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Extra Views

Magnifying Stem Cell Lineages

The Stop-EGFP Mouse

Simon Ro

Correspondence to: Simon Ro; Department of Medical Genetics; 839 Medical Sciences Building; University of Alberta; Edmonton, Alberta T6G2H7 Canada; Tel.: 780.492.3149; Fax: 780.492.1998; Email: simon.ro@ualberta.ca

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KEY WORDS

clonal cell lineage, enhanced green fluorescent protein, epidermal stem cell, fate mapping, in vivo imaging, mutation, repeated analyses

ABSTRACT

Cell fate mapping techniques which can label clonal cell lineages are of importance because they allow one to investigate the distribution and types of daughter cells arising from single precursor cells. Thus, the potential of precursor cells to generate various types of descendent cells can be studied at the single-cell level. The stop-EGFP transgenic mouse carries a premature stop codon-containing enhanced green fluorescent protein (EGFP) gene as a target gene for mutations. A cell having undergone a mutation at the premature stop codon and its descendant cell lineage will express EGFP, thus a clonal cell lineage can be traced in vivo using a fluorescent microscope. Using the stop-EGFP mouse, stem cell clonal lineages in the mouse dorsal epidermis can be investigated in vivo and repeated analyses of the same cell lineages can be performed over time. In vivo imaging studies possible with the stop-EGFP mouse provide new insights into the structure of epidermal proliferative units (EPUs). The stop-EGFP system provides a novel tool for investigating clonal cell lineages in developmental studies as well as in stem cell biology.

Investigation of the distribution and the types of daughter cells that individual precursor cells give rise to is of great importance not only in developmental studies but also in stem cell biology. Recent studies imply that stem cells in an adult tissue, which have long been thought to produce only limited cell types in a given tissue, have the capability to produce even cells of developmentally unrelated tissue types in experimentally modified conditions.[1-4] To determine how diverse a range of cell types and how expansive a clonal cell lineage a single stem cell of a particular tissue can produce, a fate mapping technique that allows one to trace clonal cell lineages in a mammalian system in vivo is required. Although various mapping techniques have been developed so far, labeling of single cells is difficult to achieve and very few systems have been efficiently applied to fate mapping of single precursor cells (Table 1).[5,6] Green fluorescent protein (GFP) has proven to be a versatile marker in developmental studies for various organisms.[7-10] An advantage of using GFP is that imaging of GFP does not require any substrate or cofactor, or any fixation process which necessitates sacrifice of tissue or animal. Thus, one can perform live imaging of GFP-expressing cells, real-time imaging, or repeated imaging of GFP-labeled cells over time.[7,9] Direct imaging of GFP can also avoid possible artifacts which might be introduced during the process of tissue fixation.[8]

Recently, a transgenic mouse system was developed to allow in vivo imaging of clonal cell lineages via GFP.[11] Individual cells are labeled by means of random mutation and cell lineages that descended from the labeled single cells express GFP, such that clonal cell lineages can be traced in vivo. A plasmid designed to express EGFP (enhanced GFP) ubiquitously[12] was modified by introducing a premature stop codon (TAG) at a neutral codon site. A mutation at any one of the three nucleotides of the stop codon can restore EGFP function (except in the case of a mutation from G to A at the third site, which produces another stop codon, TAA) because any substitution for amino acid encoded by a codon at a neutral site does not affect the protein function. The fact that multiple copies of a transgene are usually inserted in a linear array in a transgenic mouse increases the efficiency of this system for detecting mutations. The stop-EGFP transgenic mouse that we have developed also carries the wild-type EBFP gene. The usage of the EBFP gene not only allows the stop-EGFP mouse to detect all possible point mutations arising by either transitions or transversions,[11] but also minimizes the possibility of an immune response to revertant EGFP. If EGFP is first expressed via a mutation in an adult mouse, the protein could be recognized as a foreign antigen by the immune system, and cells expressing EGFP killed by cytotoxic T lymphocytes (CTLs). A strong CTL response against EGFP-expressing cells has been detected in the BALB/c strain and a moderate CTL response in the C57BL/6

strain.[13] Because EBFP differs from EGFP by only a single amino acid, transgenic mice exposed to EBFP from conception could have no immune response to revertant EGFP.

The efficiency of the stop-EGFP system for in vivo analysis of stem cell clonal lineages was demonstrated by applying the system to the mouse dorsal epidermis.[11] Mutations were induced in stop-EGFP mice using a potent mutagen, ENU (*N*-ethyl-*N*-nitrosourea) to randomly label epidermal cells. In vivo imaging after several epidermal turnovers revealed stem cell clonal lineages containing three adjacent corneocytes (i.e., epidermal cells belonging to three adjacent EPUs) suggesting that epidermal cells migrate horizontally to adjacent EPUs.[11] Thus, a single stem cell is capable of providing epidermal cells for multiple adjacent EPUs, which contradicts the classical EPU model[14-16] but is consistent with other recent reports[17,18] (see Fig. 1 for illustration). In other words, this finding suggests the actual proliferative compartment in the epidermis may be larger than a single EPU; several EPUs might constitute a large proliferative compartment whereby multiple stem cells of different EPUs cooperatively provide epidermal cells for the compartment as a whole. The in vivo imaging data also appear to suggest that epidermal cells can migrate between EPUs not only along the basal layer, but also above the basal layer. Optical sectioning of cell lineages (e.g., see Fig. 3 in ref. 11) shows no green fluorescent signal in the basal layer beneath one of three adjacent green fluorescent corneocytes. This finding is best explained by horizontal migration of epidermal cells above the basal layer (i.e., in the suprabasal layers). If epidermal cells migrate along the basal layer, in this case, the basal cells must turn off transgene expression during horizontal migration and then restore gene expression during vertical migration for terminal differentiation, which seems very unlikely.

More recent studies that we have carried out using additional stop-EGFP mice show that the number of adjacent corneocytes that a stem cell clonal lineage can produce is dynamic. Epidermal stem cell clonal lineages containing 1–4 adjacent corneocytes have now been detected more than six weeks post-ENU administration (i.e., after more than three epidermal turnovers) suggesting that the number of EPUs that a single stem cell can contribute to might be stochastic (unpublished data). One important advantage of using our stop-EGFP system in cell lineage studies is that the system can be used for repeated analyses of the same clonal cell lineages over time, which could allow one to study the dynamics of the clonal development of a particular cell lineage. In a new set of studies carried out recently, we have successfully performed repeated in vivo imaging of the same stem cell clonal lineages in the epidermis (unpublished data). At eight weeks post-ENU administration, we performed in vivo imaging of dorsal skin of two stop-EGFP mice and found two epidermal stem cell clonal lineages (Fig. 2A and C). Four days later, the same cell lineages were imaged again. The second imaging showed that during the four days, the cell lineage shown in Figure 2A expanded from containing three adjacent corneocytes to four adjacent corneocytes (Fig. 2B), while the size of the cell lineage shown in Figure 2C remained the same (Fig. 2D). These data strongly demonstrate that it is feasible to carry out repeated observations on stem cell clonal lineages over time in a live mouse.

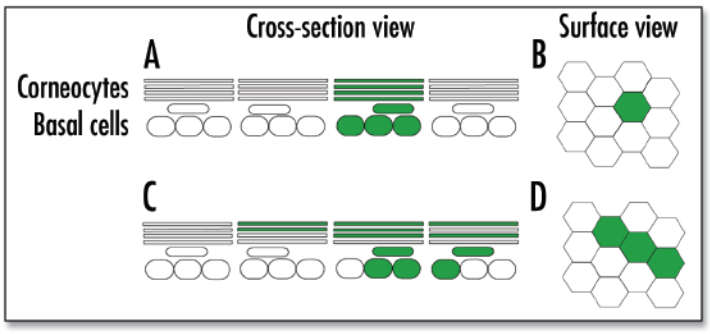

Figure 1. The two opposing models for a stem cell clonal lineage in the mouse dorsal epidermis. The classical EPU model proposes that an EPU is a clonal unit of cellular proliferation. According to the EPU model, a single stem cell having undergone a mutation at the premature stop codon of the stop-EGFP gene should generate only one green fluorescent EPU (A), which occupies area of a single corneocyte on the skin surface (B). The opposing model proposes that epidermal cells can migrate to adjacent EPUs and a single stem cell can provide epidermal cells for more than one EPU. The finding of epidermal stem cell clonal lineages containing three adjacent corneocytes (i.e., epidermal cells belonging to three adjacent EPUs) in our study[11] strongly supports the latter model. A stem cell clonal lineage detected in our study (shown in green) is schematically represented (C), which contained three adjacent corneocytes on the skin surface (D).

Cell fate mapping using the stop-EGFP transgenic mouse, which generates clonal cell lineages via mutation, is expected to be useful in revealing the potential of single stem cells to provide cells to replenish other cellular compartments, or to produce cells of diverse tissue

Table 1 **Fate Mapping Techniques Currently Used in Cell Lineage Studies**

Method	Advantages	Disadvantages	Refs.
Labeling with vital dyes	Easy to apply	Label many cells simultaneously; signals are diluted by cell division	27,28
Labeling with tritiated thymidine (^{3}H-TdR) or bromodeoxyuridine (BrdU)	Easy to apply	Label many cells simultaneously; diluted by cell division; can only label dividing cells	29
Infection of replication-deficient retrovirus carrying a reporter gene	Permanent labeling	Hard to label single cells; can only label proliferating cells	17,30
Intracellular injection of tracers	Can label single cells	Hard to label small cells or cells located deep in tissue; signals are diluted by cell division	31
The *Dlb-1* (*Dolichos biflorus*) mouse system	Single cells are labeled by random mutation	Can only be applied to the epithelium of the small intestine; requires fixation (thus, repeated analyses are not possible)	24
The *laacZ* transgenic mouse system	Single cells are labeled by rare spontaneous recombination; can be applied to diverse tissues	Requires sacrifice of tissue or animal for fixation; hard to determine when the recombination events occur during development	32,33

See also refs. 5 and 6.

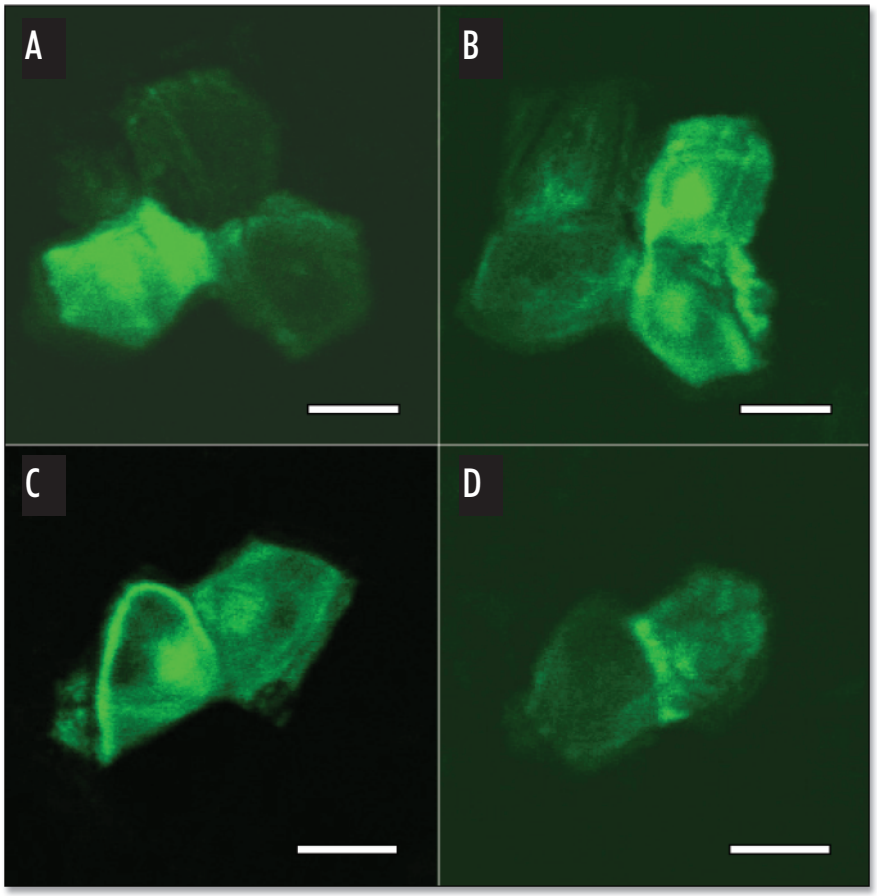

Figure 2. Repeated analyses of the same cell lineages in the epidermis over time. The first in vivo imaging performed at 8 weeks post-ENU administration revealed two epidermal stem cell clonal lineages (A and C). Four days later, the second imaging of the same cell lineages was performed in vivo (B and D, respectively). The repeated imaging shows that the stem cell clonal lineage shown in panel A expanded, while the size of the cell lineage shown in (C) remained the same. The data demonstrate that repeated analyses of specific clonal cell lineages can be carried out over time, which could allow one to investigate the dynamics of the clonal development of a cell lineage. Scale bars: 20 μm.

types. For example, there has been much interest in determining how diverse a range of types of differentiated cells a single stem cell in the respiratory tract,[19,20] the liver,[21] or the mammary gland[22] can produce, either in a normal steady state or during the regenerative processes. The stop-EGFP system could be efficiently used to address these issues of the potential of single stem (or precursor) cells to generate various types of descendent cells in those tissues. Furthermore, repeated in vivo imaging of the same tissue sites performed over time might allow a time-lapse study of the generation of various types of cells from a stem cell clonal lineage. Another advantage of using GFP as a cell marker is that external imaging of even single cells in internal organs can be performed.[23] Thus, one might be able to investigate the dynamics of the clonal development of a cell lineage in an internal organ by repeated external imaging of the stop-EGFP mouse over time.

In addition to clonal cell lineage studies in adult tissues, the stop-EGFP system also has the potential to be efficiently applied to tracing clonal cell lineages in developmental studies. Embryos can be exposed to ENU in utero at various time points during development by exposing pregnant mice to the mutagen.[24] In our system, cells can be labeled at a specific time point during development by treatment with a pulse of the mutagen, thus a clonal cell lineage can be generated that originated from a precursor cell present in a specific stage during development. Finally, the stop-EGFP system could be applied to investigating clonal cell lineages for other organisms. For example, transgenic fish that ubiquitously express GFP have been developed.[25,26] Thus, a transgenic fish which carries the stop-EGFP gene could be generated and used for tracing clonal cell lineages during development, or in constantly renewing adult tissues.

One potential weakness of the stop-EGFP mouse is that the mutation rate at the premature stop codon within the stop-EGFP gene appears to be low even after treatment with a potent mutagen, ENU. This reduces the efficiency with which labeled cells are generated via mutation in the stop-EGFP system. Imaging of a 2.5 cm X 2.5 cm area fo dorsal skin of an ENU-treated stop-EGFP mouse reveals one or fewer stem cell lineages on average. Thus, imaging of many mice might be required to detect clonal cell lineages in a tissue of interest. Repeated treatments with ENU or another mutagen could help to increase the expected frequency of mutations at the premature stop codon. Another strategy is to create a transgenic mouse carrying a very high copy number of the stop-EGFP gene. Alternatively, a transgenic mouse carrying the wild-type EGFP gene under control of a strong repressor could be generated in which a mutation at the repressor gene leads to expression of EGFP. Although the low mutation rate in the stop-EGFP system may appear disadvantageous, the extremely low rate of spontaneous mutation in the stop-EGFP mouse (e.g., no mutations were detected in the skin, kidney and liver of untreated stop-EGFP mice) is an advantage because it enables cells to be labeled at a specific time point by treatment with a pulse of mutagen.[11] Therefore, imaging experiments carried out after several tissue turnovers in an adult renewing tissue of mice treated with a mutagen could allow one to specifically trace cell lineages that descended from stem cells of the tissue. As well, treatment of embryo with a pulse of mutagen could allow clonal cell lineages to be generated that originated specifically at a time point of interest without contamination by cell lineages generated by spontaneous mutations occurring at other time points during development.

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