Editorial

Stem Cell INVESTIGATION

FOXD3 controls pluripotency through modulating enhancer activity

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Received: 27 April 2016; Accepted: 23 May 2016; Published: 27 May 2016.

doi: 10.21037/sci.2016.05.03

View this article at: http://dx.doi.org/10.21037/sci.2016.05.03

Pluripotent stem cell governs the potential for full and proper range of early development. These cell fate transitions are mostly achieved by the sequential binding of transcription factors (TFs) and the associated changes in gene expression. Enhancers are the major DNA elements that are involved in the regulation of cell-specific gene expression. Thus, to understand the drivers of these transitions and the exact mechanisms for these cells to retain their pluripotency, it is essential to look deeper into these enhancers and how they work with various TFs. Krishnakumar *et al.* recently identified that forkhead TF FOXD3 binds to a differential set of enhancers in embryonic stem cells (ESCs) and epiblast cells (EpiCs). FOXD3 acts as a pioneer TF and precedes the binding of other TFs to regulate gene expression by the regulation of local chromatin structure and nucleosomes. Notably, FOXD3 interacts with both the SW1/SNF chromatin remodelling complex ATPase BRG1 and histone deacetylase 1/2 (HDAC1/2), in order to keep expression in check by activating and simultaneously suppressing different sets of genes.

Embryonic development is a biological process that promotes the differentiation of pluripotent stem cells into specific cell lineages through several sequential inductive events. The ability of progenitor cell intermediates to respond to environmental inductive cues that drive cell-specific expression programmes is called developmental competence. The mechanism that induces developmental competence in ESCs in a context-dependent manner is as yet elusive (1).

The activity of TFs and the epigenetic priming of enhancers, are key contributors for the induction of cellular competence in development (1,2). Epigenetic patterning of enhancers, which are regulatory DNA elements that modulate gene expression, occurs prior to cell fate decisions and involves the DNA methylation status, the binding of pioneer TFs to enhancers and existing histone modifications (2). Understanding the molecular events underlying the epigenetic priming of enhancers could prove useful to develop strategies to manipulate induced pluripotent stem cells for the enormous therapeutic potential due to their remarkable ability to self-renew (2,3).

Furthermore, pioneer factors are a subset of TFs which interact with target genes prior to binding of other TFs for gene expression (4). It has been proposed that binding of pioneer TFs to specific enhancers promotes future gene expression through nucleosome repositioning which ensures the subsequent recruitment of other TFs and regulatory proteins and assembly of transcriptional complexes. Also, it is worth mentioning that these molecules access compact chromatin without the involvement of chromatin modifiers and remodellers (4,5).

Multiple pioneer factors such as FOXA1, FOXA2, and FOXD3 belong to the superfamily of Forkhead box (FOX) TFs (1,6). The Forkhead box proteins are characterised by an evolutionary conserved 'forkhead' or 'winged helix' DNA-binding domain. The FOX proteins are involved in orchestrating temporal and spatial gene expression during embryonic development and homeostasis of adult tissues. Besides, they regulate fundamental biological processes such as cell cycle progression, proliferation, differentiation, metabolism, senescence, apoptosis and survival (7). Additionally, these transcriptional regulators play a crucial role in cancer since they are implicated in cancer initiation,

progression and chemoresistance (7).

In fact, several studies have shown that pioneer factors govern the dynamics of enhancers for specific gene expression in pluripotent stem cell potential, cell fate transitions, lineage choice and differentiation (8,9). A previous study by Wang *et al.*, has demonstrated that FOXA1 and FOXA2 are involved in the epigenetic priming of enhancers that leads to the acquisition of developmental competence during endodermal lineage diversification (1). In addition, FOXA1 mainly operates through enhancers binding and trigger transcriptional competency of target genes, through initial chromatin decompaction (10). FOXA1 also plays a role in the organ differentiation and developments, and has also been involved in cancer including breast and prostate cancer (10,11). In the other hand, FOXD3 is involved in pluripotency maintenance in human ESCs, in promoting epithelial-to-mesenchymal transition during neural crest specification, in regulating the balance between melanocyte and Schwann cell development, and in the regulation of a set of differentiation-associated genes along with NFATc3 in ESCs (12-15). In addition, FOXD3 acts as tumour suppressor in a wide range of human cancers, such as hepatocellular carcinoma, non-small cell lung cancer, and breast cancer (16-18). Recently, FOXD3 is in the limelight for being involved in the regulation of stem cell pluripotency by binding to different enhancer marks and subsequently manipulating transcriptional competency of specific genes (6,19,20).

A recent study by Krishnakumar *et al.*, described the epigenetic mechanism underlying enhancer priming and regulation of pluripotent stem cell potential during differentiation of ESCs to EpiCs that takes place in the early stages of mammalian embryonic development (6). The results have uncovered a major role of FOXD3 in fine-tuning gene expression through coordinated activation and repression of enhancer activity in a context-dependent manner prior to gastrulation and germ layer foundation.

First of all, through a highly specialised reporter system that follows enhancer activity during ESCs to EpiCs transition with differential cell culture conditions and chromatin immunoprecipitation (ChIP)-sequencing, Krishnakumar *et al.* has observed the highly interchangeable nature of this developmental stage as a result of enhancer transition from active to primed states and vice versa (6). The establishment of epigenetic marks such as H3K27ac was also highly dynamic and independent of the most common HAT protein found at enhancer regions, P300. Next, ChromHMM was used to analyse the occurrence of four epigenetic marks at enhancer sites during six cell states. Again, it was observed that distinct cell states were characterised by the presence of different enhancer marks. In addition, FOXD3 binding motif was found to be highly enriched during these cell transitions, which turn the research focus towards it.

Moreover, by ChIP-seq for FOXD3-3X-FLAG, *de novo* motif finding and FIMO analysis with FOXD3 position weight matrix (PWM), it was reflected that FOXD3 translocates itself during ESCs to EpiCs transition and interacts with genomic regions close to different genes in the two cell states prior to their maximal expression (6). Furthermore, ChiP-seq analysis showed that FOXD3 binding precedes the recruitment of other crucial regulatory factors, such as OCT4, which confirms that FOXD3 regulates chromatin structures to ensure future binding of other TFs.

Evidence obtained from FAIRE-seq, DNAsel hypersensitivity (HS)-seq, high-resolution micrococcal nuclease (MNase)-ChIP-seq, a conditional KO model for FOXD3 with a Tamoxifen-inducible CRE recombinase driven off an ubiquitous promoter, IP of FOXD3 and expression microarray analysis indicated that FOXD3 promotes enhancer activity through the nucleosome repositioning at H3K4me1 sites in specific genomic regions in a precise manner during ESCs to EpiCs transition which primes them for subsequent activation (6). In the same way, FOXD3 sustains hypoacetylation of neighbouring genes to repress them which restricts their expression in a precise manner until occurrence of an appropriate scenario. ChIP-qPCR confirmed that FOXD3 is responsible for recruiting BRG1, a crucial component of the SWI/SNF nucleosome remodeling complex, to enhancers. However, BRG1 maintenance at these regulatory sites is FOXD3 independent. FOXD3 primes enhancers to modulate gene expression, so that they can be either activated or repressed in future scenarios based on specific induction signals. FOXD3 recruits and interacts with HDAC1/2 at enhancer sites to fine-tune modulation of gene expression. Finally, different combinations of IP-western blot and sequential ChIP confirmed that BRG1, HDAC1 and FOXD3 interact with each other and form a single complex. This protein complex is involved in epigenetic priming of enhancers which are simultaneously repressed by the epigenetic mark H3K27ac.

These findings have broaden the current understanding of the mechanisms that tightly regulate transcriptional activity and gene expression during stem cell transition states in embryonic development. However, a study led by Respuela *et al.* (20) that addresses the same issue was published simultaneously in the same scientific journal.

Interestingly, both studies reached different conclusions about the role of FOXD3 in pluripotency and early differentiation of mESCs (21). The main reason that explains this divergence between both studies is the fact that these studies differ in certain parameters included in their respective experimental designs selected to analyse this molecular scenario, such as distinct cell culture conditions, methodological approaches, and algorithms/criteria applied for bioinformatical data analysis. Due to opposing conclusions being reached, these results provided by both groups will have to be confirmed by further studies.

The study of the molecular events that orchestrate the complex processes, which conduct mammalian embryonic development, will provide key insights that could be applied to unravel the as yet unknown aspects that rule the onset of diseases such as cancer. The mechanisms that are involved in early development are crucial to maintain homeostasis and health of the later differentiated cells. Their unexpected perturbation is responsible for the rise of disease phenotypes. Acquiring advanced knowledge about the system at equilibrium will pave the way for the development of more effective therapeutic strategies to overcome these diseases. Finally, these insights will provide better tools to manipulate induced pluripotent stem cells (iPSCs) and their therapeutic potentials in order to improve tissue transplantation approaches to treat diseases through regenerative medicine.

Acknowledgements

Funding: EW Lam's work is supported by Breast Cancer Now (2012MayPR070; 2012NovPhD016), CRUK (A12011), and MRC/Newton Fund (MR/N012097/1), and JS Yong by an Imperial College PhD Scholarship.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Comment on: Krishnakumar R, Chen AF, Pantovich MG, *et al*. FOXD3 regulates pluripotent stem cell potential by simultaneously initiating and repressing enhancer activity. Cell Stem Cell 2016;18:104-17.

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doi: 10.21037/sci.2016.05.03
Cite this article as: Yong JS, Intriago-Baldeón DP, Lam EW. FOXD3 controls pluripotency through modulating enhancer activity. Stem Cell Investig 2016;3:17.