Symmetry breaking in development and stochastic gene expression

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Abstract

The prevailing emphasis in developmental biology since the expansion of the molecular biology age has been that developmental decisions are instructive. A cell differentiates to become a specific cell type because it receives a signal, whereas its neighbour, that does not receive the signal, adopts a different fate. This emphasis has been generally accepted, largely because of the success of this view in tractable invertebrate model organisms, and the widespread similarities in molecular regulation to the development of more complex species. An alternative emphasis, that cells make their own decisions, has until the past decade been conspicuously silent. Here I trace the re-emergence of our appreciation of single cell decision-making in development, and how widespread this phenomenon is likely to be. I will focus the discussion on the potential role of stochastic gene expression in generating differences between cells in the absence of simple instructive signals and highlight the complexity of systems proposed to involve this type of regulation. Finally, I will discuss the approaches required to fully test hypotheses that noisy gene regulation can be extrapolated through developmental time to accurately specify cell fate.

The excitement of the so-called Golden Age of developmental biology was in the unification of developmental phenomena with molecular biology. The ideas were simple and attractive. The generation of form and pattern within an embryo could begin to be explained in terms of molecules with specific localisations and activities, either within cells or embryos. A molecule localised at a particular part of an embryo would drive the cells near it into a specific fate. Cells not seeing this signal would become something else. In many situations, localised gene expression would match some previously described embryonic organiser, which was known to have the supposed activity. The emergent unity between embryology and gene was satisfying to both undergraduate and expert scientist.

Much of the early momentum behind the successes of this era can be traced to pioneering genetic screens in relatively simple invertebrate models. Mutants lacked specific structures, or had them in the wrong place. The genes identified therefore determined cell identity at the correct position in the embryo. As molecular biology became easier, and a multitude of homologous genes were found involved in analogous, and perhaps homologous processes in vertebrate development, developmental determinism- the view of genes providing a blueprint for pattern in an embryo- became real. Putting a transcription factor into a fibroblast turns it into muscle. Taking away the factor (with one of its friends) means the muscle is gone. Disrupting another transcription factor can cause the loss of eyes. Expressing it ectopically gives eyes on legs.

This was a triumphant phase in biology. The instructive processes characterised are indeed widespread, and occur over multiple scales, derived from the asymmetric partitioning of determinants in a single cell or syncytium, through to populations of cells (organisers) with some predetermined signalling potential. And as methods for imaging and measuring how genes pattern embryos have been greatly improved by the growing partnership between developmental biology and traditionally more quantitative sciences, the accuracy with which genes provide blueprints, and some very interesting mechanisms that help generate this accuracy, have been revealed. Simplicity and instruction are appealing. If something controls something, we are at least temporarily sated. That the something that controls also needs to be controlled, that it is not normally deleted or overexpressed, that a blueprint needs to deal with contingencies, can be dismissed, at least initially. The view goes well beyond biology. A standard history book still portrays the past as dominated by kings, queens, popes and their devious advisors, and not the contexts and events to which these symbols were completely enslaved.

Even during the Golden Age, the utility of the gene-centred instructive view had its non- and partial adherents (Nijhout, 1990). Indeed there were some striking counter examples, such as observations that endoderm and mesoderm precursors arise stochastically in chick embryos, without any obvious positional bias (Stern and Canning, 1990). However, time was needed before a rebalancing of our views of development. A successful concept will always require diverse and complex forms of attrition to be accurately contextualised, and alternative views need nourishment. We can cite the

lack of satisfaction emerging from years of mouse mutants with complex or minimal phenotypes. However, these were and still are put down to "redundancy", and less as a caveat to the gene-centric view. More recently, as the systems biology age has gained momentum, there has been a growing tendency to highlight not the individual genes (which of course are necessary) but how they act in concert. Sadly, there has been a tendency to exploit the systems label, but carry on working on genes and pathways in the standard way, which has retarded the incorporation of a pure systems view. Nevertheless, good things are often oversold in the short term, and undersold in the long, and the systems view has gained traction. Over the same timeline, we have seen a proliferation and remarkable improvement in genome wide technologies for measuring gene expression, combined with methods to monitor the transcriptional and chromatin complexes that provide regulation. It has understandably proven difficult to really comprehend the staggering complexity of these data sets without channelling our understanding through selected topical pathways and genes.

These developments have occurred alongside a proliferation of studies on the gene expression states of individual cells. Over the past 15 years, the widespread view has surfaced, combined with a broad range of new technology, that population average measurements do not adequately describe the gene expression of individual cells. Gene expression is highly heterogeneous, in all forms of life (Balazsi et al., 2011; Eldar and Elowitz, 2010; Raj and van Oudenaarden, 2008). Closely related, neighbouring cells can have strikingly different gene expression profiles. How can an instructive signal act reliably on its cellular substrate if the cellular substrate is not a constant?

In parallel, in part due to the strong surge in interest in stem cell biology, there has been a reinvigoration of the view that cell fate choices are not always instructive, and that many differentiation choices occur in an apparently stochastic manner, without clear deterministic instruction. For example, ES cell populations differentiate into multiple cell types, despite uniform exposure to signals promoting differentiation (Graf and Stadtfeld, 2008). Inner cells of pre-implantation mammalian blastocysts become either embryo, or non-embryonic tissue, with no clear deterministic trigger (Xenopoulos et al., 2012). Other paradigms of stochastic fate choice have again come to the fore. These include neural progenitor differentiation (He et al., 2012), haematopoiesis (Becker et al., 1963), specification of limb progenitors (Altabef et al., 1997), lateral inhibition (Cohen et al., 2010), tissue regeneration (Krieger and Simons, 2015), organoid differentiation (Ader and Tanaka, 2014) and *Dictyostelium* development (Weijer, 2004).

Two of these examples are particularly illustrative. The generation of organ-like structures (organoids) from stem cells has become widespread. Organoids can adopt high levels of organisation strongly reminiscent of their *in vivo* counterparts. Yet this can happen in a relatively unstructured environment, without spatial cues to generate multiple fates in different parts of the cell mass. Gut organoids can be derived from individual stem cells (Sato and Clevers, 2013), which indicates that all the information for generating the cell-type diversity and structure are contained within a single multipotent cell. The environment is permissive, but the cells become different not

because of some external signal operating on a subset of cells, but because the cells self pattern and self organise. One of the purest forms of developmental self-organisation is *Dictyostelium*. Upon starvation, these soil-dwelling amoebae assemble by chemotaxis into a multicellular structure. This structure then sub-divides, by cell sorting, into the two major cell fates (Thompson et al., 2004). The fates are not predetermined, and remain plastic and flexible until terminal differentiation. The cells decide amongst themselves, without a specific localised signal telling them what to do. How can this occur?

In this review, I will discuss the evidence that differences between cells during development can arise from spontaneous cellular heterogeneity in gene expression. Whilst this is an attractive concept, and promises to illuminate many developmental processes that have not been adequately understood from the perspective of instruction, it has remained a concept that has proven very difficult to test, and there are a number of caveats. Heterogeneity may often be invoked where there is insufficient evidence to be confident it is a useful interpretation for causing cell fate divergence. Indeed, I will draw on several examples where the embryo actively reduces heterogeneity to manage an otherwise instructive developmental programme. I will close by suggesting experimental strategies that could be used to test for a role of expression heterogeneity in setting up the differences between cells during development.

Some potential scenarios

This will not be an exhaustive review of the single cell gene expression field, although I will refer to many of the current themes. For an informed recent discussion, I refer readers to an excellent review by Symmons and Raj (Symmons and Raj, 2016). Individual cells, in otherwise uniform cell populations, can show tremendous spontaneous variability in their gene expression (Elowitz et al., 2002; Raj and van Oudenaarden, 2008; Raser and O'Shea, 2005). This heterogeneity is proposed to arise from random molecular collisions in gene expression processes, and natural variation between cells in influences such as cell cycle state and local environment. Levels of expression variability can be tuned, by intracellular (Battich et al., 2015; Gregor et al., 2007; Lagha et al., 2013; Padovan-Merhar et al., 2015) and extracellular (Corrigan and Chubb, 2014) control.

Cell fate regulators show pronounced levels of variability, from bacteria to mammalian cells (Losick and Desplan, 2008; Martinez Arias and Brickman, 2011). An essential regulator can be very strongly expressed in one cell, while neighbours have negligible expression (Canham et al., 2010; Losick and Desplan, 2008; Maamar et al., 2007; Stevense et al., 2010; Suel et al., 2007). This means neighbouring cells may have very different competence during differentiation, potentially resulting in different fate choices.

We can think of several simple scenarios for how this expression variability may be harnessed to generate the functional differences between cells occurring during development (Figure 1): genes heterogeneous at the onset of development prime cells

for specific fates (Figure 1A). The standard mechanism inferred for this type of process is that there is noise in the level of some key regulator, with a specific threshold of expression above which a cascade of effects ensues and locks in a cell fate. Alternatively, expression fluctuates during development, with heterogeneity at the time of commitment determining fate (Figure 1B). In this suggestion, the expression of the fate regulator is dynamic, and fluctuates from high to low in all cells at some point, with the expression level upon receipt of some additional signal locking in the effects of a specific expression level during development. Another possibility is that heterogeneity increases during development, resolving into different fates (Figure 1C). Finally, heterogeneity does not determine fate (Figure 1D), which relates to the possibility that cells have multiple trajectories to the same fate (Huang et al., 2005). An alternative hypothesis, discussed later, proposes heterogeneity allows flexibility in fate choice. The examples shown in Figure 1 are meant to be intuitive. An attractive alternative representation is to think of cells as existing in multi-dimensional attractor states (or epigenetic valleys), with noise contributing to the magnitude and direction of a cell's impetus out of one attractor and into the next (Huang et al., 2005).

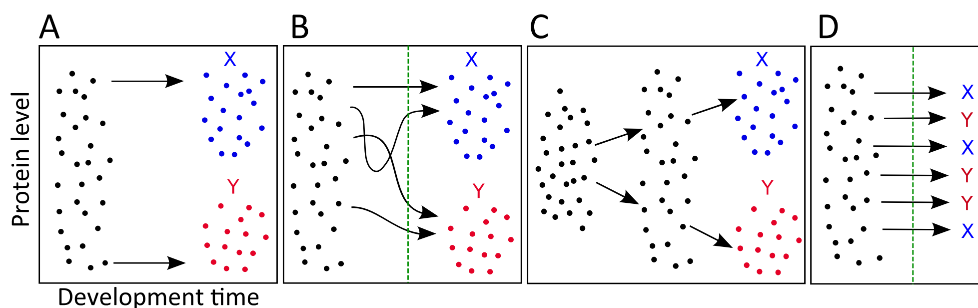

Figure 1. Different scenarios for how expression heterogeneity underlies cell differentiation to alternative fates X and Y. Each dot represents a cell. Green line in B and D shows time of fate "commitment". See text for details.

Early proofs of concept

The most compelling examples of a role for spontaneous expression variability in determining cell fate outcome are not from multicellular eukaryotic development, but from bacteria. *Bacillus subtilis* cells can develop competence to take up DNA from their environment in stationary phase. Development of competence is driven by the regulator ComK. Above a certain threshold in expression ComK activates its own expression, enforcing the decision to push the cell into the competent state. ComK expression varies between cells, even in a well-stirred culture. This variation means some cells will be below the threshold for positive feedback, and some cells will be above. Artficially reducing the variation in expression of ComK, without altering its mean level, reduces the proportion of cells that exceed the threshold level, and so impairs the differentiation to competence (Maamar et al., 2007). The essential test of the role of variability is the loss-of-function experiment- reduce the variability and see

what happens. Earlier studies had suggested that noise inversely scales with transcription rate, but is unaffected by the rate of translation. Taking advantage of these properties of gene regulation, Maamar et al reduced expression variability by increasing the *comK* transcription rate, but by weakening the translation initiation rate on the *comK* RNA, left the mean expression of ComK in the population essentially unchanged. An alternative loss-of-noise experiment was carried out by reducing the overall cellular noise, by inducing an absence of septation between dividing cells (Suel et al., 2007). Here, variability is reduced by the mixing of cellular contents between the still-connected daughter cells. The result was the same- impaired differentiation to the competent state. These studies highlight the experimental approaches required to fully test a role for expression variability. To date, and perhaps not surprisingly given the additional complexity involved in dealing with multicellular eukaryotic systems, these two studies have provided the most compelling evidence of a role for spontaneous gene expression variability as a substrate for cell fate specification.

Perhaps the strongest example in a multicellular eukaryote is the case of the *spineless* gene in the *Drosophila* eye (Wernet et al., 2006). *Spineless* is stochastically expressed in a subset of R7 photoreceptors is the developing eye. Whether the ommatidia of the eye become “pale” or “yellow” depends upon whether or not their R7 photoreceptor expresses *spineless.* The stochastic expression of this gene ensures that pale and yellow ommatidia occur in the mosaic pattern necessary for colour vision. Unlike the *Bacillus* examples, it has not been demonstrated that the presence or absence of *spineless* expression in a cell maps onto a specific end fate, nor has the variability in *spineless* expression been subject to a loss-of-noise test. It also represents a solution to symmetry breaking in an unusually highly ordered (almost crystalline) structure and is an example where stochasticity in gene expression generates a stochastic final pattern. Would an organism use stochastic gene expression to generate a more organised pattern in an organ critical for basic physiology? A similar comment may be applied to other examples where stochastic gene expression may underlie a stochastic pattern, for example, in generating the cell-type specific expression of olfactory receptors in mammalian cells (Rodriguez, 2013), or the pigmentation on a butterfly wing (Brunetti et al., 2001). However, the accusation that stochastic gene expression underlies “fate-lite” cell decisions is not likely to matter to an insect detecting colour, or a mammal trying to decipher odours, or a butterfly trying to mimic a bad-tasting neighbour, and after more than a decade, the example of *spineless* remains difficult to ignore.

Stem cell heterogeneity and stochastic gene expression- unrequited desire?
In mammalian developmental biology, the view that stochastic gene expression underlies cell decision-making has been widespread for much of the last decade. Nowhere is this more prevalent than in the study of stem cell fate choices. The fate choices that stem cells make can be highly unpredictable. Unpredictable cell fate choices lend themselves to inferences of underlying probabilistic mechanisms. The unpredictable fates can be readily observed in a culture dish of embryonic stem cells, where removal of factors promoting pluripotency generates a spectrum of different

differentiation outcomes. Even differentiation protocols optimised for a high level of cell type specificity generally fail to get a pure population of a specific progenitor fate. The diversity of fates spontaneously arising from a supposed uniform population supposedly underlies the potential for individual stem cells and unpatterned cell aggregates to develop into organ-like structures with multiple cell types, often showing organisation reminiscent of the native tissue (Sasai, 2013).

Stochastic fate choices are also a highly conserved feature of stem cell decision-making *in vivo.* The partitioning of the inner cell mass (ICM) of the mouse blastocyst into epiblast (prospective embryo) and primitive endoderm (PE; prospective extraembryonic tissue) arises independently of any clear positional cues. The epiblast and PE arise in a salt and pepper manner in the ICM, before spatially segregating prior to implantation, reminiscent of the cell fate partitioning in *Dictyostelium*. Stochastic fate choices have been revealed in a plethora of other stem cell populations in mammals. The probabilistic nature of these choices has been demonstrated by the analysis of long-term lineage tracing studies (Krieger and Simons, 2015). Based on the quantitative analysis of the resulting sizes of the labelled clones, models of deterministic asymmetric cell choices versus population asymmetric choices (stochastic, but governed by deterministic overall probabilities) can be distinguished. In the majority of cases, in a wide range of tissue types, the choices are stochastic. These lineage-tracing studies are long term, often occurring over several months, and do not report the gene expression choices of cells that might give rise to stochastic behaviour. To measure the expression changes underlying these choices requires single cell imaging of gene activity- how do expression dynamics map onto the fate of a single cell? In addressing this question, a considerable amount of study has been concentrated on a single pluripotency factor.

Nanog is a homeodomain transcription factor, identified by virtue of its ability to sustain self-renewal of mouse embryonic stem cells (mESCs)(Chambers et al., 2003). Deletion of Nanog predisposes mESCs to differentiation. Nanog became the "poster-child" of mammalian stochastic gene expression when it became clear that the protein is heterogeneously expressed in standard mESC culture conditions, and that high and low expression levels predispose mESCs to pluripotency and differentiation respectively (Abranches et al., 2014; Chambers et al., 2007). The mouse blastocyst also shows heterogeneous Nanog expression, with Nanog marking the epiblast and not the PE compartment (Xenopoulos et al., 2015). The high and low expression states, when separated, were shown to be able to repopulate the entire range of Nanog expression heterogeneity within a few days (Chambers et al., 2007). This inter-convertability of expression states led to speculation that Nanog may operate as a cell fate determinant along the lines of the scenario in Figure 1B. Analogous to the *Bacillus* competence response, it was proposed that Nanog shows noisy switch-like behaviour (Kalmar et al., 2009), which promotes the pluripotent state when high and differentiation when low. This view was not universally held. A counter argument proposed that Nanog heterogeneity was an epiphenomenon of developmental progression, with the high and

low states trapped under culture conditions favouring self-renewal and the maintenance of pluripotency (Smith, 2013).

A recent series of quantitative long-term live cell imaging studies, using a range of different Nanog expression reporters has challenged simple views of the importance of Nanog heterogeneity in cell decision-making (Cannon et al., 2015; Filipczyk et al., 2015; Singer et al., 2014). These studies are all agreed that Nanog expression is highly stable in standard mESC culture, even accounting for the different stabilities of the fluorescent protein reporters used. The rate of change in Nanog expression in culture is considerably slower than rates of change for other mammalian proteins (Sigal et al., 2006), implying Nanog levels are actively stabilised, even outside the context of an embryo. Contributions to this stability were shown to arise from a community effect phenomenon, in addition to any within-cell "epigenetic" inheritance of gene expression state (Cannon et al., 2015). In other words, cells signal to their neighbours and this maintains Nanog expression level. A parallel study imaging Nanog reporter expression in mouse blastocysts also found stable expression, with cells that initiate expression maintaining Nanog levels throughout blastocyst development, and only a few rare cells initiating expression after the initial wave of expression (Xenopoulos et al., 2015). Whilst the sum of these studies does not refute the idea that Nanog heterogeneity drives cell decision making- an early decision to express may still finalise a cell fate decision- the scenario illustrated in Figure 1B does not seem to apply.

A role for gene expression heterogeneity in providing the impetus for cell fate choice has also been proposed for an earlier fate choice in the mouse embryo. At the 8 cell stage, the cell mass undergoes compaction, resulting in the generation of inner and outer cells. Cells remaining internal become ICM, with external cells becoming TE (prospective extra-embryonic). A recent study argues that heterogeneous expression of Sox21 biases cell fate, with low Sox21 favouring extra-embryonic fates (Goolam et al., 2016). Although the variability in Sox21 protein expression is low, at least compared to Nanog in the blastocyst, and the low Sox21 cells were not tracked to their final fate, experimental knock-down of Sox21 at the 4 cell stage biased cells towards an extra-embryonic fate. A recurrent theme in approaches to test the role of expression heterogeneity in stochastic cell fate choice is to knock-down, or overexpress the candidate noisy regulator. This of course, is creating a new cell state, not necessarily simply related to the endogenous situation. Ideally one should reduce the variance whilst leaving the mean expression level in a population intact. In support of the role of expression heterogeneity in early cell fate choices in the mouse, a parallel study (White et al., 2016) used a sophisticated combination of imaging and tracking approaches and revealed that Sox2, which operates upstream of Sox21, and is also heterogeneously expressed, shows more long-lived binding to chromatin in cells destined to become embryonic. However, the debate about the nature and timing of early cell fate decisions in the mouse continues, with another recent long-term live cell tracking study clearly demonstrating that cell fate commitment is only observed at the 16 cell stage, and not especially penetrant until the 32 cell stage (Strnad et al., 2016). To make a more general comment, many mESC and embryo studies on stochastic cell fate seem to make

the implicit assumption that noisy genes provide the variance upon which cell fate bifurcations emerge. After nearly a decade of searching, the evidence for this view is still patchy, and other models have gained support. For example, a persistent and attractive alternative model for the first TE/ICM decision is not that it is driven by expression noise, but by the chance position of the cells relative to the inside and outside of the embryo after compaction (Sasaki, 2017). Inside and outside cells, by default, have different signalling and mechanical environments. It seems reasonable to assume that the genes are merely secondary to these influences, rather than isolated noise generators in their own right.

The isolated noise generator

What privilege do genes have to be the source of variation? This is perhaps the centre of the argument. Genes are regulated by signals- to transcription factors, to chromatin, at the many steps from the initiation of transcription through to the dynamic localisation, modification and degradation of the protein product. Yet genes create the noise? This argument is of course simplistic, and historically rooted in the potency attributed to early-characterised transcription factors, in addition to the comparative simplicity of measuring gene activity over most other activities in the cell. And of course, for development to progress, the genes must change. It doesn't mean they act in isolation to dictate the course of differentiation. However, the idea that any spontaneous variation emerges at the gene has become pervasive (Justman, 2015).

Although it is a concept that many papers in the single cell field include in their opening pitch, its origins were not intentional. Transcription occurs in irregular bursts, in all forms of life (Bahar Halpern et al., 2015; Chubb et al., 2006; Golding et al., 2005; Raj et al., 2006; Suter et al., 2011). These bursts have been traditionally measured by two approaches, by live cell detection of the dynamics of newly synthesised RNA or by measuring the amount of RNA (usually by single molecule RNA FISH) for a specific gene in each cell and fitting the data to a model of the transcriptional process (Chen and Larson, 2016). Both approaches have their merits and their limitations. Although the live cell approach can be used to accurately count the RNAs arising at the transcription site (Larson et al., 2011; Tantale et al., 2016), the intensive illumination required for single molecule imaging is damaging to samples over developmental timescales. In practice, the need to limit photodamage reduces the quantitative potential of live cell RNA detection and requires that strongly expressed genes are studied. The FISH approach is limited by the need to use fixed cells, and so dynamic information can only be inferred from the model. The model generates the quantitative parameters describing transcription bursts by fitting, usually, to a two state model, where the gene exists in an inactive and a permissive state, with a certain frequency of switching between the two states (Paulsson, 2005). According to the level of mean and variance in transcript counts, different bursting parameters are generated. Therefore variance comes from bursts. Genes with more variance in their expression are "bursty" (Bahar Halpern et al., 2015). Genes with low variance are not (Zenklusen et al., 2008). It follows that at a cursory glance transcription is responsible for variance.

An intuitive view of how molecular noise might be incorporated into the transcription process would relate to the many multi-subunit complexes involved in generating a mature mRNA, in addition to the vagaries of the chromatin template. With the likely number of proteins that need to be assembled, rapidly, again and again, there seems a considerable amount of room for noise to creep into the process. Indeed the process of a transcription factor finding its target, even assuming the protein is modified and ready to go, seems baffling in its overall reliability. However, the ability of some strongly expressed genes to churn out tens of RNAs in a few minutes (a small fraction of the lifetime of many RNAs) seems to argue that most of the steps of transcription do not have to be especially limiting (Corrigan et al., 2016; Garcia et al., 2013; Stevense et al., 2010).

A number of recent studies fill in much-needed counterweight to the view that functional variability emerges at the gene (rather than at the countless other places that cells can be regulated). Firstly, it is clear that transcriptional burst parameters, measured live or by FISH, are sensitive to a wide variety of cell and population-level features. These include cell size and cell cycle time (Padovan-Merhar et al., 2015), strength and frequency of extracellular signalling (Cai et al., 2014; Corrigan and Chubb, 2014; Molina et al., 2013; Senecal et al., 2014; Stevense et al., 2010), cell density (Corrigan and Chubb, 2014), developmental time (Ferraro et al., 2016; Muramoto et al., 2012) and embryonic context (Bothma et al., 2014; Garcia et al., 2013; Lucas et al., 2013), enhancer and promoter elements (Corrigan et al., 2016; Fukaya et al., 2016), chromatin state (Muramoto et al., 2010), DNA supercoiling (Chong et al., 2014) and not surprisingly, the nature of the gene itself (Muramoto et al., 2012; Suter et al., 2011). Indeed, a recent comprehensive FISH study on Hela cells showed that single cell transcript count variability could be almost entirely explained by different defined intra- and extracellular sources of variation, leaving very little room for variability to come from molecular noise (Battich et al., 2015). This result is remarkable, especially given the absence of temporal information (individual cell histories) in the FISH dataset. It is very difficult in practice to accurately apportion variability between defined sources or molecular noise (Hilfinger and Paulsson, 2011), although the Battich study went to great pains to demonstrate causality between cellular phenotype and variation. It will be very interesting to see how well this result holds in more developmental contexts. Along these lines, a recent live imaging study on Nanog heterogeneity, although not as comprehensive as the Hela study in determining sources of variation, revealed that even this single gene has a diverse range of interactions with multiple features of the cell state and local environment (Cannon et al., 2015), such as cell cycle duration, cell motility, local signalling and determinants inherited from the previous generation. A notable feature of this study was the demonstration that a second pluripotency regulator, Rex1, showed both overlapping and distinct regulatory interactions to Nanog. The implication of this finding is that even if stochastic gene expression does provide the raw variance for cell decision-making, it may not be prudent just to consider the variability for only a single gene, no matter how exposed it is in the regulatory network.

The potential fragility of the gene-only view is apparent in the diverse situations where the influences of some of the aforementioned cell and population features become penetrant in the cellular phenotype. This is perhaps most clearly emphasised by the developmental effects of the spontaneous heterogeneity in cell cycle position and timing in cell populations (Pauklin and Vallier, 2013; Primmett et al., 1989; Weijer et al., 1984). The cycle can act as a cell autonomous timer, which coupled with some global trigger- such as a differentiation signal- can lock cells into a specific state, which then triggers other downstream events, such as the induction of neighbouring cells. This would be much like the scenario in Figure 1B, placing the emphasis on a cellular process, rather than the single gene.

Reducing noise, sharpening noise, silent noise

In the most instructive developmental systems, we might be expected to see the least noise. For a signal to be accurately perceived and interpreted, the signal itself needs to reliably perceptible, and the receiving cell must be consistent enough in its responses to generate the desired effect. The early development of *Drosophila* is generally considered a model for patterning accuracy and reproducibility, and indeed reveals several mechanisms by which cells enhance their signal perception by generating comparatively predictable transcriptional responses. At a first glance, the early embryo of the fly seems well set-up for transcriptional accuracy. The embryo is syncytial, allowing spatial averaging of chance fluctuations in transcription factor concentration between neighbouring nuclei by diffusion (Gregor et al., 2007). Nuclei are restricted in their positions, which means they are unable to sample too many strong conflicting signals during the laying down of global pattern. The cell cycles are highly synchronous, meaning differences in cycle position are unlikely to be strongly present to bias the transcriptional responses of nuclei to global inducers. Finally, the embryo is seeded with localised determinants derived from the mother (Petkova et al., 2014), which ensure the nuclei do not have much opportunity to jiggle around in multi-dimensional state-space waiting for some chance event to hint at a possible fate. Overall, these features are manifest in the tremendous accuracy and reproducibility of developmental boundary formation in the early embryo. *Drosophila* has also emerged as an excellent model for live imaging of transcription (Garcia et al., 2013; Lucas et al., 2013), allowing the accuracy of the transcriptional events to be visualised in real-time, and permitting the dissection of genetic elements that contribute to transcriptional accuracy, which provides us with several additional noise-reduction concepts.

Firstly, some genes have shadow enhancers. These are enhancers that specify an overlapping temporal and spatial pattern of expression to the dominant enhancer. This allows buffering of the regulatory inputs to a gene, and given the potential unpredictability of the transcription factor search for its binding site, will increase the probability that a gene is expressed at the correct place and time. If these shadow enhancers are perturbed, pattern formation can be disrupted (Perry et al., 2010). Secondly, many early developmental genes have a promoter-proximal polymerase bound prior to the onset of strong expression. Pausing is thought to provide rapid and

reliable activation of the gene, by reducing the complexity of the transcription initiation process. If paused promoters are replaced by non-paused promoters, then transcription becomes more noisy, and developmental defects occur (Lagha et al., 2013). Interpreting this effect is not necessarily simple. Even if primed and ready to go, a polymerase is still awaiting the signal to start. If this signal is noisy, then the paused polymerase will potentially begin a surge of unwanted transcription- more noise- unless the system is somehow additionally configured to buffer unwanted signal fluctuations. Finally, genes can show a short-term memory of their transcriptional state in the previous cell cycle (Ferraro et al., 2016; Muramoto et al., 2010). Genes that were strongly expressed in the mother cell will be activated more readily than genes that were not. This effect may drive a strong and reliable induction of transcription in the next cell cycle, which may be desirable if the transcription has been accurately specified in the first place.

Without active suppression of the variability in transcription, many systems may be able to tolerate this variation- either because of redundancy in the components mediating a particular function, or because other aspects of the system allow buffering (Figure 1D). These include adaptation in cellular signalling networks and compensation by gene expression processes downstream of transcription (Cote et al., 2016; Shah and Tyagi, 2013). Transcriptional noise can also be dampened by the action of an oscillating extracellular signal (Corrigan and Chubb, 2014), at least in the context where the gene is responsive to the signal. Similarly, an intracellular oscillator based on autorepression may act to dampen transcriptional noise during somitogenesis (Lewis, 2003). We predict that secreted, diffusible signals may also tolerate a large degree of transcriptional noise in their expression, as their free-range protein products spatially mute the nuclear responses of the cell. More generally, if the RNA and protein lifetime are long, then noisy transcription is less likely to be visible. Similarly, if the timescale of the cell decision is considerably more than the lifetime of the variance in gene expression, then the variance is not likely to be particularly penetrant in the phenotype (Little et al., 2013).

Unpredictability in gene expression may also operate to regulate development in counter-intuitive ways. Superficially, we think that noise would be disruptive to boundary formation in embryos. However, the opposite seems to be true during rhombomere specification in zebrafish (Zhang et al., 2012). Here, inaccuracies in the initial sub-division of the hindbrain into segments are resolved by feedback. Cells perceive the inaccuracy in their initial specification and respond by changing their gene expression to reflect their actual position. Modelling suggests this process may be dependent upon gene expression noise. We can understand this behaviour if we allow that noise maintains an extended range of cellular sensitivities, so if by chance a cell falls on the wrong side of a segment boundary- perhaps by motility or a consequence of the orientation of cell division- then the expanded range of sensitivity to signals stops the cells getting locked into an inappropriate fate.

In biological and non-biological systems undergoing a critical transition, an increase in noise is a signature of the oncoming change (Scheffer et al., 2009). A

standard example is the volatility in stock markets during the prelude to a crash. Three recent papers describe a similar signature in the build-up to cell fate decisions (Figure 1C). Two studies used single cell transcript analysis of cultured cells to study the gene expression diversity in cell populations prior to a cell fate bifurcation (Bargaje et al., 2017; Richard et al., 2016), and revealed a significant increase in expression variance of many genes just before the split. The third study used live tissue imaging of the expression of a heterogeneous cell fate regulator during *Drosophila* eye disc differentiation (Pelaez et al., 2015), with transient peak in expression variability observed as the cells progressed to differentiation. The functional significance of this diversity is unclear, but one can use the metaphor of Waddington's landscape for an intuitive explanation (Waddington, 1957). As the ball in the valley approaches a branch point, the terrain transiently flattens out as the two new valleys take shape. With the ball on a broad valley floor, rather than confined to a narrow channel, it is more sensitive to deflection.

Prospects

The early studies showing that gene expression variability provides the cell type diversity underlying cell fate choices, such as those on bacteria and the *Drosophila* eye, have not been effectively mirrored in large animal systems. That does not mean that different mechanisms necessarily apply, and perhaps relates to how much more complex the regulation actually is, and how emphasis on a single gene, such as *comK* or *spineless*, may confound analysis. A potential roadmap for testing the hypothesis that gene expression variability prescribes the direction of a cell fate choice in your favourite system requires: 1) The gene you wish to test is highly variable in its expression, at the protein level (assuming the transcript encodes a protein) 2) The ability to monitor the expression of the gene and preferably also its protein product over the time course of the developmental transition. This requires live imaging, in the normal developmental context, to be able to test to what extent the expression level of your candidate regulator maps onto a specific cell fate outcome (Pelaez et al., 2015; Xenopoulos et al., 2015). 3) Ideally, one should experimentally test the importance of the variability by reducing it, without altering the population mean expression. This is non-trivial- the neat genetic tricks carried out for the competence studies in bacteria may not be directly transferrable to more complex developmental systems. Large-scale promoter mutagenesis to generate low variance promoters provides one route (Wolf et al., 2015). 4) Knowing that variability in your favourite gene is important is not sufficient- where does the variability comes from? There may be multiple weak sources (Battich et al., 2015; Cannon et al., 2015), which might provide robustness in cell decision-making. 5) Ideally the study would include some probabilistic modelling approaches, so that with the data in hand, one can evaluate what proportion of the functional variance in differentiation is contributed by the candidate gene. The endpoints of these analyses will likely depend on the system, the cell population size, the timescale of the decision the ability of the population to correct for errors in the initial specification events and the proportion of the control that the cell dares to channel through a single locus.

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