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Cellular reprogramming: a small molecule perspective

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Abstract

The discovery that somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) by the expression of a few transcription factors has attracted enormous interest in biomedical research and the field of regenerative medicine. iPSCs nearly identically resemble embryonic stem cells (ESCs) and can give rise to all cell types in the body, and thus have opened new opportunities for personalized regenerative medicine and new ways of modeling human diseases. Although some studies have raised concerns about genomic stability and epigenetic memory in the resulting cells, better understanding and control of the reprogramming process should enable enhanced efficiency and higher fidelity in reprogramming. Therefore, small molecules regulating reprogramming mechanisms are valuable tools to probe the process of reprogremming and harness cell fate transitions for various applications.

Introduction

Regenerative medicine aims to restore tissues damaged by trauma, aging and diseases. This can be accomplished through cell replacement, in which transplanted cells engraft and rebuild tissues, or by stimulating regenerative capacities of endogenous cells within the tissue and organ using conventional therapeutic molecules (small molecule drug or biologics) or cellbased therapy that function through paracrine mechanisms. Pluripotent stem cells, with their ability to self-renew and differentiate into every cell type of the body, have attracted significant interest for understanding basic biology and the development of biomedical applications. In contrast to embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), which are reprogrammed from somatic cells through the overexpression of four exogenously delivered transcription factors (Oct4, SOX2, KLF4, and c-Myc, i.e., OSKM), allow for the generation of patient-specific pluripotent cells and have diminished ethical concerns, and so are promising for personalized disease modeling and regenerative medicine. Over the past few years, various combinations of transcription factors (TFs), using both integrating and non-integrating strategies, and small molecules, which functionally replace reprogramming TFs and/or enhance reprogramming efficiency, have successfully been developed to create iPSCs. Inspired by the iPSC approach using multiple TFs, many studies have shown that, with the proper conditions, somatic cells can also be transdifferentiated into another cell fate both within and outside of their germ layer, which is also called lineage-specific reprogramming.

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Cellular reprogramming to iPSCs is an inefficient and slow process, and involves stepwise stochastic events. These hurdles may present issues in generating safe iPSCs. For example, although non-integrating methods, such as episomal plasmid [1], protein [2, 3], and mRNA [4] transfection, provide safer approaches to address potential problems caused by integrating factor methods (e.g., retro- and lentiviruses), it is still possible that significant genome instability will happen during reprogramming process *per se*. In addition, recent studies showed that iPSCs may be incompletely reprogrammed at the transcriptional and epigenetic levels [5–9], and that the reprogramming and culturing processes may capture and/or cause mutations and genomic instability [10–15]. These observations raise concerns about the quality of iPSCs. Consequently more work is necessary to study how to control cell reprogramming more efficiently and faithfully.

Because small molecules provide several distinct advantages in controlling protein functions (e.g., temporally controllable, reversible, tunable, modular, and tractable), they have attracted much interest for steering reprogramming toward a faster, more efficient, and directed process. Thus far, many small molecules have been identified and characterized to functionally substitute for reprogramming TFs and enhance reprogramming, including mesenchymal-epithelial transition (MET) regulators, cell senescence alleviators, and modulators of metabolism, epigenetic processes, and other signaling pathways [16–18]. Here we will review recent studies in cellular reprogramming with an emphasis on chemical approaches that advance our understanding of the molecular mechanisms involved in cellular reprogramming.

Mesenchymal-to-Epithelial Transition

MET is a reversible biological process involving the transition from motile, multipolar or spindle-shaped mesenchymal cells to planar arrays of polarized epithelial cells. Reprogramming of fibroblast cells to iPSCs inevitably involves a MET process, as cells even at the early stage of reprogramming undergo morphological changes toward epithelial-like cells and epithelial genes such as E-cadherin and Epcam are upregulated while key mesenchymal genes such as Snail and N-Cadherin are downregulated. At the transcriptional level, Sox2/Oct4 suppress the EMT mediator Snail, c-Myc downregulates TGFβ1 and TGFβ2, and Klf4 induces epithelial genes including E-cadherin. Consistently, ectopic expression of E-cadherin significantly enhances reprogramming efficiency, while TGFβ1/2/3 and overexpression of Snail leads to greatly decreased reprogramming efficiency [19••, 20••]. Given the key role of the TGFβ pathway in inducing epithelial-to-mesenchymal transition (EMT) (the reverse process of MET), several studies demonstrated as expected that inhibition of TGFβ signaling pathway by small molecules enhances reprogramming through de-repressing the epithelial phenotype and inducing MET. The combination of SB431542 (an inhibitor of TGFβ receptor) and PD0325901 (an inhibitor of MEK) was shown to dramatically enhance the efficiency of human iPSCs generation (>100 fold) and accelerate the reprogramming speed [21]. Additionally, Thiazovivin, a potent inhibitor of Rho-associated kinase (ROCK) that has been shown to promote survival of dissociated human ESCs by stabilizing E-cadherin [22], could further promote reprogramming. Another study found that a TGFβ inhibitor, E-616452 (renamed as RepSox), could functionally substitute for SOX2 in mouse fibroblast reprogramming with OKM and indirectly enhance NANOG expression during late stage of reprogramming [23]. It's also found that miR-302 and miR-372 could enhance somatic reprogramming by increasing the kinetics of MET during reprogramming [24].

Cell stress and senescence

Cellular senescence appears to be incompatible with reprogramming to pluripotency and is a barrier to this process. Consistent with this notion, fibroblasts after serial passaging have much reduced reprogramming efficiency [25]. Interestingly, expression of reprogramming factors also triggers senescence by up-regulating p53, $p16^{INK4a}$, and $p21^{CIP1}$. Mechanistically, induction of DNA damage response and chromatin remodeling of the INK4a/ARF locus were found as two reasons behind this induction of senescence [26]. Vitamin C (Vc, also known as ascorbic acid), an essential nutrient for cells, can act as an antioxidant to protect against oxidative stress, and also serves as a cofactor for several metabolic enzymes. In iPSC reprogramming, Vc not only improves the efficiency of reprogramming in both mouse and human somatic cells, but also promotes the transition of partially reprogrammed iPSCs to a fully reprogrammed state, probably by reducing reactive oxidant species (ROS) and senescence triggered by reprogramming TFs [27•]. Recently, another study demonstrated that histone H3K36 demethylases Jhdm1a/1b are key effectors of somatic cell reprogramming in a Vcdependent manner [28]. Jhdm1b activation can also accelerate cell cycle progression and suppress cellular senescence by repressing the INK4/ARF locus.

Metabolism shift: glycolysis

Many types of stem cells, including pluripotent stem cells, mainly rely on glycolysis followed by lactic acid fermentation in the cytosol to produce energy in contrast to a relatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria used by most differentiated cells. This could be advantageous for stem cells as glycolytic metabolism can more effectively produce various macromolecular precursors to meet metabolic and energy demands while generating fewer reactive oxygen species that can induce oxidative damage. Consequently, reprogramming somatic cells that utilize mitochondrial oxidation to iPSCs would entail a metabolism switch to glycolysis. Taking this into consideration, small molecules promoting glycolysis may enhance reprogramming. Consistently, we identified PS48, an activator of 3-phosphoinositide-dependent protein kinase 1 (PDK1)-PI3K/Akt pathway, that significantly induces the expression of glycolytic genes and facilitates iPSC reprogramming of human somatic cells transduced with only Oct4 [29]. It was speculated that mitochondria in ESCs probably have no functional Oxidative Phosphorylation (OxPhos) machinery. However, Zhang *et al.* showed that human pluripotent stem cells (hPSCs) actually possess functional OxPhos machinery and consume oxygen at a rate similar to differentiated cell mitochondria [30]. Unlike in differentiated cells, glucose uptake is less coupled to OxPhos in hPSCs, and instead hPSCs predominantly use glycolysis to generate ATP. Mitochondrial uncoupling protein 2 (UCP2) plays a critical role in separating oxidative phosphorylation from ATP synthesis with energy dissipated as heat accompanied by a reduction of mitochondria-derived ROS. This uncoupling regulates energy metabolism and differentiation potential of hPSCs [30]. Consequently, small molecules that uncouple the mitochondrial respiratory chain may promote reprogramming to iPSCs. Indeed, 2,4-Dinitrophenol (DNP), a well-known uncoupler, significantly increases reprogramming efficiency [29•]. These studies reveal that metabolism switch is another fundamental mechanism in somatic cell reprogramming.

DNA damage response

In contrast to somatic cells that primarily use non-homologous end joining (NHEJ) DNA repair mechanism, pluripotent cells mainly rely on homologous recombination (HR) DNA repair to safeguard genomic stability. During reprogramming, DNA damage responses are activated in cells [26], and the DNA damage marker γH2AX appears during the early stage

of the reprogramming process. Consistently, the reprogramming efficiency decreased dramatically in p53BP1- and ATM- (both are DNA repair components) knockout cells [10]. Another study demonstrated that defects in the Fanconi anemia (FA) DNA repair pathway led to poor reprogramming efficiency of murine and human primary cells. Complementation of the FA pathway by expressing *FANCA* in *Fanca*$^{-/-}$ fibroblast cells reduced senescence and restored reprogramming efficiency to the normal levels [31]. These observations indicate the important roles of DNA damage repair pathways in reprogramming. Recently, issues on the genomic quality of iPSCs have also attracted increased attention [11–15].

In iPSC generation, not only the mutations in starting cells may be captured in selected iPSC clones, but new mutations could also be generated during reprogramming and expanding processes. Furthermore, copy number variation (CNV) has been shown to be increased in early passage iPSCs, and such CNV variability becomes reduced in subsequent passages as many CNVs are selected against in the passaging and long-term culture [13]. These studies raise concerns about the genome stability and quality of iPSCs generated even by non-integrating methods. For example, an exome sequencing study of 22 hiPSC lines that were generated from three integrating methods (*e.g.*, viral delivery) and two non-integrating methods (*e.g.*, mRNA delivery) found an average of six protein-coding point mutations per exome [14]. Some of these mutations could influence cell survival and function or potentially cause tumorigenicity. These findings suggest that the whole reprogramming process (from reprogramming induction to iPSC clonal selection and expansion) may lead to iPSCs with certain mutations. However, a whole genome sequencing study revealed a lower incidence of DNA sequence variation occurred in non-integrating episomal vector generated hiPSCs when compared with traditional retrovirus generated iPSCs [32]. The divergence of results is probably due to different experimental design, data interpretation, and reprogramming approaches including materials and culture conditions. While it's generally accepted that mutations in starting somatic cells could be captured in clonally selected iPSC lines, it's still a highly debated topic concerning the genome quality of iPSCs.

In addition, the mitochondrial genome is also susceptible to mutations during the reprogramming process [33]. One reason that the mitochondrial genome is so vulnerable to mutations is that mitochondria do not have the endogenous molecular repair mechanisms found in the cell nucleus at their disposal. In addition, free radicals (particularly reactive molecules that can trigger mutations) arise during cellular respiration, which occurs in the mitochondria. Taking this into consideration, PSCs would benefit the metabolic switch from oxidative respiration to glycolysis for reduction of ROS generation and genomic mutation. Small molecules that reduce ROS levels could be useful to reduce the chances of mutation in the mitochondria during reprogramming.

In addition to its role in iPSC reprogramming to minimize genomic instability, DNA damage repair may also be important in the DNA demethylation process. During the reprogramming process, many gene loci (e.g., pluripotency genes), the DNA of which is hypermethylated in the differentiated state, must be demethylated to allow for their transcription. The Tet family of proteins catalyze the conversion of 5-methyl-cytosine (5mC) to 5-hydroxy-methyl cytosine (5hmC), 5-formyl-cytosine (5fC), and 5-carboxyl-cytosine (5caC) sequentially, all of which could be further processed and converted into cytosine directly or in part through DNA repair mechanisms such as base excision repair (BER), effectively demethylating the DNA[34]. Tet1 and Tet2, both highly expressed in PSCs, are induced shortly after the initiation of reprogramming concurrent with a global upregulation of 5hmC[35]. It is thought that this, combined with DNA damage repair, is instrumental in the active DNA demethylation of important pluripotency related genes. This process may make an attractive target for small molecule enhancement of reprogramming.

As well as their canonical role in DNA damage repair, a recent study revealed DNA repair complexes also possess noncanonical function as gene expression regulators. Using an unbiased *in vitro* transcription-biochemical complementation assay, it was found that XPCRAD23B- CETN2 nucleotide excision repair (NER) complex belongs to a multi-subunit stem cell coactivator complex (SCC) that is selectively required for the synergistic activation of the *Nanog* gene by Oct4 and Sox2 [36•]. This function is independent of the DNA repair activity of NER since the mutation of the residue in RAD23B important for DNA damage repair does not affect this newly uncovered function. Therefore, besides functioning as repair components, XPC-RAD23B-CETN2 also works as scaffold to recruit transcription coactivators, which are important for pluripotency maintenance and successful reprogramming.

Chromatin remodeling: resetting the epigenome

The eukaryotic genome has a highly organized structure comprised of DNA, histones, nonhistone proteins and RNA. This organized nucleic acid and protein structure is referred to as chromatin. The chromatin exists as a dynamic entity, shuttling between the open and closed forms at specific nuclear regions and loci to determine gene expression and cell fate. The chromatin of PSCs is in a relatively open conformation and is marked by hyperdynamic association of chromatin proteins, while the chromatin of somatic cells is mostly in an inactive and compact state called heterochromatin [37]. Chromatin remodeling is an enzyme-assisted process that modulates access of nucleosomal DNA by reshaping the structure, composition and positioning of nucleosomes. Access to nucleosomal DNA is governed by two major classes of protein complexes: histone proteins (and their covalent modifications); and ATP-dependent chromatin remodeling complexes.

During the reprogramming process the epigenome must be “reset” from the somatic cell patterns to the pluripotent cell patterns. Several proteins in chromatin remodeling complexes have been shown essential for successful reprogramming, including Chd1[38] and components of the BAF complex[39]. BAF complex components Brg1 and Baf155 help achieve a euchromatic chromatin state and enhance binding of reprogramming factors like Oct4 onto key pluripotency gene promoters, thereby enhancing reprogramming. Primarily through reprogramming TF-guided chromatin remodeling, the hallmarks of epigenetic reprogramming such as X chromosome reactivation, silencing of retroviral promoters, methylation of loci specific to the differentiated state, demethylation of pluripotency gene loci, and genomic imprinting establishment can be accomplished. Therefore, small molecules that affect epigenetic modifications such as inhibitors of DNA methyltransferases, histone deacetylases, methyltransferases (e.g., G9a, hDOT1), and demethylases (e.g., LSD1) have been identified to increase reprogramming efficiency and substitute for reprogramming factor(s)[16–18].

Full reprogramming of the epigenome of somatic cells to the PSC state is important to ensure iPSC quality. Studies showed that selected iPSC clones generated using particular reprogramming expression systems exhibited aberrant silencing in the imprinted Dlk1–Dio3 gene cluster on chromosome 12qF1. These clones contributed poorly to chimaeras and failed to support the development of entirely iPSC-derived animals in the tetraploid complementation assay. In contrast, iPSC clones with normal expression of the Dlk1–Dio3 cluster contributed to high-grade chimaeras and generated viable all-iPSC mice [40••]. Several microRNAs from the Dlk1-Dio3 cluster potentially target components of the polycomb repressive complex 2 (PRC2) and may form a feedback regulatory loop to control the expression of the genes and non-coding RNAs encoded by this region in fully reprogrammed iPSCs [41]. The Jaenisch lab showed that the epigenetic and biological properties of iPSCs are dependent on the stoichiometry of reprogramming factors. They

demonstrated that high expression of Oct4 and Klf4 combined with lower expression of c-Myc and Sox2 produced iPSCs that efficiently generated "all-iPSC mice" by tetraploid complementation, maintained normal imprinting at the Dlk1-Dio3 locus, and did not create mice with tumors [42]. Notably, treatment of an iPSC clone that had silenced Dlk1–Dio3 with a histone deacetylase inhibitor, VPA, reactivated the locus and rescued its ability to support full-term development of all-iPSC mice [40]. Later, it was shown that Vc treatment could help prevent loss of *Dlk1-Dio3* imprinting and facilitate generation of all-iPSC mice from terminally differentiated B cells [43•].

Nuclear receptors

Oct4 is arguably the most important reprogramming factor forming the core of the pluripotency network. Currently, both mouse and human iPSCs can be generated by ectopic expression of only Oct4 plus different combinations of small molecules in somatic cells [29, 44, 45]. However, an orphan nuclear receptor Nr5a2 was reported to replace Oct4 in iPSC generation in the presence of ectopic expression of KSM [46••]. This study highlighted the importance of nuclear receptors in reprogramming and the pluripotency state. Nuclear receptors are a large family of ligand-dependent or -independent transcription factors that have the ability to directly bind to DNA and regulate gene expression. Many nuclear receptors play important roles in stem cell regulation, including maintaining pluripotency and influencing stem cell differentiation [47].

A recent study added new evidence to support roles of nuclear receptors in iPSC reprogramming. It was found that ectopic expression of RARα/γ and Nr5a2 greatly enhanced reprogramming efficiency and kinetics [48]. Interestingly, both RARα agonist CD437 and RARγ agonist AM580 were further shown to significantly enhance reprogramming. It was postulated that during reprogramming the RAR ligands bind to RAR and cause the heterodimerization of RAR to RXR. This RAR:RXR complex binds to the Oct4 locus to activate and stabilize Oct4 expression and consequently facilitates further chromatin remodeling.

Another orphan nuclear receptor-Esrrb also plays an important role in ESC self-renewal. In addition to the core pluripotency circuitry consisting of Oct4, Sox2 and Nanog, Ivanova *et al.* showed that the nuclear receptor Esrrb, along with TBX3 and TCL1 could also regulate pluripotency in ESCs, thus forming a second regulatory axis [49]. When combined with Oct4 and Sox2, it could reprogram MEF cells into iPSCs [50]. Esrrb forms a complex with Oct4 and Sox2 to synergistically activate ESC specific gene expression in somatic cells.

Lineage conversion

An alternative to generating iPSCs from one somatic cell type and then differentiating them into other lineage-specific cell types is lineage conversion, a direct conversion of one cell type to another developmentally non-permissive cell type across lineage boundaries without passing through the pluripotent state. This strategy could be ultimately more attractive for *in vivo* therapy with reduced risks of tumorigenesis.

As early as 1987, Davis *et al* found that the overexpression of one transcription factor, MyoD, could convert fibroblasts into myoblasts [51]. Subsequently, many lineage conversion studies were reported, but the field became more maturely developed and widely accepted when the strategy of using multiple lineage-specific TFs was devised after the advent of iPSC reprogramming technology.

Lineage specific transcription factor-induced lineage conversion

In the conventional approach to lineage conversion, TFs or other genes (e.g., miRNAs) for lineage reprogramming are selected based on their key roles in cell fate specification in the embryonic development. The initially selected gene candidates will be pooled together and delivered to fibroblasts to test for cell lineage conversion. Later this pool is narrowed down by reducing factors to get a final best combination. Using this method, several cell types, including neurons generated from normal fibroblasts [52–58] and from Alzheimer's disease patient fibroblasts [59], cardiomyocytes [60, 61•], macrophages [62], and hepatocytes [63, 64], have been successfully converted from fibroblasts by ectopic expression of multiple lineage specific factors. This part of work has been well summarized in other reviews [65, 66].

Not only can cell fate be reprogrammed in a petri dish *in vitro*, lineage conversion was also achieved *in vivo* by virus mediated transduction of lineage specific TFs. For example, it was shown that ectopic overexpression of Ngn3, Pdx1 and Mafa by adenovirus converted pancreatic exocrine cells into β-cell like cells in adult mice. Induced β-cells had indistinguishable morphology and similar gene expression patterns as endogenous pancreatic β-cell and could ameliorate hyperglycemia in streptozotocin induced diabetic mice [67]. Recently, it was demonstrated that retroviral delivery of Gata4, MEF2C and Tbx5 in myocardium around coronary infarct zone converted cardiac fibroblasts into functional cardiomyocytes *in vivo* in adult mice [68•]. Those in vivo converted cardiomyocytes were more fully reprogrammed and more similar to endogenous cardiomyocytes than their *in vitro* reprogrammed counterparts using the same set of TFs. Another study demonstrated that the combination of Gata4, MEF2C, Hand2 and Tbx5 could achieve more robust *in vitro* and *in vivo* reprogramming of fibroblasts into cardiomyocytes [61•]. These studies revealed that the native microenvironment, including extracellular matrix, secreted cytokines, cell-cell contacts and tissue stiffness, may promote cell survival and/or maturation that further enhances lineage conversion. Therefore, small molecules regulating various aspects of lineage reprogramming may further increase efficiency of lineage conversion and, ideally, replace transcription factors to convert one resident cell type into another functional cell type *in vivo* for therapeutic applications.

A recent study found that the combination of three small molecules, CHIR99021 (a GSK3 inhibitor), SB-431542 and LDN-193189 (a BMP receptor inhibitor), could enhance reprogramming of human fibroblasts into neurons with only two transcription factors, Ascl1 and Ngn2 [69•]. As reviewed earlier, blockade of the TGFβ/SMAD pathway also enhances iPSC reprogramming through promoting MET. Further studies would be useful to address whether and how MET is involved in neuronal reprogramming from fibroblasts.

iPSC factors induced lineage conversion

An alternative strategy to the above conventional lineage conversion method was recently developed that employs transient overexpression of iPSC-TFs in conjunction with lineage specific soluble signals to reprogram somatic cells into diverse lineage-specific cell types without entering the pluripotent state.

This paradigm came from the study of iPSC reprogramming, in which iPSCs are generated through a lengthy and inefficient process with stochastic events. Only a few cells finally become pluripotent but many cells "land" in other non-pluripotent states. We hypothesized that initial overexpression of iPSC-TFs may induce an "epigenetic activation" (e.g., destabilizing and erasing starting cell's epigenetic state, and enabling more permissive states for genes in other lineages), and that temporally controlled expression of exogenous iPSC-TFs could interact with TFs downstream of lineage-specific signals (i.e., culture conditions)

to initiate transcriptional cascades and establish the reprogrammed cell's epigenetic landscape.

With this rationale, we found that mouse fibroblasts could be converted into cardiomyocytes through temporally restricted expression of Oct4, Klf4, Sox2 (as short as 4 days) followed by BMP4 treatment, without entering the pluripotent state [70••]. During this process, a JAK inhibitor (JI1) was added into the culture condition to block iPSC formation and increase the lineage conversion efficiency. Compared with previous methods of cardiac lineage conversion by overexpression of cardiac specific transcription factors, it is more efficient and less time consuming. The spontaneously beating cell patches were observed as early as 11 days post induction. Importantly, those mature (cTnT+) cardiomyocytes were generated through cardiac precursor (Flk-1+ and Isl1+) populations using this method. Due to issues of the required number of cells for transplantation and cell survivability *in vivo*, proliferating lineage-specific progenitor cells could be more promising for future regenerative therapies than terminally differentiated cells. Through a similar strategy, neural progenitor cells (NPCs) can also be reprogrammed from fibroblasts by transient expression of the iPSC reprogramming factors followed by treatment with corresponding cell lineage-specific growth factors and small molecules [71]. Remarkably, it was demonstrated that the induced NPCs could be isolated, expanded *in vitro*, and then further differentiated into functional neuronal and glial cell types. Recently, two other groups also successfully derived mouse neural stem cells (NSCs) using transduction of Sox2, Klf4, c-myc, together with either two other transcription factors, Brn4 and E47, or transient induction of Oct4 [72, 73]. Importantly, those induced NSCs can differentiate into neurons, astrocytes and oligodendrocytes, and maintain their differentiation potential over many passages.

Compared with conventional lineage conversion, this new method has several advantages. Expression of a single set of TFs could be better optimized for different cell lineages. In addition, transient expression of TFs might be more amenable to non-integrating or non-genetic methods for inducing lineage conversion, such as using miRNA, mRNA and small molecules. Furthermore, generating multipotent progenitor populations could be more useful than reprogramming directly into mature non-proliferative cells, as is common with conventional lineage reprogramming, for many applications. The mechanisms of conventional and iPSC-TF mediated lineage conversion are quite different. In the conventional paradigm, a cell is forced to adopt another fate by master transcription factors of the target cell type, whereas in iPSC-TF mediated lineage conversion, the original cell fate is destabilized and the cells are partially "dedifferentiated" by the iPSC-TFs, enabling their differentiation to be directed and patterned with soluble factors. Because the trans-differentiation of the iPSC-TF initiated cells follows, in part, natural development, this method can benefit from the field of directed differentiation. It will remain a question which paradigm will be more useful for various applications in terms of efficiency and fidelity until more thorough investigations have been performed.

Collectively, the four conventional iPSC factors not only induce reprogramming to iPSCs, but also are capable of mediating direct cell fate switching between somatic cells. Changing the duration of transgene expression and culture conditions may allow establishing a transient, plastic state and effectively serve as a cellular platform for lineage conversion toward various lineages. With continued advances in iPSC technology, many small molecules that have been identified to enhance iPSC reprogramming may also have a positive role in lineage-specific programming, especially in iPSC-reprogramming-factors-induced lineage conversion. For example, small molecules that modulate epigenetic processes may promote erasure of the original epigenetic state of initial cells and accelerate the maturation and increase the function of transdifferentiated cells.

Conclusions and perspectives

As reviewed above, small molecules are not only valuable to significantly promote cellular reprogramming and functionally substitute ectopic expression of TFs, but also provide insights into molecular mechanisms underlying this process. By manipulating these interconnected molecular mechanisms and physiological processes using small molecules, higher reprogramming efficiency and better reprogramming quality can be obtained (Fig. 1). Ultimately, complete small-molecule-based reprogramming in a directed and deterministic manner will fundamentally change the reprogramming paradigm through a mechanism that involves activation of endogenous TFs by small molecules rather than by exogenously provided reprogramming TFs. To achieve this, a better understanding of the reprogramming process and an improved ability to identify new reprogramming-inducing small molecules under new cellular contexts is required.

Unlike the array of small molecules that have been uncovered and studied in iPSC reprogramming, so far very few small molecules have been investigated in lineage conversion. Considering some similar mechanisms involved in both iPSC reprogramming and lineage-specific lineage conversion, we can speculate that some of the small molecules regulating above-mentioned pathways in iPSC reprogramming may also increase the efficiency and cell quality in lineage conversion. It is conceivable that small molecules could be identified and further developed to activate lineage conversion of resident cells into other functional cells in a highly controlled manner *in vivo* and be used as a regenerative treatment.

The concerns about genetic, genomic and epigenetic abnormalities that could have unpredictable and undesirable effects on the cells are a major challenge to future clinical applications of reprogrammed cells. Considering CNVs are generated mainly by NHEJ and can be minimized by HR, HR-promoting chemicals may substantially promote the maintenance of genomic integrity. To date, there have been no published studies reporting the genome status in transdifferentiated cells, such as induced neurons and cardiomyocytes. Extensive analysis should be established to ensure the safety of iPSCs and directly transdifferentiated cells before their clinical use. With advances in mechanistic understanding of reprogramming processes and continued developments of small molecule tools to enhance these reprogramming processes, safer and higher quality reprogrammed cells through a more efficient process are within our reach.

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• of special interest

•• of outstanding interest

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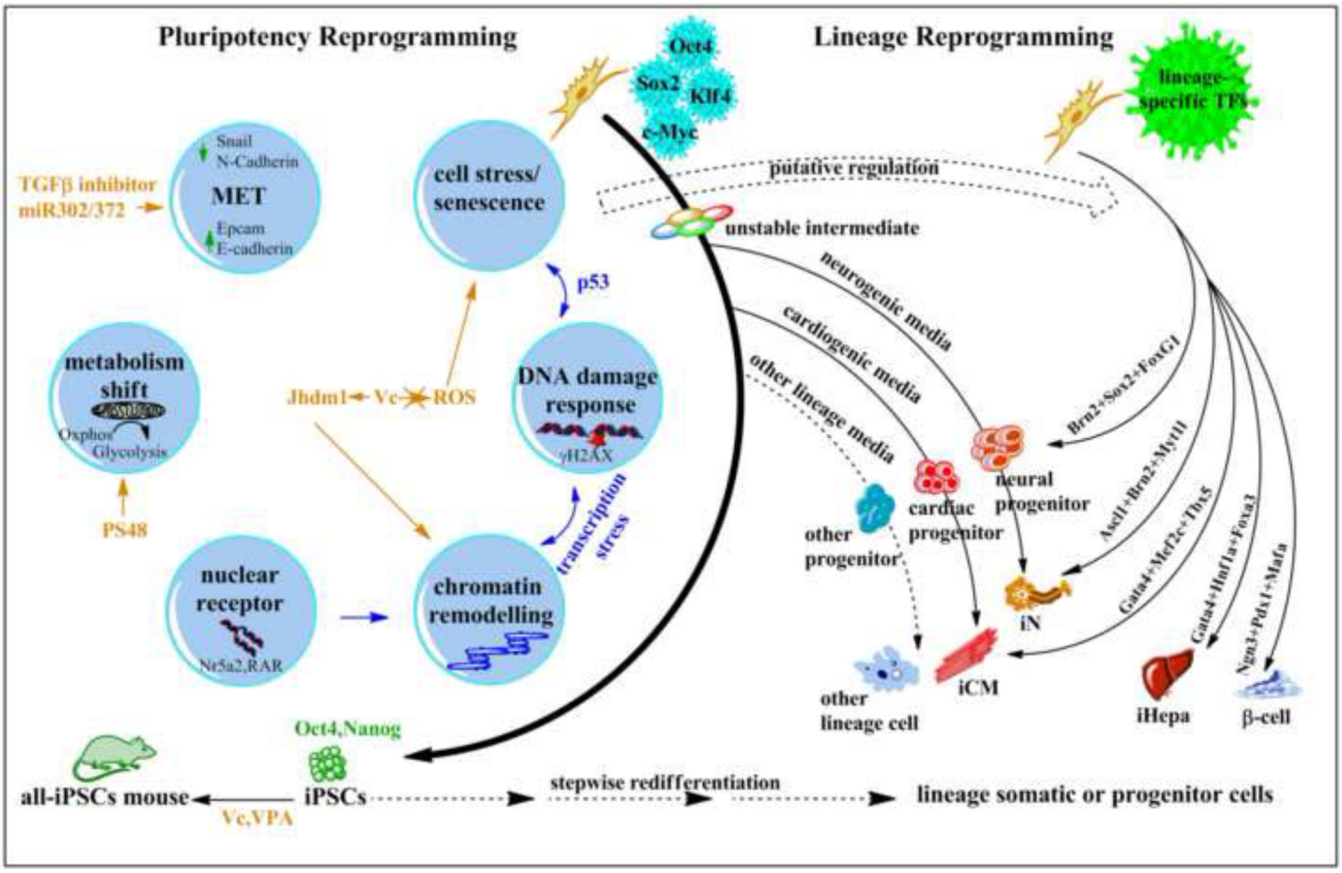

Figure 1. Schematic model of cellular reprogramming and the regulation pathways

Pluripotency reprogramming is a slow and stochastic process that is regulated by several interconnected mechanisms. Transient overexpression of reprogramming factors in fibroblasts leads to the rapid generation of epigenetically “activated” cells (unstable intermediate), which can be coaxed to various cell states by using lineage-specific conditions. iPSCs are one of the outcomes. Besides iPSCs, many lineage-specific cells and progenitor cells can be obtained. Only some representative small molecules and transcription factors are shown here.