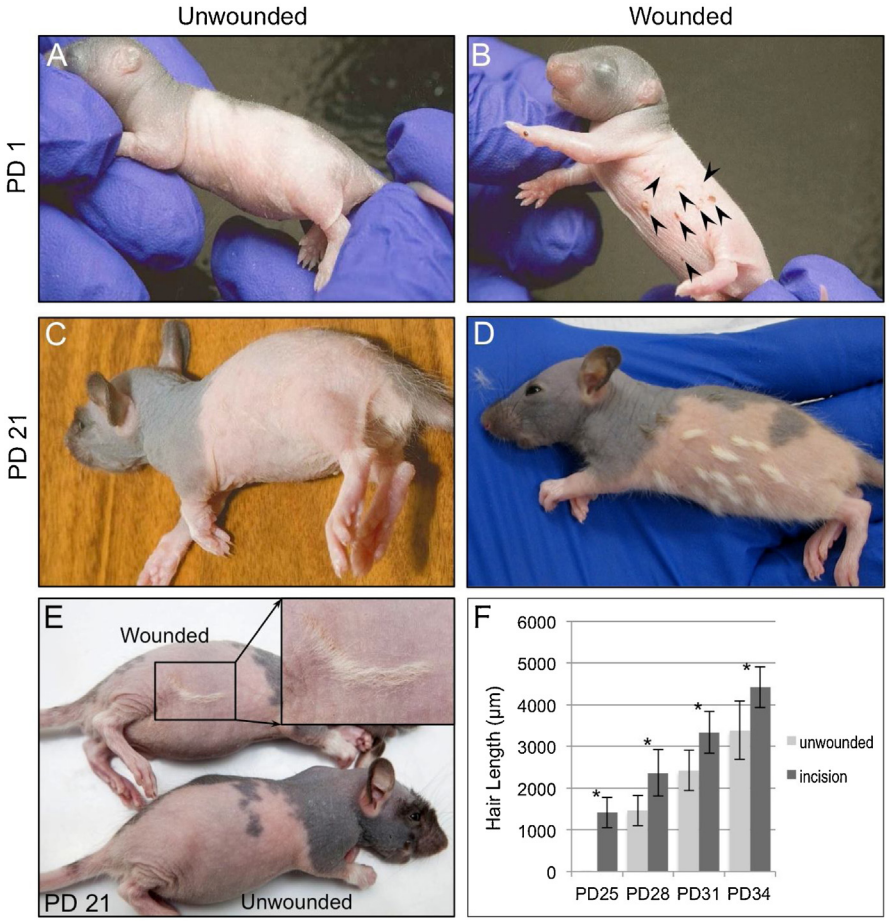

Fig. 1. Unintentional (A–D) and induced incisional wounds (E) protected from chemotherapy-induced alopecia at the wound sites. (A–D) Gross phenotype of a rat pup wounded (arrowheads) by its mother on postnatal day 1 (PD1) (B), and protection of hair loss at the wound sites on PD21 (D), compared with an unwounded littermate that developed total alopecia on the trunk (C), after treatment with etoposide on PD11-13. (E) Protection of hair loss at the site of an incisional wound (induced on PD3) on PD21, compared with total alopecia on the trunk in an unwounded littermate (n = 8 each). (F) Comparison of awl hair shaft length between the incisional sites and contralateral unwounded sites (n = 5). Bars denote standard deviation, and asterisks (*) denote statistical significance ($p < 0.05$).

expression changes in the wounded tissue and the underlying mechanism of protection. Collectively, our results indicated that wound healing created a signaling environment, characterized specifically by increased interleukin-1β production, which delayed HF morphogenesis and protected HFs from CIA. These results suggest that manipulating interleukin-1β levels in the HF microenvironment may provide a novel approach to alter the hair cycle to prevent hair loss or to treat hair disorders.

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2. Materials and methods

2.1. Animals

All animal care and use procedures were approved by the University of Miami Institutional Animal Care and Use Committee (rat study) or the University of Manchester under UK Home Office licence (mouse study). Nursing Long-Evans rat pups (mixed sex) along with their mothers were purchased from Charles River Laboratories (Wilmington, MA) and Harlan Laboratories (now Envigo, Indianapolis, IN), and were maintained under standard conditions. The rat pups were used for incisional wound induction and chemotherapy-induced alopecia experiments. Female C56BL/6 mice were purchased from Charles River Laboratories (Margate, UK), and were used for excisional wound experiments.

2.2. Incisional wound induction and chemotherapeutic agent treatment in rats

Rat litters were randomly assigned to 13 treatment groups (groups a-m, Table 1) of 10 pups each. A 3 mm incisional wound was made on the right dorsum on PD3 (groups a-d), PD8 (e-g) or PD11 (h-j), or they were left unwounded (k-m)

Table 1. Incisional wound induction and chemotherapeutic agent treatment in young rats. Rat pups (n = 10/group) were wounded by incision on days 3, 8 or 11 after birth, and subsequently (PD11-13) received various chemotherapeutic agents to induce alopecia. Groups k-m pups were unwounded, and group d pups received no chemotherapy. Two pups from each group were euthanized on PD11 for histology. Pups were monitored daily for hair loss.

Group	Wounded	Injection (n = 10 each)	Outcome on PD21 (n = 8 each)
a	PD3	Etoposide	Protection from hair loss at wound site
b		Cyclophosphamide	
c		Cyclophosphamide/doxorubicin	
d		No injection	No hair loss
e	PD8	Etoposide	Total alopecia on trunk
f		Cyclophosphamide	
g		Cyclophosphamide/doxorubicin	
h	PD11	Etoposide	Total alopecia on trunk
i		Cyclophosphamide	
j		Cyclophosphamide/doxorubicin	
k	Unwounded	Etoposide	Total alopecia on trunk
l		Cyclophosphamide	
m		Cyclophosphamide/doxorubicin	

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(Table 1). For wound induction, dorsal skin was first cleaned with Betadine (Sigma-Aldrich, St. Louis, MO), and pups were anesthetized with intraperitoneal (i.p.) injection of 50 mg/kg ketamine HCl (Sigma-Aldrich) and 5 mg/kg xylazine (Sigma-Aldrich). Anesthesia was confirmed by loss of toe pinch reflex. For chemotherapeutic agent treatment, pups received daily i.p. injections of etoposide (1.5 mg/kg; Sigma-Aldrich) on PD 11–13, or a single i.p. injection of cyclophosphamide (CYP) (37.5 mg/kg; Sigma-Aldrich) on PD13, or a combination treatment of CYP (37.5 mg/kg; Sigma-Aldrich) on PD11 and doxorubicin (DXR) (1.5 mg/kg; Sigma-Aldrich) on PD 11–13 (Wikramanayake et al., 2013). Groups k-m pups were unwounded, and group d pups received no chemotherapy and were used for weight and histological reference. Two pups from each group were euthanized on PD11 for histology. Skin specimens were harvested from euthanized rats and fixed in 10% formalin and paraffin-embedded. In a separate experiment, skin specimens were collected from wound sites and corresponding sites of unwounded rat littermates (n = 4 each) on PD4, 36 h after incisional wound induction. Skin samples were treated with RNAlater® for subsequent RNA isolation and microarray analysis.

2.3. Excisional wound induction in mice

A 6-mm full thickness excisional wound was induced in anesthetized female C57BL/6 mice on either PD32 (anagen) or PD49 (telogen) (n = 7 and 9, respectively), as previously described (Ansell et al., 2014). Skin samples at the wound sites were collected 24 h later. Harvested skin was snap frozen and stored at −80 °C until use.

2.4. Hair length measurements

Pups were monitored daily for hair changes. Hair was plucked from the site of incision on the right dorsum and from the contralateral unwounded site on the left dorsum as pups recovered from CIA (n = 8). Hairs were collected in triplicates from each pup and mounted on a slide using Permount (Fisher Scientific International, Inc., Hampton, NH). The length of individual awl hairs was measured using a Nikon Eclipse E800 microscope and NIS Elements BR3.10 software.

2.5. Histological analysis

Paraffin sections were stained with hematoxylin and eosin following standard protocol. The stained slides were subsequently evaluated using an Axio Observer D1 microscope and AxioVision software (Carl Zeiss Microimaging, Thornwood, NY). Mast cells were visualized by Giemsa staining and quantitative mast cell histomorphometry (Paus et al., 1994). Images were captured using a Keyence

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Biozero BZ–800 K Microscope and analysed using Image J image analysis software.

2.6. RNA isolation, cDNA synthesis, and microarray analysis

Total RNA was isolated from homogenized skin (snap frozen upon collection or stored in RNAlater®) using Trizol Reagent (Life Technologies, Grand Island, NY) and purified with the RNeasy micro kit (Qiagen). cDNA synthesis was carried out using the Tetro cDNA synthesis kit (Bioline, Taunton, MA). For microarray analysis, total RNA (n = 4 each of wounded and unwounded skin) of PD4 rat skin 36 h after incisional wound induction was purified and sent to Ocean Ridge Biosciences (ORB, Palm Beach Gardens, FL) for analysis using the Rat RN1100 MI-Ready Array (GPL15166 in the GEO accession browser, Microarrays Inc., Huntsville, AL). For statistical analysis, samples were binned into two treatment groups (wound vs. control). The log2-transformed and normalized spot intensities for the 16,198 detectable probes were examined for differences between the treatment groups by 1-way ANOVA. The statistical significance was determined using the False Discovery Rate (FDR) method. A total of 3,239 probes showed significant differences with $P < 0.05$ and FDR (false discovery rate) less than 0.1. Hierarchical cluster analysis was performed using Cluster 3.0 software to detect functional clusters. The microarray data can be accessed in the Gene Expression Omnibus with the accession number GSE98105.

2.7. Real-time qPCR analysis

One-step qRT-PCR was performed using RNA isolated from PD4 wounded and unwounded rat skin (36 h after wounding). Experiments were performed in triplicates using qScript One-step SYBR Green qRT-PCR Kit for iQ (Quanta Biosciences, Gaithersburg, MD) and Opticon2 thermal cycler (Bio-Rad, Hercules, CA). The primers used were: *Osteopontin*, forward 5′-TGCAGTGGCCATTTG-CATTT-3′, reverse 5′-GGCCCTGAGCTTAGTTCGTT-3′; *IL-1β*, forward 5′-GACTTCACCATGGAACCCGT-3′, reverse 5′-GGAGACTGCCCATTCTC-GAC-3′; *Cxcl2*, forward 5′-CAGGAAGCCTGGATCGTACC-3′, reverse 5′-TGAGCTGGCCAATGCATATCT-3′, *Ccl24* forward 5′-AACTCCGAGGCAA-TAGCACC-3′, reverse 5′-AGGGGAAACGAACTCAGGAC-3′; and *Gapdh* forward 5′-CACGGCAAGTTCAACGGCACAGTCA-3′, reverse 5′-GTGAA-GACGCCAGTAGACTCCACGA-3′. Real-time qPCR for mouse *IL-1β* and *Gapdh* was performed in triplicates using the Taqman probe sets Mm99999915_g1 for *Gapdh* and MM00434228_m1 for *IL1 β* (ThermoFisher Scientific). Statistical analyses of expression levels were performed using Student's *t* test.

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2.8. Cytokine injection and chemotherapeutic agent treatment in rat pups

PD3 rat pups were randomly assigned into 10 groups (groups 1–10, Table 2) of 10 pups each. Pups received daily subcutaneous injections on the dorsum on PD3-5 of one of the treatments (Table 2): 50 ng IFN-γ (R&D Systems, Minneapolis, MN), 50 ng TNF-α (R&D Systems), 50 ng EGF (R&D Systems), 50 ng FGF1 (R&D Systems), 50 ng TGF-β (R&D Systems), 1X PBS (vehicle treatment) (Sigma-Aldrich, St. Louis, MO), or 50 ng of IL-1β (R&D Systems). Pups received daily i.p. injections of etoposide (1.5 mg/kg) on PD11 ~ 13, or cyclophosphamide (37.5 mg/kg) on PD13, or daily i.p. injections of doxorubicin (1.5 mg/kg) on PD11 ~ 13 and cyclophosphamide (37.5 mg/kg) on PD13. Control group (group 10) did not receive chemotherapy and were used for weight and histological comparison. Skin specimens were collected from the corresponding injected areas after hair assessment on PD21.

Rat pups were also injected subcutaneous once daily on PD3-5 with 50 ng of IL-1β (R&D Systems) or 1X PBS (vehicle control), and euthanized on PD13 for skin histology and quantitative HF histomorphometry. N = 5 each.

2.9. IL-1β neutralizing antibody treatment

On PD2, the day before incisional wound was induced in rat pups, the area of the prospect wound was labeled, and anti-rat IL-1β/IL-1F2 antibody (100 μl of 10 μg/1 ml) (R&D Systems, Minneapolis, MN) was subcutaneously injected. Wounding was performed on PD3, and antibody injections continued daily till PD6. Control

Table 2. Effects of subcutaneously injected cytokines and growth factors on chemotherapy-induced alopecia (CIA). Of the six cytokines and growth factors injected subcutaneously, protection from CIA was only observed in IL-1β treated rats, regardless of the different chemotherapy regimens.

Group	Injected Cytokine/growth factor (PD3)	Chemotherapy	Outcome
1	IFN-γ	Etoposide	Alopecia
2	TNF-α	Etoposide	Alopecia
3	EGF	Etoposide	Alopecia
4	FGF	Etoposide	Alopecia
5	TGF-β	Etoposide	Alopecia
6	PBS (vehicle)	Etoposide	Alopecia
7		Etoposide	
8	IL-1β	Cyclophosphamide	Local protection of hair loss
9		Cyclophosphamide/doxorubicin	
10	No injection	No chemotherapy	Normal hair phenotype

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animals were injected with vehicle control (1X PBS). On PD11-13, pups received daily etoposide treatment. Rats were monitored until PD30 for the assessment of hair regrowth.

3. Results

3.1. Wound healing protects neonatal rats from CIA

To determine whether cutaneous wounding can protect neonatal rats from CIA, we induced wounds in rat pups before administering chemotherapeutic agents and monitored hair growth/loss. We induced a single incisional wound on the right dorsum of pups three days after birth (PD3). We then administered chemotherapeutic agents etoposide, or cyclophosphamide, or cyclophosphamide/doxorubicin combination (Table 1) on PD11-13, as previously described (Hussein et al., 1990). By PD21, as expected, unwounded pups developed total alopecia on the trunk (Fig. 1E). In the wounded pups, however, we observed protection from hair loss at the site of incision, regardless of the chemotherapeutic agents received, while the rest of the trunk was completely alopecic (Fig. 1E). For the next two weeks, we measured the length of awl hair shafts plucked from the wound sites and the contralateral unwounded sites, and hair shafts from the wound sites were on average at least 30% longer ($p < 0.05$) (Fig. 1F). These results confirmed that the hair shafts were retained at the wound sites when hair shafts at other locations on the trunk were completely lost in response to chemotherapy.

Histological analysis revealed significant differences in the progression of HF morphogenesis between wounded and unwounded skin in etoposide-treated rat pups (Fig. 2). Such difference was readily detected on PD11, when all the HFs in the dorsal skin from unwounded pups were at advanced growth phase, with their hair bulbs at the very bottom of the dermal adipose layer (Fig. 2A). In skin from the wounded pups, however, HFs immediately adjacent to the wound site appeared much shorter than HFs more distant from the wound, suggesting that their growth had been delayed (Fig. 2B). By PD16, many HFs in the control skin had already entered catagen, with reduced diameter and their hair bulbs away from the bottom of the dermal adipose layer (Fig. 2C). HFs immediately adjacent to the wound site, however, were at robust growth phase with their enlarged hair bulbs deep within the adipose layer (Fig. 2D). By PD21, HFs in the unwounded skin have already transitioned from telogen to early anagen, and were in anagen II as the large dermal papilla had reached the dermis-adipose boundary (Fig. 2E). HFs immediately adjacent to the wound site, on the other hand, were still in advanced growth phase with their hair bulbs deep within the adipose tissue (Fig. 2F). HFs at a distance away from the wound sites were at similar hair cycle stages as those in the skin of unwounded rats (data not shown). These results suggested that wounding on PD3 had significant impact on the progression of HF morphogenesis, causing a delay by

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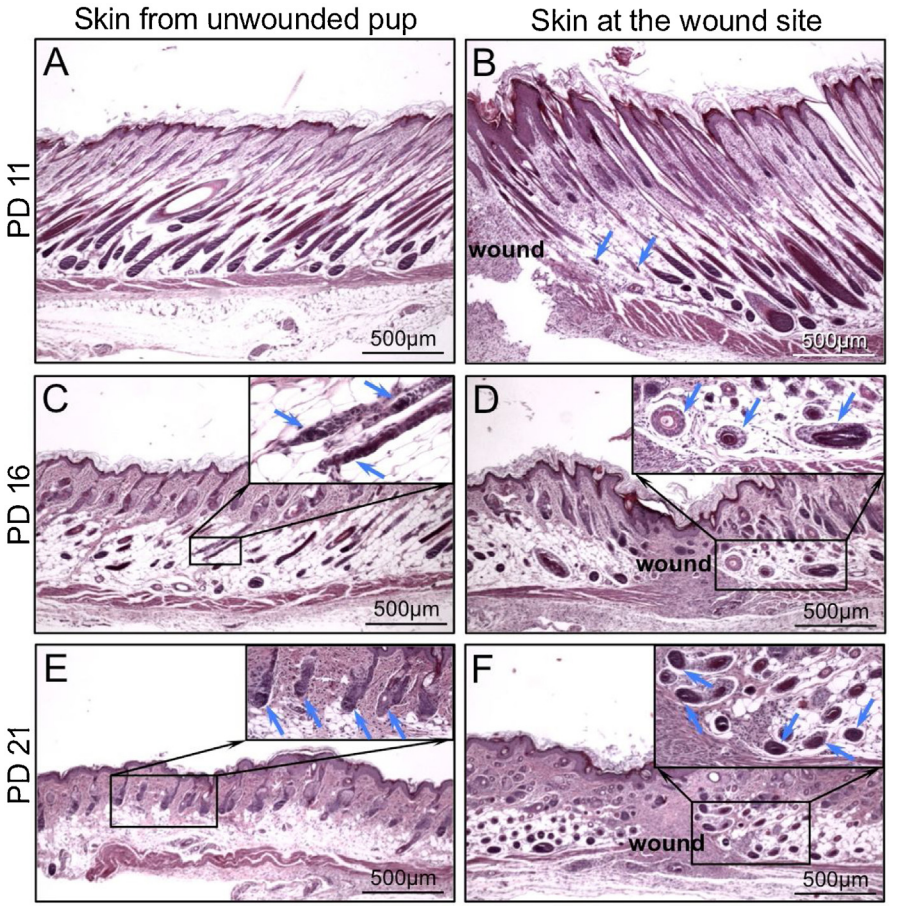

Fig. 2. Hair follicles (HFs) immediately adjacent to the wound site were at different phases of the hair cycle from HFs in unwounded skin. Hematoxylin and eosin staining showed HFs immediately adjacent to the wound site (B, D, F) were of delayed anagen stage compared with skin from unwounded pups (A, C, E). All pups received etoposide on PD11-13. Blue arrows point to the hair bulbs.

PD11, when the less advanced HF growth phase and reduced cell proliferation rendered the HFs less susceptible to chemotherapy on PD11-13. The consequence was a rapid resumption of follicular growth after chemotherapeutics agents were depleted through metabolism. This was in contrast to control unwounded skin, which encountered chemo agents when the HFs were most susceptible, and went through follicular dystrophy before recovery, showing a delay in hair regrowth.

To test whether wounding at any time point would confer protection from CIA, we also induced wounds on PD8 or PD11, and administered the three chemotherapy regimens to both unwounded and wounded pups (Table 1). By PD21, all the wounded pups developed total alopecia on the trunk, as observed in unwounded pups (data not shown). Therefore, hair preservation at the wound sites was only observed in rats wounded on PD3 and not on PD8 or PD11. We concluded that the wound-induced CIA protection was strictly time-dependent, suggesting that the signaling milieu

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generated during the early phases of wound healing greatly impacts the HF's sensitivity to chemotherapy-inflicted damage. At the same time, hair protection from chemotherapy-inflicted damage appears to be HF stage dependent.

3.2. Gene expression profiling suggests a role for IL-1β in wound healing-associated CIA protection

To better understand the intracutaneous signaling milieu that rendered wound healing-associated protection from CIA in neonatal rat skin, we obtained the gene expression signature of rat skin 36 h after wounding, using microarray analysis. Hierarchical cluster analysis of microarray data showed that wounded skin samples shared similar gene expression profiles, as did the control unwounded skin samples (Fig. 3A), but the gene signature of wounded skin differed from that of unwounded skin (Fig. 3A). As expected, we detected altered regulation of various genes known to be involved in the wound healing process (Fig. 3B). For instance, interleukins (ILs) and chemokine receptor ligands (CXCLs, CCLs), tumor necrosis factor ligands, osteopontin, and macrophage inhibitory factor were among highly up-regulated genes. Specifically, *IL-1β* was among the most strongly induced cytokine transcripts in wounded skin compared with control skin. Real-time qPCR analysis confirmed increased relative expression of *Cxcl2*, *Osteopontin*, and *IL-1β*, and decreased expression of *Ccl24*, normalized to *Gapdh*, in wounded skin compared with unwounded skin (Fig. 3C). The observed upregulation of IL-1β *in vivo* correlated with previous reports that various cell types release IL-1β during the inflammatory and proliferative phases of wound healing (Barrientos et al., 2008; Mirza and Koh, 2015; Zielins et al., 2014).

IL-1β was previously shown to be a potent inhibitor of human HF growth in organ culture (Philpott et al., 1996; Xiong and Harmon, 1997). To determine the hair cycle dependence of wound-induced *IL-1β* expression, we analyzed *IL-1β* expression after wounding by excision on PD32 (anagen) and PD49 (telogen) in C57BL/6 mice, where large differences in the rate of healing are known to occur (Ansell et al., 2011). While the *IL-1β* expression was low in both PD32 and PD49 unwounded mouse skin, high level of *IL-1β* expression was detected 24 h post-wounding, during the early phases of wound healing, in both anagen and telogen mouse skin (Fig. 3D). Although the mean *IL-1β* expression normalized to *Gapdh* was 2.7-fold higher in anagen wounds than in telogen wounds, the difference was not statistically significant ($p = 0.11$).

3.3. IL-1β provided broad protection from CIA in neonatal rat skin

We next tested whether IL-1β or a number of carefully selected cytokines/growth factors which were previously shown to exhibit hair growth-inhibitory properties

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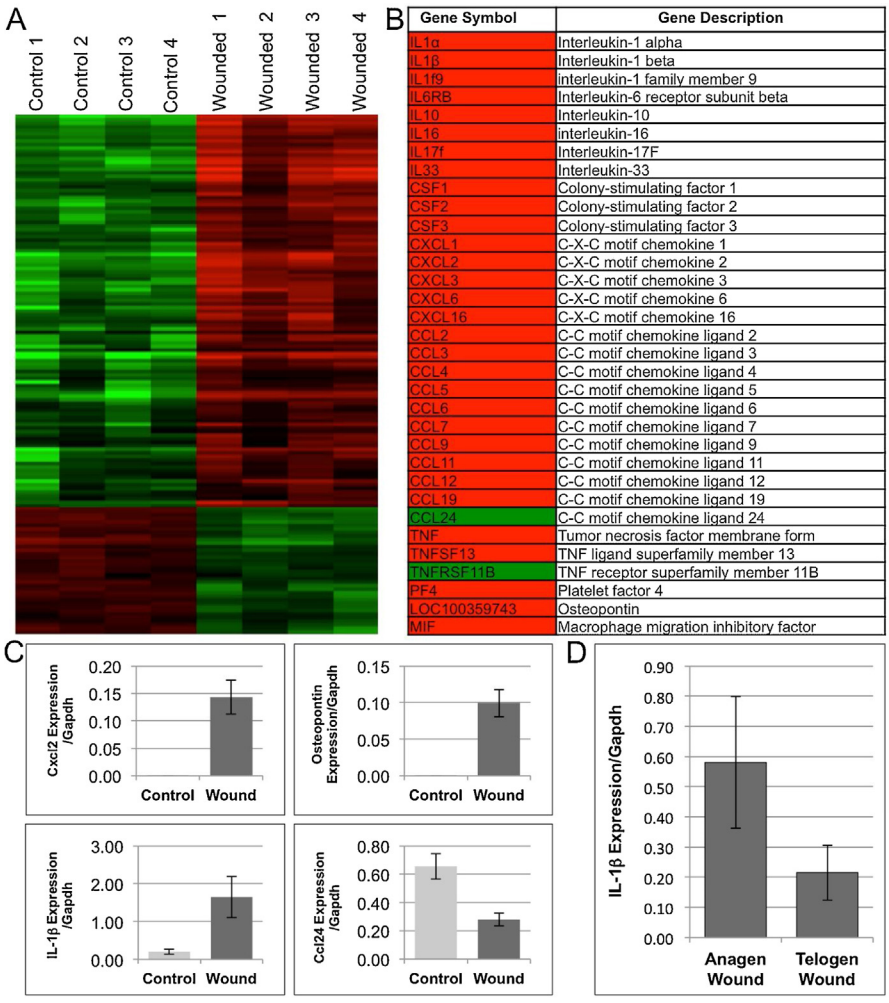

Gene Symbol	Gene Description
IL1α	Interleukin-1 alpha
IL1β	Interleukin-1 beta
IL1f9	interleukin-1 family member 9
IL6RB	Interleukin-6 receptor subunit beta
IL10	Interleukin-10
IL16	interleukin-16
IL17f	Interleukin-17F
IL33	Interleukin-33
CSF1	Colony-stimulating factor 1
CSF2	Colony-stimulating factor 2
CSF3	Colony-stimulating factor 3
CXCL1	C-X-C motif chemokine 1
CXCL2	C-X-C motif chemokine 2
CXCL3	C-X-C motif chemokine 3
CXCL6	C-X-C motif chemokine 6
CXCL16	C-X-C motif chemokine 16
CCL2	C-C motif chemokine ligand 2
CCL3	C-C motif chemokine ligand 3
CCL4	C-C motif chemokine ligand 4
CCL5	C-C motif chemokine ligand 5
CCL6	C-C motif chemokine ligand 6
CCL7	C-C motif chemokine ligand 7
CCL9	C-C motif chemokine ligand 9
CCL11	C-C motif chemokine ligand 11
CCL12	C-C motif chemokine ligand 12
CCL19	C-C motif chemokine ligand 19
CCL24	C-C motif chemokine ligand 24
TNF	Tumor necrosis factor membrane form
TNFSF13	TNF ligand superfamily member 13
TNFRSF11B	TNF receptor superfamily member 11B
PF4	Platelet factor 4
LOC100359743	Osteopontin
MIF	Macrophage migration inhibitory factor

Fig. 3. Strongly induced cytokine and chemokine gene expression in wounded skin 36 h post wounding. (A) Gene expression signature in wounded rat skin differed from the control unwounded skin, as indicated by hierarchical cluster analysis of microarray data. (B) A set of genes that were up-regulated (red) or down-regulated (green) by more than two folds in wounded skin compared with unwounded skin. (C) Quantitative real-time qPCR analysis confirmed increased expression of *Cxcl2*, *Osteopontin*, and *IL-1β*, and decreased expression of *Ccl24*, in wounded skin compared with unwounded skin, normalized to *Gapdh* expression. Bars = SD. (D) IL-1β was highly expressed 24 h post wounding in both anagen (n = 7) and telogen mouse skin (n = 9) ($p = 0.11$). Bars = SEM.

could reproduce the CIA-protective effects exerted by wounding (Table 2). The factors we tested included IFN-γ, TNF-α, EGF, FGF-1, TGF-β, and IL-1β (Schneider et al., 2009; Sugawara et al., 2010; Tong and Coulombe, 2006; Yu et al., 2008). Cytokines or growth factors were injected subcutaneously daily on PD3-5 into unwounded rats before administration of etoposide, a topoisomerase inhibitor, on PD11-13. Interestingly, only IL-1β injection protected against etoposide-induced alopecia at the injection sites (Fig. 4A, and data not shown), suggesting that the CIA-protective effect of wounding is IL-1β specific.

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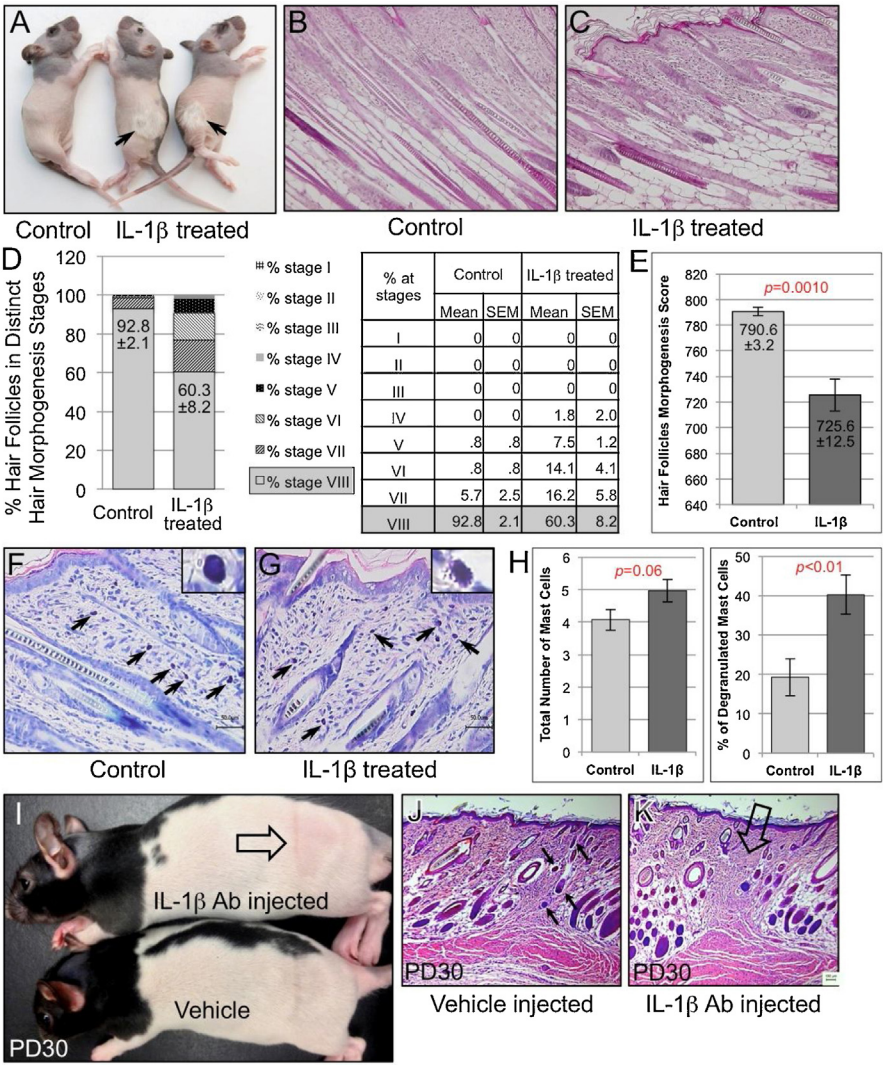

% at stages	Control		IL-1β treated	
	Mean	SEM	Mean	SEM
I	0	0	0	0
II	0	0	0	0
III	0	0	0	0
IV	0	0	1.8	2.0
V	.8	.8	7.5	1.2
VI	.8	.8	14.1	4.1
VII	5.7	2.5	16.2	5.8
VIII	92.8	2.1	60.3	8.2

Fig. 4. Local IL-1β is necessary and sufficient to protect from CIA by retarding HF morphogenesis. (A) Gross phenotype of IL-1β and vehicle pre-treated rats after etoposide treatment on PD21 (n = 6 each). (B–E) H&E staining (B, C) and quantitative HF staging (D, E) showed delayed HF cycling in IL-1β treated rats on PD13 compared with vehicle treated controls. Many fewer HFs were of stage VIII in IL-1β treated compared with control skin (D). Table shows the mean and SEM values of percentages of HFs at different stages. N = 5 each. This observation was confirmed by the lower HF morphogenesis score in IL-1β treated skin (E). Error bars = SEM. (F–H) Giemsa staining (F, G) and semi-quantitative histomorphometry (H) showed increased mast cell degranulation in IL-1β treated skin. Arrows point at mast cells (F, G). Insets show intact (in F) and degranulated (in G) mast cells. Error bars = SEM. (I–K) Gross phenotype (I) and H&E staining (J, K) showed that IL-1β neutralizing antibody (Ab) injection at the wound site inhibited hair re-growth after etoposide treatment. Block arrow points to lack of hair regrowth (I) and HF structures (K) at IL-1β antibody injected wound site, and arrows point to HFs in the vehicle injected wound site (J) (n = 6 each).

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We then tested whether IL-1β could also protect against CIA caused by other chemotherapy regimens: cyclophosphamide, an alkylating agent; or the combination of cyclophosphamide and doxorubicin, an anthracycline antitumor antibiotic. Pretreatment with IL-1β protected against hair loss caused by both cyclophosphamide and the combination of cyclophosphamide and doxorubicin**.** Thus, IL-1β injections reproduced the protective effects of wounding against CIA caused by pharmacologically distinct classes of chemotherapeutics that are currently used in the clinic for cancer treatment.

3.4. IL-1β protects HFs from CIA by retarding postnatal HF morphogenesis

IL1-β was previously shown to be a potent inhibitor of HF growth in culture and to promote catagen progression in organ-cultured human scalp HFs (Philpott et al., 1996). These observations suggest that the CIA-protective effects of IL-1β may be mediated by inhibition of postnatal HF morphogenesis, which closely resembles anagen, the cyclic HF regeneration later in life (Hoffmann et al., 1998; Muller-Rover et al., 2001; Schneider et al., 2009). Therefore, we analyzed the effects of IL-1β on HF morphogenesis, in the absence of chemotherapy.

We injected rat pups subcutaneously with either IL-1β or vehicle, on PD3-5 daily, and analyzed skin histology and hair growth. Hematoxylin and eosin staining showed delayed postnatal HF morphogenesis in IL-1β pre-treated skin compared with control skin on PD13 (Fig. 4B, C). The significant delay in HF progression by IL-1β pre-treatment was confirmed by quantitative HF histomorphometry (Fig. 4D, E). Compared with control, rats pretreated with IL-1β had many more HFs in earlier morphogenesis on PD13, with only 60.3% of HFs at morphogenesis stage VIII compared with 92.8% in the control (Fig. 4D). This delay was confirmed by a much lower hair morphogenesis score in the IL-1β pre-treated skin than control, 725.6 ± 12.5 versus 790.6 ± 3.2 ($p = 0.0010$) (Fig. 4E), indicating that vehicle-treated skin had progressed further in postnatal HF morphogenesis than IL-1β treated skin. Since HF morphogenesis closely resembles anagen progression (Botchkarev and Paus, 2003; Lee and Tumbar, 2012; Schneider et al., 2009), these data suggest that IL-1β pre-treatment on PD3-5 may have protected the developing HFs from CIA by retarding their progression towards morphogenesis stage VIII, the stage maximally sensitive to anti-proliferative drugs.

Because skin mast cell degranulation has been detected in early anagen and is associated with the modulation of anagen progression (Paus et al., 1994), we assessed the degranulation status of mast cells in IL-1β and vehicle treated skin. Whereas the total number of mast cells was comparable between IL-1β and vehicle treated skin (Fig. 4F–H), IL-1β treated skin showed a 2-fold increase in the percentage of degranulated mast cells, and the increase was statistically significant

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(Fig. 4H). IL-1β is secreted by various cell types during early wound healing, and can recruit many types of immune cells. At this point it is unclear whether the degranulated mast cells were the main cellular source of the IL-1β detected in wounded skin, or if secreted IL-1β recruited the mast cells.

3.5. IL-1β is indispensable for wound healing-associated CIA protection

To determine whether IL-1β is necessary for wound healing-associated protection from CIA, we blocked the effects of IL-1β by injecting rat-specific IL-1β neutralizing antibody at the wound site. We injected IL-1β antibodies or vehicle on PD2 at the site of prospect wound, induced wound on PD3, and injected IL-1β antibodies or vehicle again around the wound site on PD3 and for the next 3 days. Pups were subjected to etoposide chemotherapy on PD11-13 and monitored for alopecia, hair regrowth and wound healing until PD30 (Fig. 4I–K). As expected, hair loss protection was seen in vehicle-treated wounds but not in IL-1β neutralizing antibody-treated wounds (PD21, data not shown). Additionally, IL-1β neutralizing antibody treated wound sites showed no hair re-growth at PD30 (Fig. 4I). Histological analysis also revealed a lack of HF structures at the wound site at PD30 (Fig. 4K), long after HF damage by etoposide. These results indicate that IL-1β mediated signaling events are not only sufficient, but also indispensable for rendering protection against CIA if administered before chemotherapy-associated HF damage.

4. Discussion

In this study, we show that wounding of neonatal rat skin protects from chemotherapy-induced hair loss at the wound sites. We further demonstrate that an upregulation of the proinflammatory cytokine IL-1β in the cutaneous wound microenvironment is both necessary and sufficient to render such protection.

The current data agree with our previous demonstration that IL-1β protected HFs against cytarabine-induced alopecia in rats (Jimenez et al., 1991; 1992). However, in this study we administered IL-1β a week prior to chemotherapy rather than administering them simultaneously, making direct interactions of IL-1β with the damaging effects of chemotherapy on the HF unlikely (Paus et al., 2013). In support of this notion, we show that protection was achieved by delaying postnatal HF development, thereby rendering the follicle less susceptible to antiproliferative assailants. These results are consistent with previous findings in human HF organ cultures that IL-1β is a highly potent inhibitor of HF growth (Xiong and Harmon, 1997). IL-1β knockout mice are viable and fertile, despite an impaired acute-phase inflammatory response (Zheng et al., 1995). Loss of protection from CIA in

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wounded IL-1β knockout mice under our experimental settings would validate our conclusions.

In this study, we show that wounding or IL-1β-induced protection against CIA occurs with pharmacologically distinct classes of chemotherapy agents currently used in the clinic for cancer treatment. CIA is one of the most distressing adverse effects of cancer chemotherapy, with significant negative psychological repercussions for patients (Paus et al., 2013). While reversible in most patients, CIA can also be permanent (Miteva et al., 2011; Palamaras et al., 2011). On a cellular level, CIA is caused by the cytotoxicity of anticancer drugs on the rapidly proliferating HF matrix cells during late anagen (Paus et al., 2013). Although some therapies, such as scalp cooling and the immunomodulator AS101, have reduced the CIA burden in clinical trials, fully satisfactory preventive measures remain to be developed (Paus et al., 2013). In this study, maintaining HFs in the early postnatal morphogenetic stages through wounding rendered protection from CIA in young rats. Although HF morphogenesis is a developmental process quite distinct from the regenerative anagen during each HF cycle, they share many cellular and molecular pathways. The Wnt/β-catenin pathway, TGF-β, Hedgehog, Notch, BMPs and FGFs have all been implicated in both HF morphogenesis and postnatal HF cycling, as well as wound healing (Akita et al., 2013; Bielefeld et al., 2013; Lewis et al., 2014; Sennett and Rendl, 2012; Shi et al., 2015).

Because HFs of earlier anagen stages are less susceptible to CIA (Paus et al., 2013), manipulation of the HF cycling should be explored as a novel approach to prevent CIA. In this study, wounding of neonatal rat skin caused a delay in HF morphogenesis, arresting the HFs in earlier stages, therefore providing protection against CIA. Such protective effects were recapitulated by IL-1β injections, and abrogated by IL-1β antibodies. To translate such findings into the clinic to prevent CIA, one could mimic the wounding process or use transdermal deliveries of small molecules to activate/inhibit related pathways.

The potential applications of our findings are not necessarily limited to the prevention of CIA. Manipulation of the microenvironment by wounding or IL-1β treatment may be applicable to promote or inhibit hair growth. Wounding by microneedling may be used to stimulate hair growth, as it has been shown in men with androgenetic alopecia who failed to respond to conventional therapy of finasteride and 5% minoxidil solution (Dhurat and Mathapati, 2015). Additionally, accelerated hair regrowth was documented when the skin was wounded with ablative fractional laser treatment in a murine model (Bae et al., 2015). Furthermore, manipulation of the IL-1β pathway, e.g., treatment with IL-1β, its pathway components, or small molecules that activate or inhibit the pathway, may be therefore used to interfere with the HF cycle. Such manipulation may provide

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therapeutic benefits to a variety of conditions, from prevention of alopecia to inhibition of hair growth and the treatment of hirsutism.

Declarations

Author contribution statement

Olivera Stojadinovic, Tongyu C Wikramanayake: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Marjana Tomic-Canic, Joaquin Jimenez: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ralf Paus: Conceived and designed the experiments; Analyzed and interpreted the data.

Alexandra C. Villasante Fricke: Performed the experiments; Wrote the paper.

Natalie C. Yin: Performed the experiments.

Liang Liang, Eleanor Hinde, Julia Escandon, David Ansell: Performed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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