Negative Evidence of Direct Differentiation from Bone-Marrow Cells to Keratinocytes in Normal and Wounded Skin Using Keratin 5-Specific Reporter Mice

TO THE EDITOR

It remains controversial whether bone marrow (BM) cells can directly differentiate into keratinocytes in vivo (Fan et al., 2006; Graf et al., 2014; Krause et al., 2001). Some previous studies have shown the existence of BM-derived keratinocytes using BM chimeric mice with ubiquitous promoter-driven reporter mice such as CAG-GFP mice (Borue et al., 2004; Brittan et al., 2005; Chino et al., 2006; Graf et al., 2014; Krause et al., 2001). Distinguishing GFP⁺ keratinocytes from GFP⁺ immune cells is challenging, however, because large numbers of BM-derived immune cells such as Langerhans cells and γδ T cells are recruited into the epidermis. In addition, it is difficult to distinguish these two populations by morphology or by surface markers with conventional histological analysis because morphological information can be lost and cells are overlapped in two-dimensional vertical sections.

To solve these problems, we crossed keratin 5 (K5)-Cre mice, which express Cre recombinase under the control of K5 promoter (Tarutani et al., 1997), with R26-stop–TdT mice (Madisen et al., 2010), in which cells are labeled with a red fluorescent protein (TdT) at the time when they express Cre recombinase. All mice experiments were performed under institutional approval. Using these K5-reporter mice, we observed ear skin with two-photon microscopy (IX-81; Olympus, Tokyo, Japan) to visualize the three-dimensional spatial distribution of TdT⁺ cells (Kabashima and Egawa, 2014). Interfollicular epidermal keratinocytes (Figure 1a, left), follicular keratinocytes, and sebaceous glands (Figure 1a, right), as well as eccrine sweat glands in the footpad (Figure 1b) were labeled with TdT, suggesting that these cells are derived from K5⁺ cells. Thymic epithelial cells were also labeled with TdT (Figure 1c), because these cells are well known to express K5. To our surprise, we found a duct-like TdT⁺ structure in the dermis (Figure 1d, red). This structure was distinct from the blood capillaries, which were labeled with an intravenous injection of fluorescein isothiocyanate-dextran (Figure 1d, green) and was surrounding the isolectin-labeled neurons (Figure 1e, green), suggesting that these cells are Schwann cells. In the epidermis, dendritic cells were TdT⁻ (Figure 1f), and flow cytometric analysis showed that these cells were CD45⁺ immune cells (Figure 1g, left). There were no TdT⁺ cells in the BM of K5-reporter mice (Figure 1g, right).

To distinguish BM-derived K5⁺ cells from BM-derived immune cells, we generated BM-chimeric mice using K5-reporter mice as donors. Lethally irradiated (600 rad, two times) C57BL/6-albino mice were transplanted with one million BM cells each from K5-reporter mice. As a comparison, another group of BM chimeric mice was generated using BM cells from CAG-GFP mice, in which not only BM-derived keratinocytes but also BM-derived immune cells were labeled with GFP. Twelve weeks after BM transplantation, the ear skin of each group was observed with two-photon microscopy. In BM chimeras with K5-reporter mice, we could not detect any TdT⁺ cells among either interfollicular or follicular keratinocytes (Figure 2a, left). Likewise, no TdT⁺ cells were observed.

Abbreviations: BM, bone marrow; K5, keratin 5

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in the dermis (Figure 2a, right). In BM chimeras with CAG-GFP mice, in contrast, a small number of GFP+ cells exhibiting a dendritic morphology were observed in the epidermis (Figure 2b, left), and large numbers of GFP+ cells were found in the dermis (Figure 2b, right). These observations suggest that, in the steady state, BM-derived cells in the skin are immune cells and that no K5+ keratinocytes are of BM origin. It must be mentioned that a very small number of dendritic cells in the epidermis were labeled with TdT in BM chimeras with K5-reporter mice (Figure 2a, left, arrowheads). These TdT+ cells were CD45+ MHC class II+EpCAM+ (Figure 2c), suggesting that a small population of Langerhans cells express K5 at least in a certain period of their development or that few Langerhans cells may have obtained Cre recombinase protein from keratinocytes.

We also tried to detect TdT+ cells during wound healing, because some previous studies have reported that BM-derived keratinocytes are detectable only during this process (Badiavas et al., 2003; Borue et al., 2004). BM chimeric mice were anesthetized, and a wound was made on the back skin using an 8-mm round scalpel. At 2–3 weeks later, when the wounds had shrunk and re-epithelialized, the back skin was harvested and observed under two-photon microscopy. In BM chimeras with K5-reporter mice, no TdT+ cells were found in the re-epithelialized area (Figure 2d), as in steady state. In GFP BM-transplanted controls, in contrast, large numbers of GFP+ cells were observed in the re-epithelialized area (Figure 2e). These results suggest that, during the wound healing process, many BM-derived immune cells accumulate around the wound and intermingle in the re-epithelialized area, although no direct conversion of BM cells to keratinocytes occurs.

This study provides evidence against BM cells’ plasticity for keratinocytes in both normal and wounded skin and shows a massive intraepidermal infiltration of immune cells during the wound healing process. These infiltrating immune cells may be misidentified as BM-derived keratinocytes in vertical sections, particularly in BM chimera with ubiquitous promoter-driven reporter mice. Here, we clearly distinguished BM-derived

Figure 1. TdT+ cells in the K5-reporter mice. (a) The ear skin of a representative K5-reporter mouse. Epidermal side (left) and dermal side (right) are shown. Blue represents dermal collagen fibers detected with second harmonic generation. (b) Eccrine sweat gland in the footpad (arrow). (c) TdT+ cells in the thymus (red). (d, e) TdT+ cells in the dermis (red). (d) Blood capillaries (green), (e) neurons (green), and hair follicles (asterisks) are shown. (f) High-magnification view of the epidermis. (g) Flow cytometry of epidermal (left) and BM cells (right) from K5-reporter mice. Scale bars = 100 µm in a and c and 20 µm in b, d, e, and f. BM, bone marrow; K5, keratin 5.
keratinocytes from BM-derived immune cells using K5-reporter mice. Furthermore, we obtained high-resolution horizontal images of the skin by means of two-photon microscopy to examine cell morphology and confirmed the absence of BM-derived keratinocytes.

Our study supports a previous report that denied the occurrence of cell fusion between BM cells and keratinocytes (Harris et al., 2004) because no TdT\(^+\) keratinocytes were found in BM chimeras with K5-reporter mice (Figure 2a and d). However, cell fusion or cell engulfment may occur between keratinocytes and Langerhans cells, because TdT\(^+\) cells with dendritic morphology were observed in BM chimeras with K5-reporter mice (Figure 2a).

This study does not oppose the efficacy of BM transfer, especially the transfer of mesenheric stem cells in the BM, for the promotion of wound healing (Chino et al., 2008; Sasaki et al., 2008). BM-derived cells accumulate in the wounded area and provide cytokines that are beneficial for wound closure, although direct differentiation into keratinocytes is undetected, at least in our model. Further studies are needed to address whether BM-derived keratinocytes can be found in other different injury conditions such as bullous disease models.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Gyohei Egawa\(^1,*\) and Kenji Kabashima\(^1,2,*\)

\(^1\)Department of Dermatology, Kyoto University Graduate School of Medicine, Kyoto, Japan; and \(^2\)Singapore Immunology Network (SIgN) and Institute of Medical Biology, Agency for Science, Technology and Research (A*STAR), Biopolis, Singapore

*Corresponding author e-mail: gyohei@kuhp.kyoto-u.ac.jp or kaba@kuhp.kyoto-u.ac.jp

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