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Regenerative Chemical Biology: Current Challenges and Future Potential

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Abstract

The enthusiasm surrounding the clinical potential of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is tempered by the fact that key issues regarding their safety, efficacy, and long-term benefits have thus far been suboptimal. Small molecules can potentially relieve these problems at major junctions of stem cell biology and regenerative therapy. In this review, we will introduce recent advances in these important areas and the first-generation of small molecules used in the regenerative context. Current chemical biology studies will provide the archetype for future interdisciplinary collaborations, and improve clinical benefits of cell-based therapies.

Promise and Challenges for Regenerative Medicine

Life expectancy has increased dramatically in the modern era. Along with it, there is the observed increase in chronic diseases such as heart disease, neurodegenerative disorders, and diabetes. These progressively degenerative conditions are largely irreversible and incurable, except for rare cases where organ transplantation is an option. The recognizable need to correct or replace defective and failing tissues has led to a surging interest in cell-based regenerative therapy. The main goal is to produce a reliable source of replacement biomaterials and tissues *ex vivo* and supplant the current donor-based system, which is always in limited supply. Furthermore, an *ex vivo* source can potentially be tailored to specific individuals, which may prevent rejection due to donor-recipient incompatibility and the accompanying risks of immunosuppressive drugs, which are necessary components of organ and tissue transplant procedures (Teo and Vallier, 2010; Rolletschek and Wobus, 2009).

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Cell-based therapy has been used as a blanket term that encompasses the usage of significantly different varieties of pluripotent cells. Each variation possesses unique properties that are not fully characterized, and has different implications under each therapeutic context. The establishment of *in vitro* cultures of mouse embryonic stem cells (mESCs) (Evans and Kaufman, 1981), human embryonic stem cells (hESCs) (Thomson et al., 1998), inducible pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006), and the discovery of adult somatic stem cells in various tissues initiated a flurry of studies into their respective therapeutic potential for basic research and for cell replacement (Figure 1). Recent characterization of different pluripotency states put forth intriguing possibilities for further refining lineage specification and increasing their utility (Brons et al., 2007; Bao et al., 2009).

The initial body of research revealed a number of technical obstacles against the practical usage of embryonic and induced pluripotent cell types. The most pressing challenges are: developing a stable and renewable source of pluripotent cells, reliably maintaining pluripotency without compromising genomic integrity, and efficiently directing differentiation to eliminate cellular heterogeneity. Guiding cell fate determination is especially important as it relates directly to the feasibility and safety of exogenous cell transplants. This is because undifferentiated cells can result in tumor formation as they spontaneously differentiate (Cooke et al., 2006; Blum and Benvenisty, 2008). It is also obvious that established methods are inadequate due to the inconsistent and haphazard nature of current maintenance and directed differentiation approaches (Nagy et al., 1993; Reubinoff et al., 2000). For example, mESCs require the addition of Leukemia Inhibitory Factor (LIF) in the medium to maintain pluripotency and cell proliferation. By contrast, hESCs do not respond to LIF, but instead require Transforming Growth Factor-β (TGF-β)/ Nodal and Basic Fibroblast Growth Factor (bFGF) in the medium to sustain pluripotency (Vallier et al., 2005). Another problem is the reliance of growth factors from feeder layers or animal-derived serums in culture protocols, which inevitably introduces batch variability. In addition, the high costs of growth factor additives are prohibitive to the large scale production of pluripotent cells and further limit clinical applications.

A potential alternative source of pluripotent cells is to reprogram differentiated somatic cell types to a pluripotent state. There are two practical advantages to this approach: 1) it circumvents ethical concerns of using embryo-derived stem cells, and 2) it employs a patient's own cells and would limit immune rejection. A landmark study from Yamanaka's group identified four transcription factors (Sox2, Oct4, Klf4 and c-Myc) that, when introduced via viral-mediated transduction, re-established pluripotency in adult fibroblasts (Takahashi and Yamanaka, 2006). The resulted iPSCs were shown to closely resemble ESCs, as they were pluripotent and could be induced to differentiate into every cell type (Takahashi and Yamanaka, 2006; Yu et al., 2007). It has since been reported that another combination of genetic factors (Sox2, Oct4, Lin28 and Nanog) can also induce pluripotency (Yu et al., 2007), and that c-Myc can be omitted (Nakagawa et al., 2008). While these results are exciting, some major issues must be resolved before iPSCs become a viable option for cell replacement therapy. The first is the introduction of reprogramming factors using viral transduction systems, which raise reasonable concerns for oncogenic risks in patients. This issue has been partly addressed with the availability of plasmid-based, protein-based, and modified RNA-based strategies that have resulted in successful virus-free cellular reprogramming (Cho et al., 2010; Okita et al., 2008; Warren et al., 2010). The second issue is the extremely low reprogramming efficiency of 0.001 to 0.005% (Hasegawa et al., 2010), which remains unresolved because reprogramming mechanisms are imperfectly understood.

Endogenous somatic stem cells have been scrutinized as an alternative to *ex vivo* sources. They are resident pools of lineage-restricted multipotent cells that are responsible for tissue

turnover, and have been identified in the brain (Doetsch, 2003), skin (Jones, 1993), heart (Messina et al., 2004), skeletal muscle (Martin et al., 2006), and intestines (Casali and Batlle, 2009). They are a tempting source because it is theoretically possible to direct them towards tissue repair, all without risking immunological incompatibility problems. However, they are currently on the fringes of therapeutic options as their isolation, propagation and usage are unrefined and may be restricted to local niches.

Another concern for cell-based therapies is post-transplantation events. The capacity of exogenous cells to integrate with host tissue and restore normal physiological functions is low using current methods. While free floating, suspension cells like bone marrow-derived hematopoietic stem cells (HSCs) are less resistant to integration, the actual integration rate is highly dependent on the stage of HSC differentiation and its affinity for the local microenvironment (Lo Celso et al., 2009). The situation is more complicated when stem cells are introduced to highly-structured organs like the heart, where synchronous contractions are crucial for function. Implanted cells either die from the initial onslaught of inflammatory cytokines, or fail to integrate into the host tissue; instead establishing themselves as a separate or hybrid entity at the transplant site (Reinecke et al., 1999; Alvarez-Dolado et al., 2003). Furthermore, the impact of paracrine cell signaling cannot be understated. Initial benefits described from transplantation studies using various sources of stem cells including bone marrow, mesenchymal, and neural stem cells offered temporary functional improvements. These benefits were subsequently characterized as the result of paracrine factors secreted by the stem cells rather than true cell autonomous repair (Kim et al., 2010a; Perez-Ilzarbe et al., 2008).

Future technologies must take advantage of the synergistic interactions between the transplanted cells and the local microenvironment in order to harness the full potential of regenerative therapy. Advances in chemical biology can conceivably serve first as a tool to dissect the complicated pathways regulating self-renewal and cell fate decisions, and second as the means to manipulate those same pathways for the desired therapeutic outcome.

Chemical Biology: Advantages of Small Molecules

The guiding principal in chemical biology is to discover and develop synthetic bioactive molecules. It presents several advantages over traditional protein- or gene-based tools. The usage of traditional biomolecules is limited as they are difficult to produce and manipulate. Their effects are unstable and cannot be fine tuned since genetic switches are generally all-or-none. They can modulate only a single target at a time and introduction of multiple biomolecules into a specific tissue is technically challenging. In contrast, small molecules can be delivered efficiently into the cell, can be targeted to specific tissues and their effects are reversible. The dosage of the compounds can be modulated for maximum benefit, and individual molecules can be further modified via medicinal chemistry to increase potency, safety, or stability. Small molecules can target the biology of a desired phenotype by stimulating multiple druggable categories via intersecting signaling nodes. Small molecules are also relatively inexpensive to produce, and can be scaled to particular needs. These attributes place small molecules on a favorable position for regenerative medicine developments.

Small molecules can participate at several junctions of regenerative medicine as many key cellular events utilize the same pathways and form druggable nodes. These key pathways are Hedgehog (Hh), Wnt, Transforming Growth Factor -β (TGF-β), Notch, and Fibroblast Growth Factor (FGF) (Jiang and Hui, 2008; MacDonald et al., 2009; Wu and Hill, 2009; Bolós et al., 2007; Beenken and Mohammadi, 2009). Specific information on how these key pathways function in pluripotency and differentiation is discussed in other excellent reviews

(Loebel et al., 2003; Pera and Tam, 2010). Furthermore, small molecules have been identified that target these pathways singly and in combination with other small molecules or genetic factors. As an emerging area of study, small molecules have provided early proof that cell fate is dynamic and can be manipulated using artificial means. The attributes of pluripotent and lineage-restricted progenitor cells, when combined with the practical utility of small molecules, are powerful assets for realizing the full potential of regenerative therapy.

Small Molecules in Regenerative Medicine

Many small molecules relevant to stem cell biology were identified from chemical libraries using high-throughput cell phenotype-based, reporter-based, or organism-based screens (Figure 2). Lead compounds were further examined to identify associated targets and relevant pathways, which then guide future optimization based on biochemical, pharmacological, and physiological requirements (Ding and Schultz, 2004). Compounds that promote self-renewal, facilitate reprogramming, and direct differentiation pathways have been identified using the above methods. These chemical biology discoveries, detailed below, are shaping the future of regenerative medicine.

Self renewal and Pluripotency

A major obstacle for the practical usage of stem cells has been maintaining their pluripotent state in culture. Spontaneous differentiation occurs due to constant bombardment from undefined and varied amounts of growth factors found in traditional culture protocols that use animal serum and feeder cells. For this reason, a number of serum- and feeder-free protocols have been developed that use commercially available supplements. Many of these supplements still use animal-derived and recombinant growth factors, but they are optimized for pluripotent cell culture and reduce batch variability. They include KnockOut (Cheng et al., 2004) or N2 and B27 (N2B27) (Ying and Smith, 2003) that consist of essential recombinant growth factors for mESCs and iPSCs using GIBCO's proprietary formula. Another formulation is to add LIF and bone morphogenetic protein (BMP) to N2B27 media, which also drive continuous self-renewal in serum-free conditions (Nichols and Ying, 2006). A feeder-free, serum-free method has successfully maintained hESCs and iPSCs in culture (Ludwig et al., 2006). It replaces the feeder layer with an animal-derived extracellular matrix called Matrigel (BD Bioscience), and a serum-free medium called mTeSR1 (STEMCELL Tech) supplemented with recombinant proteins (Ludwig et al., 2006). As promising as these new feeder-free, serum-free approaches are, they are not yet compatible with wide-scale clinical applications since they still require complex animal-derived products or recombinant protein modulators, which can be expensive and unstable.

The above-mentioned issues motivate the development of synthetic media formulations that minimizes or eliminates animal-derived or recombinant bioactive products. The first-generation collection of small molecules has been identified for this purpose. Pluripotin (Table 1) was the first compound identified in a chemical screen that propagates mESCs in an undifferentiated state (Chen et al., 2006). This discovery was especially remarkable because it showed that pluripotent cells can indeed be maintained in chemically defined conditions, without the use of animal-derived products or LIF. Furthermore, pluripotin did not stimulate the predicted pluripotency pathways, i.e. LIF-STAT3 (Niwa et al., 1998), BMP4-Smad-Id (Ying et al., 2003) or Wnt signaling (Sato et al., 2004). Instead, pluripotin blocked two major differentiation-inducing pathways, i.e. MEK-ERK and Ras-GAP signaling (Johnson and Lapadat, 2002; Lypowy et al., 2005). This was significant because it demonstrated the existence of a basal, self-renewing stem cell state that can be maintained by inhibiting differentiation.

A different approach to boost pluripotency is to upregulate known pluripotency pathways. 6-Bromoindirubin-3'-oxime (BIO, Table 3), which activates the canonical Wnt signaling by inhibiting GSK3, could maintain pluripotency in human and mouse ESCs (Sato et al., 2004). However, this compound is strictly a stabilizer of pluripotency signals, and requires LIF to initiate pluripotency. Another Wnt signal modulator called IQ-1 (Table 1) can replace exogenous LIF and feeder cell requirements in mouse stem cell culture (Miyabayashi et al., 2007). While these studies suggest Wnt signaling is a fundamental component in regulating pluripotency, one must caution against generalization since the role of any single signaling pathway may be highly dependent on cellular context (Sato et al., 2004; Dravid et al., 2005).

Combining Wnt activation with inhibition of the differentiation-inducing signals, particularly the FGF (fibroblast growth factor)/MEK-ERK pathway, has proven to be highly effective for maintaining pluripotency of ESCs. For example, a cocktail of small molecule inhibitors, dubbed "3i," composed of GSK3 inhibitor CHIR99021 (Table 1), MEK inhibitor PD0325901 (Table 1) and FGF receptor inhibitor SU5402 (Table 1) added to the basal N2B27 media effectively inhibited spontaneous differentiation and ensured homogeneous Nanog expression, a key component for pluripotency maintenance (Mitsui et al., 2003; Ying et al., 2008). A similar strategy combining CHIR99021 (Table 1), PD0325901 (Table 1) and TGF-β receptor inhibitor A-83-01 (Table 1) was also capable of maintaining rat and human iPSCs (Li et al., 2009a).

Although significant progress has been made in understanding the basic mechanisms and characteristics of pluripotency, current understanding is far from complete and basal pluripotency requirements remain undefined. Overcoming these obstacles through the discovery of additional pluripotency pathways and their chemical modulators could enable large-scale production of pluripotent cells and finally provide a stable source of starting materials for tissue regeneration.

Reprogramming

Major hurdles against the practical usage of iPSCs include low reprogramming efficiency, and safety concerns raised by the use of viral transduction in the process. The fast moving pace of the reprogramming and iPSC field has yielded numerous reports of chemicals that either improve reprogramming efficiency or can substitute for specific reprogramming factors. Mechanistically, the chemicals generally function by altering signal transduction pathways, or modify chromatin structure to remove epigenetic barriers. These properties, when known, will be described below.

Several small molecules improve the reprogramming process by lowering the epigenetic barrier to initiate pluripotency. The accompanying side effect is eliminating the need for one or two Yamanaka factors (c-Myc or Sox2) in the reprogramming cocktail. This is because those two factors are not necessary to initiate pluripotency, but are responsible for its maintenance (Masui et al., 2007). These initial experiments focused on mouse embryonic fibroblast (MEF) reprogramming. Valproic acid (VPA, Table 2) is a histone deacetylase (HDAC) inhibitor that dramatically improved reprogramming efficiency by 100-fold without the use of c-Myc (Huangfu et al., 2008). A G9a histone methyltransferase (HMTase) inhibitor, BIX01294 (Table 2), substantially increased reprogramming efficiency of Sox2-expressing mouse neural progenitor cells (NPCs) transduced with Oct4 and Klf4 (OK) to levels obtained with canonical Yamanaka factors (Shi et al., 2008b). A similar example used a combination of DNA methyltransferase (DNMT) inhibitor called RG108 (Table 2), an L-type calcium channel agonist called BayK8644 (Table 2), and BIX01294 to promote reprogramming in MEFs transduced with OK (Shi et al., 2008a). Another chemical screen identified a TGF-β signaling inhibitor called RepSox (Table 2) as a potent Sox2 replacement (Ichida et al., 2009). This molecule was shown to promote reprogramming by

inducing Nanog expression, which is a transcription factor known to drive self-renewal in ESCs (Pan and Thomson, 2007).

The chemical cocktails perfected in MEFs were quickly adapted for human somatic cell reprogramming. A combination of SB431542 (Table 2) and PD0323901 (Table 1), which inhibit TGF-β and MAPK/ERK pathways respectively, and thiazovivin, which improves the survival of hESCs upon trypsinization, increased reprogramming efficiency in human fibroblasts by 200-fold (Lin et al., 2009). The GSK3 inhibitor, CHIR99021 (Table 1), when combined with a lysine-specific demethylase 1 (LSD1) inhibitor called Parnate (also called tranylcypromine) (Table 2) enhanced reprogramming of human keratinocytes transduced with OK (Li et al., 2009b). A more recent report described a stepwise chemical treatment protocol that required only OCT4 transduction (Zhu et al., 2010). This protocol used a combination of TGF-β receptor inhibitor A-83-01 (Table 1), HDAC inhibitor sodium butyrate (NaB, Table 2), and PDK1 activator called PS48 (Table 2) for the first four weeks. For the next four weeks, PD0323901 (Table 1) was combined with the aforementioned mixture to complete reprogramming. The report identified a switch in metabolic state from mitochondrial oxidation to glycolysis, known in oncology as the “Warburg Effect” (Robey et al., 2008), which may be critical during reprogramming (Zhu et al., 2010). The reprogramming efficiency using this protocol was extremely low, as only 4 to 6 iPSC colonies formed for every 1×10^6 cells seeded (Zhu et al., 2010). Nevertheless, this initial report demonstrates the power chemical biology can hold for the future of human somatic cell reprogramming.

There is another class of compounds that functions by de-differentiating lineage-committed cells to a more primitive, multipotent state. Multiple studies have described reversine (Table 2) as a potent dedifferentiation agent, which facilitated transformation of differentiated cells to other lineages via a lineage-restricted progenitor (Chen et al., 2004; Lee et al., 2009). Reversine was characterized as an Aurora kinase inhibitor (D'Alise et al., 2008), possibly specific for Aurora kinase B (Amabile et al., 2009). Another Aurora kinase inhibitor, VX-608, was also found to dedifferentiate mouse myoblasts (Amabile et al., 2009). The partially reprogrammed state induced by these compounds confirmed that cell fate commitment is a stepwise process, and more importantly, some of the intermediate steps can be manipulated chemically and may even be reversible.

These chemical biology studies suggest that somatic cells are highly plastic and can be induced to assume multiple de-differentiated states. This notion has parallels in the emerging concept of distinct “naïve” and “primed” pluripotent states, which has gained serious consideration since the recent identification of murine epiblast stem cells (EpiSCs) (Nichols and Smith, 2009). EpiSCs derived from postimplantation epiblast represent a more mature stage than mESCs, derived from the inner cell mass of preimplantation blastocysts (Tesar et al., 2007; Bao et al., 2009; Chou et al., 2008; Brons et al., 2007). EpiSCs resemble hESCs in many ways, including their dependence on bFGF/Activin A signaling instead of LIF/STAT signaling, flattened colony morphology, their X-inactivation status, and their inability to be passaged as single cells (Tesar et al., 2007; Brons et al., 2007). These similarities suggest that hESCs are analogous to the more mature, primed pluripotent state of EpiSCs. Recent chemical biology studies demonstrated that EpiSCs, as well as hESCs and hiPSCs, can be converted to the earlier, naïve pluripotent state. The converted cells become functionally, transcriptionally and epigenetically similar to mESCs. Taking cues from the 3i strategy described above, Ding and colleagues successfully converted EpiSCs to the mESC phenotype using a cocktail of small molecule inhibitors of TGF-β receptor (A-83-01), FGF receptor (PD173074), MEK (PD0325901), GSK3 (CHIR99021) and LSD1 (Parnate) added to the LIF-containing media (Zhou et al., 2010). Using a similar chemical biology approach, Jaensich and colleagues demonstrated that the combination of 3 compounds PD173074,

CHIR99021 and forskolin, an activator of the enzyme adenylate cyclase, added to the LIF-containing basal N2B27 media induced the naïve pluripotent state in both hESCs and hiPSCs (Hanna et al., 2010). Distinction between the naïve and primed pluripotent states is important because human iPSCs derived using current protocols are thought to represent the later, characterized by significant biases in differentiation potential (Nichols and Smith, 2009). Thus conversion of hiPSCs to the naïve pluripotent state may be necessary for efficient generation of diverse patient-specific tissues (Hanna et al., 2010).

The naïve and primed pluripotent states have important implications for reprogramming and directed differentiation especially in light of the recent discovery of the epigenetic memory in iPSCs (Kim et al., 2010b; Ji et al., 2010). Epigenetic memory is a phenomenon in which iPSCs derived from adult tissues retain DNA methylation signatures characteristic of their somatic tissue of origin, unlike the classical ESCs. Furthermore, it may sustain a residual gene expression signature from the parental cell and account for gene expression differences between iPSCs and ESCs (Ghosh et al., 2010; Chin et al., 2009). Consequently, iPSCs preferentially differentiation along lineages related to the parental cell, with restricted potential for alternative cell fates (Kim et al., 2010b). While the epigenetic memory of parental tissues could at least partially be reset by treatment with chromatin-modifying drugs Trichostatin A (TSA), an inhibitor of histone deacetylase, and 5-azacytidine (AZA), a cytosine analog resistant to methylation (Kim et al., 2010b), it represents a significant barrier against full reprogramming and directed differentiation toward many desired cell types.

There are now many examples of chemical modulators facilitating cellular reprogramming by increasing reprogramming efficiency, eliminating potentially oncogenic factors in the reprogramming cocktail, or increasing cell fate plasticity. These observations have also fueled new questions and challenges. In most cases, the precise mechanisms by which synthetic chemicals influence reprogramming are yet to be discovered. Additional chemical biology advances are needed to completely erase epigenetic memory, and to eliminate the need for exogenous reprogramming factors altogether. Given tremendous advances in the past few years, we anticipate that future developments will substantially improve the process of iPSC generation and provide better understanding of reprogramming.

Directed Differentiation

Achieving and maintaining a pluripotent stem cell population is one element in regenerative therapy improvement. Another requirement is to produce large quantities of stage-specific cells in a controlled manner *in vivo*. A pluripotent cell population cannot be used directly in patients because they can form tumors. Therefore, it is necessary to pre-differentiate pluripotent cells to a desired cell type prior to transplantation. Compounds for this purpose have been identified in high-throughput assays based on lineage-specific gene expression profiles (Figure 2), and will be discussed below in relations to the three germ layers: endoderm, mesoderm, and ectoderm. The net effects of these chemicals have been to functionally promote lineage commitment, or to block self-renewal maintenance and spur differentiation.

The endoderm lineage has been the least characterized of the three lineages due to the lack of early endoderm markers, but imperfect markers, like Sox17 and Foxa2, exist for later endoderm commitment (Iwamuro et al., 2010). Two chemical compounds named IDE1 (Table 3) and IDE2 were identified from a library of putative HDAC inhibitors based on Sox17 induction (Borowiak et al., 2009). IDE1 and IDE2 activated close to 80% endoderm progenitor production in mouse and human stem cells, which is above that of known TGF-β modulators Activin A and Nodal (Borowiak et al., 2009). The compounds upregulated Nodal signaling, and increased endoderm lineage commitment and developmental

competence (Borowiak et al., 2009). Another compound called Indolactum V (ILV, Table 3) increased development of Pdx-1 expressing pancreatic progenitors from endoderm-biased progenitors (Borowiak et al., 2009; Chen et al., 2009). Proliferation studies showed ILV did not drive proliferation of existing Pdx-1 expressing cells, but rather committed other cell types of the heterogeneous endoderm-restricted pool towards the pancreatic progenitor lineage via activation of protein kinase C (PKC) (Chen et al., 2009). However, there was no mechanism proposed on how PKC activation increases pancreatic progenitors.

Similarly, there are examples of chemical cocktails that direct mesoderm lineage commitment but the mechanisms are unknown. Cardiogenol C (Table 3) was identified in a high-throughput, cardiac-specific ANF reporter assay (Wu et al., 2004). It specifically induces cardiomyocyte formation in mESC culture after 3 days of treatment (Wu et al., 2004). However, the compound appears to only be effective in certain cell types (Jasmin et al., 2010), which would suggest that it is a non-specific compound that requires a specific cellular context to support pro-cardiac effects.

Modulation of BMP signaling can direct ectoderm and mesoderm formation during embryonic differentiation (Winnier et al., 1995; Finley et al., 1999). A BMP selective inhibitor identified using an unbiased, high-throughput *in vivo* screen in zebrafish, called dorsomorphin (Table 3), induced beating cardiomyocyte formation in mouse ESC culture (Yu et al., 2008; Hao et al., 2008). Unlike cardiogenol C, dorsomorphin is required only during the first 24 hours of induction to specify cardiomyocyte commitment. This timeframe makes the pro-cardiomyogenic mechanism of dorsomorphin especially interesting as it occurs prior to the expression of known early mesoderm markers like BryT and Mesp1 (Kubo et al., 2004; Bondue et al., 2008). This strongly suggests that dorsomorphin increases cardiomyogenesis by way of an unknown progenitor.

Chemical modulation of the Wnt signaling also has profound effects on mesoderm specification and cardiomyocyte induction. GSK3 inhibitor BIO (Table 3), which activates Wnt signaling, significantly induced cardiomyocyte formation in mESCs when introduced during the first three days of ESC differentiation (Naito et al., 2006). BIO was also reported to markedly expand the specific subset of ESC-derived, embryonic and postnatal cardiovascular progenitor cells which express the Isl1 marker (Qyang et al., 2007). These results outlined the stage-specific role of Wnt signaling in cardiac progenitor specification and proliferation. Paradoxically, cardiomyocyte differentiation was suppressed when mESCs were exposed to BIO after day 5 of differentiation, following the formation of cardiovascular progenitor cells (Naito et al., 2006). Similar biphasic role of Wnt signaling was demonstrated in hESCs (Paige et al., 2010), whereby inhibiting Wnt signaling in multipotent mesodermal progenitor cells promoted cardiac differentiation (Kattman et al., 2011). Consistent with these results, XAV939, a selective small molecule inhibitor of Tankyrase required for Wnt signaling, markedly induced cardiomyogenesis in mESCs when introduced after mesoderm formation (Wang et al., 2011). In summary, these studies suggest that stage-specific chemical modulation of Wnt signaling is a promising strategy for directed differentiation of cardiac cell types in human pluripotent stem cells.

In contrast to the endoderm and mesoderm lineages, ectoderm differentiation of ESCs is commonly considered a default developmental pathway (Reubinoff et al., 2000). Ectoderm commitment can be further promoted by inhibiting BMP (Winnier et al., 1995; Finley et al., 1999) and downstream SMAD signaling (Sirard et al., 1998) that are required for mesoderm formation (Finley et al., 1999). This signaling pathway can be targeted using chemical means in conjunction with other protein modulators. The TGF-β inhibitor SB431542 (Table 2), when used in combination with BMP inhibitor Noggin, induced neural differentiation in human ESC and human iPSC (Chambers et al., 2009). There are other compounds that can

generate the neurogenic phenotype, but the mechanism is not well characterized. Neuropathiazol (Table 3) can induce differentiation of human hippocampus progenitors into neurons (Warashina et al., 2006), which may hold therapeutic potential as a treatment for Parkinson's disease. An additional pro-neurogenesis cocktail was described recently; GSK3 inhibitors SB216763 and kenpaullone (Table 3) were found to stimulate human neural progenitor cells commitment by upregulating Wnt signaling without changing cell cycle progression or proliferation (Lange et al., 2011). This observation would suggest that the neurogenic effect of SB216763 and kenpaullone may be to increase lineage commitment rather than to expand existing neural cells.

A study performed in rats showed the compound KHS101 (Table 3) can induce neural differentiation by inhibiting neural progenitor cell maintenance, while also stabilizing pro-neurogenesis transcriptional pathways (Wurdak et al., 2010). KHS101 was shown to negatively affect cell cycle exit and proliferation, thereby blocking the maintenance of the undifferentiated neural progenitor cell phenotype. Concurrently, KHS101 physically interacted with TACC3 (a structural component of the centrosome and mitotic spindle) and prevents its ability to sequester ARNT2 (a pro-neurogenesis transcription factor) in the cytoplasm (Wurdak et al., 2010). The net effect was KHS101 accelerated neural differentiation from the progenitor pool by blocking self-renewal.

It would be extremely useful if protein modulators could be excluded completely from the cell fate decision process, and stepwise lineage commitment was achieved chemically. In the event that such compounds or protocols failed to arise, ESCs can be chemically manipulated to increase their sensitivity to exogenous differentiation signals. Stauprimide (Table 3) was reported to prime mouse and human ESCs for differentiation via interaction with NME2, a metastatic factor with a possible role in promoting pluripotency (Zhu et al., 2009). The initial report did not demonstrate that the compound stimulates differentiation towards any particular lineage, but rather amplifies induction efficiency (Zhu et al., 2009). The mechanism is unclear at this time, but it may be that NME2 inhibition limits self-renewal maintenance by reducing c-Myc expression (Thakur et al., 2009). In this case, stauprimide may function by making self-renewal an unfavorable condition for the cell and thus lowering the energy requirements for extracellular differentiation signals. It would be interesting to see whether modulating self-renewal can be a general approach to promote directed differentiation.

The above examples introduced some of the potential advantages of using small molecules for directed differentiation. Not only do they help uncover novel differentiation mechanisms and interact with known development pathways, but they also offer fine temporal control to the investigator. Given our limited knowledge of the temporal regulation of individual pathways, chemical biology has yielded intriguing insights into the dynamic interactions that drive cell fate commitment, and continues to be integrated with developmental and cellular biology. The foreseeable trajectory for all these areas is to promote basic understanding of developmental mechanisms and to apply this knowledge towards improving clinical outcomes.

The Future of Bioactive Compounds in Cell-based Therapy

An emerging area of interest for both regenerative medicine and chemical biology is the manipulation of the local tissue microenvironment following transplantation. Transplanted cells are generally introduced to a hostile environment in injured or diseased tissues, resulting in massive cell loss and depleting much of their therapeutic potential. Identification of signaling pathways that improve the survival of newly introduced cells either by inhibiting apoptosis or sustaining proliferation would be of special interest. In addition,

transplanted cells will likely require extracellular cues to fully mature and undertake their functional role in regeneration or repair. Therefore, understanding and controlling these extracellular factors is required to extract long-term benefits from any cell-based system.

A paradigm shift has occurred in regenerative medicine that will require a multifaceted approach to cell-based therapies. It would require focus not only on robust pluripotency maintenance and appropriate cell differentiation, but also on their interactions with the extracellular milieu. The future of this field will require a fully coordinated view that will match specific pluripotent /progenitor states to the desired differentiated population, and their incorporation into the optimal extracellular support matrices for downstream applications. At this point, the usage of bioactive compound is in the early stages and questions remain concerning their cellular effects compared to that produced by natural processes. Additional studies are required to better correlate *in vitro* benefits to *in vivo* applications as well as their pharmacological properties. Nevertheless, small molecules have shown themselves as enablers of regenerative therapy and have reduced many of its challenges. They will undoubtedly play a critical role in many areas of stem cell research and help mobilize those discoveries towards therapeutic options.

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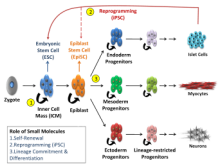


Figure 1. Applications of small molecules in cell-based therapies

Small molecules can intervene at many junctions of the stepwise differentiation process. They can 1) promote self-renewal in culture, 2) enhance reprogramming of adult somatic cells, and 3) direct differentiation of pluripotent or lineage-committed progenitor cells. These features will be valuable for harnessing the full potential of pluripotent cells in regenerative therapy.

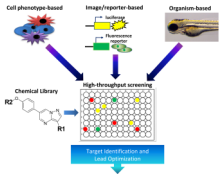
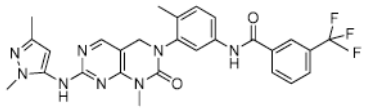
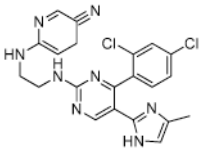
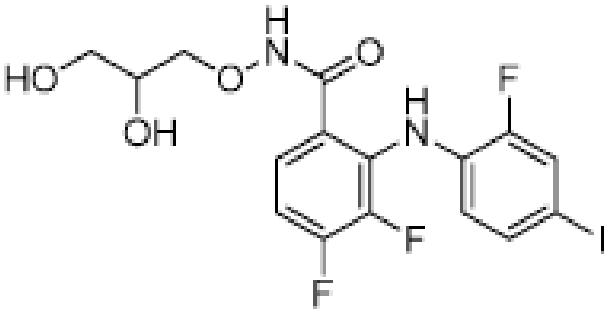
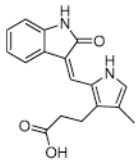
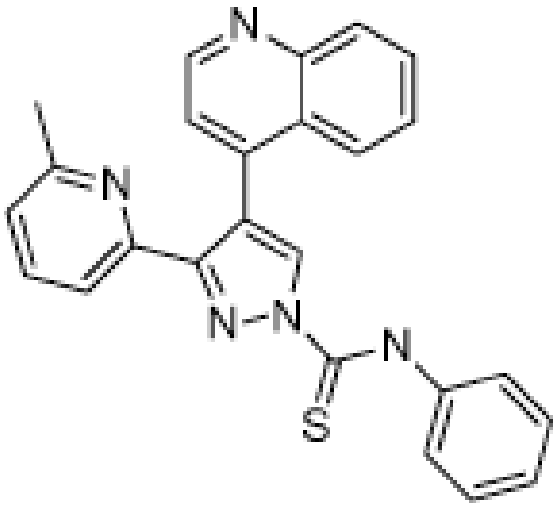


Figure 2. Strategies for high-throughput chemical screening

Small molecules from a chemical library are identified in high-throughput screening assays using stem cells. Compounds are evaluated for the desired effects using cellular images, specific promoter driven reporters, or organism phenotypes as readout.

Table 1

Small molecules for self-renewal in stem cells and iPSCs

Molecule	Name	Target	Reference
	Pluripotin/SC1	Dual Inhibitor RasGAP/ERK1	Chen (2006)
	CHIR99021	GSK3 inhibitor	Ying (2008) Li (2009a) Li (2009b)
	PD0325901	MEK inhibitor	Lin (2009) Zhu (2010)
	SU5402	FGF inhibitor	Ying (2008)
	A-83-01	TGF-β Inhibitor	Li (2009a) Zhu (2010)

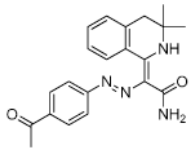
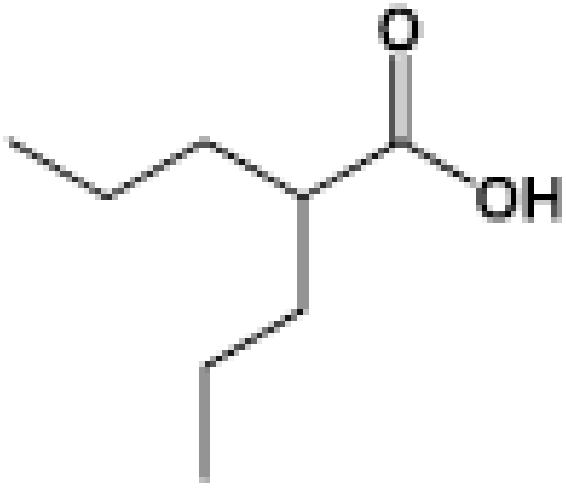
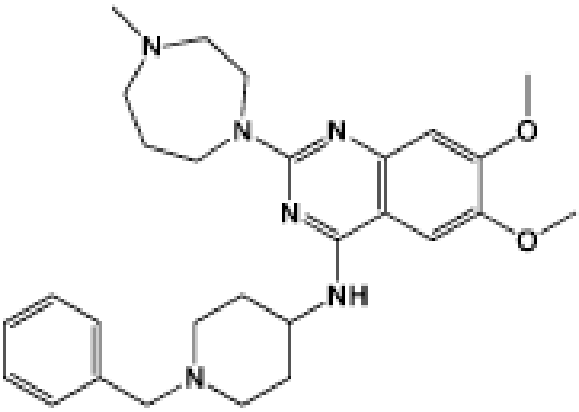
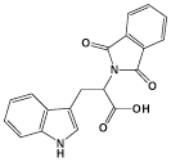
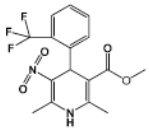
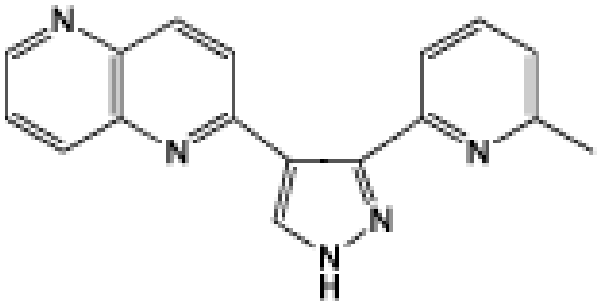
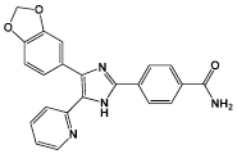
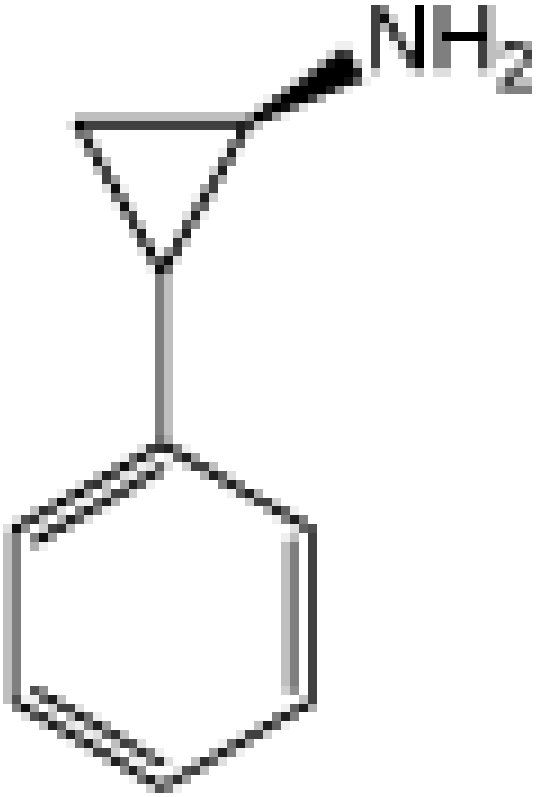
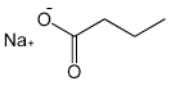
Molecule	Name	Target	Reference
	IQ-1	Phosphatase PP2A Inhibitor (Wnt modulator)	Miyabayashi (2007)

Table 2

Small molecules for enhanced reprogramming efficiency

Molecule	Name	Target	Reference
	Valproic Acid (VPA)	Histone deacetylase (HDAC) Inhibitor	Huangfu (2008)
	BIX01294	AG9a histone methyltransferase (HMTase) inhibitor	Shi (2008b)
	RG108	DNA methyltransferase (DNMT) Inhibitor	Shi (2008a)
	BayK8644	L-type calcium channel agonist	Shi (2008a)

Molecule	Name	Target	Reference
	RepSox	TGFβ inhibitor	Ichida (2009)
	SB431542	TGFβ inhibitor	Lin (2009) Chambers (2009)
	Parnate	lysine-specific demethylase 1 (LSD1) inhibitor	Li (2009a)
	Sodium Butyrate (NaB)	Histone Deacetylase (HDAC) inhibitor	Zhu (2010)

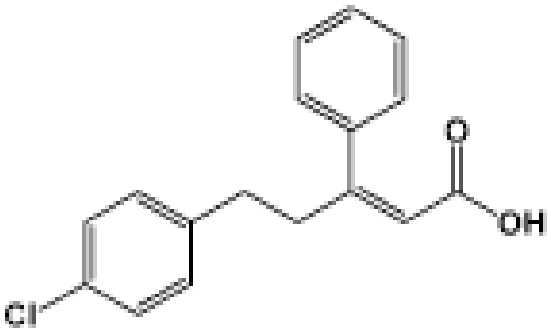
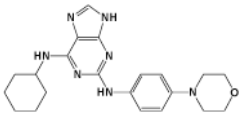
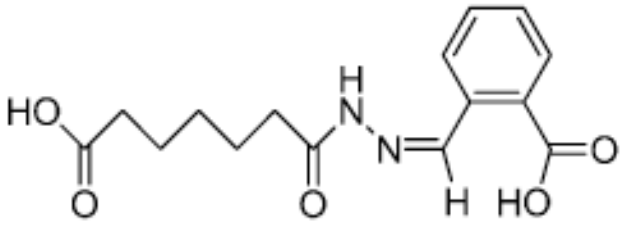
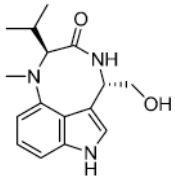
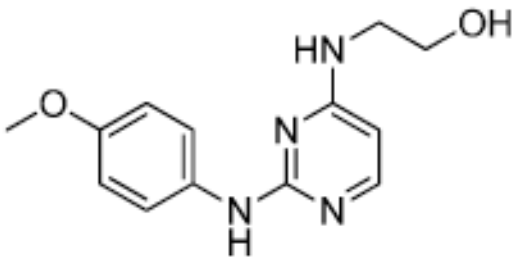
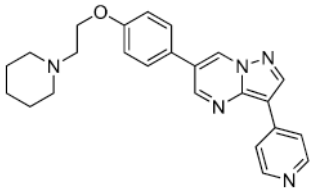
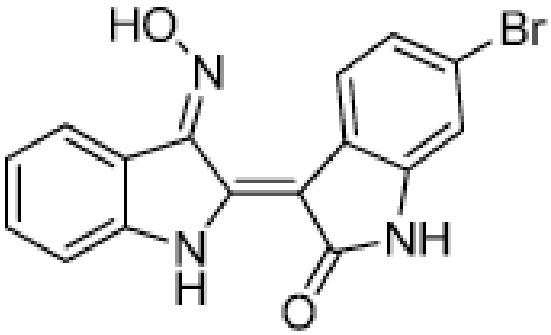
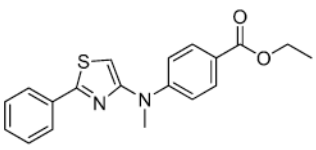
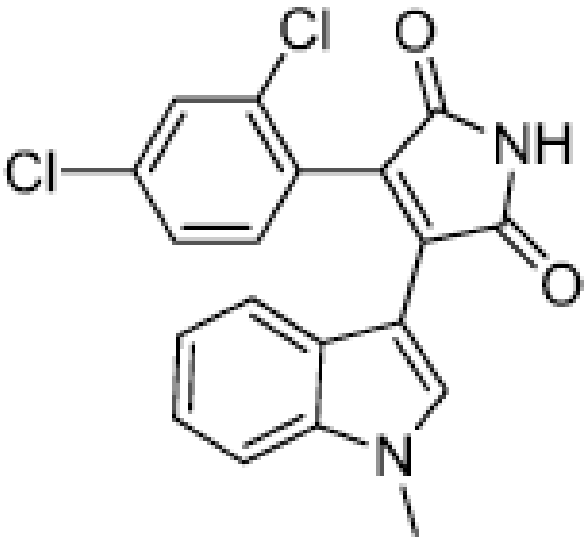
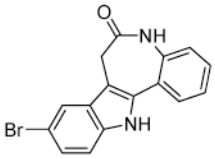
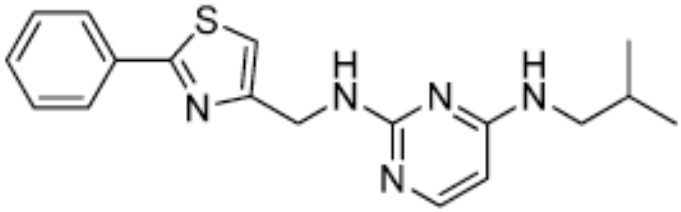
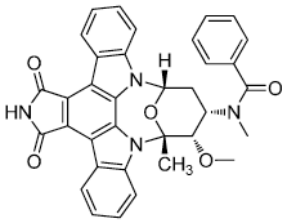
Molecule	Name	Target	Reference
	PS48	PDK1 activator	Zhu (2010)
	Reversine	Aurora B Kinase Inhibitor	Chen (2004) D'Alise (2008)

Table 3

Small molecules for directed differentiation

Molecule	Name	Target	Reference
	IDE1	Unknown	Borowiak (2009)
	Indolactam V(ILV)	PKC Activator	Borowiak (2009) Chen (2009)
	Cardiogenol C	Unknown	Wu (2004)
	Dorsomorphin	BMP Type 1 receptor Inhibitor	Hao (2008)
	6-Bromoindirubin-3'-oxime (BIO)	GSK3 Inhibitor (Wnt activator)	Sato(2004) Naito (2006) Tseng (2006) Qyang(2007)
	Neuropathiazol	Unknown	Warashina (2006)

Molecule	Name	Target	Reference
	SB216763	GSK3 Inhibitor (Wnt activator)	Lange (2011)
	Kenpaullone	GSK3 Inhibitor (Wnt activator)	Lange (2011)
	KHS101	TACC3 (Mitotic spindle)	Wurdak(2010)
	Stauprimide	NME2(metastatic factor)	Zhu (2009)