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Evidence from the stop-EGFP mouse supports a niche-sharing model of epidermal proliferative units

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Abstract: The classical model of epidermal proliferative units (EPUs) postulates that each EPU is composed of a single column of corneocytes plus epidermal cells directly below the column and is maintained by a single stem cell within the unit. Using the stop-enhanced green fluorescent protein (stop-EGFP) transgenic mouse system, we previously showed epidermal stem cell clonal lineages could produce multiple adjacent corneocytes (i.e. epidermal cells belonging to multiple adjacent EPUs), contradicting the classical EPU model. One possible problem with our earlier study was that *N*-ethyl-*N*-nitrosourea (ENU) was used to generate mutations for clonal analysis. This could alter the normal environment of the epidermal tissue and might lead to an artificial expansion of stem cell clonal lineages. In this study, we replicate our earlier findings using untreated stop-EGFP mice and relying on spontaneous mutations to generate clonal cell lineages. We propose an alternative to the classical EPU model to explain the dynamic nature of epidermal proliferation. Our niche-sharing model of EPUs allows epidermal cells to horizontally migrate among EPUs, so that multiple stem cells cooperatively maintain a larger proliferative compartment.

Simon Ro[1] & Bruce Rannala[2]

[1]Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada;
[2]Genome Center, University of California Davis, Davis, CA, USA

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Corresponding author: Simon Ro
Department of Medical Genetics
839 Medical Sciences Building
University of Alberta
Edmonton, Alberta
Canada T6G2H7
Tel.: +1 780 492 3149
Fax: +1 780 492 1998
e-mail: simon.ro@ualberta.ca

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Introduction

In many constantly renewing tissues, such as the epidermis and the epithelium of the small intestine, a distinct compartment of cellular proliferation is thought to exist which functions as a unit of tissue renewal (1). A single or a few stem cells located within a proliferative compartment (also referred to as a proliferative unit) maintain the unit by constantly producing cells, which make up for the cells being lost. In the mouse dorsal epidermis, it has been proposed that a proliferative unit is composed of a single column of corneocytes plus epidermal cells directly below the column (2,3). According to the model proposed by Potten and colleagues (4,5), each epidermal proliferative unit (EPU) contains 10.6 cells on average in the basal layer (with a stem cell in the center) and surrounding transit amplifying (TA) basal cells that descended from a central stem cell move directly upward to terminally differentiate into corneocytes. The model specifies that an EPU is a unit of cellular proliferation maintained by a single central stem cell, and thus, two neighboring EPUs (or two adjacent columns of corneocytes) will be maintained by two different stem cells located within each respective EPU.

The concept of the EPU was experimentally verified in a study by Mackenzie (6) investigating cell lineages in the mouse epidermis using retroviral transduction encoding the *lacZ* gene. In the study, it was observed that *lacZ*-labeled cell clusters derived from single stem cells corresponded to single columns of corneocytes plus epidermal cells beneath them as suggested by the EPU model. Ultra-structural morphology and kinetic data of the epidermal tissue also have supported the EPU concept (4,7,8). However, several recent reports bring the EPU model into question. Using chimeras of mice made from strains that express different H-2 antigens, Schmidt et al. (9) observed that epidermal cells of both strains were located under a single column of corneocytes (i.e. within a single EPU), suggesting that epidermal cells might migrate between EPUs, and thus, an

EPU might not be a proliferative compartment. As well, recent epidermal cell lineage studies using replication-deficient *lacZ*-carrying retrovirus suggest that a single basal stem cell might maintain multiple EPUs (10,11). The method using retroviral transduction, however, has the limitation that cell lineages expanding to multiple EPUs might be generated from multiple stem cells which are independently labeled by the retroviral infection, and thus, the clonality of cell lineages detected in the studies is not guaranteed.

Using a novel transgenic mouse system, Ro and Rannala (12) presented additional evidence to suggest that epidermal cells can migrate horizontally to adjacent EPUs and that a single stem cell can contribute to multiple EPUs. The transgenic mouse [referred to as the stop-enhanced green fluorescent protein (stop-EGFP) mouse] carries a premature stop codon-containing EGFP gene as a target gene for mutations. In the stop-EGFP system, single cells are labeled by means of random mutations that have very low frequency, and thus, the clonality of cell lineages generated in this system is certain. Using the system, we randomly labeled individual epidermal cells via treatment with a pulse of mutagen, *N*-ethyl-*N*-nitrosourea (ENU). *In vivo* imaging after several epidermal turnovers revealed green fluorescent stem cell clonal lineages containing three adjacent corneocytes (i.e. epidermal cells belonging to three adjacent EPUs). The finding strongly suggests that a single stem cell can provide epidermal cells beyond its own EPU, which contradicts the classical EPU model. More recent studies using the stop-EGFP mouse suggest that the number of EPUs that a single stem cell can contribute to can be larger than three (13). However, the question can be raised whether the stem cell lineages we observed reflect the characteristics of epidermal stem cells under the normal steady-state condition. Treatment of cells with mutagenic and cytotoxic ENU, as was done in our earlier studies, might alter the local cellular environment of epidermis or induce hyperproliferation of epidermal cells, in which case the normal behavior of stem cells might be altered. To test this hypothesis, we imaged the dorsal epidermis of 26 untreated stop-EGFP mice and investigated clonal cell lineages generated by spontaneous mutations.

Methods

Animal experiments

All experiments using live mice were performed in compliance with the recommendations of the Canadian Council on Animal Care and have been approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta.

Imaging of the mouse dorsal skin in vivo

At least 1 week before the *in vivo* imaging experiment, 4–10 months old stop-EGFP mice (26 mice in total) were anesthetized by inhalation of isoflurane gas. After the mice were sedated, the hair (2.5×2.5 cm area) in the lower part of the dorsal skin was clipped using electric clippers (Oster Golden A5, small animal clippers with a number 40 blade, Oster Professional Products, McMinnville, TN, USA). A depilatory agent (Nair, Church & Dwight Co., Inc., Princeton, NJ, USA) was applied to the clipped area for 2.5 min. On the day of the imaging experiment, mice were anesthetized with a mixture of ketamine and acepromazine administered intra-peritoneally. To facilitate the *in vivo* imaging, we used a mouse restraint with a window on the bottom plate. The procedures for the *in vivo* imaging study are shown in Fig. 1. The depilated area of the epidermis was illuminated by a 50 W mercury lamp and scanned using a Zeiss Axiovert 200M inverted microscope with a $\times 10$ F-Fluar lens (NA 0.5) and LP 520 emission filter (Carl Zeiss, Jena, Germany). With the emission filter (LP 520), EGFP-expressing cells exhibited typical green color while autofluorescence from the skin revealed yellowish color. Images of green fluorescent cells were collected with a confocal laser scanning microscope (LSM 510 NLO, software version 3.0, Carl Zeiss) mounted on the Zeiss Axiovert 200M with a $\times 10$ F-Fluar lens (NA 0.5). The 488-nm laser line was used to image EGFP with a band pass filter (505–530 nm) for detecting emissions. The animals had their eyes lubricated while under the microscope.

Results

To test whether expanded epidermal clonal cell lineages containing multiple adjacent corneocytes can be observed in stop-EGFP mice in the absence of treatment with ENU, we performed *in vivo* imaging of the dorsal epidermis using untreated stop-EGFP mice. In this case, any green fluorescent clonal cell lineages that are observed will have been generated by spontaneous mutations occurring at the premature stop codon within the stop-EGFP gene. Such mutations will occur at an extremely low frequency; thus, imaging of many mice is required to detect clonal cell lineages in the tissue.

In vivo imaging of the dorsal epidermis of 26 untreated stop-EGFP mice revealed four epidermal clonal cell lineages containing multiple adjacent corneocytes (Fig. 2a,b; Table 1). These observations strongly suggest that, under the normal steady-state condition, epidermal cells can migrate horizontally between EPUs, which is counter to the classical EPU model (4). The clonal cell lineage shown in Fig. 2b, in particular, reveals five (or possibly six) adjacent corneocytes (i.e. epidermal cells belonging to five, or possibly six, adjacent EPUs) which exceeds the numbers of adjacent corneocytes in clonal lineages found in the previous *in vivo* imaging studies using

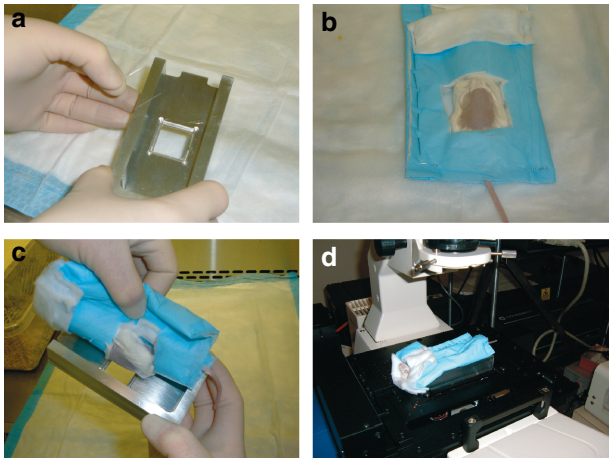

Figure 1. Procedures for the *in vivo* imaging study of the mouse dorsal epidermis. (a) A 25 × 25 mm cover slip is placed on the window of a mouse restraint. (b) To prevent a mouse from directly contacting metal surface of the restraint (and also to keep it warm), we wrap the mouse with cotton pad. (c) The mouse is placed with the depilated dorsal skin on the cover slip of the mouse restraint. (d) The constrained mouse is placed on the microscope stage of an inverted fluorescence microscope, and the depilated skin is scanned using the microscope.

ENU-treated stop-EGFP mice. In those studies, the largest clonal cell lineage observed appeared to have expanded to four EPUs (13). The *in vivo* imaging experiment using 26 untreated stop-EGFP mice also revealed single columns of corneocytes which better fit the classical EPU model (Fig. 2d,e). In total, six green fluorescent patches

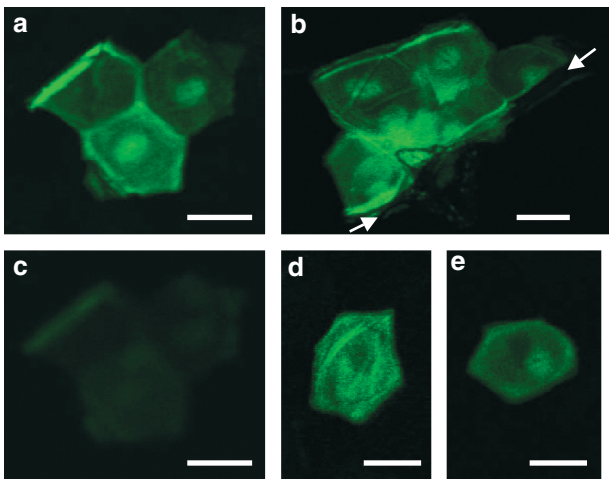

Figure 2. *In vivo* imaging of epidermal clonal cell lineages found in the dorsal epidermis of untreated stop-enhanced green fluorescent protein mice. (a, b) The *in vivo* imaging experiment revealed epidermal clonal cell lineages containing multiple adjacent corneocytes. In panel b, the morphology of corneocytes located lower is not completely seen because of the line generated by skin fold (indicated by arrows). (c) Longitudinal optical sectioning detected no green fluorescent signals at deeper layers below the three corneocytes shown in panel a. (d, e) Single columns of corneocytes which better fit the epidermal proliferative unit model were also detected. Scale bar: 20 μm.

Table 1. *In vivo* imaging of the dorsal epidermis of 26 untreated stop-EGFP mice

	Single corneocyte	Two corneocytes	Three corneocytes	Five (or six) corneocytes
Number of cell lineages found	6	2	1	1

The numbers of clonal cell lineages observed containing different numbers of adjacent corneocytes are listed.

containing single columns of corneocytes were observed (Table 1). The proportion of clonal cell lineages that contain multiple adjacent corneocytes (which contradict the classical EPU model) is therefore 40 vs. 60% of clonal cell lineages that contain single corneocytes (which fit the classical EPU model). Thus, we suggest that clonal expansion of cell lineages to multiple neighboring EPUs is a normal feature of epidermal cells under the steady-state condition.

The clonality of epidermal cell lineages found in our *in vivo* imaging experiment is guaranteed, because cells were labeled by means of random and very infrequent spontaneous mutations. The green fluorescent cell lineages that we observed in this study are less likely to be stem cell clonal lineages than those found in our previous studies, however. Epidermal clonal cell lineages observed in our previous studies contained green fluorescent signals in the basal layer where stem cells and their early descendent cells are presumed to be located (12). However, longitudinal optical sectioning of the cell lineages observed in this study detected no green fluorescent signals in the basal layer (Fig. 2c). Presumably, this would occur if a TA cell had undergone a mutation at the premature stop codon within the stop-EGFP gene, and after having undergone several rounds of cell division, the daughter cells migrated beyond the boundary of the original EPU to adjacent EPUs, as well as moving upward for terminal differentiation. Although the clonal cell lineages containing multiple adjacent corneocytes found in this study appear to have originated from mutant TA cells rather than mutant stem cells, the finding still implies that a single stem cell can contribute to multiple EPUs, because a TA cell that has undergone a mutation must originate from a single stem cell (i.e. a TA cell can always be traced back to a single stem cell).

Discussion

The findings of clonal cell lineages containing multiple adjacent corneocytes (epidermal cells

belonging to multiple adjacent EPUs) in the present study strongly suggest epidermal cells can migrate horizontally between EPUs under the normal steady-state condition. The idea of horizontal migration of epidermal cells between EPUs has been previously proposed (2,9). Although Potten (2) favored the classical EPU model in describing the cellular proliferative structure in the mouse epidermis, he also considered the possibility of horizontal migration of suprabasal cells between EPUs. The observed frequency of clonal cell lineages expanding to multiple EPUs of 40% in this study (Table 1) suggests horizontal migration of epidermal cells between EPUs occurs frequently under the normal steady-state condition.

Observations of epidermal clonal cell lineages larger than a single EPU have been reported previously in circumstances where hyperproliferation in the mouse epidermis was induced by epidermal tissue transplantation or by chronic irradiation with 280–320 nm UV light (6,14). Cell lineages generated in the UV-induced hyperproliferative epidermis show clonal expansion of basal cells to as many as 100 adjacent EPUs (14). ENU is a mutagenic and cytotoxic agent (15), and treatment with the mutagen might therefore be expected to induce some cell death with subsequent hyperproliferation of epidermal cells leading to an artificial expansion of stem cell clonal lineages. In the epithelium of the small intestine, for example, treatment with a mutagen led to an increase in the mean size of clonal cell lineages by comparison with those found in untreated normal tissue (16). Furthermore, it has been shown that application of a carcinogen can induce transient epidermal hyperplasia and affect the tissue homeostasis significantly (17,18). Thus, it is possible that our previous observations that stem cell clonal lineages expanded to multiple EPUs may have been induced by treatment with ENU (12,13). The present findings, obtained using untreated stop-EGFP mice, strongly suggest that expansion of clonal cell lineages to multiple EPUs arises under the normal steady-steady condition.

On the basis of the findings of the present study as well as our previous studies, we propose an alternative to the classical EPU model (Fig. 3). Our niche-sharing model of EPU structure specifies that, under the normal steady-state condition, a single stem cell not only maintains its own EPU but also can contribute to adjacent EPUs by providing differentiated cells. In other words, the effective proliferative compartment in the epidermis may be larger than a single EPU.

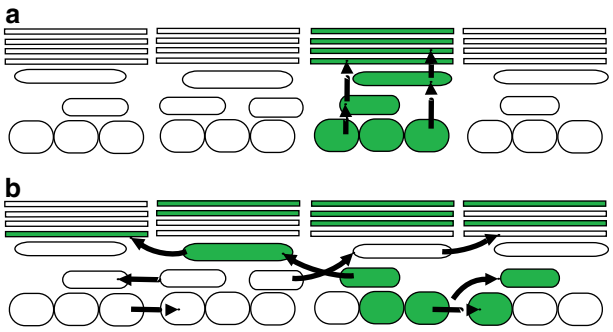

Figure 3. Comparison of the classical epidermal proliferative unit (EPU) model and the niche-sharing model of EPUs. Cells in green represent a clonal cell lineage that originated from an epidermal basal stem cell under each model. (a) The classical EPU model identifies an EPU as a clonal unit of cellular proliferation, and no horizontal migration between EPUs is expected. Thus, cellular migration occurs only within an EPU, mainly vertical migration from the basal layer to the cornified layer for terminal differentiation (see arrows). (b) The niche-sharing model proposes that epidermal cells can migrate to adjacent EPUs, and thus, a single stem cell can contribute to multiple EPUs. Arrows indicate horizontal migration of epidermal cells among EPUs.

In this model, several EPUs constitute a larger proliferative compartment whereby multiple stem cells of different EPUs cooperatively provide epidermal cells for the compartment as a whole (Fig. 3b).

The stop-EGFP system allows individual cells to be labeled by means of infrequent and random mutations, and thus, the clonality of resulting cell lineages is virtually certain. This overcomes limitations of other lineage-tracing methods, such as the retroviral transduction method, in which clonality is not certain (10,19). Furthermore, the retroviral transduction method requires hyperproliferation of epidermal cells to label quiescent stem cells in the epidermis *in vivo* (e.g. by applying dermabrasion). An alternative method uses lentiviral vectors carrying reporter genes, which allow epidermal stem cells to be labeled without induced proliferation (20). Using the lentivirus-mediated genetic marking system, Ghazizadeh et al. (21) traced epidermal stem cell lineages in human foreskin xenografted onto nude mice and found that stem cells were distributed in the basal layer in a non-uniform manner, and the sizes of labeled compartments were considerably variable. Recently, it has been reported that upper follicular cells can migrate to the epidermis and differentiate into epidermal cells in response to a penetrating wound (22), and it has been suggested that stem cells located in the bulge of the hair follicle might maintain the normal epidermis (23,24). Epidermal cellular organization might be more dynamic than previously thought with

epidermal cells migrating from one EPU to other adjacent EPUs and possibly even cells in other tissues migrating to the epidermis and differentiating into epidermal cells.

In this study, the dorsal skin of untreated stop-EGFP mice was scanned in an attempt to investigate epidermal clonal cell lineages under the normal steady-state condition. Using untreated mice for the *in vivo* imaging study allows the potentially cytotoxic effects of a mutagen to be avoided. However, one could argue that the epidermis investigated in our current study may still not represent the normal steady-state epidermis, because depilation was performed before the *in vivo* imaging study. However, the cornified layer renews every 5 days and the entire epidermal structure (from the basal to the cornified layer) renews every 2 weeks in the mouse dorsal skin (25,26). We performed the *in vivo* imaging study at least 1 week after depilation. For example, the clonal cell lineage containing three adjacent corneocytes shown in Fig. 2a was detected 12 days after depilation. Thus, even if the columnar structure of the cornified layer were disrupted by depilation, the normal epidermal structure is expected to be restored during the time period between depilation and the imaging experiment. Although it seems unlikely, the possibility that depilation could affect the normal behavior of epidermal cells cannot be completely ruled out. Investigation of the dorsal skin of hairless stop-EGFP mice could be carried out to address this issue. By successive breeding of stop-EGFP mice with nude mice, hairless stop-EGFP mice could be generated which do not require depilation before the *in vivo* imaging experiment. It would therefore be interesting to investigate whether the characteristic clonal cell lineages expanding to multiple neighboring EPUs are also observed in undepilated hairless stop-EGFP mice.

A cell fate-mapping technique that allows clonal cell lineages to be traced (such as the stop-EGFP system) would be useful for addressing various issues in stem cell biology (e.g. the potential of stem cells to produce cells of diverse tissue types). If stem cell markers were identified, one might be able to specifically trace clonal lineages that originated from stem cells in various tissues using the stop-EGFP mouse. With the aim of identifying stem cell markers, cell populations enriched (or isolated) from human skin using putative stem cell markers have been tested for prolonged self-renewal potential and proliferative capacity in various surrogate *in vitro* assays (27,28). Recently, Pouliot et al. (29) developed a technically simple method in which the capability of isolated cells to regenerate fully stratified human epidermis can be tested using an *in vivo* tracheal transplantation model. Such a system would have the potential to test putative markers for stem cells and thus to provide a better understanding of stem cell biology.

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