Gating pluripotency via nuclear pores

Jiping Yang1*, Ning Cai1*, Fei Yi2*, Guang-Hui Liu1,3,4, Jing Qu1, and Juan Carlos Izpisua Belmonte2,5

1National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
2Gene Expression Laboratory, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
3State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
4Beijing Institute for Brain Disorders, Beijing, China
5Center for Regenerative Medicine in Barcelona, Dr. Aiguader 88, 08003 Barcelona, Spain

In recent years, growing evidence has pointed to the interesting idea that pluripotency might be regulated by a nuclear-pore-coordinated network that controls the level of pluripotency factors in the nucleus. A thorough understanding of this process might improve our comprehension of cell pluripotency and differentiation during embryogenesis, as well as aiding the development of novel models for studying human diseases.

Introduction

Nuclear-pore complexes (NPCs) are multi-protein channels that are embedded in the nuclear envelope and are comprised of approximately 30 different nucleoporins (Nups). NPCs serve as the primary conduit for communication between the nucleus and cytoplasm. Nucleocytoplasmic trafficking via NPCs is a complex yet precise mechanism that is mediated by many transport factors, such as importins, exportins, the GTP-binding nuclear protein Ran, and Nups (Box 1). In addition to their primary function in nucleocytoplasmic trafficking, nuclear pores are involved in a number of important cellular processes, including gene regulation and chromatin organization [1]. Recently, an emerging role of nuclear pores in regulating pluripotency and differentiation has been revealed [2–7].

Regulating nuclear transport of pluripotency factors via post-translational modifications

Pluripotency factors such as POU class 5 homeobox 1 (POU5F1, also known as Oct4), SRY(sex determining region Y)-box 2 (Sox2), and Nanog homeobox (Nanog) are transcription factors that are required for the maintenance of pluripotency and suppression of lineage-specific differentiation in pluripotent stem cells (PSCs), including embryonic stem cells (ESCs). In order to perform their functions, these factors need to access the nucleus, which is gated by nuclear pores. Recent studies have shown that post-translational modifications of pluripotency factors influence their nuclear trafficking through nuclear pores and subsequently regulate cell-fate determination.

Oct4

Lin et al. reported that the phosphorylation of Oct4 at T235 by an Akt protein kinase promoted self-renewal and survival of embryonal carcinoma cells (ECCs) — the malignant counterpart of ESCs [2]. Akt-mediated phosphorylation prevented Oct4 from being degraded and increased the stability of intracellular Oct4. Stabilized Oct4 exhibited predominant nuclear localization (Figure 1). However, when cells were treated with MG132, a proteasome inhibitor, the Oct4-T235A mutant, which cannot be phosphorylated by Akt, accumulated more in the cytoplasm as compared to wild-type Oct4 or the Oct4-T235D phosphorylation mimic. These observations indicate that phosphorylated Oct4 is exported from the nucleus and degraded in the cytoplasm. Importantly, treatment with a specific Akt inhibitor, Akti-1/2, together with MG132 markedly accelerated the cytoplasmic accumulation of Oct4, a phenomenon that could be partially reversed by the nuclear export inhibitor leptomycin B (LMB) [2]. Therefore, Akt-mediated phosphorylation stabilizes Oct4 in the nucleus and prevents it from being exported from the nucleus via nuclear pores, a mechanism that presumably involves the export factor CRM1 (also known as exportin-1). Although most of the previous evidence suggests that the nuclear export of Oct4 is an active process that is regulated by CRM1, a recent study proposed an alternative model, claiming that most Oct4 is exported from the nucleus by passive diffusion [8]. Despite the possible controversy, it remains to be elucidated how different mechanisms coordinately regulate nuclear export of Oct4. In addition, it has been suggested that Oct4 requires Akt-mediated phosphorylation to maintain the association with other pluripotency factors, such as Sox2 and Kruppel-like factor 4 (KLF4) [2]. By regulating the binding affinity of Oct4 to Sox2 at gene promoters, Akt-mediated phosphorylation might directly increase the transcription of other pluripotency genes, such as Nanog [2]. Thus, Akt-mediated phosphorylation causes both nuclear accumulation of Oct4 and increased Oct4 transcriptional activity by promoting its partnering with Sox2, and together these processes contribute to the self-renewal and survival of ECCs [2].

The phosphorylation of Oct4 at different sites might also determine its nucleocytoplasmic redistribution or
Box 1. Nucleocytoplasmic trafficking mechanism

Nucleocytoplasmic trafficking via nuclear-pore complexes (NPCs) includes energy-independent passive diffusion and energy-dependent active transport. Small molecules undergo passive diffusion to enter the nucleus without regulation and use of energy. However, macromolecules, such as most transcription factors, histones, mRNA, or non-coding RNAs, are actively transported, and these processes are mediated by transport factors, such as importins, exportins, the GTP-binding nuclear protein Ran, and nucleoporins. Importins are the most important participants in nuclear importing. These proteins are able to recognize the specific amino acid sequences — nuclear localization signals (NLSs) — on cargos. Importins, which bind cargos in the cytoplasm, interact with NPCs to get through the channel. As long as the cargos enter the nucleus, importins disassociate from the cargos and return to the cytoplasm. Importin-α and importin-β are the best-known importins and have been shown to import many proteins, such as POU class 5 homeobox 1 (POUSF1, also known as Oct4) and SRY(sex determining region Y)-box 2 (SOX2). Conversely, exportins bind to the nuclear export signals (NESs) of cargos and exit from the nucleus through the pores to the cytoplasm, where the exportin–cargo complexes disassemble. The export factor CRM1, also known as exportin-1, exports cargos with leucine-rich NESs; exportin-5 specifically mediates the exit of microRNA. Ran proteins are responsible for the supply of energy. Ran hydrolyzes GTP to GDP and produces energy for nuclear transport. Nucleocytoplasmic trafficking precisely controls the localization of macromolecules, which in turn might regulate processes related to cell pluripotency and differentiation.

degradation. Ferro et al. showed that Oct4 was phosphorylated at serines 105 and 107 and became mainly cytoplasmic after the differentiation of dental pulp stem cells (DPSCs) into osteoblastic, hepatic, myocytic, and neural lineages. Conversely, Oct4 was localized not only in the cytoplasm but also in the nucleus in undifferentiated DPSCs [3]. Furthermore, phosphorylation-motif analysis showed that serine 105 of Oct4 was a putative phosphorylation site for casein kinase II (CK-II), which has been previously proven to have a central role in facilitating phosphorylation of nuclear proteins and mediating their nucleocytoplasmic shuttling via a CRM1-dependent mechanism (Figure 1). More recently, the same group demonstrated that Oct4 was phosphorylated at serine 111 by the Mitogen-activated protein kinase kinase/Extracellular signal-regulated kinases 1 (also known as MEK/ERK1) signaling pathway. Instead of increasing the nuclear localization and stabilizing the protein, this site-specific phosphorylation of Oct4 triggered its CRM1-mediated nuclear export and ubiquitin-proteasomal degradation [9] (Figure 1). Although the essential role of Oct4 in governing pluripotency is well established, a recent study has uncovered differential roles for Oct4 in maintaining or reconstructing pluripotency through Oct4 nucleocytoplasmic shuttling. Data demonstrated that transient retention of Oct4 in the nucleus was sufficient to maintain an undifferentiated state in ESCs, whereas the long-term intranuclear localization of Oct4 was critical for cell reprogramming-associated chromatin remodeling and epigenetic modifications [8]. This finding revealed an underappreciated role of Oct4 nucleocytoplasmic shuttling in cell-fate determination. However, the underlying mechanism remains to be elucidated.

Sox2
Another pluripotency factor, Sox2, has also been proposed to be governed by nuclear-pore-mediated regulation, which might affect cell-fate determination. For example, protein acetylation had been identified to have a direct effect on nuclear transport of Sox2 [10]. Sox2 had been shown to
translocate from the cytoplasm into the nucleus during the early stages of embryogenesis, indicating that subcellular shuttling might be important for its activity [10]. Baltus et al. discovered that CREB-binding protein (CBP/p300 acetylated lysine 75 on the nuclear export signal (NES) of Sox2, which mediated the nuclear export and subsequent proteasomal degradation of Sox2 in the cytoplasm (Figure 1).

Other pluripotency-related factors

The link between post-translational modifications and nucleocytoplasmic trafficking has been well studied for Oct4 and Sox2 owing to their importance in maintaining pluripotency. However, the regulated nucleocytoplasmic transport of many other pluripotency-related factors — such as Klf4 and cMyc, as well as the downstream targets of Oct4 and Sox2, which are also involved in somatic cell reprogramming — has not yet been studied to the same depth. In addition, the nuclear transport of histones and basal transcriptional machinery is seldom reported in the context of pluripotency regulation. Therefore, many gaps in our knowledge remain to be filled before we have a comprehensive understanding of how nuclear pores regulate pluripotency through post-translational modifications of transcription factors.

Mechanisms of nuclear transport

Nuclear localization signal, nuclear export signal, and nuclear localization

As mentioned above, the NES and nuclear localization signal (NLS) sequences within transcription factors are critical for the nuclear transport machinery and their nuclear localization. So far, many groups have identified NESs or NLSs within Oct4, Sox2, and Nanog. The presence of both NES and NLS sequences in pluripotency factors suggests that dynamic nuclear transport might constitute a new layer of regulation in pluripotency maintenance and cell differentiation. For example, Oct4 has been found to have a classic NLS localized at amino acid residues 195–199 within the N-terminal portion of the homeobox domain (HD) [11]. Mutations in this region prohibited the protein from entering the nucleus via nuclear pores and thus induced the differentiation of P19 cells (mouse ECCs) into trophoblast-like giant cells.

Sox2 has also been reported to contain a leucine-rich NES and two functional NLSs within the high mobility group (HMG) domain [12]. Li et al. demonstrated that mutations within these two NLSs of Sox2 induced the differentiation of ESCs into the trophobectoderm lineage.

In addition, one putative NES and two NLSs have been identified in Nanog. Do et al. revealed that the HD of Nanog is essential for its nuclear localization, and they further validated two basic NLSs within the N terminus and C terminus of the HD [13]. Moreover, the same group also identified a putative NES within the HD in which mutations prevented Nanog from exporting [14]. The treatment of the CRM1-specific nuclear export inhibitor LMB blocked the export of Nanog, suggesting that the export of Nanog might be mediated by the recognition of CRM1 and NES. Nevertheless, the impact of NES mutations on PSCs has not yet been specifically discussed.

These studies indicate that nuclear localization of pluripotency factors is critical for the maintenance of ESCs and serves as a molecular switch for cell differentiation.

Importins and exportins

The trafficking of pluripotency factors through nuclear pores is mediated by different importins and exportins, which recognize cargos by specific NLS or NES sequences. Importin-α1 has been proven to interact with Oct4 and mediate its nuclear localization. Sox2 has also been reported to import into the nucleus by three parallel pathways, through exportin-4, importin-9, or importin-β/7 heterodimer in mouse ESCs [15]. Interestingly, Yasuhara et al. found that the expression of different subtypes of importin-α changed the nuclear import of a specific set of transcription factors, including Oct4, Sox2 and Brn2, a neural lineage-specific transcription factor, during neural differentiation of mouse ESCs [4]. Expression of importin-α1 was high in undifferentiated ESCs but shut down rapidly during induced neural differentiation, whereas expression of importin-α3 and importin-α5 gradually increased to high levels in differentiated cells. Based on further investigations using in vitro nuclear-transport assays, they proposed that in undifferentiated ESCs, Oct4 is imported into the nucleus by importin-α1/β, whereas Sox2 is imported by importin-β alone (Figure 2). When neural differentiation occurs, downregulation of importin-α1 and upregulation of importin-α3/5 are coordinated with increased nuclear import of Sox2 and a decreased import of Oct4 to initiate differentiation of ESCs. Meanwhile, Brn2 is imported into the nucleus in an importin-α5/β-dependent manner, leading to directed neural differentiation (Figure 2). Although importin-α5 seems to be essential for the neural differentiation of mouse ESCs, a report showed that importin-α5-null mice had normal brain development [16]. Moreover, a significant upregulation of importin-α4 was detected in the brain of importin-α5-null mice. Thus, it is likely that importin-α4 could compensate for the loss of importin-α5 to ensure normal differentiation. Furthermore, Young et al. demonstrated that importin-α2-mediated Oct4 nuclear import directly maintained the pluripotency of mouse ESCs. However, given that importin-α4 was upregulated in differentiated ESCs and overexpression of importin-α4 could dramatically decrease the Oct4 protein levels, it is clear that importin-α2 and importin-α4 have contradictory roles in determining the cell fate of ESCs [17]. Perez-Terzic et al. also discovered that during the differentiation of mouse ESCs into cardiomyocytes, the expression of nuclear transport factors, including importins, exportins, transportins, nucleoporins, and Ran-related factors, was downregulated globally, whereas the expression of transportin-2 and Ran-binding protein 6 was upregulated [18]. These changes were proposed to correlate with the nuclear entry of cardiac transcription factors such as myocyte enhancer factor 2C (Mef2C), NK2 homeobox 5 (Nkx2.5), and GATA binding protein 4 (Gata4).

Additionally, exportin-5 participates in precursor microRNA (pre-miRNA) translocation during miRNA maturation [19]. Some miRNAs have been linked to key regulators of stem cells. For example, miR-21 targets the pluripotency factors Nanog and Sox2, and thus specifically suppresses
the self-renewal of ESCs [19]. Importantly, the maturation of miRNAs depends on exporting pre-miRNA from the nucleoplasm into the cytoplasm. Knocking down exportin-5 blocked the export of pre-miRNA 30 [20], whereas overexpression of exportin-5 enhanced RNA interference mediated by miRNAs [21]. It is suggested that exportin-5 might somehow be able to regulate pluripotency factors indirectly by controlling certain miRNAs. Besides exportin-5, it is unclear whether there are any other specific exportins that mediate the transportation of miRNA. All these observations suggest that importins and exportins act as important regulators of pluripotency and cell differentiation by controlling key factors either directly or indirectly.

Taken together, it is conceivable that a nuclear-pore-dependent protein-shuttling mechanism is controlling the nuclear levels of crucial transcription factors involved in pluripotency maintenance and differentiation in a precisely tuned manner.

**Nuclear-pore components**

**Nup133**

The involvement of individual Nups in regulating pluripotency and differentiation is another aspect that needs to be discussed. Certain studies have shown that several nuclear-pore components exhibit cell-type-specific expression and that NPC compositions vary between stem cells and differentiated cells. Lupu et al. found that the pluripotency of mouse ESCs was regulated by changes in peripheral NPC composition [6] (Figure 3). They showed that the depletion of Nup133 in mouse ESCs caused an abnormal pluripotent state and disrupted differentiation into neural lineages. Furthermore, they even demonstrated that the loss of a single Nup133 allele affected the efficient development of ESCs into neurons. These observations clearly indicate that nuclear-pore components might be critical for cell differentiation.

**Nup210**

D'Angelo et al. demonstrated that Nup210 is another NPC component that is critical for the differentiation of ESCs towards specific lineages [5]. They showed that the expression of Nup210 was absent in ESCs but increased during myogenesis or neural differentiation (Figure 3). Knockdown of Nup210 blocked neural differentiation but had no effect on ESC survival, whereas overexpression of Nup210 accelerated the differentiation process. Interestingly, even though Nup210 is an important transmembrane protein, deficiency in Nup210 did not compromise nucleocytoplasmic transport, but it did contribute to the dysregulated expression of several differentiation-associated genes. These observations further suggested a role of nuclear-pore components in regulating ESC functions [5].

**Nup98**

Nup98, which acts as a docking-site Nup in nucleocytoplasmic trafficking, was recently found to be important for human ESC differentiation [7]. Through a genome-wide binding analysis, Hetzer et al. identified a cluster of Nup98-bound genes that correlate with differentiation, especially of the neural lineage. Overexpression of the dominant negative form of Nup98 repressed the expression of these Nup98-binding genes, which further confirmed the activation of such genes through Nup98 binding during
differentiation [7]. Considering that Nup98 is a mobile Nup with the capability of shuttling on and off nuclear pores, the authors proposed that Nup98–gene interactions are dynamic and might have diverse patterns during different stages of differentiation [7]. At the initial stage, the interactions between Nup98 and chromatin occur in the NPC periphery for stable microenvironment with the aid of NPC structures, whereas for genes activated in later stages of

Figure 3. Changes in nuclear-pore components during neural differentiation. The transmembrane nucleoporin Nup210 and the outer-ring nucleoporin Nup133 are absent in embryonic stem cells and become expressed during neural differentiation. Both proteins are required for neural lineage commitment.

Figure 4. Perspectives of nuclear-pore complexes (NPCs) in regulating cell pluripotency (A), as well as mediating human genetic disorders (B) and ageing (C). (A) Increased nuclear POU class 5 homeobox 1 (POU5F1), also known as Oct4, contributes to a pluripotent stem cell (iPSC) identity. Nucleoporin 133 (Nup133) and Nup210 positively regulate neural differentiation of embryonic stem cells (ESCs). Downregulation of importin-α1 and upregulation of importin-α3 and importin-α5 promote neural differentiation of ESCs. Knockdown of lamin A facilitates reprogramming of mouse fibroblasts to induced pluripotency stem cells (iPSCs). Nups and/or nuclear-transport receptors might be targeted to facilitate reprogramming. (B) Mutations in Nups are linked to various human disorders. Somatic cell reprogramming combined with gene targeting might pave the way for using patient-derived autologous cells in cell therapy. (C) In aged post-mitotic cells, dysfunction of NPCs leads to an increased nuclear permeability and leakage of cytoplasmic proteins into the nucleus. Abbreviations: AAAS, triple A syndrome; AF, atrial fibrillation; ANE, acute necrotizing encephalopathy; IBSN, infantile bilateral striatal necrosis.
Box 2. Nuclear pore components and human genetic disorders

Mutations of nuclear-pore components as well as the nuclear envelope have been implicated in various human genetic disorders [29,30]. The first serious human genetic disorder that was discovered to be caused by a single nuclear-pore component was achalasia-addisonianism-alacrima syndrome (AAAS; triple A syndrome). Triple A syndrome is caused by mutations in Aladin (H160R, S268P, V313A, Q15K), a poorly understood component of nuclear-pore complexes [25]. Moreover, mutations in nucleoporins (Nups) have been linked to neurological disorders. Missense mutation of Nup62 causes autosomal recessive infantile bilateral striatal necrosis (IBSN) [26]. The mutation Q391P is highly conserved in patients. It is suggested that the mutation influences the function of Nup62 in the degeneration of the basal ganglia in humans. Nup358 has also been connected with neurological disorders [28]. Patients with the mutation (T585M) are susceptible to acute necrotizing encephalopathy (ANE). However, the pathogenesis of the disease remains unclear.

Lastly, in Nup155 (R391H) affects its nuclear localization, reduces nuclear-envelope permeability and ultimately results in atrial fibrillation, which connects nuclear-pore complexes to human cardiovascular diseases [27].

differentiation, Nup98 interacts with the chromatin away from NPCs for an open surrounding. Currently, very little is known about how the Nup98–chromatin interaction is able to enhance gene transcription.

Collectively, certain nuclear-pore components are able to regulate the transcription of a wide range of genes during differentiation. In spite of the reported evidence connecting nuclear-pore components to stem-cell fate determination, a comprehensive study of underlying mechanisms remains to be conducted.

Concluding remarks and future perspectives

In summary, increasing evidence in recent years has supported an emerging role of nuclear pores in modulating pluripotency. Studies of nuclear pores have paved the way to a better understanding of pluripotency and have provided insights into somatic cell reprogramming (Figure 4A). It might be possible to manipulate the nuclear-pore-dependent shuttling mechanism along with key nuclear-pore components in order to increase the efficiency of somatic cell reprogramming. For instance, modulating the nuclear export of Oct4 and/or Sox2 by pharmacological intervention would help to prolong their retention in the nucleus, which might facilitate reprogramming. Furthermore, better understanding of how individual nuclear-pore components recruit chromatin [22] and regulate gene expression might result in an alternative strategy for targeting nuclear pores to obtain higher reprogramming efficiency. Although there is no direct evidence yet to support such a possibility, the involvement of nuclear-pore components in the regulation of cell differentiation has been widely reported [5,6]. Moreover, knockdown of lamin A, a key nuclear lamina neighbor of the NPC, in mouse fibroblasts has been reported to increase reprogramming efficiency [23]. Similar ideas might also be applicable to facilitate cell transdifferentiation [24]. In addition, mutations in nuclear-pore components as well as nuclear envelopes have been implicated in various human genetic disorders [1,25–30] (Box 2). Modeling these diseases using induced-PSC technology might be an important route towards unveiling roles of Nups in development, as well as towards developing potential therapies (Figure 4B). Last but not least, dysfunction of nuclear pores has been implicated in both accelerated and normal ageing processes [31,32]. In postmitotic neurons, accumulated damage to long-life nuclear-pore components during an organism’s lifespan could cause increased nuclear permeability and compromised cellular functions, which ultimately result in neurodegeneration (Figure 4C) [31]. Overall, the results reviewed here indicate that a better understanding of how nuclear pores regulate cell pluripotency and differentiation might provide insights into human ageing, as well as ageing-associated disorders [33].

Acknowledgments

G.H.L. is supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA10102312), the National Basic Research Program of China (973 Program, 2014CB964600), the National Natural Science Foundation of China (NSFC) (81330008, 81271266, 31222039, and 31201111), the Thousand Young Talents program of China, the National Laboratory of Biomacromolecules, and the State Key Laboratory of Drug Research (SIMM1302KF-17). J.C. is supported by the National Basic Research Program of China (973 Program, 2014CB910500) and NSFC (81371342). J.C.I.B. is supported by TERCEL-ISCIII-MINECO, CIBER, Fundacion Cellex, the G. Harold and Leila Y. Mathers Charitable Foundation, the Leona M. and Harry B. Helmsley Charitable Trust, The Glenn Foundation and the Ellison Medical Foundation.

References

23 Zuo, B. et al. (2012) Influences of lamin A levels on induction of pluripotent stem cells. Biol. Open 1, 1118–1127