METHODS and TECHNIQUES for SKIN RESEARCH

Genetic Tools for Cell Lineage Tracing and Profiling Developmental Trajectories in the Skin

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The epidermis is the body's first line of protection against dehydration and pathogens, continually regenerating the outermost protective skin layers throughout life. During both embryonic development and wound healing, epidermal stem and progenitor cells must respond to external stimuli and insults to build, maintain, and repair the cutaneous barrier. Recent advances in CRISPR-based methods for cell lineage tracing have remarkably expanded the potential for experiments that track stem and progenitor cell proliferation and differentiation over the course of tissue and even organismal development. Additional tools for DNA-based recording of cellular signaling cues promise to deepen our understanding of the mechanisms driving normal skin morphogenesis and response to stressors as well as the dysregulation of cell proliferation and differentiation in skin diseases and cancer. In this review, we highlight cutting-edge methods for cell lineage tracing, including in organoids and model organisms, and explore how cutaneous biology researchers might leverage these techniques to elucidate the developmental programs that support the regenerative capacity and plasticity of the skin.

Keywords: CRISPR, DNA barcoding, DNA typewriter, Genome editing, Prime editor

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INTRODUCTION

As the external armor of the body, the skin plays a vital role in protecting terrestrial organisms from the assaults of diverse and swiftly changing environments to prevent dehydration, thermal instability, pathogen invasion, and UV damage (Hsu and Fuchs, 2022; Sun et al, 2014a). To restore the body's external barrier after wounding, the skin can rapidly rebuild the epidermis, but cutaneous appendages such as sweat glands or hair follicles are lost in scarred adult tissue (Chen et al, 2020; Gay et al, 2013). Understanding the precise order in which skin stem cells divide during development and wound repair and tracking the cell-type identities of their progeny would allow researchers to build a regenerative blueprint of the skin. Such a developmental roadmap of conserved morphogenetic programs and how they are disrupted in dermatologic disease could be harnessed to enhance the healing of the epidermis but also to engineer fully functional skin appendages, a long-standing goal in the field of cutaneous biology.

THE PROMISE AND LIMITATIONS OF LINEAGE-TRACING METHODS

Historical methods of lineage tracing in the skin include classic pulse-chase experiments in which cells or mice were injected with a labeled probe that can be tracked over time. These methods provided key insights into fundamental processes such as the cyclic stages of hair growth and regression and the organization of epidermal proliferative units (Alcolea and Jones, 2013; Cotsarelis et al, 1990; Potten and Allen, 1975). Later lineage-tracing experiments utilized fluorescent markers (eg, histone H2B-GFP) to pulse epidermal cells and calculate fluorescence dilution to estimate cell divisions (Tumbar et al, 2004). To add a further level of pulse control, investigators used Cre recombinase driven by tissue- and cell type–specific promoters to drive expression of a fluorescent reporter (eg, tdTomato) in select cells that could then be visually chased over time (Alcolea and Jones, 2013; Sada et al, 2016). However, important limitations of these methods include their low-throughput nature, eventual dilution of the marker used in

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Abbreviations: BCC, basal cell carcinoma; DSB, double-stranded break; gRNA, guide RNA; iPSC, induced pluripotent stem cell; MOI, multiplicity of infection; NHEJ, nonhomologous end-joining; pegRNA, prime editing guide RNA; scRNA-seq, single-cell RNA sequencing; tRNA, transfer RNA; UTR, untranslated region

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pulse-chase experiments over many cell divisions, and leaky expression of Cre causing off-target labeling (Wang et al, 2023). More recently, optical imaging methods that rely on fluorescently labeled cells, advanced microscopy, and computational tracking algorithms have allowed researchers to follow individual cells in vivo to determine their fates (Cockburn et al, 2022; Rompolas et al, 2012). Live microscopy traces a continuous trajectory of cell fate, offering one of the highest temporal resolution methods of lineage tracing. However, light microscopy–based methods are limited in the number and type of cells they can track within an optical window using spectrally distinct fluorophores.

Approaching the challenge of lineage tracing from a different angle, scientists have developed computational tools that can approximate cellular differentiation trajectories through analysis of single-cell profiling methods. Transcriptional profiling through single-cell RNA sequencing (scRNA-seq) can richly describe a cell's state and nominate specific biological pathways as important for its development through gene ontology analysis. By comparing transcriptional similarities, these methods can be used to define specific cell types, infer differentiation states, and estimate signaling pathway activation (Calderon et al, 2022; Herring et al, 2018; Pollen et al, 2014). Although computational methods for scRNA-seq can retrospectively infer developmental trajectories and intercellular communication networks, gene expression alone is a static measurement of cell state, does not directly reveal the routes taken to achieve a specific fate, and requires destruction of the sample—this is akin to taking a single high-resolution photograph rather than a movie. Thus, laborious time courses must be performed to map cellular trajectories, which are still marred by considerable uncertainty (Efremova et al, 2020; Hu et al, 2021; Jin et al, 2021; Lun and Bodenmiller, 2020). Although RNA velocity analysis methods can be used to generate pseudotime estimates (or inferred trajectories), including in murine skin (Joost et al, 2018), their conclusions are limited to inference rather than a direct and prospective recording of lineage relationships (Fleck et al, 2023; Haghverdi et al, 2016; Heumos et al, 2023).

In recent years, enormous strides in genome engineering methods as well as in nucleotide sequencing capacity, efficiency, and cost have enabled the development of DNA sequence–based lineage-tracing methods that can more directly capture the lineage relationships among thousands or potentially millions of cells (National Human Genome Research Institute, 2019). The improved accuracy of high-throughput DNA sequencing has also made it feasible for scientists to track cell lineage through naturally occurring somatic mutations in the nuclear or mitochondrial genome. The phylogenetic concepts used to derive lineage trees from natural and engineered mutations are similar; however, locus-specific engineered single mutations or short nucleotide sequence insertions (barcodes) only require sequencing a defined locus or set of loci rather than many scattered loci to extract sufficient information to build complete lineage maps. Methods to directly track cell lineage using engineered barcodes have emerged through creative applications of serine and tyrosine recombinases, CRISPR/Cas9 editors, base editors, and prime editors to alter endogenous DNA sequences in a defined manner (Choi et al, 2022; Chow et al, 2021; Frieda et al, 2017; Hwang et al, 2019; Loveless et al, 2021a[1]).

Importantly, these lineage-tracing technologies can still be multiplexed with single-cell and spatial mRNA sequencing at the time of tissue or organism harvest to add historical context, not only capturing the end-fate of cells but also revealing where they came from and how they arrived at their final destination. Moreover, adaptation of these methods has been used to engineer DNA-based recording systems (akin to a tape recorder). These catalog biologically meaningful data, such as signaling cascade activation, in a sequential manner within intact tissues or organisms over time to document the type and order of intrinsic and extrinsic cues received by cells (Chen et al, 2021b[2]; Frieda et al, 2017; Tang and Liu, 2018). In this paper, we summarize how novel DNA editing tools work; how they have been utilized to map cell lineage in other organ systems; and how they could be leveraged to draft a comprehensive morphogenetic blueprint of normal skin, to engineer fully functional grafts and appendages, and to understand cutaneous malformations, disease pathology, and carcinogenesis.

PRINCIPLES OF DNA SEQUENCING–BASED PLATFORMS FOR LINEAGE TRACING

Sophisticated tools for genome engineering have increasingly allowed scientists to precisely insert, delete, and modify nucleotides at specific loci (Anzalone et al, 2019; Doudna and Charpentier, 2014; Komor et al, 2016). Although the proposed use of these tools has primarily been focused on editing native DNA sequences, such as the correction of disease-associated mutations, they have also enabled completely new methods for lineage tracing. By inserting DNA barcodes, researchers can now edit the genome to distinctly label cells and their progeny in a manner that can be deciphered through routine nucleotide sequencing (Frangoul et al, 2021; Masuyama et al, 2019; Urnov, 2021). In methods that apply static barcodes, lineage relationships (similar to family trees) can be retroactively constructed by labeling individual cells in a founder population with a unique marker (Figure 1a), for example, through lentiviral delivery of a single, unique DNA barcode or a fluorophore (Bhang et al, 2015; Hampel et al, 2011; Livet et al, 2007). In the Brainbow method, founder cells undergo fluorescent labeling using stochastic Cre/loxP recombinase activity to randomize the expression of genetically encoded fluorophores in individual cells, thus marking a founder and its progeny with a distinct color combination (Livet et al, 2007). However, because the label remains unchanged (ie, static) in a founder clone's descendants across generations, one can link all progeny only by their shared founder. This is analogous to mapping one's ancestry using only a surname that was passed down, which restricts the ability to reconstruct the more complicated branching of family trees. In contrast, lineage-tracing methods that utilize dynamic generation of barcodes to cumulatively label cells over multiple generations are able to accurately define intricate branching lineage hierarchies (Figure 1b). In this paper,

[1] Loveless TB, Carlson CK, Hu VJ, Dentzel Helmy CA, Liang G, Ficht M, et al. Molecular recording of sequential cellular events into DNA. bioRxiv 2021a.
[2] Chen W, Choi J, Nathans JF, Agarwal V, Martin B, Nichols E, et al. Multiplex genomic recording of enhancer and signal transduction activity in mammalian cells. bioRxiv 2021b.

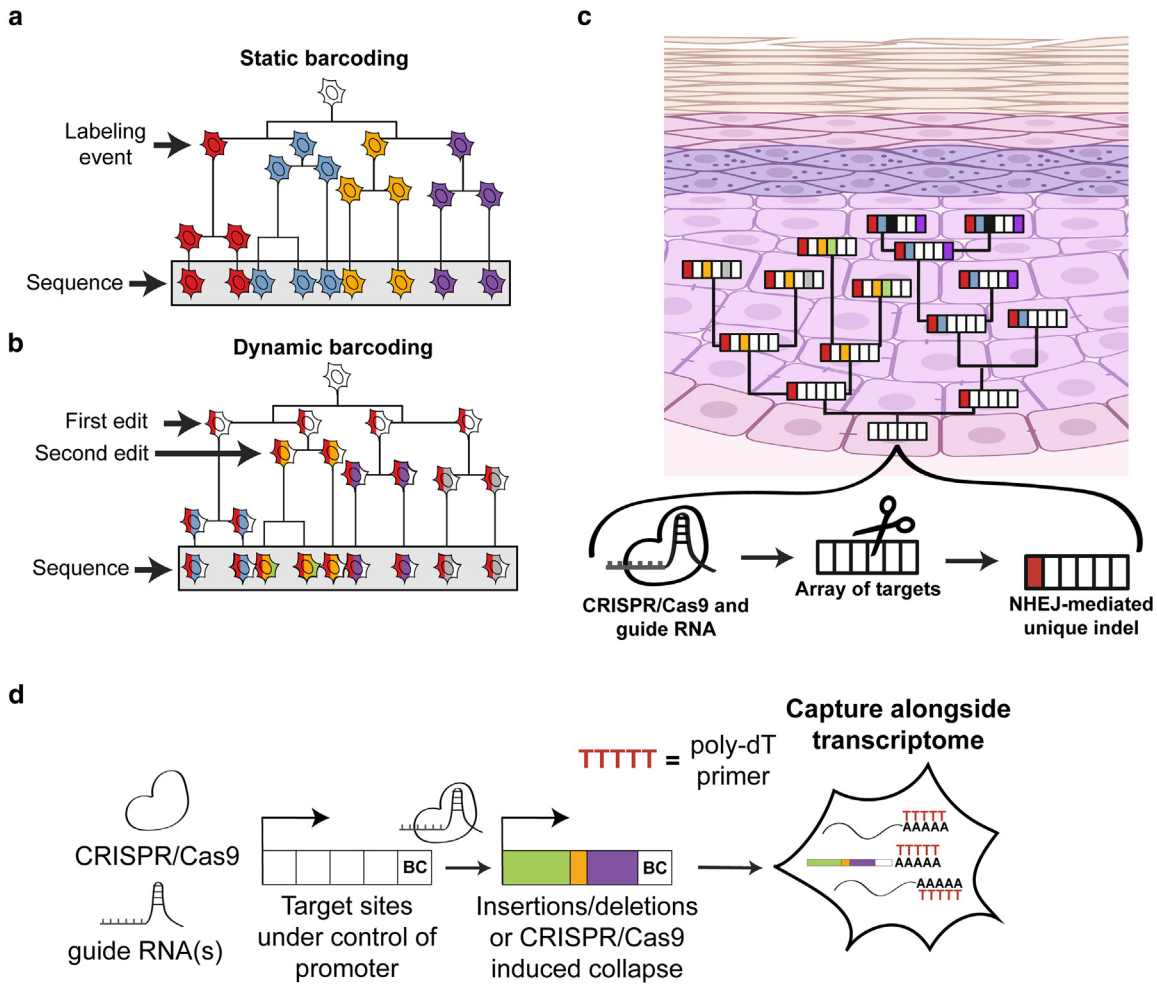

Figure 1. Dynamic DNA barcoding enhances lineage-tracing capacity and can be multiplexed with single-cell transcriptomics. (**a**) Static barcoding introduces a unique barcode per cell in a population during a single labeling event (eg, lentiviral transduction); the founder cell's barcode is then stably passed on to all its progeny. (**b**) Dynamic barcoding continually diversifies a DNA barcode over multiple generations of cells to permit reconstruction of a more complex lineage tree. (**c**) Dynamic lineage tracing in tissues such as the epidermis requires introduction of a genome editor (eg, Cas9), an array of DNA targets that become the edited barcode, and guide RNA(s) to direct the genome editor to the targets; the lineage of all cells within the tissue can be deciphered by the similarity of their final edited barcode. (**d**) By embedding a DNA barcode (denoted as BC) under the control of a promoter that recruits RNA polymerase-II (eg, placing the barcode within the 5′ or 3′ UTR of a fluorescent protein or other selection marker driven by a constitutive promoter), the barcode itself is transcribed into mRNA including a poly-A tail; thus, when capturing the entire transcriptome with a poly-dT primer in scRNAseq, the barcode is read along with all other mRNAs so that a cell's transcriptional state can be captured along with its lineage. NHEJ, nonhomologous end-joining; scRNAseq, single-cell RNA sequencing; UTR, untranslated region.

we primarily discuss dynamic barcoding and the genome editing tools commonly used to generate cumulative barcode diversity.

Although nested recombinase systems and multiple rounds of lentiviral barcode delivery have both been utilized to generate more complex lineage trees (He et al, 2017; Jindal et al, 2023; Kong et al, 2020), most dynamic barcoding methods heavily rely on CRISPR/Cas9 and its derivative editor systems. CRISPR/Cas9 requires guide RNAs (gRNA) to direct DNA-cutting enzymes to a specific genomic locus, where Cas9 cleaves both strands of DNA (Doudna and Charpentier, 2014). The resulting double-stranded break (DSB) will usually be corrected by endogenous repair processes such as nonhomologous end-joining (NHEJ), but DNA repair will occasionally generate mutations at the site of the DSB, typically nucleotide insertions and/or deletions (collectively referred to as indels). These changes typically remain stable because they no longer match the gRNA and are not targeted again by Cas9; indels that disrupt a gene's function are commonly used in a knockout strategy. Instead of relying on random indels, CRISPR/Cas9 users can add a donor DNA template that includes the desired changes from the host sequence. The donor DNA contains complementary regions on both sides of the host target sequence, which allows homology-directed repair machinery to use the donor DNA as the template for repair, thus incorporating the desired changes to the host DNA sequence. For a more thorough review of basic CRISPR gene editing methods, we direct the reader to a previous article in this series in the *Journal of Investigative Dermatology* (Guitart et al, 2016).

Subsequent iterations of genome engineering technologies allow investigators to insert, delete, or modify specific short nucleotide sequences in a chosen genomic location. When fused to DNA modifiers such as transfer RNA (tRNA)

adenosine deaminases, cytidine deaminases, or reverse transcriptases, the CRISPR–Cas9 system acts as a navigator to direct these enzymes to a specific locus, which allows targeted adenine base editing, cytosine base editing, and prime editing, respectively. Prime editing was developed by fusing a reverse-transcriptase domain to a Cas9-nickase (Anzalone et al, 2019), which nicks DNA, generating a single-stranded DNA break within the genomic target site that is encoded by the prime editing gRNA (pegRNA). The pegRNA also contains a homology arm, which helps guide the insertion to the correct location in the genomic DNA, and a reverse transcriptase template that contains a primer binding site, which primes the enzyme to reverse transcribe the pegRNA template into the nicked DNA strand, including the desired insertion or deletion. Thus, the endogenous target locus and the desired nucleotide edits are both encoded in the same pegRNA. Endogenous repair mechanisms then incorporate the edited DNA strand into both native strands of genomic DNA, thus maintaining the desired nucleotide edits in subsequent rounds of DNA replication during mitosis. Later iterations of prime editing have offered increasing editing efficiency (Chen et al, 2021a; Doman et al, 2023).

DNA sequencing–based lineage-tracing methods capitalize on the stochastic nature of target-site editing by enzymes such as CRISPR/Cas9 or more precise insertion of specific DNA sequences with prime editors, both of which result in diverse DNA edits among progenitor cells that are then passed down and can accumulate additional DNA edits in subsequent generations (Figure 1c). The first Cas9-based methods to generate dynamic DNA barcodes for tracking cell lineages relied on NHEJ to accumulate random deletions and/or insertions in an engineered DNA barcode over many cellular generations (Chan et al, 2019; Kalhor et al, 2017; Loveless et al, 2021b; McKenna et al, 2016; Perli et al, 2016; Schmidt et al, 2017; Spanjaard et al, 2018) (Figure 1d). Relationships between cells could then be computationally inferred from how similar or different the resultant barcode sequences are to one another. These methods require that a tandem array of Cas9-target sites be inserted into the genome to expand the number of available target sites that can be stochastically edited. After cells grow or differentiate and are harvested, routine DNA sequencing, scRNA-seq, or FISH can be used to read the resultant edits within the target sites (Askary et al, 2020; Chow et al, 2021; Frieda et al, 2017) and compare them among different cells within the biological system (eg, organoid, tissue, tumor, or whole-model organism) to infer a cell lineage tree. Despite its benefits over pulse-chase and optical forms of lineage tracing, this technology has important limitations. Cas9-based editing relies on double-stranded DNA breaks, which if not properly repaired can potentially compromise cellular health or promote carcinogenesis (Carroll, 2018; Haapaniemi et al, 2018). In addition, owing to multiple DSBs occurring at once, larger portions of the barcode can be unintentionally deleted, resulting in loss of lineage information.

Prime editing–based methods of lineage tracing, such as DNA Typewriter and peCHYRON (prime editing Cell HistorY Recording by Ordered iNsertion), overcome many key limitations (Choi et al, 2022; Loveless et al, 2021a[1]) (Figure 2a). In each of these methods, a pegRNA targets a synthetic locus inserted into the genome that has been optimized for controlled rather than stochastic DNA editing. The synthetic target sequence is designed to require that the first portion of DNA must be edited before any subsequent edits can occur, thus ensuring a unidirectional editing process that can be read out at the end of an experiment in which cells have

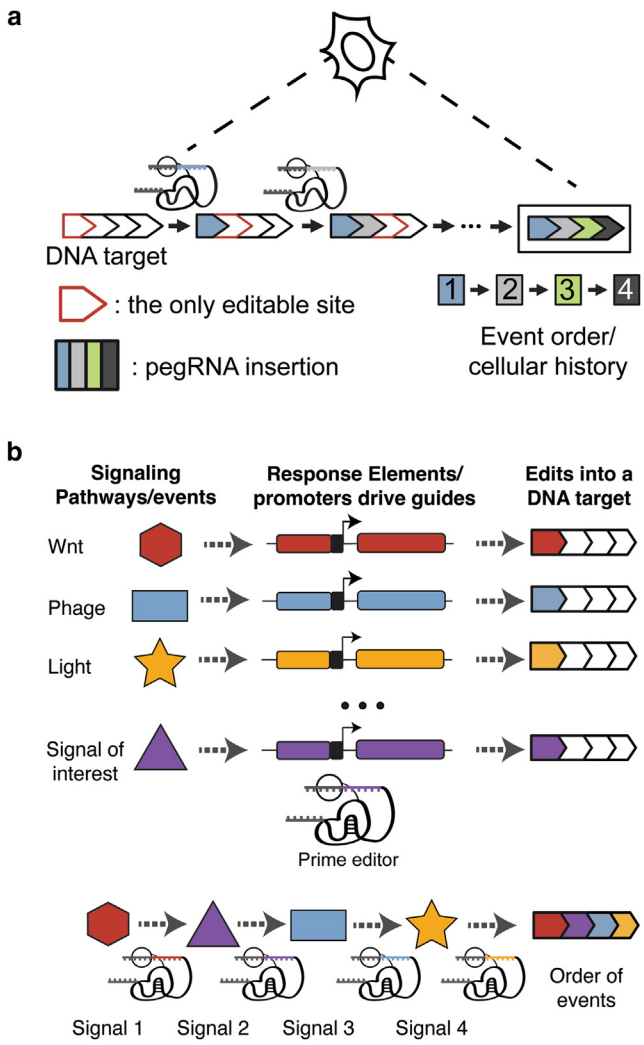

Figure 2. Prime editing–based lineage tracing records the order of DNA edits and events linked to edits. (**a**) Prime editing–based lineage tracing can record the order in which edits were introduced using a synthetic DNA target introduced into the cell's genome. DNA Typewriter uses a tandem array of identical prime editing–target sites in which all but the first in the series are truncated to prevent editing. The pegRNA edits the first target site with high specificity; the resulting edit disrupts the first target site (preventing subsequent editing) and completes (unlocks) the second target site so that it is now recognized by the pegRNA; similar editing and unlocking of the subsequent target sites occur such that cells accumulate defined DNA edits in a sequentially ordered fashion within the barcode. (**b**) Prime editing–based recording methods chronicle the temporal order of biological events by linking production of a pegRNA to a specific signal or event (eg, Wnt/β-catenin signal activity, phage infection, light exposure, or other signals of interest); pegRNAs are placed under transcriptional control of a signal/event-responsive promoter and encode a specific edit to the barcode when a biological event of interest is experienced by the cell. Using DNA Typewriter, biological events are thus sequentially recorded into the editing target, allowing for reconstruction of event order on the basis of the final edited sequence of the barcode. pegRNA, prime editing guide RNA.

accumulated edits in a sequential fashion. DNA Typewriter and peCHYRON have significant advantages over CRISPR/Cas9-based lineage tracing: (i) no double-stranded DNA breaks are generated, such that edited DNA at the locus is not at risk of subsequent deletion, and (ii) the order in which edits were introduced is explicitly captured owing to unidirectional editing. Although the efficiency of these more controllable prime editing–based methods has been limited by low editing rates of barcode insertions longer than a few base pairs, researchers are rapidly identifying means to improve prime editing's capacity and efficiency (Koeppel et al, 2023; Mathis et al, 2023; Yu et al, 2023; Zhao et al, 2023).

Base editing methods have also been used for lineage tracing. The adenine base editor was developed by fusing a Cas9 nickase to a tRNA adenosine deaminase to catalyze the conversion of A•T to G•C at the target site. In a similar approach, Cas9 nickase was fused to a single-stranded DNA–specific cytidine deaminase, which can catalyze the conversion of cytosine to uracil for C•G to A•T editing (Gaudelli et al, 2017; Huang et al, 2021). More advanced base editing methods such as DOMINO (DNA-based Ordered Memory and Iteration Network Operator), ZOMBIE (Zombie is Optical Measurement of Barcodes by In situ Expression), and CAMERA (CRISPR-mediated Analog Multi-Event Recording Apparatus) have been applied to control the order of DNA editing events to facilitate lineage tracing (Askary et al, 2020; Farzadfard et al, 2019; Tang and Liu, 2018). However, because these methods are reliant upon predefined target sites and make only single-nucleotide changes, they generally do not produce the stochastic edits and barcode diversity required for more extensive and complex lineage tree reconstruction.

Applications of several of the methods described earlier have aimed not just to record lineage through stochastic cellular barcoding but to chronicle biological activities such as signal transduction or key protein interactions by linking the biological event to insertion of a predefined DNA barcode or edit (Figure 2b). For example, recent methods such as ENGRAM (Enhancer-driven Genomic Recording of transcriptional Activity in Multiplex) and P3 (Protein-Protein Proximity) editing can couple expression of a pegRNA with the activation of certain signaling cascades and upon specific protein–protein interactions, respectively (Chen et al, 2021b[1]; Choi et al, 2023[3]). Activation of Wnt/β-catenin signaling, which has long been known to play a key role in the development of normal hair follicles and follicular tumors (Chan et al, 1999; Hile and Harms, 2021; Huelsken et al, 2001; Matos et al, 2020), was successfully recorded by DNA editing through both the prime editing–based ENGRAM system and the CRISPR/Cas9-based MEMOIR (Memory by Engineered Mutagenesis with Optical In situ Readout) system (Chen et al, 2021b[1]; Frieda et al, 2017). CAMERA base editing has been used to record other biological events such as exposure to visible light and infection by a phage (Tang and Liu, 2018). These genomic editing tools offer the potential to link any transcriptionally active event of interest to the production of a specific gRNA, which acts as a cellular historian to edit the DNA barcode to record the event. If coupled with unidirectional editing methods such as DNA Typewriter or peCHYRON, signal-responsive DNA edits can record not just whether a biological event occurred but can chronicle the order in which events linked to distinct pegRNAs occurred in a cell (Figure 2b). These capabilities represent a powerful new dimension for lineage-tracing research, which could be applied to understand the complex sequence of biological signals that direct the morphogenesis of the skin and its appendages (Fuchs, 2016; Sun et al, 2020; Zhang and Chen, 2024) and how these go awry in models of disease or carcinogenesis.

In this section, we introduced the diverse genome editing–based lineage-tracing technologies that generate barcode diversity in vivo, allowing for high-resolution lineage tracing from a monoclonal population of cells. For more details about these methods, we direct the reader to several excellent reviews (Sankaran et al, 2022; VanHorn and Morris, 2021; Yao et al, 2022). In the following sections, we focus on existing and potential applications of CRISPR/Cas9-based lineage and molecular recording technologies.

LINEAGE TRACING IN ORGANOIDS, ORGANS, AND MODEL ORGANISMS

Starting with Sulston et al (1983)'s monumental achievement of visually tracking and recording the lineage of each individual cell in the model organism *Caenorhabditis elegans*, developmental biologists have aimed to generate comparable complete maps of the development of more complicated biological systems, including human organoids and whole organs such as the brain (Chan et al, 2019; He et al, 2022; McKenna et al, 2016; Raj et al, 2018). Over the last decade, great strides in single-cell indexing methods and high-throughput DNA-sequencing technologies have allowed researchers to develop atlases that catalog developmental gene expression programs during organismal development at single-cell resolution (Aldridge and Teichmann, 2020; Svensson et al, 2018). Lineage-tracing technologies have the potential to complement these efforts by allowing scientists to generate lineage trees for tracking the diverse states that pluripotent cells traverse to reach their ultimate fate in a mature tissue in models that are not amenable to continuous visual tracking as done by Sulston et al (1983). Although still in their nascent phase, these technologies have so far shed light on how healthy development progresses and promise to illuminate how congenital disorders and malformations may result from disrupted developmental lineages.

The first demonstration that Cas9 editing could be used to generate sufficient barcode diversity to allow in vivo lineage tracing was termed GESTALT (Genome Editing of Synthetic Target Arrays for Lineage Tracing) and was applied to zebrafish (Figure 3). The authors generated transgenic zebrafish harboring arrays of CRISPR/Cas9 target sites and injected Cas9 preloaded with gRNAs into zygotes (McKenna et al, 2016). Owing to the injection of the editing machinery at the zygote stage and saturation of the genomic target sites, most editing took place during the early stages of development. Interestingly, the authors found that blood lineages in particular were bottlenecked during development, with 98% of all blood in the adult zebrafish attributable to just 5 early

[3] Choi J, Chen W, Liao H, Li X, Shendure J. A molecular proximity sensor based on an engineered, dual-component guide RNA. bioRxiv 2023.

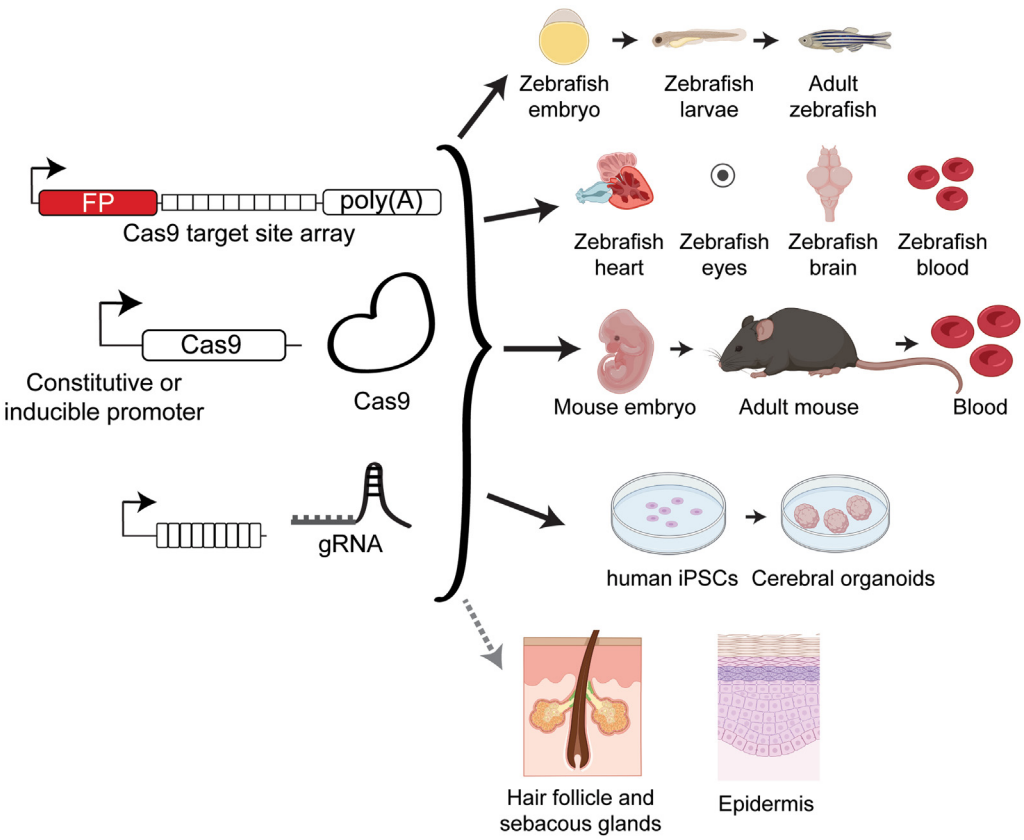

Figure 3. Cas9-based lineage tracing can map the fate of progenitor cells during the development of organoids, organs, and whole organisms. Cas9-based methods dynamically generate diverse barcodes over many cellular generations and have been applied to map the development of whole zebrafish and its organs, mouse embryogenesis and hematopoiesis, and human iPSC-derived cerebroids. An array of Cas9 targets (often linked to a transcribed FP) and gRNAs are delivered into stem or progenitor cells. Subsequent editing generates the barcode diversity needed for complex lineage tracing; importantly, the Cas9 editor itself can either be constitutively or inducibly expressed (eg, under tetracycline- or heat shock–inducible promoters) to allow temporal control over when editing occurs during development. Such technology could be applied to animal or organoid models of skin to comprehensively map the developmental plan of the epidermis and its appendages. FP, fluorescent protein; gRNA, guide RNA; iPSC, induced pluripotent stem cell.

ancestors. In other tissues, over half of the cells in each organ were represented by <7 tissue-specific lineages, indicating that the majority of each organ arose from just a handful of founder cells. These dominant lineages were generally organ specific, although not exclusively so; for example, the same edit was found in >10% of both cardiomyocytes and intestinal bulb cells, indicating that these divergent cell types shared a common progenitor. To determine the ancestral origins of distinct brain regions during neurogenesis, an improved version of GESTALT coupled to single-cell transcriptional profiling was applied to the zebrafish brain with a heat shock–inducible Cas9 to permit temporal control of DNA editing (Raj et al, 2018). This study found that although most cells in the forebrain, midbrain, and hindbrain arose from distinct lineages, some lineages spanned multiple brain regions, suggesting that edits either accumulated early in multipotent progenitors or that some later lineages retain the ability to contribute to >1 brain region. Cutaneous biology researchers have posed similar questions aiming to understand how common progenitor cells contribute to the development of the epidermis and its appendages (hair follicles, sebaceous glands, sweat glands, and sensory structures such as touch domes) as well as how malignant clones arise from various skin compartments to form the wide variety of cutaneous tumor types (Alcolea and Jones, 2014; Andersen et al, 2019; Arwert et al, 2012; Clayton et al, 2007; Ge et al, 2017). DNA editing–based lineage tracing tools promise to make asking such questions more tractable.

Mammalian development has more recently been probed using Cas9-based barcoding methods. Mouse gastrulation was studied using a method similar to GESTALT by injecting a target array, Cas9, and multiple gRNAs into murine oocytes before they developed into embryos (Chan et al, 2019). Importantly, the researchers found that cells having a very similar state at the time of harvesting, as measured by scRNAseq, did not always share the same developmental origin; for example, although scRNAseq classified a cell population as embryonic endoderm, the lineage of a subset of these cells was clearly traced to an extraembryonic progenitor population that likely migrated during development and later adopted an endodermal phenotype. This study underscores the enhanced precision of developmental mapping afforded by combining lineage tracing with transcriptomic profiling. Lineage recording has also been used successfully to trace blood cell expansion and maturation in adult mice by introducing barcodes through retroviral delivery into hematopoietic stem cells and then tracking their progeny over time by reading out a static barcode (Chan et al, 2019; Gerrits et al, 2010; Lu et al, 2011; Sun et al, 2014b). Another study added an array of loxP sites for Cre recombinase to randomly recombine to create a unique cellular barcode in the 3′ untranslated region (UTR) of tdTomato (Pei et al, 2020). Reading the barcodes alongside the transcriptome allowed researchers to stratify adult blood cell populations by their progenitors. Although sufficient for cataloging cell fates within the hematopoietic system, the use of static barcodes restricted the resolution at which lineage relationships could be

determined, and hematopoietic stem cells had to be re-engineered for each experiment. To overcome these limitations, researchers generated a more versatile mouse model for joint lineage tracing and transcriptomic profiling using a framework similar to GESTALT. This model showed that after bone marrow ablation, only a few hematopoietic stem cell clones generated the majority of progeny during hematopoiesis in adult mice (Bowling et al, 2020). Although these investigators benchmarked Cas9-based lineage-tracing methods in mouse hematopoietic lines, similar methods could be used to trace the fate of stem cells in the skin and determine whether a similar clonal bottleneck occurs during skin development, tissue regeneration after wounding, or tumorigenesis.

Lineage-tracing tools can also be used to analyze organoid systems that model human tissue and are more easily manipulated ex vivo. In a study of human induced pluripotent stem cell (iPSC)—derived brain organoids that applied spatial RNA sequencing in tandem with lineage tracing, researchers developed a method termed iTracer (He et al, 2022). This system provides 2 channels of barcoding to expand lineage-tracing capability: the construct used for DNA editing contains a U6 promoter—driven gRNA as well as a fluorophore gene (to allow positive cell sorting) with a random barcode in its 3′ UTR. The fluorophore gene itself contains the gRNA target within its 3′ coding region, but it is not edited until expression of Cas9, which is controlled by a doxycycline-inducible promoter. Thus, gene editing could be induced after initiating cerebroid formation by adding doxycycline to the medium at various days during the protocol to generate edits. The researchers then used a spatial transcriptomics platform, Visium, to recover cell lineage and spatial relationships in tandem (Ståhl et al, 2016). Analysis of the permanent CRISPR-induced edits and unique barcodes revealed that each barcode was clustered on the basis of location within the organoid, demonstrating that lineages were spatially constrained early in tissue differentiation. Investigators seeking to understand how stem cells residing in the bulge area of the hair follicle contribute to wound repair, for example, could use such a system to map the precise lineage, transcriptome, and the ultimate physical destination of cells in the healed tissue (Aragona et al, 2017; Joost et al, 2018; Phan et al, 2021; Sada et al, 2016).

Developmental biologists have more recently appreciated the importance of epigenetic modifications as a driver of differentiation (Cantone and Fisher, 2013; Meissner, 2010). Thus, profiling a cell's lineage and transcriptome as well as epigenetic modifications would provide a more comprehensive roadmap of developmental programs (Lu et al, 2020). Toward this goal, a group recently developed a mouse model called DARLIN (Cas9-TdT CRISPR Array Repair LINeage tracing) (Li et al, 2023). Similar to GESTALT, this system generated barcode diversity for lineage tracing by inserting an array of Cas9 gRNAs and targets into safe harbor loci in a mouse line. Cell lineage, gene expression, and epigenetic modifications were simultaneously profiled by splitting single cells into cytoplasmic and nuclear fractions; the cytoplasmic fraction was used to capture all mRNAs, including lineage-specific edited transcripts, whereas the nuclear fraction was labeled with GpC methyltransferase to mark open chromatin domains. Using bisulfite DNA sequencing, the researchers were then able to determine the open versus closed chromatin status at genomic loci and compare these among clones.

TRACING THE ORIGINS AND DRIVERS OF CANCER

Identifying the cells of origin in tumors is essential to delineate the early mechanisms driving carcinogenesis. Moreover, understanding the states that a cell must traverse during malignant transformation and its primary driver signals could allow pharmacologic targeting of malignant stem cells to prevent cancer initiation or recurrence, including in the skin (Sánchez-Danés et al, 2016). Applying the dynamic barcoding methods outlined earlier, researchers have engineered tumor cells ex vivo, introduced them into model systems, and subsequently harvested the primary tumor or sites of metastases for lineage tracing (Aalam et al, 2023). Metastases are of particular interest to clinicians because treatment effectiveness and survival are minimal for metastatic disease. Moreover, 2−5% of cancers are diagnosed as metastases of unknown primary tumor (Varadhachary, 2007), which reduces clinicians' ability to choose the most effective treatment for the underlying cancer. Using lineage-tracing methods to directly link metastasized cells to ancestral cells in cancer models, we can enhance our understanding of tumor origin, progression, and spread (Figure 4a).

Researchers focused on a wide variety of cancers have applied Cas9-based lineage-tracing methods to tumor xenograft models to probe metastatic origins (Quinn et al, 2021; Simeonov et al, 2021; Yang et al, 2022). Aggressive human *KRAS*-mutant lung adenocarcinoma lines were engineered with synthetic arrays of Cas9 sites and gRNAs along with luciferase as an optical signal to locate where the cancer had metastasized (Quinn et al, 2021) (Figure 4b). The results showed that 30,000 profiled metastatic lung cancer cells had nearly as many unique lineage states and that individual cell clades had varying degrees of metastatic behavior. Some clades of related cells remained entirely in their original location, whereas others seeded several metastatic progenies into one other tissue, and some seeded many metastatic cells across multiple tissues. Lineage tracing allowed identification of clades that were then transcriptionally compared to identify genes directly or inversely correlated with metastatic potential. To validate these findings, candidate driver or suppressor genes were either upregulated or downregulated to directly test their effect on invasiveness of multiple different cell types. This study demonstrates the power of coupling lineage tracing with transcriptional profiling to simultaneously trace a tumors' origin and delineate key controllers of its metastatic potential.

Similar lineage tracing was performed in a mouse pancreatic cancer transplant model using a variant of GESTALT with doxycycline-inducible Cas9 to initiate lineage recording in a desired temporal window (Simeonov et al, 2021). This allowed the investigators to define the origin of rare metastatic cells and identify bottlenecks during tumor implantation because only a small minority of cells were successfully engrafted. Another mouse model of lung adenocarcinoma used mouse embryonic stem cells engineered with a similar Cas9-based lineage-tracing system that also allowed Cre-inducible activation of oncogenic *Kras* and *p53* mutation

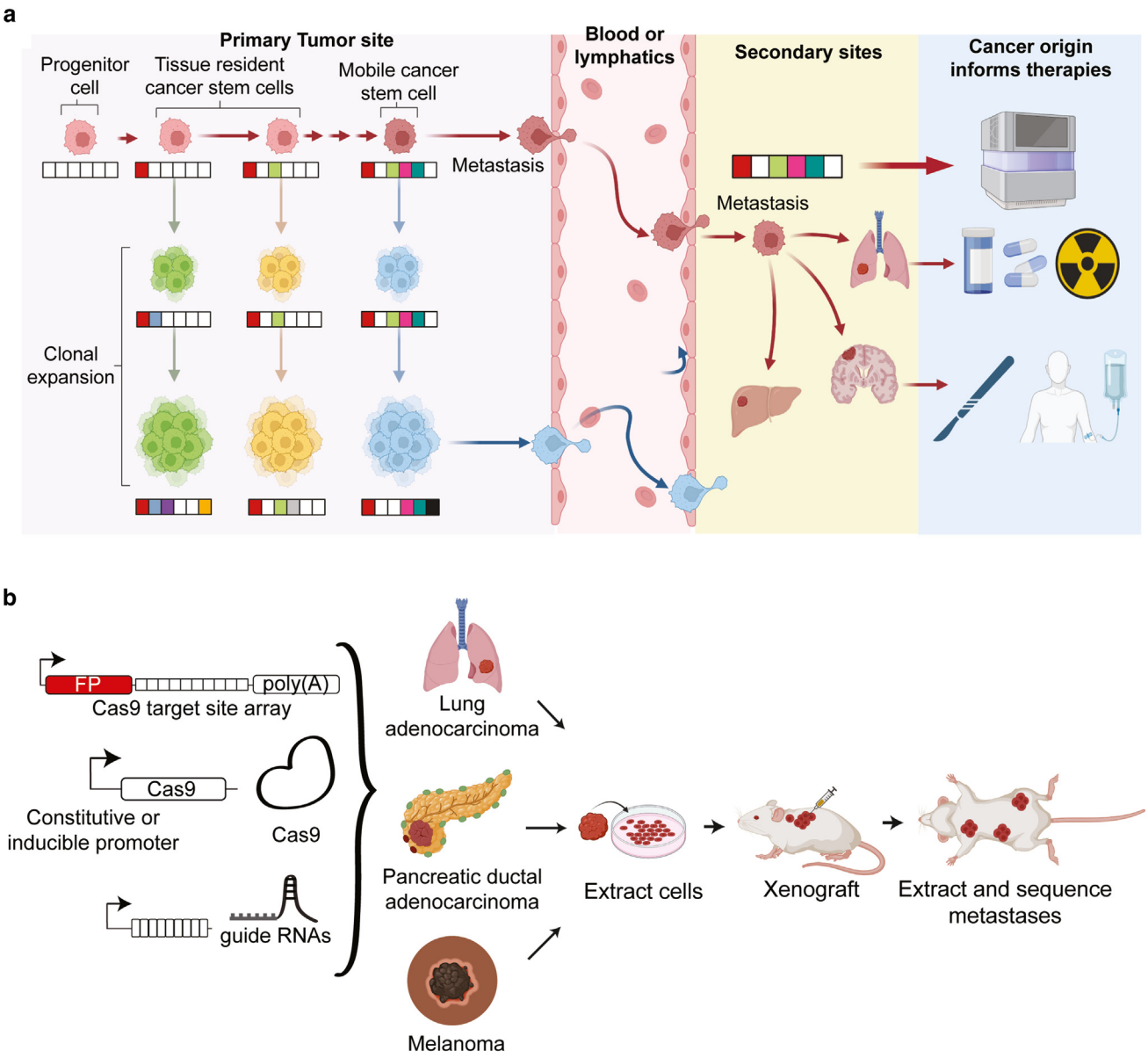

Figure 4. Lineage tracing in cancer models can reveal key oncogenic steps and drivers of metastatic behavior. (**a**) Lineage tracing allows investigators to track a cancer progenitor cell through its clonal expansion, epithelial-mesenchymal transition, and leaving the site of origin through the vasculature to metastasize, seed, and grow in distant sites. The resultant barcode sequence at secondary metastatic sites can identify the clone of origin and can be coupled with transcriptomic analysis to understand the critical drivers of its metastatic behavior, which could dictate the therapeutic strategy. (**b**) Cancer cells can be engineered to express the machinery for DNA editing–based lineage tracing studies; this approach has revealed the origins and drivers of metastases in mouse xenograft models of lung and pancreatic carcinomas and could be adapted to study metastatic behavior in melanoma or other invasive skin cancers.

(Yang et al, 2022). Their study produced a high-resolution lineage map of lung cancer progression and found that the most aggressive metastatic clones were enriched in mesenchymal genes, showing a direct correlation between metastatic potential and progression along the epithelial-mesenchymal transition (Figure 4b) (Kalluri and Weinberg, 2009).

LEVERAGING DNA SEQUENCE–BASED LINEAGE TRACING IN THE SKIN

In summary, CRISPR/Cas9-based lineage tracing has been leveraged to study development at both early and late time points and cancer metastases in diverse model systems and human organoids. Although many of these innovative technologies were pioneered in extracutaneous systems, we propose that they can be readily applied to many unanswered questions in cutaneous biology and dermatology to better understand and treat genodermatoses, congenital nevi, and vascular malformations, as examples. Applying DNA sequencing–based lineage-tracing methods to models used to study cutaneous biology, including mice, human organotypic cultures (Simpson et al, 2010; Smits et al, 2017), and more recently described iPSC-derived appendage-bearing organoids (Lee et al, 2020), would allow us to understand how the epidermis and its appendages continuously renew the skin, hair, and nails and how its stem cells respond to

external insults and injuries (Figure 1c and Figure 3). Although advanced lineage-tracing tools have been used up till now to understand the development of internal organs such as the brain, the external location of the skin and the potential for xenografting human stem cell–derived tissues or organoids onto mouse skin further amplifies the experimental possibilities. By combining lineage-tracing tools with intravital imaging (Obeidy et al, 2018), investigators can elucidate both the genetic lineage and spatiotemporal fate of progenitor cells during human skin morphogenesis and regeneration or scarring after wounding or other damage such as radiation. Recent studies have applied scRNA-seq to skin organoid systems both as ex vivo and as in vivo xenografts (Lee et al, 2020) in which one can both define cell types and infer lineage by computationally derived RNA velocities (Stabell et al, 2023). However, the accuracy of such methods can be greatly enhanced when supplemented with and verified by direct lineage tracing using the dynamic barcoding methods described earlier (He et al, 2022).

In dermatology, the study of melanoma could benefit greatly from the application of advanced lineage-tracing methods multiplexed with transcriptomics to define the subsets of this cancer with invasive behavior that pose true risk to patients to help determine the need for staging and the aggressiveness of therapy. Lineage tracing methods could also help map the origins of melanoma, which may arise de novo or from existing nevi or lentigines, and to define the early fate–determining signals that dictate outcomes ranging from atypical melanocytic lesions that may remain in situ indefinitely to rapidly progressive nodular tumors that readily metastasize (Figure 4a and b). Melanocytic stem cells have been tracked using a fluorescent label and a tyrosinase marker, which allowed investigators to localize invasive tumor cells over time in a mouse model of melanoma driven by *BRAF* V600E mutation and UVR (Moon et al, 2017). Applying more advanced CRISPR/Cas9-based lineage-tracing methods would allow investigators to elucidate more details about the ancestral origin of melanoma in this and other in vivo models in addition to mapping the epigenetic and transcriptional landscape of melanocyte stem cells that primes certain cancer progenitors for malignant transformation.

Although many keratinocyte carcinomas can be treated with local surgical methods or topical chemotherapy, these are the most numerous human cancers by far, and a subset of squamous cell carcinomas (and rarely basal cell carcinomas [BCCs]) does invade and metastasize. However, it is unknown whether the cell of origin or lineage differs between more and less aggressive keratinocyte tumor subtypes, for example, superficial spreading BCCs, which are easily treated, versus basosquamous carcinoma, which exhibits much more invasive behavior (Chiang et al, 2019). Even less is known about the origins and signaling drivers of more aggressive tumors arising from the skin such as sebaceous carcinoma and Merkel cell carcinoma, which have very high mortality rates once they have spread beyond the skin. Applying lineage-tracing methods to murine (Lowry et al, 2016; Verhaegen et al, 2022) and/or xenograft (Fang et al, 2020; Patel et al, 2012) models of these aggressive skin cancer types could provide important advances to understand their cellular origins, define the signaling pathways that drive their aggressive behavior, and identify preventive strategies and effective treatments. Building on recent evidence showing that specific chromatin modifiers exert a major influence on keratinocyte carcinomas in the skin (Ko et al, 2022[4]), tracing cell lineage alongside epigenetic modifications could be applied to mouse models of skin carcinogenesis to understand how epigenetic factors influence cancer stem cells (Li et al, 2023).

Dermal fibroblasts are known to play key roles in epidermal differentiation, appendage development, and wound repair but also drive fibrotic pathologies such as keloids and scleroderma and can even fuel inflammatory disorders (Driskell et al, 2013; Leask et al, 2004). The marked heterogeneity of skin fibroblast populations has been characterized both by Cre-based lineage tracing and single-cell RNA-sequencing–based pseudotime trajectory analysis (Driskell et al, 2013; Guerrero-Juarez et al, 2019). Although these novel studies identified crucial divergences in fibroblast lineage, such as distinct fibroblast populations responsible for a first and second wave of wound healing, an understanding of the precise signals that drive fibroblasts into different lineages remains elusive. Using the methods described herein, investigators have the tools needed to map lineage hierarchies of fibroblasts throughout cutaneous development, during regeneration versus scar formation after wounding, and within models of fibrotic (or nonfibrotic) disorders. Understanding fibroblast fate, function, and pathogenic behavior could uncover the much-needed treatments for sclerosing disorders and advance long-standing goals such as hair follicle regeneration and scarless wound healing (Mascharak et al, 2021).

SUMMARY

DNA editing–based methods have rapidly expanded researchers' ability to interrogate cell lineage relationships required to reconstruct complex morphogenetic programs. Newer dynamic barcoding strategies allow investigators to generate detailed maps of cell lineage, which can now be overlaid with single-cell gene expression and epigenetic information. Even greater diversity of DNA barcodes afforded by Cas9-based editing or arrays of truncated prime editing targets that must be edited in sequence has provided additional bandwidth to construct more expansive lineage trees to map cellular origins and relationships in higher-order biological systems such as organoids, organs, or even whole organisms. Moreover, methods such as peCHYRON and DNA Typewriter provide a temporal directionality to barcoding, which allows more accurate mapping of lineages by reading sequential edits inserted over time. Finally, linking sequential DNA editing to specific biological events, investigators can translate the edited DNA template into a historical record of signals that a cell has received over the course of development, tissue repair, or carcinogenesis. Although the use of these more advanced DNA-editing tools has been limited in cutaneous biology, their application to the fields of hematology, immunology, and neurobiology has yielded deep insights into the developmental trajectories of

[4] Ko EK, Anderson A, Zou J, Huang S, Cho S, Alawi F, et al. H3K36M provokes cellular plasticity to drive aberrant glandular formation and squamous carcinogenesis. bioRxiv 2022.

Table 1. Comparison of Lineage Tracing Methods

Lineage Tracing Methods

Method	Advantages	Disadvantages
Fluorescence-based optical lineage tracing	High temporal and spatial resolution Can be coupled with fluorescent biosensors to capture physiologic signals along with lineage Readily applied to the skin surface using 2-photon microscopy	Low throughput (number of cells traced is limited by the visual window and available fluorophores) Data collection is limited to the labeled cell population
RNA sequencing–based lineage inference	Provides a broad description of cell state through transcriptomics Analysis can be performed using many widely available software tools No delivery of fluorescent markers or complicated editing machinery	Data are limited to the time point/s chosen for RNA collection Inferred lineages have considerable uncertainty
CRISPR/Cas9-based lineage tracing	Can be coupled with RNA sequencing to provide a broad description of cell state through transcriptomics Continuous DNA editing allows dynamic barcoding to construct more complicated lineages	DNA barcodes can be inadvertently deleted Double-stranded DNA breaks may have deleterious effects Order of DNA edits is not explicitly captured
Prime editor–based lineage tracing	Can be coupled with RNA sequencing to provide a broad description of cell state through transcriptomics Continuous DNA editing allows dynamic barcoding to construct more complicated lineages Can engineer the barcode to be unidirectional to prevent loss of edits Can be used to record the temporal order of edits or biological signals No double-stranded DNA breaks	Editing rate is generally low Analysis software is nascent and not widely available Delivery of large editing machinery into cells can be challenging

stem and progenitor cells and the origins and metastatic potential of cancerous cells derived from their respective organ systems. By introducing cutaneous biologists to the remarkable toolkit of DNA editors capable of advanced lineage tracing and cataloging of biological events, we hope to have expanded the notion of what is possible by applying these cutting-edge techniques to the study of skin biology. Together, these remarkable technologies in DNA editing offer cutaneous biologists the opportunity to reimagine our capacity to create a blueprint of the morphogenetic programs that support skin development and regeneration and to understand how these go awry in cutaneous malformations, diseases, and malignancies and how they might be reversed. Perhaps most excitingly, prime editing–based molecular recording tools can now, using the nucleotide alphabet, chronicle cellular responses to external signaling cues or injury. These advances in DNA sequence–based recording should allow investigators to optimize stem cell mobilization and differentiation protocols and aspire to build fully functional skin grafts and appendages, bringing real-world regenerative medicine within reach in the field of dermatology.

PRACTICAL CONSIDERATIONS

Each specific method of lineage tracing offers both benefits and limitations (Table 1). For example, optical lineage tracing through fluorescence offers optimal spatial resolution, but the time-consuming nature of live microscopy can be limiting, whereas CRISPR/Cas9 edits can accumulate over many cellular generations and be interpreted retrospectively. Although prime editor–based tracing affords sequential barcoding to delineate complex lineages, it requires delivery of multiple large transgenes that are not needed for RNA sequencing–based inferred lineages. In the following sections, we describe additional practical considerations to help users appropriately plan and optimize their use of DNA editing–based lineage-tracing strategies.

HIGH COPY NUMBER DELIVERY METHODS

To generate high-resolution lineage maps, it is best to integrate high copy numbers (termed multiplicity of infection [MOI]) of lineage-tracing components into cells prior to expansion and differentiation. High copy numbers of components are desirable to provide more DNA editing targets per cell and higher expression of the editor protein to enhance the rate of successful editing. Together, these factors provide more potential barcode diversity, which enhances the ability to distinguish subclades for high-resolution lineage tracing.

Transduction is the most widely used method to establish cell lines with high copy numbers of editing components. Optimization of MOI is feasible for viral packaging vectors such as lentivirus and adenovirus; however, their packaging capacity of ~9 kb and ~4.7 kb, respectively, limits their ability to carry large transgenes required for more complex editing platforms (Dong et al, 1996; Kumar et al, 2001). Recent advances in optimizing smaller or split prime editors have enhanced viral delivery of lineage-tracing cargo (Davis et al, 2024; Doman et al, 2023).

Transposases are also utilized for high copy number transgene delivery but can integrate into nondesirable sites, potentially disrupting native genes that encode critical proteins or tumor suppressors. PiggyBac and sleeping beauty transposases preferentially integrate at specific genomic sequences, and the cells in which a deleterious integration event occurs are generally diluted out through reduced fitness over several generations. The upper bound for transposon cargo is >100 kb, making them suitable for large transgene

integration (Li et al, 2013; Rostovskaya et al, 2012). Larger transgene capacity allows editing components to be integrated alongside selection markers, which can be used to increase the copy number of integrants by selecting cells with the highest puromycin resistance or sorting those with highest fluorescence intensity (Chardon et al, 2023[5]). However, the reversible nature of DNA transposition makes it possible for later excision of the editing machinery, which can preclude further editing.

TIMING AND SATURATION OF EDITING ACTIVITY

The lineage-tracing methods outlined in this review can be restrained using inducible Cas9 expression to control the precise timing of DNA editing. This is important because sustained editing by Cas9 or its derived editing systems can lead to saturation of DNA target sites. For example, in the original description of GESTALT, the authors found that the majority of editing happened very early in zebrafish development owing to exhaustion of DNA target sites (McKenna et al, 2016). To address this, investigators can employ systems that initiate Cas9 editing only during discrete temporal windows to reduce the risk of target-site saturation and to strategically time editing to occur during periods of particular interest in the model system. Under the Tet-ON system (Das et al, 2016), application of a tetracycline-class drug such as doxycycline leads to specific activation of a transcription factor that drives expression of Cas9. This system has been utilized in lineage-tracing systems ranging from cerebral organoids to full mouse development from embryonic stem cells (He et al, 2022; Li et al, 2023). Although the Tet-ON platform has been frequently used to control Cas9-based lineage-tracing experiments, other tunable protein induction and degradation methods have been developed for mammalian systems, which could permit more precise control over editing enzymes to match the biology under study (Kallunki et al, 2019; Mullick et al, 2006; Yesbolatova et al, 2020).

LIBRARY GENERATION

The majority of lineage-tracing methods described in this review are reliant upon high-fidelity DNA sequencing to accurately read the target or tape in which editing or barcoding has occurred. Moreover, optimal primer design is necessary to sufficiently amplify the edited loci from genomic DNA with few off-target products. To optimize the protocol for sequencing the DNA tape (where edits have been recorded), the region can be amplified through quantitative PCR to capture amplified sites before PCR saturation, and then nonquantitative thermocyclers can be used once an optimal PCR protocol has been determined. For primer design, Primer-BLAST is an excellent resource to generate primers specific to the target with minimal off-target amplifications as well as primer designing tools available on softwares such as Snapgene and Benchling (Sofi et al, 2022). The primers should include sequences specific to the region of interest as well as adaptors for a second round of PCR to index the sample. Gel electrophoresis through agarose gel or Tapestation devices can be used to validate the correct size of the amplicon. The desired library can be isolated through gel extraction or size selected through purification with magnetic beads. We suggest cleaning and checking library size after each PCR step during initial protocol optimization to ensure reliable results.

DATA ANALYSIS

Lineage-tracing methods that utilize Cas9 to generate random, nonordered edits are analyzed through phylogenetic analysis methods, which can be mathematically complex. Data from these assays are analyzed comparably to phylogenetic analysis of accumulated mutations in the genome to determine phylogenetic maps of cell ancestry and lineage (similar analysis is used to generate evolutionary trees) (Pavlopoulos et al, 2010). These data can be analyzed with assistance from software available on Github developed in several of the methods described in this review (Bowling et al, 2020; McKenna et al, 2016; Raj et al, 2018; Yang et al, 2022). The Cassiopeia pipeline for single-cell lineage-tracing data may be useful as well (Jones et al, 2020).

Because the tape in DNA Typewriter and peCHYRON is sequentially edited and the sequence of the tape is highly repetitive, the editing data produced from these experiments must be analyzed differently from those produced with GESTALT or related methods. The scripts used in the original DNA Typewriter and peCHYRON papers are similarly available on Github and Figshare (Choi et al, 2022; Loveless et al, 2021a[1]). Familiarity with Regular Expressions is also advised for analysis of this type of sequencing data.

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CONFLICT OF INTEREST

The University of Washington has filed a patent application for several technologies related to molecular recording, including DNA Typewriter, ENGRAM, and P3 editing, on which JS is listed as inventor. JS is on the scientific advisory board, a consultant, and/or a cofounder of Adaptive Biotechnologies, Cajal Neuroscience, Camp4 Therapeutics, Guardant Health, Maze Therapeutics, Pacific Biosciences, Phase Genomics, Prime Medicine, Scale Biosciences, and Sixth Street Capital.

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AUTHOR CONTRIBUTIONS

Conceptualization: JFN, CLS; Formal Analysis: JFN, JLA, CLS; Resources: CLS; Supervision: JS, CLS; Visualization: JFN, JLA; Writing - Original Draft Preparation: JFN, CLS; Writing - Review and Editing: JFN, JLA, JS, CLS

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