Modulation of embryonic stem cell fate and somatic cell reprogramming by small molecules

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Abstract  Embryonic stem cells (ESC) are pluripotent cells and have the ability to self-renew in vitro and to differentiate into cells representing all three germ layers. They provide enormous opportunities for basic research, regenerative medicine as well as drug discovery. The mechanisms that govern ESC fate are not completely understood, so a better understanding and control of ESC self-renewal and differentiation are pivotal for therapeutic applications. In contrast to growth factors and genetic manipulations, small molecules offer great advantages in modulating ESC fate. For instance, they could be conveniently identified through high-throughput screening, work across multiple signalling pathways and affect epigenetic modifications as well. This review focuses on the recent progress in the use of small molecules to regulate ESC self-renewal, differentiation and somatic cell reprogramming.

Introduction

Embryonic stem cells (ESC), which are derived from the inner cell mass of blastocysts, have both the ability to self-renew in vitro and to differentiate into cells from the three germ layers. These characteristics suggest that ESC hold great potential for application in tissue engineering and regenerative medicine (Murry and Keller, 2008), as well as in drug discovery and toxicity testing (Sartipy et al., 2007). Such uses of ESC, however, require further understanding of the mechanisms that govern their self-renewal and differentiation and better control of ESC fate. ESC are usually maintained or undergo directed differentiation with combinations of growth factors or genetic manipulations, which make the culture conditions incompletely defined and variable and lead to inconsistent results. More recently, small molecules (organic compounds with molecular weight less than 800 Da) have been used successfully to regulate ESC fate and enhance somatic cell reprogramming with distinct advantages. First, they could be conveniently identified by high-throughput screening of compound libraries. Second, a small molecule might work across multiple cell signalling pathways to achieve a synergistic effect. Third, small molecules can be synthesized and potentially enable...
the formulation of non-animal-derived chemically defined culture systems, which are necessary to manipulate ESC for therapeutic applications. Thus, the use of small molecules to modulate ESC fate will facilitate the applications in research and therapy. This article focuses on recent developments in the use of small molecules to modulate ESC self-renewal, differentiation and somatic cell reprogramming. In addition, the signalling pathways regulating ESC fate are also briefly reviewed.

**Embryonic stem cell self-renewal**

ESC are routinely cultured with feeder cells, serum and exogenous factors. Although autogeneic feeder cells could avoid potential viral transfer (Stojkovic et al., 2005), feeder cells secret a large number of unidentified proteins, which makes the culture system complex, variable and unsuitable for generation of consistent ESC. Serum, containing animal-derived components, limits the therapeutic application of ESC. Therefore, elucidation of the mechanisms of ESC self-renewal and simplification of the culture conditions are required.

Leukaemia inhibitory factor (LIF) was first found to sustain mouse ESC self-renewal in the presence of serum via activation of signal transducers and activators of transcription protein 3 (STAT3) (Niwa et al., 1998; Smith et al., 1988; Williams et al., 1988). LIF also activated the extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) pathway, which, on the contrary, seemed to favour mouse ESC differentiation (Burdon et al., 1999a; Ernst et al., 1996). In the absence of serum LIF induced mouse ESC neural differentiation, however, bone morphogenetic proteins (BMP) could replace serum and act in combination with LIF to maintain mouse ESC self-renewal via induction of Id genes and inhibition of ERK and p38 MAPK pathways. Neural differentiation induced by LIF was blocked by Id genes. In parallel, the capacity of BMP4 to induce mesodermal and endodermal differentiation was constrained by STAT3 (Qi et al., 2004; Ying et al., 2003). Consistent with these findings, small molecules PD98059 (12.5 μmol/l), an inhibitor of mitogen-activated protein kinase kinase (MEK) with an IC50 of 0.2 μmol/l (Almeida et al., 2000) and SB203580 (1–2 μmol/l), an inhibitor of p38 MAPK with an IC50 of 0.6 μmol/l (Cuenda et al., 1995), could mimic BMP4 in supporting mouse ESC proliferation in vitro in combination with LIF (Qi et al., 2004). Interestingly, the inhibition of mouse ESC differentiation by PD98059 was in a dose-dependent manner, with a peak at 12.5–25 μmol/l and at concentrations greater than 50 μmol/l, the mouse ESC growth was impaired, which may be due to nonspecific inhibition by PD98059 (Burdon et al., 1999b). Similar to PD98059, 30 μmol/l SB203580 exerted no positive effects on self-renewal of mouse ESC in combination with LIF (Ying et al., 2003). According to the report by Davies et al. (2000), many compounds could inhibit multiple protein kinases with similar or different potency and they may inhibit other protein kinases at high concentrations.

In contrast to mouse ESC, the LIF/STAT3 pathway cannot maintain the pluripotency of human ESC (Daheron et al., 2004; Thomson et al., 1998). Basic fibroblast growth factor (FGF) has been reported to play a pivotal role in maintaining human ESC identity (Amit et al., 2004; Wang et al., 2005; Xu et al., 2005a) through stimulating MEK/ERK MAPK signalling cascade (Kang et al., 2005). Inhibition of MEK/ERK activity by specific MEK inhibitors PD98059 and U0126 rapidly caused the loss of human ESC pluripotency (Li et al., 2007). However, basic FGF signal is necessary but not sufficient for human ESC self-renewal. Noggin, the BMP4 antagonist, could suppress the differentiation of human ESC induced by BMP4 (Xu et al., 2002) and co-operate with basic FGF in sustaining undifferentiated proliferation of human ESC (Wang et al., 2005; Xu et al., 2005b). Activin/Nodal signalling acts in concert with FGF signalling to maintain human ESC pluripotency through activation of Smad2/3 and SB431542 and SU5402, inhibitors of activin receptor-like kinase (ALK) 4/5/7 and tyrosine kinase, could cause human ESC differentiation via inhibition of the above signalling pathways (Vallier et al., 2005).

Based on current knowledge of signalling pathways involved in ESC self-renewal, it becomes possible to combine several small molecules that target these signalling pathways to maintain ESC pluripotency. This strategy is indeed supported by some recent studies. For instance, the Wnt/β-catenin signalling pathway was shown to play an important role in human and mouse ESC self-renewal revealed by comparative analysis of undifferentiated human ESC and mouse ESC-enriched genes (Sato et al., 2003). Thus, Sato et al. (2004) used 6-bromoiridouribin-3’-oxime (BIO) (2 μmol/l), a specific inhibitor of glycogen synthase kinase 3 (GSK3) (with IC50 of 0.005 μmol/l), to activate Wnt pathway, which successfully sustained human ESC and mouse ESC proliferation. However, BIO was not sufficient to maintain ESC identity in long-term culture, it could also cross-react with cyclin-dependent kinase 1 (CDK1) (IC50 of 0.320 μmol/l). Therefore, Ying et al. (2008) used a more selective GSK3 inhibitor CHIR99021 (3 μmol/l) (with IC50 of 0.007 μmol/l for GSK3 and IC50 of 8.8 μmol/l for CDK1) in combination with MEK inhibitor PD0325901 or with FGF receptor inhibitor SU5402 and MEK inhibitor PD184352 to activate Wnt pathway and inhibit ERK MAPK pathway synchronously and successfully sustained mouse ESC self-renewal. These compounds together with their targets, the protein affinity (represented by IC50 or effector concentration for half-maximum response; EC50), effects on ESC and effective concentrations are listed in Table 1.

The above examples demonstrate that small molecules could modulate ESC fate by targeting some known signalling pathways, which could be considered as a target-based approach. As there is still no complete understanding of the mechanisms controlling ESC self-renewal, a phenotype-based approach involved in high-throughput screening of chemical libraries has been carried out to identify small molecules capable of producing desired phenotypes. The identified compounds are then characterized to elucidate their mechanisms of action, which provide a useful tool to examine the underlying mechanisms of ESC regulation. For instance, Chen et al. (2006) used an established transgenic Oct4-green fluorescent protein (GFP) reporter mouse ESC line (which loses GFP expression completely in 4–6 days in the absence of feeder cells and LIF), screened a compound library containing 50,000 discrete heterocycles and discovered a heterocycle SC1 (pluripotin) that could maintain mouse ESC self-renewal in a chemically defined system.
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<th>Small molecule</th>
<th>Target</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µmol/l)</th>
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<td>IQ-1</td>
<td>PR72/130 subunit of protein phosphatase 2A</td>
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<td>Mouse ESC self-renewal</td>
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<td>CHIR99021</td>
<td>GSK3 inhibition</td>
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<td>MEK inhibition</td>
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<td>MEK inhibition</td>
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<td>Y-27632</td>
<td>ROCK inhibition</td>
<td>0.8 (Davies et al., 2000)</td>
<td>Human ESC survival</td>
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<td>Fasudil</td>
<td>ROCK inhibition</td>
<td>1.9 (Davies et al., 2000)</td>
<td>Human ESC survival</td>
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<td>ID-1, ID-8</td>
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<td>SB431542</td>
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<td>Retinoic acid receptor</td>
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<td>Mouse ESC and human ESC differentiation</td>
<td>0.001–1 (Dani et al., 1997; Geijsen et al., 2004; Guan et al., 2001; Schuldiner et al., 2001; Wobus et al., 1997)</td>
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<td>Hh-Ag1.3</td>
<td>Sonic hedgehog agonist</td>
<td>ND</td>
<td>Mouse ESC and human ESC neuronal differentiation</td>
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<td>Purmorphamine</td>
<td>Sonic hedgehog agonist</td>
<td>0.5 (EC&lt;sub&gt;50&lt;/sub&gt;) (Wu et al. 2004b)</td>
<td>Human ESC neuronal differentiation</td>
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<td>H-1152</td>
<td>ROCK inhibition</td>
<td>0.0016 (Ki) (Hwang et al. 2008)</td>
<td>Mouse ESC neuronal differentiation</td>
<td>0.1–2 (Hwang et al., 2008)</td>
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<td>Small molecule</td>
<td>Target</td>
<td>$IC_{50}$ (μmol/l)</td>
<td>Effects</td>
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<td>TWS119</td>
<td>GSK3β inhibition</td>
<td>0.03 (Ding et al., 2003)</td>
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<td>IDE1/IDE2</td>
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<td>5 (Borowiak et al., 2009)</td>
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<td>NME2 inhibition</td>
<td>0.3</td>
<td>Mouse ESC and human ESC differentiation</td>
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<td>(−)-Indolactam V</td>
<td>PKC activation</td>
<td>0.142 (EC$_{50}$) (Chen et al., 2009)</td>
<td>Human ESC pancreatic differentiation</td>
<td>0.3 (Chen et al., 2009)</td>
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<td>Ascorbic acid</td>
<td>ND</td>
<td>ND</td>
<td>Mouse ESC cardiomyogenesis</td>
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<td>Cardiogoneol C, D</td>
<td>ND</td>
<td>0.1 (EC$_{50}$) (Wu et al., 2004a)</td>
<td>Mouse ESC cardiomyogenesis</td>
<td>0.25 (Wu et al., 2004a)</td>
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<td>Verapamil</td>
<td>L-type Ca$^{2+}$ channel blocker</td>
<td>1.06 (EC$_{50}$) (Sachinidis et al., 2006)</td>
<td>Mouse ESC cardiomyogenesis</td>
<td>1 (Sachinidis et al., 2006)</td>
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<td>Dorsomorphin</td>
<td>BMP type 1 receptors inhibition</td>
<td>0.47 (Yu et al., 2008)</td>
<td>Mouse ESC cardiomyogenesis</td>
<td>2 (Hao et al., 2008)</td>
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<td>Rosiglitazone</td>
<td>Peroxisome proliferator-activated receptor gamma 2</td>
<td>0.022 (EC$_{50}$) (Doebber et al., 2004)</td>
<td>Human ESC adipocyte differentiation</td>
<td>1 (Xiong et al., 2005)</td>
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<td>SU6656</td>
<td>Src family kinase inhibition</td>
<td>0.28 (Cuneo et al., 2006)</td>
<td>Mouse ESC differentiation</td>
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<td>5-Azacytidine</td>
<td>DNA methylation inhibition</td>
<td>ND</td>
<td>Human ESC differentiation, reprogramming</td>
<td>0.1 (Yoon et al., 2006), 1 (Wernig et al., 2008a), 2 (Huangfu et al., 2008a)</td>
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<td>Valproic acid</td>
<td>HDAC inhibition</td>
<td>700–1000 (HDAC1–3), 1000–1500 (HDAC4,5,7) (Gurvich et al., 2004)</td>
<td>Reprogramming</td>
<td>2000 (Huangfu et al., 2008a)</td>
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<tr>
<td>Suberoylanilide hydroxamic acid</td>
<td>HDAC inhibition</td>
<td>0.01 (HDAC1), 0.02 (HDAC3) (Richon et al., 1998)</td>
<td>Reprogramming</td>
<td>5 (Huangfu et al., 2008a)</td>
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<td>Trichostatin A</td>
<td>HDAC inhibition</td>
<td>0.006 (HDAC1), 0.038 (HDAC4), 0.0086 (HDAC6) (Yoshida et al., 2001)</td>
<td>Reprogramming</td>
<td>0.02 (Huangfu et al., 2008a)</td>
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<td>BIX-01294</td>
<td>G9a histone methyltransferase inhibition</td>
<td>1.7 (Kubicek et al., 2007)</td>
<td>Reprogramming</td>
<td>0.5–1 (Shi et al., 2008b)</td>
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<tr>
<td>Parnate</td>
<td>Lysine-specific histone demethylase 1 inhibition</td>
<td>2 (Mimasu et al., 2008)</td>
<td>Reprogramming</td>
<td>2 (Li et al., 2009b)</td>
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<td>RG108</td>
<td>DNA methylation inhibition</td>
<td>0.115 (Brueckner et al., 2005)</td>
<td>Reprogramming</td>
<td>0.04 (Shi et al., 2008a)</td>
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<td>Bay K 8644</td>
<td>L-channel calcium agonist</td>
<td>0.77 (Velasquez et al., 2003)</td>
<td>Reprogramming</td>
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<td>E-616452</td>
<td>TGFβ1 receptor inhibition</td>
<td>ND</td>
<td>Reprogramming</td>
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<td>Kenpaullone</td>
<td>GSK3β and CDK inhibition</td>
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<td>Reprogramming</td>
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</table>

ALK = activin receptor-like kinase; BMP = bone morphogenetic protein; CDK = cyclin-dependent kinase; EC$_{50}$ = effector concentration for half-maximum response; ERK = extracellular receptor kinase; ESC = embryonic stem cells; GSK3 = glycogen synthase kinase 3; HDAC = histone deacetylase; MAPK = mitogen-activated protein kinase; p38 MAPK = p38 mitogen-activated protein kinase; MEK = mitogen-activated protein kinase kinase; ND = data not available; PKC = protein kinase C; ROCK = Rho-associated kinase; TGF β = transforming growth factor β.
without feeder cells, serum and LIF. SC1 were shown to activate the phosphoinositol 3-kinase pathway and block the ERK pathway through dual inhibition of RasGAP and ERK1. Miyabayashi et al. (2007) identified small molecule IQ-1 that could maintain the pluripotency of mouse ESC in long-term culture from screening a chemical library and further studies elucidated that IQ-1 acted through an increase of β-catenin/CREB binding protein (CBP)-mediated transcription at the expense of β-catenin/p300 interaction. More recently, Miyabayashi et al. (2008) discovered indole derivatives (ID-1 and ID-8) that sustained mouse ESC long-term self-renewal in a feeder-, serum- and LIF-free system by screening chemical libraries.

ESC self-renewal are likely to result from a delicate balance of signalling networks and it is practicable to maintain ESC identity through inhibiting the pathways that favour their differentiation and activating those involved in their proliferation with small molecules. The use of small molecules, avoiding the variables brought with feeder cells and serum, would make it easier to culture ESC in a more controllable environment and facilitate large-scale expansion of ESC in an animal component-free, chemically defined medium to meet the needs of therapeutic application.

Embryonic stem cell differentiation

ESC has the potential to differentiate into cells representing all three germ layers. They can differentiate spontaneously under appropriate conditions. However, directed differentiation of ESC into distinct cell types in high purity is the prerequisite of the potential application of ESC in regenerative medicine. Consequently, dissecting ESC signalling pathways and identifying crucial factors involved in tissue specification are required. A number of small molecules (Table 1) have been identified that regulate tissue-specific differentiation of ESC.

Retinoic acid (RA) is widely used to induce both mouse and human ESC neural differentiation (Guan et al., 2001; Schuldiner et al., 2001) and Sonic hedgehog (Shh) was demonstrated to be involved in neuronal subtype specification (Litingtung and Chiang, 2000). Recently it was shown that specific neuronal subtypes could be generated from mouse and human ESC in a stepwise fashion. For example, RA followed with Shh or Shh agonist Hh-Ag1.3 induced mouse and human ESC to differentiate into motor neurons (approximately 20%). These ESC-derived motor neurons could induce acetylcholine receptor clustering and establish functional neuromuscular transmission in vitro, as well as populate the embryonic spinal cord, extend axons and form synapses with target muscles in vivo, which would be useful for screening pharmaceutical agents targeting motor neuron-related disorders (Li et al., 2005; Wichterle et al., 2002). Furthermore, Li et al. (2008) reported an efficient differentiation of motor neurons (about 50%) from human ESC by a simple sequential application of RA and Shh in a chemically defined suspension culture. Moreover, they also discovered that a small molecule purmorphamine, a purine derivative which activates Shh pathway, could replace Shh to generate motor neurons in a similar way. Most recently, Osakada et al. (2009) used casin kinase I inhibitor CKI-7 and SB-431542 to block Wnt signalling and Nodal signalling and induce retinal differentiation in human ESC. The ESC-derived retinal progenitors were competent to differentiate into retinal pigment epithelia and photoreceptors treated with RA and taurine. These experiments suggest that multiple sequential or combination of signals may be helpful for enriching specific cell populations. However, it is worth noting that RA induced mouse ESC differentiation in a time- and concentration-dependent manner (Rohwedel et al., 1999).

Treatment of embryoid bodies between day 2 and 5 with $10^{-7}$ μmol/l RA induced neural differentiation, treatment with $10^{-8}$ μmol/l RA induced adipogenesis (Dani et al., 1997), whereas treatment with $10^{-8}$ μmol/l and $10^{-9}$ μmol/l RA between day 5 and 15 enhanced cardiomyocyte differentiation (Wobus et al., 1997) in mouse ESC. Further studies indicate that ERK and p38 MAPK pathways are involved in these processes (Aouadi et al., 2006; Bost et al., 2002).

Considerable cell-based phenotypic screens have been carried out and made great progress in identifying compounds that enhance cell specification in ESC. For instance, Ding et al. (2003) identified a heterocyclic compound TWS119 that could induce differentiation of mouse ESC to neurons through inhibition of GSK3β by screening kinase-directed combinatorial libraries. In fact, inhibition of GSK3β leads to the accumulation and nuclear translocation of β-catenin, which promotes neuronal differentiation of mouse ESC (Otero et al., 2004). Most recently, Hwang et al. (2008) screened 41 protein kinase inhibitors to alter the orchestration of multiple signalling pathways involved in differentiation of mouse ESC on P6 feeder layer. H-1152, a derivative of isoquinolinesulphonamide, known as a Rho kinase inhibitor, was found to significantly enhance mouse ESC differentiation to midbrain dopamine neurons that could form synapses. In accordance with these findings, Rho kinase was shown to be involved in the regulation of neuronal morphogenesis, and inhibition of Rho kinase promoted neurite outgrowth (Luo 2000). It is worth noting that H-1152 increased dopaminergic differentiation in a dose-dependent manner at a concentration between 0.1 and 2 μmol/l and exerted cytotoxic effects at high concentrations (>5 μmol/l) (Hwang et al., 2008). Chen et al. (2009) recently identified a small molecule, (–)-indolactam V, through a high-content chemical screen and it could activate protein kinase C (PKC) signalling and direct the pancreatic specification of endoderm cells derived from human ESC. More than 45% of the differentiated cells are Pdx1-expressing pancreatic progenitors and some of them form insulin-expressing cells after transplantation into the kidney capsule of nude mice. The function of (–)-indolactam V is elusive and it may act through a related mechanism with RA which is involved in pancreatic development via activation of the PKC pathway. The directed differentiation of ESC into pancreatic progenitor cells using small molecules would provide a new source of insulin-secreting beta cells for cell replacement.

ESC has the ability to differentiate into specific cells including cardiac myocytes, but the efficiency is quite low and the process is not completely understood. Takahashi et al. (2003) screened 880 compounds for their ability to induce cardiac differentiation of ESC using α-myosin heavy chain (αMHC) promoter-driven enhanced GFP (EGFP)-infected mouse ESC. Ascorbic acid was discovered to markedly enhance cardiac differentiation in an
under the control of the myogenesis using a transgenic mouse ESC expressing EGFP. Sachinidis et al. (2006) investigated the effects of 33 small molecules on cardiomyogenesis in ESC, which shows significant cellular toxicity at concentrations greater than 25 \( \mu \text{mol/l} \). To identify signalling cascades which are involved in cardiomyogenesis in ESC, Sachinidis et al. (2006) investigated the effects of 33 small molecules on cardiomyogenesis using a transgenic mouse ESC expressing EGFP under the control of the \( \alpha \text{HCM} \) promoter. The results showed that the protein phosphatase 2B inhibitor verapamil had a striking pro-cardiomyogenic effect. Interestingly, the most prominent effect was observed after stimulation of 1-day-old embryoid bodies for 48 h and no promoting effect was observed when the compound was added at other time points. It was revealed that verapamil promoted cardiomyocyte differentiation of ESC through inhibiting intracellular Ca\(^{2+}\) signalling, which is involved in cardiac valve formation.

Previous reports have shown that the p38 MAPK pathway plays a critical role in cardiomyocyte differentiation of mouse ESC and P19 embryonal carcinoma cell line and SB203580 (10 \( \mu \text{mol/l} \)) significantly inhibits cardiac differentiation (Davidson and Morange, 2000; Ding et al., 2008). Aouadi et al. (2006) suggested that the control of p38 MAPK activity constituted an early switch in mouse ESC commitment into cardiomyocytes (p38 on) and neurons (p38 off). In contrast, SB203580 was shown to promote cardiomyogenesis in human ESC in serum-free medium conditioned with END2 cells (a mouse endoderm-like cell line) in a time- and dose-dependent way. It enhanced cardiomyogenesis at concentrations \(<10 \mu \text{mol/l}\) and inhibited cardiomyogenesis and increased neural differentiation at concentrations \(\geq 15 \mu \text{mol/l}\). Furthermore, SB203580 exerted the promoting effect on cardiomyogenesis only when it was added during the first 3 days of ESC differentiation (Graichen et al., 2008). Biochemical analysis shows that prostaglandin I2 (PGI2) concentration is higher in END2 cell-conditioned medium. PGI2 and SB203580 (5 \( \mu \text{mol/l}\)) could enhance cardiomyogenic differentiation in human ESC in a chemically defined medium. The concentration-dependent response of SB203580 in cardiomyocyte differentiation in human ESC was also observed, equivalent to Graichen’s findings (Xu et al., 2008). The conflicting effects that SB203580 exhibits on mouse and human ESC cardiomyogenic differentiation might be caused by differences between species. Another signalling pathway involved in cardiomyogenesis is the BMP pathway and Noggin was reported to induce mouse ESC to differentiate into cardiomyocytes via inhibition of the BMP pathway (Yuasa et al., 2005). Recently, Hao et al. (2008) found that dorsomorphin, a small molecule inhibitor of BMP type 1 receptors, promoted cardiomyogenesis in mouse ESC at the initial 24 h of ESC differentiation at the expense of other mesodermal lineages. The results indicate that small molecule modulators of developmental pathways could enhance ESC differentiation into cardiomyocytes. The ESC-derived cardiomyocytes could express cardiomyogenic markers, spontaneously contract or even exhibit electrical characteristics, which would become a reliable cell source for cardiomyocyte-specific drug discovery, safety pharmacology and cell replacement therapy.

These findings demonstrate that small molecules can promote differentiation of ESC into functional cell types, especially those cells that rarely arise during spontaneous differentiation. Germ cells and gametes are one of such cells that are difficult to obtain by ESC spontaneous differentiation. Recently, considerable progress has been made in derivation of germ cells and gametes from both mouse and human ESC (Bucay et al., 2009; Yu et al., 2009b). Although several growth factors such as BMP4 and basic FGF have been suggested to promote germ cell differentiation (Kee et al., 2006; West et al., 2008; Yamauchi et al., 2009), the efficiency is low and the mechanisms by which germ cells generate remain undefined. RA (1 \( \mu \text{mol/l}\)) has also been shown to stimulate development of male gametes (Geijsen et al., 2004; Nayernia et al., 2006), but RA is a multipurpose factor and it is not convenient to understand the process of germ cell differentiation. It could be expected that screening for more compounds that increase germ cell differentiation in ESC would help elucidate gametogenesis.

Some conclusions can now be drawn from the above examples where small molecules were used to induce ESC to differentiate. First, as tissue specification usually occurs within a discrete time window during embryoid body differentiation, it is important to modulate the related signalling pathways with small molecules at the appropriate time. Second, as the induction of ESC differentiation by small molecules occurs in a dose-dependent fashion, a range of different concentrations of small molecules should always be explored in the experimental plan. Third, directed differentiation of ESC to terminally differentiated cell types is a complex and stepwise process that involves connected cellular pathways. Thus the application of sequential and/or combinations of small molecules may therefore be required to better modulate ESC fate.

### Somatic cell reprogramming

ESC, which are derived from the inner cell mass of preimplantation embryos, and epiblast stem cells, which are derived from the late epiblast layer of post-implantation embryos (Brons et al., 2007; Tesar et al., 2007) are pluripotent stem cells that are related to embryos and evoke difficult ethical issues. Fortunately, it has recently been reported that mouse (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007), rat (Li et al., 2009a; Liao et al., 2009), rhesus monkey (Liu et al., 2008) and human (Takahashi et al., 2007; Yu et al., 2007) somatic cells could be reprogrammed to induced pluripotent stem (IPS) cells through viral introduction of four transcription factors: Oct4 and Sox2 with either Klf4 and c-Myc or Lin28 and Nanog. These IPS cells possess morphological, molecular and developmental features close to those of ESC. They express key markers of ESC, exhibit a normal karyotype and have the potential to differentiate into all three germ layers (Amabile and Meissner, 2009). These characteristics provide IPS cells with great potential for cell therapy, drug screening and disease modelling.

Reprogramming somatic cells to IPS cells would broaden the source of pluripotent stem cells, avoid the ethical issues...
and facilitate generation of patient-specific stem cells for studying human disease. However, initial reprogramming strategies involve viral vector transfection to deliver reprogramming factor transgenes including oncogenes c-Myc and Klf4, which would cause unpredictable genetic dysfunction and tumour formation and limit the utility of these cells in both research and clinical applications. Many efforts have been made to improve the safety of IPS cells through omitting the transfection of c-Myc or using a non-integrating gene delivery system (shown in Figure 1). For instance, Wernig et al. (2008b) suggested that c-Myc was dispensable and mouse and human fibroblasts were successfully reprogrammed to IPS cells with retroviral transduction of three transcription factors in the absence of c-Myc, which reduced the risk of tumourigenicity of IPS cells (Nakagawa et al., 2008). The generation of IPS cells without c-Myc is slower and the efficiency is much lower than with c-Myc. Adult mouse neural stem cells, which endogenously express Sox2, c-Myc and Klf4, could be converted to IPS cells by transduction of Oct4 with a low efficiency (0.014%) (Kim et al., 2009). To generate non-integrating IPS cells, Stadtfeld et al. (2008) used non-integrating adenoviruses to deliver four reprogramming genes (efficiency of 0.1–0.2%), Okita et al. (2008) transiently transfected mouse cells with two plasmids containing the four reprogramming factors (efficiency of 1–29 colonies/10^6 transfected cells) and Soldner et al. (2009) reprogrammed patient-derived fibroblasts using Cre-recombinase excisable viruses to deliver three factors (lacking c-Myc) (efficiency of 0.005%) or four factors (efficiency of 0.01%). In addition, Kaji et al. (2009) produced viral-free and factor-removable human IPS cells using a single plasmid comprising the four factors combined with piggyBac transposons which excised the transgenes when the reprogramming was achieved (efficiency of 2.5% with different estimation method from others). With this approach, residual vector sequences cannot be removed, which may cause insertional mutations. Vector- and transgene-free mouse and human IPS cells have been successfully produced using piggyBac transposition or non-integrating oriP/Epstein-Barr nuclear antigen-1-based vectors (efficiency of 3–6 colonies/10^6 cells) (Woltjen et al., 2009; Yu et al., 2009a), which removes one obstacle to the application of IPS cells. Most recently, mouse embryonic fibroblasts (MEF) have been reprogrammed to IPS cells with four or three (lacking c-Myc) recombinant reprogramming proteins in combination with valproic acid (VPA) without exogenous genetic modifications (efficiency of 1–3 colonies/5 × 10^6 cells) (Zhou et al., 2009), which demonstrates that it is possible to produce IPS cells by completely chemical methods.

Although IPS cells have been generated through viral or non-viral methods, the reprogramming is a slow and inefficient process. Some recent reports demonstrated that small molecules could improve the reprogramming efficiency and replace one or more transcription factors. For instance, Huangfu et al. (2008a) discovered that the DNA demethylating agent 5-azacytidine and the histone deacetylase inhibitors suberoylanilide hydroxamic acid, trichostatin A and VPA promoted the reprogramming of MEF to IPS cells. Moreover, VPA was shown to enhance the reprogramming markedly without introduction of c-Myc and microarray analysis showed that VPA acted by up-regulating ESC-specific genes and down-regulating MEF-specific genes. In addition, VPA enabled the reprogramming of human fibroblasts with two factors Oct4 and Sox2, omitting the oncogenes c-Myc and

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**Figure 1** The schematic illustration of somatic cell reprogramming to induced pluripotent stem cells. Somatic cells are reprogrammed to induced pluripotent stem cells through viral or non-viral introduction of exogenous transcription factors or protein transduction. Small molecules can improve reprogramming efficiency and replace certain transcription factors through epigenetic modifications or signalling pathway regulation. 5-AzaC = 5-azacytidine; EBNA1 = Epstein-Barr nuclear antigen-1; IPS = induced pluripotent stem; SAHA = suberoylanilide hydroxamic acid; TSA = trichostatin A; VPA = valproic acid.
Klf4 (Huangfu et al., 2008b). Most recently, Lyssiotis et al. (2009) screened 500,000 compounds and found that kenpaullone, an inhibitor of GSK3β and CDK, could increase MEF reprogramming with the four factors as well as facilitate generation of IPS cells in the absence of Klf4.

Sox2 is one of the key transcription factors that induce reprogramming of somatic cells to IPS cells. Several small molecules have been found that could replace Sox2 in reprogramming. For example, Shi et al. (2008b) reported a combined chemical and genetic approach to induce reprogramming of mouse neural progenitor cells that endogenously express Sox2. The results showed that small molecule BIX-01294, an inhibitor of the G9a histone methyltransferase, could compensate for Sox2 or Oct4 and increase the reprogramming efficiency in neural progenitor cells transduced with Oct3/4 and Klf4 or in fetal neural progenitor cells transduced with Klf4, Sox2 and c-Myc. In addition, BIX-01294, acted with Bay K 8644 (a L-channel calcium agonist) or RG108 (a DNA methyltransferase inhibitor) could substitute for Sox2 in reprogramming MEF to IPS cells with Oct4 and Klf4 (Shi et al., 2008a). Li et al. (2009a) reported that CHIR99021 could allow the reprogramming of both MEF and human neonatal keratinocytes that were transduced with Oct4 and Klf4 in combination with parnate, a lysine-specific histone demethylase 1 inhibitor. More recently, Ichida et al. (2009) transduced MEF, Klf4 and c-Myc and screened for compounds that could complete the reprogramming. Small molecule E-616452 was identified as screening compound libraries, which would provide insights into the mechanisms regulating reprogramming. As Sox2 plays a critical role in reprogramming, the replacement of Sox2 brings much hope for the generation of IPS cells by chemical methods. More efforts are required to screen for compounds that could substitute for Oct4.

The basic difference between somatic cells and pluripotent cells is the epigenetic state, for instance, many tissue-specific genes are active in the former while pluripotency-associated genes are active in the latter. To regulate gene expression, global epigenetic reprogramming is required for the conversion (Amabile and Meissner, 2009). That is why 5-azacytidine, suberoylanilide hydroxamic acid, trichostatin A, VPA, BIX-01294, parnate and RG108 could promote the reprogramming efficiency. It could be expected that small molecules involved in chromatin modification would enhance the generation of vector- and transgene-free IPS cells.

It is worth noting that, during the conversion, cells are maintained in culture for several weeks with significant proliferation and possibly under selective pressure. Small molecules capable of promoting ESC proliferation might exert positive effects on IPS cells and improve reprogramming efficiency. In fact, PD0325901 has been reported to inhibit growth of non-IPS cell colonies and promote growth of reprogrammed IPS cells through stabilization of the IPS cell state (Shi et al., 2008b). CHIR99021 has been shown to increase the reprogramming of MEF transduced with Oct4, Sox2 and Klf4 and allow MEF reprogramming with Oct4 and Klf4 in the absence of Sox2 (Li et al., 2009b). Rho-associated kinase (ROCK) inhibitors such as Y-27632 and fasudil (5-(1,4-diazepane-1-sulfonyl)isoquinoline) have been demonstrated to diminish human ESC dissociation-induced apoptosis markedly, increase human ESC cloning efficiency and facilitate human ESC subcloning after gene transfer (Watanabe et al., 2007). It would be of considerable value to evaluate their effects on IPS cells. It could be expected that more compounds which target signalling pathways involved in ESC fate modulation would be identified to enhance somatic cell reprogramming.

The great potential of IPS cells in cell therapy, drug screening and disease modelling has attracted enormous attention and led to extremely rapid progress in this field. Many researchers expect to generate IPS cells without genetic modification, instead using combination of small molecules. Further efforts are required to screen more compounds to promote the reprogramming efficiency, making the use of IPS cells safer and more practical.

Conclusions

ESC biology is a fast-growing field providing ample opportunities for basic research, drug discovery and therapies. Better understanding of signalling pathways controlling ESC self-renewal and differentiation is crucial to these applications. Chemical approaches and small molecules have been used successfully in modulating ESC fate, enhancing somatic cell reprogramming and played important roles in setting up chemical-defined culture systems as well as elucidating the underlying mechanisms of ESC regulation. Moreover, small molecules will be useful tools in the analysis of disease-specific stem cells to better understand the mechanisms of disease and pathology as well as for drug screening. In addition, there are prospects in using small molecules to reprogramme one somatic cell type into another, without reverting back to an embryonic-like state. However, there are some points worth noting when using small molecules. First, most small molecules have the ability to target multiple pathways, so they work in a dose-dependent manner, and at high concentrations they might cross-react with other targets or even exert cytotoxic effects. Choosing an appropriate concentration is important to get the desired result and fortunately, the protein affinity (IC50 or EC50) of a small molecule provides a good reference for it. Second, ESC fate is likely to be controlled by an elaborate orchestration of multiple signalling pathways, multiple sequential and/or combination of small molecules seems to be more efficient in regulating ESC fate. Taken together, small molecules, with their good cell penetration, low cost and lack of animal-derived components, would become useful tools for ESC research and regenerative medicine. More chemical libraries and screening strategies are needed to design systems to regulate ESC proliferation, differentiation into desired functional cell types and reprogramming of somatic cells into IPS cells.

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