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Review

Simplifying cell fate map by determining lineage history of core pathway activation during fate specification

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ABSTRACT

A fundamental question in developmental biology is how a single genome gives rise to the diversity of cell fates. In essence, each cell fate in the human body is a unique but stable output state of the genome, maintained by positive and negative feedbacks from both inside and outside the cell (a stable cell state). Traditionally, defining a cell fate means identifying a unique combination of transcriptional factors expressed by the specific cell type. The hundreds of transcriptional factors in the genome, however, have complicated the task of simplifying cell fate representation and obtaining insights into its regulation. Moreover, results from this approach provide only a static picture, with each cell fate/state disconnected from one another. An alternative approach instead defines cell fates by determining their relationship to each other, through identifying the signaling pathways that control each step of their lineage transition from a common progenitor during development. Decades of studies have shown only a handful of signaling pathways are sufficient to specify all cell fates in the body, simplifying the execution of such a strategy. In this review, it is argued that this alternative approach is not only feasible but also has the potential of simplifying the cell fate landscape as well as facilitating the engineering of different cell fates for regenerative medicine.

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KEYWORDS: cell fate, cell state, signaling pathway, cell fate transition, lineage history, pathway activity recorder, pathway activity reporter.

INTRODUCTION

The human body contains hundreds of different cell types, each with a different gene expression pattern and performing a different function. Despite these differences, however, they are all derived from a single common progenitor, the fertilized egg, and all carry an identical genome. Each of these cell types/fates in the body is therefore in essence simply one of the output states of a single identical genome. Under physiological conditions, these different cell states are quite stable. Some are maintained by cell-intrinsic positive and negative feedback mechanisms, apparently independent of extrinsic inputs, while others rely to a different degree on positive and negative feedback signals from neighboring cells and the environment, for maintenance of a stable state. Under diseased conditions, however, many of these cell states become functionally impaired and their replacement by regenerative medicine is increasingly a promising potential therapeutic option. Regenerative medicine requires the engineering of specific cell fates from pluripotent cells and thus in-depth knowledge of the normal developmental mechanisms that specify these cell fates. This may not pose a problem for common cell types. However, it will likely be an issue for

many rare cell types that are poorly studied. Thus, a simplified cell fate map of all cell fates in the body that not only indicates their relationship to each other but also shows how they are derived from a common progenitor would likely greatly promote the application of regenerative medicine. Traditionally, cell fates are defined by the unique combination of transcriptional factors that these cells express. The hundreds of transcriptional factors in the genome, however, have complicated the task of simplifying cell fate representation. Moreover, such representation is also static, with each fate disconnected from one another. In this review, it is argued that by determining the activation history of the handful of core signaling pathways involved in cell fate specification during development, one may derive a simplified cell fate map that may not only reveal novel insights into cell fate landscape regulation but may also facilitate the engineering of these cell fates.

Defining cell fates using the lineage history of core pathway activation

Decades of research have revealed there exist only two mechanisms of cell fate determination during normal development, extrinsic regulation and intrinsic patterning. Intrinsic patterning typically depends on asymmetric cell division in which cell fate determinants are differentially segregated to two daughter cells to specify their different cell fates. Since *pure* intrinsic patterning invariably leads to the same combination of output cell states from one progenitor, as is best illustrated during Drosophila neuroblast division [1], such events can each, in aggregate, be considered one cell fate. As a result, one can essentially ignore intrinsic cell fate patterning and focus on the regulation by extrinsic signals. Viewed in this light, each cell fate in the body can be considered the product of a unique sequence of extrinsic signals that the common progenitor is exposed to during development. As such, one may define any specific cell fate using *the identity and the order* of the series of signals that the cell is exposed to and/or the pathways that are subsequently activated in the lineage history of that specific cell type (Figure 1). Since only about eight core pathways are involved in and sufficient for all cell fate decision in the body, such an approach may greatly simplify the representation of the cell fate landscape and reveal the underlying logic of cell fate diversification and specification. It may also provide a blueprint for differentiating pluripotent stem cells into potentially any cell type, thereby potentially serving an equivalent role in biology as the periodic table in chemistry.

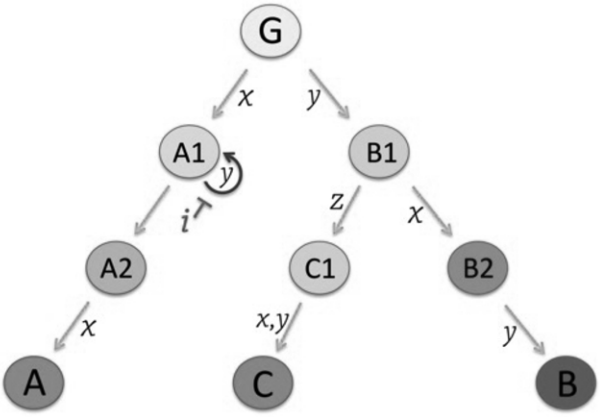

Figure 1. Defining cell fates using lineage history of core pathway activation. Each cell fate would be defined as the order and the identity of the core pathways activated during the multi-step specification of that cell fate from a common progenitor **G**. For example, the cell fates illustrated above would be defined as: **A**: *x, -y, x*; **B**: *y, x, y*; **C**: *y, z*, (*x*,*y*); **A1**: *x, y*; **A2**: *x, -y*; etc. Pathway *y* is used by intermediate fate **A1** for self-maintenance, the inhibition of which by *i* triggers transition to **A2**. The combinatorial activation of pathways *x* and *y* (*x*,*y*) instead triggers the last step in the specification of cell fate **C**.

Studies across many vertebrate and invertebrate species have shown that only a handful of pathways, including Hedgehog, Wnt/β-catenin, receptor tyrosine kinase (RTK), Notch, TGFβ, BMP, JAK/STAT, and retinoic acid (RA) signaling, a total of eight, have evolved and appear sufficient for specifying all the myriad of cell fates in the body [2]. At first glance, this may appear puzzling. Yet, if viewed through the lens of cell lineage, this is in fact a beautiful solution for cell fate specification. In the lineage tree of all cell fates, each step may be considered a different cell state (Figure 1). Since it is a different state, even the same signal that induces that specific state could theoretically be used again to trigger its transition to another cell state. This has indeed

been observed for RTK signaling in Drosophila eye development, where a 'prepattern' determines the outcome of many fates while all the transitions are triggered by RTK signaling [3, 4]. Thus, with 8 pathways, one could theoretically generate from 1 ground state $8 \times 8 \times 8 \times 8 = 4096$ different cell fates in only 4 transition steps, without even involving the combinatorial use of the core pathways. In practice, of course there are many constraints. For example, as a safety mechanism, the use of the same signal to activate two consecutive steps of cell fate transition should probably be avoided, to prevent spurious lineage progression, unless perhaps, for example, there is a big enough response latency separating these two steps. Pathways may also be needed, at the same time or place, for other biological processes or in adjacent cells. Nevertheless, even with these constraints, a handful of pathways likely still suffice to generate the diversity of cell fates in the body. Defining cell fates by their *lineage history of pathway activation* may thus provide a simplifying view of how a single genome generates a diversity of cell fates.

The feasibility of this approach is supported by several lines of evidence. First, compelling evidence indicates that unlike physiological signaling, developmental signaling is mostly linear, with minimal pathway crosstalk. This may stem from the need to transmit a clear signal for cell fate specification during development, due to the quick temporal pace of these processes [5]. As a result, it substantially removes the complication of pathway crosstalk and justifies the representation of the activity of each pathway (at least for most of the pathways, see below for discussion on morphogens) in only one binary dimension (either on or off).

Second, for many of the eight core pathways involved in cell fate specification, there indeed exist a plethora of ligands, receptors, and other modulators. However, studies show that these ligands, receptors, and modulators mostly evolve to provide spatial/temporal target specificity, redundancy, robustness, and/or for other optimization during development, but they eventually all converge on the same intracellular signaling cascade of one of the core pathways during cell fate specification, either turning it on or turning it off (e.g. [6-9]). This therefore obviates the need to represent any of these upstream complexities. Instead, since they converge on the core pathways, one only need to report whether these pathways are on or off in the transition steps. One exception to this is JAK/STAT signaling, which, because of its simplicity, entails less ligand-specific-signal degradation [10, 11] (i.e., less pathway convergence) and each ligand may of itself transmit a different signal. However, it is conceivable that with additional reporters generated for each of these JAK/STAT pathways, their potential distinctions may be further mapped. Several RTK ligands also induce different dynamics of pathway activity (transient vs. sustained), leading to different outcomes (proliferation vs. differentiation) [12-15]. However, since cell proliferation alone does not amount to a bona fide cell fate decision, it is of less relevance to the goal of simplifying the cell fate map. There have also been reported cases of combinatorial use of RTK ligands during cell fate specification in the Drosophila eye. However, studies show that the different RTK ligands mainly confer spatial and other information, while the pathway remains binary (either on or off) during the cell fate specification process [16, 17]. Thus, by focusing on the core pathways, it is possible to isolate the common denominators of cell fate decision, while removing the complexities of upstream ligands/receptors and modulators.

Third, it is well known that the same ligand can induce very different responses in different cell types. However, in the absence of combinatorial use with other ligands, the different responses can only be due to the different states that the responding cells are in, a variable already accounted for as lineage history in the proposed model (Figure 1). Exceptions to this include morphogens such as Hedgehog [18, 19] and Nodal [20], which appear indeed capable of inducing different cell fates from the same precursor cells based on the morphogen dosage. However, recent studies have shown that the length of time the precursor cells are exposed to plays a key role in morphogen gradient interpretation and fate determination in these cells [21-23]. Thus, while it may be technically challenging at present, one may determine the

duration of morphogen-responding pathway activation during cell fate lineage progression, incorporate this information into the linage history, and provide a cell fate map that also includes regulation by morphogen gradients. As such, for the vast majority of core pathways, a temporal-sensitive binary code may suffice to represent all the inputs into their lineage history.

Fourth, for the ligands or other components of nearly every canonical core pathway, there are also notably non-canonical cascades activated both during development and in adulthood. In cases of cell fate decision, however, studies show these non-canonical events all appear to converge on the canonical intracellular cascade of another core pathway [24-26]. Many of these cases presumably result from unique cellular contexts that elevate pathway crosstalk at the expense of canonical signaling. For example, in the Drosophila eye, photoreceptor R4 specification depends on Wnt pathway signaling yet the Notch pathway has been found activated in R4. Studies show this in fact result from Wnt pathway inducing a Notch ligand in neighboring R3, which then activates Notch pathway in R4. Thus, recording Notch pathway activation alone would suffice to represent this step in R4 lineage history. Meanwhile, it also removes the complications from indirect Wnt pathway involvement. In another case, Notch has been found to inhibit the activity of β-catenin, a canonical Wnt-pathway component, to regulate stem cell differentiation. The bona fide signal in this step is thus the loss of Wnt pathway activity in the cell of interest, and not the turning-on of Notch pathway activity. The loss of Wnt pathway activity, not the gain of Notch activity, should therefore be recorded. These examples demonstrate that, even though a core pathway may be activated or inactivated by a non-canonical signal due to pathway crosstalk, it is still a valid approach to record the identity and the activation state of the core pathway in the cell of interest for defining its cell fate lineage history.

Lastly, for some lineages, as alluded to above, cell fate progression is achieved not through core pathway activation, but through inhibition. For example, inhibition of TGFβ signaling is known to induce neural fate in the embryonic ectoderm [27, 28]. Another way of interpretating this is that the TGFβ pathway normally provides a positive feedback loop that maintains the intermediate ectodermal fate. Interruption of this feedback loop triggers the cell fate transition and the induction of the new neural fate. These events in lineage history may be recorded by the loss of the specific core pathway activity. Altogether, these lines of evidence thus strongly argue that by determining the activation state (on and off), the activation duration, and the activation order of each of the core pathways during lineage progression, it is possible to produce a simplified map of most cell fates in the body.

Strategies for producing a simplified cell fate map

To design a reporter system for recording core pathway activity during lineage progression, one may adopt a strategy combining the use of pathway-specific regular or destabilized tamoxifen-inducible Cre ($CreER^{T2}$) with loxP-STOP-loxP reporters that converts transient pathway activation into permanent changes at the genomic level (Figure 2). By systematically delivering tamoxifen during development using a battery of pathway specific $CreER^{T2}$ lines while examining reporter expression at mature stages, one may obtain a lineage history of any cell type of interest in the body. Similar approaches have already been used successfully for examining individual pathway activities during development. For example, in one case, $CreER^{T2}$ has been inserted into the locus of Gli1, a transcription factor induced by Hedgehog signaling, revealing dynamic changes of Hedgehog signaling during limb and brain development [29, 30]. In another case, $CreER^{T2}$ has been inserted into the Axin2 locus for tracking Wnt/β-catenin signaling during development [31, 32].

One key to this strategy is the identification of pathway- but non-tissue-specific (i.e., ubiquitous) inducible genes or promoters for driving $CreER^{T2}$. Decades of research have indeed identified many such genes and/or DNA response elements for each of the eight core pathways (Table 1). Thus, it is feasible to generate $CreER^{T2}$ lines for all core pathways (and in the long term, potentially multiple independent lines for each pathway). Another key to this approach is to activate $CreER^{T2}$ in a temporally precise manner. Tamoxifen clearance takes time *in vivo* [33]. One may therefore expect

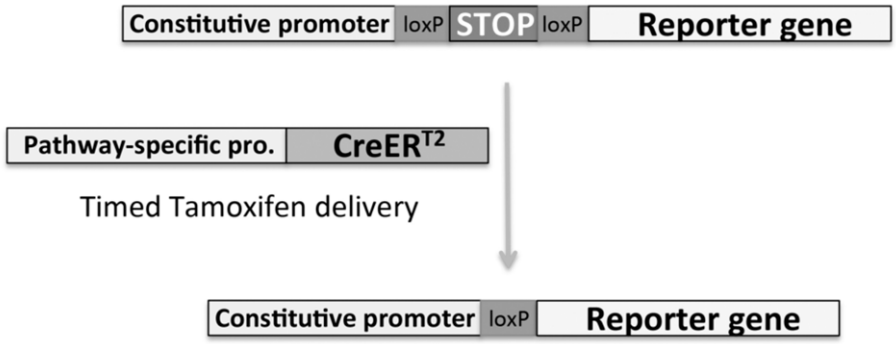

Figure 2. A reporter system for recording core pathway activity during lineage progression. Temporally precise Tamoxifen delivery, together with pathway activity-driven CreER^{T2}, will convert temporary pathway activity into permanent changes in the genome.

Table 1. Pathway-specific inducible genes and DNA response elements.

Pathways	**Inducible genes**	**DNA response elements**
Hedgehog	Gli1 [29]	Gli-binding site [34]
Wnt/β-catenin	Axin2 [35, 36]	TCF binding motif (e.g. [37])
Receptor tyrosine kinase	Sprouty2 [38, 39] DUSP6 [40-42]	Sprouty2 promoter 0.4 kb region [43] DUSP6 Ets-binding site [44]
TGFβ	Smad6/7 [45, 46], TMEPAI [47]	PAI-SBE [48-50]
BMP	Smad6/7 [45, 46]	BRE [51, 52]
Notch	Nrarp [53, 54]	CBFRE [55, 56]
JAK/STAT	SOCS3 [57]	SOCS3-SBE [58, 59], Drosophila SBE [60, 61]
Retinoic acid (RA)	RIP140 [62, 63]	RARE (e.g. [64, 65])

this will broaden the time window when the reporters appear active. However, by comparing just the *onset point* of each of these active time windows, the order of pathways involved can likely still be deduced. In addition, by employing different recombinases such as Cre and Flp under the control of different pathways of interest, together with the use of special reporters designed to distinguish their order of activation, the order of pathway activation can conceivably be further refined.

In analyzing results from using this reporter system, several classes of signals may be encountered: a) bona fide fate transition signals that, individually or in combination, promote lineage progression (some of which may also simultaneously regulate cell cycle); b) autocrine/paracrine signals involved in the maintenance of intermediate cell fates, the inhibition of which may trigger cell fate transition; c) pure cell cycle-regulating signals that also utilize the core pathways; d) developmental coordinating signals that use the core pathways (e.g., signals that regulate expression of ligands of another pathway). On the other hand, the myriad of signals that regulate cell localization, migration, and/or other aspects of subcellular/intercellular spatial organization (such as axon/dendrite growth and guidance) will likely be removed (except in cases when they also engage the core pathways). Thus, although additional strategies (e.g., testing during embryonic stem cell differentiation of the

roles of specific pathways) may be needed to further parse out potentially unrelated signals, this system may establish at first a coarse-grained lineage history of the vast majority of cell types in the body. In turn, this may help reveal higher-order insights into the biology of cell fate regulation.

Potential specific steps for producing a simplified cell fate map

1) Generate and validate CreER^{T2} lines for all core pathways

As mentioned, two CreERT2 lines have already been generated for the Hedgehog and Wnt/β-catenin pathways and are likely sufficient for the initial determination of major lineages. To generate CreER^{T2} lines for the remaining pathways, one may take two approaches. For RTK signaling, since DNA response elements are not as well defined, one may generate CreER^{T2} knockin into the Sprouty2 locus (Table 1). For other pathways, since a large number of transgenic lines have been successfully generated employing DNA response elements, one may employ these same DNA elements (Table 1). This may also avoid potential complication due to uncharacterized promoter elements in pathway-inducible genes (e.g., [59, 66-68]). To ensure specific CreER^{T2} expression, one may generate these transgenic lines by knockin into the Hprt locus, which has been shown to provide a neutral transgene environment [69-72]. Although X-inactivation may affect reporter use, the Hprt locus may still be better than Col1A1 (autosomal) locus, since the latter appears to repress brain transgene expression [73-75]. To validate these lines, one may take three approaches. First, derive cell lines from each to determine ligand/pathway specificity as well as dosage response. Second, evaluate Cre expression *in vivo* throughout development in tissues with known pathway activity (see references in Table 1). Lastly, employ pathway-specific mutations to determine *in vivo* dependence of Cre expression on pathway activity (e. g. [39]).

2) Test the system through determining lineage histories of midbrain dopaminergic (DA) neurons and pancreatic β-cells

Because of intense medical interest, DA neurons and β-cells are among the cell types best characterized regarding developmental regulation and stem cell differentiation. They are thus well suited for testing the system. In addition, this may also yield additional insights for improving the engineering of these cell types and their application in medicine. Several pathways have been implicated in DA neuron development, including Hedgehog, FGF8, and Wnt signaling [76]. Notably, the recent identification of Wnt/β-catenin regulation in DA neuron development has dramatically improved efficiency of DA neuron *in vitro* differentiation [77, 78], validating the importance of elucidating their lineage history of core pathway activation. However, many questions still remain. Are other pathways completely irrelevant? If not, what are they and when are they active? Wnt signaling is known to regulate floor-plate neurogenesis in the midbrain [79]. Is it also involved in earlier lineage specification of DA neurons [80, 81]? Besides the midbrain, DA neurons are also produced in the forebrain. What is the difference in the lineage history that produces these different classes of DA neurons? Employment of the proposed reporter system may not only validate pathways already identified, but also provide answers to these questions. Similarly, in β-cell development, although early steps through pancreatic endoderm induction are relatively well understood, little is known about later steps. As a result, efficient differentiation of fully functional β-cells *in vitro* has not yet been achieved [82, 83]. Engrafted pancreatic progenitors, however, can mature *in vivo* into insulin-expressing, glucose-responsive cells (e.g., [84, 85]). This suggests yet-to-be-identified signals that promote β-cell differentiation. Employment of the reporter system may help identify these signals and facilitate *in vitro* generation of fully functional β-cells.

3) Elucidate mechanisms of neocortical fate decision through determining major cell lineage histories

How the diverse cortical cell types are generated is a fundamental question in neurobiology and has attracted intense interest in the field (e.g., [86-90]). The proposed reporter system provides a potential means to address this question at a single-cell resolution and elucidate how external signals regulate cell fate decision by each cell,

at each step. To this end, one may first focus on intralaminar and interlaminar cell fates. To study intralaminar fates, one may choose layer V, which contains well-characterized neuronal subtypes that account for the morphological and physiological diversity of layer V [91-94]. This knowledge may help eliminate potential complication of lineage merging in experiments. Similarly, one may analyze neuronal subtypes in other layers, especially rare populations (to minimize chances of lineage merging) [95, 96], to identify signals directing interlaminar fates. Furthermore, one may track cell positions along the medial-lateral and anterior-posterior axes, and identify signals directing arealization (e.g., [97-99]). Recent studies have revealed a significant stochastic contribution to neurogenesis in the retina [100, 101]. The same may apply to brain neurogenesis. To investigate this possibility, one may integrate all data and deduce any signal(s) that may influence the probabilities of specific fates. Altogether, by mapping all the extrinsic inputs regulating cell fate during corticogenesis, one may substantially elucidate the contributions of external signals in coordinating the overall pattern of cortical neurogenesis and gliogenesis as well as the specification of individual cell fates. This may help resolve one of the fundamental questions in neurobiology.

In the long run, it is conceivable that one may determine the lineage history of all major cell types in the body, provide an extensive map of nearly all cell fate transition connected by core pathways during development, and begin to better understand the logic of cell fate decision at a higher level. This may help answer a number of fundamental questions in biology. For example, what signals do prospective adult stem cells receive that distinguish them from other progenitors? Is there a general rule by which tissues control the proliferation of stem cells and how its disruption may relate to cancer? At the genome level, how do specific core pathways orchestrate the transition of one stable cell state into another and how are distinct cell states maintained? Lastly, by delineating similar fate maps from different species, one may even potentially begin to understand how cell fate regulation evolves.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest.

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