Quantitative stem cell biology: the threat and the glory

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MEETING REVIEW

Quantitative stem cell biology: the threat and the glory

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ABSTRACT

Major technological innovations over the past decade have transformed our ability to extract quantitative data from biological systems at an unprecedented scale and resolution. These quantitative methods and associated large datasets should lead to an exciting new phase of discovery across many areas of biology. However, there is a clear threat: will we drown in these rivers of data? On 18th July 2016, stem cell biologists gathered in Cambridge for the 5th annual Cambridge Stem Cell Symposium to discuss 'Quantitative stem cell biology: from molecules to models'. This Meeting Review provides a summary of the data presented by each speaker, with a focus on quantitative techniques and the new biological insights that are emerging.

KEY WORDS: Bioinformatics, Computational biology, Epigenetics, Genome editing, Stem cells, Transcription

Introduction

Quantitative approaches have been boosted across many areas of biology by the relentless improvements in sequencing, imaging, computational methods and computational power. Stem cell biology sits at the intersection of classical developmental biology and cell biology, and a central question is how gene activity shifts in space and time to direct specific cell fates. The 5th annual Cambridge Stem Cell Symposium 'Quantitative stem cell biology: from molecules to models' brought together researchers from around the world to discuss their use of quantitative techniques to address this question. A key theme for the meeting was the varied uses of next-generation sequencing technologies to monitor gene transcription, epigenetics and three-dimensional (3D) chromosome topology. These advances have enabled us to begin to unearth the decision-making mechanisms that govern specification and commitment to distinct cell fates. Other interesting quantitative approaches, such as those relating to the physical biology of stem cells, have been covered in previous meetings in this series (Lowell, 2013).

Azim Surani (Gurdon Institute, University of Cambridge, UK), who co-organised the meeting with Paul Bertone and Elisa Laurenti (University of Cambridge), began by emphasising the power of new quantitative methods and analytic tools. But there was a clear caveat: how do we best make sense of all this data? The meeting offered the chance to hear just how far we have come with regard to quantitative data analysis, with some wonderful examples from pioneers in this area. In this Meeting review, I outline the major ideas and topics that emerged, focusing on the key processes that influence stem cell fate: transcription, mitosis, epigenetics and fate specification.

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Transcription and pluripotency

The paradigm experimental models for studying mammalian stem cells were on full display throughout the meeting: pluripotent stem cells and reprogramming, neural stem cells, haematopoietic stem cells and epidermal stem cells all featured throughout the talks. Rick Young (Whitehead Institute, USA) provided a superb overview of mammalian transcriptional regulation based on work carried out primarily on pluripotent stem cells. His group has been one of the pioneers in establishing and exploiting techniques that enable genome-wide analysis of transcription. In his plenary talk, Young discussed the importance of genome organisation: the arrangement of DNA in 3D space, and how this influences gene expression. Mapping the topological organisation of chromosomes has been one of the areas of research that has been boosted by next-generation sequencing. Interacting sites are captured in their 3D conformation and ligated before being mapped by deep sequencing. This approach has revealed topologically associating domains (TADs) as a fundamental structural unit of genome organisation (Gorkin et al., 2014). Boundaries between TADs are largely shared between different cell types, ruling out the idea that reconfiguration of these boundaries could be regulating lineage commitment and differentiation. However, Young presented evidence that within each TAD the situation is more dynamic because of the presence of 'insulated neighbourhoods'. These insulated neighbourhoods do vary between cell types and are defined by highly conserved nuclear protein CCCTC-binding factor (CTCF) sites that are secured by cohesin (Dowen et al., 2014). Insulated neighbourhoods offer a solution to the long-standing conundrum of how enhancers identify the right target: they simply have no choice where to loop. How these insulated neighbourhoods are set up in the first instance and how they can be dynamically controlled are important questions for the field.

Analysis of pluripotent stem cells by Young and co-workers has revealed that there are approximately 10,000 insulated neighbourhoods across the genome, which are, on average 200 kb in size, but can range from 4 kb to 3 Mb, and contain one or two genes. This new knowledge has immediate and broad significance for human disease studies. For example, many of the disease-associated single nucleotide polymorphisms (SNPs) identified in human genome-wide association studies are located within enhancers. This is also the case for many somatic cancer mutations.

The pluripotency transcriptional network was a topic also covered in computational modelling talks from both Sara-Jane Dunn (Microsoft Research, Cambridge, UK) and Ingo Roeder (IMB, Dresden, Germany). Roeder discussed how he has been using computational strategies to model the emergence of transcription factor heterogeneity from the core pluripotency gene regulatory network (GRN) (Herberg and Roeder, 2015). He provided a clear example of how modelling generates useful and testable hypotheses – in this instance whether the core pluripotency GRN would result in oscillatory behaviour or whether it is more likely to generate uncoordinated fluctuations. Comparing experimental observations with his modelling results suggest the

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latter. A major problem, however, is that even with the most powerful computers it would be impractical to compute and explore all possible network topologies. So how do we reduce the complexity? One solution is to filter out candidate GRNs that do not fit with known experimental data. This cannot be performed manually, and so new computational approaches are needed. Dunn has been tackling this together with Austin Smith (Dunn et al., 2014). She described a new tool – the reasoning engine for interaction networks (RE:IN) – which was devised to sidestep the need for computationally intensive simulations (Yordanov et al., 2016). Taking a mathematical approach, experimental observations are encoded as constraints, allowing the user to synthesise only those networks that are consistent with experiments. This is a computationally efficient way to quickly filter models that do not make sense. The power of this strategy was demonstrated in the establishment of a more accurate and extended core pluripotency GRN, and the identification of new regulatory interactions that would otherwise have been impossible to deduce. Importantly, these have been functionally validated to provide confidence in the approach (Dunn et al., 2014). Her ongoing efforts are now focussed on how this network is established during reprogramming.

Diagrams that illustrate core GRNs often imply a very static and stable cell state. But this is inappropriate, as cells often display significant cell-cell variability and fluctuations in gene expression. Such heterogeneity may underpin the diverse differentiation paths available to otherwise phenotypically equivalent cells. Naomi Moris (Pina and Martinez-Arias laboratories, University of Cambridge) discussed her efforts to explore the possible causes of cellular heterogeneity and presented results indicating that the chromatin remodeller Kat2a – the mammalian orthologue of yeast Gcn5 – could be involved. In yeast, Gcn5 has been identified as a candidate regulator of transcriptional noise. Intriguingly, Moris has found evidence that inhibition of Kat2a in mouse embryonic stem cells enhances Nanog transcriptional heterogeneity, destabilising the pluripotent state and promoting differentiation.

GRNs undergo dynamic change as cells pass through the cell cycle. Transcription ceases on mitotic chromosomes and a longstanding idea has been that this mitotic interlude in transcription provides a window of opportunity for new GRNs to emerge. At the same time, however, self-renewal requires persistence of the parental GRN. So how is the same transcriptional state propagated to the daughter cells? An attractive idea, which first emerged many years ago, is the concept of mitotic bookmarking (John and Workman, 1998). This hypothesis proposes that a subset of the critical lineage determining transcription factors remain bound to the mitotic chromosomes, thereby enabling rapid reactivation of their targets once mitosis is complete. David Suter [École polytechnique fédérale de Lausanne (EPFL), Switzerland] discussed his group's efforts to determine whether any of the members of the core pluripotency circuit might act as mitotic bookmarkers. Immunocytochemistry has been problematic for addressing this question, mainly because of fixation artefacts. Suter presented an impressive systematic assessment of fluorescent fusion variants for each of the factors in the core network defined by Dunn and Smith (Dunn et al., 2014). Using live cell imaging, he found that many of the core pluripotency transcription factors do indeed remain localised to the condensed chromosomes, consistent with their putative role as mitotic bookmarking transcription factors. In ongoing studies, Suter is exploring exactly where the transcription factors are bound, as well as the tricky task of determining the functional consequences of reducing bookmarking in mitotic pluripotent stem cells.

During direct reprogramming to pluripotency, the pluripotency GRN must be established *de novo*. Noa Novershtern (Hanna Lab, Weizmann Institute of Science, Israel) has been gaining mechanistic insights into this process and discussed how a more efficient reprogramming system – using cells mutant for Gatad2a and Mbd3, which are both components of the NuRD complex – allows a survey of transcriptional and chromatin changes without the need for sorting or selection. The integrative analysis of these data uncovered two distinct programs of gene expression changes during reprogramming. In one, a cohort of chromatin regulatory genes predominates, which is enriched for cell fate determinants. The second program contains more cell maintenance-associated genes that have a consistent active epigenetic configuration and includes many Myc targets. This rich dataset will be an invaluable tool in the effort to dissect the order of events during reprogramming and the many layers of regulation therein.

Epigenetics

DNA methylation is the best understood example of epigenetic control. It has long been considered to be critical for developmental decision-making and stabilisation of differentiated phenotypes (Smith and Meissner, 2013). Several talks covered this topic, including a plenary talk from Alex Meissner (Harvard Medical School, USA). Meissner first presented an overview of the distribution and dynamics of DNA methylation in a normal developmental context with specific examples during the differentiation of human embryonic stem cells. He then described his lab's efforts to more closely inspect the mitotic inheritance of DNA methylation using a genomic approach and presented his hypothesis on the practical and biological relevance of these findings. These results were further integrated with preliminary results on how the pioneer transcription factor FoxA2 engages with its targets and whether the observed changes in DNA methylation are the result of active or passive mechanisms.

The Fox transcription factor family was also discussed in another context by Steve Pollard (University of Edinburgh, UK). Cancer stem cells derived from glioblastoma – a type of brain tumour – frequently express high levels of FOX and SOX factors. Using ATAC-Seq, a tractable and powerful method to define chromatin accessibility genome-wide, Pollard presented evidence that FOX and SOX factors are responsible for limiting terminal differentiation (Caren et al., 2015). Furthermore, he was able to show that these factors control many core cell cycle and epigenetic regulators. In this way, cancer cells can be seen to hijack and subvert the molecular apparatus used by stem and progenitor cells to fuel unconstrained self-renewal.

Dynamic changes in epigenetic memory are essential for cells to undergo dramatic changes in cell fate, whether during early development, in the germ line or in direct reprogramming experiments. Wolf Reik (Babraham Institute, UK) discussed his recent data on how demethylation occurs during transition from the primed embryonic stem cell state to the naïve state, which can be imposed by culturing the cells under specific conditions. Despite their pivotal role in demethylation, Tet proteins were found to be dispensable (von Meyenn et al., 2016). Instead, demethylation appeared to involve downregulation of Uhrf, which is the protein responsible for recruiting Dnmt1 to the replication fork, thus suggesting a failure of the DNA methylation maintenance apparatus. Reik also discussed the role of DNA methylation in contributing to the generation of cellular heterogeneity during cell fate decision making in the early embryo. Underlying the heterogeneity might be the competing influences of TET and

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DNMT3 coexpression, a strategy that Reik hypothesised could create the heterogeneous patterns of methylation needed to seed diverse cell fate options. He entranced us with the oscillating dance of the dynamic methylation model he has developed with Ben Simons. Moving forward, it will be necessary to track these changes in live cells.

A battle between Tet and Dnmt3a has also been identified in haematopoietic stem cells, and was discussed by Margaret Goodell (Baylor College of Medicine, Houston, TX, USA). Haematopoietic stem cells lacking Dnmt3a fail to differentiate efficiently, and recent studies have found recurrent mutations in this gene as an early event in various leukaemias. Goodell discussed how her laboratory has identified large blocks of low DNA methylation called 'canyons', which at >3.5 kb are much broader than CpG islands. There are around 1000 of these DNA methylation canyons and they are highly enriched in developmental regulatory transcription factor binding motifs. In Dnmt3a mutant haematopoietic stem cells, the methylation boundaries at these canyons, which are enriched for hydroxyl methylation, become eroded. By extension, then, the maintenance methylase Dnmt1 is not sufficient to protect them (Jeong et al., 2014). The boundaries appear to be restored when Tet2 is mutated, which suggests a model in which TET and DNMT3 compete at the edges of these canyons. It will be interesting to define how these canyons relate to super-enhancers, TADs and insulated neighbourhoods as described by Rick Young.

Discussions on Dnmt3 were continued by Salvador Aznar-Benitah (IRB, Barcelona, Spain), who presented work exploring the differentiation of adult stem cells in the absence of DNA methylation – this time in the skin. Aznar-Benitah showed how conditional knockout of Dnmt3a results in exacerbated ageing and increased tumour initiation and he discussed some of the underlying molecular mechanisms that contribute to the phenotype. He explained how Dnmt3a and Dnmt3b activate super-enhancers that regulate the expression of genes associated with stem cell self-renewal and differentiation (Rinaldi et al., 2016). Interestingly, Dnmt3a-mediated activation of super-enhancers requires their subsequent hydroxymethylation by Tet2, supporting a synergistic role of both proteins in gene regulation.

Fate specification

While the various 'Seq' and 'omics approaches have proved their value, it is also clear that they have limitations, as they can only provide snapshots and are often population-based averages. Direct visual inspection of cell behaviours is essential to appreciate the dynamics and kinetics of molecular and cellular changes. Timm Schroeder (ETH, Zurich, Switzerland) presented his ongoing efforts to continuously monitor gene activity in single cells. One of the key challenges with this approach is the associated huge imaging datasets that are collected and required to monitor fate and differentiation. This is a problem we are all facing: the ease of data collection is outstripping the ease of data analysis and new tools are needed to make sense of it all. To this end, Schroeder has assembled an impressive team of engineers, physicists, statisticians and computer scientists to work together, and his presentation was a demonstration of what can be achieved when such collaborations are formed. Schroeder described how his team has tracked the lineage choices of blood progenitors *in vitro* over the course of 2 weeks. Focusing on the proposed bi-stable switch of PU.1 and GATA, he measured the amount of protein every 30 min across many thousands of cells over time. These data immediately revealed that it is not a battle between these two factors that determines cell fate; instead they serve to reinforce decisions that have already been made (Hoppe et al., 2016). Indeed, the decisions appear to have been made much earlier than anticipated – occurring five or more cell divisions before fate commitment occurs. The molecular explanation for this remains unclear.

While Schroeder presented the power of *in vitro* culture for tracking cell fate over many days and weeks, it remains problematic in intact organisms and tissue explants. However, technologies enabling live cell imaging *in vivo* with cellular resolution have emerged. A study using intravital imaging was presented by Saskia Ellenbroek (van Rheenen lab, Hubrecht Institute, The Netherlands), who has been using this approach to analyse intestinal stem cell dynamics. Using live cell tracking in Lgr5 confetti mice, it appears that a cell's position within the intestinal crypt has an influence on fate (Ritsma et al., 2014). Now, using similar technology, she is exploring cancer cell differentiation and plasticity in breast cancer models.

Multilayer 'omics analysis of haematopoietic stem cells and their more lineage-restricted progenitors was presented by Nina Cabezas-Wallscheid (Andreas Trumpp laboratory, DKFZ, Heidelberg). Multipotent progenitors are the immediate progeny of haematopoietic stem cells, but the regulatory factors that maintain and distinguish these two populations remains unclear. In previous work, Cabezas-Wallscheid has shown how extensive molecular profiling – incorporating proteome, transcriptome and methylome analyses – can identify key regulatory elements in haematopoietic stem cells, which can then be linked to their functional potential *in vivo* (Cabezas-Wallscheid et al., 2014). In her talk, Cabezas-Wallscheid presented data derived from bulk and single-cell RNA-Seq analysis comparing dormant and active haematopoietic stem cells. These data demonstrated a clear role for Myc in upregulating biosynthetic processes to prime stem cells towards re-engagement of the cell cycle. Interestingly, she showed how this process follows a continuum rather than an on/off switch – something that has become increasingly clear across many tissue stem cells. In functional experiments, Cabezas-Wallscheid also identified retinoic acid/vitamin A as an important regulator of haematopoietic stem cell dormancy.

Haematopoietic stem cells were also the focus of a talk from Sergei Doulatov (George Daley laboratory, Harvard Medical School, USA), who provided an excellent example of how useful it can be to generate haematopoietic stem cells from human induced pluripotent stem cells (iPSCs). Doulatov showed how the *in vitro*-generated haematopoietic stem cells could be exploited for disease modelling and drug discovery, with a focus on patients with Diamond–Blackfan anaemia. Gerald de Haan (University Medical Center Groningen, The Netherlands) continued on the haematopoietic stem cell theme, discussing how his group is exploring the relationship between ageing and haematopoietic stem cell function. He has used barcoding to track haematopoietic stem cell clones and then measured clonal output in those derived from either young or old mice that are co-transplanted (Verovskaya et al., 2013). Interestingly, no loss of old haematopoietic stem cells was observed, suggesting that their decreased output was due to cell intrinsic deficits compromising their function.

Joaquina Delas (Hannon lab, Cancer Research UK Cambridge Research Institute) was one of the few talks that covered the role of long noncoding RNAs (lncRNAs) in the haematopoietic system. She identified the set of lncRNAs expressed in mouse – both in the normal and disease state – using *de novo* transcriptome assembly. These data formed the basis of a specific functional *in vivo* assessment of the role of these lncRNAs in acute myeloid leukaemia (AML) progression. Among the hits identified were several lncRNAs required to maintain the expression of signature

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leukaemic stem cell genes. Upon depletion of these lncRNAs, cells upregulated myeloid differentiation markers. These lncRNAs seem to operate through the Myc transcription factor, as Myc levels were reduced upon lncRNA knockdown, and enforced expression of Myc rescued the phenotype. The future focus of this work will be to resolve mechanistically how lncRNAs can influence Myc, as well as their role in normal fate choice.

Concluding remarks: Seq and ye shall find

The meeting ended with a plenary talk from Alexander Van Oudenaarden (Hubrecht Institute, The Netherlands). Single-cell analysis has opened the door to a more refined understanding of cell states and transitions, and Van Oudenaarden provided some impressive examples of the power of these approaches to define the repertoire of cell types and states. He has also been exploiting the CRISPR/Cas system, repurposing it to knock-in barcodes that enable the parallel tracking of lineages in whole organisms. This a powerful approach that has also been successfully used by others (McKenna et al., 2016). Van Oudenaarden made an important point concerning the recent rapid convergence of many different single cell assays and approaches: the time has come where these distinct methods can be integrated to gain a more coherent view of stem cell states and behaviours. Stem cell biologists working with 'classic' single-cell analyses, such as immunocytochemistry, flow cytometry and live-cell imaging now have the opportunity to link these methods with the powerful single-cell 'omics approaches. As the barriers to these technologies are reduced and analytical tools become quicker and more reliable, we will undoubtedly witness a new phase of integrative quantitative analysis in which a coherent picture of cell states can be readily obtained.

One obvious point to emerge from the meeting was the rapid adoption of CRISPR/Cas across many areas. A number of elegant and clever approaches demonstrated how this new-found ability to edit the genome can be used for lineage tracing, library screens and epigenome editing. There were also several examples of how CRISPR/Cas genome editing enables functional annotation of gene regulatory elements; an impressive new toolkit for the dissection of regulatory circuits within the human genome. Another theme to emerge was the requirement for better tools to monitor the dynamics of gene regulation in live cells, ideally at single-cell resolution. It was also striking how many speakers are making insights that directly inform our understanding of human cancer: the longstanding promise of developmental and stem cell biology to help tackle human cancer perhaps finally coming to fruition.

After dinner at Magdalene College, meeting attendees strode into town for some well-earned refreshment, passing by Portugal Place, the former home of Francis Crick. It is now over half a century since he and Watson uncovered the structure of DNA – the secret of life – and over 15 years since the human genome was deciphered. Crick would surely be astonished by our new-found ability to routinely sequence and edit human genomes, and to globally survey gene expression patterns. These new rivers of data do not necessarily mean we will drown; but more than ever we need collaborative approaches to take advantage of the opportunities they offer.

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Competing interests

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