Using Single-Cell Techniques to Understand Noise in Gene Expression

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CHAPTER I

INTRODUCTION

Population vs Single Cell Measurements

Gene expression is an inherently stochastic process. This is in part due to transcription and translation being reliant on stochastic biochemical reactions. This is further amplified by the low cellular concentration of DNA. Because of the low cellular concentration of DNA, the fluctuation rates of transcription and translation do not average away and become easily detectable even in isogenic cells (Raj & van Oudenaarden, 2008). These stochastic fluctuations, also known as intrinsic noise, directly impact cellular functions in both detrimental and beneficial ways. An early study on penicillin highlights the benefit of noise in gene expression. Bigger and colleagues found that a random fraction of cells entered a state of seemingly reduced fitness, which increased the likelihood of the subpopulation surviving current and future environmental stress (Bigger, 1944). Due to noise in gene expression, this subpopulation survived additional antibiotics; however, the antibiotic resistance was nonheritable and bacterial cultures derived from the antibiotic-resistant subpopulation reverted to their antibiotic sensitive state (Moyed & Broderick, 1986). Albeit the antibiotic resistance was temporary, it served to ensure the survival of the bacterial culture as a whole, thus showing a beneficial way that noise effects gene expression.

Other early studies of isogenic cells showed that single cells varied in their enzyme production, chemotactic behavior, and phage burst size (Delbruck, 1945; Novick & Weiner, 1957; Spudich & Koshland, 1976). These pioneering studies demonstrate how cellular heterogeneity, often caused by regulatory noise, plays an important role in cellular response to environmental changes; however, they were unable to identify the mechanism of adaptation due to technological limitations. Recent advances have enabled the accurate measurement of protein and RNA levels in single cells, which has led to greater insight into cellular heterogeneity and regulatory noise.

A key example that shows the benefits of single cell measurements over population measurements is NF-κB dynamics. Following population studies, tumor necrosis factor (TNF) induced NF-κB was thought to have dampened oscillations (Hoffmann et al., 2002). However, Nelson and colleagues revealed that FP-RelA, the transcriptionally active subunit of the NF-κB pathway, exhibits persistent oscillations in individual cells (Nelson et al., 2004). Additionally, Hughey and colleagues observed that oscillation period varied greatly between cells, however, the intracellular oscillation variability was significantly smaller than the intercellular oscillation variability (Hughey et al., 2015). These findings suggest that these differences are due to pre-existing cell-to-cell differences, also referred to as extrinsic noise. These features of the NF-κB pathway were only found using single cell techniques and were masked when analyzing population dynamics.

Noise in Gene Expression

There are two major types of noise in gene expression, intrinsic and extrinsic. Intrinsic noise refers to the fluctuations of stochastic biochemical reactions within a cell such as random

binding of transcription factors. Extrinsic noise refers to differences between different cells in a shared environment such as cell size, stage in cell cycle, and the abundance of genetic expression machinery. Elowitz and colleagues were the first to introduce the idea of intrinsic and extrinsic gene expression (Elowitz et al., 2002). To study gene expression noise, they introduced two identical promoters into *E. coli*. One promoter drove the expression of yellow fluorescent protein (YFP), and the other drove the expression of cyan fluorescent protein (CFP). Extrinsic fluctuations would influence the expression of both YFP and CFP, while intrinsic fluctuations would affect the expression of either independently. They found that intrinsic and extrinsic noise contribute substantially to cellular heterogeneity. Following a similar approach, many researchers began using single cell microscopy-based approaches to monitor expression levels. One particularly interesting phenomenon that was identified by such techniques was transcription bursting, a phenomenon now known as a major source of noise in gene expression (Golding et al., 2005). These findings, give insight into the mechanisms that promote cellular heterogeneity.

CHAPTER II

ROLE OF TRANSCRIPTION IN GENE REGULATION

RNA levels vary among individual cells

Cellular heterogeneity is in part due to variable RNA levels among isogenic cells (Lenstra et al., 2016). Single cell methods have continued to give invaluable insight into regulatory and functional mechanisms that are concealed by bulk, population-level studies that measure average cellular response. An early example is the dose dependence of glucocorticoid-inducible gene expression (Ko et al., 1990). This study was one of the first to use an expression reporter to examine the stochastic foundation of single-cell expression variability. Increasing dosage led to increased frequency of cellular expression, but not an increase in expression in all cells. This indicates that the dose dependence led to a higher probability of an individual cell expressing high levels of the gene.

Transcriptional Bursting

One reason that RNA abundance varies among isogenic cells is due to transcriptional bursting, the process by which DNA is transcribed into RNA in stochastic bursts. Transcriptional bursting has been observed in various organisms ranging from bacteria to mammals (Bahar Halpern et al., 2015; Chubb et al., 2006; Golding et al., 2005; Raj et al., 2006; Suter et al., 2011). This phenomenon was identified following the advent of MS2 tagging (Johansson et al., 1997)

and single-molecule RNA fluorescence *in situ* hybridization (smRNA FISH) (Femino et al., 1998). These technologies allow for simultaneous detection, localization, and quantification of individual RNA molecules in single cells. This information has been used to monitor the distribution of RNA transcripts in a population and shed light on otherwise undetectable expression dynamics (Raj & van Oudenaarden, 2008). Golding and colleagues utilized the *in vivo* MS2-GFP tagging method (Bertrand et al., 1998; Peabody, 1993) to monitor real time RNA transcription in *E. coli*. They observed transcriptional bursting which is consistent with the gene activation/inactivation model (Golding et al., 2005). The phenomenon of transcriptional bursts adds another source of stochasticity beyond the random biochemical interactions involved in transcription and translation.

Another study utilized single-cell bioluminescence measurements to observe transcriptional bursting in mammalian cells (Suter et al., 2011). Using this method, they found that transcriptional bursts are gene-specific and often associated to cis-regulatory elements, regions of non-coding DNA that regulate the transcription of neighboring genes. Recently, Chong and colleagues utilized smRNA FISH in fixed cells to characterize sources of noise in gene expression (Chong et al., 2014). They found that different degrees of supercoiling during transcription is a physical cause of bursting in *E. coli*. Another interesting study utilized a synthetic biology approach, incorporating smRNA FISH to identify mechanisms of cell-to cell variability in gene expression (Jones et al., 2014). It determined that different promoter architectures are associated with different levels of gene variability. This indicates that transcriptional noise is tunable and may be targeted by evolutionary selection pressures.

Transcriptional bursting provides significant variability in the number of RNA molecules from a given gene, even among isogenic cells. In turn, this can directly influence cellular behavior; consequently, it is hypothesized that cells have mechanisms to mitigate transcriptional noise. Battich and colleagues utilized an innovative smRNA FISH technique to show that mammalian cells do indeed utilize mechanisms to suppress RNA variability (Battich et al., 2015). They measured the transcript abundance of ~1000 genes while simultaneously measuring 183 cell state features and microenvironments, and they found that cytoplasmic mRNA has significantly less stochasticity in comparison to nuclear mRNA. This indicates that cells utilize mRNA nuclear export mechanisms to reduce stochastic fluctuations that arise from transcriptional bursting.

Transcriptional Memory

Another important mechanism contributing to regulatory noise in gene expression is transcriptional memory, where cells modify transcription patterns following a stimulus to better respond to similar stimuli in the future. Transcriptional memory was first observed in *S. cerevisiae* (Acar et al., 2005). Another example of transcriptional memory has been observed in *Arabidopsis.* Following heat shock, certain genes are upregulated to allow for survival (Ding et al., 2013). Additionally, a similar response has been shown in HeLa cells (Gialitakis et al., 2010; Light et al., 2010). Following exposure to Interferon-γ (IFN-γ), IFN-γ-inducible genes exhibit faster and stronger induction. This effect occurs for up to seven cell divisions (Light et al., 2010). In all these examples, transcriptional memory led to heritable changes in transcriptional output. These findings suggest that transcriptional memory is an essential phenomenon for adaptation.

A well characterized system for studying transcriptional memory is the *S. cerevisiae* high osmolarity glycerol pathway. In this system, previous low osmolar stress conditions prime cells

to respond to high osmolar stress conditions (English et al., 2015). Upon exposure to high osmolar conditions, cells shrink followed by HOG pathway activation which works to restore the osmotic gradient by producing glycerol (Blomberg & Adler, 1989; Reed et al., 1987) and increasing expression of osmotic stress related genes (De Nadal & Posas, 2010). This is accomplished in part by nuclear localization of Hog1, a mitogen-activated kinase (MAPK), which then activates stress-responsive transcription factors (TFs). These TFs bind to gene promoters and induce changes in chromatin structure (De Nadal et al., 2004; Mas et al., 2009) as well as binding of RNA polymerase II (RNAPII) which promotes transcriptional reactivation (Mas et al., 2009). Interestingly, single cells exhibit homogenous Hog1 nuclear localization; however, they still display heterogenous gene expression (Muzzey et al., 2009; Pelet et al., 2011). Thus, differences in expression are due to nuclear regulation rather than signal transduction variability.

One innovative way that the HOG pathway has been used to understand transcriptional memory mechanisms utilizes stimulation kinetics which vary the rate of change, duration, and time between stimuli. The goal is to dissect the unique role of proteins in gene regulation and signal transduction. Johnson and colleagues varied the rate at which osmotic stress was administered to *S. cerevisiae* (Johnson et al., 2020). Hog1 nuclear localization was monitored using time-lapse microscopy while administrating different rates of osmotic stress. They observed that there is both a concentration and rate threshold for Hog1 nuclear localization. Additionally, they observed that cells that experienced osmotic stress at rates lower than the identified rate threshold likely did not develop transcriptional memory and remained more prone to secondary osmotic stress. Additionally, they showed that the rate threshold is due to protein tyrosine phosphatase (PTP2). This innovative study represents a paradigm shift in how gene

expression and signal transduction mechanisms can be unveiled using kinetic stimuli. Overall, these studies show that mRNA levels fluctuate in single-cells in part due to transcriptional bursting and memory. These fluctuations promote cellular heterogeneity between cells and result in variable protein levels which directly influences cellular behavior.

CHAPTER III

CORRELATING RNA AND PROTEIN LEVELS

RNA and Protein Levels in Isogenic Cells

Considering the variability in transcription dynamics we discussed previously, it is not surprising that protein levels vary among isogenic cells. Generally, there is a weak correlation between mRNA count and the encoded protein. Since only about 40% of protein variance among isogenic cells can be attributed to stochasticity in transcription (De Sousa Abreu et al., 2009; Maier et al., 2009), there must be additional regulatory processes that occur after transcription. Some known processes that add to the complexities of noise propagation in gene regulation include post-transcriptional, translational, and protein/RNA degradation regulation.

With all these processes that result in variation among isogenic cells, how do cells coordinate the expression of multiple genes that code for multimeric proteins? To answer this question, Gandhi and colleagues used smRNA FISH to determine whether mRNA abundance of functionally related genes was more correlated than functionally unrelated genes (Gandhi et al., 2011). Interestingly, they found that functionally related and unrelated genes were both weakly correlated. This suggests that the stochastic nature of transcription does not provide a mechanism for cells to coordinate production of stoichiometric concentrations for multimeric

proteins. Thus, mechanisms must exist downstream of transcription that regulate protein abundance.

To better understand the relationship between mRNA and protein abundance, Taniguchi and colleagues applied smRNA FISH methods (Taniguchi et al., 2010). They utilized a yellow fluorescent protein (YFP) fusion library for *E. coli* to quantify mRNA and protein abundance for each of over 1000 genes. They found that lower abundant proteins vary due to intrinsic noise such as transcriptional bursts while higher abundance proteins vary due to extrinsic noise such as cell size. They found that a single cell's protein abundance and mRNA transcript count are uncorrelated. One of the reasons for variation in protein abundance may be due to production and degradation of mRNA and protein. For example, in mammalian cells, transcription occurs at slower rate than translation. On average, mammalian cells produce two copies of an mRNA transcript per hour. In comparison, the same cells produce dozens of proteins from these transcripts per hour (Gandhi et al., 2011; Taniguchi et al., 2010). Consequently, there is not a one-to-one correlation of transcript to protein. Additionally, a potential flaw in Taniguchi and colleagues' study is that they compared protein and mRNA abundance at a fixed time point. This would likely skew the data because transcription and translation are spatially and temporally isolated. Future studies should compare protein and their respective mRNA template at various time points.

RNA Localization Effect on Transcription and Translation Dynamics

Eukaryotic cells evolved to localize individual macromolecules and organelles to specific areas in the cell. This process is essential for proper development, differentiation, and physiological activity. Therefore, it is not surprising that cells may also have mechanisms to

regulate gene expression by ensuring proper mRNA localization, which then allows for efficient and timely protein synthesis. In particular, localized mRNA allows for local synthesis and accumulation of protein which promotes cellular compartment specialization and cell polarization (Rodriguez et al., 2008).

Epithelial tissue is commonly used to study macromolecule localization due to its inherent functional polarization of apical and basal sides. The intestinal epithelium has a monolayer of enterocytes that specialize in absorbing nutrients from the apical side and excreting them into the blood stream on the basal side. Importantly, there are two specialized transporters used for absorption and excretion that localize to the apical and basal side (Ljungqvist, 2011).

To study the intracellular localization of mRNA and their effect on protein abundance, Moor and colleagues adopted a transcriptome-wide approach to analyze mRNA localization in enterocytes (Moor et al., 2017). They used laser capture microdissection to isolate apical and basal subcellular domains and RNA sequencing (RNA-seq) to determine mRNA abundance. They found that nearly 30% of the 2000 most highly expressed transcripts exhibit polarized localization. As expected, mRNA localization corresponded well with respective protein localization, including apical transporter transcripts. However, many mRNAs did not correlate with their respective protein localization. This observation led to further analyzing the correlation of mRNA and protein localization, and the unexpected finding that localization of many mRNA groups was anticorrelated to their respective protein, meaning they were found in the opposing part of the cell. A finding that was particularly interesting was that mRNAs coding for ribosomal proteins, translation machinery needed to produce proteins, was basally enriched. This is interesting because the actual ribosomal proteins are known to be apically enriched. Importantly,

with ribosomes being apically enriched, transcripts located in the apical domain should undergo translation at faster rates. This was indeed the case. They found that apically enriched transcripts were translated nearly twice as frequently as basally enriched transcripts.

Another interesting finding is that in mice ribosome coding mRNA localize to the apical side. This apical enrichment is due to the polarization of microtubules and specialized motor proteins. The change in localization of the ribosomal transcripts increases translational frequency of ribosomes, thus, increasing the ribosome abundance in the apical side. This formed a feed-forward loop, where investing resources into transcription machinery increases translational efficiency in the future. These results explain how intestines quickly adapt to digestion and absorption. Overall, this work has unveiled new mechanisms of mRNA regulation and spatial dynamics and its effect on translational dynamics.

Conclusion and Future Outlook

Understanding mechanisms of noise propagation in gene expression will provide major insight into cellular heterogeneity and its role in physiology as well as disease progression. Current limitations for elucidating these mechanisms include spatial and temporal resolution in microscopy. One particular area that would benefit from these advances is single-cell correlation analyses. Currently, mRNA and protein abundance has been compared at single time points; however, this fails to account for the spatial and temporal differences in transcription and translation. Modelling studies comparing mRNA and protein abundance at different time points can shed light on this inherently stochastic process.

While transcription of individual genes can be quantified in real time, it remains difficult to quantify translation. This is in part because current fluorescent tags, such as GFP, take too long

to fluoresce and/or the signal is too weak to monitor translation in real time. However, recent studies have developed methods to overcome these limitations. Moon and colleagues recently developed a technique to distinguish between translating and non-translating transcripts (Moon et al., 2019). This was accomplished by labelling mRNA with an MS2 coat protein and utilizing fluorescent antibody fragments (Fab) that specifically bind to the N terminus of the peptide being translated. Thus, labeling translating mRNA with both an MS2 coat protein and Fab. New methods such as this will pave the way to understanding mRNA dynamics.

Another challenge when studying real-time gene expression and signaling in single cells is the low multiplexing capability. As shown, scRNA-seq has made progress in overcoming this barrier. However, scRNA-seq only provides RNA abundance of cells at a single time point. Additionally, it does not allow for monitoring of RNA dynamics. Mass cytometry is another recently developed technique that allows for measurement of over 40 molecular species in fixed single cells (Spitzer & Nolan, 2016). However, this fails to provide information for real-time dynamics. Nonetheless, combining these techniques with emerging technology in transcriptomics, proteomics, and imaging technology, will continue elucidating mechanisms of regulatory noise and its role in signal transduction.

As we continue to gain insight into the mechanisms of noise propagation in gene expression, we will continue to expand our understanding of cell biology and heterogeneity in gene expression. Additionally, we do not have an in depth understanding of how ever-changing physiological conditions affect cellular response, why people respond to medical treatments differently, or why cancers vary in treatment efficacy. To accomplish this; more research should involve stimulation kinetics by varying rates of stimuli and monitoring downstream dynamics in

real time. Such studies may allow for dissection of previously unknown mechanisms involved in uncontrolled cell growth, regulation of therapeutic targets, and even modifying cellular function. Learning to modify cellular function could be useful in such things as re-sensitizing beta cells to glucose in patients with diabetes. By elucidating mechanisms of noise propagation and regulation, we are one step closer to personalized medicine.

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