Journal of Dermatological Science xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Opinion article

Pigmentation of regenerated hairs after wounding☆

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ARTICLE INFO

Article history:
Received 24 May 2016
Received in revised form 20 June 2016
Accepted 6 July 2016

Keywords:
De novo hair regeneration
Melanocyte stem cell
Keratinocyte stem cell
Wnt signaing
Wnt7a
Kitl

ABSTRACT

Background: After severe wounding, hair follicles were known to be regenerated *de novo* along with the re-epithelialization. However, the regenerated hairs lack pigmentation.
Objective: We aimed to find out the condition to regenerate pigmented hairs after severe wounding.
Methods: De novo hair regeneration was observed during the re-epithelialization process after the full thickness excision of dorsal skin. Hair pigmentation mechanism was assessed by the modulation of Wnt and Kit signalings.
Results: Stable regeneration of pigmented hairs was demonstrated when a wound was created to the mice during the anagen stage of the hair cycle. A significant increase in the number of melanocyte stem cells in the postnatal 1st anagen interfollicular skin of 5-week-old mice was observed. An increase of Wnt7a of the keratinocytes was observed in the skin at this stage, which may direct melanocyte stem cells to produce pigmented hairs in the regenerating follicles. This was supported by the finding that transgenic mice expressing the melanocyte stimulatory factor Kitl in their skin promoted the regeneration of pigmented hairs irrespective of the stage of the hair cycle.
Conclusion: Our results provide a new insight into the intimate regulation process between two follicular stem cell systems, keratinocyte stem cells and melanocyte stem cells, during *de novo* hair regeneration after wounding.

1. Introduction

Hair formation is a complex, but well-regulated process that starts from multipotent embryonic progenitor keratinocytes. Once hair follicles have been formed, they regularly regenerate from hair follicle-associated keratinocyte stem cells throughout adult life [1–4]. Although hair follicle neogenesis is rarely observed in adult [5], recent findings on involvement of the molecular mechanisms in hair follicle development have provided solid evidence for hair follicle neogenesis associated with re-epithelialization during skin wound healing process in mice [6].

De novo hair follicle regeneration started in the center of large healing wound which was created by the excision of 1 cm^2 of full-thickness skin. These regenerated hair follicles were barely indistinguishable from normally developing hair follicles, except the fact that no pigmentation was seen on them, including the colored skin mice. While the very early stage of *de novo* hair follicle regeneration is hard to follow, *de novo* regenerating follicles of 2 weeks after the wounding resemble normally developing hair follicles from the viewpoint of molecular and morphological characteristics [6–11]. As we used black hair C57BL/6 mice, we did not expect the complete lack of follicular melanocytes in these *de novo* regenerated hair follicles. During regular hair cycles, hair follicles arise from follicular keratinocyte stem cells residing in the bulge region and these stem cells may provide a niche for follicular melanocyte stem cells [4,12–17]. Therefore, the close relationship between follicular keratinocytes and melanocytes, both of which are essential to generate pigmented hairs, must be established during normal hair development and subsequent cyclic regeneration.

Our experiments using hair follicle reconstitution assay with follicular keratinocytes and melanocytes revealed that only epidermal melanocytes were integrated into hair follicles even under conditions that both epidermal and dermal melanocytes were equally interactive physically with keratinocytes [18]. By investigating the reason why only hairs lacking pigmentation were

☆ This study was supported in part by Grants-in-Aid for Scientific Research of JSPS (25670092) to T.K. and Gifu University KASSEIKA-KEIHI to H.A.
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http://dx.doi.org/10.1016/j.jdermsci.2016.07.004
0923-1811/

formed during the hair follicle neogenesis after wounding, we might be able to understand the nature of the coordinated interaction between follicular keratinocyte stem cells and follicular melanocyte stem cells. We initially identified the condition allowed to regenerate pigmented hairs after wounding and then observed the behavior of melanocytes in the re-epithelializing skin after wounding.

2. Methods

2.1. Experimental mice

All animal experiments were approved by the Animal Research Committee of the Graduate School of Medicine, Gifu University (Form 20-138 issued 05 Feb 2009; Form 22-32 issued 06 Jan 2011). C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). The transgenic mice were maintained in our animal facility: those generated with human cytokeratin 14 promoter (hk14) driving cytokine/growth factor cDNAs (*hk14-Kitl*-Tg mice [19]; *hk14-ET3*-Tg mice [20]) were maintained as hemizygous on the *DCT-LacZ* transgenic mouse [21] background and littermate wild-type animals were used as controls.

All the mice were housed in standard animal rooms with food and water *ad libitum* under controlled humidity and temperature ($22 \pm 2\,^{\circ}C$) conditions. The room was illuminated by fluorescent lights that were turned on from 8:00 to 20:00 daily.

2.2. Wounding

The mice were anesthetized with 2,2,2-Tribromoethanol and the full thickness excision of skin was made on the dorsal trunk region using scissors. Skin areas of $1\,cm^2$ and $2.25\,cm^2$ were excised from 3-, 5-, and 7 week-old mice, respectively. Thereafter, left untreated open wounds were allowed to heal spontaneously.

2.3. LacZ staining

LacZ staining was performed as reported in detail previously [22]. In brief, skins were fixed in 2% paraformaldehyde supplemented with 0.2% glutaraldehyde and 0.02% Tween-20 for 30 min. After three washes in PBS, the skins was stained overnight at 37.1 °C in 10 mM phosphate buffer (pH 7.2) containing 1.0 mM $MgCl_2$, 3.1 mM $K_4Fe(CN)_6$, 3.1 mM $K_3Fe(CN)_6$, and 2 mg/ml X-Gal. The staining reaction was stopped by washing in PBS. Then the specimens were post-fixed overnight in 10% formalin in phosphate buffer (pH 7.2).

2.4. Hair plucking

Hair follicles were allowed to be regenerated after the wounding at 5 week-old anagen skin. When the hair follicles were fully regenerated after 3 months, we plucked the hair from the regenerated hair follicles.

2.5. Application of lithium chloride during wound healing

20 mM LiCl in 50% of EtOH was applied topically to the open wounds of the mice. 200 μL of the solution was gently ejected from the sterile pipette tip directly to the wound daily for 7 days. Thereafter, wounds were allowed to heal spontaneously [23].

2.6. Histology

The skin was fixed by immersion overnight in 10% formalin in phosphate buffer (pH 7.2). The methods used for the histological analysis have been described in detail previously [24]. Briefly, skin was dehydrated with ethanol, soaked in xylene, and embedded in paraffin. Vertical 3-μm-thick serial sections were prepared and stained with hematoxylin and eosin (HE). Morphological aspects of the hair follicles during the hair cycle were referenced from Muller-Rover et al. [25].

2.7. qRT–PCR analysis

Total RNA from freshly isolated mouse (n = 3) full thickness dorsal skin was purified using RNeasy (Qiagen, Germany). PrimeScript RTKit (Takara, Japan) was employed for cDNA synthesis, according to the manufacturer's instructions. Quantitative real-time PCR analyses were conducted using SYBR premix Ex Taq (Takara, Japan) and the Thermal Cycler Dice RealTime system II (Takara, Japan). The following PCR conditions were applied: 10 min 95 °C initial denaturation, cyclic denaturation at 95 °C for 5 s followed by an annealing step at 60 °Cfor 30s. The primer pairs were designed as shown in Supplementary Table 2. Fold differences between P21 telogen, P35 anagen, and P49 2nd telogen mice as well as age and sex-matched wild-type controls were calculated based on the DDCt method, adjusted to Gapdh expression, and are depicted as fold changes normalized to wild-type expression.

3. Results

3.1. Hair cycle-dependent pigmentation of wounding-induced hair follicle neogenesis

Previous studies demonstrated that hair follicles that formed *de novo* after full-thickness dorsal skin excision exclusively contained unpigmented hairs [6,13,26,27]. This finding was attributed to melanocyte precursors distributed in the skin being promptly lost after birth except those in the hair follicles [28]. Since the nascent hair follicles regenerated during wound healing arise from epithelial keratinocyte stem cells outside of the existing hair follicles [6], absence of the melanocyte precursors from the nearby skin may naturally result in the formation of unpigmented hairs. However, we frequently observed interfollicular melanocyte precursors marked by the melanocyte lineage-specific marker *DCT-LacZ* in the skin of the postnatal 1st anagen stage (5-week-old; Fig. S1). Thus, we hypothesized that the *de novo* regeneration of pigmented hair follicles occurred if full-thickness skin was excised during the anagen stage. In addition, previous de novo hair follicle regeneration experiments were exclusively performed only in the telogen stage of the hair cycle [6].

We therefore firstly studied wound healing-induced *de novo* hair regeneration starting from various stages of hair cycles: full-thickness dorsal skins were excised from 3-week-old (1st telogen), 5-week-old (1st anagen) and 7-week-old (2nd telogen) mice (Fig. 1A). To detect melanocyte lineage cells including melanocyte precursors (described below), we used *DCT-LacZ* transgenic mice expressing *LacZ* reporter gene under the melanocyte lineage specific *DCT* promoter [21,19]. No significant difference was observed in the rate of *de novo* hair regeneration or the morphology of each hair follicle in the dorsal skin of the 5-week-old (1st anagen) *DCT-LacZ* transgenic mice with a C57BL/6 background or their litters without transgenes (Fig. S2). To quantify and compare the pigmentation of wound-induced regenerated hairs in various conditions, we shaved the hair around the re-epithelialized areas and then photographed the regenerated hair. We only counted hairs of the re-epithelialized areas containing at least 10 regenerated hairs, and only those more than one third of the regenerated hairs were pigmented were designated as areas containing pigmented regenerated hairs. Under this classification, 15 re-epithelialized areas were found to contain no pigmented hairs and only 1 contained pigmented hairs in 76 re-epithelialized

Please cite this article in press as: M. Yuriguchi, et al., Pigmentation of regenerated hairs after wounding, J Dermatol Sci (2016), http://dx.doi.org/10.1016/j.jdermsci.2016.07.004