Review Article

Stem Cell INVESTIGATION

Regulatory factors of induced pluripotency: current status

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Abstract: Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) through enforced expression of four transcription factors [Oct4, Sox2, Klf4, and c-Myc (OSKM)]; however, the reprogramming efficiency is extremely low. This finding raises fundamental questions about the regulators that influence the change in epigenetic stability and endowment of dedifferentiation potential during reprogramming. Identification of such regulators is critical to removing the roadblocks impeding the efficient generation of safe iPSCs and their successful translation into clinical therapies. In this review, we summarize the current progress that has been made in understanding cellular reprogramming, with an emphasis on the molecular mechanisms of epigenetic regulators in induced pluripotency.

Keywords: Cellular reprogramming; induced pluripotent stem cells (iPSCs); epigenetic reprogramming; epigenetics

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Introduction

Reprogramming is the process by which a differentiated somatic cell reverts to a pluripotent state from which it can adopt any cellular identity (1,2). During development, cell fate is established and maintained by complex regulatory networks of transcription factors that promote the expression of cell-type specific gene products and repress regulators of other lineages. Despite numerous intrinsic and extrinsic perturbations, cellular identity is remarkably stable once established. This stability is likely the result of a combination of multiple molecular features including cis-acting epigenetic modifications, such as DNA methylation, post-translational modifications of histone tails, nucleosome positioning, incorporation of histone variants into nucleosomes, and trans-acting regulatory factors, such as sequence-specific DNA-binding transcription factors, transcriptional co-activators, non-coding RNAs, and chromatin remodeling complexes (3). Although generally stable *in vivo*, differentiated cell fate can be dominantly reprogrammed to pluripotent status by various methods (*Figure 1A*). These methods include: (I) somatic cell nuclear transfer (SCNT); (II) cell fusion; (III) enforced expression of transcription factors [Oct4, Sox2, Klf4, and c-Myc (OSKM)] to generate induced pluripotent stem cells (iPSCs) (4-6). A team of researchers from Japan and Boston reported a cellular reprogramming phenomenon that sublethal stress, such as low pH medium, can induced neonatal somatic cells into pluripotency (STAP, *Figure 1B*) (7,8). But this method is still controversial. iPSC has less ethical and legal issues than SCNT (5). Moreover, iPSCs offer invaluable sources of patient-specific pluripotent stem cells for disease modeling, drug screening, toxicology tests, and regenerative medicine (9).

There are, however, several hurdles need to be overcome before iPSCs used in a therapeutic setting (10,11). Currently, iPSC induction is typically slow. The reprogramming of somatic cells from accessible adult tissues is particularly inefficient, because the cells are at a late stage of differentiation (10). Acquisition of induced pluripotency is a slow (usually more than 2 weeks in human) and inefficient (0.1-3%) process. It indicates that cellular reprogramming need to overcome a series of barriers (12). Increased understanding of the molecular and regulatory mechanisms of the reprogramming process is essential to improve the quality of resulting iPSCs for potential

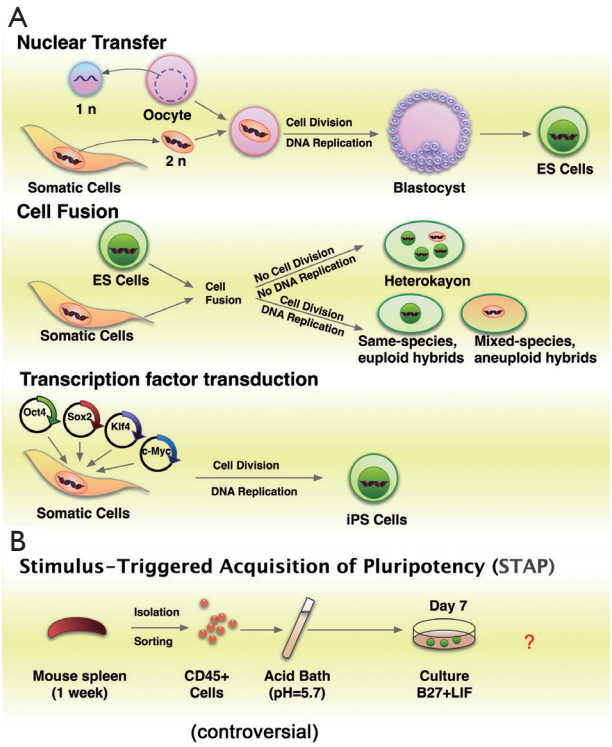

Figure 1 Methods of reprogramming to pluripotency. (A) schematic presentation of 3 approaches of reprogramming t pluripotency [adopted from Yamanaka *et al.* (4)]; (B) The "STAP" cells were claimed to be made by exposing bodily ls t cid pressure to acquire the characteristics of embryoni ste s.

therapeutic applications and to address fundamental questions about the control of cell identity (13). In this review, we briefly summariz the current understanding of induced reprogramming and focus on the roles of regulators in this process. We also discuss the future directions of reprogramming research.

Molecular events of OSKM-induced reprogramming

Given the fact that only few starting cells become iPSCs, a number of models have been developed to explain the inefficiency of iPSC generation (*Figure 2*) (14). In 2009, two contending models were initially proposed, namely the stochastic model and elite model. In the elite model, small numbers of cells are predetermined for reprogramming even before transduction of OSKM. However, in the stochastic model, most differentiated cells have the potential to become iPS cells after OSKM transduction. The cells become pluripotent dependent on "a sufficient push" from proper expression of OSKM and success of overcoming epigenetic block (15). In the same year, Hanna and his colleagues showed that the stochastic model is more favorable to explain the iPS reprogramming process with inducible reprogramming systems (16). Later on the same group modified their model by combining stochastic and hierarchical model. They demonstrated that stochastic events of gene expression were in early stage of reprogramming. It is followed by a late hierarchical phase with Sox2 being the upstream factor in a gene expression hierarchy (17). Therefore, epigenetic priming events early in the reprogramming process might be critical for pluripotency induction. However, recently, Tanabe's group demonstrated that maturation and not initiation is the limiting step during human fibroblast reprogramming (18). Disparities in the reprogramming process between mouse and human cells are likely due to the fact that conventional mouse and human iPSCs represent different states of pluripotency these cells differ epigenetically as highlighted by their X chromosome inactivation state. Recently, Guo *et al.* identified a privileged somatic cell state in which acquisition of pluripotency occurred in a non-stochastic manner. They showed that granulocyte monocyte progenitors (GMP, "privileged cells") are highly efficient in reprogramming. And they think the privileged state is different from the conventional "elite" cells (19).

iPSC reprogramming proceeds in a stepwise manner (2,14). Early works showed that fibroblasts initially reduce somatic state markers and subsequently activate pluripotency genes, suggesting an ordered process. Fully reprogrammed iPSCs activate endogenous pluripotency genes including *Oct4*, *Sox2*, and *Nanog* to acquire a self-sustaining pluripotent state in which exogenous factors are no longer required (12,20,21). Current evidence showed that iPSC reprogramming is a multistep process in which failure to transition through any of the steps would lead to the low overall reprogramming efficiency (2,22). Utilizing specific surface marker combinations, cells poised to become iPSCs can be enriched at different times during reprogramming. For example, $Thy1^-$ and $SSEA1^+$ intermediate cells generated iPSCs with significantly higher efficiency compared with $Thy1^+$ and $SSEA1^-$ cells (22). O'Malley *et al.* demonstrated that, in mouse embryonic fibroblasts (MEFs), reprogramming follows an orderly sequence of stage transitions marked by a decrease in CD44 and an increase in ICAM1 expression (23). Similarly, Quintanilla *et al.* validated that CD44 is a negative cell surface marker for human fibroblast reprogramming (24).

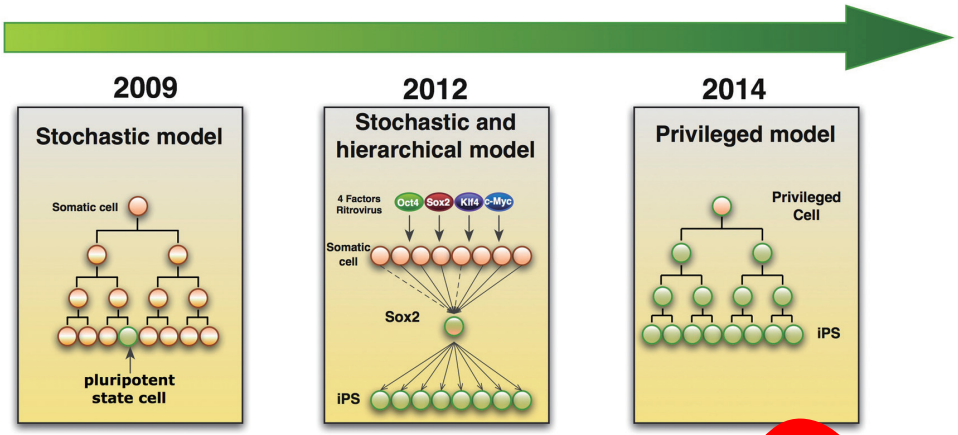

Figure 2 Models of cellular reprogramming. Three models of reprogramming are represented to account for the latency of somatic cells in reprogramming to iPSC following by the expression of OSKM.

These findings improve the understanding of a detailed reprogramming process, and may lead to new reprogramming strategies.

Genome-wide transcriptional profiling has been used to further delineate the sequence of events that drive reprogramming. Initially, cells appear to respond relatively homogeneously to the expression of the reprogramming factors and robustly silence typical mesenchymal genes expressed in fibroblasts, such as *Snai1*, *Snai2*, *Zeb1* and *Zeb2*. These events lead to the activation of epithelial markers (such as Cdh1, Epcam, and Ocln) in a process called mesenchymal-to-epithelial transition (MET). MET appears to be critical for the early reprogramming phase and is accompanied by morphological changes, increased proliferation, and the formation of cell clusters (25-27). The key characteristic of the subsequent reprogramming phase is the gradual activation of pluripotency-associated genes. The pluripotency loci *Nanog* and *Sall4* are transcriptionally upregulated at a late intermediate stage, whereas others, such as *Utf1* and endogenous *Sox2*, are induced even later, closely mirroring the acquisition of the full pluripotency expression programming (17,28). Although detailed time course of transcriptional studies describing the stage transitions in reprogramming cells have been performed at the single-cell level, facilitators and inhibitors of reprogramming are not easily identified from these data. Because fundamental changes in gene expression during reprogramming occur at the epigenetic level. In the next section, we focus on the epigenetic regulators of the OSKM-induced reprogramming process (*Figure 3*).

Epigenetic regulation of iPSC reprogramming

DNA methylation regulators

DNA methylation maintains long-lasting cell memories and therefore is considered to be a pivotal epigenetic barrier to cell reprogramming (29,30). High resolution mapping of DNA methylation has revealed an intriguing distribution of methylated cytosine in pluripotent stem cells (31). Intriguingly, DNA hypermethylation at the promoters of tissue-specific genes with low CpG density is accompanied by bivalent chromatin in embryonic stem cells (ESCs) and iPSC. And DNA methylation changes mostly occur at the end of the reprogramming process (22). The inhibition of DNA methylation by chemical compounds or RNA interference that target DNA methyltransferases (Dnmts) can increase the efficiency of iPSC generation (32). These finding indicated that changes in DNA methylation and hydroxymethylation play important roles in genome-wide epigenetic remodeling during reprogramming.

Dnmts in cellular reprogramming

DNA methylation is preserved by the maintenance methyltransferase Dnmt1 and established by the *de novo* methyltransferases Dnmt3a and Dnmt3b. The loss of Dnmt1 causes the loss of two-thirds of total DNA methylation, thus leading to embryonic lethality (33). Embryos with mutant Dnmt3b appear to be normal in early developmental stages but show multiple developmental defects in the later stages (34). Although the Dnmt family plays an essential role in both developmental and germ cell reprogramming processes, Dnmt3a- and Dnmt3b-mediated

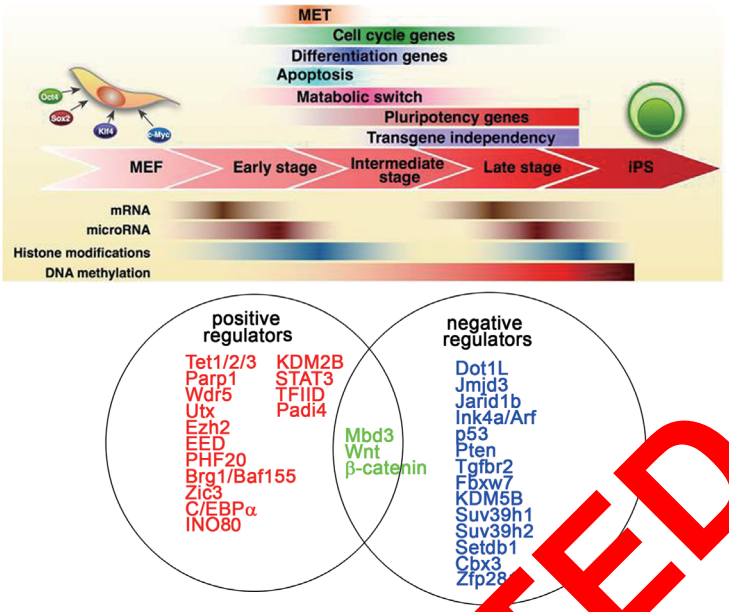

Figure 3 Molecular events and regulators during cellular reprogramming. MET denotes mesenchymal-to-epithelial transition.

de novo methylation is dispensable for iPSC induction (35). Dnmt3b conditional deletion in MEFs leads to a partial los of DNA methylation (36). Dnmt3a knockdown promote iPSC formation in human cells, whereas deletion of murine Dnmt3a and Dnmt3b has no consequence o cellular reprogramming (35,37).

Ten-eleven translocations (TETs) in cellular reprogramming

TET proteins have emerged as important regulators of DNA demethylation. TETs catalyze the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which serves as a substrate for thymine DNA glycosylase (TDG)-mediated base excision repair into unmodified cytosine (38). The TET1-binding sites overlap with Polycomb group (PcG) target sites (39). Knockdown of *TET1* decreases the expression of PcG target genes and pluripotency-related genes, indicating that gene regulation by TET1 cannot be completely explained by the collaborative functioning with PcG (40). Moreover, *TET1* overexpression can replace *Oct4* (TSKM induction system) during cellular reprogramming, providing genetic evidence that TET1 contributes to the activation of endogenous pluripotency genes. In the TSKM reprogramming system, Tet1 facilitated endogenous *Oct4* demethylation and reactivation through 5hmC conversion (41). Although Tet1 plays important role in cellular reprogramming *in vitro*, ESCs from *TET1* knockout (KO) mice did not show any abnormalities in the maintenance of pluripotency. Moreover, *TET1* deficient mice are viable and fertile (42). The Mbd3/nucleosome remodeling and deacetylation (NuRD) complexes directly recognize 5hmC and therefore, may control the expression of TET1 target genes. Yildirim *et al*. showed that Mbd3 knockdown preferentially affected expression of 5hmC-marked genes and Mbd3 preferentially binds to 5hmC relative to 5mC *in vitro* (43). Depletion of the Mbd3 surprisingly yielded reprogramming efficiencies of up to 100% within days (44), which we will talk about later.

TET2 has been shown to induce hydroxymethylation of key pluripotency genes such as *Nanog* shortly after OKSM overexpression. Knockdown of *TET2* prevents the reprogramming synergy of Nanog with a catalytically deficient mutant of *TET1*. Genome-wide Chromatin Immunoprecipitation Sequencing (ChIP-seq) analyses further revealed that TET1 and TET2 co-occupy many pluripotency targets in ESCs (45). In agreement with these analyses, Zhu *et al*. develop a combination of modified reprogramming factors (OySyNyK) which significantly increased Tet1 expression at the early stage and interact with TET1/2 to promote reprogramming (46). Doege *et al*. identified poly (ADP-ribose) polymerase-1 (Parp1) and TET2 necessary for iPSC generation, which were recruited to the Nanog and Esrrb loci during the early

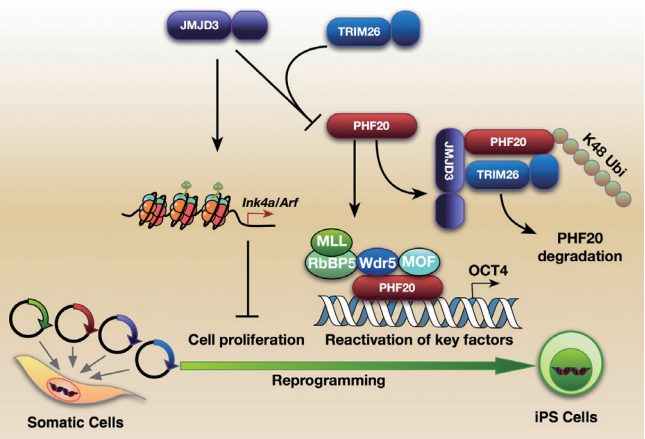

Figure 4 Jmjd3 functions as a roadblock to somatic cellular reprogramming. Jmjd3 upregulated Ink4a/Arf by modulating H3K27 methylation through its demethylase activity. Increased amounts of Ink4a and Arf induced cell senescence and reduced cell proliferation, thus decreasing the efficiency and kinetics of reprogramming; Jmjd3 also targeted PHF20 for ubiquitination and degradation by recruiting an E3 ligase, Trim26, in an H3K27 demethylase activity-independent manner. The resultant decrease in PHF20 protein led to the loss of endogenous Oct4, thereby greatly reducing reprogramming efficiency.

stage of reprogramming. They further showed that [illegible]-1 functioned in the regulation of 5mC modification ([illegible]).

Tet1 and Tet2 are highly expressed in [illegible] ES cells but Tet3 is more enriched in oocytes and one-cell zygotes (48). Gu *et al*. showed that Tet3-mediated DNA hydroxylation is involved in epigenetic reprogramming of the zygotic paternal DNA following fertilization and may also contribute to somatic cell nuclear reprogramming during animal cloning (49). Recently, TET1/2/3 triple KO MEFs were derived by the same research group. They found that MEFs deleted in all three Tet genes cannot be reprogrammed because of a block in the MET step (50).

Histone-modifying enzymes in reprogramming

Histone marks and chromatin structure are regulated by histone modifying enzymes including "reader", such as PHD finger proteins, "writers", such as histone methyltransferases (HMTs) and histone acetyltransferases, and "erasers" such as histone demethylases (HDMs) and histone deacetylases (HDACs) (51). These enzymes function as co-activators or co-repressors of OKSM at different stages of reprogramming and can profoundly influence iPSC derivation (12). Recent technical advances have allowed us to map chromatin modifications throughout the genome by combining ChIP with DNA microarray analysis or high-performance sequencing. These methodologies have revealed that pluripotent stem cells have a unique expression pattern for histone modifiers and distinct distributions of modified histones.

Trimethylation of histone 3 lysine 4 (H3K4), an active marker of transcription, is frequently observed in promoter regions of pluripotent stem cells (52). ESC pluripotency is regulated in part by H3K4 methylation; however, it is still unclear whether H3K4 methylation is involved in iPSC reprogramming. Studies have shown the Trithorax group (TrxG) complexes with the activity of H3K4 methylation to promote iPSC reprogramming, thus providing a functional link between H3K4 methylation and reprogramming. Wdr5, a key component of TrxG, interacts with H3K4me2 to mediate the transition of H3K4me2 to H3K4me3. The expression of Wdr5 is the highest in undifferentiated ESCs and iPSCs and decreases during the differentiation process. Wdr5 has been shown to enhance the efficiency of OSKM-mediated iPSC generation by interacting with [illegible]4 (53). Kidder *et al*. found that the H3K4-specific demethylase KDM5B is a barrier to the reprogramming process as evidenced by the accelerated reprogramming of differentiated cells in the absence of KDM5B (54).

The methylation of histone 3 lysine 27 (H3K27) is mediated by polycomb repressive complex 2 (PRC2), which is composed of PcG proteins such as enhancer of zeste 2 (EZH2), embryonic ectoderm development (EED), and suppressor of zeste 12 homolog (Suz12) (55). Onder *et al*. used short hairpin RNAs (shRNAs) to target genes in DNA and histone methylation pathways, and identified that *EZH2* and *EED* KD reduced reprogramming efficiency (37). The JmjC domain-containing proteins UTX and JMJD3 demethylate trimethylate H3K27 (56). Our group showed that Jmjd3 blocks reprogramming not only by activating the *Ink4a/Arf* locus but also by targeting the methyl-lysine effector protein PHF20 for ubiquitination (*Figure 4*). We also found that PHF20 in collaboration with Wdr5 is required to activate *Oct4* transcription (57). Interestingly, Utx promotes somatic and germ cell epigenetic reprogramming. Hanna and his colleagues showed that *Utx* deficient somatic cells failed to robustly reprogram back to the ground state of pluripotency. Utx directly interacted with OSKM to facilitate iPSC generation in a HDMs activity-independent manner (58).

The histone 3 lysine 9 (H3K9) methyltransferases (HMT) maintain the refractory heterochromatic state of somatic cells, thus acting as major barriers to reprogramming. The H3K9 methyltransferase G9a is essential for embryonic development and has been shown to prevent reprogramming by recruiting Dnmt3a and Dnmt3b to the promoters of *Oct4* and *HP1β* (59). Pei and colleagues show that bone morphogenetic protein (BMP) signaling to H3K9 methylation is a barrier to reprogramming somatic cells into iPSCs. *Setdb1* knockdown led to ~100% efficiency in the reprogramming of pre-iPSCs into iPSCs in the presence of vitamin C (60). Consistent with this notion, knockdown of *Suv39h1*, *Suv39h2*, *Setdb1*, or *heterochromatin protein-1γ* (*Cbx3*), increase transcription factor accessibility and result in more efficient iPSC generation from somatic cells (37,61).

Activation of the histone 3 lysine 36 (H3K36) demethylases (HDMs), Jhdm1a and Jhdm1b promote intermediate to late stages of iPSC generation by suppressing the *Ink4/Arf* locus, which is essential for the acquisition of immortality (62,63). An additional early role for Jhdm1b in epithelial gene activation has recently been reported. Liang's group showed that KDM2B, a histone H3 Lys 36 dimethyl (H3K36me2)-specific demethylase, functioned at the beginning of t reprogramming process and promoted activation of earl responsive genes in reprogramming. This capacity ends on its demethylase and DNA-binding activities a is l ely independent of its role in antagonizing senesce e (

Inhibition of the histone 3 lysine 79 H3K79) MT *DOT1L* significantly increased reprogramming fficiency and substituted for *KLF4* and *c-Myc*. In bition of *DOT1L* early in the reprogramming process l to marked increase of *Nanog* and *LIN28*, which pla ntia unc onal roles in the enhancement of reprogr mmin (37). K79 methylation plays a critical role in t rogr sion of G1 phase, S phase, mitosis, and meiosis. D letion of *DOT1L* results in reduced cell proliferation in mouse ESCs and human cancer cells. By contrast, the cardiac-specific deletion of *DOT1L* leads to increased proliferation of heart tissues (65). The specific function of *DOT1L* in cell proliferation of cellular reprogramming requires further elucidation.

Chromatin remodelers also play important role in cellular reprogramming (12). Singhal *et al*. identified components of the ATP-dependent BAF chromatin-remodeling complex, *Brg1* and *Baf155*, which significantly increases reprogramming efficiency when used together with the four factors (66). Wang *et al*. showed that INO80 complex, a SWI/SNF family chromatin remodeler facilitates pluripotency gene activation in ESC self-renewal, reprogramming, and blastocyst development. INO80 co-occupied pluripotency gene promoters with the master transcription factors. At the pluripotency genes, INO80 promoted the recruitment of RNA polymerase II complex for gene activation by maintaining open chromatin architecture (67). Ingrid Grummt and colleagues showed that downregulation of the NuRD complex is required for efficient reprogramming. Overexpression of *Mbd3*, a subunit of NuRD, inhibits induction of iPSCs. Almost at same time, Jacob Hanna group showed that depletion of *Mbd3* yielded reprogramming efficiencies of up to 100% within days, suggesting that elimination of a single gene suffices to render reprogramming a inistic process (44). However, another research tea n UK, ported that overexpression of *Mbd3/NuRD* f ilit s re ogramming in a context-dependent ma er. Mbd3 only facilitated the initiation of neural st m c epro mming but also was required for efficient SC gen n from EpiSCs and pre-iPSCs (68). There ore, eper investigation is needed to understand the m ular mec nism of Mbd3 in cellular reprogramming.

N ng RNAs in reprogramming

mprove the quality of generated iPSCs, researchers have also focused on using non-coding RNAs such as miRNAs, which are associated with regulation of the epigenome. Two groups reported that the transfection of *microRNA (miR)-302* and *miR-367* clusters successfully reprogramed mouse and human somatic cells to iPSCs without the use of exogenous transcription factors (69,70). Similarly, KO of the *miR-302/367* cluster by TALE nucleases (TALENs) completely blocked iPSC generation (71). The molecular mechanism of *miR-302* and *miR-367* induced pluripotency is via activating endogenous *Oct4* and accelerating MET (69,72,73). Moreover, expression of exogenous *miR-302* cluster (without *miR-367*) is efficient in achieving a fully reprogrammed iPS state in partially reprogrammed cells by relieving Mbd2-mediated inhibition of *Nanog* expression (74).

Many miRNAs have been shown to promote OSKM-induced reprogramming. The miRNAs *miR-291-3p*, *miR-294* and *miR-295* increase the efficiency of reprogramming by Oct4, Sox2 and Klf4, but not by these factors plus c-Myc (75). It was also reported that the activation of BMP signaling induced the expression of *miR-205* and *miR-200* family members and enhanced MET (27). Li *et al*. systematically studied small RNA-mediated regulation of iPS cell generation by KD miRNAs during cellular reprogramming. They found that *miR-17*, *miR-25*, *miR-*

106a and *miR-302b* clusters were induced during the early stage of reprogramming. And *miR-93* and *miR-106b* enhance iPSC induction and MET step of reprogramming (76).

miRNAs also suppress reprogramming. For example, Yamanaka's group showed that inhibition of *let*-7 during reprogramming leads to an increase in the level of the *let*-7 target *LIN-41/TRIM71*, which in turn promotes reprogramming and is important for overcoming the *let*-7 barrier to reprogramming (77). Another important miRNA barrier for reprogramming is the p53-mediated pathway, which induces the expression of *miR-34* family members and suppression of the pluripotency factors Nanog and Sox2. Genetic deletion of *miR-34a* increases the efficiency and kinetics of reprogramming and establishes pluripotency at a late stage (78). Additionally, the suppression of p53 through the overexpression of *miR-138* or repression of *miR-21* and *miR-29a*, enhances reprogramming (79,80).

Other signaling pathways and regulators in reprogramming

Tumor suppressor genes have been found to inhibit reprogramming. p53 has been implicated as an enforcer differentiation by virtue of its ability to limit the cardina stem cell characteristic of self-renewal in several syst Dr. Zhao's team first found that *p53* siRNA and und erent ted embryonic cell transcription factor 1 (*UTF1* enh ed the efficiency of iPSC generation up to 100 ld (81). ter, four research teams demonstrated that p is a potent reprogramming barrier by pro ting cell s escence (63,82-84). Pten is one of most t mor suppressors in human cancer. Recently, Lia al. nd at *Pten* deletion promotes reprogrammin of M Fs in iPSCs. They also showed that the Pten i tor a potassium bisperoxo (5-hydroxypyridine-2-carboxy oxovanadate could be used to improve the efficiency of germline-competent iPSC derivation from mouse somatic cells (85).

Wnt/β-catenin signaling pathway plays a pivotal role in the maintenance of pluripotency as well as somatic cell reprogramming. As early as 2008, Marson's team reported that soluble Wnt3a can be used to enhance the efficiency of reprogramming in combination with nuclear factors, Oct4, Sox2 and Klf4 (86). Recently, Zhang *et al.* found that β-catenin signaling enhances reprogramming efficiency primarily at the initial stage. β-catenin interacts with reprogramming factors Klf4, Oct4 and Sox2, further enhancing expression of pluripotency circuitry genes (87). Another study demonstrated that increase of β-catenin promoted the activity of Oct4 and Nanog, and enhanced pluripotency (88). However, a series of reports indicate that β-catenin may not be required for pluripotent stem cell self-renewal and expansion. It has been reported that OCT4 represses β-catenin signaling during self-renewal and knockdown of OCT4 activates β-catenin signaling in hESCs (89). Ho *et al.* demonstrated that active Wnt signaling inhibits the early stage of iPSC reprogramming but is required and even stimulated during the late stage (90). These findings suggest that the effect of β-catenin may be context dependent.

It is well known that the TGF superfamily member BMP4 cooperates with mia inhibitory factor (LIF) to maintain the plur tency mouse ESCs (91). Jeffrey Wrana group hi lig d th important role of BMP signaling in pr moti the ET stage of reprogramming. BMP coul i e mi -205 and miR-200 family of microR s to m te MET (27). Chen group further demo str d that BMP4 and BMP7 enhanced the e sion of ithelial genes (*Cdh1*, *EpCAM*, etc.) and hibit th expression of mesenchymal genes (*Zeb1*, *Twist1*,) in S-infected MEFs (92). In 2009, Maherali and colleagues showed that inhibition of TGF-β signaling anced both the efficiency and kinetics of OSKM-reprogrammed MEFs, whereas activation of the TGF-β signaling blocked reprogramming (93). It was further demonstrated that TGF-β inhibitor could replace *Sox2* in reprogramming through induction of the transcription factor *Nanog* (94).

Recent progress in reprogramming research now points to an important role for transcription factors in the establishment and maintenance of pluripotent phenotypes. Yang *et al.* (95) discovered that STAT3 activation can directly convert epiblast stem cells into naïve iPSCs in 2i medium. They also demonstrated that STAT3 activation plays a vital role in late-stage somatic cell reprogramming (i.e., activation of endogenous *Oct4* gene). Thus, STAT3 activity is essential for converting the primed state to naïve pluripotency state in the mouse. Pijnappel *et al.* showed that knockdown of the transcription factor IID complex affects the pluripotent circuitry in mouse ESCs and inhibits reprogramming of fibroblasts. Transient expression of TFIID subunits greatly enhanced reprogramming (96). Padi4, a member of peptidylarginine deiminases (PADIs), increased during reprogramming in mouse. Padi4 could bind to regulatory elements of key stem cell genes to activate their expression. Padi4 inhibition significantly reduced reprogramming efficiency (97). Similarly, CCAAT/enhancer

binding protein-α (C/EBPα) enhanced reprogramming when co-expressed with OSKM. Ectopic expression of C/EBPα is essential in reprogramming of mature B cells (98). Overexpressing C/EBPα with OSKM induces a 100-fold increase in iPS cell reprogramming efficiency. Pluripotency and epithelial-mesenchymal transition (EMT) genes were markedly upregulated during this conversion. Moreover, C/EBPα transiently made the chromatin of pluripotency genes more accessible to DNase I and induced the expression of TET2 after OSKM induction (99). Zinc finger protein of the cerebellum (Zic)3, a member of Gli family of transcription factors is essential for maintaining ESC pluripotency. Declercq *et al*. showed that MEFs transduced with Zic3 plus OSK enhanced iPSC formation compared with OSK alone. Zic3 also enhanced the expression of genes known to enhance iPSCs derivation including *Nanog* and *Tbx3* (100). Fidalgo *et al*. identified Zfp281as a roadblock to efficient somatic cell reprogramming. Zfp281 recruited the NuRD repressor complex onto the *Nanog* locus and mediated *Nanog* transcription in repression (101).

Many other signaling pathways are also reported to regulate reprogramming. For example, protein ubiquitination system (UPS) mediates the rapid and high specific degradation of intracellular proteins and thereby contributes to the dynamic regulation of protein abundance. Using UPS-targeted RNA interference screen, Buckley *et al*. identified a significant number of ubiquitin enzymes essential in pluripotency regulation, including Psmd14 and Fbxw7. Psmd14 expression is regulated during ESC differentiation and its silencing affects ESC pluripotency and abrogates cellular reprogramming. On the other hand, the depletion of E3 ligase Fbxw7 leads to the inhibition of differentiation and enhancement of iPSC generation (102). Interestingly, biochemical and biophysical factors can also help reprogram somatic cells into pluripotent stem cells. Downing *et al*. showed that parallel microgrooves on the surface of cell-adhesive substrates can replace the effects of small molecule epigenetic modifiers and significantly improve reprogramming efficiency (103).

Small chemicals to promote OSKM-induced reprogramming

Stem cell fate is regulated by both intrinsic/extrinsic regulators and the extracellular niche. Because these regulators have limitations in their efficiency and selectivity for controlling stem cell fate, a new strategy is to use small molecules. Surprisingly, a research team lead by Hou showed that pluripotent stem cells can be generated from mouse somatic cells at a frequency up to 0.2% using a combination of seven small molecule compounds (104). Before that, Esteban and colleagues show that vitamin C enhances iPSC generation from both mouse and human somatic cells (105). They further showed that vitamin C induced H3K36me2/3 demethylation during reprogramming and identified KDM2A/2B, two known vitamin-C-dependent H3K36 demethylases, as potent regulators of reprogramming (62). PD0325901 (mitogen-activated protein kinase kinase inhibitor) and CHIR99021 (glycogen synthase kinase-3 inhibitor) (2i) plus LIF have been shown to induced stable pluripotent regulation of *Oct4* and *Nanog*, reactivation of the X chromosome, transgene silencing, and competence for somatic and germline chimaerism (106). The Rho-associated kinase inhibitors Y-27632 and thiazovivin enhance the survival of human ESCs, whereas a combination of PD0325901, CHIR99021, and Y-27632 supplemented with basic fibroblast growth factor supports the maintenance of human ESCs (107). Recently, Hanna and colleagues have established defined conditions that facilitate the derivation of human naïve ground state pluripotent stem cells with a chemical cocktail (108). Compared to genetic manipulations, small molecule approaches have a number of advantages: (I) the biological effects of small molecules are rapid, reversible, and dose-dependent; (II) small molecules will not cause instability of genome; and (III) a variety of chemical libraries provide data for the optimization of small molecule effects. Small chemicals could be useful for the iPS technology in clinic.

Future directions

The medical applications of human iPSCs in disease modeling and stem cell therapy have been progressing rapidly. Elucidation of the details and mechanisms of the reprogramming process during iPSC generation has resolved many problems regarding the clinical use of iPSCs. The first human clinical trial using autologous iPSCs has been approved by the Japan Ministry Health and will be conducted in 2014 in Kobe. Although the clinical application of iPSC technology has a bright future, challenges remain including concerns regarding the safety of OSKM-induced reprogramming. Numerous alternative methods for inducing pluripotency without the use of viral vectors have been reported, but their efficiency remains problematic (109). In conclusion, we need to identify more deterministic regulators, in particular the small chemicals,

which lead to global changes in the epigenetic regulation of somatic cells from a differentiated state to a pluripotent state. Further research is needed to efficiently generate high-quality and safe iPSCs for clinical use.

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None.

Footnote

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