Viewpoint Article

Title

Single Cell Pluripotency Regulatory Networks

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Abstract

Pluripotent stem cells (PSCs) are a popular model system for investigating development, tissue regeneration and repair. Although much is known about the molecular mechanisms that regulate the balance between self-renewal and lineage commitment in PSCs, the spatiotemporal integration of responsive signalling pathways with core transcriptional regulatory networks are complex and only partially understood. Moreover, measurements made on populations of cells reveal only average properties of the underlying regulatory networks, obscuring their fine detail. Here, we discuss the reconstruction of regulatory networks in individual cells using novel single cell transcriptomics and proteomics, in order to expand our understanding of the molecular basis of pluripotency, including the role of cell-cell variability within PSC cell populations, and ways in which networks may be controlled in order to reliably manipulate cell behaviour.

Keywords: Pluripotency, Cellular Reprogramming, Single Cell Networks, Cell-to-Cell Variability, Controllability of Complex Networks

1 Introduction

Our understanding of pluripotency has grown tremendously since pioneering studies described the derivation and *in vitro* culture of pluripotent stem cells (PSCs) [1-9]. It is now well known that PSCs display two characteristic features: 1) indefinite self-renewal in vitro, and 2) tri-lineage commitment to ectoderm, endoderm and mesoderm, once released from the self-renewing regime. Knowledge of both these properties has been predominantly generated from aggregates of cellular material, and therefore represents the average behaviour of hundreds or thousands of cells. Nevertheless, from a wealth of regulatory relationships, a limited set of core transcription factors have been inferred and validated, resulting in the construction of a now reliable regulatory model for the pluripotent state [10]. However, more recent measurements at the single cell level have highlighted the presence of significant phenotypic variability within clonal PSC populations [11], suggesting that subtle cell-cell variations in network configurations may have an important role in regulating pluripotency [12-16]**.** These results stress the importance of reconstructing regulatory networks at the individual cell level in order to uncover the refined mechanisms that balance self-renewal and differentiation *in vitro* and cellular propensities for different developmental states. In this article, we sketch out our current understanding of the integrated regulatory network (IRN) that controls the transient developmental state of pluripotency and discuss the ways in which more refined single cell regulatory networks are enhancing our understanding of PSC states and the ways in which PSCs balance self-renewal and lineage commitment at the individual cell level.

2 Combinatorial control of pluripotency by regulatory networks

The pluripotent state in mouse and human cells is regulated by a number of integrated regulatory networks (previously reviewed [17-21]), including transcriptional [22], epigenetic [23], signalling [24] and metabolic [25] sub-networks. These networks may be visualized schematically using simple graphical models in which nodes represent the fundamental molecular building blocks of cells – such as genes, transcripts and proteins – and arcs represent interactions between these elements (see Figure 1 for examples). It is commonly observed that biological networks are characterised by a limited number of central nodes (hubs), which integrate and disseminate information throughout the network and hence significantly control cell behaviour [26]. In the presence of defined extrinsic stimuli [27,28], the pluripotent state is maintained by a cell-intrinsic set of transcription factors (TFs) that constitute a self-

sustaining gene regulatory network (GRN) that is rich in feedback [29]. Central to this GRN lies a core network of TFs composed of Oct4, Sox2, Nanog, with significant support of secondary factors such as also Klf4, Myc and Lin28 [30]. Combinations of these TFs were originally found to revert the cell identity of terminally differentiated somatic cells towards the pluripotent cell identity [6-8], however subsets of these factors are also sufficient to reconstitute pluripotency in somatic cells [31,32]. The members of this core GRN interact with a range of auxiliary transcription factors via protein-protein interactions (PPI) [33-38], and collectively control transcription of a large number of genes. Transcriptional control is exerted either directly, by binding to gene promoters [22,35,39,40], or indirectly, by mediating the effects of epigenetic remodelling complexes [21,41,42]. The latter help maintain pluripotency by producing a permissive chromatin state that allows for widespread nonspecific transcription [43], in which important developmental genes are sporadically expressed at low levels, yet remain poised for robust expression under the appropriate differentiation cues [44-46]. To buffer this "noisy" environment, a network of microRNAs [47-50] and ribosome specific mechanisms [51], ensure appropriate protein levels are robustly maintained. In addition to these cell-intrinsic regulatory mechanisms, a layer of signalling pathways integrates cell-extrinsic information to the central pluripotency GRN. While the core transcriptional circuitry is broadly similar in mouse and human cells [52], mouse embryonic stem cells (mESCs), mouse epiblast stem cells (mEpiSCs) and human embryonic stem cells (hESCs) display marked differences in their dependence on extrinsic signalling factors. In mESCs, Lif/Stat signalling [53,54], Bmp [55] and canonical Wnt [27] promote self-renewal, while Fgf/Erk signalling disrupts pluripotency [27,56-58]. In contrast, hESCs and mEpiSCs require Activin and Fgf [59,60] signalling for self-renewal and cells in this "primed" pluripotent state undergo differentiation when exposed to Bmp [60], while Lif/Stat signalling has no measureable effect on their self-renewal *in vitro* [61]. Importantly, the flow of information between signalling and transcriptional regulatory networks is not one-way: signalling networks mediate external environmental information to the core GRN, while the core GRN affects the expression of the pathway components themselves, or of key miRNAs that in turn regulate signalling pathway components [62]. Collectively, these reports indicate that pluripotency is regulated by mechanisms that act at both the transcriptional and translational levels and involve layers of combinatorial regulatory control, including complex feedback relationships between the transcriptional, epigenetic and signalling strata. However, while this model has been tremendously successful, much of this information has been inferred from

bulk properties of large ensembles of cells, for which proteomics techniques, such as chromatin- or co-immunoprecipitation followed by mass spectrometry (IP/MS), have proven instrumental [22,33-40]. Within individual cells, regulatory networks may adopt a variety of different states – for example, individual cells may express differing subsets of key proteins – and hence may deviate dramatically from this ensemble model. Thus, a better understanding of how cell-cell variation in network structure affects cell population function is now needed.

3 Regulatory networks at the single cell level

In contrast to ensemble networks from bulk cell material, single cell measurements of co-expression patterns are able to reveal a more nuanced picture of regulatory networks within cell populations [63]. Nucleic-acid-based techniques such as RNA-FISH [64,65], qRT-PCR [66] and RNA-seq [67] are able to assess multi-dimensional transcript expression patterns at single cell resolution. These emerging single cell technologies are now enabling broad expression profiles across a large number of cells to be obtained [68], from which single cell regulatory networks can be inferred statistically [69,70]. Such nucleic-acid-based techniques are convenient tools to study gene expression and have provided valuable insights, for instance into the extent of cell-cell variability in embryonic stem cells [71,72]. Although the majority of functional properties of a gene only emerge at the protein level, gene expression is often used as a proxy for protein expression, despite the well established discrepancy between gene expression and protein expression caused, for instance, by post-transcriptional and post-translational regulation, or fundamentally dissimilar transcript and protein turnover rates [73-75]. Moreover, nucleic-acid-techniques cannot be used directly to infer important properties, such as the connectivity between elements of the IRN, which may depend critically on protein-protein interactions, or the activity of phosphorylation cascades. Therefore, there is now a pressing need to extend proteomics techniques to the single cell level [76,77].

Traditionally, flow-cytometry (FC) has been used to quantify protein co-expression patterns within individual cells. However, FC methods are intrinsically limited in the number of factors that can be co-assessed (currently up to about 18), mainly due to the spectral resolution of fluorescently labelled antibodies [78]. These limitations are gradually being overcome, for instance using new methods such as Cytometry by Time of Flight (CyTOF), which, at present, is able to quantify co-expression of up to

approximately 50 different proteins in individual cells via immuno-labelling with elemental isotopes [78-80]. Using conventional methods such as FC, evidence of heterogeneity in clonal cell populations has accumulated for individual factors of the integrated pluripotency regulatory network [15,71,81-84] and it is generally agreed that variable quantities of mRNA and protein can be present within individual cells of a clonal cell population, for instance due to noise inherent to transcription and translation [85,86]. In many cases, such variability is not functional. However, for important members of the IRN such variation can strongly affect the efficiency with which cell–extrinsic stimuli are processed by individual cells and thereby drive widespread differences in multivariate expression patterns within clonal populations (for example amongst the descendants of individual PSCs – see Figure 2) [16]. Moreover, variability in expression of central regulatory factors can also affect network structure by differentially activating particular sub-networks of the IRN within individual cells [87]. Such structural changes have been shown to have an important role in regulating cell-cell variability in PSC fate changes, for example upon Nanog withdrawal [29,88]. Ultimately, differential expression of important sub-networks may impose constraints on cellular signal processing, which in turn may restrict degrees of freedom of cell-fate decisions. Although not yet fully demonstrated, it is likely that similar mechanisms, involving subtle variations in network structure between individual cells, are important for regulating the collective dynamics of PSC populations [89] .

4 Plasticity of pluripotency networks in development and reprogramming

The regulatory networks that control particular cellular identities undergo dramatic changes as cells progress from one developmental state to another [90,91]. Such changes can be exploited to classify cellular identities based on properties of their underlying regulatory network [92,93]. Four instances of cellular identity changes are of particular interest with respect to understanding cell-cell variability and network-plasticity in pluripotency: three of these instances are associated with the native developmental programmes, starting with blastulation (the origin of pluripotency) and proceeding through gastrulation (establishment of different pluripotent states, followed by exit from pluripotency); while the fourth is related to the reverse process of establishing the pluripotent regulatory network in somatic cells during cellular reprogramming. In all four cases, variation in cell-cell expression of regulatory networks has an important role.

A *Network plasticity during blastulation*

During pre-implantation development, the inner cell mass (ICM) forms two strata, the epiblast (EPI) and primitive endoderm (PE), in preparation to the formation of the embryo proper from the EPI. Initially, mosaic expression patterns of central regulatory factors for EPI (Nanog) and PE (Gata6) emerge seemingly at random [94]. The apparent spatial randomness of this process suggests that cell intrinsic stochastic mechanisms are responsible for the initial EPI–PE stratification process [95], while sub-networks centred on Nanog or Gata6 subsequently reinforce these initial stochastic variations before cell re-arrangements, coordinated through juxtacrine signalling, lead to tissue-organisation into the two strata [96,97]. However, other evidence suggests that the mosaic expression of Nanog and Gata6 is preceded by asymmetric cell division leading to an unequal distribution of Fgf-signalling components [98-100]. Very recently, single cell gene expression data obtained during pre-implantation development did not provide conclusive evidence for either model in humans, but rather indicated widespread co-expression of EPI, PE and even trophectoderm associated genes prior to segregation into distinct lineages [101]. The reconstruction of single-cell regulatory networks from data such as these could be instrumental in consolidating existing models by inferring the logical sequence of events from unbiased single cell expression data.

B *Network plasticity during the naïve pluripotent to primed pluripotent transition*

Two pluripotent states exist that display distinct differences in their IRN [4,5]: a naïve pluripotent state present in the EPI of the pre-implantation embryo, from which mESCs are derived; and a primed state, characteristic of the late stage of the EPI in the post-implantation embryo (the egg cylinder in mice), from which mEpiSCs are derived [28]. While the transition from the naïve state to the primed state corresponds to the natural developmental progression in the embryo, the primed states can be artificially reverted to the naïve state *in vitro* through ectopic expression of individual factors such as Klf4 [102], Nanog [103], Stat3 [104], Nr5a1 or Nr5a2 [105] in mEpiSCs, and, in hPSC, combinations of Klf4 and Oct4 or Klf2 [106], or Nanog and Klf2 [107]. These observations reveal a remarkable property: only few key nodes are necessary to alter the processing logic of the IRN towards accepting contrasting extrinsic signalling inputs (i.e. LIF and BMP in mESCs versus Activin and Fgf/Erk in mEpiSCs) in order to arrive at the same outcome: self-renewal. This phenomenon could also explain the spontaneous reversion from primed to naïve pluripotency that is

observed at low frequencies in variations of naïve culture conditions for mouse [108-110] and human cells [111], and hybrid (naïve/primed) conditions for human cells [112,113]. In this view, stochastic expression of key genes (most likely an effect of specific epigenetic modulators, facilitating widespread gene expression [105,110,114,115]) trigger the spontaneous rewiring of signalling pathways into the GRN, thereby effecting a change in cell fate. We previously used conventional ensemble proteomic techniques to compare the naïve and primed pluripotent states, and identified DNA methylation and chromatin-regulatory networks as primary differentiators of the two pluripotent states [116]. This type of approach however only provides a snapshot of each state, and future single cell profiling techniques should provide the opportunity to study the dynamics of such stochastic transitions between alternative pluripotent states. In this regard, single cell protein expression data will be of particular interest, as expression variability observed at the transcript level does not necessarily translate to the protein level [73,74].

C *Decay of the IRN: From pluripotency and lineage commitment*

In development, the transient pluripotent state ceases with the formation of the germ layers during gastrulation. The spatial organisation of the peri-implantation embryo contains various localised sources of signalling molecules [117] and it has been demonstrated that such localised extrinsic signals can cause asymmetric division, leading to two daughter cells with different sets of active signalling networks and GRN components [118]. If key GRN components such as Nanog are lost – with accompanying changes in transcription factor binding [119] and chromatin reorganisation [120] – then the self-sustaining properties of core GRN in one daughter may be compromised, leading to destabilisation of the pluripotent state and spontaneous differentiation [29]. Thus, changes in expression of key factors subsequent to cell division can lead to divergent fates in paired daughter cells, via reorganization of intracellular regulatory networks (see Figure 2). Moreover, it is likely that periodic expression of key factors, such as regulated oscillations of cell cycle genes, act as mediators between components of the IRN and hence provide adequate integration of differentiation signals only during specific phases of the cell cycle [121]. Similar mechanisms have been used to explain the increased differentiation propensity of PSCs during the G1 cell cycle phase as well as alternative processing of Activin/Nodal signalling dependent on the levels of cyclin D proteins [122,123]. Single

cell proteomic data on cell cycle status, and the activity of the Smad signalling cascade would be instrumental in further dissecting these processes.

D *Induced pluripotency*

The most dramatic changes to the IRN occur during reprogramming *in vitro*, which results in the establishment of the specific pluripotency IRN in terminally differentiated cells [124]. The principle of cellular reprogramming is to establish pluripotency in somatic cells by transiently inducing the activity of key parts of the self-sustaining pluripotency network, for instance by ectopic overexpression of core factors in direct reprogramming [6-8]. The more components a somatic cell IRN and the pluripotent IRN have in common, the fewer factors are required for this identity-remodelling, since relevant co-regulators are already active (such as ample levels of Sox2 in neuronal progenitor cells, which allow reprogramming using Oct4 only) [31,32]. Moreover, redundancies in the promoter binding activity and mutual transcriptional activation among members of the pluripotency GRN allow the replacement of individual reprogramming factors without affecting the final pluripotent cell identity [125]. Full reprogramming typically takes a number of weeks, and along the way to the pluripotent state, cells transition through a range of intermediate signalling [126] and chromatin states [127,128]. Although the specific trajectory taken depends upon the cells initial identity, it has been shown, using a drug inducible system for comprehensive dedifferentiation [129], that the progeny of the majority of cell types are able to undergo an identity conversion towards pluripotency [130]. While early reports indicated that reprogramming is a stochastic process [130-132], in the case that overexpression of key factors is supplemented with additional knockdown of Mbd3, deterministic reprogramming with synchronised emergence of pluripotent colonies has been observed [110], suggesting that reprogramming progresses through a fixed sequence of remodelling events. Thus, the balance between stochastic and deterministic mechanisms has yet to be fully elucidated, and the precise role of Mbd3 in this context remains a topic of active debate [133]. Single cell based expression data, addressing the sequence of reprogramming events have very recently become available [134-136]. A common observation between these single cell based studies was the apparent initial stochastic expression of individual genes, which subsequently resulted in the consolidation of the endogenous pluripotency IRN through an incompletely understood chain of molecular events. Such stochastic expression would normally be masked as low-level expression

when ensembles of cells are examined. The detailed single cell analysis by Zunder et al. (and Lujan et al.) further highlighted the presence of an intermediate population predisposed for reprogramming and characterised by high expression of transcription factors Oct4 and Klf4 [135] (and membrane proteins CD73, CD49d and CD200 [136]). While not all components of the pluripotency IRN are present in this sub-population, concerted action of multiple members of the GRN eventually leads to the complete induction of the PSC state. Using these data and similar experiments to study the corresponding topological changes to the IRN that occur in individual cells during this transition, will likely inform important questions surrounding the nature of reprogramming and the role of variability in establishing the full pluripotency IRN.

Overall, our understanding of processes A-D will benefit significantly from single cell expression data (some early results are summarised in Table 1). In particular, our understanding of these processes at the protein level within single cells is severely limited, and new methods to accurately profile protein co-expression patterns in individual cells are vitally needed. Additionally, conventional proteomics techniques are also vital to this endeavour, for instance by refining existing IRN connectivity data through high-resolution assessment protein-protein interactions. Such a refined ensemble cell based IRN, which not only includes interactions between core transcription factors but also between auxiliary factors, could redress our current 'hub-heavy' understanding, due to the sampling bias that naturally arises by focussing on well-established factors such as Oct4. These more refined models will provide a complete IRN backbone against which single cell co-expression data can be mapped.

5 Controllability of single cell networks

In summary, many efforts have been made to decipher the components within the IRN that control average cell behaviour [137-139]. The structure of these ensemble networks can explain this population-level behaviour [10], however, due to variability in the expression levels, and, necessarily, cell-to-cell differences in the IRN, the response to the provided stimuli will vary greatly among individual cells, leading, for instance, to impurities in the resulting cell population following 'directed' differentiation, or, as another example, incomplete cellular reprogramming. An intuitive strategy to develop better experimental protocols is to identify important driver nodes, in order to reduce the undesirable by-products that emerge from these processes. These driver nodes are the set of nodes that

must be manipulated in order to control a system completely [140], for instance to steer the IRN of a particular cell state into the desired alternative state. In the absence of a full understanding of the structure and dynamics of the IRN this is a challenging task, although recent developments in the theory of controllability of networks may help [141,142]. One strategy to address this problem is the computational inference of regulatory networks from single cell data. For this purpose, a number of methods are available [143,144]. Individual studies that have already started to adopt similar strategies employed Boolean networks to reconstruct pluripotent IRN from single cell qPCR data [88] or Bayesian network inference in order to extract the sequence of events during reprogramming [134]. We predict that such methods – which combine high-throughput single cell profiling, with advanced network analysis routines – will lead to a more complete understanding of pluripotency in general and the development of better protocols for stem cell maintenance, differentiation and reprogramming in particular.

6 References

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Conflict of Interest Statement

The authors have declared no conflict of interest.

Figure and Table Legends

Figure 1

Pluripotency is regulated by a multiplex network composed of several sub-networks, such as signalling cascades (yellow), metabolic networks (orange), interactions among transcription factors (light green), or genome wide networks (dark green) that are characterised by qualitatively different interactions. However, each sub-network is connected, for instance through the directional flow of information from genome to the proteome, but also via complex feedback interactions between the different “layers”. Collectively, these sub-networks form an integrated regulatory network (IRN). Example interactions are annotated as follows: S, receptor binding of a signalling molecule; +P, phosphorylation; PPI, protein-protein interaction; PDI, protein-DNA interaction; D, dimerization; DDI, 3D organisation of the genome; CDMI, co-factor mediated interaction.

Figure 2

Divergence of sister cell fates upon an asymmetric division. Differential inheritance (e.g. due to low copy number effects) of factors associated with important nodes in the IRN can lead to profound differences in regulatory network structure in the daughter cells. For instance, loss of a central transcription factor (blue) in one of the daughter cells can lead to the dissociation of connectivity between signalling cascades (yellow), transcriptional networks (light green) and genome-wide networks (dark green). In this example, loss of connectivity results in the differential processing of extrinsic stimuli between descendent cells, with one daughter becoming susceptible to differentiation stimuli (red nucleus).

Table 1

Available datasets for reconstructing single cell regulatory networks. Cellular processes of interest are: **A** developmental origins of pluripotency in the inner cell mass; **B** alternative pluripotent states; **C** exit from pluripotency and; **D** establishing pluripotency ectopically in somatic cells.

Tables

Table 1

Method	Process of Interest			
	A	**B**	**C**	**D**
RNA-FISH		[15,71,72]		
qRT-PCR		[15,28,71,87,88]	[29]	[134]
RNA-seq	[101]	[84,145,146]		
CyTOF				[135,136]

Figure 1

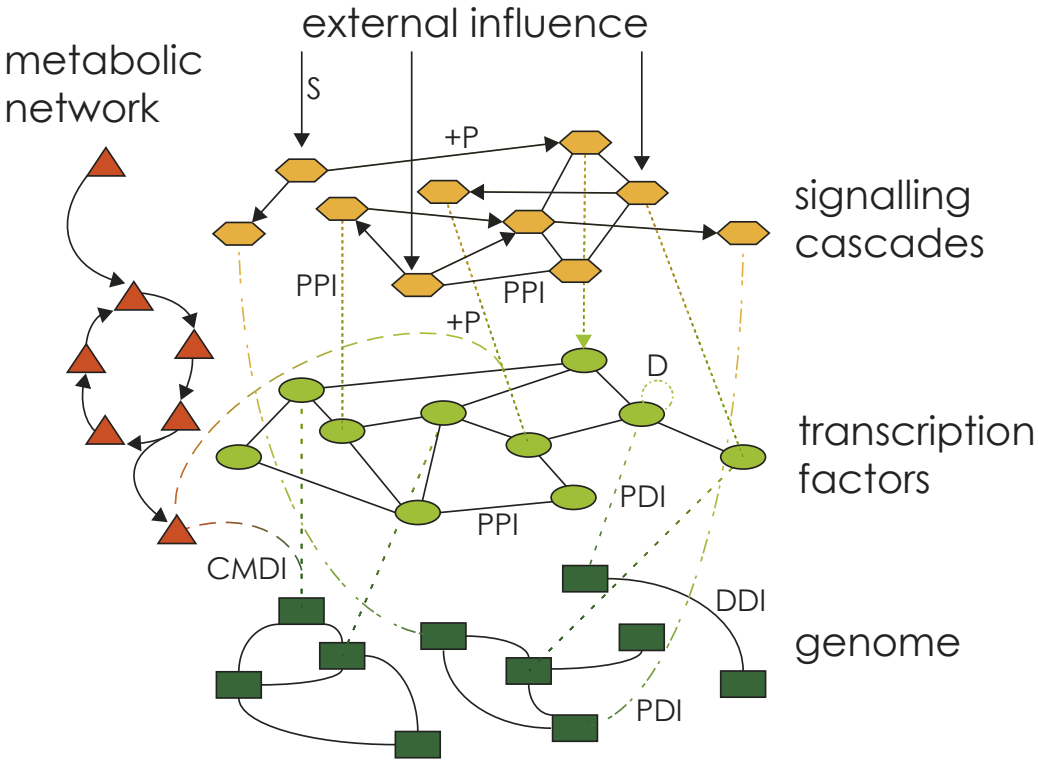

Figure 2