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MEETING REVIEW

Transcriptional and epigenetic insights from stem cells and developing tissues

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ABSTRACT

In March 2015, over 200 scientists gathered in Steamboat Springs, Colorado, USA, for the Keystone Symposium 'Transcriptional and Epigenetic Influences on Stem Cell States' to discuss the molecular mechanisms of pluripotency, cell differentiation, cell reprogramming and transdifferentiation, among other topics. In this meeting, translational research on stem cells for disease modeling and therapy was also presented. This Meeting Review describes key themes and selected findings, providing a timely update on this fast-moving area of research.

KEY WORDS: Developing tissues, Epigenetic regulation, Single cells, Stem cells, Transcriptional regulation, Transdifferentiation

Introduction

A fundamental goal in biology is to understand how the genetic material of a stem cell encodes the information necessary for the production of the distinct cell types that form the tissues and organs of metazoans. The knowledge gained from the pursuit of this goal not only contributes important insights into human development, but also advances our ability to model diseases and develop regenerative therapies. At the recent Keystone Symposium on 'Transcriptional and Epigenetic Influences on Stem Cell States', the organizers – Thomas Zwaka (Icahn School of Medicine at Mount Sinai, USA), Rudolf Jaenisch (Whitehead Institute, Massachusetts Institute of Technology, USA) and Joanna Wysocka (Stanford University, USA) – brought together over 200 scientists from 20 countries to present work that addresses this overarching, fundamental goal. The diverse research approaches and perspectives provided attendees with a broad view of recent advancements in this interdisciplinary field.

The symposium covered insights gained from basic research fields, such as transcriptional regulation, chromatin modifications and higher-order genome organization. These topics were discussed in the context of normal development (maintenance of pluripotency and cell differentiation) as well as cellular reprogramming and transdifferentiation. Several talks highlighted the value of single-cell genomic analysis to understand heterogeneity and dynamics within cell populations. Discoveries regarding cell engineering for disease modeling and cellular therapies were also presented, providing a realistic measure of hope that this research will directly benefit human patients and society. This Meeting Review highlights a selection of presentations that relate to these major themes.

Transcriptional regulation of cell state transitions

The acquisition of specific cell states is in part regulated by the action of transcription factors, and a number of speakers presented work regarding the transcriptional control of three specific state transitions: cell differentiation, cell reprogramming and transdifferentiation. One recurrent theme was the importance of specific gene repression during cell lineage specification. For instance, Margaret Fuller (Stanford University, USA) showed that during sperm cell differentiation in *Drosophila*, a zinc-finger protein is required for the repression of a large set of somatic genes. Raga Krishnakumar (University of California, San Francisco, USA) found that in differentiating mouse ESCs, FOXD3 has a dual role, recruiting BRG1 to activate enhancers as well as the histone deacetylases HDAC1/2 to mediate gene repression. Along the same lines, Tonis Org (University of California, Los Angeles, USA) showed that in the developing mouse mesoderm, Scl binds and activates hematopoietic enhancers but also interferes with the activation of the enhancers that drive the cardiac lineage, preventing ectopic cardiogenesis (Org et al., 2015).

Whereas cellular reprogramming has attracted much attention for the engineering of cells for transplantation and disease modeling, studying the process itself has also proven to be useful for understanding the transition between different cell states during development. Notably, the transcription factor-induced reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) involves genome-wide changes to the chromatin. Kathrin Plath (University of California, Los Angeles, USA), who has been exploring the mechanisms by which the four 'OSKM' Yamanaka factors (Oct4, Sox2, Klf4 and cMyc) induce pluripotency in mouse fibroblasts, showed that during iPSC generation, the reprograming factors 'shift' their binding from somatically active sites to those more characteristic of embryonic stem cells (ESCs).

The work of Ken Zaret (University of Pennsylvania, USA) addresses the question of how the OSK reprogramming factors can serve as 'pioneer' factors, binding to DNA sequences within closed chromatin domains where the nucleosome structure would generally impair transcription factor binding through steric constraints. Interestingly, despite lacking canonical cognate binding sites, human reprogramming factors can bind nucleosomes when the DNA is wrapped around the core octomer. Thus, contrary to the popular belief that nucleosomes are inherently repressive to transcription factor binding, Zaret showed that the structural changes induced to the DNA by being wrapped around the core histones result in the presentation of partial DNA motifs to which pioneer factors can bind by adapting their DNA binding domains (Soufi et al., 2015).

Whereas Zaret's work showed that OSKM factors can apparently access and initiate the activation of genomic regions lacking a definitive cognate binding site, Marius Wernig (Stanford University, USA) had previously found that the pioneer activity of other factors like ASCL1, which reprograms mouse fibroblasts into neurons, is more directed to cognate binding sites (Wapinski et al., 2013). At this meeting, Wernig showed that MYTL1, another

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Development (2015) 142, 2549-2553 doi:10.1242/dev.122424

transcription factor that promotes direct neuronal reprogramming, plays an important role in the repression of non-neuronal genes such as the Notch effector HES1 during transdifferentiation, mirroring the gene repression observed during lineage specification (see above). Interestingly, Konrad Hochedlinger (HHMI, Harvard University, USA) found that the transdifferentiation of mouse fibroblasts into neural stem cells involves transient passage through an iPSC-like state.

Chromatin-based regulation of stem cells and their differentiation

Given the importance of specific chromatin states in the establishment and maintenance of cellular identity, there has been great interest in identifying chromatin regulators involved in stem cell maintenance and cell fate specification. Jacob Hanna (Weizmann Institute, Israel) reported that early and controlled (50-80%) depletion of the NuRD repressor core component MBD3 dramatically increases the efficiency of iPSC generation (Luo et al., 2013; Rais et al., 2013), whereas others have described a seemingly opposite effect upon complete MBD3 ablation (dos Santos et al., 2014). One possible explanation for these conflicting results is that, as the Hanna lab had originally reported (Rais et al., 2013), *Mbd3*-knockout cells abort proliferation. By further dissecting the composition of the MBD3/NuRD complex, the Hanna lab has been able to identify other NuRD-specific components, the complete ablation of which does not inhibit somatic proliferation but yields an increase in the efficiency of iPSC generation similar to that reported for incomplete MBD3 depletion. Further work is required to understand the exact contribution of the different NuRD conformations to the reprogramming process and somatic cell cycle regulation. Konrad Hochedlinger also presented work on small molecules such as ascorbic acid and a GSK3b inhibitor that enhance the speed and synchrony of mouse iPSC generation (Bar-Nur et al., 2014).

Ulrich Elling (Institute of Molecular Biotechnology, Vienna, Austria) performed an shRNA screen for chromatin regulators in an inducible OSKM mouse cell line, identifying those that facilitate iPSC generation. Knockdown of *Chaf1a* (part of a complex required for histone deposition on replicating DNA) resulted in a dramatic increase in the efficiency of iPSC generation, apparently due to increased accessibility of enhancers, correlating with increased SOX2 binding.

Kristian Helin (University of Copenhagen, Denmark) presented an important update to our understanding of the role of the JMJD2 histone demethylase family in the transcriptional regulation of stem cell self-renewal. A previous analysis of *Jmjd2b* and *Jmjd2c* shRNA knockdown in mouse ESCs suggested a crucial function of JMJD2 in ESC self-renewal and iPSC generation (Das et al., 2014). By contrast, using genetic knockouts, the Helin lab, which had previously published that *Jmjd2c*-null mouse ESCs proliferate normally, now found that *Jmjd2b*-null and *Jmjd2a*-null mouse ESCs also show normal proliferation. However, proliferation was defective in *Jmjd2a/c* double-knockout cells, and these enzymes appear to be required for H3K9me3 demethylation and the expression of target genes. Although there are many possible reasons for this apparent disparity in results, these data were presented as a cautionary tale about the use of shRNAs for the study of highly related proteins.

Developing primordial germ cells (PGCs) – the precursors of sperm and eggs – show unique characteristics in terms of chromatin dynamics, related to the fact that, as these cells are preserved across generations, their genome has to be 'reset' between each. This occurs through a wide-scale demethylation of the DNA across the genome, and how this process is regulated in different species is a topic of active investigation. In his Keynote presentation, Azim Surani (Gurdon Institute, Cambridge, UK) presented data showing that the transcriptional program that specifies human PGCs is very different from that of mice (Irie et al., 2015). Whereas PRDM14 is a crucial factor for mouse PGC development, human PGC specification requires SOX17, which induces BLIMP1. Additionally, PGC specification is accompanied by the induction of TET1/2 [enzymes that catalyze the conversion of 5-methylcytosine (5 mC) to 5-hydroxymethycytosine (5 hmC), a step that can subsequently lead to DNA demethylation] and the repression of the *de novo* DNA methyltransferases *Dnmt3a/b*. Thus, SOX17 and BLIMP1 are central regulatory factors of the DNA methylation reset 'switch' in PGC development. Interestingly, not all loci undergo DNA demethylation, and it is intriguing to consider whether such 'escapee' loci contribute to transgenerational epigenetic inheritance.

Once specified, PGCs give rise to adult germline stem cells (AGSCs) during postnatal development. How are pluripotency genes silenced in mature sperm, and are these genes 'poised' for reactivation following fertilization? To address these questions, Bradley Cairns (HHMI, University of Utah School of Medicine, USA) analyzed the chromatin state dynamics and the transcriptional profile of mouse and human AGSCs at different stages of gametogenesis. Cairns found that key pluripotency genes (e.g. *Nanog*) are silenced in AGSCs, but have chromatin features that might 'poise' them for later reactivation (Hammoud et al., 2014). In addition to specific enhancers having a 'bivalent' histone signature, the corresponding promoters lack DNA methylation. Finally, Cairns showed that during the postnatal establishment of AGSCs, the DNA demethylation of the genome is very focal, occurring at particular sets of genes involved in spermatogenesis, and also at particular clustered genes.

In addition to having a crucial function in the germ line, regulators of DNA methylation are also important in the soma and other stem cell populations. By inactivating the different DNA methyltransferases in hESCs using CRISPR/Cas9, Alex Meissner (Broad Institute of MIT and Harvard University, USA) found that DNMT3A and DNMT3B act redundantly at many genomic targets and that DNMT3A is required for *de novo* methylation during lineage specification. Meissner also showed that DNMT1, which maintains 5 mC after DNA replication, is necessary for the maintenance of CpG methylation and required for cell survival (Liao et al., 2015). Margaret Goodell (Baylor College of Medicine, Houston, USA) discovered that, in addition to gene body methylation, DNMT3A is responsible for DNA methylation at the edges of large hypomethylated regions termed 'canyons,' contributing to the regulation of genes required for mouse blood cell differentiation (Jeong et al., 2014). Whereas the inactivation of *Dnmt3a* causes a loss of 5 mC at the edges of these canyons, mutations in *Tet2* cause an increase in 5 mC at the canyon borders. Furthermore, *Dnmt3a* is epistatic to TET enzymes in the passive loss of DNA methylation that occurs during DNA replication. Interestingly, *DNMT3A* and *TET2* are mutated in a subset of B-cell and myeloid lineage diseases, and these cancer cells have phenotypic similarities to blood stem cells with *TET2* loss. Although *DNMT3A* mutations alone might not be sufficient to drive blood cell cancers (Yang et al., 2015), it is worrisome that blood stem cells with clonal mutations in *DNMT3A* are found in increased proportions in the bone marrow during aging.

Development (2015) 142, 2549-2553 doi:10.1242/dev.122424

Chromatin structure in pluripotency and lineage commitment

In the nucleus, chromatin is organized in higher-order three-dimensional (3D) structures. Chromosome-conformation-capture (3C) and related 4C and 5C techniques have helped researchers to identify interactions between chromosome regions. Within each chromosome, intra-chromosomal interactions organize the genome into 'compartments' (3 Mb median size), and within such compartments, several smaller (~800 kb) topologically associating domains (TADs) are typically found. Within TADs, intra-chromosomal 'looping' interactions represent an additional level of genome organization. Jennifer Phillips-Cremins (University of Pennsylvania, Philadelphia, USA) used 5C to study how the 3D topology of the genome 'unfolds' during the reprogramming of mouse neural precursor cells (NPCs) into iPSCs. She presented evidence suggesting that some genome-folding configurations found in somatic cells are retained in induced pluripotent stem cells.

Work from Rick Young's lab (Massachusetts Institute of Technology, Cambridge, USA) on mouse ESCs has identified so-called 'super-enhancers' – domains containing large clusters of enhancers that play prominent roles in cell identity (Whyte et al., 2013). Genes driven by super-enhancers are often located within 'insulated neighborhoods' which correspond to large DNA loops consisting of two CTCF sites co-occupied by cohesin. These insulated neighborhoods are important for maintaining proper gene expression both inside and outside of the loop (Dowen et al., 2014). Young also presented more recent data on normal human colon tissue and colorectal cancer cells, indicating that mutations that create new super-enhancers – or those that disrupt insulated neighborhoods – can drive oncogenesis (Hnisz et al., 2015; Mansour et al., 2014).

By studying human ESCs at different stages of the cell cycle (Singh et al., 2013), Stephen Dalton's group (University of Georgia, Athens, USA) found that promoter-enhancer looping interactions at developmental genes in human pluripotent stem cells (PSCs) are cell cycle-dependent. During G1 phase, the H3K4 methyltransferase MLL2 undergoes phosphorylation by CDK2, which triggers epigenetic changes required for 'priming' of developmental genes. These epigenetic priming events underpin the previous observation that pluripotent cells initiate cell fate decisions from G1 phase.

Insights from single-cell transcriptomics and epigenomics

Recent advances in single-cell genomic and transcriptomic sequencing are rapidly changing our understanding of cell identity. For instance, using single-cell sequencing, Fredrik Lanner (Karolinska Institutet, Sweden) described a transcriptional 'roadmap' leading to the emergence of the first cell lineages in the human embryo, generating new insights into the transcriptional mechanisms of lineage segregation. The new depth of information provided by single-cell analysis has also revealed tremendous heterogeneity among cell populations. Alex Van Ourdenaarden (Hubrecht Institute, University Medical Centre Utrecht, The Netherlands) has pioneered new methods for sequencing the genome and transcriptome from single mouse and human cells (Dey et al., 2015). Van Ourdenaarden described how his group has overcome some of the computational challenges in understanding how gene expression differences observed in single cells can reflect different cell identities. Commonly used methodologies (e.g. *K*-means clustering) group cells according to their gene expression patterns, but outlier cells can sometimes 'drive' the clustering inappropriately, essentially 'hiding' the presence of rare cell types. By analyzing sequencing data of single mouse intestinal cells in a very high-dimensional space, the Van Ourdenaarden group has been able to identify outliers as very rare cell types, confirming their existence with single-molecule FISH. Using single-cell analysis of the methylome with whole-genome bisulfite sequencing, Wolf Reik (Babraham and Sanger Institutes, Cambridge, UK) discovered enormous heterogeneity of DNA methylation patterns in mouse pluripotent cell populations. Interestingly, the greatest heterogeneity was found in enhancer sequences (Smallwood et al., 2014) and was linked to transcriptional heterogeneity with single-cell RNA-seq. These findings suggest that the transcriptional heterogeneity observed in pluripotent stem cells results, at least in part, from DNA methylation heterogeneity.

Planarians are flatworms that can regenerate any missing body region, and a proliferative cell population called neoblasts mediates this remarkable regenerative capacity. Neoblasts were formerly thought to represent a single cell type. However, using single-cell transcriptional profiling, Peter Reddien (Whitehead Institute, MIT, USA) discovered that this population is actually composed of at least two cell types with distinct developmental potentials (van Wolfswinkel et al., 2014).

Long noncoding RNAs (lncRNAs) are transcripts greater than 200 nucleotides in length that have little evidence of protein coding potential. After presenting recent data regarding the neurodevelopmental function and mechanisms of action of a novel lncRNA called *Pnky* (Ramos et al., 2015), Dan Lim (University of California, San Francisco, USA) presented an analysis of lncRNA expression in single cells isolated from the developing human brain. In individual cells, lncRNAs were found to be unexpectedly abundant and discretely expressed across different cell populations, indicating that lncRNAs might help to define the vast diversity of cell types of the human brain.

New inroads and ongoing controversies

The human genome contains a large amount and variety of endogenous retroviral sequences, which are remnants of ancient viral infections of the germ line. Joanna Wysocka recently discovered a dynamic expression of different classes of endogenous retroviruses in early human embryos, including the evolutionary youngest class HERV-K, which retained protein-coding potential (Grow et al., 2015). Furthermore, Wysocka found that HERV-K transcription during pre-implantation development is synergistically facilitated by OCT4 and DNA hypomethylation. Intriguingly, electron microscopy analyses revealed viral-like particles containing HERV-K capsid protein within cells of the human blastocyst. It is therefore thought-provoking to consider the possibility that infectious retroviral particles are produced during normal human development and could influence this process.

Cell competition is an emerging developmental concept regarding the control of organ size during development and tissue homeostasis. By performing a genome-wide screen on murine iPSCs, Thomas Zwaka identified a network of genes that controls the relative contribution of pluripotent epiblast cells to the final organism (Dejosez et al., 2013). *Edar* (a member of the tumor necrosis factor family) is a master regulator of this cell-competition gene network, and, interestingly, *Edar* is among the top SNPs implicated in human evolution, suggesting that cell competition plays a role in natural selection in humans.

After the blastocyst stage, when the embryo is implanted into the uterine wall, the ICM segregates into the hypoblast and epiblast layers. Shortly after implantation, the naïve epiblast cells polarize and organize into a 3D rosette structure. How these changes in

Development (2015) 142, 2549-2553 doi:10.1242/dev.122424

cellular architecture, coupled to an alteration of the pluripotency state, occur is poorly understood. To shed light on this process, Magdalena Zernicka-Goetz (Gurdon Institute, Cambridge, UK) studied the maturation of mouse blastocysts *in vitro* and found that apoptosis is not essential for peri-implantation morphogenesis, as was previously believed (Bedzhov and Zernicka-Goetz, 2014). Instead, basal membrane-stimulated integrin signaling drives epiblast cell polarization and rosette formation.

PSCs exist in at least two distinct states *in vitro*, naïve and primed, which probably represent sequential 'snapshots' of the pluripotency state found in normal embryonic development (Nichols and Smith, 2009). Whereas naïve PSCs share many properties with ICM cells, primed PSCs more closely resemble epiblast cells of the post-implantation embryo. Until recently, most human ESC lines were found to closely resemble mouse primed ESCs. Rudolph Jaenisch and Austin Smith (Cambridge Stem Cell Institute, UK) were among several investigators who presented ongoing work to define and generate human naïve ESCs better. Using an Oct4-deltaPE-GFP reporter, the Jaenisch lab found that many published methods do not robustly maintain the naïve human PSC state (Theunissen et al., 2014). Interestingly, Jaenisch also found that naïve human PSCs appeared to have undergone X chromosome inactivation, resulting in one active and one inactive X chromosome, in contrast to naïve mouse PSCs. Additionally, by using specific culture conditions (2i medium and chemical aPKC inhibition), Smith reported derivation and characterization of new human ESCs in a naïve-like state. However, as mentioned by a number of speakers, significant work is still required to characterize naïve pluripotency both in mouse and human ESCs.

Advances in translational research

Some of the great excitement about stem cell research relates to the use of cell engineering for regenerative medicine or other translational purposes. In this area, Lorenz Studer (Memorial Sloan Kettering Cancer Center, USA) made a number of recent contributions that bring stem cell research closer to clinical utility. Using iPSC technologies in human cells, the Studer lab modeled familial dysautonomia (Riley–Day syndrome), a disorder that affects both the development and survival of neurons in the autonomic nervous system (Lee et al., 2009). With this cell culture model, he identified molecules that prevent the neurodegenerative aspect of the disease. Studer also generated human iPSC-derived enteric neural crest stem cells (eNCSCs) in the hope of treating Hirschsprung's disease, a disorder in which the lack of certain neural cells in the intestine impairs gastrointestinal motility and leads to obstruction. Upon transplantation into a Hirschsprung mouse model, the iPSC-derived eNCSCs gave rise to cells capable of rescuing aspects of the disease phenotype. Along the same lines, Ihor Lemischka (Icahn School of Medicine at Mount Sinai, USA) has been looking into factors that might reprogram human iPSCs and ESCs into long-term hematopoietic stem cells that give rise to blood and immune cells when engrafted in mice. This work, and that of others in the field, opens the possibility of generating patient-specific blood-derived tissues for regenerative purposes.

Another emerging strategy for regenerative medicine is the *in vivo* conversion of differentiated tissue-resident cells into cell types that are lost due to disease. For the treatment of diabetes, several groups have successfully generated insulin-secreting cells by expressing various sets of transcription factors in differentiated cells *in vivo* (Pagliuca and Melton, 2013). However, when reprogramming a mature cell directly into another cell type, the cell of origin appears to be an important consideration. By combining ATAC-seq, a method to assess regions with accessible chromatin, and motif analysis of beta-cell reprogramming factors, Qiou Zhou (Harvard University, USA) predicted mouse stomach endocrine cells to be particularly amenable for direct conversion into insulin-secreting cells. The Zhou lab then confirmed this prediction *in vivo*, creating a stomach 'pouch' containing endocrine cells reprogrammed into insulin-secreting cells, which was able to properly regulate blood glucose.

Another crucial aspect of cellular reprogramming for regenerative purposes is the 'quality' of the resulting engineered cells. To address whether reprogrammed cells are similar to their *in vivo* counterparts, George Daley (HHMI, Boston Children's Hospital, USA) has developed a web resource called CellNet (cellnet.hms.harvard.edu), which can be used to assess the quality of engineered cells and also propose ways in which the engineering process can be improved (Cahan et al., 2014; Morris et al., 2014). CellNet uses network analysis to identify gene regulatory networks (GRNs) in a variety of reprogrammed cell types and compares them with that of native cells isolated from the human body. By making such comparisons, one can begin to assess how closely reprogrammed cells resemble their native counterparts on a transcriptional level and infer ways in which the fidelity of cell conversion can be improved.

Concluding remarks

After surveying the broad range of research presented at this meeting, it is clear that many key advances have arisen from productive collaborations among researchers with expertise in different systems and experimental approaches. In particular, major insights included the significance of chromatin modifications and higher-order genomic structures in the regulation of cell-state transitions, the underappreciated heterogeneity of cell populations, and the importance of understanding mechanistic differences between human and mouse developmental programs, particularly for the development of therapeutic applications. In addition to disseminating recent and often unpublished findings, this Keystone Symposium has assuredly served as another catalyst for such collaborative interactions, perhaps inspiring research directions that will reveal new principles of transcriptional and epigenetic regulation of cellular fate.

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Competing interests

The author declares no competing or financial interests.

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