Lineage Tracing Mediated by Cre-Recombinase Activity

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INTRODUCTION

Clinical research aims to unravel mechanisms leading to disease. Looking at a disease state, frequently asked questions are when and how did cells change their native behavior and how do these cells circumvent regulation by the healthy environment? Answers to these questions can often be identified through the use of a powerful technique called lineage tracing. A cell lineage describes a single stem or progenitor cell that gives rise to progeny that can adopt differential cell fates. This cellular fate is represented through differential cell properties or migration to specific regions within an organ or the organism. Lineage tracing allows us to study these dynamic processes through visualization of the cell lineage within an organism. Lineage tracing originated in the field of developmental biology. In the early twentieth century, Walter Vogt and his colleagues developed a novel method by injection of "vital dyes" into single cells of an amphibian embryo to trace the cells' fate during gastrulation using light microscopy (Vogt, 1929). This technique was restricted by dilution of the dye following each cell division, and ultimately it was lost from the cell lineage. Although various ways to trace cells were described in past decades, advances in mouse transgenesis led to genetic lineage tracing techniques, which are now the preferred approaches. We will discuss how the Cre–loxP system can circumvent the problem of label dilution through induction of reporter gene expression in specific cell populations and how these reporter genes can be used to visualize cell lineages and analyze their behavior *in vivo*.

WHAT LINEAGE TRACING DOES

- Lineage tracing allows us to follow cell fate decisions of stem/progenitor cell populations and their descendants within a living organism.
- The technique is based on visualization of the cell lineage via a time and spatially controlled reporter gene expression in stem/progenitor cells that is passed on to their cell progeny.
- Lineage tracing enables us to study the dynamics of proliferation, differentiation, and migration of these cells in an *in vivo* context during development, homeostasis, and disease.

LIMITATIONS

- A detailed characterization of the Cre-recombinase and the reporter genes, in terms of both activity and specificity, is required to prevent system leakage.
- The expression of reporter genes and Cre-inducing agents such as tamoxifen may also have a direct effect on cell behavior, owing to the lack of suitable controls.

BASICS OF LINEAGE TRACING TECHNOLOGY

Several reporter systems are suitable for the labeling of cell lineages. In general, reporter genes are used for the visualization of a cell without undesired interference with intracellular processes that might affect the cell's behavior *in vivo*. To achieve controlled expression of reporter genes, the Cre–loxP system is commonly used, which allows site-specific DNA recombination and can be induced in a space- and time-dependent manner. The two most common Cre-induced reporters are β-galactosidase (β-gal) and fluorescent proteins.

The β-gal enzyme, encoded by the lacZ gene, is derived from the *Escherichia coli* bacterium, where it is involved in the hydrolysis of β-galactosides (Jacob and Monod, 1961). Cells expressing β-gal can be visualized via staining with the substrate X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside). Here, cleavage of X-gal causes a dark blue precipitate in β-gal-expressing cells and thereby allows their identification (Soriano, 1999). The main disadvantage of the β-gal reporter system is the specific staining method of the induced cells, which requires their fixation and impedes analysis of living cells within the organism. By contrast, the advantage of a fluorescent reporter is its easy visualization. The first fluorescent

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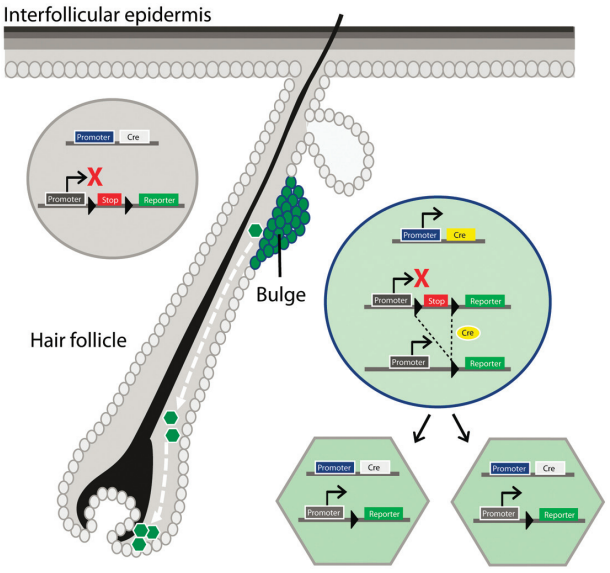

Figure 1. Lineage tracing of hair follicle bulge stem cells. Cell population–specific expression of the Cre-recombinase is achieved by placing the Cre gene under the control of a promoter specific for the cell population of interest. In cells in the bulge cell lineage (green cells), the Cre-recombinase mediates the deletion of the loxP site–flanked stop codon in front of the reporter gene and thereby induces reporter gene expression. This permanent genetic modification is passed on to the cell progeny, independent of Cre expression, and facilitates tracing of the cell lineage. In cells that are not in the bulge cell lineage (gray cell), the Cre gene is not expressed and therefore the reporter gene will not be expressed.

reporter used for lineage tracing was the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Mao *et al.*, 2001). GFP emits green fluorescent light upon exposure to UV light. Nowadays, many spectral derivates of GFP are available that allow the visualization of cells within the organism via live-cell imaging technologies and multicolor lineage tracing.

The Cre–loxP system is commonly used to control gene expression and was the topic of a previously published Research Techniques Made Simple article (Scharfenberger *et al.*, 2014). Briefly, the Cre-recombinase is a site-specific DNA recombinase that mediates the deletion or inversion of a loxP site–flanked DNA region. To induce reporter gene expression, the most common method is to introduce a loxP site–flanked stop codon in front of the reporter gene. Whereas under normal conditions the expression of the reporter is prevented by the stop codon, upon Cre expression this stop codon is excised and the reporter gene is expressed, resulting in labeling of the cell. Importantly, this permanent genetic manipulation circumvents the problem of label dilution due to the constitutive reporter gene expression in the daughter cells after a cell division.

REGULATION OF Cre ACTIVITY

To achieve cell population or tissue-specific Cre activation, the Cre-recombinase is expressed under the control of a promoter that is only active in the cell population of interest, whereas the reporter gene is expressed under control of a ubiquitous expressed gene such as ROSA26 or β-actin. As an example, if the Cre-recombinase is expressed under control of a hair follicle bulge stem cell–specific promoter, excision of the stop codon, and thereby reporter gene expression, is mediated only in the stem-cell population and its cell progeny (Figure 1).

If single cells need to be labeled to follow not only the cell fate decisions of whole cell populations but also single cells within the population and/or at defined developmental time points, an inducible Cre-recombinase is favorable (Günschmann *et al.*, 2014). Here, the Cre activity can be temporally regulated on the transcriptional or posttranscriptional level. The expression of the Cre-recombinase can be transcriptionally controlled by the Tet-On/Off system. Using the Tet-Off system, the gene of interest is expressed under control of the tetracycline response element, consisting of TetO operator sequences, which are placed in front of the gene promoter. In addition, the expression of a tetracycline transactivator (tTA) protein, which is able to bind to the TetO sequence, is controlled by a promoter, which is chosen according to the cell population of interest. If tetracycline is present, it binds the tTA protein, thus impairing the binding to the TetO operator sequence and the transcription of the Cre-recombinase. Using the Tet-On system, binding of tetracycline to the tTA protein enables binding to the TetO operator sequences and induces Cre expression. tTA is responsive not only to tetracycline but also to its derivates, such as doxycycline,

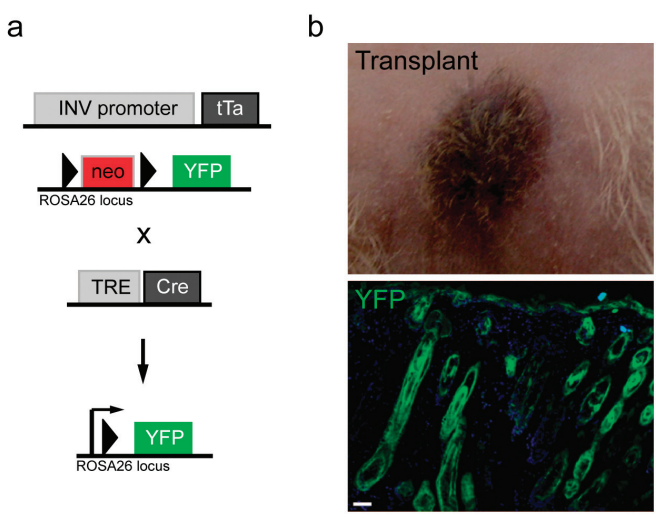

Figure 2. Lineage tracing example: inducible Cre-recombination via the Tet-Off system. Mannik *et al.* (2010) used a lineage tracing approach to demonstrate that differentiated keratinocytes are able to regenerate a fully functional epidermis when transplanted onto nude mice. The investigators induced Cre expression in differentiated keratinocytes, placing the tetracycline transactivator (tTA) protein under control of the involucrin (INV) (keratinocyte differentiation marker) promoter. Using this system, tetracycline administration activates Cre-recombinase expression and thereby the excision of a loxP site–flanked neomycin cassette (**a**). Through induced expression of the fluorescent reporter YFP, a yellow fluorescent protein, differentiated keratinocytes could be isolated via FACS and transplanted onto nude mice (**b**). Adapted from Mannik *et al.*, 2010. FACS, fluorescence activated cell sorting

which is mostly used in genetic mouse models (Bujard and Gossen, 1992). To induce Cre activity in single cells, the Tet-On system is favorable to the Tet-Off system owing to its faster responsiveness and better control of dosage-dependent induction of the tTA protein in single cells. As an example, Mannik *et al*. (2010) used the Tet-Off system to temporally induce reporter gene expression during keratinocyte differentiation using Cre-recombinase under the control of the involucrin promoter (Figure 2). This promoter is only active in differentiated keratinocytes residing in the spinous and granular layer of the epidermis. Using this technology, the investigators showed that differentiated keratinocytes, which were positive for the yellow fluorescent protein (YFP), can regenerate the complete epidermis, including its appendages, when transplanted onto nude mice (Mannik *et al*., 2010).

For regulation of Cre activity on the posttranscriptional level, Cre can be fused to a mutated form of the human estrogen receptor (CreER). In the absence of tamoxifen, the ligand of the mutated estrogen receptor, CreER, resides in the cytoplasm as a result of binding to the heat-shock protein HSP90. In the presence of tamoxifen, the ligand binding causes a conformational change of the receptor, its release from HSP90, and the nuclear translocation of CreER, where it can recombine LoxP sites. In this way, CreER can be used to excise a loxP-flanked STOP codon in front of the reporter gene, which induces its expression. The activation of transcriptional and posttranscriptional regulated Cre-recombinases is dose dependent of tetracycline or tamoxifen, which means that low doses of the substances induce fewer cells and the induction can be optimized down to the single-cell level in specific cell populations (Hayashi and McMahon, 2002). The administration of tetracycline and tamoxifen can be achieved via food, water, topical treatment, or injection.

ADVANTAGES AND LIMITATIONS

Lineage tracing enables us to gain detailed information about the migration and cell fate decisions of cell populations or single cells within their native (patho-)physiological environment, which makes it an advantageous tool. Beside the expression of a reporter gene and thereby the visualization of cell lineages within the living organism, additional gene modifications can be introduced into these lineages. Thereby we can learn whether genetic manipulation of specific genes affects the interaction of cells with their environment, migratory behavior, or intrinsic cell fate decisions during development or under homeostatic conditions. For example, the mutation or deletion of tumor suppressor genes can induce tumor formation; here lineage tracing enables the analysis of the interaction of tumor cells within the healthy tumor environment and during the metastatic processes. But although lineage tracing seems to be a straightforward technique, a detailed characterization of the Cre-recombinase and the reporter genes, in terms of both activity and specificity, is required to prevent system leakage. In addition, the expression of reporter genes and Cre-inducing agents such as tamoxifen may have a direct effect on cell behavior because of the lack of suitable controls.

CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of the Duke University School of Medicine and Society for Investigative Dermatology. The Duke University School of Medicine is accredited by the ACCME to provide continuing medical education for physicians. To participate in the CME activity, follow the link provided. Physicians should only claim credit commensurate with the extent of their participation in the activity.

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QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

For each question, more than one answer may be correct.

1. What is a cell lineage?

A. A group of cells aligned next to each other.
B. The progeny of one cell or a specific cell type.
C. The border between epidermis and dermis.

2. Which kind of reporter systems *cannot* be used to visualize a population of cells?

A. Fluorescent reporters.
B. β-Galactosidase.
C. Vital dyes.
D. DMSO.

3. The advantage of a fluorescent reporter compared to a β-gal reporter is which of the following?

A. The fluorescent reporter is expressed in all tissues.
B. Live cell imaging within tissues.
C. The possibility of multicolor labeling.
D. It does not alter cell functions.

4. The Cre-recombinase should be expressed under control of a promoter that is which of the following?

A. Active in terminally differentiated cell populations.
B. Active in proliferative active cell populations.
C. Active in a distinct cell population.
D. Ubiquitously activated.

5. Which systems are commonly used to induce Cre-recombinase activity?

A. CreER.
B. Tet-On.
C. Tet-Off.
D. β-Galactosidase.

SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2014.472.

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