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A role for chemistry in stem cell biology

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Although stem cells hold considerable promise for the treatment of numerous diseases including cardiovascular disease, neurodegenerative disease, musculoskeletal disease, diabetes and cancer, obstacles such as the control of stem cell fate, allogenic rejection and limited cell availability must be overcome before their therapeutic potential can be realized. This requires an improved understanding of the signaling pathways that affect stem cell fate. Cell-based phenotypic and pathway-specific screens of natural products and synthetic compounds have recently provided a number of small molecules that can be used to selectively control stem cell proliferation and differentiation. Examples include the selective induction of neurogenesis and cardiomyogenesis in murine embryonic stem cells, osteogenesis in mesenchymal stem cells and dedifferentiation in skeletal muscle cells. Such molecules will likely provide new insights into stem cell biology, and may ultimately contribute to effective medicines for tissue repair and regeneration.

Stem cells are unspecialized precursor cells with the ability to self-renew and differentiate into specialized cells in response to appropriate signals[1]. Traditionally stem cells are classified as either embryonic or tissue-specific stem cells. Embryonic stem (ES) cells are derived from the inner cell mass of the early embryo (blastocyst) and can give rise to any differentiated cell type found in the three primary germ layers of the embryo (endoderm, mesoderm and ectoderm), as well as germ cells[2–4]. Tissue-specific stem cells (adult stem cells) are unspecialized cells found in differentiated tissues, which can self-renew for long periods of time and differentiate into specialized (mature) cell types of the tissue in which they reside. ES cells generally can be maintained in an undifferentiated state indefinitely without losing differentiation potential, although they may develop karyotypic abnormalities when cultured over long periods[5]. In contrast, adult stem cells can only proliferate for a limited number of generations, and their response to differentiation signals declines after each generation. The differentiation of adult stem cells is also generally restricted to certain tissue types (multipotent), although a type of multipotent adult progenitor cell derived from bone marrow appears to have greater differentiation potential[6]. In contrast, ES cells are generally regarded as pluripotent and will develop a benign tumor (teratoma) containing multiple tissue types when injected into a host. Consequently, ES cells must be differentiated into the desired tissue and subtype specific cells before they can be used safely and effectively in clinical applications.

Although ethical debates surround the isolation of human ES cells, significant technical challenges surround the isolation and expansion of tissue-specific adult stem cells. For example, hematopoietic stem cells are relatively easy to isolate from bone marrow, but are difficult to expand without differentiation. Conversely, neural stem cells can be expanded in the presence of growth factors (such as basic fibroblast growth factor, bFGF), but the noninvasive isolation and purification of significant numbers of neural stem cells from the brain remains challenging. Histocompatibility raises another challenge in the use of allogeneic cells for therapeutic applications, although progress has been made in using one's own adult stem cells for tissue repair, as well as in generating human ES cells via nuclear transfer cloning using human adult somatic cells[7].

Stem cell fate is controlled by both intrinsic regulators and the extracellular environment (niche). Under appropriate conditions in cell culture, stem cells can differentiate spontaneously. For example, when ES cells are grown in suspension in the absence of leukemia inhibitory factors, they form aggregates called embryoid bodies, which begin to differentiate spontaneously into various cell types, including hematopoietic, endothelial, neuronal and muscle cells. However, spontaneous differentiation is generally inefficient and leads to heterogeneous populations of differentiated and undifferentiated cells, which are not useful for cell-based therapy and also complicate biological studies of particular differentiation programs. Thus stem cell expansion and differentiation *ex vivo* are generally controlled by 'cocktails' of growth factors, signaling molecules and/or genetic manipulation. Clearly, more efficient and selective methods are needed to direct the proliferation and the differentiation of stem cells, especially ES cells, to produce homogenous populations of particular cell types. This may be essential not only for the therapeutic use of stem cells, but will also greatly facilitate studies of the molecular mechanism of development.

Cell-based phenotypic assays and, more recently, pathway screens of synthetic small molecules and natural products have historically provided useful chemical tools to modulate and/or study complex cellular processes[8]. Cell permeable small molecules (**Fig. 1**) such as dexamethasone (a glucocorticoid receptor agonist), ascorbic acid, 5-azacytidine (5-aza-C, a DNA demethylating agent) and *all-trans* retinoic acid have proven useful for inducing the differentiation of various stem cells (*e.g.*, embryonic, neural and mesenchymal stem

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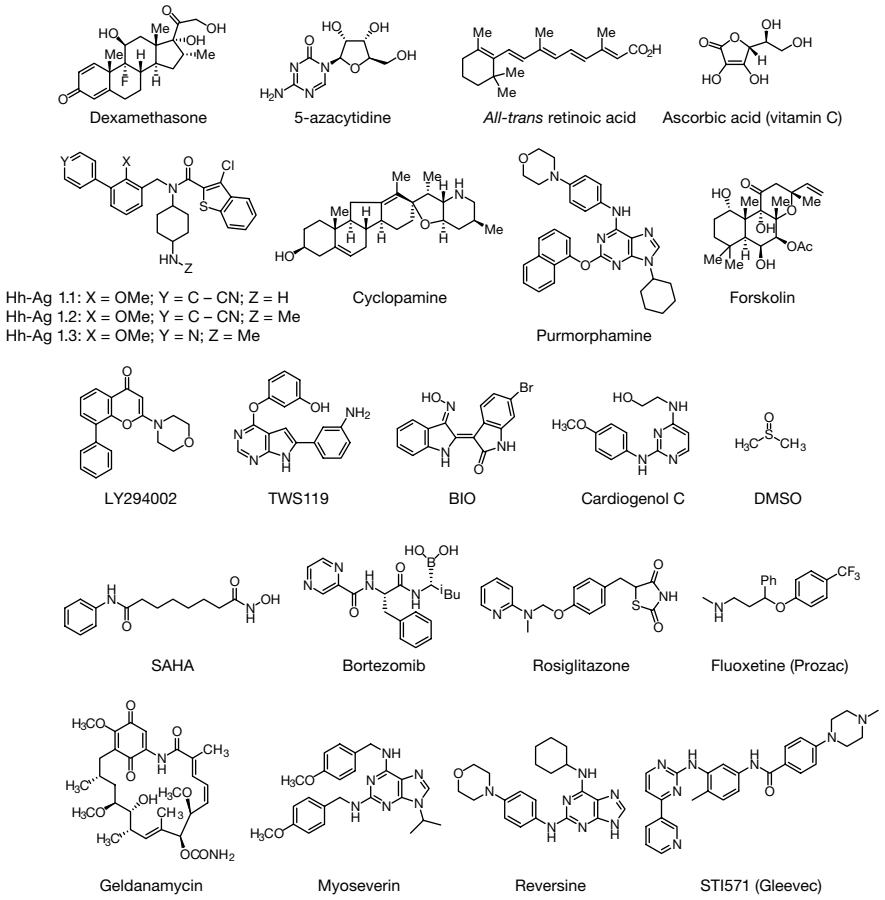

Figure 1 Structures of small molecules that regulate stem cell fate.

cells). For example, studies with 5-aza-C, which induces myogenic differentiation of a mouse mesenchymal progenitor cell line, led to the discovery of a master transcription factor, MyoD[9], responsible for skeletal myogenic fate determination. Moreover, small molecule inhibitors, such as suberoylanilide hydroxamic acid (SAHA, inhibitor of histone deacetylase-HDAC)[10], geldanamycin (Hsp90 inhibitor)[11], imatinib mesylate (Gleevec; kinase inhibitor)[12] and bortezomib (proteasome inhibitor)[13] induce differentiation of various progenitor and transformed cells and are used clinically for the treatment of cancers. In this article, we focus on recent advances in the use of small molecules to control cell proliferation, differentiation and dedifferentiation/transdifferentiation in various embryonic and adult stem cell systems, as well as approaches to studying their underlying mechanisms of action.

Strategies

One approach to generating functional small molecules that control stem cell fate involves the use of cell-based phenotypic or pathway-specific screens of synthetic chemical or natural product libraries (**Fig. 2**). With recent advances in automation and detection technologies, millions of discrete compounds can be screened rapidly and cost-effectively. However, although combinatorial technologies allow the synthesis of a large number of molecules with immense structural diversity, it is impossible to saturate chemical space. Consequently, the careful design of chemical libraries becomes a critical aspect of combinatorial synthesis.

Ideally, the properties of a chemical library are optimized to interact with the specific biomolecules or collection of biomolecules of interest. Previously, we developed the concept of using molecular scaffolds themselves as a diversity element for combinatorial synthesis[14]. In this approach, various naturally occurring and synthetic heterocycles known to interact with proteins involved in cell signaling (*e.g.*, kinases and cell surface receptors) comprise the core molecular scaffolds. These included substituted purines, pyrimidines, indoles, quinazolines, pyrazines, pyrrolopyrimidines, pyrazolopyrimidines, phthalazines, pyridazines and quinoxalines. General synthetic schemes were then developed that could be used in parallel reactions to introduce a variety of substituents into each scaffold to create diverse chemical libraries. For example, a second diversity element could be introduced into heterocyclic scaffolds using solution-phase alkylation or acylation reactions. This was followed by capture of the modified heterocycles onto solid support using different immobilized amines to introduce a third diversity element. The resin-bound heterocycles could then be further modified (introducing a fourth diversity element) through various chemistries including acylation, amination and palladium-mediated cross-coupling reactions with amines, anilines, phenols and boronic acids. Using these chemistries in conjunction with the 'directed-sorting' method, we have generated a privileged heterocycle library consisting of over 25 distinct structural classes and 100,000 discrete small molecules, which has proven to be a rich source of biologically active small molecules.

To systematically identify small molecules that can generate a cellular phenotype of interest (for example, the directed differentiation of ES cells to neurons), high-throughput screens of large diverse chemical libraries are carried out in a desired model system—a cell line (primary or immortalized) or a simple organism (*Xenopus laevis* or zebrafish) with an appropriate readout such as luminescence (*e.g.*, a luciferase reporter), fluorescence (*e.g.*, an enhanced green fluorescence protein (EGFP) reporter), or absorbance (enzymatic reactions to generate chromophores). However, such assays only provide limited information and require a battery of secondary assays to determine the precise cellular pathways or processes being affected. With recent advances in high-content imaging technologies, high resolution microscopy/image-based screens allow the capture of multiple parameters from a single reading at the single cell level, facilitating the identification of molecules with a desired biological activity (**Fig. 2**).

Pluripotent ES cells

Pluripotent ES cells represent a potentially unlimited source for all types of specialized cells and are ideally suited for studies of differentiation and early embryonic development. The most commonly used method to differentiate ES cells is to grow them as aggregates in suspension without leukemia inhibitory factors (LIF) to form embryoid bodies, which spontaneously differentiate into various cell lineages.

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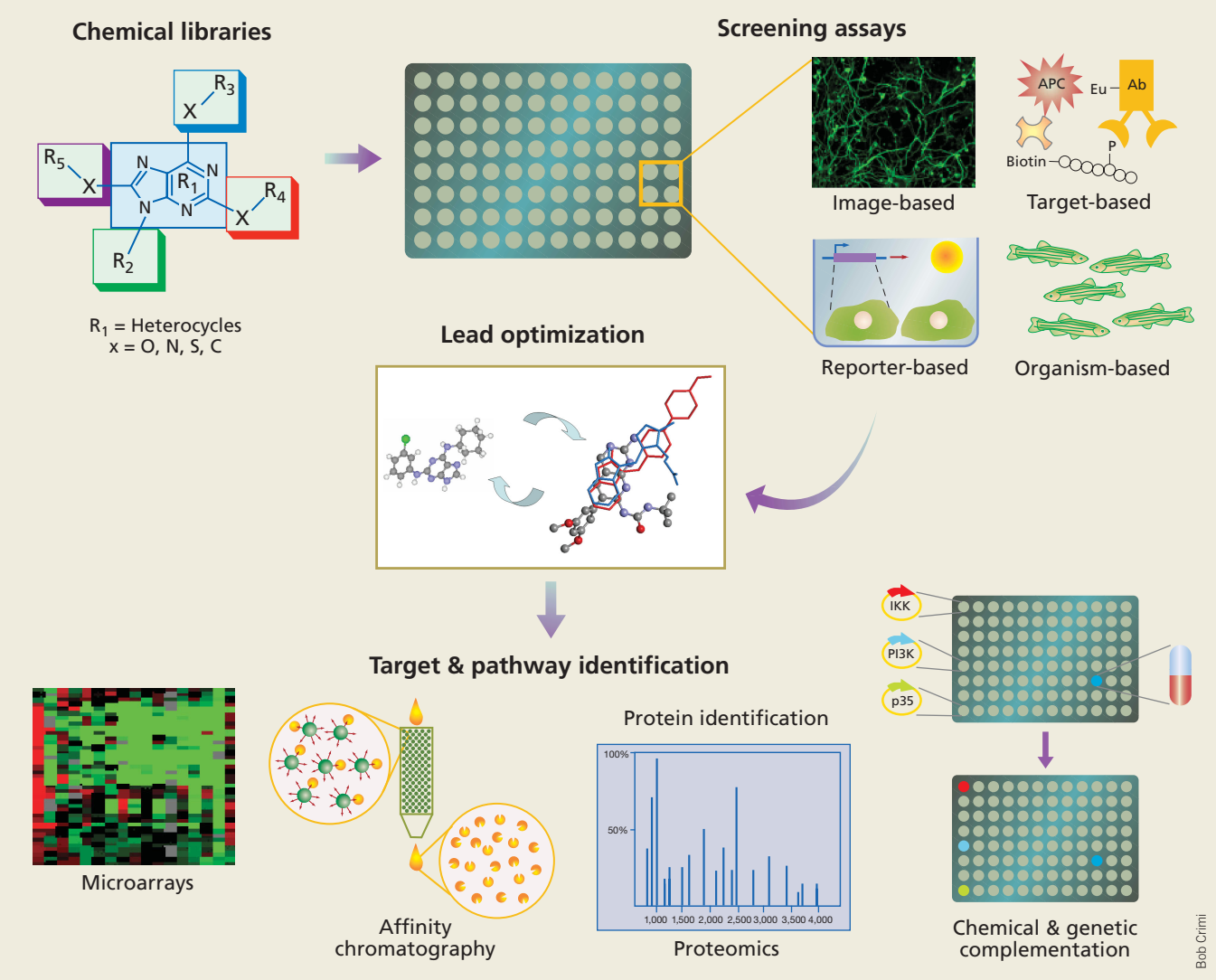

Figure 2 Chemical screens and characterizations of small molecules that control stem cell fate. Chemical libraries are screened with various target-based, cell-based (phenotypic or pathway-specific) and organism-based phenotypic assays, and identified hit molecules are optimized and characterized by target identification via affinity chromatography, genomic and proteomic profiling, and chemical and gene complementations.

The differentiation programs of embryoid bodies can be shifted toward cardiomyogenic, neuronal or primordial germ cells by treatment with small molecules, such as dimethyl sulfoxide (DMSO) or retinoic acid, at specific stages of differentiation. However, this approach is not very efficient, and normally requires selection to enrich for specific cell lineages. To overcome these difficulties, several small molecules have been recently identified that can be used to selectively control ES cell fate.

Neuronal differentiation. The directed differentiation of mouse ES cells into motor neurons[15] has been carried out in a stepwise fashion first by embryoid body formation with concomitant retinoic acid treatment to neuralize ES cells to neural progenitors. These cells were further caudalized by retinoic acid, followed by treatment with a specific small molecule agonist (Hh-Ag1.3) of Sonic hedgehog (Shh) signaling to ventralize the caudalized neural cells to become motor neurons. Most importantly, motor neurons generated from ES cells using this process can repopulate the ventral spinal cord and innervate the muscle cells *in vivo* after implantation into the spinal cord of chick embryos[15]. This experiment also suggests that multiple sequential or combinations of signals may be required to generate a terminally differentiated and/or subtype-specific cell type (*e.g.*, dopaminergic neurons or β cells). It may therefore be necessary to carry out screens for directed differentiation of these functional cells in a sequential fashion and/or with combinations of drugs.

We recently undertook a cell-based phenotypic screen to identify synthetic small molecules which induce neuronal differentiation of mouse ES cells[16]. In contrast to the above approach, which was designed based on our understanding of neural pattern formation, we anticipated that unbiased cellular screens might lead to discoveries of new signaling pathways and mechanisms involved in the neuronal differentiation of ES cells. To screen the large library of heterocyclic compounds described above, we used a pluripotent mouse embryonal carcinoma cell line, P19, stably transfected with a neuronal-differentiation-specific luciferase reporter (pTα1-Luc)[17] (Tα1 tubulin is an early neuronal specific marker). Although resembling ES cells, P19 cells have a low frequency of spontaneous neuronal differentiation[18]. The

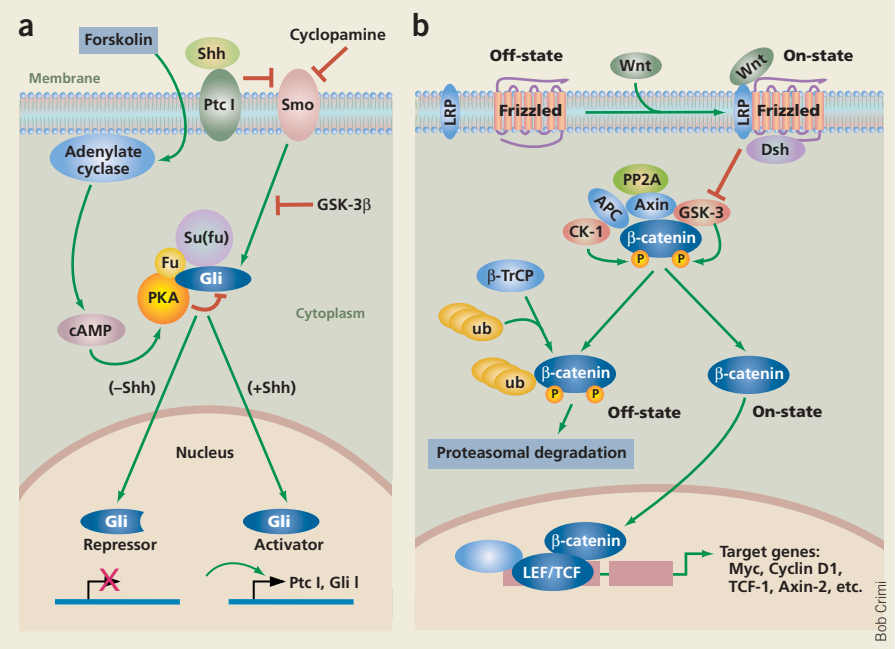

Figure 3 Signaling pathways. (**a**,**b**) Selected elements in the Shh (**a**) and Wnt (**b**) signaling pathways.

ability of selected compounds that upregulated luciferase activity to induce neuronal differentiation was confirmed by direct immunofluorescence staining with multiple neuronal markers and observation of characteristic neuronal morphology. Focused libraries of active 2,4-disubstituted-pyrrolopyrimidines were then generated to investigate the structure-activity relationship in this series of compounds, and an analog, TWS119, was found that potently induces neuronal differentiation in both mouse embryonal carcinoma and ES cells. Affinity chromatography of cell lysates with TWS119 immobilized on solid support, together with affinity supports made from inactive compounds (with minimal chemical modifications relative to the active analogs), revealed that one target of TWS119 is GSK-3β. The mode of action of TWS119 may involve GSK-3β alone or in combination with other as-of-yet unidentified cellular targets.

Although highly specific transgenic reporter assays are well suited for studying signaling pathways and offer the advantages of facile development, higher throughput and sensitivity for differentiation screens, alternative approaches, such as knock-in reporter assays, may better recapitulate endogenous gene expression. In addition, immunocytochemical, morphological and physiological assays offer more information about complex multistep cellular process, but are less amenable to high-throughput implementation.

Cardiomyogenic differentiation. The mammalian adult heart, like the brain, is mainly composed of postmitotic, terminally differentiated cells. Recent genetic tracing studies[19–21] of bone-marrow-derived stem cells transplanted in injured heart showed that the transplanted cells are unable to transdifferentiate into cardiomyocytes. Rather, cell fusion at a low frequency[21] contributed to the observed bone marrow–derived cardiomyocytes. In addition, although evidence[22–24] suggests the existence of a resident population of self-renewing cardiac stem cells capable of contributing to heart repair, the scarcity of these cells and their intrinsically poor regenerative response to heart injury remain obstacles for their therapeutic application.

Alternatively, pluripotent ES cells represent a possible unlimited source of functional cardiomyocytes. Cardiomyogenesis of ES cells occurs spontaneously upon embryoid body differentiation *in vitro*, but involves a poorly defined, inefficient and relatively nonselective process. Consequently, directed differentiation of ES cells into cardiomyocytes could facilitate therapeutic applications of ES cells, and increase our understanding of the molecular mechanism underlying cardiomyocyte differentiation and heart development.

Using ES cells stably transfected with the cardiac muscle–specific α-myosin heavy chain (MHC) promoter–driven EGFP as a reporter, Takahashi *et al.* screened 880 known drugs in monolayer culture and found that ascorbic acid (vitamin C) significantly enhances spontaneous cardiac differentiation of ES cells[25]. Interestingly, other antioxidants such as *N*-acetylcysteine or vitamin E do not have a similar effect, suggesting that the cardiomyogenesis-inducing activity of ascorbic acid may be independent of its antioxidative property. In addition, it was shown that ascorbic acid has no significant effect on spontaneous cardiomyogenesis via embryoid body formation, suggesting ascorbic acid mimics the permissive environments of embryoid bodies, rather than inducing autonomous cardiac muscle differentiation.

Concurrent with this work, we screened large combinatorial chemical libraries for synthetic small molecules that can selectively and efficiently induce the differentiation of murine ES cells to cardiomyocytes[26]. The primary screen was again carried out in P19 embryonal carcinoma cells which were stably transfected with the cardiac muscle–specific atrial natriuretic factor (ANF) promoter–driven luciferase reporter gene. A series of diaminopyrimidine compounds (cardiogenol A-D) were identified that efficiently and selectively induce mouse embryonal carcinoma and ES cells to differentiate into cardiomyocytes. The differentiated cells expressed multiple cardiac muscle markers and formed large areas of spontaneous contracting patches. In the case of human ES cells, 5-aza-deoxycytidine (a DNA demethylating agent that induces differentiation of mesenchymal stem cells to cardiomyocytes) was found to enhance spontaneous cardiomyocyte differentiation[27], whereas other small molecules such as retinoic acid and DMSO, known to have cardiomyogenesis-promoting effects on mouse ES cells, have no such activity.

β-cell differentiation. One treatment for type 1 diabetes, which results from autoimmune destruction of the insulin-secreting β cells in the pancreatic islets, is the transplantation of functional glucose-responsive islets with simultaneous prevention of their immune destruction. However, obstacles to this approach include the shortage of engraftable donor tissues and lifelong immune suppression. Alternatively, it may be possible to stimulate endogenous β-cell regeneration *in vivo*, especially in light of a recent study[28] demonstrating that β cells themselves are responsible for β-cell maintenance and regeneration in adult mice. Although identification of renewable cell sources of engraftable functional β cells is desired for transplantation therapy, both approaches require an improved ability to manipulate β-cell regeneration *in vitro* and *in vivo*.

ES cells could provide a source of pancreatic islet cells, but the spontaneous differentiation of ES cells produces only small numbers of insulin-producing cells. Recently large numbers of insulin-secreting

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structures resembling pancreatic islets were derived from mouse ES cells by a sophisticated multistage cell culture manipulation[29]. Hori *et al.* later modified this protocol and demonstrated that a phosphoinositide 3-kinase (PI3-K) inhibitor, LY294002, promotes differentiation of insulin-secreting β cells from ES cells[30] in culture. They further demonstrated that these differentiated β cells can function *in vivo* to increase circulating insulin levels, reduce weight loss, improve glycemic control and completely rescue mice with diabetes mellitus.

Glucagon-like peptide-1 (GLP-1) and exendin-4 (a peptide from lizard venom), GLP-1 receptor agonists, increase islet neogenesis, enhance β-cell proliferation and survival *in vivo*, as well as promote β-cell proliferation and differentiation *in vitro*[31]. A synthetic exendin-4, AC2993, is currently in clinical trials for the treatment of type 1 and 2 diabetes[32] .

Proliferation. The identification of factors and signaling pathways that maintain self-renewal of ES cells is also of considerable interest. Mouse ES cells are conventionally maintained on feeder cells and/or mixtures of exogenous factors (including LIF and serum). However, complex media may contain factors that promote differentiation and other activities. Although the factors that control self-renewal of human ES cells are still largely unknown, recent work by Ying *et al.* showed that mouse ES cells can be grown in a defined medium supplemented with LIF and bone morphogenetic protein (BMP)[33]. Interestingly, 6-bromoindirubin-3′-oxime (BIO), a natural product derived from mollusk Tyrian purple, has been recently shown to maintain human and mouse ES cells in a pluripotent state in the absence of conditioned medium[34]. BIO may function by inhibiting GSK3 and activating the canonical Wnt signaling pathway (**Fig. 3**).

Multipotent mesenchymal stem cells

Multipotent mesenchymal stem cells (MSCs) can differentiate into a variety of nonhematopoietic tissues such as osteoblasts, adipocytes and chondrocytes. Previous studies have shown that adult MSCs, when injected into animals, are capable of homing to a site of injury and restoring tissue function[35]. MSCs are also relatively easy to isolate from small aspirates of bone marrow, and expand in culture.

In addition to primary MSCs, the mouse mesenchymal progenitor cell line, C3H10T1/2, as well as a series of lineage-committed progenitor cell lines (*e.g.*, 3T3L1/preadipocytes and MC3T3-E1/preosteoblasts), are widely used to study cell fate determination. Numerous small molecules have been found that can be used to control the differentiation of these progenitor cells for a variety of applications. For example, 5-aza-C can induce C3H10T1/2 cells to differentiate into myoblasts, osteoblasts, adipocytes and chondrocytes. 5-Aza-C does not directly activate a specific differentiation program, but rather converts the cells into a competent spontaneous differentiation state. Peroxisome proliferator-activated receptor γ (PPARγ) agonists (such as rosiglitazone) and antagonists are widely used as adipogenesis modulators; dexamethasone, ascorbic acid and β-glycerophosphate induce osteogenesis or adipogenesis of MSCs under carefully defined conditions[36,37].

To identify small molecules that selectively differentiate MSCs into defined lineage-committed progenitor cells, we screened the combinatorial heterocyclic compound library in the C3H10T1/2 cell line for molecules that induce osteogenesis[38]. A high-throughput fluorescence-based enzymatic assay was used to detect the bone-specific marker, alkaline phosphatase (ALP). A 2,6,9-trisubstituted purine compound, purmorphamine, was identified as a potent osteoblast differentiation-inducing agent. Purmorphamine can activate Cbfa1/Runx2 (a master regulator of bone development), as well as upregulate other bone-specific markers, such as osteopontin and collagen-I. Cells treated with purmorphamine also have the characteristic osteoblast morphology.

To uncover the molecular mechanism by which purmorphamine induces osteogenesis, genome-wide mRNA expression profiling was carried out on cells treated with purmorphamine. This expression analysis revealed that purmorphamine treatment upregulates key downstream members of the hedgehog (Hh) signaling pathway including Gli1 and Patched. To validate whether purmorphamine's effect is dependent on Hh pathway activation, two different Hh pathway inhibitors, which act at different points along the pathway, were used. Cyclopamine, a natural alkaloid isolated from California corn lily and a specific Hh antagonist, acts at the level of Smoothened (Smo). Another inhibitor, forskolin, activates protein kinase A (PKA), which converts Gli proteins to transcriptional repressors by phosphorylation (**Fig. 3**). Both cyclopamine and forskolin block purmorphamine's ability to induce osteogenesis and Hh pathway activation, but have no inhibitory effect on BMP-4-induced osteogenesis in C3H10T1/2 cells. This result suggests that purmorphamine acts through the Hh pathway, either at the level of Smo or an earlier point in the pathway.

Neural stem cells

Recent discoveries of neural stem cells (NSCs) in the adult central nervous system (CNS) and their regenerative roles in brain damage may make possible new approaches to the treatment of neurodegenerative disease and CNS injury[39]. These could involve cell replacement therapy and/or drug treatment to stimulate the body's own regenerative mechanisms by promoting survival, migration, proliferation and/or differentiation of endogenous CNS stem cells. However, such approaches require the identification of renewable cell sources of engraftable functional neurons, and an improved understanding of and ability to manipulate neuronal development.

CNS stem cells can be isolated from embryonic and adult brains, and are normally maintained in an undifferentiated state *in vitro* with bFGF. They are generally considered as tripotent, self-renewing progenitors that can generate neurons, astrocytes and oligodendrocytes, the three major cell types of the CNS. The fact that neural progenitors that are restricted to generate specific types of CNS cells *in vivo* can self-renew and differentiate into all three CNS cell types in cell culture, suggests that environment (niche) can influence or reprogram the fate of these progenitors[40].

Pathway-specific modulators also play critical roles in regulating neural stem cell fate. For example, Shh has recently been shown to regulate proliferation of adult hippocampal neural progenitors both *in vitro* and *in vivo*. Cyclopamine, an antagonist of the Shh signaling pathway, inhibits proliferation and initiates differentiation of murine medulloblastoma cells[41]. Moreover, cyclopamine can shrink murine tumor allografts *in vivo* and induce rapid cell death of human medulloblastoma cells. Consistent with Shh's function in maintaining stem cell self-renewal in various tissues, these findings support the notion that certain tumors may arise from cancer stem cells with deregulated proliferation/differentiation signaling pathways. Targeting such pathways specifically to induce apoptosis and/or differentiation of cancer stem cells may have therapeutic benefits[42].

A series of Hh pathway–specific agonists and antagonists have been identified through screens of synthetic compounds using 10T1/2 cells stably transfected with a plasmid containing a luciferase reporter downstream of multimerized Gli binding sites and a minimal promoter[43]. One agonist (Hh-Ag 1.2) was shown to stimulate proliferation of neural progenitor cells. Chemical epistasis and binding assays showed that Hh-Ag 1.2 acts at the same level as cyclopamine, possibly as a Smo ligand. Synthetic Hh pathway specific–agonists and antagonists were also used to dissect the effect of bFGF on CNS stem cells

in vitro. This study[37] demonstrated that bFGF can ventralize CNS stem cells, at least in part, through induction of Shh expression and downstream signaling, which converts the *in vivo* fate-restricted bipotent neural precursors to the *in vitro* tripotent neural progenitor cells with three fates. Forskolin, which acts with retinoic acid to promote neuronal differentiation of adult neural stem cells[44], activates adenylate cyclase and leads to activation of cAMP signaling and PKA, resulting in blocking Shh signaling through inhibition of Gli-dependent transcriptional activation, which is involved in neural stem cell proliferation pathways. In another example, chronic antidepression drugs, such as fluoxetine, were shown to stimulate neurogenesis in the hippocampus, possibly establishing a link between the regeneration of neurons and depression[45].

We have used high-content imaging in conjunction with immunofluorescent labeling of neuronal (βIII tubulin, TuJ1) and astroglial markers (glial fibrillary acidic protein) to screen chemical libraries for molecules that can direct the differentiation of primary NSCs isolated from adult rat hippocampus specifically into neurons or astroglia (S.D. and P.G.S., unpublished data). Treatment of adult rat primary NSCs in monolayer with a 2-substituted aminothiazole compound induced up to 80% of cells to differentiate into TuJ1 positive neurons with the characteristic neuronal morphology. Expression of the neuronal bHLH transcription factors NeuroD1 was also shown to be upregulated, and expression of Sox2 (a neural progenitor marker) decreased after compound treatment. Importantly, the compound was also shown to suppress astroglial differentiation induced by BMP2 and LIF combination treatment, whereas retinoic acid failed to do so. We are currently examining the *in vivo* effects of this molecule.

Regeneration, reprogramming and dedifferentiation

Regeneration involves either epimorphosis where pre-existing stem cells or dedifferentiation-generated progenitor cells proliferate and differentiate to replace the lost cells, and/or morphallaxis where the regenerative processes are mainly mediated by the remodeling of remaining tissues with little cellular proliferation. Among organisms that have regenerative capabilities, the urodele amphibians uniquely use a cellular dedifferentiation mechanism at the damaged site to form a blastema which contains dedifferentiated progenitor cells[46]. These cells can proliferate and redifferentiate under developmental control to regenerate a wide variety of tissues, such as limbs, tails and lens. In mammals, epimorphic regeneration is largely limited by an irreversible differentiation process.

A long-standing notion in developmental biology has been that organ/tissue-specific stem cells are restricted to differentiating into cell types of the tissue in which they reside. In other words, they have irreversibly lost the capacity to generate other cell types in the body. Recent studies have demonstrated that the observed *in vivo* 'plasticity' of bone marrow–derived stem cells may be largely attributed to cell fusion events[21,47–51]. However, evidence suggests that tissue-specific stem cells may overcome their intrinsic lineage-restriction upon exposure to a specific set of signals *in vitro*[52] and *in vivo*[53] (although this reprogramming may not reflect potentials that are normally exercised *in vivo*). An extreme example is the reprogramming of a somatic cell to a totipotent state by nuclear transfer cloning, where either the nucleus of a somatic cell is transferred into an enucleated oocyte[54,55], or the extracts of the oocyte are fused with a somatic cell[56].

Although in mammals neither transdifferentiation nor dedifferentiation has yet been identified as a naturally occurring process (except in certain diseases[57]), the discovery of stem cell plasticity raises the possibility of reprogramming restricted cell fate. The ability to dedifferentiate or reverse lineage-committed cells to multipotent progenitor cells might overcome many of the obstacles associated with using ES cells and adult stem cells in clinical applications. With an efficient dedifferentiation process, one's own healthy, abundant and easily accessible adult cells could conceivably be used to generate different functional cell types to repair damaged tissues and organs.

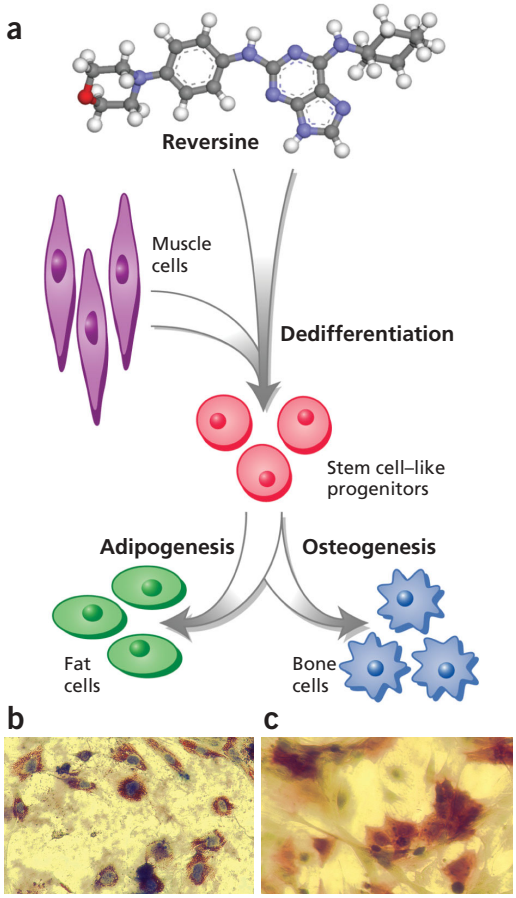

Figure 4 Reversine, a 2,6-disubstituted purine, dedifferentiates lineage-committed myoblasts to multipotent mesenchymal progenitor cells. (**a**) Dedifferentiation scheme. (**b**,**c**) Dedifferentiated C2C12 myoblasts can differentiate into adipocytes under adipogenesis-inducing conditions (**b**) or into osteoblasts under osteogenesis-inducing conditions (**c**).

Recent *in vitro* studies suggest that terminally differentiated C2C12 myotubes can be induced to undergo dedifferentiation into mesenchymal progenitor cells by the ectopic expression[58] of *Msx1* or addition of extracts[59] from regenerating newt limb. We have shown that a small molecule, myoseverin, induces cleavage of multinucleated myotubes to generate myoblast-like cells, which can proliferate and redifferentiate into myotubes[60]. Affinity chromatography of cell extracts and examination of cytoskeletal proteins revealed that myoseverin binds and disassembles microtubules. Genome-wide mRNA expression analysis further demonstrated the downregulation of myogenic differentiation markers and upregulation of proliferation-associated genes. These results suggest that the effects of myoseverin most likely stem from cytoskeletal remodeling rather than the dedifferentiation to multipotent progenitor cells. Nonetheless, although the myoblasts generated by myoseverin are still unipotent, and the observed cleavage of myotubes may only represent a single step of lineage-reversal, this experiment demonstrated that terminally differentiated states can be altered by a small molecule.

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To identify small molecules that induce true dedifferentiation of C2C12 myoblasts, a new screen[61] was designed based on the notion that lineage-reversed myoblasts should regain multipotency; dedifferentiated myoblasts should acquire the ability to differentiate into multiple mesenchymal cell lineages that are not permitted for untreated C2C12 myoblasts under conditions that typically induce differentiation of mesenchymal progenitor cells into adipocytes, osteoblasts or chondrocytes. To assay multipotency of dedifferentiated myoblasts in the primary screen, a two-stage screening protocol was used (**Fig. 4a**): C2C12 cells were treated with compounds to induce dedifferentiation, the compounds were then removed and cells were assayed for their ability to undergo osteogenesis upon addition of known osteogenesis-inducing agents (which only affect mesenchymal progenitor cells). Among a series of 2,6-disubstituted purine analogs, 2-(4-morpholinoanilino)-6-cyclohexylamino-purine (reversine) had the highest activity in the primary screen. Reversine inhibits myotube formation and treated myoblasts continue to grow to form a confluent culture of mononucleated cells, which can redifferentiate into osteoblasts (**Fig. 4c**) and adipocytes (**Fig. 4b**) upon exposure to appropriate differentiation conditions. This dedifferentiation effect of reversine on C2C12 cells can be shown at the clonal level. In addition, at the most effective concentration of reversine, no significant cell death was observed during dedifferentiation although slower growth was observed. In the absence of osteogenesis-inducing medium, continuous reversine treatment alone has no osteogenesis activity; transdifferentiation of C2C12 myoblasts to osteoblasts or adipocytes was also not observed under the conditions used to induce osteogenesis or adipogenesis. These observations suggest that reversine acts as a dedifferentiation-inducing agent rather than simply enriching certain progenitor cells by selectively killing myoblasts. However, considerable work is required to determine the mechanism by which this molecule functions. Moreover, it is unclear whether a universal molecule can be found that can reprogram multiple types of somatic cells back to ESC-like cells, in a fashion similar to egg extracts.

Although numerous reports demonstrate the use of growth factors and cytokines to transdifferentiate cells in culture, relatively little work has been done with small molecules. Shen and coworkers showed that dexamethasone converts pancreatic cells into hepatocytes, in a pancreatic cell line, AR42J-B13, and in organ cultures of pancreatic buds from mouse embryos[62]. They further characterized the molecular mechanism underlying such transdifferentiation, and identified the transcription factor C/EBPβ as a master switch by both gain-of-function (overexpression of C/EBPβ induces transdifferentiation to hepatocytes in AR42J-B13 cells) and loss-of-function studies (overexpression of liver inhibitory protein, which acts as a dominant-negative form of C/EBPβ, can block transdifferentiation of AR42J-B13 to hepatocytes induced by dexamethasone and oncostatin M). In another example, Skillington and coworkers, in examining the extracellular signaling cues that direct the conversion of adipocytes (3T3-F442A preadipocyte) into osteoblasts[63], found that retinoic acid can synergize with BMP-2 to stimulate cell proliferation, repress adipogenesis and promote osteoblast differentiation. Similarly, we previously showed that purmorphamine can function synergistically with BMP-4 to induce transdifferentiation of 3T3-L1 preadipocytes to osteoblasts[35].

Concluding remarks

Clearly, stem cell biology is a fast growing field that is providing new insights into the molecular mechanisms that control developmental processes. At the same time stem cells may have potential uses in the treatment of devastating diseases such as cardiovascular disease, neurodegenerative disease, musculoskeletal disease, diabetes and cancer. However, significant obstacles remain that must be overcome before the therapeutic potential of stem cells can be realized. This requires a better understanding of the signaling pathways that control stem cell fate and an improved ability to manipulate stem cell proliferation and differentiation. Ultimately, it may be possible to stimulate the body's own regenerative mechanisms through drug treatment by promoting survival, migration and homing, proliferation and differentiation of endogenous cells.

Although cell-based screens have been used for decades to identify small molecules for use in drug discovery and cell biology, the value of such small molecule approaches in the stem cell field is just now beginning to be realized. As exemplified above, synthetic molecules have been identified that regulate stem cell differentiation and can be used as probes of the underlying biology. They may function by modulating the activities of one or more proteins. However, challenges remain and many basic questions still need to be answered including: (i) what are the global effects of these small molecules on cellular process and through what specific mechanisms are these effects mediated; (ii) are there functional differences in the cell fates generated by the small molecules and natural processes; (iii) to what degree can the examples described above be generalized to other cell types and cell fates; (iv) can the *in vitro* effects of these small molecules be recapitulated *in vivo*; and (v) can a sufficient degree of selectivity and therapeutic window be achieved to allow clinical applications. Nonetheless, clearly stem cell biology and regenerative medicine will be significantly advanced by the identification of additional molecules that control stem cell fate.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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