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SPOTLIGHT

Cell state transitions: definitions and challenges

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ABSTRACT

A fundamental challenge when studying biological systems is the description of cell state dynamics. During transitions between cell states, a multitude of parameters may change – from the promoters that are active, to the RNAs and proteins that are expressed and modified. Cells can also adopt different shapes, alter their motility and change their reliance on cell-cell junctions or adhesion. These parameters are integral to how a cell behaves and collectively define the state a cell is in. Yet, technical challenges prevent us from measuring all of these parameters simultaneously and dynamically. How, then, can we comprehend cell state transitions using finite descriptions? The recent virtual workshop organised by The Company of Biologists entitled 'Cell State Transitions: Approaches, Experimental Systems and Models' attempted to address this question. Here, we summarise some of the main points that emerged during the workshop's themed discussions. We also present examples of cell state transitions and describe models and systems that are pushing forward our understanding of how cells rewire their state.

KEY WORDS: Cell state transitions, Definition of cell states, Heterogeneity, Modelling

Introduction

The term 'cell state transition' refers to the process by which cells change states over time. Such transitions are an intrinsic part of embryonic development as cells progressively differentiate. They are also crucial during homeostasis and tissue repair, as damaged and worn cells are replaced to maintain tissue function. Moreover, many pathologies, from developmental disorders to cancers, involve aberrant transitions in cell states. Thus, understanding these transitions is of crucial importance.

The Company of Biologists virtual workshop on 'Cell State Transitions: Approaches, Experimental Systems and Models' brought together experimentalists and theorists from different backgrounds who are studying cell state transitions across various systems. In themed discussions, we tackled three topics: the definition of cell states and the role of heterogeneity; the role of autonomous and non-autonomous regulation in informing cell states and transitions; and the technical challenges and opportunities facing the field. In this brief Spotlight article, we summarise some of the main messages that emerged from the discussions.

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Defining and identifying cell states

A starting point of discussion was how the definitions of cell states have evolved over time. With limited tools, cell states were initially assigned based on observable and phenotypic features, such as location, morphology and inferred function. Indeed, the phrase 'cell states' (*Zellenstaat*), akin to cell 'societies', was a metaphor that emerged in the late nineteenth century to describe the grouping of cells based on their functions, where each 'state' fulfilled an essential role and contributed to the 'economy of the organism' (Reynolds, 2007). Over time, the definition of cell state became increasingly reliant on the description of molecular features. As technology progressed, non-specific dyes that could broadly mark populations of cells or organelles were replaced by antibodies that could recognise specific epitopes (Coons et al., 1941), and then by hybridisation-based techniques that could detect an ever-expanding repertoire of markers that underpin cell state. Global profiling using high throughput technologies, such as next generation sequencing, has further expanded the number of descriptive parameters available. Today, we generally identify cell states using complementary approaches: by molecular characterisation, i.e. the description of different molecules (whether transcripts, distribution of chromatin marks or proteins), and by functional characterisation i.e. the description of what a specific cell can do.

Molecular characterisation of cell states

The most common descriptor of cell state relies on the annotation of specific molecules that compose a particular cell. Traditionally, cell states were defined using a small number of parameters or key markers that either showed strong correlation with a functional cell state or were functionally required (Mojtahedi et al., 2016; Wheat et al., 2020). For example, in the context of mouse development, pluripotency is generally characterised by expression of the transcription factor Oct4. Increasing the number of markers allows pluripotency to be further subdivided into distinct states. For example, naïve pluripotency, which is restricted to the pre-implantation epiblast, is characterised by the co-expression of Oct4, Nanog, Sox2 and Klf2/4, whereas formative pluripotency, associated with early post-implantation development, is associated with Oct4, Sox2 and Otx2 expression (Kinoshita and Smith, 2018; Nichols and Smith, 2009).

Global profiling has enabled the identification of a much larger set of defining molecular characteristics. A series of technical advances, in particular in single cell approaches, has allowed us to characterise an ever-greater number of single cells and parameters, tackling systems of increasing complexity and size. This increased capacity has been incredibly useful for identifying and characterising rare populations (e.g. hematopoietic stem cells or primordial germ cells) and very heterogeneous or complex systems (e.g. the brain).

Molecular characterisation of cell states does not, in principle, require previous knowledge of the system. However, annotation of such datasets often relies on knowledge of marker expression. With single cell assays, we can obtain many parameters describing

Development (2021) 148, dev199950. doi:10.1242/dev.199950

very complex multicellular systems. However, there is a significant limitation: as we cannot characterise all types of molecules at the same time (e.g. genes, proteins, etc.), we are often forced to pick one type of measurement, most commonly gene expression. Therefore, we assume that cell states are accurately characterised or identified by that measurement. Although multi-omics approaches are now allowing us to analyse multiple features in parallel (e.g. gene expression and chromatin accessibility), they largely remain confined to exploring regulation at the DNA level. However, a cell state is more than the sum of its parts, meaning that multiple regulatory levels are often fundamental for determining and maintaining cells in a given state. Finally, in the context of molecular characterisation of cell states, it can be challenging to identify which molecules, amongst all those present, contribute to regulating that particular state.

Functional characterisation of cell states

Functional assays are a powerful tool for identifying and defining cell states based on what cells can do. For example, the functional characterisation of cells as mature pancreatic β-cells requires cells to respond to high glucose concentrations by depolarising, increasing calcium influx and secreting physiological levels of insulin (Pagliuca et al., 2014). Immature or wrongly-specified cells fail one or more of these functional tests. In the case of stem and progenitor cells, clonal lineage tracing *in vivo* is a powerful method to reveal both self-renewal and tissue contributions of single cells over time (Blanpain and Simons, 2013). For example, transplantation of single haematopoietic stem cells (HSCs) proved their ability to reconstitute all lineages long-term in mice (Osawa et al., 1996) and subsequent single cell transplants uncovered functional heterogeneity within the stem cell pool (Dykstra et al., 2007). Similarly, the ability of cells to integrate into a host pre-implantation embryo and contribute to normal development in chimera assays is a defining functional property of mouse embryonic stem cells (Bradley et al., 1984; Masaki et al., 2016).

In vitro functional assays can also be very powerful. For example, cell culture assays have demonstrated the ability of single Lgr5+ cells to generate intestinal organoids (Sato et al., 2009). Culture systems similarly validated a distinct functional cell state during early embryonic development in which cells transiently acquire the competence to form primordial germ cells (Ohinata et al., 2009; Hayashi et al., 2011; Kinoshita and Smith, 2018; Mulas et al., 2017).

Functional characterisation requires appreciation of the biology of the system and can be challenging at the single cell level. Moreover, in complex and dynamic contexts, it can be difficult to link the functional response to a molecular phenotype. Often, linking function to molecular profiling relies on dividing the cell pool into subpopulations based on a limited set of markers. However, as functional assays probe cell behaviour, we can identify cell states and transitions that might arise from a complex interaction of gene expression, chromatin and protein changes; these states and transitions might not be apparent when looking at each regulatory level in isolation. A further benefit of functional assays is that theyprovide powerful readouts for phenotypic screens and thus can be used to identify potential regulators of cell states and transitions.

Multiscale descriptions

Although molecular descriptions and functional assays are powerful tools to describe cell states, the workshop emphasised that the next technical challenge is to combine different techniques to attain a multiscale description of cell states. The development of multi-omics approaches is now allowing us to characterise cells, cell states and transitions between cell states across multiple levels of regulation (Lee et al., 2020). In parallel, spatial transcriptomic methods are becoming increasingly useful in characterising cellular gene expression in systems in which function correlates with spatial location (Waylen et al., 2020). However, integrating data across regulatory levels remains challenging. For example, it would be very beneficial to merge functional and molecular descriptions of cell state. However, most molecular/high throughput sequencing techniques destroy cells. Thus, it is generally not possible to simultaneously measure the transcriptional state of a cell as well as its functional potential. New approaches are emerging to meet this challenge, either by labelling cells with markers and reporters, or by sampling labelled populations over time and integrating clonal lineage tracing with single cell transcriptomics (Wagner and Klein, 2020). Moreover, live-cell RNA-sequencing represents a major technological advance that could allow for the combination of functional and molecular assays in single cells (Chen et al., 2021 preprint).

Discrete versus continuous cell states

Defining the state of a cell based on its constituents is already a challenge in terminally differentiated tissues or tissues with limited turnover (e.g. the cerebral cortex). In such systems, cells typically maintain stable patterns of gene expression, chromatin modifications, etc., yet show staggering diversity. The challenge of defining cell states is further compounded in dynamic systems, such as during embryonic development or homeostatic tissue turnover. In such systems, it is not straightforward to determine, for example, when a cell becomes differentiated and is no longer a stem or progenitor cell. Moreover, thousands of genes and loci, and hundreds of proteins, can change over a short period of time. During the themed discussions, we debated whether cell states were discrete or continuous, and how much the categorisation of cells into states was dependent on the assay used.

Classical studies of embryonic development and haematopoiesis have supported the notion of discrete states, with cells passing through ‘commitment points’ – points in which cells have irreversibly committed to a fate and lose the ability to revert back and respond to signals in a different way (with the earliest evidence summarised in the 1980s; Heasman et al., 1985). However, technical challenges (e.g. the viability of single transplanted cells) have hindered the mapping of functional transitions at the single cell level with high temporal resolution. As such, it is currently difficult to determine whether abrupt boundaries exist, or whether properties may change gradually with intermediate phenotypes.

Conversely, more recent analysis of transitions by single cell RNA-sequencing have suggested continuous transcriptional trajectories. These observations have led many scientists to rethink one of the oldest models of stem cell differentiation: the haematopoietic hierarchy (Laurenti and Göttgens, 2018). The observations of continuous trajectories is not unexpected: even if cells abruptly switch states, mRNA and protein decay timescales are likely to result in intermediate expression values. Moreover, single cell RNA-sequencing is particularly sensitive to technical noise and batch effects. A major challenge is that computational methods, such as dimensionality reduction and pseudo-temporal ordering, while powerful visualisation tools, can also bias how we perceive the data.

Finally, intrinsic cell dynamics, such as cell cycle or circadian rhythms, and dynamic interactions between the cell and its environment, can further complicate the distinction between

Development (2021) 148, dev199950. doi:10.1242/dev.199950

discrete and continuous cell states. Reliable information on the dynamics of cell state transitions is fundamental for identifying the appropriate mathematical tools that can be applied to model transitions, and it also impacts how we interpret and understand the underlying molecular logic that controls cell states. In turn, appropriate mathematical and computational tools can lead to a better understanding of these dynamics. This is an exciting area in which experimental evidence combined with new analytical approaches might help resolve how molecular and functional dynamics overlap.

Environmental context: are cell states autonomous or non-autonomous?

Whether the transitions in cell state are temporally continuous or discrete, they are also influenced by the context in which the cell is found. Indeed, the key parameters defining cell state can be cell-autonomous, but they can also be extrinsic, modulated by the environment the cell is in. In a themed discussion, we debated the extent to which cell states are dependent on their niche.

In 1924, Spemann and Mangold performed a classic experiment that is now discussed in every developmental biology manual: they grafted a part of the dorsal blastopore of a *Xenopus* embryo, which they suspected induced the formation of the dorso-ventral axis, onto other part of the embryo, creating an ectopic dorso-ventral axis (Spemann and Mangold, 1924). These transplantation experiments highlighted a key observation about cell states: some cells can maintain their state and, in particular, their signalling potency, irrespective of their cellular context, i.e. their state is autonomous. On the contrary, other cells can become induced towards a different fate when put in contact with a different set of neighbours, showing non-autonomous control of their fate.

A key question that arose during the workshop related to the identification of autonomous or non-autonomous cell states. This is a complex question that might have as many answers as developmental contexts and cell types. A typical example of this complexity is found in developing vertebrate somites, in which the so-called segmentation clock produces waves of transcription (Hubaud et al., 2017; Oates, 2020). The waves travel from the developing tail towards the anterior part of the embryo and stop with the formation of each somite. Single cells from this tissue (the presomitic mesoderm) can oscillate autonomously but are poorly coordinated, and coordination is only achieved at the population level (Hubaud et al., 2017; Oates, 2020). Several mechanisms have been proposed to allow coordination, including quorum sensing of signalling molecules, adhesion and mechanics-mediated signalling. Here, the cell state is both autonomous and non-autonomous, as it operates in each cell, but can only be maintained in a coordinated manner by cells in a population. Thus, to comprehend the cell state, it is important to consider the cell context, for example its neighbours and the mechanics of the surrounding environment. This calls for the development of *in toto* models that fully recapitulate the context the cell is in. In contrast, a complementary approach is to dissect the singular constituents of the 'niche' and then reconstitute the niche using a bottom-up approach.

Does the road cells take matter?

By using such bottom-up approaches, we have realised that different cell types occasionally converge towards the same state, despite the fact that they have different origins and might have taken different trajectories. In such cases, distinguishing between cell types and cell states is not always straightforward. For example, in the mouse embryo, definitive endoderm is specified when cells from the epiblast intercalate with the underlying visceral endoderm (VE) during gastrulation. Despite epiblast and VE fates segregating early during mouse embryonic development, their transcriptional profiles converge to some extent as the definitive endoderm is specified (Nowotschin et al., 2019; Pijuan-Sala et al., 2019). Schwann cells present another curious example. These cells typically arise from the neural crest and are responsible for myelinating axons in the peripheral nervous system (PNS). However, it has been shown that Schwann cells can also originate from oligodendrocyte precursor cells (OPCs), which reside exclusively in the central nervous system (CNS) and are derived from the neuroepithelium during gastrulation. Despite their different origins, both CNS- and PNS-resident Schwann cells share many defining characteristics (Chen et al., 2021 preprint). The comparison between microglia (brain-resident macrophages) and tissue-resident macrophages was also discussed. Despite sharing many molecular characteristics, it is still debated whether these two cells are the same cell type. They have different developmental origins; in the mouse microglia are specified from the embryonic yolk sac at ∼7.5 days post-fertilisation, whereas macrophages arise from multipotent progenitors 3 days later. Moreover, transplanted bone marrow-derived macrophages fail to completely converge to a microglia phenotype when they graft in the brain and instead retain many molecular characteristics of their cell of origin (Shemer et al., 2018). It therefore appears that, in some cases, the road that cells took towards their current state can be important for defining that state and potential, highlighting the value of analytical approaches that integrate lineage tracing.

Transitions between states

Reversible transitions, irreversible transitions and plasticity

Throughout the talks and discussions, the issue of 'spontaneous' cell state reversibility, as opposed to experimentally induced reprogramming, was also highlighted. Most biological transitions have an intrinsic directionality under homeostatic conditions. For example, a progenitor cell is more likely to give rise to a differentiated cell than a differentiated cell is to give rise to a progenitor or stem cell. Similarly, development progresses until cells become more specialised, and they generally do not spontaneously revert. Remarkable exceptions exist, however, as observed in *Dictyostelium*, in which dedifferentiation occurs rapidly in response to damage, following a trajectory that is remarkably similar to differentiation in reverse (Nichols et al., 2020). In mammalian systems, the most common examples of dedifferentiation occur in response to damage and activation of a regeneration response, and can result in disease if unconstrained (Yao and Wang, 2020). Are such cells that revert states in response to specific stimuli (e.g. damage), without experimentally-induced genetic or epigenetic resetting, separate cell states or are they part of a single 'meta-state'? In the context of stem cells, Greulich and colleagues have argued for a hierarchy in which different molecularly-defined cells should all be considered stem cells if they are interconvertible and can adopt a state with the same lineage potential (Greulich et al., 2021). Clearly, how cell states are defined and modelled must account for instances of reversion.

Coordination of transitions

Most often, cell state transitions occur in a multicellular context. Coordinated transitions ensure that the right number of cells are specified at the correct time and in the correct place. During the workshop, we also discussed the strategies typically employed to achieve such coordination both in time and space.

Development (2021) 148, dev199950. doi:10.1242/dev.199950

Clonal history

Coordination of cell state transitions can be achieved through cell-intrinsic temporal patterns, such as the cell cycle. Across a number of systems, sister cells have been shown to be highly correlated, undergoing transitions and subsequently dividing at very similar times, as seen in the context of mouse embryonic stem cell differentiation (Chaigne et al., 2020; Strawbridge et al., 2020 preprint). As it has also been proposed that the G1 phase is permissive of cell fate transitions in early mammalian development (Chaigne et al., 2020; Gonzales et al., 2015; Pauklin and Vallier, 2013; Singh et al., 2015; Waisman et al., 2017; Wang et al., 2017), coordinated cell cycles can potentially lead to coordinated signalling responses. To add a layer of complexity, key drivers of cell state transitions, such as the bHLH transcription factor Neurogenin 2, have been shown to regulate (Ali et al., 2011) and be regulated by (Lacomme et al., 2012) the cell cycle machinery during neurogenesis. Thus, clonal history and cell states are tightly intertwined.

The role of the microenvironment in coordinating transitions

Tissue mechanics can also be an effective way to coordinate cell state transitions. For example, in several cell types, mechanical stretch can induce DNA methylation, which in turn influences cell state (Maki et al., 2021; Nava et al., 2020). Tissue stretch has also been shown to induce a coordinated switch between proliferation and differentiation between post-natal and adult homeostasis in the mouse oesophagus (McGinn et al., 2021), and tissue mechanics have been shown to have an influence on the ability of CNS progenitor cells to proliferate and differentiate (Segel et al., 2019). The mechanical properties of cells can regulate cell signalling, for example by influencing ERK signalling, and in turn modify cell states (Boocock et al., 2021; De Belly et al., 2021). Similarly, we saw examples of how morphogenesis and patterning of villus and crypt regions in intestinal organoids are coordinated via osmotic changes (Yang et al., 2021). New tools are being developed to allow modulation of tissue mechanics using optogenetics, proving even greater experimental control (Martínez-Ara et al., 2021 preprint).

Beyond mechanical regulation, we also saw examples in which access to the niche or fate determinants can direct and coordinate cell fate decisions (Corominas-Murtra et al., 2020; Kitadate et al., 2019). Similarly, we discussed cases in which the *in vivo* environment achieves a level of coordination that is not recapitulated *in vitro*. During mouse embryonic development, for example, neural markers appear simultaneously as a consequence of switching from E-Cadherin- to N-Cadherin-based cell-cell adhesions, but this process is heterogeneous *in vitro* (Punovuori et al., 2019). Although it is possible to increase the synchrony of differentiating cells by directly modifying the activity of signalling pathways (for example by modulating negative feedback loops; Nett et al., 2018), it is not clear what factors determine the difference in synchronicity between the embryo and *in vitro* culture conditions.

The role of heterogeneity/asynchrony

Although transitions have to be coordinated to ensure the right cells are generated at the right time and in the correct location, asynchrony, heterogeneity and noise might play a fundamental role in cell state changes. One of the key points debated in the themed discussion was the challenge of measuring true biological noise, and the need for better methods to distinguish between technical and biological sources of variability. We also saw how heterogeneity can be used by a group of cells to increase the efficiency of information flow. For example, during the workshop we discussed how collective information processing in the context of calcium flux in cell monolayers is rendered more efficient by heterogeneity in the ability of cells to sense and receive signals (Zamir et al., 2020 preprint).

From data to models

Mathematical models allow us to obtain insights and make predictions about the inner workings of a system that might not be intuitive. As many parameters key to cell state transitions, such as heterogeneity, noise and information processing, are fundamentally mathematical concepts, it is unsurprising that mathematical models are being used increasingly to study transitions between states and are becoming integral to understanding basic biology.

Throughout the workshop, it was clear that the definitions of cell states that are employed determine the way we approach and model transitions. Defining cell states transcriptionally leads to largely descriptive analyses of cell state transitions. These analyses often leverage dimensionality reduction techniques to identify trajectories or paths that cells follow, for example during embryonic development or when stem cells become reactivated after injury. These methods also rely on the assumption that transcriptionally similar cells are likely to represent sampled timepoints within a trajectory (Saelens et al., 2019). Combining lineage tracing with sequencing shows that computationally inferred trajectories can accurately identify the paths cells follow. However, branching or commitment points (i.e. when cells choose/change fate) cannot be accurately inferred from transcriptional data alone (Weinreb et al., 2020).

Other definitions of cell states and transitions rely on stronger assumptions. We saw how cell states could be modelled as ‘attractors’, or valleys, that become destabilised as gene regulatory networks change or as noise increases, allowing cells to hop over ‘hills’ to the next valley, parameterising a model first proposed by Weddington (Camacho-Aguilar et al., 2021). Such an approach, which combines marker-based identification of cell states and experimental perturbations, has been used to construct a ‘landscape’ of cell fate decisions during pluripotent stem cell differentiation with high predictive power (Sáez et al., 2021).

Conversely, defining cell states as discrete entities leads to stepwise transitions through more or less defined macro- and micro-states (Stumpf et al., 2017). The power of discrete definitions of cell states, combined with accurate measurements of population dynamics, can be used to identify a stochastic tissue renewal program based on competition for fate determinants, such as niche access (Krieger and Simons, 2015) or growth factors (Kitadate et al., 2019). The workshop also clearly highlighted how gene-based models can be powerful tools for explaining fate transitions, and the importance of accounting for dynamics and changes in dynamics to address changes in cell states (Negrete et al., 2021).

As each model has important implications for the underlying biology, it is necessary to test the extent to which the underlying assumptions are valid. For example, describing a biological process as a phase transition implies cells must go through a critical point, characterised by the appearance of power-law patterns (i.e. when one variable changes as a power of another, independent of the initial conditions) and sharp increases in variance/disorder, as shown recently in tissue remodelling (Petridou et al., 2021). Similarly, describing a transition as a Markov-chain process (Wheat et al., 2020) implies the future state (e.g. of a differentiated cell) depends exclusively on the previous state (e.g. of the progenitor), without any previous memory of the states that preceded it.

Finally, the type of data used to describe the system ultimately constrains the models we can use. For example, a Markov process assumes we know and can measure all the variables that influence cell states and transitions. However, this is generally not possible. Moreover, even if the key parameters are known, most biological data is sparse and subject to sampling. Such datasets instead call for computational methods built upon on non-Markovian dynamics (Wang and Klein, 2021 preprint). The challenge is for theorists and experimentalists to work together to go beyond determining whether the data fit a model, and to test experimentally the assumptions behind and predictions from the models.

Perspectives

Much of the discussion around the concept of cell state was essentially philosophical. How do you define cell state? There are probably as many definitions as there are biological, biochemical and biophysical parameters that can be used to describe a cell. Furthermore, the parameters used to describe cell state are not necessarily those that are important, or sufficient, to control it. However, fully understanding transitions between states calls for dynamic, multiscale measurements combined with formal mathematical and computational modelling. Ultimately, this virtual workshop served to demonstrate the necessity and incredible power of bridging scales and disciplines to tackle the fundamental issue of how cells establish and rewire their states.

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