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A Protein Roadmap to Pluripotency and Faithful Reprogramming

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Embryonic stem cells · Pluripotency · Proteomics · Protein interaction network · Reprogramming

Abstract
Embryonic stem (ES) cells are of great interest because of their capability of unlimited self-renewal and multilineage differentiation, thus serving as a potentially unlimited source for tissue replacement in regenerative medicine. ES cells possess factors that maintain and induce pluripotency, as demonstrated by successful reprogramming of somatic cells by fusion with ES cells. Understanding the complex molecular mechanisms underlying ES cell pluripotency should illuminate fundamental properties of stem cells and the process of reprogramming. Proteomics has proven to be a powerful approach to gain insight into key intracellular signals governing ES cell self-renewal and differentiation. We have recently employed a proteomics approach to explore the regulatory protein networks in which Nanog, a fundamental ES cell transcription factor, operates and have constructed the first protein interaction network in mouse ES cells. The network is highly enriched for factors known to be critical in ES cell biology and appears to function as a module for pluripotency. Here we will review current ES cell proteomic studies and provide insights into how a pluripotency protein network will advance recent efforts in cellular reprogramming.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of preimplantation mouse embryos with the unique capacity to proliferate extensively while maintaining pluripotency [Evans and Kaufman, 1981; Martin, 1981]. Mouse ES cells are indispensable for the generation of engineered animal models and their human counterparts have potential in regenerative medicine. An improved understanding of how pluripotency is established and maintained at the molecular level should provide insights into fundamental aspects of stem cells and suggest alternative options for reprogramming somatic cells to an ES-like state.

Much work [Ivanova et al., 2002; Ramalho-Santos et al., 2002] has been done to evaluate the transcriptome of mouse ES cells to identify genes whose expression is enriched in certain stem cell populations. Inherent problems associated with such a transcriptional profiling ap-

Abbreviations used in this paper

ES	embryonic stem
ICM	inner cell mass
iPS	induced pluripotent stem
MS	mass spectrometry

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proach include the following: first, the analysis is obviously limited to genes present on the microarray, and it is possible that there are 'stemness' genes that have not yet been identified and are not represented in the chips used; second, genes that are important for stem cell pluripotency may also be expressed in non-stem cells (for example, *stat3, gp130* or *myc*) and are therefore unlikely to be identified by a comparative microarray approach [Fortunel et al., 2003]. Proteomic analyses of embryonic stemness have been probed using mass spectrometry (MS)-based protein profiling of both undifferentiated and differentiated ES cells [Elliott et al., 2004; Nagano et al., 2005; van Hoof et al., 2006]. These studies resulted in a large proteome dataset for ES cells that serves as a fundamental protein catalogue complementary to the mRNA data. The protein dataset contains many components derived from ES cell-specific and stemness genes defined by the transcriptome analyses, in addition to a number of components that are expressed specifically in ES cells, such as Oct4 and UTF1 [Baharvand et al., 2007]. Moreover, direct analysis of a large number of peptides using two-dimensional liquid chromatography-MS/MS permitted the identification of peptides carrying posttranslational modifications such as Lys acetylation (trimethylation) and site-specific phosphorylation in the ES cell proteome [Nagano et al., 2005].

Several transcription factors, notably the nonclassical homeobox proteins Nanog [Chambers et al., 2003; Mitsui et al., 2003] and Oct4 [Nichols et al., 1998] along with Sox2 [Avilion et al., 2003], are thought to act in combination to maintain ES cell identity. In part, Nanog and Oct4 sustain pluripotency by blocking differentiation to specific fates, and Sox2 stabilizes ES cells in a pluripotent state by maintaining the requisite level of Oct4 expression [Masui et al., 2007]. ES cells are sensitive to the dosage of Nanog [Hatano et al., 2005] and Oct4 [Niwa et al., 2000], such that inappropriately low expression leads to differentiation to primitive endoderm and trophoblast, respectively, whereas overexpression promotes pluripotency in the absence of leukemia inhibitory factor [Chambers et al., 2003] or drives primitive endoderm differentiation [Niwa et al., 2000], respectively. Dosage sensitivity suggests that the ES cell state reflects a balance of multiple transcriptional inputs. In specifying lineages, critical transcription factors cooperate or antagonize each other's function directly, often through protein-protein interaction [Graf et al., 1992; Orkin, 2000]. For example, Oct4 and Cdx2 counteract each other's functions to shift the balance between trophectoderm and ICM fates [Niwa et al., 2005], and Nanog and Gata6 antagonize each other to define epiblast and primitive endoderm lineages [Chazaud et al., 2006]. The ES cell state, therefore, is likely to be maintained by the continuous and direct interplay of multiple nuclear factors, acting in cooperative and antagonistic modes.

Putative transcriptional targets of Nanog, Oct4 and Sox2 have been identified by assessing genome-wide occupancy of mouse [Loh et al., 2006] and human [Boyer et al., 2005] ES cell chromatin. Nanog binds up to ~10% of expressed genes in ES cells, whereas Oct4 and Sox2 occupy fewer genes. Many genes exhibit cooccupancy for these factors in all combinations. Genes encoding lineage-specific transcription factors are highly represented among the bound genes, and presumably direct targets of repression [Boyer et al., 2005]. While genome-wide occupancy studies implicate auto- and cross-regulatory circuits, they focus on a limited number of core factors and fail to reveal how they act in a protein network, or how many other critical regulators need to be considered. Recently, using an integrated functional genomics approach, Ivanova et al. [2006] have demonstrated that *Esrrb*, *Tbx3* and *Tcl1*, as well as previously identified *Nanog*, *Oct4* and *Sox2*, are required for efficient self-renewal of ES cells in vitro. Downregulation of each gene induces differentiation of ES cells along specific lineages.

Given the intricate network apparent from the consideration of just a handful of these components, one may ask how many other key pluripotency factors with properties overlapping those of Oct4, Sox2 and Nanog remain to be identified. In this regard, the proteomic study of ES cells will be important and promises to bear fruit [Baharvand et al., 2007]. For example, large-scale quantitative surveys of protein expression, modification and protein interactions will help uncover and further define signaling pathways and molecular mechanisms involved in the maintenance of the undifferentiated state and initial loss of pluripotency during differentiation. Ultimately, they will serve to elucidate the dynamic aspects of functional protein networks that play critical roles in ES cell pluripotency.

A Protein Interaction Network for Pluripotency

Defining the functional relationships between proteins is critical for understanding virtually all aspects of cell biology. Since protein complexes mediate the majority of cellular processes, large-scale identification of protein complexes has provided an important step towards this goal [Collins et al., 2007]. To understand how pluri-

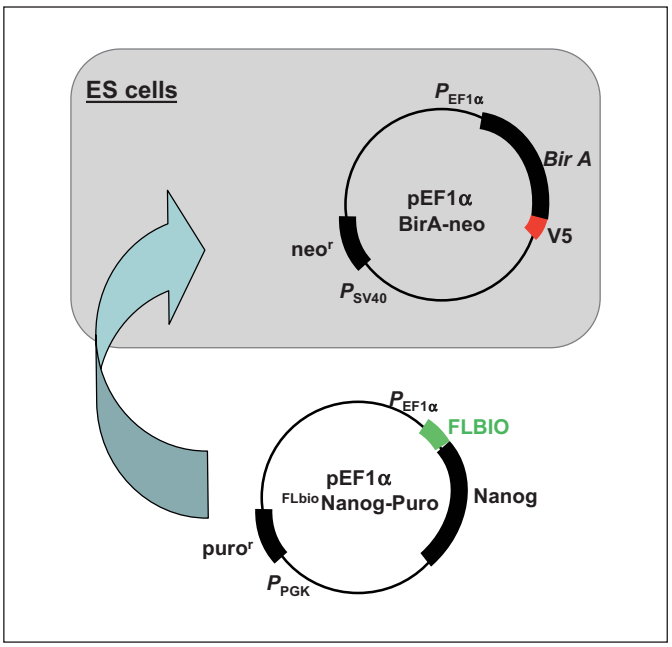


Fig. 1. Establishment of a biotinylation system in ES cells. A stable ES cell line expressing the bacterial BirA enzyme was first established by transfection with a BirA-expressing plasmid bearing the neomycin resistance (neo^r) gene followed by G418 selection. A second plasmid containing Nanog cDNA with an N-terminal FLAG biotag (FLBIO) and a puromycin resistance (puro^r) gene was introduced and cells were selected with puromycin. The resulting stable lines are resistant to both G418 and puromycin and express FLAG-tagged, biotinylated Nanog that can be immunoprecipitated by anti-FLAG antibodies and streptavidin beads.

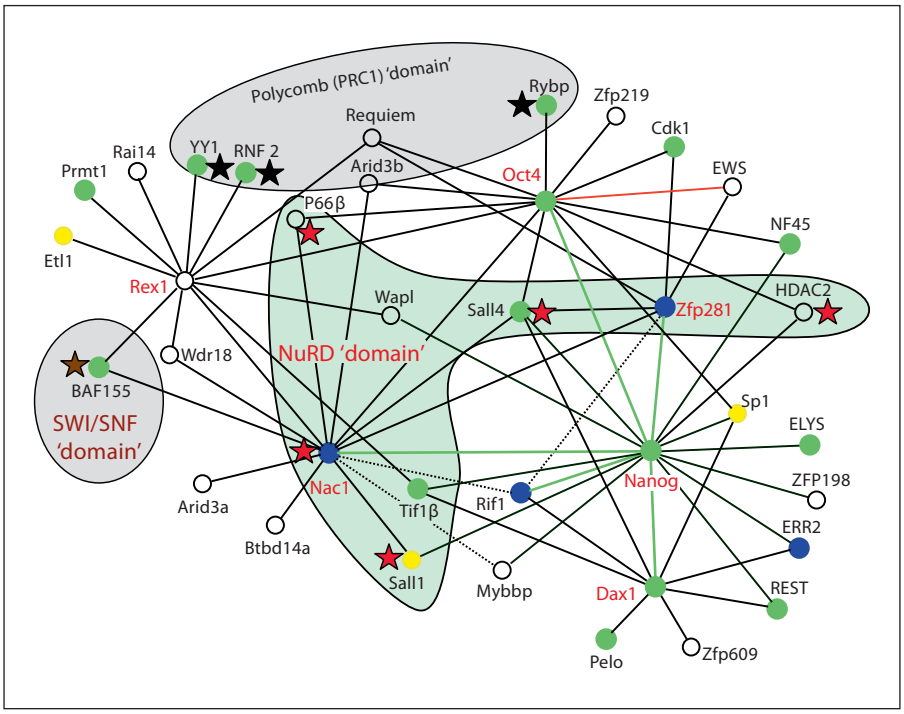


Fig. 2. A protein interaction network in mouse ES cells. Proteins with red labels are tagged baits for affinity purification. Green and red lines indicate confirmed interactions by coimmunoprecipitation or published data. Dotted lines indicate potential association. Green circles indicate proteins whose knockout results in defects in proliferation and/or survival of the ICM or other aspects of early development; blue circles indicate proteins whose reduction by RNAi (or shRNA) results in defects in self-renewal and/or differentiation of ES cells; yellow circles are proteins whose knockout results in later developmental defects; white circles denote proteins for which no loss-of-function data are available. Also indicated within the network are 3 major chromatin-modifying complexes whose components are marked with black stars (polycomb 'domain'), red stars (NuRD 'domain') and a brown star (SWI/SNF 'domain'), respectively.

potency is programmed and maintained in ES cells, we have utilized a proteomic approach to isolate protein complexes and we constructed a protein interaction network surrounding the pluripotency factor Nanog [Wang et al., 2006].

The approach takes advantage of the extraordinary affinity of streptavidin for biotin, and obviates reliance on antibodies of inherently lower affinity for purification. It has been reported that single-step streptavidin capture of tagged transcription factors is sufficient to isolate spe-

cifically associated proteins with minimal nonspecific contamination [de Boer et al., 2003]. In this system, BirA-expressing ES cells serve as a recipient for other tagged cDNAs. A construct bearing the pluripotency factor Nanog with a FLAG tag as well as a peptide tag that serves as a substrate for in vivo biotinylation was expressed in ES cells (fig. 1). The tagged protein was recovered from nuclear extracts with streptavidin beads together with its potential interacting partners. For tandem purification, the nuclear extracts were first subjected to immunoprecipitation with anti-FLAG antibodies and the recovered protein complexes were further purified by streptavidin beads. Protein complexes recovered from either one-step streptavidin or tandem purification were subjected to microsequencing by MS. Not surprisingly, many of the candidates identified were other transcription factors or components of transcriptional complexes, some of which had already been associated with ES cell functions in previous studies. A number of novel (for example, Dax1, Nac1 and Zfp281) and known (for example, Oct4) critical factors were validated, both physically and functionally, for association with the bait Nanog and were used (together with another well-known ES marker, Rex1) for purification of a second tier of complexes. The resulting dataset was used to generate a complex network of interacting proteins that is concisely depicted in figure 2. This iterative, bottom-up strategy reveals a tight, highly interconnected protein network greatly enriched in nuclear factors individually required for maintenance of ES cell properties and coregulated on ES cell differentiation [Wang et al., 2006].

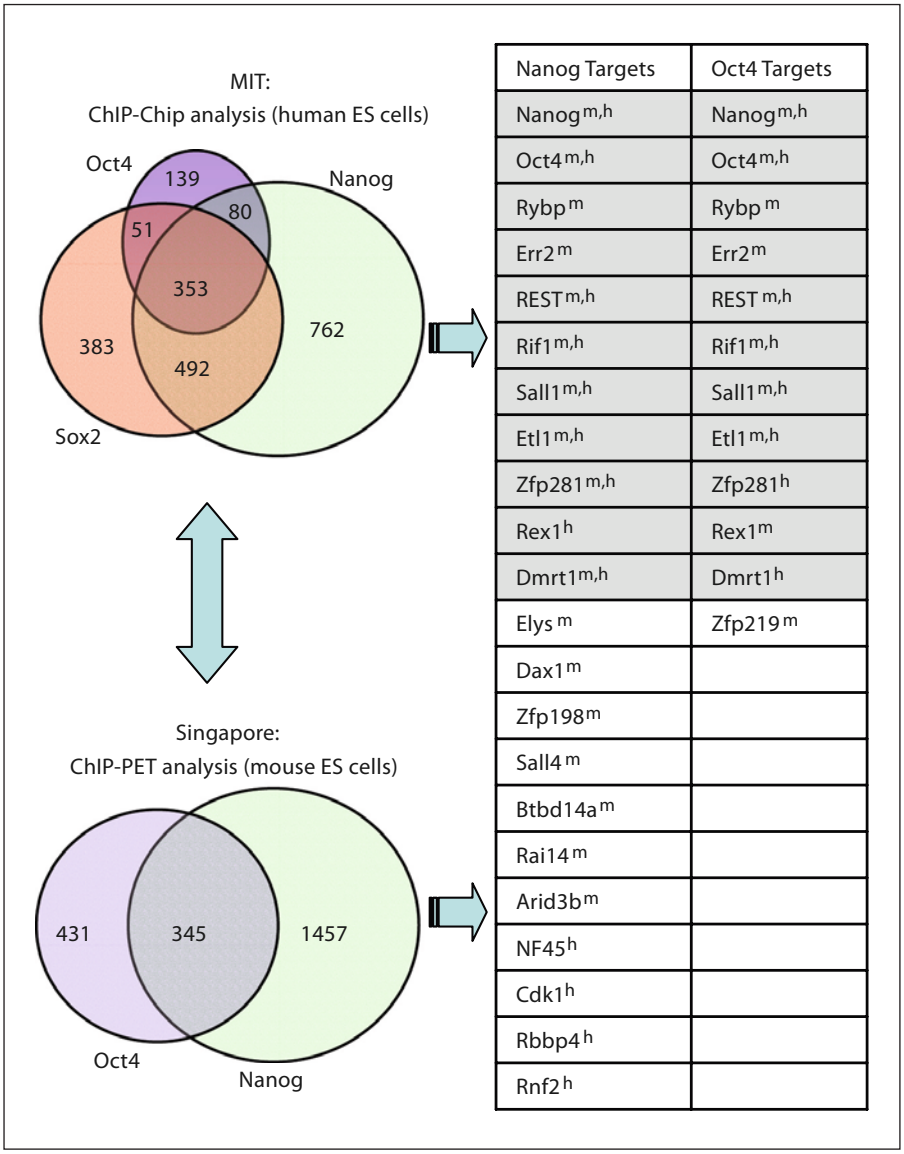
An important finding from our study is that over 80% of the network proteins with available loss of function data are essential for, or associated with, early development and/or ES cell properties. The knockout of several network proteins including Prmt1, YY1, Rnf2, BAF155, Rybp, Oct4, Cdk1, NF45, Sall4, Elys, Tif1β, Pelo, Dax1 and REST is known to result in defects in proliferation and/or survival of the ICM or other aspects of early development [Wang et al., 2006]. The knockdown of Err2 (also known as Esrrb [Loh et al., 2006]), Rif1 [Loh et al., 2006], Nac1 and Zfp281 [Wang et al., 2006] has been shown to result in defects in self-renewal and/or differentiation of ES cells.

The second striking feature of this network is that more than half of the proteins function as both targets and effectors (fig. 3), suggesting that it represents a tightly controlled functional module. Given the numerous gene targets of the proteins within the network, it is likely that subsets of targets are regulated by different complexes. For example, *Gata6* promoter sequences are bound by Nac1 and Zfp281 in addition to Nanog [Wang et al., 2006]. Presumably, proteins within the network, such as Dax1 and Sall4, also bind other targets with Nanog, Oct4 and/or Sox2. Accordingly, identification of the target genes of these newly identified pluripotency factors will reveal that the combinatorial control of target genes is far greater in complexity than suggested by previous chromatin occupancy studies [Boyer et al., 2005; Loh et al., 2006].

A third feature of the network is that it is linked to several different cofactor pathways largely involved in mediating transcriptional repression. These include the histone deacetylase NuRD (P66β and HDAC2) and its related components (Sall1, Sall4 and Nac1), polycomb group (YY1, Rnf2 and Rybp) and SWI/SNF chromatin remodeling (BAF155) complexes (fig. 2). Of particular interest, Rex1 and Oct4 are associated with polycomb components, whereas Nanog and its closest partners (Dax1, Sall4 and Nac1) are linked to HDAC/NuRD chromatin remodeling complexes. Polycomb complexes have been shown to directly repress developmental regulators in murine ES cells [Boyer et al., 2006], and NuRD is required for ES cell pluripotency and represents a potential link between maintaining the undifferentiated state and the capacity to differentiate [Crook et al., 2006; Kaji et al., 2006]. The involvement of multiple corepressor complexes rather than reliance on a single pathway may provide a mechanism for regulating different sets of target genes and/or a fail-safe mechanism to prevent differentiation along different lineages, which is a requisite for maintaining the pluripotent state.

Implication of the Protein Network in Reprogramming

Generation of pluripotent stem cells directly from somatic tissue is one of the ultimate goals in regenerative medicine. In mouse models, there are currently 4 methods that have been reported to induce pluripotency [Hochedlinger and Jaenisch, 2006; Yamanaka, 2007]. The first 2 reprogramming regimens, nuclear transfer to oocytes [Hochedlinger and Jaenisch, 2002; Li et al., 2003; Eggan et al., 2004; Hochedlinger et al., 2004; Li et al., 2004] and fusion with ES cells [Tada et al., 2001; Cowan et al., 2005; Silva et al., 2006] are those most extensively studied. The recent demonstration of developmental reprogramming after chromosome transfer into mitotic mouse zygotes [Egli et al., 2007] and enhanced transfer of



Nanog Targets	Oct4 Targets
Nanog^{m,h}	Nanog^{m,h}
Oct4^{m,h}	Oct4^{m,h}
Rybp^{m}	Rybp^{m}
Err2^{m}	Err2^{m}
REST^{m,h}	REST^{m,h}
Rif1^{m,h}	Rif1^{m,h}
Sall1^{m,h}	Sall1^{m,h}
Etl1^{m,h}	Etl1^{m,h}
Zfp281^{m,h}	Zfp281^{h}
Rex1^{h}	Rex1^{m}
Dmrt1^{m,h}	Dmrt1^{h}
Elys^{m}	Zfp219^{m}
Dax1^{m}	
Zfp198^{m}	
Sall4^{m}	
Btbd14a^{m}	
Rai14^{m}	
Arid3b^{m}	
NF45^{h}	
Cdk1^{h}	
Rbbp4^{h}	
Rnf2^{h}	

Fig. 3. Targets of pluripotency factors are highly represented in the network. The left panels show the targets of Nanog, Oct4 and Sox2 in human ES cells [Boyer et al., 2005] and the targets of Nanog and Oct4 in mouse ES cells [Loh et al., 2006]. The right table summarizes the targets of Nanog and Oct4 from the 2 ChIP studies (left) that are present in the protein network (fig. 2). $X^{m,h}$ indicates that gene X identified as targets of Nanog and/or Oct4 in both mouse (m) and human (h) ES cells. The targets of both Nanog and Oct4 are shaded.

pluripotency by Nanog after cell fusion provide exciting new avenues to improve and perfect these types of approach. A third method appears to involve spontaneous reprogramming by culture. This includes generation of multipotent germline stem cells from neonate mouse testes [Kanatsu-Shinohara et al., 2004], multipotent adult germline stem cells from adult mouse testes [Guan et al., 2006] and parthenogenetic ES cells [Kim et al., 2007].

Perhaps the most exciting progress in the field was made by the introduction of the fourth reprogramming regimen by Yamanaka and associates last year [Takahashi and Yamanaka, 2006]. Following the lead from fusion-based reprogramming (that is, the ES cell phenotype is dominant to that of somatic cells), they reasoned that ES cells have factors that induce pluripotency and also play important roles in the maintenance of pluripotency. Through systematic screening of 24 major ES cell factors previously identified by digital display [Mitsui et al., 2003], they narrowed these down to a set of 4 factors (Oct4/Sox2/c-Myc/Klf4) that directly reprogram fibroblast cells into an ES cell-like state upon retroviral transfer. Although the induced pluripotent stem (iPS) cells were not identical to ES cells in terms of global gene expression, epigenetic modifications and germline transmissibility, this iPS cell system has been further improved dramatically in several recent reports [Maherali et al., 2007; Okita et al., 2007;

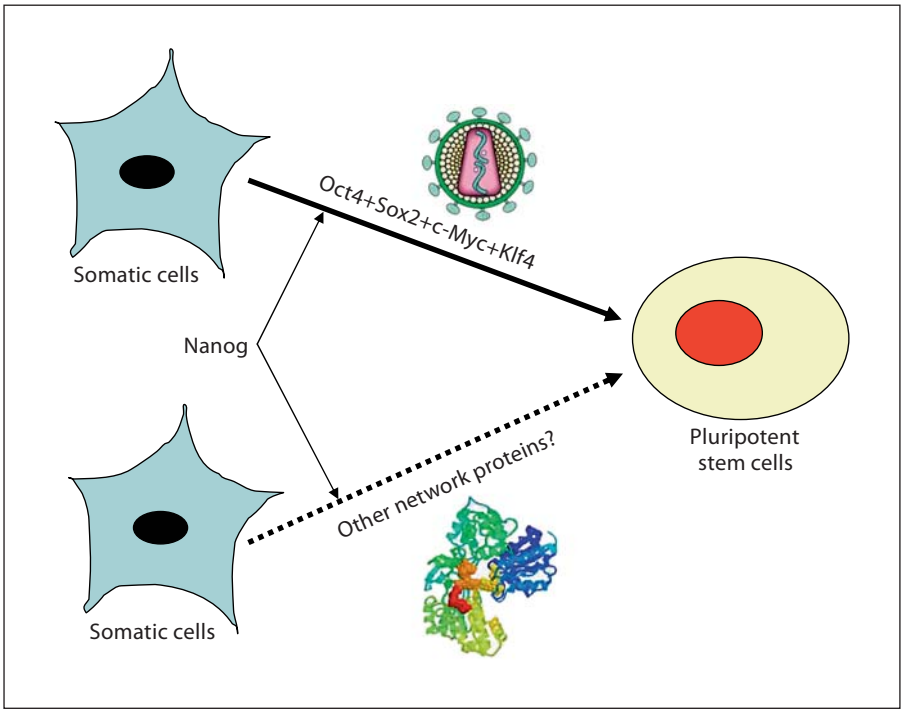


Fig. 4. Alternative pathways for direct reprogramming by defined factors. Current studies indicate that 4 factors (Oct4/Sox2/c-Myc/Klf4) can reprogram somatic cells to pluripotent stem cells by retroviral transduction and selection with Nanog. To avoid potential insertional mutagenesis caused by retroviral transduction and oncogenic c-Myc reactivation associated with the current methodology, an alternative way to reprogram somatic cells by direct protein transduction or transient expression of selected pluripotency network factor(s) is proposed.

Wernig et al., 2007]. The modified system utilizes the same quartet of factors (Oct4/Sox2/c-Myc/Klf4) but with a more stringent selection marker Nanog, compared with the previous Fbx15 marker, which is dispensable for pluripotency. This fourth reprogramming strategy eliminates the need for oocytes and destruction of embryos required by nuclear transfer, maintains the diploid chromosome and genome stability that are compromised in fusion due to tetraploidy, and does not have the problems imposed by male- or female-specific imprinting during culturing of spontaneous reprogrammed multipotent adult germline stem or parthenogenetic ES cells. Needless to say, this methodology also needs further optimization/improvement to increase the efficiency and minimize the potential oncogenic properties of retroviral insertion and c-Myc reactivation in the reprogrammed cells.

The absence of Nanog in the quartet of factors came as a surprise, but is not without a plausible explanation. First, both the Oct4/Sox2 duo and Klf4 have been shown directly [Kuroda et al., 2005; Rodda et al., 2005] or indirectly [Lin et al., 2005; Rowland et al., 2005] to activate Nanog gene expression. Second, the demonstration that Nanog selection allows the generation of high-quality iPS cells that are close ES cell counterparts [Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007] indicates that Nanog is a major determinant of quality in cellular pluripotency. Third, Nanog has been demonstrated to be a facilitator in fusion-based cellular reprogramming [Silva et al., 2006]. Taken together, Nanog likely acts as a master transcriptional organizer that entrains the hierarchy of pluripotent gene expression after erasure of the differentiated epigenome, although further work needs to be performed to show that this is indeed the case. Nevertheless, our work of defining components of a protein network surrounding Nanog that maintains ES cell pluripotency is directly relevant to cellular reprogramming.

How Nanog and the quartet of factors (Oct4/Sox2/c-Myc/Klf4) function in the reprogramming process is not well understood. Several lines of evidence suggest that they do not operate alone but act in conjunction with additional ES cell machinery. First, Oct4 and Sox2 in addition to Nanog have been shown to function as core transcription factors in maintaining pluripotency [Boyer et al., 2005; Loh et al., 2006]. Second, the variability of germline competence among Nanog iPS cell clones [Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007] suggests the existence of other important determinants in addition to Nanog for full/faithful reprogramming. Third, the low efficiency of Nanog iPS cell induction and the reliance on retroviral transduction in the system [Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007] suggest other fortuitous gene activation by retrovi-

ral integration might be required in addition to the 4 factors for successful reprogramming.

Our protein interaction network indicates that Nanog may orchestrate the activity of a number of other transcription factors (such as Oct4, Sall4, Zfp281) in conjunction with chromatin remodeling factors, such as HDAC/NuRD and polycomb complexes [Wang et al., 2006]. Although the 4 factors Oct4, Sox2, c-Myc and Klf4 were not all included within the network we constructed, we believe that our ongoing and future efforts in identifying interacting partners of these 4 factors will extend the network and be helpful in illuminating their mode of action in reprogramming. A fully expanded protein interaction network will lead us to a better understanding of the reprogramming process and help explore alternative ways of direct cellular reprogramming through either transduction of genes (or proteins) or inhibition of specific proteins beyond these 4 factors (fig. 4).

Concluding Remarks

Extensive analyses of numerous human and mouse ES cell lines have shown generic similarities and intrinsic differences at both the transcriptional and functional levels. Comprehensive proteome analyses have now produced a wealth of data identifying proteins and mechanisms involved in stem cell proliferation and differentiation [Baharvand et al., 2007]. Applying proteomics to investigate the programs that control self-renewal, differentiation and reprogramming will provide valuable insights into how the factors involved induce differentiation of stem cells to specific lineages, and how they reprogram differentiated cells back to an embryonic state.

Starting from a central regulator of transcription in ES cells, Nanog, followed by iterative tagging and purification of Nanog-associated proteins, we have developed a protein interaction network that is remarkable for its concentration of proteins individually vital to maintenance of pluripotency [Wang et al., 2006]. This mini interactome will be further developed to include the 4 reprogramming factors as well as other critical factors to be identified in the future. This expanded network will provide a framework for developing approaches to directed reprogramming of more differentiated cells to an ES cell state.

Whereas proteins of the network cooperate to maintain pluripotency, their interdependency renders the cell susceptible to rapid loss of pluripotency upon the down-regulation, or inactivation, of any of its multiple components. These properties offer the possibility that cellular reprogramming to an ES cell state might be achieved by a limited number of carefully chosen proteins within the network coupled with direct transduction or transient expression with higher efficiency and without the need for retroviruses (fig. 4). A protein roadmap to pluripotency will guide us through this long journey to finally achieve faithful reprogramming without recourse to nuclear transfer or cell fusion.

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