Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming

Wenlin Li and Sheng Ding*

Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Recent breakthroughs in stem cell biology, especially the development of induced pluripotent stem cell technique, have generated tremendous enthusiasm and efforts to explore the therapeutic potential of stem cells in regenerative medicine. Improved understanding of stem cell biology, in addition to better control of stem cell fate, is critical to realize this potential. Small molecules, targeting specific signaling pathways and/or mechanisms, have been shown to be useful chemical tools in manipulating cell fate, state and function. These small molecules are starting to play increasingly important roles in both elucidating the fundamental biology of stem cells and facilitating the development of therapeutic approaches toward regenerative medicine. Such approaches could involve cell replacement therapies using homogenous functional cells produced under chemically defined conditions in vitro and the development of small-molecule drugs that can stimulate patients’ endogenous cells to repair and regenerate. Here, we review recent progress in using small molecules to sustain pluripotency, or induce differentiation of embryonic stem cells. We also highlight small molecules that can replace transcription factors and/or enhance efficiency during somatic cell reprogramming.

Introduction

Stem and progenitor cells are less differentiated/specialized cells that have the ability to self-renew for an extended period of time and to differentiate into more specialized cell types under appropriate conditions. On the basis of their differentiation potential, stem cells can be pluripotent or multipotent. Pluripotent stem cells can be expanded indefinitely and give rise to all cell types in the body; they typically include embryonic stem (ES) cells derived from the inner cell mass (ICM) of pre-implantation embryos [1,2], germline stem cells and derivatives [3-6], epiblast stem cells (EpiSCs) derived from the late epiblast layer of post-implantation embryos [7,8], and induced pluripotent stem (iPS) cells from somatic cells [9]. Multipotent stem cells usually have limited life span in culture and can develop only into cells within the same cell lineage. These cells typically play a critical role in maintaining tissue homeostasis by giving rise to new cells of the tissue where they originated and reside.

Stem cells not only are excellent model systems in which to study the fundamental biology of human development and tissue homeostasis, but also offer significant promise for developing treatments for devastating human diseases and injuries. Understanding the mechanisms and devising improved approaches to control cell fate and function in vitro and in vivo are crucial steps toward translating stem cells and their modulators into the clinic. Small molecules that can modulate specific target(s) in signaling and epigenetic mechanisms have been shown to be useful chemical tools for manipulating cell fate [10,11], and clearly offer some distinct advantages over genetic manipulation. For example, in contrast to genetic manipulation, the effects of small molecules are typically fast and reversible, providing more precise temporal regulation of protein function. These effects can also be finely tuned by varying the concentration and combination of the small molecules of interest. Furthermore, the almost unlimited structural and functional diversity endowed by synthetic chemistry provides small molecules with unbounded potential to precisely control molecular interactions and/or recognition, a feature that can be extensively explored by design and screening.

Glossary

**Totipotent**: Having the ability to give rise to an entire functional organism. In mammals, the fertilized egg and early embryonic cells are totipotent. Totipotent cells not only have the potential to give rise to all of the cell types of the body, but also the cell types of entire extraembryonic tissues.

**Pluripotent**: Having the potential to give rise to all of the cell types of the body, but not the cell types that make up the extraembryonic tissues such as the placenta. Embryonic stem cells derived from the inner cell mass of pre-implantation embryo are pluripotent. Pluripotent stem cells also include germline stem cells and derivatives, epiblast stem cells (EpiSCs) derived from the late epiblast layer of post-implantation embryos, and induced pluripotent stem (iPS) cells from somatic cells. Particularly, human embryonic stem cells (or mouse EpiSCs) can differentiate into trophoblast by BMP4 treatment. Whether it is a kind of artificial potential caused by in vitro conditions is still unknown.

**Multipotent**: Having the ability to develop into different cell types within the same cell lineage. For example, hematopoietic stem cells that are multipotent can give rise to all the blood cell types.

**Blastocyst**: A pre-implantation embryo of about 150 cells produced by cleavage of the fertilized egg. The blastocyst is a hollow sphere made up of an outer layer of cells called trophoblast (forming the placenta eventually), a fluid-filled cavity, and a cluster of cells called inner cell mass.

**Inner cell mass (ICM)**: The cluster of cells attached to the wall of the blastocyst. These cells eventually give rise to all the organs and tissues of the whole organism, but do not give rise to the extraembryonic placenta tissues. The ICM cells are used to generate embryonic stem cells.

**Epiblast**: The cell population in the post-implantation embryo that develops from the inner cell mass. The epiblast represents the final embryonic founder cells with the potential to give rise to all cell types of the adult body.

*Corresponding author:Ding, S. (sding@scripps.edu)

0165-6147/$ – see front matter © 2009 Elsevier Ltd. All rights reserved. doi:10.1016/j.tips.2009.10.002 Available online 4 November 2009
In this review, we discuss chemical approaches to controlling the self-renewal and differentiation of ES cells. In addition, we also discuss recent developments in using small molecules to replace transcription factors and/or enhance efficiency during somatic cell reprogramming. The mechanism of some small molecules will also be discussed.

Self-renewal of ES cells

ES cells are usually maintained and expanded on feeder cells in the presence of serum or serum fractions (e.g. serum replacement) which contains animal serum or undefined components derived from serum, and additional exogenous factors, for example, leukemia inhibitory factor (LIF) for mouse ES (mES) cells [12], and basic fibroblast growth factor (bFGF) for human ES (hES) cells [2]. These undefined culture conditions present many problems. In particular, the use of feeder cells and serum products (which can differ from batch to batch) can compromise the consistency of the ES cell culture, and complicate biological studies of a particular cellular process. In practice, the use of animal products to culture hES cells will also hinder the clinical utility of these cells. Consequently, identifying small molecules that can support ES cell self-renewal in serum- and feeder-free conditions is highly desirable for both basic research and regenerative medicine.

Usually, ES cells are derived from the ICM of blastocyst, the pre-implantation embryo with a hollow sphere structure formed by cell division following fertilization (Figure 1). However, although mES and hES cells can be typically derived by in vitro culture of blastocysts [1,2], hES cells show significant differences in phenotype and signaling response as compared with mES cells [13], and correspond closely to EpiSCs derived from the post-implantation egg cylinder stage epiblasts of mouse and rat [7,8] (Figure 1, the properties of different pluripotent cells are summarized in Table 1). For example, LIF is insufficient to support either hES cells or EpiSCs, whereas bone morphogenetic protein 4 (BMP4), which is required in conjunction with LIF for self-renewal by mES cells [14,15], induces hES cells or EpiSCs to differentiate into trophoblasts or primitive endoderm [7,8,16]. In addition, bFGF and Activin A (a cytokine of the transforming growth factor β superfamily involved in a wide range of biological processes including tissue morphogenesis, repair, fibrosis, inflammation, and carcinogenesis) seem to be dispensable for mES cell self-renewal, but are required by EpiSCs and hES cells for self-renewal [17] (The signaling pathways controlling pluripotent stem cell self-renewal is described in Figure 2). These data support the notion that mES cells represent pre-implantation ICM-stage pluripotent stem cells, whereas hES cells and EpiSCs represent a later epiblast stage of pluripotent cells. Because EpiSCs show little competency to incorporate into the ICM of the blastocyst following microinjection and give rise to chimaeras (which are comprised of cell populations and tissues arising from both the host blastocyst strain and the injected cells), it is important to identify the conditions and/or small molecules that can sustain self-renewal of ICM-state ES cells from other invaluable model animals, such as rats and other refractory mouse strains, for use in various applications.

**Maintenance of mES cell self-renewal**

To identify small molecules that can sustain self-renewal of mES cells in chemically defined conditions, we previously carried out a high-throughput screen of synthetic small molecule libraries under a feeder-, serum- and LIF-free environment.
We identified a novel compound named pluripotin/SC1 (Table 2), which can maintain long-term homogeneous self-renewal of mES cells under a chemically defined condition in the absence of feeder cells, serum, LIF or BMP4 in vitro without the cells losing their competence to incorporate into various tissues in chimeric mice, including germline tissues [18]. Mechanistically independent from activation of the self-renewal pathways that have been conventionally regarded to be essential (i.e. LIF/STAT3 [12], BMP4/Smad-Id [14] and Wnt/β-catenin [19–21]), pluripotin has been characterized as a dual inhibitor of RasGAP and extracellular signal-regulated kinase-1 (ERK1), two endogenously expressed differentiation-inducing proteins. Inhibition of RasGAP promotes ES cell self-renewal by enhancing the phosphoinositide-3 kinase (PI3K) signaling pathway [22], whereas ERK1 inhibition blocks ES cell differentiation [15,23] (Figure 2).

From a stem cell perspective, maintenance of self-renewal can be viewed as a balanced activity of continued proliferation and inhibition of differentiation and cell death through cross-regulation among positive and negative regulators. The fact that pluripotin can maintain ES cell self-renewal independent of the exogenous activation of conventional self-renewal pathways by simply inhibiting the activity of endogenous differentiation-inducing proteins has provided a fundamental new view on the mechanism of ES cell self-renewal: ES cells possess an intrinsic ability to maintain pluripotency and do not require exogenous stimulation of additional gene expression. At the same time, endogenous expression of some differentiation-inducing proteins at a certain level in undifferentiated ES cells may poise cells to differentiate. Therefore, the key to sustaining ES cells (or other types of stem cell) in a self-renewal state is to inhibit the negative effects of endogenously expressed inducers of differentiation or cell death. This conceptual advance in stem cell self-renewal is also supported by a more recent study, in which a combination of specific chemical inhibitors (CHIR99021 and PD0325901; Table 2) of glycogen synthase kinase-3 (GSK3) and mitogen-activated protein kinase (MEK) similarly supported the derivation and long-term self-renewal of mES cells in the absence of exogenous cytokines [24]. It should be noted that inhibition of GSK3 activates the Wnt/β-catenin pathway, and on its own induces robust mesoderm differentiation of ES cells in the absence of other self-renewal cytokines or small molecules. Those small molecules that can support ES cell maintenance not only are useful for delineating the self-renewal regulatory mechanisms, but also provide a platform for a more consistent and robust cell culture as well as derivation of new cell lines from difficult strains or species. For example, pluripotin and GSK3/MEK inhibitors can facilitate the derivation of mES cell lines from refractory strains [25,26], such as non-obese diabetes/severe combined immunodeficiency
<table>
<thead>
<tr>
<th>Molecule Name</th>
<th>Target(s)</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluripotin/SC1</td>
<td>RasGAP and ERK1</td>
<td>Supports mES cell self-renewal [18]</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3</td>
<td>Activates Wnt signaling, Supports ES cell self-renewal [24,27]</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK</td>
<td>Blocks differentiation pathway of mES cells [24]</td>
</tr>
<tr>
<td>A83-01</td>
<td>ALK5, ALK4 and ALK7</td>
<td>Supports riPS cell long-term self-renewal when combined with CHIR99021 and PD0325901 [27]</td>
</tr>
<tr>
<td>IDE1</td>
<td>Unknown</td>
<td>Induces Smad2 phosphorylation in ES cells and induces endoderm differentiation [49]</td>
</tr>
<tr>
<td>(-) Indolactam V</td>
<td>PKC</td>
<td>Enhances the pancreatic differentiation of ES cell derived definitive endoderm [50]</td>
</tr>
<tr>
<td>Stauprimide</td>
<td>NME2</td>
<td>Enhances differentiation of ES cells [52]</td>
</tr>
<tr>
<td>SB431542</td>
<td>ALK5, ALK4 and ALK7</td>
<td>Induces high efficiency neural differentiation of hES cells together with Noggin [53]</td>
</tr>
<tr>
<td>BIX-01294</td>
<td>G9a HMTase</td>
<td>Promotes reprogramming of NPCs and MEFs transduced by Oct4/Klf4 [66,71]</td>
</tr>
</tbody>
</table>
(NOD-SCID) and SCID beige mice whose ES cells are difficult to derive under conventional conditions.

**Capture of pluripotent cells from another species**

Although ES cells have been established from mice since 1981 [1], attempts to generate their counterparts from rats were not completely successful until recently [27–29]. The generation of authentic rat pluripotent cells is another excellent example that highlights the power of small molecules to capture and sustain the right pluripotent state of cells from species that were previously thought to be non-permissive [30–33]. By combining genetic reprogramming and cell signaling modulation using small molecules [27], we successfully induced, captured and maintained “mES cell-like” rat pluripotent cells from rat somatic cells. A diploid rat liver progenitor cell line WB-F344 [34] was transduced with Oct4, Sox2 and Klf4. Compact, alkaline phosphatase (ALP)-positive ES cell-like colonies were observed, but could not be maintained under the conventional cell culture condition used for mES cells. By using a combination of MEK inhibitor (PD0325901), GSK3 inhibitor (CHIR99021) and LIF, rat induced pluripotent stem cells (riPS cells) could be short-term maintained in culture but displayed extensive spontaneous differentiation. By the addition of another small-molecule TGFβ receptor inhibitor (A-83-01; Table 2), spontaneous differentiation of riPS cells was substantially inhibited and the cells were maintained as a more homogeneous self-renewing population. Because the TGFβ/Activin A/Nodal signaling cascade is essential for maintaining undifferentiated hESCs and EpiSCs, but is dispensable for mES cell self-renewal and induces differentiation of mES cells, the TGFβ pathway inhibitor might be able to shield riPS cells from differentiation-inducing effects mediated by TGFβ signaling. Under this condition, riPS cells are akin to the mES cells in forming typical domed colonies in culture, expressing conventional pluripotency markers, and being competent to generate teratoma and contribute to chimeras. Concurrent studies also showed that rat ES cells could be derived from rat blastocysts under a chemically defined condition in the presence of PD0325901, CHIR99021 and LIF [28,29]. Rats are better suited for physiological and behavioral studies than mice and have the potential to provide excellent models for multigenic human diseases. Establishment of the above authentic rat pluripotent stem cells [27–29] will pave the way to generate gene-targeted rats for biomedical research. Using the same conditions for generating the riPS cells, we have also established mES cell-like (i.e. ICM-state) human induced pluripotent stem (hiPS) cells [27], which can be maintained long term in the presence of MEK inhibitor, TGFβ receptor inhibitor and GSK3 inhibitor. These novel mES cell-like hiPS cells, which express typical pluripotency markers and generate teratoma after transplantation into SCID mice, seem to bypass the requirement of bFGF and TGFβ signaling for self-renewal, representing a new pluripotent

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Name</th>
<th>Target(s)</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG108</td>
<td>DNA MTase</td>
<td>Promotes MEF reprogramming [71]</td>
<td></td>
</tr>
<tr>
<td>(+)-Bayk 8644</td>
<td>L-type Ca²⁺ channel</td>
<td>Promotes MEF reprogramming [71]</td>
<td></td>
</tr>
<tr>
<td>Parnate</td>
<td>Lysine-specific demethylase 1</td>
<td>Enable the reprogramming of human keratinocytes transduced by Oct4/Klf4</td>
<td></td>
</tr>
<tr>
<td>Kenpaullone</td>
<td>CDKs and GSK3</td>
<td>Replace Klf4 to induce MEF reprogramming [76]</td>
<td></td>
</tr>
<tr>
<td>Valproic Acid</td>
<td>Histone deacetylase</td>
<td>Promotes reprogramming, enable the reprogramming of human fibroblasts transduced by Oct4/Sox2 [73,74]</td>
<td></td>
</tr>
<tr>
<td>Reversine</td>
<td>Nonmuscle myosin II heavy chain and MEK1</td>
<td>Induces dedifferentiation of muscle or fibroblast cells to a more primitive multipotent state [78–81]</td>
<td></td>
</tr>
</tbody>
</table>
state for human ES cells that is similar to conventional mES cells but different from conventional hES cells. Recent studies show that mouse EpiSCs can be converted into an ES cell state by ectopic expression of Klf4 [35]. Whether mES cell-like human pluripotent stem cells can be derived from human blastocysts or converted from hES cells under similar conditions, however, requires further investigation.

**Self-renewal by physiological small molecules**

Recent studies have also shown that physiological small molecules can enhance the self-renewal of ES cells. Chen et al. reported that retinol (vitamin A) can support feeder-independent self-renewal of mES cells in long-term culture by modulating the expression of Nanog [36]. Nanog is a master transcriptional factor in maintaining ES cell pluripotency. Garcia-Gonzalo et al. showed that albumin-associated lipids in knock-out serum replacement/KSR are responsible for the effects of KSR on promoting hES cell self-renewal [37]. However, the direct targets and/or mechanisms of these physiological small molecules remain unclear.

The discovery of small molecules in maintaining ES cell self-renewal not only provides valuable tools to explore ES cell biology, but also enables better conditions to practically expand ES cells as a cell source for various applications.

**Differentiation of ES cells**

Differentiation is a process in which unspecialized cells progress to become more specialized and functional cells with restricted developmental potential. Conventionally, the differentiation of ES cells involves growing the cells in suspension to form aggregates called embryoid bodies (EBs), which then differentiate spontaneously into various cell types, including derivatives of all of the three germ-layers of ectoderm, mesoderm, and endoderm. However, such spontaneous differentiation in vitro is typically an inefficient and non-specific process, and the cell types of interest usually have to be selected from a very heterogeneous cell population by various means such as specific marker expression. From the same practical and mechanistic perspectives discussed earlier, developing small molecules for their ability to induce mES cells to express endogenous Sox17 [52], a compound, named staurprimide (Table 2), was found to promote efficient induction of Sox17 in both mouse and human ES cells in the presence of low concentrations of Activin A. Interestingly, staurprimide acts synergistically with Activin A and does not induce endoderm differentiation in the absence of Activin A. Affinity-based target identification revealed that staurprimide can interact with NME2, a c-Myc-activating transcription factor, and inhibit its nuclear localization. Upon staurprimide treatment, the resulting down-regulation of c-Myc can destabilize the self-renewal state of ES cells, which renders ES cells more vulnerable to differentiation cues. Indeed, further studies showed that staurprimide enhances not only endoderm differentiation of ES cells, but also differentiation toward mesoderm and ectoderm lineages under appropriate lineage-specifying conditions.

Another recent study by Chambers et al. described an efficient neural induction method for hES cells that bypasses EB formation [53]. They found that a combination of Noggin (a secreted protein that binds to and

**Enhanced differentiation of stem cells**

As discussed above, inhibition of differentiation and cell death can promote self-renewal of ES cells. Conversely, blockade of the self-renewal (e.g. anti-differentiation) mechanism can also enhance differentiation of ES cells. In a recent study, Zhu et al. screened more than 20,000 small molecules for their ability to induce mES cells to express endogenous Sox17 [52]. A compound, named staurprimide (Table 2), was found to promote efficient induction of Sox17 in both mouse and human ES cells in the presence of low concentrations of Activin A. Interestingly, staurprimide acts synergistically with Activin A and does not induce endoderm differentiation in the absence of Activin A. Affinity-based target identification revealed that staurprimide can interact with NME2, a c-Myc-activating transcription factor, and inhibit its nuclear localization. Upon staurprimide treatment, the resulting down-regulation of c-Myc can destabilize the self-renewal state of ES cells, which renders ES cells more vulnerable to differentiation cues. Indeed, further studies showed that staurprimide enhances not only endoderm differentiation of ES cells, but also differentiation toward mesoderm and ectoderm lineages under appropriate lineage-specifying conditions.

Another recent study by Chambers et al. described an efficient neural induction method for hES cells that bypasses EB formation [53]. They found that a combination of Noggin (a secreted protein that binds to and

**Directed differentiation of stem cells**

Improved understanding of signaling mechanisms governing cell specification in development has guided the design of directed differentiation strategies of stem cells. Through recapitulation of appropriate developmental steps with stage- and lineage-specific modulators (e.g. cytokines and small molecules) in vitro, various cell types have been generated from ES cells via more defined and stepwise differentiation [44–48]. Recent efforts have focused on identifying small molecules that can mimic the signal requirement during development and direct differentiation.

Using mES cells stably transfected with a pSox17-dTomato reporter (Sox17 is a marker for endoderm progenitor cells), Borowiak et al. screened a collection of 4,000 compounds for their ability to induce Sox17 expression under low serum differentiation condition without Activin A, which typically was used to induce endoderm specification. The primary hits were then filtered in secondary assays that confirmed the generated Sox-17-dTomato positive epithelial cells were negative for extraembryonic cell markers. Two structurally similar small molecules, IDE1 (Table 2) and IDE2, were identified to induce definitive endoderm differentiation in up to 80% of mES cells (or 50% of hES cells) treated in the absence of Activin A [49]. Similar to Activin A and Nodal (a natural inducer of definitive endoderm), both IDE1 and IDE2 induce Smad2 phosphorylation in mES cells through unknown targets. Interestingly, the endoderm-like cells induced by IDE1 and IDE2 can further differentiate into a pancreatic lineage when exposed to another small molecule Indolactam V (Table 2), an activator of protein kinase C (PKC). Indolactam V was identified by screening compounds for their ability to induce Pdx1 expression from hES cell-derived definitive endoderm cell [50]. Pdx1 is a master transcription factor regulating pancreas development and mature beta cell functions [51]. It was found that Indolactam V enhanced the pancreatic differentiation of definitive endoderm, rather than promoting the proliferation of Pdx1-positive cells already present in the hES cell-derived endodermal population. The Pdx1-positive cells can further differentiate into more mature beta cells in vitro and in vivo.

**Review**

Trends in Pharmacological Sciences Vol.31 No.1
inhibits BMP4) and SB431542 (a small-molecule inhibitor of the TGFβ receptor; Table 2) can promote rapid neural induction of more than 80% of hES cells in a monolayer fashion, although other undefined medium supplements (e.g. serum fractions) were also used. Those two pathway inhibitors might function synergistically to destabilize self-renewal (e.g. TGFβ signaling is essential for self-renewal of hES cells) and to prevent cells from differentiating into trophectoderm, mesoderm and endoderm lineages (for which BMP signaling has an inductive effect). These studies reinforce the notion that directed ES cell differentiation toward a specific lineage can be enhanced by the deliberately combining inductive treatment of the cell lineage and self-renewal inhibitory signals.

To increase substantially the homogeneity, yield and functionality of the cell types of interest, identifying additional small molecules that can regulate various types of lineage specification and determination would be highly desirable toward the ultimate attainment of specific, efficient and completely chemically defined ES cell differentiation conditions. Such small molecules would not only hold promise for the development of regenerative medicines, but also serve as useful tools with which to investigate the fundamental biology of cell fate regulation.

Somatic cell reprogramming

iPS cells generated from somatic cells by defined genetic factors have attracted enormous interest [9,54–58]. iPS cells closely resemble ES cells in gene expression, epigenetic signature and functional pluripotency. The simplicity of such a genetic reprogramming approach has opened up tremendous opportunities to generate patient-specific cells for disease modeling as well as potential therapeutic applications without the controversies associated with conventional hES cells. However, there are critical concerns about the genetic technique currently used to generate iPS cells. This technique is limited by the use of virus-mediated delivery of reprogramming factors, which could result in permanent integration of oncogenes (e.g. c-Myc and Klf4) and potential harmful genomic alterations. Some key advances aimed at overcoming these safety concerns have been achieved by using non-integrating gene delivery approaches (such as adenovirus or episomal plasmid transfection) [59–61], or using cell penetrating recombinant proteins to trigger the reprogramming [62,63]. However, reprogramming is extremely slow and inefficient under those conditions. The low efficiency and slow kinetics also may present ‘hidden’ risks in iPS cells, such as the accumulation and selection of subtle genetic and epigenetic abnormalities during the reprogramming process, where cell growth pathways are activated and tumor suppressor pathways are suppressed [64]. Consequently, it is highly desirable to identify new conditions and small molecules that can promote reprogramming and ultimately replace all reprogramming transcription factors.

Recently, we and others have shown that the endogenous expression of certain gene(s) in somatic cells can substitute for the over-expression of exogenous reprogramming factors and facilitate the generation of iPS cells [65–67]. Neural progenitor cells (NPCs), which express Sox2 endogenously [68], were reprogrammed into authentic iPS cells with transduction of only Oct4 and Klf4. Such a proof-of-principle demonstration points to an attractive strategy for generating iPS cells with better quality using less genetic manipulation by exploiting other, more practically accessible cell types (than fibroblasts) that endogenously express certain relevant reprogramming factors and/or have a more reprogramming-favorable epigenetic state via the cell intrinsic program (e.g. cell type- and state-specific) and/or ex vivo culture manipulation [66]. Using a phenotypic screen in the NPCs, we also identified a small-molecule inhibitor of G9a histone methyltransferase, BIX-01294 [69] (Table 2), that can significantly improve reprogramming efficiency. Importantly, BIX-01294 treatment also enabled the reprogramming of NPCs transduced with Sox2/Klf4/c-Myc, therefore bypassing the need of Oct4. Because G9a mediates the epigenetic repression of Oct4 during embryonic development [70], G9a inhibition by BIX-01294 might facilitate the derepression of Oct4 and promote reprogramming. In parallel, we also found that BIX-01294 can facilitate the reprogramming of mouse embryonic fibroblasts (MEFs) in the absence of Sox2 expression under the Oct4 and Klf4 two-factor condition [71]. A subsequent chemical screen in fibroblasts in the presence of BIX-01294 was also performed to identify small molecules that could synergize with BIX-01294 to increase reprogramming efficiency under the Oct4 and Klf4 two-factor condition [71]. A DNA methyltransferase inhibitor, RG108, and an L-type calcium channel agonist, BayK8644, were identified to have significant reprogramming promoting activity. Whereas RG108, like BIX-01294, acts as a direct epigenetic modifier to facilitate shifting epigenetic landscapes for cell reprogramming, it is interesting to find that BayK8644, which acts on up-stream signaling pathways that have not been previously linked to pluripotency and reprogramming, can significantly enhance reprogramming efficiency. Such a molecule might induce a more robust reprogramming or regeneration with better specificity in vivo for cells that are already undergoing a form of reprogramming resembling the one associated with tissue regeneration after injury, without reprogramming other healthy cells and/or tissues systemically. Consistent with a general reprogramming mechanism, several studies have also shown that other commonly used, small-molecule direct modifiers of epigenetics, including histone deacetylase inhibitors (e.g. valproic acid, trichostatin A and suberoylanilide hydroxamic acid) and a DNA methyltransferase inhibitor (e.g. 5-azacytidine), can improve mouse and human somatic cell reprogramming [72–74]. In particular, valproic acid (Table 2) facilitated induction of mouse and human pluripotent stem cells without introduction of c-Myc and enabled human fibroblasts to be reprogrammed with two factors (Oct4 and Sox2) [73]. By producing the four purified and refolded recombinant reprogramming proteins that were tagged with 11-arginine at the C-terminal and simply adding them to the cell culture media with valproic acid, Zhou et al. reported the generation of mouse protein-induced pluripotent stem (piP) cells from MEFs without the use of any genetic material and genetic manipulation [63]. Such
proof-of-concept protein transduction method represents an alternative chemically defined method to avoid introducing exogenous genetic modifications to target cells. Wnt/β-catenin signaling has been shown to promote self-renewal of ES cells under specific conditions [19–21]. Previous studies showed that Wnt3a conditioned medium promotes reprogramming of MEFs [75]. In our studies, we found that the GSK3 inhibitor CHIR99021 can significantly improve the reprogramming efficiency of MEFs transduced by Oct4, Sox2 and Klf4, and also facilitate the reprogramming of MEFs transduced by only Oct4 and Klf4. When combined with Parnate (Table 2), a lysine-specific demethylase 1 inhibitor, CHIR99021 can also result in the reprogramming of human primary keratinocytes transduced with only Oct4 and Klf4. Using MEFs derived from Nanog-luciferase reporter mice, Lyssiotis et al. screened 500,000 compounds and identified another GSK3/CDKs inhibitor, kenpaullone (Table 2), that can replace CHIR99021 in reprogramming of MEFs transduced with Oct4, Sox2 and cMyc. However, kenpaullone has demonstrated inhibition toward various other kinases and a more specific GSK3 inhibitor, CHIR99021, failed to produce the same effects on reprogramming of MEFs under the same Oct4, Sox2 and cMyc transduction, the mechanism of action by which kenpaullone facilitates reprogramming remains elusive. We had found that the MEK inhibitor PD0325901, when applied at a late stage of reprogramming, can stabilize and select the true iPS cells [66]. Most recently, we found that the dual inhibition of MEK and TGFβ inhibitor, kenpaullone (Table 2), that can replace CHIR99021 in reprogramming of MEFs transduced with Oct4, Sox2 and cMyc transduction, the mechanism of action by which kenpaullone facilitates reprogramming remains elusive. We had found that the MEK inhibitor PD0325901, when applied at a late stage of reprogramming, can stabilize and select the true iPS cells [66]. Most recently, we found that the dual inhibition of MEK and TGFβ inhibitor, kenpaullone (Table 2), that can replace CHIR99021 in reprogramming of MEFs transduced with Oct4, Sox2 and cMyc transduction, the mechanism of action by which kenpaullone facilitates reprogramming remains elusive. We had found that the MEK inhibitor PD0325901, when applied at a late stage of reprogramming, can stabilize and select the true iPS cells [66].

Perspective

Stem cell research and development are still in an early stage, but have been experiencing substantial growth in recent years. In addition to the focus on pluripotent stem cell reviewed here, understanding and controlling more relevant adult cell fate, state and function in vitro and in vivo represent both another area of active research and a significant challenge for developing better therapeutic approaches to regenerative medicine. For example, maintaining long-term self-renewal of multipotent tissue-specific stem cells remains challenging. New lines of research, especially using the chemical approaches outlined above, have generated promising outlook for the field. For example, a chemical screen in zebrafish to find small molecules that could modify hematopoietic stem cell (HSC) number in vivo has led to the clinical development of a small molecule to improve the overall efficiency of HSC transplantation in adults who have undergone nonmyeloablative conditioning therapy, by enhancing HSC proliferation and homing to the bone marrow [77].

With respect to reprogramming, continued efforts in identifying and characterizing additional small molecules that modulate the reprogramming process will ultimately lead to the development of highly efficient and precisely directed reprogramming to IPS cells (us. current inefficient and non-specific reprogramming). On the other hand, given the complexity and challenges of differentiating IPS cells for specific applications, an alternative and perhaps more attractive direction for reprogramming is to generate intermediate lineage-specific stem cells or progenitors or differentiated functional cells through chemically defined conditions (e.g. small-molecule treatment), which had attracted significant efforts before the discovery of IPS cells and is being revisited with more enthusiasm and rigor. For example, reversine, a substituted purine analogue (Table 2), was identified from a chemical screen and can induce lineage-restricted muscle or fibroblast cells to a more primitive multipotent state [78–81].

Chemical approaches have become increasingly accessible and irreplaceable in discovery biology, and are particularly useful in the area of stem cell and regenerative biology, partly because the regulation of complex stem cell phenotypes might require a much higher level of precise control uniquely offered by using small molecules. There is no question that it is a fertile area to identify and characterize small molecules that can control cell fate, state and function, as well as to advance them toward regenerative medicine.

References


Dimos, J.T. et al. (2008) Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 321, 1218–1221


Shi, Y. et al. (2008) A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell* 2, 525–528


79 Shan, S.W. et al. (2007) Induction of growth arrest and polycomb gene expression by reversine allows C2C12 cells to be reprogrammed to various differentiated cell types. *Proteomics* 7, 4303–4316
