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Regenerative Medicine

Chemical Control of Stem Cell Fate and Developmental Potential

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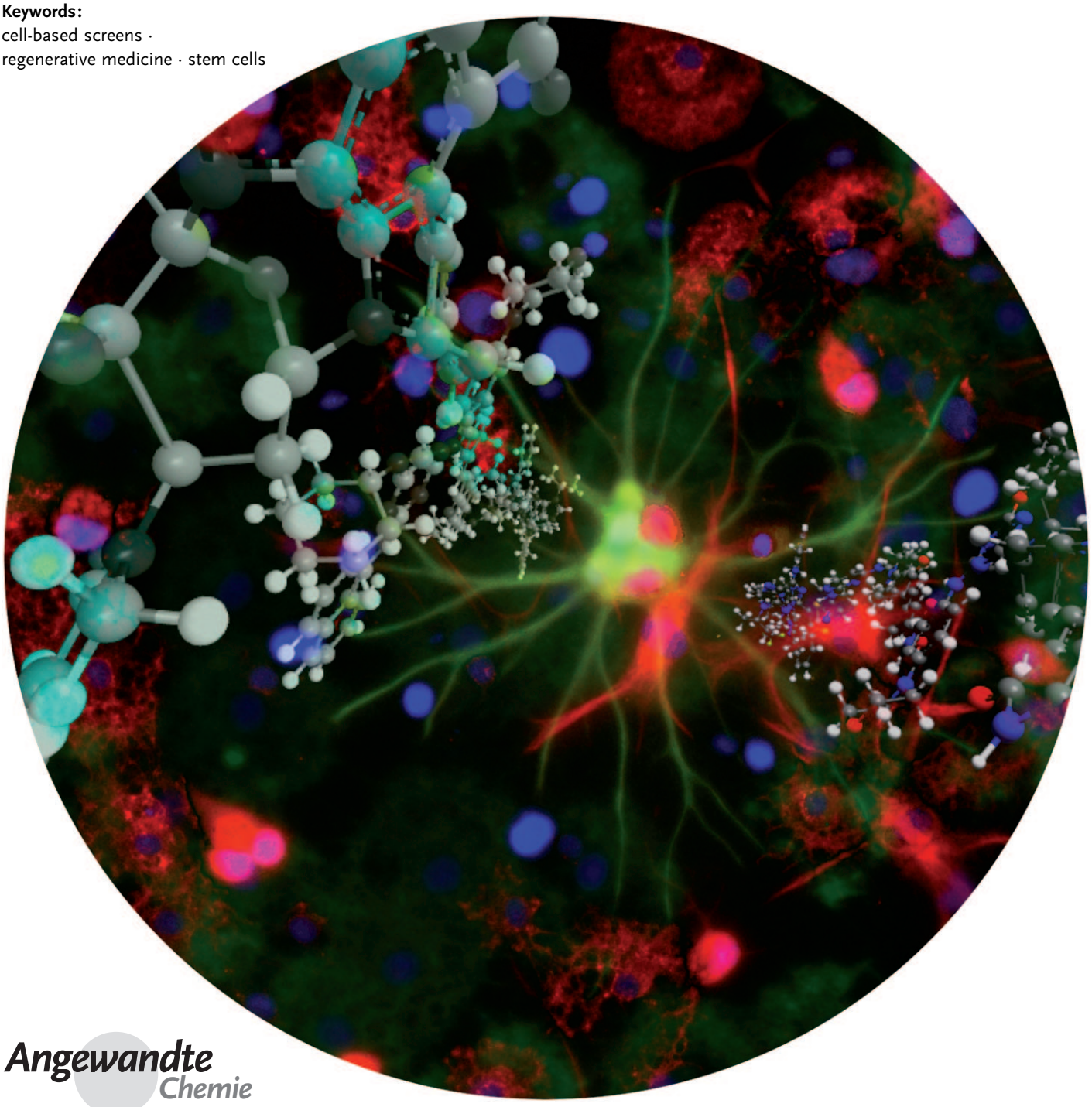

Potential applications of stem cells in medicine range from their inclusion in disease modeling and drug discovery to cell transplantation and regenerative therapies. However, before this promise can be realized several obstacles must be overcome, including the control of stem cell differentiation, allogeneic rejection and limited cell availability. This will require an improved understanding of the mechanisms that govern stem cell potential and the development of robust methods to efficiently control their fate. Recently, a number of small molecules have been identified that can be used both in vitro and in vivo as tools to expand stem cells, direct their differentiation, or reprogram somatic cells to a more naive state. These molecules have provided a wealth of insights into the signaling and epigenetic mechanisms that regulate stem cell biology, and are already beginning to contribute to the development of effective treatments for tissue repair and regeneration.

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1. Introduction

Pluripotent embryonic stem (ES) cells represent an inexhaustible cell source that can, in theory, be differentiated into any desirable cell type.[1] Examples include cardiomyocytes for cardiovascular disease, neural cells for neurodegenerative disease, muscle and cartilage for genetic or age-related musculoskeletal defects, and pancreatic β-cells for diabetes. However, the direct injection of ES cells into a host results in tumor formation. Consequently, pluripotent ES cells must be differentiated into a desired tissue or specific population of cells before they can be used safely and effectively in clinical applications. Thus the development of a cell replacement-based therapy requires efficient and reproducible methods to expand ES cells and induce their differentiation into a desired cell type. Small molecules provide one possible solution to this challenge.[2] In contrast to most genetic methods, small molecules are able to reversibly perturb specific functions of a single protein (or multiple proteins) with exquisite temporal control. This is a particularly useful property in stem cell biology, where differentiation into a given lineage is controlled by a specific sequence of cellular events. Another advantage of small molecules is that they can be rapidly translated from primary cells to in vivo models to test biological hypotheses in the complex setting of a whole organism. Small molecules can also serve as useful probes to further our understanding of the mechanisms that control developmental potential and cell fate.

In addition to ES cells, other cell sources can also form the basis for regenerative and related therapies. For example molecules are being identified that 1) direct the expansion, homing and/or differentiation of multipotent stem cells that persist throughout adulthood (somatic stem cells); 2) revert somatic cells into less differentiated cells that can subsequently be directed to distinct cell fates; 3) selectively ablate or differentiate tumor initiating stem cell-like populations of various cancer types; and 4) promote the reversible cell cycle re-entry of terminally differentiated cells (e.g., pancreatic β-cells or cardiomyocytes). In this Review we hope to illustrate the exciting opportunities that exist for chemists in harnessing and understanding the complex biology that controls cell fate.

2. Stem Cell Basics

Stem cells are unspecialized precursor cells with the ability to maintain the undifferentiated state and to differentiate into more specialized cells in response to instructive signals. As a stem cell proliferates, it can give rise to a daughter cell that is identical to itself (symmetric division), or

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a cell with a more limited developmental potential (asymmetric division).[3] The former is termed self-renewal and is responsible for maintaining stem cell identity, while asymmetric division results in lineage specialization into a more differentiated cell. It is customary to classify stem cells according to their differentiation potential (Figure 1). For example, ES cells can give rise to any cell type found in the three primary germinal layers of the embryo (endoderm, mesoderm, and ectoderm), and are thus classified as pluripotent.[1] Tissue-specific stem cells (also referred to as somatic or adult stem cells) are located in differentiated tissues and organs. They can give rise to all cell types within one particular lineage (e.g., hematopoietic stem cells from the bone marrow can give rise to all the blood lineages). Accordingly, somatic stem cells are classified as multipotent. Accumulating evidence now suggests that certain cancers, like organs and organisms, may also have a cellular hierarchy.[4] At the apex of this hierarchy lie cancer stem cells, which, like normal stem cells, possess the ability to self-renew and differentiate.

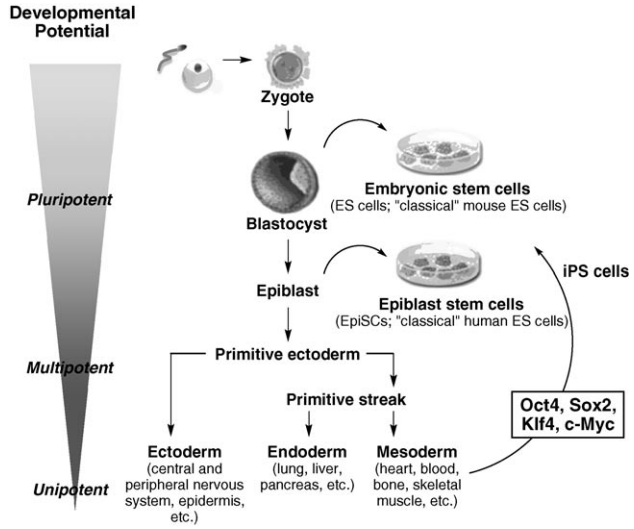

Figure 1. Mammalian development: a hierarchical depiction of development highlighting the gradual decrease in potential that accompanies differentiation from a totipotent zygote to the postmitotic somatic cells that make up an adult organism. Pluripotent cells used in culture can be derived 1) from the inner cell mass of the blastocyst (ES cells); 2) from the epiblast (EpiSCs); or 3) by reprogramming differentiated cells with Oct4, Sox2, Klf4 and c-Myc (iPS cells).

2.1. Epigenetics and Lineage Specification

The differentiation of stem cells into a given lineage during development occurs as a result of tissue-specific gene activation and silencing of "stemness" genes and those associated with alternative cell fates. A stem cell does this by imposing a distinct and heritable pattern of gene expression on a daughter cell without altering the primary DNA sequence.[5] Instead, changes in the higher order structure of chromatin result in differing accessibility of the primary DNA sequence to the transcriptional machinery. The molecular details of this process involve the reorganization of chromatin architecture (i.e., nucleosomal remodeling, nuclear compartmentalization and dynamics) and chemical changes to the chromatin, including DNA methylation and a variety of post-translational histone modifications.[6] Together these processes determine gene expression and, as a consequence, cell/lineage specification. Thus, even though the vast majority of cells in a multi-cellular organism share an identical genotype, organismal development generates a diversity of cell types with disparate, yet stable, profiles of gene expression and distinct cellular functions. Analysis of these processes has given rise to the field of epigenetics, and lineage specification can be considered an epigenetic phenomenon that plays a deterministic role in establishing and maintaining cell identity during development and throughout the lifetime of an organism.

2.2. Pluripotent Stem Cells

ES cells derived from the inner cell mass (ICM) of a developing embryo are the classical and most well studied example of pluripotent cells. However, a variety of other pluripotent cell types exist, as do numerous methods to derive or create such cells. This Review will only focus on three types of pluripotent stem cells: ICM-derived ES cells, epiblast-derived stem cells (EpiSCs), and induced pluripotent stem

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Peter Schultz received his B.S. and Ph.D. (1984) from Caltech. He joined the Berkeley faculty in 1985 and moved to the Scripps Research Institute in 1999 where he is currently the Scripps Family Chair Professor of Chemistry. His interests focus on chemical and genomics studies of stem cell biology, the development of new therapeutics for orphan and neglected diseases, and the development and application of methods for adding new building blocks to the genetic codes of various organisms. He is a founder of numerous companies, and his awards include the Alan T. Waterman Award (NSF), the Wolf Prize in Chemistry, the Paul Ehrlich Prize, and the Arthur C. Cope award.

(iPS) cells (Figure 1). For a more thorough overview of pluripotent cells and methods for their creation and derivation, one can refer to several excellent reviews.[7]

The importance of ES cells to modern biology and medicine results from two unique characteristics that distinguish them from all other stem cells. First, they can be maintained and expanded as pure populations of undifferentiated cells for extended periods of time, possibly indefinitely, in culture. Unlike transformed tumor cell lines, ES cells retain normal karyotypes following extensive passaging in culture. Second, they possess the capacity to generate any of the more than 200 different cell types that make up an adult organism, as well as the many transient cell types that arise during development.[1,8] Studies during the past 20 years have led to the development of culture conditions and protocols for the generation of a number of these lineages in vitro.[9] The ability to derive multiple lineages from ES cells has provided opportunities to model embryonic development in the laboratory and investigate the events that regulate the earliest stages of lineage induction and specification. In addition to providing a model of early development, the ES cell differentiation system is viewed by many as a novel and unlimited source of cells and tissues for transplantation which can in theory be used to treat a broad spectrum of diseases.

Though mouse ES cells have been used for decades,[10] the derivation and routine use of human ES cell lines has only occurred recently.[11] This was due in part to the fact that human ES cells require different culture conditions than mouse ES cells to maintain the pluripotent state. For example, mouse ES cells are responsive to, and thus maintained in, media containing leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP).[12] In contrast, human ES cells do not respond to LIF, and rather depend on fibroblast growth factor (FGF) and Activin/Nodal signaling to maintain the undifferentiated state.[11,13] Furthermore, the application of human embryo-derived stem cells in laboratory and clinical settings is complicated by ethical and sociopolitical considerations.[14]

It was originally assumed that the differences between human and mouse ES cells resulted from species-specific differences in the mechanisms used to maintain the pluripotent state. However, it is now appreciated that the cell(s) from which mouse and human ES cells are derived do not represent equivalent stages of development (Figure 1).[7d] Evidence for this concept was provided recently when self-renewing, pluripotent cell lines were derived from the late epiblast of post-implantation mouse embryos. These cells, referred to as EpiSCs, behave much more like human ES cells than traditional ICM-derived mouse ES cells in several regards, most notably patterns of gene expression and signaling responses.[15] The development of an alternative pluripotent mouse cell type that provides a more appropriate corollary to human ES cells will significantly aid the translation of findings in mouse to human. Importantly, because of the different stages from which human and mouse ES cells are derived, there is likely to be little overlap in the utility of small-molecule tools that control their potential.

In addition to embryo derivation, pluripotent stem cells can also be created from differentiated cells directly by over-expressing unique sets of transcription factors. These cells are induced to become pluripotent and are appropriately named iPS cells.[16] Such cells are identical to ES cells in many regards. For example, they respond to derivation stage-specific lineage-inductive cues and can give rise to entire organisms when transplanted into a developing embryo.[7a,17] The technology used to create iPS cells has received a great deal of attention because it can be readily applied to create disease-specific pluripotent cell lines.[18] Such lines are now being used to monitor disease onset and progression in vitro and as surrogate to transgenic animal models for the development of new drugs. Furthermore, iPS cells have encouraged renewed interest in the notion of creating patient-specific pluripotent cell banks because they circumvent the sociopolitical issues surrounding the use of human embryo-derived cells.

2.3. Multipotent Stem Cells

Multipotent stem cells are found among differentiated cells in a tissue or organ. Throughout the lifetime of an organism, they play a critical role in maintaining homeostasis by giving rise to new cells in the tissue/organ where they originated and reside. For these reasons, they are also referred to as tissue-specific, adult or somatic stem cells. The differentiation potential of somatic stem cells is limited to the major specialized cell types of the tissue/organ in which they reside. For example, bone marrow-derived hematopoietic stem cells (HSCs) give rise to the blood lineages (e.g., red and white blood cells).[19] Other well characterized somatic stem cells exist in the brain (neural stem cells, NSCs),[20] in the bone marrow stroma (mesenchymal stem cells, MSCs),[21] skeletal muscle (satellite cells),[22] and the epithelium of the digestive tract.

Several characteristics of somatic stem cells make them an attractive alternative to pluripotent stem cells for use in cell-based therapies. For example, unlike pluripotent cells, they are not generally tumorigenic when transplanted. They can also be isolated directly from a patient for later use in autologous therapy, thereby circumventing issues associated with histocompatability. However, somatic stem cells have a finite lifespan ex vivo and their response to differentiation signals declines after each generation in a laboratory setting. Even the most routinely used experimental protocols only afford weeks of culture. Significant technical challenges also surround the isolation of somatic stem cells due both to their restricted location (e.g., the brain) and their relatively low abundance (e.g., HSCs only constitute 1 in 10000 bone marrow cells). Nevertheless, the first clinical use of stem cells involved allogeneic bone marrow transplant of HSCs, a strategy that is now used routinely for various blood-related disorders.[19]

2.4. Cancer Stem Cells

Cancerous cells within certain tumors (e.g., acute myelocytic leukemia) are heterogeneous and are composed of

several types of differentiated and undifferentiated cells. The various stages of differentiation exhibited by cells in these tumors support a hierarchical model in some types of cancer.[4a,b] At the top of this hierarchy lie the least differentiated cells. These cells lead to tumor initiation and are thought to be responsible for relapse following treatment. Such tumor-initiating cells (also popularly known as cancer stem cells, CSCs) exhibit the ability to self-renew and differentiate into the cells of the tumor bulk.[4c–e] CSCs also share other characteristics with normal stem cells including a long lifespan, relative quiescence, resistance to drugs and toxins through the expression of multi-drug resistance transporters, an active DNA-repair capacity, and a resistance to apoptosis. As a result, insights from stem cell biology are now being applied to the study and treatment of cancer.[23]

3. Chemical Approaches to Identify Small Molecules That Control Cell Fate

Small molecules with well characterized biological activities and mechanisms of action can be used in a hypothesis-driven, target-based approach to study developmental potential and stem cell fate. Examples include the use of molecules that target epigenetic modifying enzymes and specific developmental signaling pathways. This targeted approach has proven extremely valuable in providing insights into the complex regulatory circuits involved in developmental and stem cell biology. It is, however, limited by the need for a priori knowledge of the molecular pathways involved in biological processes of interest. This is particularly problematic in stem cell biology where biological mechanisms are still poorly understood. An alternative approach relies on the use of cell-based phenotypic or pathway-based screens of chemical libraries to identify molecules that affect stem cell biology in a defined way.[2,24] Unbiased, cell-based screens of this sort are likely to reveal new genes and pathways that control cell fate.

3.1. High-Throughput Screen Design

A range of assay formats have been developed to identify molecules that affect stem cell fate and vary significantly in their scalability and levels of complexity. For example, relatively simple reporter-based cellular assays in which the expression of a luminescent or fluorescent reporter gene is driven by the promoter of the gene of interest (in the appropriate host cell) are particularly suited for screening large libraries of compounds. However, such promoter-driven screens require robust secondary assays to reduce the large number of false positives or non-specific molecules obtained from these methods.[25] Alternatively, multi-parametric high-content image-based assays can be used in which parameters associated with a desired phenotype (e.g., the presence of multiple immunofluorescent markers, cell morphology, organelle localization, etc.) can be simultaneously analyzed at the single cell level. However, these screens are generally more time intensive and costly. Screens have also been carried out at the whole organism level (e.g., zebrafish) but these require significant amounts of compound. While a more thorough discussion of high-throughput screening strategies is beyond the scope of this Review, a number of reviews have been written on the subject.[26]

Small-molecule libraries vary from the large lead discovery collections (>2 million members) found in pharmaceutical settings to the smaller ($<10\,000$) focused collections (e.g., known drugs, natural products collections, known kinase inhibitors, etc.) typically used in academic settings. The number of compounds screened in a particular assay is most often determined by the cost and availability of reagents and the degree of miniaturization that is possible using a particular screening platform. For example, organism-based screens or those using primary cells are limited by cell/organism availability and are therefore often screened against more focused compound collections. Immortalized cell lines that can be readily miniaturized to 384- or 1536-well format, on the other hand, are frequently assayed against very large compound libraries. However, these large scale screens are typically carried out in single-dose formats, which can mask a desired phenotype with anti-proliferative or other off-target effects.

Various arguments have been put forth regarding the chemical diversity and structural motifs that should be represented in chemical libraries. The possibilities range from complex natural products[27] to the simpler heterocycles (e.g., purines, benzimidazoles, benzothiazoles, indoles, quinazolines, quinolines, and other "privileged" scaffolds)[28] found in many known drugs.[29] The latter have the advantages of facile synthetic access and a preexisting wealth of pharmacological information. These attributes facilitate subsequent structure–activity studies for affinity-based target identification, as well as rapid chemical optimization to improve the selectivity and pharmacokinetic properties required for in vivo studies. Our experience is that heterocycle libraries are an extremely rich source of selective and potent hits whose properties can be rapidly optimized.

In addition to small molecules, arrayed protein libraries (e.g., secreted proteins, antibodies, etc.) can also be readily screened in the assay formats described above. Indeed, we recently developed a large-scale process with which a library of secreted proteins were expressed in mammalian cells, purified, and arrayed into 384-well format for use in various biological assays.[30] In contrast to large chemical libraries, the entire secreted proteome consists of only a few thousand proteins and, as such, can be readily applied in low-throughput, multi-parameter assays in primary cell or organism-based screening platforms. Application of this and similar platforms have provided new insights into natural factors that control cell fate and offer an additional source of therapeutic candidates in regenerative medicine.[30,31]

3.2. The Mechanism of Action of Small Molecules

Although unbiased cell-based screens are a powerful strategy for identifying new pathways that regulate complex cell processes, they also present the challenge of unraveling

the precise mechanism through which a molecule exerts its activity. The elucidation of the molecular mechanism of small molecules is complicated by a number of issues.[25b] For example, compounds of interest may interact with multiple, non-relevant targets that confound analysis.[32] Hit molecules may also have relatively low potency, solubility, cytotoxic activity, or poor pharmacokinetics. Thus detailed structure–activity studies are usually required to optimize the properties of a molecule and determine its mechanism of action.

3.2.1. Gene Expression Analysis

Microarray gene expression analysis can provide useful insights into the mechanisms underlying the activity of a small molecule.[33] In practice, cells are treated with active and structurally similar, inactive analogues and the gene expression signature is analyzed. Genes whose expression levels are changed upon small-molecule treatment (and not affected by the closely related inactive analogue) are identified and signaling pathways/networks related to these changes can be determined using bioinformatic analysis. For example, the osteogenic activity of purmorphamine was found to result from the activation of the hedgehog (Hh) signaling pathway based on the observation that purmorphamine induced a number of known Hh signaling genes.[33,34] From this knowledge, the biological target of purmorphamine was later identified as the Hh receptor Smoothened (Smo).[35] More recently, a similar approach was used to identify the relevant target of StemRegenin 1 (SR1), a compound that promotes the self-renewal of HSCs. Here, gene expression analysis demonstrated that several direct downstream targets of the aryl hydrocarbon receptor (AhR) were differentially expressed in the presence of SR1.[36] Subsequently, the direct interaction of SR1 and AhR was confirmed using biochemical methods.

3.2.2. Functional Genomic Screens

Functional genomic screens, including gain-of-function cDNA screens and loss-of-function RNAi screens, are widely used to identify gene products involved in biological processes of interest. Hits in these screens are proteins or miRNAs that can be easily identified based on the annotation of the well on the screening plate. Indeed, genomic screens provide an alternative approach to identify small molecules with a given phenotype in cases where throughput is limited (e.g., organism-based screens or with rare primary cells). In these instances, the genomic screen can be used to identify a novel target and in a second step a known drug or bioactive compound can be used to modulate the activity of the protein of interest. An example of this approach comes from Wurdak et al. who screened a small collection of kinase targeting short hairpin (sh)RNAs against glioblastoma tumor initiating cells. In this assay, a number of genes were identified, which when knocked down, prevented cell growth.[37] This target list was then used to select kinase inhibitors that could be used in place of the shRNAs (discussed in greater detail in Section 10.3.2). In this case, drug-like small molecules with the desired biological activity were identified using a genomic screening platform that was not amenable to high-throughput small molecule-based methods.

3.2.3. Affinity-Based Target Identification

Affinity based approaches with small molecule-immobilized solid matrices or photocrosslinkers can also be used to identify the targets of small-molecule screen hits. With this method, the compound of interest is attached to a solid matrix using a flexible, inert linker (such as polyethylene glycol) at a permissive moiety that allows for the retention of compound activity. Proteins that bind the immobilized compound can be isolated from the cell lysate (typically in the presence or absence of free competitor compound), analyzed by SDS-PAGE, visualized by Coomassie blue or silver staining, and identified by LC/MS/MS. Indeed, using this approach, we have previously identified targets for several small-molecule regulators of cell fate, including pluripotin,[38] reversine[39] and TWS119.[40]

Cell-permeable affinity probes (e.g., with a biotin handle or alkyne for "click" chemistry) are also widely used and allow the probe to bind its targets in a living cell. In certain instances, this is critical as the interaction between the compound and its target is compromised upon cell lysis. For example, we successfully identified the target of stauprimide as NME-2 (non-metastatic cell protein 2) by incubating a biotinylated stauprimide analogue in ES cell cultures.[41] Strategies that covalently fix the interaction between a small molecule and its target(s), (i.e., photo-activated cross-linking) may also be useful, particularly in the case of low affinity compound–target interactions.[42] However, not all small molecules maintain their activity upon attachment to affinity labels. Under these circumstances, radioactive isotope labeling strategies provide an alternative method and allow the protein target of a given compound to be visualized on denaturing gels (for covalent interaction), non-denaturing gels, or in chromatographic fractions (for a non-covalent interaction). The identification of Raf kinase inhibitor protein (RKIP) as the target of locostatin provides an example of this strategy.[43]

The biological function of potential targets must be validated, especially if multiple targets are identified for a given small molecule. This can be accomplished with functional genomic methods such as gain-of-function protein overexpression, loss-of-function protein knockdown, and/or the use of constitutively active and dominant negative mutants. The modulation of the activity of a protein by a compound in living cells can be analyzed in enzymatic assays (e.g., assays of kinase activity in Baf cells)[44] or by examining phosphorylation of downstream signaling components by Western blot. Lastly, the physical interaction between the protein(s) and the compound can be determined using biochemical methods such as surface plasmon resonance (SPR) or in vitro enzymatic assays.

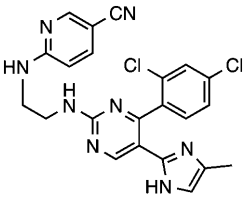
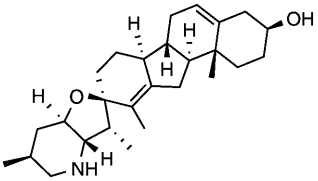
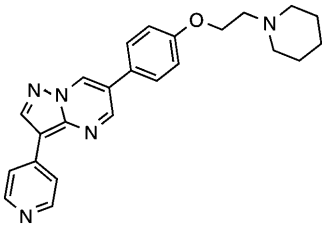
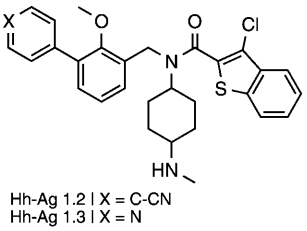
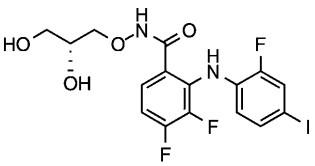
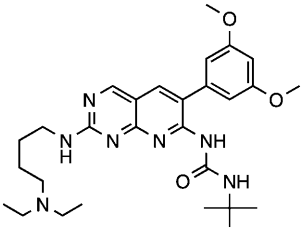
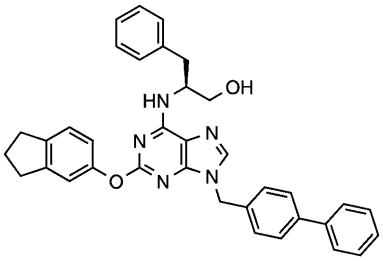
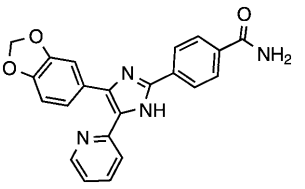
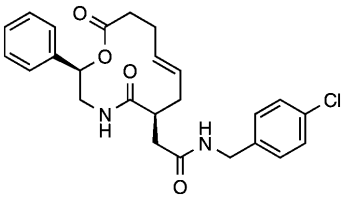
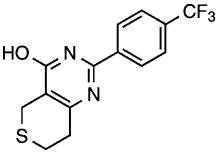
4. Developmental Signaling Pathways

Developmental signaling pathways control embryonic patterning and cell behavior and play critically important roles in stem cell regulation. Thus, molecules that selectively activate or inhibit these pathways are important tools for modulating and investigating stem cell biology. In this Section, a number of molecules that modulate developmental signaling pathways and have been used to discern various aspects of stem cell biology are described (Table 1). For a more thorough overview of small molecules that affect canonical developmental signaling pathways, readers are encouraged to examine comprehensive reviews on the topic.[45]

4.1. Canonical Wnt/β-Catenin Signaling

The Wnt/β-catenin signaling pathway is involved in a wide range of embryonic-patterning events and mediates cell fate in a variety of somatic stem cells.[46] In fact, a number of small molecules that activate Wnt signal transduction have been described and used to study stem cell biology.[45] The most widely used of these are GSK-3β inhibitors that directly activate the Wnt/β-catenin pathway by blocking the phosphorylation and subsequent destruction of β-catenin (Figure 2a).[47] For example, GSK-3β inhibitors have been used to promote the self-renewal of human ES cells[48] and can be used (in combination with MEK inhibitors) to maintain mouse ES cell self-renewal in chemically defined conditions.[49] GSK-3β inhibitors have also been used to facilitate the formation of iPS cells[50] and to expand murine cardio-

Table 1: Selected small-molecule modulators of developmental signaling pathways.

Compound	*Target* Function	Compound	*Target* Function
	**CHIR99021** *GSK-3β* Activator of canonical Wnt/β-catenin signaling		**Cyclopamine** *Smo* Inhibitor of Hh signaling
	**Dorsomorphin** *Type I Receptor (ALKs 2, 3, 6)* Inhibitor of BMP signaling	 Hh-Ag 1.2 I X = C-CN Hh-Ag 1.3 I X = N	**Hh-Ag 1.2/1.3** *Smo* Activator of Hh signaling
	**PD0325901** *MEK/ERK1* Inhibitor of MEK signaling		**PD173074** *FGFR* Inhibitor of FGF signaling
	**QS11** *ARFGAP1* Wnt/β-catenin synergist		**SB-431542** *Type I Receptor (ALKs 4, 5, 7)* Inhibitor of TGF-β signaling
	**Robotnikinin** *Shh Ligand* Inhibitor of Hh signaling		**XAV939** *Tankyrase* Inhibitor of Wnt signaling

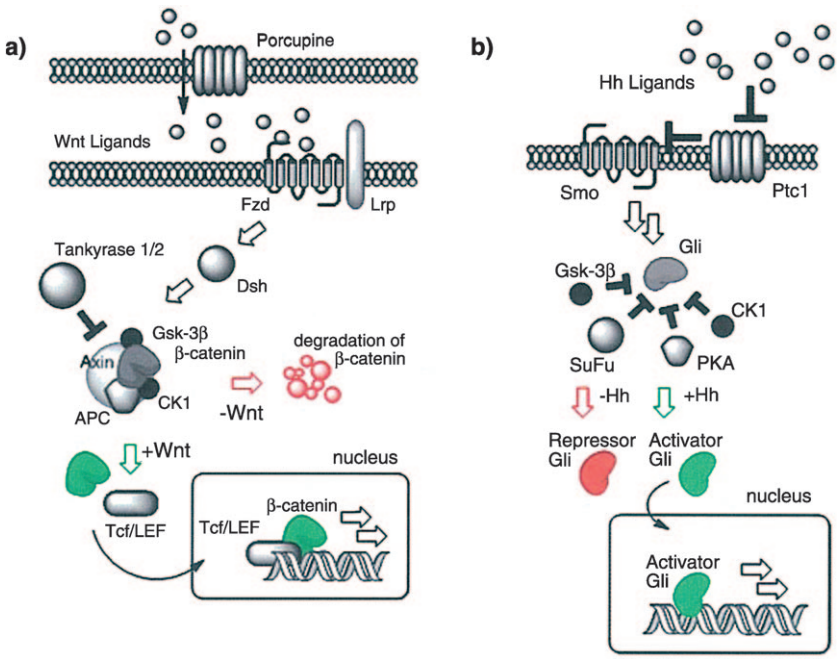


Figure 2. Canonical Wnt/β-catenin and Hedgehog signaling pathways: a) The canonical Wnt-signaling cascade can be initiated by the binding of Wnt proteins to the membrane receptor Frizzled (Fzd) and low-density lipoprotein receptor-related proteins (Lrps). In the absence of ligand-mediated pathway activation, β-catenin protein is phosphorylated by a cytoplasmic complex containing the proteins Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase-3β (GSK-3β). These phosphorylation events ultimately target β-catenin for proteosomal degradation. The Axin/APC/CK1/GSK-3β complex is antagonized by Dishevelled (Dsh), a cytoplasmic protein that is activated upon binding of Wnt to its receptor. Wnt signaling thus stabilizes β-catenin, which acts as a transcriptional co-activator by associating with the Tcf/LEF family of transcription factors. b) Hh signaling is controlled by two transmembrane proteins, Patched-1 (Ptc1) and Smoothened (Smo), and is initiated upon binding of a secreted Hh protein. In the resting state, Smo activity is suppressed by Ptc, which permits the phosphorylation of Gli transcription factors by protein kinase A (PKA), GSK-3β, and CK1. These phosphorylation events allow the processing of Gli into an inactive transcriptional repressor. Upon Hh ligand binding, Smo releases the inhibitory effect of Ptc and subsequently transduces downstream signals that activate the Gli family of transcription factors. Translocation of the Gli activator protein into the nucleus, initiated by Hh binding to Smo, promotes the expression of Hh signaling target genes.

myocytes and β-cells (two cell types that are otherwise mitotically inert).[51]

Wnt/β-catenin activators have also been discovered through cell-based assays and whole-organism screens. For example, using a Wnt pathway specific screen we identified a 2-amino-4,6-disubstituted pyrimidine compound that activates Wnt signaling.[52] Unlike many known Wnt activators, this compound does not inhibit GSK-3β, and its activity can be blocked by a dominant negative Tcf4, suggesting that the compound functions upstream of Tcf factors in the canonical Wnt/β-catenin signaling pathway. In another example, a cell-based screen was used to identify a purine derivative (QS11) that synergizes with Wnt-3a ligand.[53] Through affinity chromatography and subsequent functional assays, it was shown that QS11 binds and inhibits the GTPase activating protein of ADP-ribosylation factor 1 (ARFGAP1), which suggests that QS11 modulates Wnt/β-catenin signaling through an effect on protein trafficking. Because this molecule is the only ARFGAP inhibitor reported to date, it may not only be useful in studies of the Wnt/β-catenin signaling pathway, but it may also provide a useful tool to explore novel functions of ARFGAPs in living cells.

Small molecules that inhibit Wnt/β-catenin signaling at various points in the signal transduction pathway have also been discovered from cell-based screens. Among these are a family of benzothiazole derivatives (IWP)[54] that antagonize the Wnt ligand-secreting protein Porcupine; molecules that stabilize Axin proteins (XAV939) through the inhibition of tankyrase 1/2;[55] and molecules (PKF115-584) that prevent the formation of β-catenin-Tcf/LEF complexes.[56] Indeed, the latter are being used to target the less differentiated cells in multiple myeloma that depend on constitutive Wnt/β-catenin signaling.[57] Collectively, these molecules are providing powerful chemical tools to dissect the role of Wnt signaling in various stem cell contexts.

4.2. Hedgehog Signaling

Hedgehog (Hh) signaling regulates a diverse range of biological processes, including cellular proliferation, differentiation, and organ formation in a tissue-specific and dose-dependent manner.[58] Like the Wnt/β-catenin signaling pathway, a variety of small molecule agonists (e.g., SAG, Hh-Ag1.3, and purmorphamine)[33,35,59] and antagonists (e.g., cyclopamine and SANT1-4)[59b,60] have been identified that modulate Hh signaling. However, nearly all known small molecule modulators of the Hh pathway target the Hh receptor protein Smoothened (Smo; Figure 2b). Nevertheless, these compounds have served as useful biological probes in various stem cell and CSC systems. For example, the Smo agonists Hh-Ag1.3 and purmorphamine have been used to modulate various neural patterning events in ES cells and NSCs.[59a,61] The Hh antagonist cyclopamine has been used to induce specific lineages from ES cells and has facilitated our understanding of Hh signaling in CSCs.[59a,62]

More recently, cell-based screens have also been used to identify compounds that modulate Hh signaling independent of Smo. For example, we developed a protocol using mesenchymal progenitor cells stably transfected with a promoter of Gli-driven luciferase reporter to identify compounds that inhibited Hh signaling. Using this assay, we identified a class of 2,4-disubstituted thiazoles, one of which (JK184) inhibited Gli-dependent transcriptional activity in a dose-dependent manner.[63] Subsequent biochemical and functional assays revealed that JK184 did not bind to Smo,

but rather bound class IV alcohol dehydrogenase 7 (Adh7). Another example is the 12-membered macrocycle robotnikinin, which was discovered with a screen for compounds that bind recombinant sonic Hh (Shh).[64] Robotnikinin inhibits Hh pathway activation induced by Shh ligand, but it has no inhibitory effect on Hh target gene expression in patched null fibroblasts or in cells activated with purmorphamine. These results indicate that robotnikinin may prevent Shh from binding to Ptch1, although the molecular details of this mechanism have not yet been established.

4.3. Transforming Growth Factor-β and Bone Morphogenetic Protein Signaling

The transforming growth factor-β (TGFβ) superfamily of secreted proteins consists of a large collection of extracellular factors that regulate cell growth, cell differentiation, apoptosis, cellular homeostasis, and other functions in both the adult organism and the developing embryo. The more than 30 TGFβ family ligands are organized into the TGFβ (which comprises TGFβ and Activin/Nodal ligands), bone morphogenetic protein (BMP), and growth differentiation factor (GDF) subgroups.[65]

While TGFβ- and BMP-signaling are responsible for modulating a significant number of developmental processes and are active in various cancers, relatively few small molecules exist that regulate their activity. Among these are kinase inhibitors that block ligand-mediated SMAD phosphorylation.[66] For example, the ALK4/5/7 inhibitor SB-431542 blocks the phosphorylation of SMAD2/3, and as a result, activation of canonical TGFβ signaling (Figure 3). Indeed, SB-431542 has been used to 1) demonstrate the necessity for TGFβ signaling in the maintenance of undifferentiated human ES cells;[13] 2) replace the reprogramming factor Sox2 during the formation of murine iPS cells;[67] and 3) promote the differentiation of glioblastoma CSCs.[68] Dorsomorphin, an inhibitor of BMP-mediated signal transduction through SMAD1/5/8 (Figure 3), was identified in a zebrafish developmental screen.[66b] This molecule has been used to enhance myocardial differentiation from mouse ES cells by blocking BMP signaling.[69] Furthermore, the combination of SB-431542 and dorsomorphin in human ES cells promotes robust and efficient neural differentiation at the expense of other non-neural lineages.[70]

4.4. Fibroblast Growth Factor Signaling

The fibroblast growth factor (FGF) system comprises one of the most versatile growth factor signaling families in vertebrates and plays critical roles in a wide variety of biological processes.[71] Binding of FGFs to their cognate receptors results in receptor dimerization, tyrosine kinase autophosphorylation, and the assembly and recruitment of signaling complexes. FGF signal transduction ultimately proceeds by one, or a combination, of three main pathways: a) PLCγ/Ca^{2+}; b) phosphoinotitide-3-kinase (PI3K)/Akt; or c) Ras/mitogen-activated protein kinase (MAPK) signaling

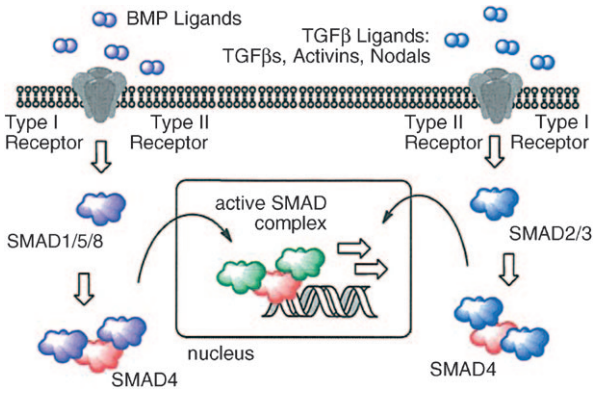


Figure 3. Bone morphogenetic protein and transforming growth factor-β signaling. Ligands of the TGFβ superfamily are secreted as proteolytically processed homodimers and signal through a heterotetrameric receptor complex composed of two types of serine/threonine kinases. TGFβ superfamily ligands bind to a type II receptor (ACVR2, ACVR2B, TGFBR2, BMPR2, and AMHR2), which recruits and phosphorylates a type I receptor (Activin receptor-like kinase receptors 1–7); different receptor combinations are used to recognize specific ligands and achieve diverse signaling outputs. The type I receptor phosphorylates receptor-regulated SMADs (SMAD1/5/8 for BMP-signaling and SMAD2/3 for TGFβ-signaling) which then bind the coSMAD–SMAD4 (illustrated in red). SMAD–coSMAD complexes accumulate in the nucleus (illustrated in green/red) where they act as transcription factors and participate in the regulation of target gene expression. Selected elements of the BMP- and TGFβ- signaling pathways are illustrated in purple and blue, respectively.

(Figure 4). Among the most notable examples of FGF signaling in stem cell biology are the maintenance of NSC and human ES cell self-renewal. Indeed, nearly all protocols described to date for NSCs or human ES cells require inclusion of basic FGF (bFGF).[11,72]

A large number of small molecules are available that can be used to specifically control the various aspects of FGF signal transduction. For example, FGF receptor (FGFR) inhibitors have been used to dissect aspects of ES cell self-renewal in mouse, rat and human;[13b,49,73] PI3K inhibitors have been used to differentially inhibit the proliferation of prostate CSCs;[74] and MEK/ERK inhibitors are used routinely to maintain mouse ES cell self-renewal and to promote the formation of iPS cells.[49,50]

5. Chemical Control of Mouse ES Cell Potential

Mouse ES cells can be maintained indefinitely or differentiated into all cell types of the adult organism.[5c,10,75] In the following Section, we review molecules that are being used to control the fate of mouse ES cells (Table 2).

5.1. Self-Renewal of Mouse ES Cells with Known Target- and Pathway-Based Modulators

Mouse ES cell self-renewal can be regulated by LIF-dependent activation of signal transducer and activator of transcription 3 (STAT3), which has long been regarded as the

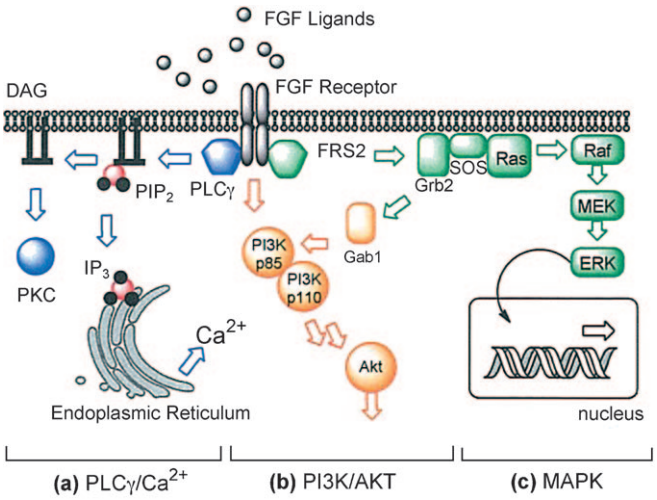
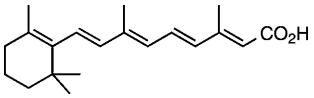
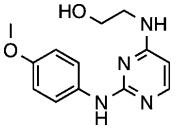
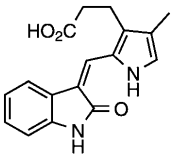
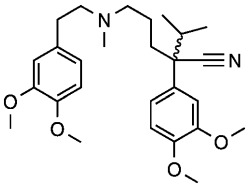
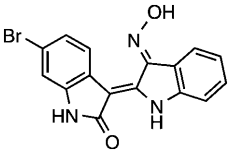
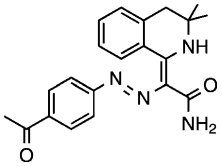
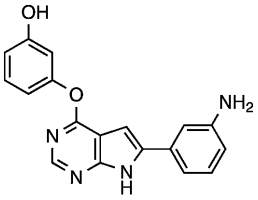
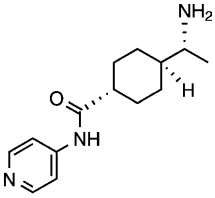


Figure 4. Fibroblast growth factor signaling can trigger a) the PLCγ/Ca^{2+} signaling (blue), b) the PI3K signaling (orange), and/or c) the MAPK signaling pathways (green). a) FGF pathway activation leads to trans-autophosphorylation and creates docking sites for downstream effectors, enabling the activated FGFRs to trigger several intracellular signaling pathways. In the case of the PLCγ/Ca^{2+} signaling pathway, binding of PLC to the FGFR activates PLCγ-mediated hydrolysis of phosphatidylinositol-4,5-diphosphate (PIP_2) to form two second messengers, inositol-1,4,5-triphosphate (IP_3), and diacylglycerol (DAG). Protein kinase C (PKC) is activated by diacylglycerol, whereas IP_3 stimulates the release of intracellular Ca^{2+} from the endoplasmic reticulum. b) The phosphoinositide-3-kinase (PI3K)/Akt pathway can be activated by three mechanisms after FGFR activation: 1) Gab1 can bind to FRS2 indirectly through Grb2, resulting in activation of the PI3K/Akt pathway through p85; 2) the PI3K-regulatory subunit p85 can bind to a phosphorylated tyrosine residue of the FGFR; 3) or activated Ras can activate the p110 catalytic subunit of PI3K. c) MAPK signaling is initiated when the FGFR substrate 2 (FRS2) is sequestered by the activated FGFR homodimer and phosphorylated, enabling it to recruit the growth factor receptor-bound 2 (Grb2) adaptor protein and guanine nucleotide exchange factor Son of sevenless (Sos) to the plasma membrane. Membrane-localized Sos then promotes Ras-dependent activation of Raf kinase, triggering the MEK/ERK kinase cascade and culminating in the actuation of MAPK-responsive transcription factors. MAPK pathway activates genes that promote entry into the G1 phase of the cell cycle and cell cycle progression, where prolonged MAPK signaling is a potent inducer of cell differentiation.

Table 2: Selected small molecule modulators of ES cell fate.

Compound	*Target* Function
	***All Trans* Retinoic Acid** *RAR/RXR* Promotes cellular differentiation in various contexts
	**Cardiogenol C** Directs cardiomyocyte differentiation of mouse ES cells
	**SU5402** *FGFR and VEGFR* Used to determine the role of FGF signaling in ES cell fate
	**Verapamil** *L-type Ca^{2+} channel* Promotes cardiomyocyte differentiation of mouse ES cells
	**BIO** *GSK-3β* Promotes self-renewal of mouse and human ES cells
	**IQ-1** *PP2A* Maintains mouse ES cells in the undifferentiated state
	**TWS119** *GSK-3β* Directs neuronal differentiation of mouse ES cells
	**Y-27632** *Rho Kinase* Increases the clonal expansion efficiency of human ES cells

pivotal mechanism through which mouse ES cells maintain the undifferentiated state.[12] LIF acts by promoting the heterodimerization of gp130 and the LIF receptor (LIFR), resulting in activation of Janus-associated (JAK) tyrosine kinases and, subsequently, STAT3. However, LIF signaling also up-regulates ERK1/2 activity. ERK activation promotes differentiation,[76] indicating that the balance between LIF-induced STAT3 and ERK signals is important in determining the fate of a dividing, undifferentiated ES cell.[75b] Consistent with this observation, application of the MEK/ERK inhibitor PD098059 in the presence of LIF enhanced the self-renewal of mouse ES cells, supporting the idea that activation of ERK impairs self-renewal.[76]

A number of additional pathways have also been identified that contribute to the regulation of self-renewal in mouse ES cells. These include the BMP, PI3K, Wnt/β-catenin, and Nanog signaling pathways. For example, in serum-free culture, LIF is insufficient to block neural differentiation and maintain pluripotency. Qi et al. found that feeder layer-derived BMP4 played a prominent role in maintaining mouse ES cell self-renewal.[77] In fact, mouse ES cells can be

propagated from single cells and derived de novo without serum or feeders using LIF plus BMP4.[77,78] Dissection of this pathway suggests that BMP4 promotes mouse ES cell self-renewal by inhibition of ERK and p38-MAPK pathways, both of which have differentiation-inducing activities. This mechanism was validated using MEK (PD098059) and p38-MAPK inhibitors (SB203580), which also allowed derivation of mouse ES cell lines from embryos that lacked the BMP4 receptor.[77] Like BMP, the PI3K pathway also affects mouse ES cell self-renewal through MEK/ERK signaling. For instance, treatment of mouse ES cells with a PI3K inhibitor (LY294002) led to a reduction in the ability of LIF to maintain self-renewal, with cells concomitantly adopting a differentiated morphology. Inhibition of PI3Ks augmented LIF-induced phosphorylation of ERKs, thereby driving differentiation through the ERK arm of LIF-induced signaling. Moreover, inhibition of MEKs reversed the effect of PI3K inhibition on self-renewal, suggesting that the elevated ERK activity observed upon PI3K inhibition contributed to the functional response observed.[79]

Wnt/β-catenin pathway activation by 6-bromoindirubin-3′-oxime (BIO)—an inhibitor of GSK-3β and other kinases—can promote self-renewal of mouse ES cells in the absence of LIF or feeder layers[48] and enhances the derivation of mouse ES cells from different mouse strains.[80] Later work with BIO showed that the maintenance of cellular c-Myc levels is a critical factor in BIO-mediated self-renewal—inhibition of GSK-3β suppresses c-Myc-T58 phosphorylation, and its subsequent degradation.[81] This observation provides a direct link between LIF and Wnt signaling, (c-Myc expression is activated by LIF-mediated STAT3 nuclear translocation). However, it is possible that BIO may also function through alternative signaling pathways since the inhibition of GSK-3β is central to signal transduction in Hh-, PI3K/Akt-, non-canonical Wnt-, and cAMP-signaling pathways, among others.[47b] By applying a series of GSK-3β specific inhibitors in mouse ES cell self-renewal assays, Bone et al. found that GSK-3β inhibition enhanced self-renewal of mouse ES cells in serum and/or LIF-containing media.[82] However, they demonstrated that GSK-3β inhibition alone was unable to supersede the requirement for LIF. Furthermore, they found that previous data with other GSK-3β inhibitors (e.g., BIO and TWS119) was confounded by the fact that such compounds interfered with other notable ES cell self-renewal signaling pathways (e.g., PI3K and MAPK).

Small-molecule modulators of signal transduction pathways have also aided in our understanding of some of the differences between mouse and human ES cells. EpiSCs, which represent a mouse counterpart to human ES cells, are not responsive to LIF and, rather, depend on media supplementation with bFGF and Activin (human ES cell conditions; see Section 2.2). Also like human ES cells, inhibition of the Activin/Nodal pathway in EpiSCs using the ALK4/5/7 inhibitor SB-431542 promoted differentiation toward the neuroectodermal lineage.[83] In contrast, mouse ES cells maintained their pluripotency markers upon inhibition of Activin/Nodal signaling. Alternatively, inhibition of STAT3 phosphorylation at tyrosine 705 with a JAK inhibitor (Jaki I) supported the undifferentiated state of EpiSCs and promoted differentiation in mouse ES cells. Furthermore, when both JAK and Activin/Nodal inhibitors were used, mouse ES cells were indistinguishable from EpiSCs treated with the Activin/Nodal inhibitor alone.[15a] This series of experiments demonstrated a stepwise differentiation of mouse ES cells from a blastocyst-derived molecular signature to that of an epiblast-derived signature and finally to neuroectodermal cells. Importantly, human ES cells also rapidly down-regulated pluripotency genes and acquired neural characteristics when treated with the Activin/Nodal inhibitor alone.[15a,83] These data are consistent with the notion that EpiSCs and human ES cells are functionally similar (Figure 1).

Based on these and other mechanistic insights, numerous target-based kinase inhibitors are now being used to further dissect the intricacies in mouse ES cell self-renewal. For instance, p38, MAPK and MEK inhibitors are being used to identify downstream effectors of BMP4 and LIF signaling;[76,77,84] Src family protein tyrosine kinase inhibitors have been used to tease apart the individual contributions of different Src family kinases;[85] a gamma secretase inhibitor has been used to study the role of Notch signaling;[86] and inhibitors of FGF signaling have been used to separate the downstream contributions to self-renewal and/or neural differentiation.[84c,d,86,87] Clearly, use of small-molecule inhibitors of defined pathway members is leading to a more detailed understanding of the complex regulatory circuits involved in stem cell biology.

5.2. Identification and Characterization of Novel Modulators of Mouse ES Cell Self-Renewal

Unbiased phenotypic screens of chemical libraries are also useful for the identification of molecules that modulate ES cell self-renewal and differentiation. Using this approach, one is likely to discover molecules that act by novel or unexpected mechanisms. For example, to identify compounds that mediate mouse ES cell self-renewal, a transgenic reporter mouse ES cell line that expresses GFP under the control of the *Oct4* regulatory elements (a marker of pluripotency) was used to screen compounds under LIF-free differentiation conditions. This strategy led to the identification of pluripotin, which can sustain long-term homogenous self-renewal of mouse ES cells in chemically defined media (that is, without feeder cells, serum, LIF or BMP4 for >10 passages).[38] Pluripotin-expanded mouse ES cells expressed multiple pluripotency-associated markers (Oct4 and alkaline phosphatase, ALP), were able to differentiate into tissue types representing each of the three primary germ layers in vitro, and were germ line-competent in vivo (Figure 5a). Subsequent experiments showed that pluripotin functioned independently of canonical mouse ES cell self-renewal pathways (including LIF-STAT3, BMP and Wnt/β-catenin), and instead acts by simultaneously inhibiting ERK1 and Ras GTPase-activating protein (RasGAP; Figure 5b). Pluripotin has also been used to derive mouse ES cell lines from refractory strains, providing further evidence that inhibition of ERK1 and RasGAP promote the maintenance of the undifferentiated, pluripotent state.[88]

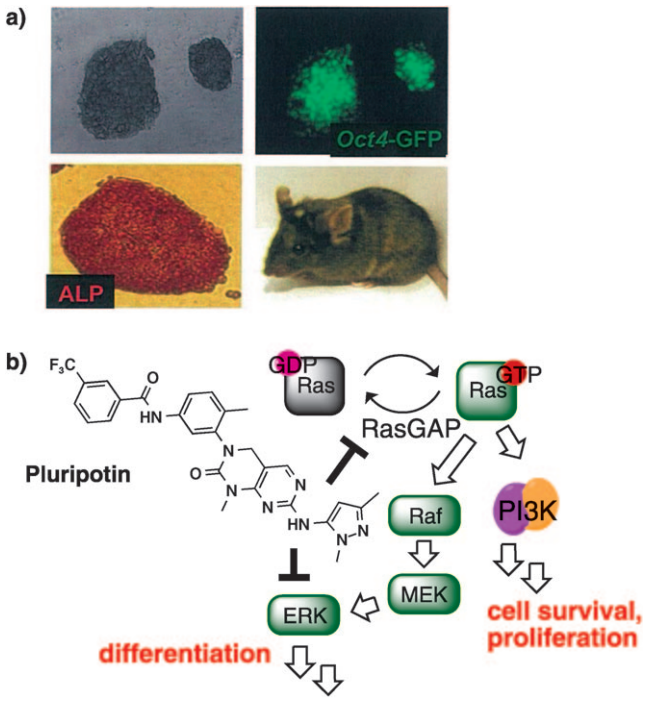


Figure 5. Pluripotin maintains self-renewal in mouse ES cells: a) Pluripotin-expanded mouse ES cells maintained under LIF/feeder free conditions exhibit gene expression characteristic of the self-renewing, pluripotent state (Oct4 and ALP) and are able to contribute to mouse development. Images reprinted with permission from Ref. [38]; Copyright 2006, National Academy of Sciences, USA. b) Proposed mechanism of action for pluripotin.

More recently, a screen based on ALP expression identified IQ-1, a compound that induces long-term expansion of ES cells in Wnt-3a containing media without feeder layers or LIF.[89] IQ-1 inhibits PR72/PR130, thereby blocking the association of β-catenin with p300 and promoting the interaction of β-catenin with CBP. Thus, it appears that the increase in β-catenin/CBP-mediated transcription at the expense of β-catenin/p300-mediated transcription is critical for the maintenance of mouse ES pluripotency. Interestingly, IQ-1 targets a completely different pathway than pluripotin, demonstrating the power of unbiased screens to identify novel mechanisms. Moreover, work with IQ-1 has provided further evidence for the role of Wnt/β-catenin signaling in mouse ES self-renewal.

Recently, Ying, Smith, and colleagues demonstrated that mouse ES cells can be maintained in growth factor-, feeder-, and serum-free conditions in media containing small molecule inhibitor cocktails (MEK and GSK-3β or MEK, GSK-3β and FGFR inhibitors).[49] These data provided the first chemically defined method(s) to grow mouse ES cells and revealed that they have an innate program for self-replication which does not require extrinsic instruction. More recently, Ying et al. derived the first germline competent rat ES cell lines,[73b,c] again using GSK-3β, MEK and FGF inhibitors for establishment, expansion and maintenance of self-renewal. Clearly chemical approaches have expanded both our knowledge of the pathways and our ability to control ES cell self-renewal.

5.3. Directed Differentiation of Mouse ES Cells

Mouse ES cells are competent to produce all fetal and adult cell types.[75a] However, the utility of mouse ES cells as a developmental model or as a source of defined cell populations (e.g., for pharmaceutical screening) is compromised because their differentiation in vitro is poorly controlled.[9,90] One method that has been developed to initiate differentiation involves removing mouse ES cells from LIF and transferring them to suspension culture. Under these conditions, mouse ES cells form aggregates composed of both differentiated and undifferentiated cells known as embryoid bodies (EBs).[91] Cell-to-cell interactions, together with differential access to nutrients and growth factors, leads to heterogeneous differentiation into a variety of lineages. In another method, ES cells are cultured directly on stromal cells, and differentiation takes place in contact with these cells.[92] However, undefined factors produced by these supportive cells may influence the differentiation of mouse ES cells to undesired cell types. A third protocol involves differentiating mouse ES cells as a monolayer on extracellular matrix proteins.[93] Differentiation in monolayers on known substrates can minimize the influence of neighboring cells and, in this regard, provides a simple reproducible method to differentiate ES cells into desired lineages. However, this strategy can only be applied to a handful of the greater than 200 lineages that make up an adult organism. Clearly then, specification of primary lineages is not well understood and, more often than not, differentiation protocols yield variable and heterogeneous outcomes. Thus, more efficient and selective methods are needed to direct the differentiation of ES cells to produce homogenous populations of particular cell types. To this end, we and others have screened small-molecule libraries in a variety of contexts to identify tools that can be used to elucidate and control the mechanisms governing lineage commitment in mouse ES cells.

5.4.1. Neuronal Differentiation

The nervous system is composed primarily of cells with limited or no expansion potential, including glia (e.g., astrocytes and oligodendrocytes) and neurons (of which many subtypes exist).[94] Loss or damage to these cells can lead to devastating diseases. For example, the loss of dopaminergic neurons is associated with Parkinson's disease; repeated damage to oligodendrocytes results in multiple sclerosis; and the loss of medium spiny projection neurons in the striatum is associated with Huntington's disease.[95] Recently a number of labs have begun to design stepwise differentiation schemes to produce neural precursors,[1,87,96] mixed populations of neurons,[40,62b,97] and homogenous subtype-specific neuronal populations[61c,62b] from mouse ES cells. Small molecules used in these chemically defined differentiation schemes have helped to delineate the signaling pathways and mechanisms governing neural differentiation from ES cells. Ultimately, such protocols and cells can be used to study neurological diseases in the laboratory and may provide methods to generate cells for regenerative therapies.

In one example we undertook a cell-based phenotypic screen to identify small molecules that induce neuronal differentiation of mouse ES cells. A pluripotent mouse cell line was stably transfected with a neuronal differentiation-specific promoter (tubulin, alpha-1a)-driven luciferase reporter to screen large numbers of discrete small molecules. Primary hits were then confirmed by immunofluorescent staining for multiple neuronal specific markers, including Map-2ab, βIII-tubulin, neurofilament-M, and synapsin. From these assays, and subsequent SAR studies, a disubstituted pyrolopyrimidine, TWS119, was identified that potently induced neuronal differentiation of mouse ES cells.[40] TWS119 was then linked at a permissive position to an affinity matrix for pull-down assays, and found to bind to GSK-3β. Western blots of TWS119-treated cells displayed an increase in β-catenin levels, suggesting that the mechanism of action may be through canonical Wnt/β-catenin signaling (Figure 2a). More recently, a number of studies have demonstrated that contextual inhibition of GSK-3β can play a role in neuronal subtype-specific differentiation[98] and neural precursor expansion.[99] While the mechanistic intricacies of GSK-3β in neuronal development are still a point for further investigation, it is clear that GSK-3β plays a role.

A major challenge in the generation of highly specified neuronal subtypes from pluripotent ES cells is the requirement for sequential signals as cells mature through various progenitor lineages. To address this limitation in the case of motor neurons, Jessell and co-workers derived a stepwise protocol from mouse ES cells.[61c] Initially, EBs were formed with inductive cues from the PA6 stromal cell line. The EBs were then directed to differentiate into neural progenitors with retinoic acid (RA), and the neural progenitors were subsequently converted into motor neuron progenitors with a specific small molecule agonist (Hh-Ag1.3) of the Hh signaling pathway. By monitoring this process over time, they observed that the developing motor neurons expressed the expected developmental markers in a sequential and time-dependent manner. Importantly, the ES cell-derived motor neurons were able to populate the embryonic spinal cord, extend axons, and formed synapses with target muscles following transplantation into chick embryos. More recently, Vanderhaeghen and co-workers employed a similar strategy to develop cortical pyramidal neurons from ES cells.[62b] Again, this process required stepwise differentiation through several intermediate progenitor fates before eventual specification of the cortical neurons. A primary step in this differentiation cascade was the inhibition of Hh signaling with cyclopamine—if Hh signaling was not appropriately blocked, the majority of neural precursors became GABAergic precursors, as opposed to cortical precursors.

Based on the results from these latter studies, it seems that an effective strategy to direct the differentiation toward mature cell lineages may require iterative steps through intermediate cell fates. As such, screening strategies carried out in a sequential fashion and/or with combination(s) of drugs may provide a means to direct differentiation to lineages that cannot otherwise be derived efficiently from ES cells.

5.4.2. *β-Cell Differentiation*

Type 1 diabetes results from the autoimmune destruction of insulin-producing pancreatic β-cells. Although insulin can directly replace the need for β-cells, this strategy requires regular blood glucose monitoring and complications develop.[100] A cure for diabetes will require the replacement of lost β-cells and abrogation of autoimmunity. Clinical studies show that islet transplantation allows temporal insulin independence and some relief from diabetes-related symptoms,[101] providing proof-of-principle that restoration of functional β-cell mass reverses disease progression. The lack of high quality donor cells has prompted the search for alternative sources of β-cells. To date, several approaches have been pursued to generate functional β-cells for cell based therapies. One strategy involves expanding primary β-cells (discussed in Section 10); a second aims to trans-differentiate other mature lineages into β-cells;[102] and a third involves generating β-cells from pluripotent precursor cells.[103]

Efforts to differentiate pluripotent cells into β-cells have also focused on stepwise differentiation protocols that aim to recapitulate the developmental processes observed in vivo. This requires first inducing pluripotent cells to differentiate toward definitive endoderm, then toward pancreatic progenitors, followed by endocrine progenitors and finally mature β-cells (Figure 6a). However, such methods are long and labor intensive, generate β-cells in poor yield ($<1\%$ of starting population) and, while the resultant cells resemble β-cells, they are often unable to functionally respond to glucose stimulation.[104] To overcome these challenges a number of laboratories are now attempting to identify small molecules that potentiate individual developmental steps during the conversion of pluripotent cells into functional β-cells.

In one example, we screened for molecules that increase the efficiency of the first step in pancreatic β-cell development from mouse ES cells, the formation of definitive endoderm. In addition to definitive endoderm, ES cells also have the potential to differentiate into extra-embryonic endodermal lineages (i.e., primitive endoderm and visceral endoderm) that are not capable of generating the β-cell lineage. To identify compounds that promote the formation of definitive endoderm specifically, mouse ES cells were plated as a monolayer in serum-free conditions with Activin A and treated with compound libraries. An image-based screen was used to identify molecules that differentiate ES cells into Sox17-positive, Sox7-negative cells (Sox17 is a transcription factor for all endoderm lineages, while Sox7 is a transcription factor specific for primitive and visceral endoderm, but not definitive endoderm). A staurosporine derivative—termed stauprimide—was found that is able to significantly promote the formation of definitive endoderm (both from mouse and human ES cells; Figure 6b).[41] Moreover, compound-directed definitive endoderm was able to produce mature endoderm-derived lineages including hepatocytes and pancreatic β-cells upon further stimulation.

Stauprimide requires Activin A to promote definitive endoderm formation. In the absence of this lineage promoting morphogen, endodermal lineages are not generated effi-

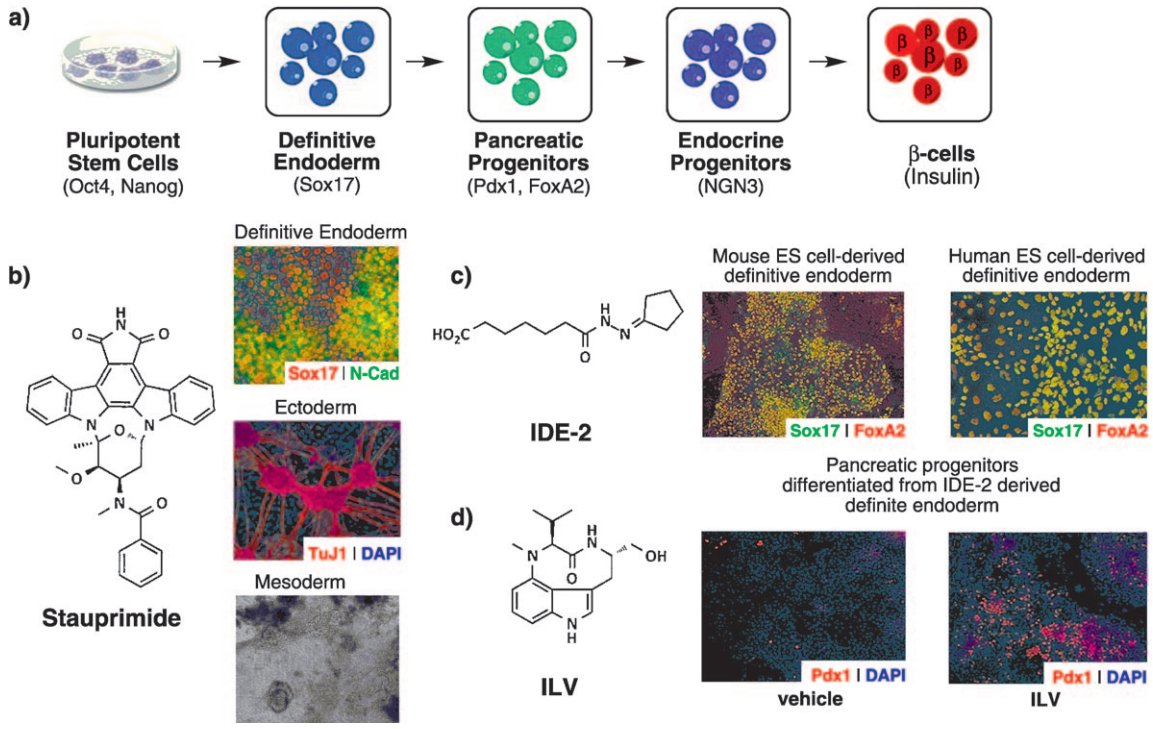


Figure 6. Deriving β-cells from pluripotent stem cells. a) Pluripotent human and mouse cells can be directed to differentiate into β-cells through stepwise in vitro recapitulation of embryonic pancreas development. b) Stauprimide potentiates the differentiation of pluripotent stem cells toward multiple lineages (definitive endoderm; neural ectoderm; and mesoderm, beating cardiomyocytes) when combined with lineage specification cues. Images reprinted with permission from Ref. [41]; Copyright 2009, Elsevier. c) IDE-2 induces lineage specification of mouse or human ES cells into definitive endoderm. d) (−)-Indolactam V (ILV) matures definitive endoderm cells into Pdx1-expressing pancreatic progenitor cells. Images in (c) and (d) reprinted with permission from Ref. [106]; Copyright 2009, Elsevier.

ciently. Similar to Activin A conditions, the compound promoted lineage-specific differentiation in response to ectoderm or mesoderm lineage-specifying conditions, suggesting that stauprimide "primes" cells for differentiation, rather than directing their differentiation toward any one given fate (Figure 6b). A biotin-tagged analogue of stauprimide was used to identify NME2, a transcription factor that regulates c-Myc expression, as the cellular target of stauprimide. c-Myc plays an important role in maintenance of the proliferative, self-renewing ES state; its down-regulation reduces this proliferative capacity. Subsequent biochemical studies demonstrated that stauprimide binds to NME2 and prevents its nuclear translocation and, as a consequence, the activation of c-Myc transcription. Because stauprimide primes ES cells to differentiate in response to external stimuli, it has received a great deal of attention for use in ES cell differentiation techniques as a means to enrich a desired output population. Furthermore, since stauprimide inhibits c-Myc expression directly, it will likely prove to be a useful tool in a variety of other biological contexts (e.g., other stem cell systems, c-Myc driven cancers, etc.).[105]

Melton an co-workers used a similar approach to identify small molecules that direct mouse ES cell differentiation toward definitive endoderm.[106] Like our study, they used Sox17 expression as a readout for definitive endoderm. Unlike our study, they did not prime the cells with Activin A, and as a result found two compounds—IDE1 and IDE2—that induce definitive endoderm differentiation (Figure 6c). Interestingly, these compounds activate SMAD2 phosphorylation by up-regulating Nodal signaling. Moreover, the differentiation activity of either compound can be enhanced by Activin A or Nodal (both morphogens that signal through phospho-SMAD2), whereas the activity of compound can be blocked by inhibitors of SMAD phosphorylation (in this case the TGFβ-inhibitor, SB-431542). Collectively, these studies suggest that IDE1 and IDE2 promote definitive endoderm formation through canonical Activin/Nodal signaling pathways, although the exact molecular targets are unknown.

5.4.3. *Cardiomyocyte Differentiation*

The mammalian adult heart is composed mainly of postmitotic, terminally differentiated muscle cells termed cardiomyocytes with no intrinsic regenerative capacity. Currently, the only effective treatment for patients suffering from severe heart failure is organ transplantation. Due to the scarcity of organs, heart disease represents a prime target for the application of ES cell-based therapies. Indeed, many groups have reported that growth factors that are active during heart development significantly increase cardiogenesis in cultures of differentiating mouse ES cells. For instance, TGFβ1,[107] BMPs[107b] or their endogenous antagonists,[108] FGFs,[109] nitric oxide, and various members of the Wnt-signaling family have been implicated in cardiac differentiation from ES cells.[110]

An increasing number of small molecules have also been reported to promote cardiogenesis in mouse ES cells, includ-

ing RA,[110,111] dorsomorphin,[69] ascorbic acid,[112] cardiogenol A–D,[113] and several others.[114] RA-mediated cardiogenesis is largely dependent on the concentration and timing of application. Late application of RA to mouse ES cells at low concentrations has a robust pro-cardiogenic effect, whereas earlier application of higher concentrations has been shown to suppress cardiac differentiation.[111] Dorsomorphin, a selective small-molecule inhibitor of BMP signaling that was identified in a screen for compounds that perturb dorsoventral axis formation in zebrafish,[66b] also induces myocardial differentiation of mouse ES cells.[69] In this case, dorsomorphin increased the yield of spontaneously beating cardiomyocytes 20-fold in a time- and dose-dependent manner. Moreover, dorsomorphin treatment, unlike the BMP-inhibiting morphogen Noggin, reduced differentiation to other mesodermal lineages. These results illustrate that small molecules, in certain instances, can be more effective than developmental cues at promoting lineage-specific differentiation.

In another example we screened combinatorial libraries for synthetic small molecules that selectively and efficiently induce differentiation of mouse ES cells into cardiomyocytes.[113] In our primary screen, we used embryonic carcinoma cells stably transfected with cardiac muscle specific atrial natriuretic factor promoter-driven luciferase. Sarcomeric myosin heavy chain (MHC), one of the essential motor proteins responsible for cardiac muscle contractibility, was used as a secondary assay for cardiac differentiation. Four diaminopyrimidines, cardiogenol A–D, were the most potent at inducing MHC expression. The differentiated cells also expressed multiple cardiac markers (GATA-4, Nkx2.5, and MEF2). Furthermore, application of cardiogenol to mouse ES cells cultured in a monolayer without LIF resulted in the formation of beating cardiac muscle, whereas no beating cells were evident in the untreated controls.

Similarly, Takahashi and co-workers screened compounds in mouse ES cells stably transfected with a cardiac-specific α-cardiac myosin heavy chain-GFP promoter-reporter.[112] They found that ascorbic acid markedly increased spontaneous cardiac differentiation of mouse ES cells. In addition, ascorbic acid induced the expression of cardiac genes, including GATA4 and α and β-myosin heavy chain in a developmentally controlled manner. The effect of ascorbic acid on cardiac differentiation was not mimicked by other antioxidants such as *N*-acetylcysteine, Tiron, or vitamin E, suggesting that the activity of ascorbic acid may be independent of its antioxidant property. Interestingly, ascorbic acid had no effect on spontaneous cardiomyogenesis through EB formation, which may suggest that ascorbic acid mimics the permissive environments of EBs, rather than inducing autonomous cardiac differentiation.

Sachinidis and co-workers have also identified a series of small molecules that induce the differentiation of murine ES cells into cardiomyocytes.[114d] Specifically, using a transgenic mouse ES cell line that expresses enhanced GFP under the control of the alpha-myosin heavy chain promoter, they found that verapamil (an L-type Ca^{2+} channel blocker) and cyclosporine (an inhibitor of protein phosphatase 2B) exerted a strong cardiomyogenic effect. On the other hand. forskolin, an adenylate cyclase stimulator, inhibited cardiomyocyte differentiation. The cardiomyogenic effect of cyclosporine and verapamil was confirmed by staining for the early cardiac markers Nkx2.5 and GATA4. These data suggest that inhibition of Ca^{2+} signaling and/or the calcineurin pathway may increase the capacity of murine ES cells to differentiate into cardiomyocytes.

6. Chemical Control of Human ES Cell Potential

Human ES cells were first isolated in 1998 by James Thomson and colleagues,[11] and are now routinely used in many laboratories. Like their murine counterparts, human ES cells are capable of karyotypically stable, prolonged self-renewal and can give rise to all of the more than 200 lineages that make up an adult organism.[11] Indeed, human ES cells have served as an immensely powerful tool to study development.[1] Furthermore, pluripotent cell lines modeling a given disease can be established from genetically abnormal embryos or by reprogramming diseased cells into pluripotent cells (the latter approach is discussed in Section 8.3)[115] and then differentiated into a desired cell type for biological studies and/or drug screens.[7b] Ultimately, it is conceivable that, with a thorough understanding of human ES cell biology, banks of specific cell types could be generated to treat degenerative diseases.[116]

6.1. Self-Renewal of Human ES Cells with Known Target- and Pathway-Based Modulators

Undifferentiated human ES cells are maintained and expanded in the presence of bFGF, feeder cell-derived Activin and serum.[11,72a] These conditions, while effective, have a number of limitations including variations in feeder cell formulation and serum, which make the maintenance of consistent and robust long-term ES cell cultures challenging. Moreover, the presence of unknown and/or variable factors in maintenance and differentiation cocktails both complicates the comparison of experimental results from lab to lab and hinders the use of ES cells in clinical applications. Another challenge is that pluripotent human cells are notoriously difficult to transfect or transduce, and, as such, few reports of knock-in lines exist.[117] This is particularly striking when compared to the large collection of genetically engineered mouse ES cell lines.[118] This limitation also makes the study of gene over-expression and/or knock-down in human ES cells very difficult, since uniform populations cannot be generated easily.

Small molecules have the ability to overcome many of the above difficulties, and have thus become useful probes in human ES cell biology and as tools for manipulating these cells (Table 2). For example one of the primary mechanisms by which human ES cells maintain the undifferentiated state is through SMAD2/3 activation, and its subsequent signaling.[13] This was demonstrated by inhibiting the TGFβ/Activin/Nodal branch of the TGFβ signaling pathway with SB-431542.[119] Application of SB-431542 to human ES cells,

maintained under self-renewing conditions, induces rapid differentiation. In addition, treatment abolished SMAD2/3 phosphorylation and, as a result, its nuclear localization. This experiment demonstrated that the Activin/Nodal pathway is essential for maintenance of pluripotency in human ES cells.[13] Interestingly, the ability of human ES cells to form EBs was drastically reduced in SB-431542 treated cells as compared to untreated cells[13a] indicating that SMAD2/3 activation is necessary but only partially sufficient for maintenance of pluripotency. In contrast, TGFβ/Activin/Nodal signaling through SMAD2/3 is dispensable for mouse ES cells as treatment with SB-431542 decreased SMAD2/3 phosphorylation, but had no effect on their ability for prolonged self-renewal.[13a]

FGF signaling also plays a critical role in human ES self-renewal, and the activating ligand, bFGF, is included in nearly all reported human ES growth media formulations.[11,120] Moreover, inhibition of FGF signaling with an FGFR inhibitor, SU5402, promoted human ES cell differentiation,[13b,73a] consistent with the idea that supplementation of human ES media with bFGF is required for maintenance of the undifferentiated state. However, the positive effect of bFGF on maintaining pluripotency strictly depends on TGFβ signaling.[13b] This was demonstrated by inhibiting Activin/Nodal signaling with SB-431542. Under these conditions, bFGF could not maintain the undifferentiated state. As such, it appears that the Activin/Nodal pathway maintains pluripotency through mechanism(s) in which FGF acts as a competence factor.[13b]

While it is clear that supplementation of human ES growth media with bFGF has a positive effect on maintaining the undifferentiated state, the exact mechanistic details governing this activity are still not clear. Initially, it was proposed that the major function of FGF signaling in human ES cells was to inhibit BMP activity.[121] This notion was based on the understanding that BMP signaling activates SMAD1/5/8 nuclear translocation which is known to drive *Id* expression and, subsequently, trophectodermal differentiation. Through the use of the FGFR inhibitor SU5402 in one experiment, and high dose bFGF in another, Vallier et al. demonstrated that FGF signaling does not have an inhibitory effect on BMP signaling in human ES cells.[13b] Phospho-SMAD1/5/8 was not affected in either experiment, which suggests that FGF signaling acts independently of BMP signaling. Moreover, an increase in BMP-induced nuclear SMAD1/5/8 was not observed upon inhibition of downstream FGF signaling with a MEK inhibitor (U-0126). Collectively, the function of FGF in human ES cell self-renewal does not appear to occur through the inhibition of BMP activity.

Nevertheless, inhibiting BMP signaling does constitute a means to promote human ES cell self-renewal in the absence of exogenous growth factors. Indeed, we recently demonstrated that a small-molecule inhibitor of BMP signaling, dorsomorphin,[66b] identified in an Oct4-GFP reporter based screen for molecules that promote human ES cell self-renewal, maintains the self-renewing, pluripotent state.[122] Furthermore, we demonstrated that dorsomorphin treatment acts by blocking autocrine secretion of BMP ligands (which act in a feed-forward loop to promote trophectodermal differentiation). Human ES cells grown in the presence of dorsomorphin could be maintained feeder- and growth factor-free for several passages while retaining the potential to differentiate into multiple lineages in vitro and to form teratomas in vivo.

Bendall and colleagues provide evidience that FGF signaling is not directly operative in pluripotent human ES cells, but rather promotes paracrine production of insulin growth factor (IGF)-II from autologously derived fibroblast-like cells (termed human dermal fibroblasts; hDFs).[73a] Through a series of careful experiments in which purified populations of ES cells and hDFs were examined, they demonstrated that the FGFR inhibitor SU-5402 acts on hDFs by preventing the production of IGF-II, and to a lesser effect TGF-β1. On the other hand, SU-5402 had no effect on purified ES cells. Furthermore, media conditioned by SU-5402-treated hDFs was unable to maintain human ES self-renewal. Indeed, the authors showed that human ES cells could be maintained in the pluripotent state without bFGF if basal media was, instead, supplemented with IGF-II. This particular finding has made a significant contribution to our understanding of signaling mechanisms that govern self-renewal in human ES cells.

Among the major developmental signaling pathways, Wnt/β-catenin is the only one that has been suggested to operate in both human and mouse ES cell self-renewal.[48] Wnt/β-catenin signaling can be activated by extracellular Wnt ligands or inhibition of GSK-3β (Figure 2a). Treatment of human ES cells with the GSK-3β inhibitor BIO activates Wnt/β-catenin signaling and maintains the undifferentiated phenotype. Moreover, BIO-mediated Wnt activation is functionally reversible, as withdrawal of the compound leads to normal multi-differentiation programs.[48]

Work on Wnt/β-catenin signaling in human ES cells has demonstrated that specifically engaging the Wnt pathway promotes hemato-endothelial differentiation.[123] This data suggests that BIO-mediated GSK-3β inhibition does more than simply activate Wnt, consistent with the role of GSK-3β in a diverse range of cellular processes and its positioning at a nexus of several signaling pathways (e.g., Hh, PI3K, non-canonical Wnt, cAMP, among others).[47b] Follow-up work from Brivanlou and co-workers provided insight into an additional role for BIO in self-renewal in which BIO treatment of human ES cells led to the activation of SMAD2/3 activation, which could be blocked by the TGFβ inhibitor, SB-431542.[13a] A likely explanation for the observed convergence of TGFβ/Activin/Nodal and BIO-induced Wnt activation may be that treatment with BIO results in the expression of TGFβ ligands from human ES cells, or autologously derived cells, which are capable of promoting self-renewal. These findings are consistent with recent data demonstrating that Wnt signaling does not directly promote self-renewal or differentiation of human ES cells, but rather enables either outcome dependent on the lineage specifying factors present.[124] Thus, in the presence of "self-renewal" factors (i.e., exogenous bFGF and/or BIO-triggered Activin production), Wnt signaling promotes proliferation of self-renewing ES cells.

More recently, it was demonstrated that inhibition of histone deacetylase (HDAC) activity can also maintain human ES cell self-renewal and, moreover, that this mechanism is operative in mouse ES cells too.[125] Interestingly, HDAC inhibition seems to work on human ES cells by reverting them to an earlier developmental stage. As discussed earlier, human ES cells appear much more like mouse EpiSCs than traditional ICM-derived ES cells (see Figure 1). Upon expansion in media containing the HDAC inhibitor butyrate, self-renewing mouse and human ES cells appear to converge upon a developmental state that is intermediate between their "normal" states based on the following observations: 1) the gene expression profile of butyrate-treated human and mouse ES cells are strikingly similar, whereas vehicle-treated controls show very little overlap; 2) X chromosome inactivation is prevented in butyrate-treated human ES cells, a characteristic of cells in an earlier stage of development; 3) butyrate-treated human ES cells form teratomas with a much greater degree of complexity; and 4) butyrate-treated mouse EpiSCs become chimera competent, a feature that is only observed in ICM-derived mouse ES cells. Collectively, these results both identify a chemically defined method to culture human ES cells and provide an ES cell derivation-stage independent mechanism that can regulate the pluripotent state.

6.2. Directed Differentiation of Human ES Cells with Known Target- and Pathway-Based Modulators

The application of human ES cells in regenerative medicine and drug discovery depends on developing techniques that allow for the reliable in vitro production of desired stem cell-derived cell types. Conventional differentiation protocols rely on EB formation, stromal feeder coculture and/or "cocktails" that contain growth factors or pleiotropic small molecules (e.g., RA) to promote particular cell fates. However, as is the case with mouse ES cells, most of these methods lead to heterogeneous differentiation outcomes, and the desired cell type has to be selected from a mixture of various other cell types. Moreover, differentiation of specialized lineages can require months of culture,[96c,126] which drastically limits the number of cells that can be generated due to cost and maintenance constraints. At present, very few efficient and robust protocols exist for the generation of ES cell derivatives. The development of such methods will accelerate the application of human ES cells in disease modeling and as therapeutic agents.

Toward this end, several small molecule-based strategies have been developed to selectively control human ES cell fate. For instance, the peroxisome proliferator-activated receptor-γ (PPARγ) agonist rosiglitazone has been used to enhance the percentage of adipocytes from human ES cells under specific differentiation conditions.[127] In another study, McLean et al. demonstrated that small-molecule suppression of PI3K signaling efficiently specifies definitive endoderm from human ES cells when used in conjunction with Activin A.[128] Combinatorial application of small molecules has also been used to generate particular lineages. For example, sequential application of RA and the hedgehog agonist purmorphamine leads to efficient motor neuron differentiation in chemically defined culture.[61b] In another recent study, Chambers et al. described an efficient neural induction method by combining the TGFβ pathway inhibitor SB-431542 with noggin, a secreted protein that binds to and inhibits BMP4.[83] This protocol directs neural induction in high yield (> 80 %) and reduces differentiation periods by 2-fold, compared with stromal feeder-mediated techniques (19 versus 30–50 days, respectively). Moreover, this combination of inhibitors can generate neurons from either the central nervous system (when ES cell are plated at high cell density) or the peripheral nervous system (when ES cells are plated at low density). The continued development of defined protocols, such as those described above, will greatly facilitate the derivation of human ES cell-derived lineages and their eventual application in disease modeling and transplantation studies.

6.3. Identification and Characterization of Novel Modulators of Human ES Cell Fate

Clearly, the use of small molecules that modulate known developmental signaling pathways is proving an effective strategy to control human ES cell fate. However, it is conceivable that human ES cell differentiation in vitro may not mimic the patterning events of early development in vivo. As such, attempts to recapitulate developmental processes in vitro may not yield the expected results. Coupled with this, in vivo differentiation depends on a complex interplay of signals from a plethora of cells at various stages of differentiation. Removal or selection of a desired cell type from the bulk heterogeneous differentiating population may also alter proper lineage commitment. Unbiased small-molecule screens, on the other hand, provide a means to identify modulators of ES cell fate beyond the known developmental techniques. Synthetic molecules identified in this way can both be applied in directed differentiation schemes and used as probes of underlying biology.

Despite the successes in identifying small molecules that modulate mouse ES cell fate, few of these compounds are active in human ES cells. A notable exception is stauprimide, which efficiently directs the differentiation of human ES cells into endodermal progenitors in combination with Activin A (Figure 6b).[41] Direct screens on human ES cells are hampered by the fact that they are difficult to propagate as homogenous populations in miniaturized formats (those necessary to run screens), even though efficient feeder-free and chemically defined conditions have been developed.[72a] Central to this problem is the fact that human ES cells must be passaged as aggregates due to their sensitivity to clonal expansion. Recent work by Watanabe and colleagues has suggested that small molecules may alleviate the inefficiencies associated with clonal expansion of human ES cells. They found that inhibitors of Rho-associated kinase (ROCK; Y27632) can increase the clonal expansion efficiency of human ES cells from ca. 1 % to 27 % by inhibiting apoptosis.[129] Importantly, the clonally expanded cells are identical to

human ES cells passaged as aggregates and faithfully differentiate into lineages representing all three germ layers when injected into nude mice. Application of ROCK inhibitors and/or similar strategies should greatly facilitate the use of human ES cells in chemical screens.

The aforementioned limitation notwithstanding, Studer and co-workers screened a focused library of biologically active small molecules on human ES cells.[130] In their assay, maintenance or loss of pluripotency was monitored by expression of Oct4. From this screen, four compounds were found to promote self-renewal and four were found to promote differentiation. Among the compounds that maintained Oct4 expression were theanine, sinomenine, gatifloxacin, and flurbiprofen. Each of these was able to promote the short-term self-renewal of human ES cells in the absence of mitogen or feeder cell conditioned media. However, none of the compounds were able to promote prolonged self-renewal, either individually or in combination. Among the compounds that induced differentiation were selegiline, cymarin, sarmentogenin, and RA, which all induced a dose-dependent reduction in Oct4 expression.

Using a similar screening platform, in which Oct4 expression was used as a reporter of pluripotency, we screened human ES cells against a library of 529 purified and arrayed secreted proteins to identify candidates that maintained the pluripotent state.[30] We found that the pigment epithelium-derived factor (PEDF) can promote long term pluripotent growth of human ES cells without bFGF or TGFβ/Activin/Nodal ligand supplementation. Our results further indicate that activation of the PEDF receptor-Erk1/2 signaling pathway by PEDF is sufficient to maintain pluripotency in human ES cells.

In another example, Melton and co-workers carried out a screen to identify chemicals that direct human ES cell differentiation into functional insulin-secreting β-cells in a stepwise fashion. They employed a high-content image-based screen to identify compounds that increase the total number of Pdx1-expressing pancreatic progenitors from ES cell-derived definitive endoderm.[131] This screen identified one compound, (−)-indolactam-V (ILV), which, when combined with growth factors, could direct the differentiation of human ES cells such that 44.5% of the cells become Pdx1-expressing pancreatic progenitors (Figure 6d). Moreover, ILV was functional in several human ES cell lines and on IDE1-directed definitive endoderm (see Section 5.4.2).[106] Together, these compounds provide a two-step protocol for in vitro generation of pancreatic progenitors that uses small molecules first as inducers of endoderm (i.e., IDE-1) and later to generate pancreatic progenitors (i.e., ILV). Importantly, the ILV-derived pancreatic progenitors were capable of differentiation into pancreatic lineages in vitro and were able to contribute to endocrine, exocrine, and ductal cells in vivo.

Earlier studies with ILV demonstrated that it activates protein kinase C (PKC) signaling. To test the role of PKC signaling in pancreatic progenitor differentiation, PKC antagonists (bisindolylmaleimide I, Go 6983, or Go 6976) were applied to ILV-treated human ES cell-derived definitive endoderm. PKC antagonists blocked ILV's effect; indeed the percentage of Pdx1-expressing cells in definitive endoderm treated with both PKC inhibitors and ILV was even lower than the DMSO-treated controls. Furthermore, treatment with PKC agonists (PMA or TPB)—in the absence of ILV—mimicked the effect of ILV in human ES cell-derived definitive endoderm. Together these data suggest that ILV induces pancreatic differentiation of human ES cell-derived definitive endoderm, at least in part, through the activation of PKC signaling.

7. Chemical Control of Somatic Stem Cell Potential

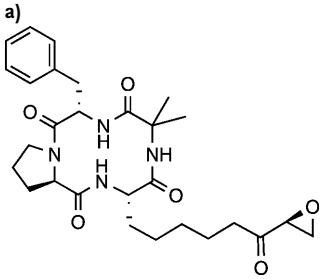
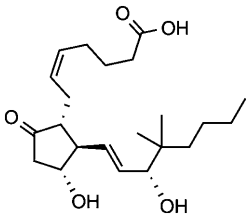
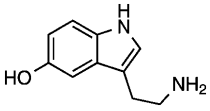
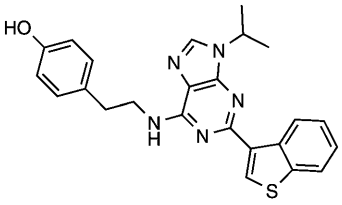
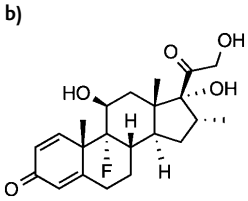
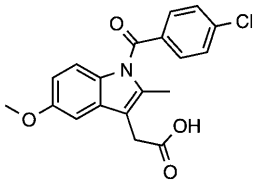
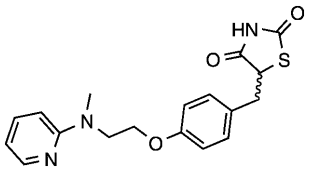
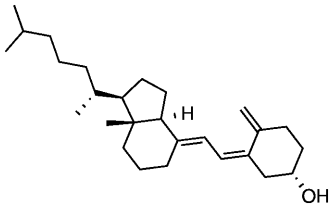
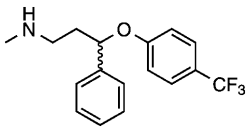
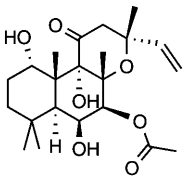
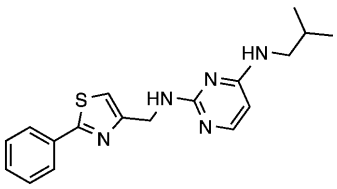
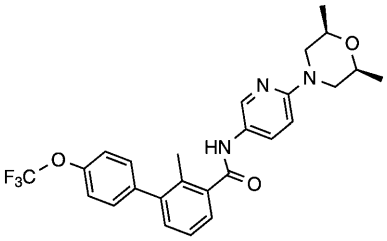
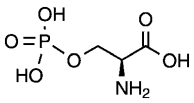
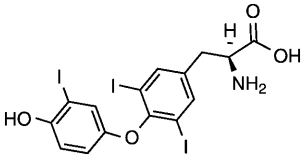
7.1. Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are multipotent stem cells located in the bone marrow with the ability to self-renew and differentiate into all blood lineages (red blood cells, platelets, granulocytes, macrophages, and B- and T-lymphocytes).[132] HSCs were the first stem cell population to be isolated, are the most well characterized adult stem cell, and are the only stem cells used routinely in cell based therapies (e.g., bone marrow transplant).[19] Despite their clinical success (e.g., in blood cancers and autoimmune disease), the full clinical impact of HSCs has yet to be realized.[133] This is due largely to a shortage of clinically available HSCs—roughly 50% of allogeneic bone marrow transplant candidates cannot find a matched donor.[134] Although cord blood-derived HSCs provide a possible solution, methods for their expansion to sufficient quantities for adult transplant do not exist. The identification of pharmacological agents that control the expansion and fate of HSCs, either in vivo or ex vivo, will greatly facilitate the application of HSC therapies to a host of human diseases.

7.1.1. Self-Renewal of HSCs with Known Target- and Pathway-Based Modulators

Several target-based approaches have yielded small-molecule modulators of HSC self-renewal (Table 3a). For example, Young and colleagues demonstrated that murine and human HSCs cultured with HDAC inhibitors maintained a more primitive phenotype than control cultures.[135] Of the HDAC inhibitors tested, chlamydocin was found to be the most effective at maintaining Thy-1 (a marker of the undifferentiated state) expression in human HSCs. Interestingly, in a non-obese diabetic-severe combined immunodeficient (NOD-SCID) repopulation assay, cells exposed to chlamydocin for 24 h displayed an average of four-fold higher engrafting ability over control cells. A more recent study examined the effects of two chromatin-modifying agents (the DNA methyl-transferase inhibitor 5-AzaC and the HDAC inhibitor trichostatin A; TSA) on HSCs isolated from the umbilical cord ($CD34^+/CD90^+$).[136] Cotreatment of these cells with TSA and 5-AzaC resulted in a 12.5-fold expansion of treated cultures as compared to the input cell numbers. Expansion of $CD34^+/CD90^+$ cells was also associated with a 9.8-fold increase in the number of colony-forming units and an 11.5-fold increase in cobblestone area forming cells (assays used to determine the number of multipotent

Table 3: Selected small-molecule modulators of somatic stem cell fate: a) HSCs, b) MSCs, and c) NSCs.

Compound	*Target* Function	Compound	*Target* Function
a)			
	**Chlamydocin** *HDACs* Maintains a primitive phenotype in HSCs		**16,16-dimethyl prostaglandin E2 (dmPGE2)** *Prostaglandin E2 Receptor* Increases engraftement potential of HSCs
	**Serotonin** *Serotonin Receptor* Enhances the expansion of HSCs		**SR1** *AhR* Promotes the self-renewal of HSCs
b)			
	**Dexamethasone** *Glucocorticoid Receptor* Promotes the osteogenic differentiation of MSCs		**Indomethacin** *COX-1 & COX-2* Promotes adipocyte differentiation of MSCs
	**Rosiglitazone** *PPARs* Promotes adipocyte differentiation of MSCs		**Vitamin D3** *Vitamin D Receptor* Potentiates osteoblast differentiation of MSCs
c)			
	**Fluoxetine** *Monoamine Transporters* Stimulates hippocampal neurogenesis		**Forskolin** *Adenylate Cyclase* Promotes neuronal differentiation of NSCs
	**KHS101** *TACC3* Promotes neuronal differentiation of NSCs		**NVP-LDE225** *Smo* Hh antagonist; induces meduloblastoma cell death
	**Phosphoserine (P-Ser)** *mGluR4* Promotes neuronal differentiation of NSCs and human ES cells		**Triiodothyronine (T_3)** *Thyroid Hormone Receptor* Promotes oligodendroglial differentiation of OPCs

HSCs). While HDAC inhibitors are capable of expanding human HSCs, their use in clinical applications is limited since their effects are only observed at narrow concentration ranges (5–10 nM), where higher concentrations result in growth arrest and cytotoxicity.

Serotonin, a monoamine neurotransmitter, has also been shown to enhance the expansion of human HSCs at near physiological concentration (200 nM).[137] Serotonin-expanded HSCs infused into sublethally irradiated NOD-SCID mice engrafted much more efficiently than vehicle-treated controls. The involvement of serotonin in promoting HSC expansion has added a new dimension to the multiple functions of this neural transmitter and warrants further investigation to identify the pathways regulating HSC self-renewal.

Clinical observations have suggested that copper ion (Cu^{2+}) may play a role in regulating HSC development.[138] Indeed Peled and co-workers demonstrated that a Cu^{2+} chelator (tetraethylenepentamine; TEPA) enabled preferential proliferation of naive HSCs, resulting in an increase in their long-term ex vivo expansion and engraftment capabilities. In preclinical development—following a three-week expansion under large-scale culture conditions—TEPA increased the median output value of naive HSCs by 89-fold and total HSCs by 172-fold over input value. Transplantation of TEPA-treated HSCs into NOD-SCID mice indicated that the engraftment potential of the ex vivo expanded cells was superior to that of unexpanded cells.[139] The chelator-based ex vivo expansion technology is currently being tested in a phase II/III clinical trial in patients undergoing cord blood transplantation for hematological malignancies.[138a]

7.1.2. *Identification and Characterization of Novel Modulators of HSC Self-Renewal*

Our group and others have attempted to identify novel compounds that expand HSCs using unbiased, cell-based small-molecule screens. Although highly specific transgenic reporter assays are well suited for small-molecule screens (discussed in Section 3), these methods are not amenable to human HSCs due to difficulties associated with engineering and expanding transgenic HSC lines. Therefore, to identify small molecules that promote the self-renewal of HSCs, we used an image-based assay that measures undifferentiated cells following in vitro culture.[36] Primary human $CD34^+$ HSCs were grown in serum free media supplemented with thrombopoietin, stem cell factor, Flt3 ligand, and interleukin-6. These conditions lead to robust proliferation accompanied by differentiation and loss of HSC activity.[135,140] HSC differentiation was visualized by maintenance of undifferentiated cell surface marker expression (CD34 and CD133; Figure 7a).

From a screen of a heterocycle library,[29] a purine derivative, SR1, was identified that increased the total number of cord blood (CB)-derived HSCs > 50-fold after 5 weeks, as compared to control cultures (vehicle + cytokines).[36] Biochemical labeling studies suggest that SR1 promotes symmetric cell divisions without the loss of primitive characteristics. Importantly, cells cultured with SR1 for 21 days afford a 17-fold increase in the number of NOD/SCID engrafting cells (compared to starting cells or control cultures) and these cells retain the ability to sustain multi-lineage reconstitution and engraft secondary recipients (Figure 7b).

mRNA expression analysis of SR1 and a closely related inactive analogue suggested that SR1 controls HSC expansion by antagonizing AhR signaling. In support of this hypothesis, SR1 treatment prevented the induction of AhR target genes (CYP1B1, AHRR) in response to the AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) in HSCs. To show a direct role for AhR in HSC expansion, lentivirus encoding shRNAs targeting AhR were used to decrease AhR expression in CB-derived HSCs resulting in sustained expression of CD34 during ex vivo culture, a phenotype similar to cultures treated with SR1 (Figure 7c). Furthermore, it was shown that the antagonizing effect of SR1 on the AhR signaling pathway is mediated by direct interaction with this nuclear receptor ($IC_{50} \approx 40$ nM; Figure 7d). Because the success of bone marrow transplants correlates with HSC numbers, the identification of SR1 (and AhR signaling as a means to control HSC self-renewal) should greatly facilitate the clinical use of CB-derived HSCs for both autologous and allogeneic transplantation therapy.

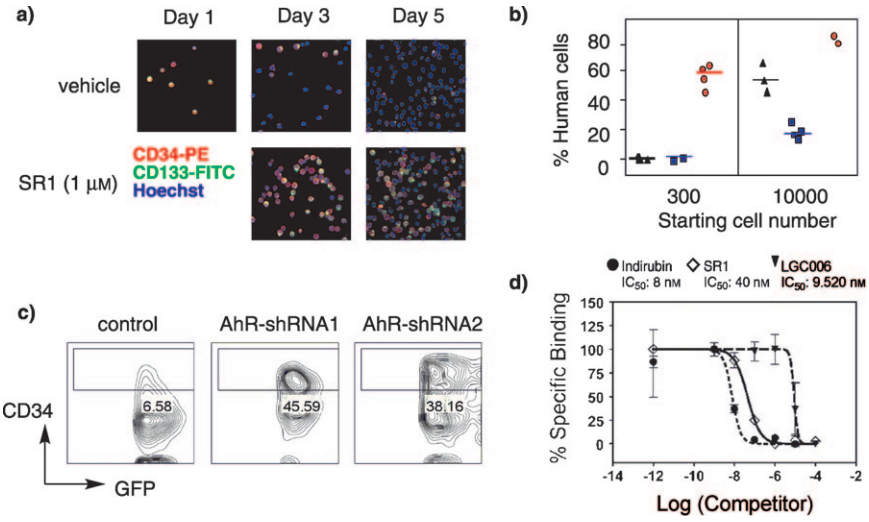


Figure 7. SR1 promotes the expansion of CD34-positive cells by antagonizing the AhR. a) SR1-treated CD34-positive cells maintain expression of stem and progenitor cell markers. b) NOG mice were injected with 300 or 10000 uncultured (▲) CD34-positive CB-derived cells; a fraction of the final culture equivalent to the progeny of 300 or 10000 CB-derived CD34-positive cells that were cultured for 21 days with cytokines and SR1 (0.75 μM, ●); or a fraction of the final culture equivalent to the progeny of 300 or 10000 CB CD34-positive cells that were cultured for 21 days with cytokines and vehicle (DMSO 0.01%, ■). The percent of human CD45-positive cells in bone marrow at 13 weeks is shown. c) shRNA-mediated knock-down of the AhR recapitulates the activity of SR1. d) SR1 binds directly to AhR as compared with the high affinity human AhR agonist (indirubin) and a less active SR1 analogue (LGC006). Figure adapted with permission from Ref. [36]; Copyright 2010, American Association for the Advancement of Science.

North et al. also recently screened a library of biologically active compounds in search of molecules that affect hematopoiesis.[141] In particular, they utilized a developmental zebrafish model and monitored for altered HSC numbers. In situ hybridization analysis 36 h post-fertilization revealed a number of molecules that increased HSC counts by enhancing prostaglandin (PG) E2 synthesis. Consistent with these results, small molecules that blocked PGE2 synthesis decreased stem cell numbers. Addition of a long-acting derivative of PGE2 (16,16-dimethyl prostaglandin E2; dmPGE2) during embryoid body expansion increased the number of multipotent HSCs 2.9-fold over control. Furthermore, murine whole bone marrow exposed to dmPGE2 ex vivo and transplanted into irradiated recipients had a 3-fold increase in spleen colony-forming units 12 days after transplant. The frequency of short-term repopulating HSCs was also 4-fold higher 12 weeks after transplant in the dmPGE2-treated whole bone marrow recipients. However, it should be noted that the observed increase in dmPGE2-treated HSC number in the murine studies was a result of an increase in HSC engraftment potential, rather than from a true expansion of HSC number ex vivo.

Clearly, compounds identified with unbiased approaches have provided significant insights into the signaling pathways that control HSC self-renewal (Table 3a). Moreover, these findings are already leading to the development of drugs that will expand the clinical use of HSCs in transplantation therapy.

7.1.3. Directed Differentiation of HSCs

Recent efforts have also suggested that HSCs and/or their progeny may be useful for the repair or replacement of blood-derived tissues (e.g., erythrocytes, megakaryocytes). Thus, efforts are focused on the directed differentiation and/or expansion of cells derived from hematopoietic lineages. For example, thrombocytopenia is characterized by abnormally low platelet counts and remains a serious problem in patients treated with intensive high-dose chemotherapy or those with rare genetic diseases. It has been suggested that the infusion of ex vivo expanded megakaryocytic progenitor cells (MPCs) may be an efficient strategy for accelerating platelet recovery. In fact, several clinical trials have been successful in alleviating thrombocytopenia by infusing MPC-rich peripheral blood stem cells[142] and bone marrow.[143] As such, there is a need for methods to direct the differentiation of HSCs toward the megakaryocyte fate either ex vivo or in vivo.

Current methods of megakaryocyte differentiation, ex vivo, rely on signaling molecules identified from studies on megakaryocytopoiesis. Among these, thrombopoietin is the most effective, even though it activates multi-lineage differentiation programs.[144] To address the shortcomings associated with current pleiotropic differentiation strategies, we set out to identify molecules that direct the differentiation of HSCs to megakaryocytes. The screen was carried out in a similar fashion to the HSC expansion screen with the exception that cells were labeled with megakaryocyte and erythrocyte markers. From this screen, a class of naphthyridinones was identified that dose-dependently increase megakaryocyte differentiation. Human HSCs treated with compound for 8 days, re-plated under megakaryocyte differentiation conditions and then cultured for an additional 12 days generate a 3- to 4-fold increase in the number of megakaryocytes, as compared to control cultures. Importantly, when compound is removed, the megakaryocyte precursors remain functional and are able to complete differentiation and form colonies. The compound was shown to act on common myeloid progenitors (CMPs) to increase megakaryocyte erythrocyte progenitors (MEPs). Indeed, purified CMPs treated with the compound had an increase in megakaryocyte differentiation similar to what was seen in the mixed population of HSCs.[145] Similar screens are ongoing for small molecules and secreted proteins that affect erythrocyte maturation; such molecules may ultimately allow the ex vivo production of blood cells for platelet and red blood cell deficiencies.

7.2. Mesenchymal Stem Cells

In addition to HSCs, bone marrow is home to another population of multipotent stem cells, those that contribute to the regeneration of mesenchymal tissues such as osteoblasts (bone), chondrocytes (cartilage) and adipocytes (fat).[21,146] The differentiation potential of these cells resembles that of mesodermal progenitors during embryonic development, and are thus named, mesenchymal stem cells (MSCs). Besides bone marrow stroma, MSCs have now also been found in other tissues, including adipose, muscle, vascular tissue, and dermis.[147] Recent studies on a variety of diseases and disease models have shown that adult MSCs, when injected, are capable of homing to a site of injury and helping to restore tissue function.[148] Moreover, MSCs do not express HLA class II antigens and are considered non-immunogenic, meaning that transplantation into an allogeneic host may not require life-long immunosuppression. MSCs are also relatively easy to isolate, expand readily in culture, and thus are receiving considerable attention for cell-based therapies.[149]

The identification of potent and specific small-molecule modulators of MSC differentiation should provide useful chemical tools to study developmental mechanisms, and ultimately may lead to new therapeutics for the treatment of a variety of degenerative diseases including osteoarthritis (OA) and bone loss. Thus, in an effort to identify small molecules that selectively induce osteogenesis, we developed a high-throughput screen which uses an enzymatic assay that detects expression of ALP, a marker expressed on osteoblasts but not on mesenchymal progenitors. Screening of the mouse mesenchymal progenitor cell line C3H10T1/2 with a chemical library identified a 2,6,9-trisubstituted purine, purmorphamine, with potent osteoblast differentiation-inducing activity.[34] The compound up-regulates Cbfa1/Runx2 expression (a master regulator of bone development), as well as other bone-specific markers, including osteopontin and collagen-I. Cells treated with purmorphamine also have the characteristic osteoblast morphology (Figure 8). In a subsequent study, purmorphamine was found to induce terminal maturation of

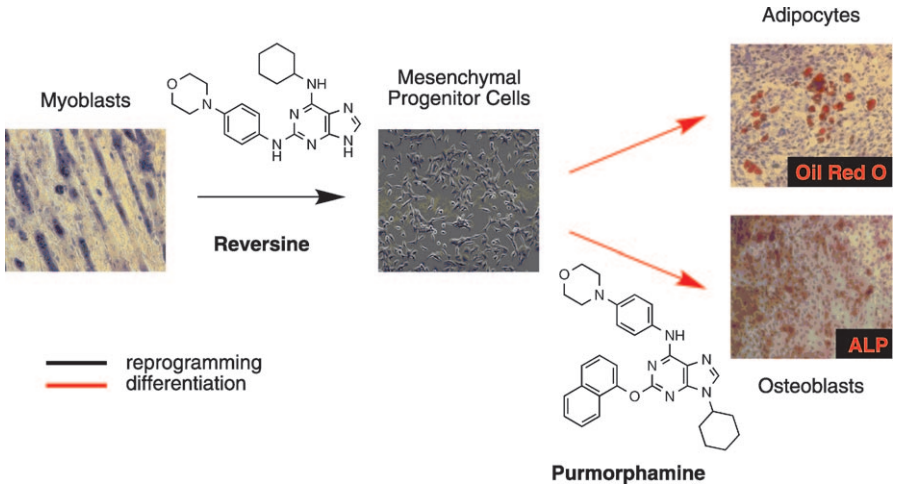


Figure 8. Chemical control of adult mesodermal cells. Reversine reprograms myoblasts into multipotent progenitor cells that can differentiate into fat (adipocytes) and bone (osteoblasts). The differentiation of mesenchymal progenitor cells into osteoblasts is enhanced by the Hh agonist purmorphamine. Lineage specific stains are depicted in red; nuclear staining with hematoxylin is depicted in blue. Figure adapted with permission from Ref. [39]; Copyright 2007, National Academy of Sciences, USA, and Ref. [33]; Copyright 2004, Elsevier.

mouse pre-osteoblasts, and when combined with BMP4, could trans-differentiate the 3T3L1 pre-adipocyte and C2C12 myoblast cell lines into osteoblasts.[150]

BMP signaling is known to be involved in osteoblast differentiation. Indeed, when C3H10T1/2 cells are treated with BMP4, the majority of cells differentiate into osteoblasts. However, a significant number of cells also differentiate into adipocytes, indicating that BMP-induced differentiation is not entirely lineage specific. To gain insights into the cellular response associated with purmorphamine treatment, transcriptional analysis was carried out on C3H10T1/2 cells treated with purmorphamine or BMP4.[33] The gene expression data suggested that purmorphamine had a distinct biological activity relative to BMP4, and that purmorphamine treatment up-regulated key downstream members of the Hh signaling pathway, including Gli1 and Ptc. Subsequent studies showed that Hh pathway antagonists block purmorphamine's ability to activate the Hh pathway and to induce osteogenesis, and recent work by Chen and Sinha confirmed that purmorphamine activates Hh signaling by directly binding to the Hh receptor Smo.[35]

In addition to purmorphamine, a variety of other small molecules and chemical cocktails with MSC differentiation inducing activity have been identified (Table 3b). For instance, 5-Aza-dC promotes the multi-lineage differentiation of C3H10T1/2 cells into osteoblasts, adipocytes, and chondrocytes.[151] Interestingly, 5-Aza-dC does not directly activate a specific differentiation program, but rather converts the cells into a competent spontaneous differentiation state accounting for its pleiotropic effect. Other small molecules that modulate osteogenesis or adipogenesis from MSCs are often included in basic differentiation cocktails. Some of these include PPARγ agonists (such as rosiglitazone) and antagonists, which are widely used as adipogenesis modulators; dexamethasone (a glucocorticoid receptor agonist), ascorbic acid, isobutylmethylxanthine (IBMX, a non-specific phosphodiesterase inhibitor), RA, vitamin D3, and indomethacin, which, under carefully defined conditions, can induce osteogenesis or adipogenesis of MSCs.[152]

OA affects 40 million people in the U.S. alone and currently there is no disease modifying therapy. The principal events in the pathogenesis of OA are abnormal activation and chondrocytic differentiation of MSCs in the injured or diseased joint. However, when adult MSCs are exposed in vitro to appropriate stimuli, they are capable of forming new functional cartilage extracellular matrix. Thus, molecules that activate the tissue regenerative potential of cartilage stem cells present in arthritic cartilage might potentially prevent further cartilage destruction and stimulate repair of cartilage lesions. To this end, we used both image-based and alcian blue screens of small-molecule libraries to identify molecules that promote chondrogenic nodule formation in primary human MSCs and murine C3H10T1/2 cells. Using this platform, a molecule was identified, chrondrogenin, which selectively induces chondrogenesis (up-regulating Sox9, lubricin, type II collagen and aggrecan expression but not osteocalcin or type X collagen). Interestingly, this molecule was also protective (glucosaminoglycan and NO release) in cartilage explant cultures grown in the presence of TNFα and oncostatin M. In both collagenase VII and surgical ligament lesion rodent models of OA, intra-articular administration of chondrogenin effectively alleviated pain and statistically improved the histological joint score compared with vehicle alone. Currently the mechanism of action of this molecule and potential therapeutic applications are under investigation.[153]

7.3. Neural Stem Cells

Neural stem cells (NSCs) exist in discrete regions of the adult brain and are capable of self-renewal and differentiation into neurons, astrocytes, and oligodendrocytes.[154] Neurons are the functional components of the nervous system that are responsible for information processing and transmission; astrocytes and oligodendrocytes are collectively known as glia and play supporting roles that are essential for the proper functioning of the nervous system. In the brain, NSCs can respond to external stimuli and, when instructed, functionally integrate into the existing neural circuitry.[20] The existence of multipotent cells in distinct regions of the adult mammalian brain raises the possibility for noninvasive endogenous brain repair. Potential therapies might involve drug treatment to stimulate the body's own regenerative mechanisms by

promoting survival, migration, proliferation, and/or differentiation of the endogenous NSCs.

NSCs can also be expanded readily ex vivo in the presence of growth factors (such as epidermal growth factor and/or bFGF) or induced to differentiate in response to mitogen withdrawal or upon exposure to various lineage-specifying stimuli (e.g., BMP4 or RA). However, the use of such cells for transplantation faces several obstacles: NSC isolation is difficult and invasive; efficient methods to generate pure neuronal subtypes from NSCs are lacking; and robust methods to integrate engrafted tissue into existing neural circuitry are still being developed. Another complication is that NSCs isolated from different areas of the brain and/or at different developmental timepoints exhibit varying responses to signaling cues.[155] Moreover, there have been significant difficulties applying the lessons learned from rodents to human systems. As such, the development of chemical tools that control NSC fate should increase our understanding of NSC biology and may ultimately result in therapeutic applications.

7.3.1. *Self-Renewal of NSCs*

A diverse number of signaling pathways are also involved in dictating whether a NSC self-renews or differentiates. Among these are epidermal growth factor, vascular endothelial growth factor, BMP, LIF-JAK-STAT, Notch, and others, where a given signal can either promote self-renewal or differentiation dependent on the context.[72b] A great deal of insight into these complex processes has come from the use of small molecules that disrupt signaling and the cell cycle (Table 3c). For instance, activation of the Notch pathway is known to promote self-renewal of NSCs and can be antagonized by JAK-STAT signaling through the LIF receptor (LIFR). McKay and co-workers demonstrated that inhibition of JAK or p38 (downstream LIFR mediators) promoted self-renewal in NSCs, and that this comes at the expense of differentiation.[157] Hh signaling has also been shown to regulate proliferation of adult hippocampal NSCs both in vitro and in vivo.[61a] Porter and co-workers demonstrated that a Hh pathway-specific agonist (Hh-Ag1.2) was able to stimulate proliferation of NSCs.[59a] Consistent with this observation, Berman et al. demonstrated that the small-molecule Hh antagonist, cyclopamine, was able to inhibit proliferation and initiate differentiation of murine brain-derived CSCs.[62a] Application of cyclopamine or the novel Hh antagonist NVP-LDE225 have both demonstrated efficacy in murine tumor allografts in vivo and induce rapid cell death of human medulloblastoma cells.[59a,158] These findings have conclusively demonstrated that Hh signaling plays a predominant role in the self-renewal of various brain-derived stem cells.

To discover novel mechanisms involved in NSC self-renewal, Diamandis et al. conducted a cell-based screen with a library of pharmacologically active compounds for those that inhibit neurosphere proliferation of mouse NSCs.[159] A number of neuromodulatory compounds were revealed including dihydrocapsaicin, an agonist of the vanilloid receptor; apomorphine, an agonist of the dopamine receptor; *p*-aminophenethyl-*m*-trifluoromethylphenyl piperazine (PAPP), a serotonin agonist; as well as numerous other modulators of the serotonin, opioid, and glutamate pathways. Importantly, this screen also identified known modulators of NSC self-renewal and differentiation, including the Hh antagonist, cyclopamine, as well as p38 and JAK inhibitors (SB-202190 and WHI-P131, respectively). The authors showed that neurosphere formation was inhibited by many of the compounds indicating that the replicative stem cells—those that re-establish new neurospheres—had been depleted. Notably, many of the compounds identified as antiproliferative for neurospheres also inhibited tumor sphere growth in vitro, including known modulators of the dopamine, opioid, vanilloid, and serotonin pathways. These results suggest that clinically approved neuromodulatory agents may remodel the mature central nervous system and prove useful in the treatment of brain cancer.[160]

7.3.2. *Directed Differentiation of NSCs with Known Target- and Pathway-Based Modulators*

Central nervous system development and homeostasis are influenced by a variety of small molecules in vivo. These can act through nuclear receptors or by one of several cell surface receptor-mediated signal cascades (e.g., thyroid hormone, retinoids, glucocorticoids, biogenic amines). For instance, RA is an established signaling molecule involved in neuronal patterning, neural differentiation and axonal outgrowth,[161] and thyroid hormone can mediate oligodendrogenesis and maintenance.[162] Due to the well established roles that these small molecules play in development, many are now used as tools to direct the differentiation of neural stem/progenitor and ES cells in vitro. In addition to these well known compounds, a variety of other compounds are being used as tools to study lineage progression and commitment in NSCs, both in vitro and in vivo (Table 3c). An early example was the natural product and PKA signaling agonist forskolin (FSK). In vitro, FSK treatment of NSCs promotes neurogenesis by increasing intracellular cAMP concentration, thereby blocking Hh signaling through inhibition of Gli-dependent transcriptional activation.[59a] Moreover, when FSK is used in conjunction with RA, a synergistic increase in neuron formation from NSCs is observed.[163] More recently, a number of studies have suggested that most antidepressants and environmental interventions that confer antidepressant-like behavioral effects stimulate adult hippocampal neurogenesis in vivo. Indeed, treatment with chronic anti-depression drugs, such as fluoxetine, has been used to stimulate neurogenesis in the hippocampus.[164] This and other studies have established a convincing link between the regeneration of neurons and their role in reducing the symptoms of depression.[20,165]

There is increasing evidence that the alteration of epigenetic marks is an important determinant in lineage specification of neural stem and progenitor cells.[166] For instance, HDAC inhibitors have been used to demonstrate the necessity of HDAC activity during a specific time interval in oligodendrocyte differentiation[167] and process outgrowth[168] in vitro and in vivo. In another report, Gage et al. found that

small-molecule inhibition of HDAC activity promoted neuronal differentiation of adult NSCs at the expense of astrocyte and oligodendrocyte differentiation. Moreover, HDAC inhibitor-treated NSCs differentiated into neurons even under astrocyte and oligodendrocyte lineage-specific differentiation conditions.[169] Mechanistic analysis revealed that HDAC inhibitors (i.e., TSA, butyrate, and valproic acid (VPA)) neither directly activate nor inhibit known neurogenic or gliogenic pathways, respectively, pointing to another mechanism of action. Interestingly, HDAC inhibitors were found to up-regulate the master neurogenic transcription factor NeuroD, suggesting that HDAC inhibition promotes neuronal fate and inhibits glial fate simultaneously through the induction of neurogenic transcription factors. Similarly, Schneider and colleagues characterized a neurogenic isoxazole that activates CaMKII (the major HDAC kinase), leading to export of HDAC5 from the nucleus and derepression of neurogenic transcription factors, including NeuroD.[170] These studies provide evidence that blockade of HDAC activity plays a major role in neuronal lineage specification. Indeed, Hsieh et al. demonstrated that VPA treatment decreased proliferation and increased neuronal differentiation in the dentate gyrus of adult rats in vivo.[169] Hao et al. have also demonstrated that HDAC inhibition promotes neurogenesis in vivo.[171] However, they suggest that the effect observed with VPA does not result exclusively from HDAC inhibition. VPA, in addition to modulating HDAC activity, is also known to inhibit GSK-3β and activate the MEK/ERK pathway. Selective inhibition of GSK-3β, HDACs or both GSK-3β and HDACs did not produce the same neurogenic activity as VPA, indicating that activation of MEK/ERK may also be pro-neurogenic in vivo. In a more recent study, Fischer et al. provided further evidence that the inhibition of HDACs is neurogenic in vivo. Treatment of mice with butyrate induced sprouting of dendrites, increased synapse number, and reinstated learning behavior and access to long-term memories.[172] Collectively, these data demonstrate that HDAC-mediated gene regulation is an important determinant for lineage progression and maintenance in the brain, and that HDAC inhibition may ultimately serve as a clinical means to stimulate the brain's regenerative mechanisms in vivo.

The regenerative process of remyelination in the CNS following neuronal loss or axonal damage involves oligodendrocyte precursor cells (OPCs) that generate the mature oligodendrocytes that ensheath demyelinated axons with new myelin.[173] In several demyelinating diseases, including multiple sclerosis (MS), neurological decline is associated with progressive failure of the remyelination process. This failure results, at least in part, from an observed inhibition of OPC differentiation at sites of demyelination injury and inflammatory insult. The mechanisms governing inhibition of OPC differentiation are not completely clear but are thought to involve inflammatory cytokines, excitatory amino acids, myelin proteins, and possibly neurotrophic growth factors.[173] Small molecules that promote the differentiation of OPCs into a mature myelinating cell fate in such environments would have great promise in the development of effective combination therapies for the treatment of MS. As such, we and others have conducted screens aimed at identifying small molecules that induce the differentiation of OPCs and/or enhance the process of myelination. For example, Buckley et al. screened a small collection of known bioactive compounds in vivo using transgenic *Olig*2-GFP zebrafish to identify molecules that affect the recruitment of oligodendrocyte lineage cells and then assessed the effects of primary hits on oligodendrocyte differentiation using qRT-PCR for myelin basic protein (MBP).[174] However, all of the confirmed hits from the primary recruitment assay negatively affected MBP expression. In another example, Joubert et al. identified compounds that induce OPC differentiation using a high-content image-based screen of a focused collection of biologically active molecules in an OPC cell line.[175] In addition to the classes of known inducers of OPC differentiation, including cAMP/PKA agonists (i.e., forskolin, dbcAMP), nuclear receptor ligands (i.e., retinoic acid, glucocorticosteroids) and nucleoside analogues (i.e., ribavirin, leflunomide), the ErbB inhibitors PD174265 and 4557W were identified as novel regulators of OPC cell fate.

Using primary rat optic nerve derived OPCs, we have also performed a high-content image-based screen against an extensive collection of known biologically active compounds. Confirmed hits from this effort include diverse members of the above mentioned classes, as well as others (most notably Rho/ROCK Kinase inhibitors and ion channel blockers). Consistent with our findings, a recent report indicates that the Rock II inhibitor Fasudil (one of the most potent inducers of OPC differentiation in our assays) drives OPC differentiation in the presence of inhibitory myelin proteins.[176] Interestingly, several of the known drugs identified in our screen are currently in clinical trials for the treatment of MS (e.g., the β_2 agonist levalbuterol).[177] The proposed mechanism of action for these molecules is attributed to their immunomodulatory effects. Intriguingly, our results suggest that the observed clinical efficacy of such molecules could be, at least in part, attributed to direct modulation of the remyelination process.

7.3.3. *Identification and Characterization of Novel Modulators of NSC Differentiation*

Although targeted modulation of known pathways and the epigenome have identified a variety of operative mechanisms in NSCs, the field is still far from a complete understanding of, or the selective ability to control NSC fate. As such, we and others have designed unbiased cell-based screens to identify novel mechanisms that control NSC differentiation. Toward this end, we isolated primary neural precursor cells (NPCs) from the adult rat hippocampus, plated them in monolayer culture conditions in the absence of mitogen stimulation and treated with chemical libraries.[178] After four days, the cells were fixed and immunostained with a neuronal (βIII-tubulin) and an astroglial (GFAP) marker to identify compounds that direct the differentiation of adult NPCs specifically into neurons or astrocytes. A 2-substituted aminothiazol, named neuropathiazol, induced up to 80% of cells to differentiate into βIII-tubulin positive neurons with characteristic neuronal morphology. Neuropathiazol treatment induced expression of NeuroD, concomitant with a

decrease in expression of Sox2 (a neural progenitor marker). Importantly, unlike RA-treated cells, neuropathiazol blocked proliferation and BMP-induced astrocyte differentiation.

A focused SAR study afforded a molecule (KHS101) that had a higher neurogenic activity in vitro compared to the previously reported compound,[178] and increased brain penetration. Upon systemic exposure to adult rats, this molecule increased neuronal differentiation in vivo. This molecule results in an increase in neuronal differentiation upon systemic exposure to adult rats.[179] Affinity-based studies revealed that KHS101 promotes neurogenesis by interacting with transforming acidic coiled-coil containing protein 3 (TACC3). Knockdown of TACC3 caused a strong neuronal differentiation phenotype in cultured NPCs in a comparable fashion to KHS101 treatment. Interestingly, KHS101 treatment led to increased nuclear localization of the nervous system-specific transcription factor ARNT2 (a TACC3 interacting protein), and over-expression of ARNT2 markedly favored neuronal differentiation over the astrocyte cell fate, even under astrocyte-inducing conditions (BMP treatment). Taken together, these findings indicate that KHS101 accelerates neurogenesis by interacting with the TACC3 protein and support a functional link between KHS101 and the TACC3-ARNT2 axis. This occurs through negative regulation of the cell cycle and concomitant activation of a neuronal differentiation program in NPCs. Indeed, a body of literature suggests that TACC3 is playing a crucial role in progenitor cell maintenance and is down-regulated upon differentiation, although the molecular mechanisms downstream of TACC3 have yet to be determined.[180]

In a similar study, Saxe et al. identified a series of inhibitors and enhancers of neuronal differentiation from a library of bioactive compounds.[181] Among these was the orphan ligand phosphoserine (P-Ser), which inhibited murine NSC proliferation and self-renewal, enhanced neurogenic fate commitment, and improved neuronal survival. Importantly, the activity of P-Ser was found to be dependent on its binding to the group III metabotropic glutamate receptor 4 (mGluR4), thereby de-orphanizing this receptor. Previous work with a different mGluR4 enhancer, PHCCC, suggested the mGluR4 agonism had an inhibitory effect on NSC proliferation and promoted neurite outgrowth.[182] Thus the identification of P-Ser provides a developmental link between an endogenous ligand and a receptor that is known to play a role in neurogenesis. Interestingly, P-Ser also increased neurogenesis in human ES cell-derived neural progenitors, demonstrating a convergent signaling pathway in human and rodent neurogenesis.[181]

8. Chemical Control of Cellular Plasticity: De-Differentiation and Reprogramming

During mammalian development, unspecialized stem cells are programmed into lineage-restricted cell fates. Lineage specification, however, occurs at a developmental cost. Once a cell becomes specialized it irreversibly loses the capacity to generate other cell types in the body (Figure 1). This is in contrast to the regenerative potential observed in other non-mammalian organisms. For instance, the cells on the outer edge of a severed salamander arm can de-differentiate, form a multipotent blastema, and regenerate a lost limb in its entirety (including the bone, blood vessels, and nerves).[183] Much of the early work in the field of regenerative medicine focused on identifying the mechanisms that govern de-differentiation in fish and amphibians with the hope that such mechanisms could be translated to mammals.[184]

More recently, work on understanding mammalian cellular plasticity has relied on alternative, exogenous means to expand developmental potential. In general, two methods have been applied: pharmacological intervention and/or genetic modification. The latter involves expressing stem cell factors in somatic cells,[102b] whereas the former is done by extrinsically influencing somatic cells to behave like stem cells by pharmacological perturbation of signaling pathways and/or the epigenetic architecture.[185]

In addition to de-differentiation back to a multipotent state, a variety of methods now exist to reprogram mammalian cells all the way back to the pluripotent state. This was first demonstrated in sheep by transplanting a differentiated genome into an enucleated oocyte, a process referred to as somatic cell-nuclear transfer (SCNT) or cloning.[186] More recently, simpler and more tractable techniques have also been developed to reprogram mammalian cells, including somatic cell–ES cell fusion,[187] environment-induced reprogramming of germ cells,[188] and retroviral delivery of defined transcription factor cocktails.[16,189] The identification of these alternative reprogramming methods has rapidly accelerated this field.

The discovery of cellular plasticity in mammals raises the possibility that one's own healthy, abundant and easily accessible adult cells could conceivably be used to generate different functional cell types. Such cells could then be used to repair damaged tissues/organs and circumvent many of the obstacles associated with using allogeneic stem cells in clinical applications. Reprogramming technologies also permit the creation of disease-matched cellular models that can be used to study various genetic diseases in a tissue culture setting or screens for drugs that modify disease state.[18] Clearly then, there is considerable interest in understanding these complex processes, as well as ultimately controlling them for regenerative therapies. Below we discuss the chemical approaches that have been used to direct lineage interconversion and reprogramming to the pluripotent state.

8.1. Methods to Establish Cellular Plasticity

Cellular plasticity is the term used to describe the ability of one cell type to convert to another across lineage- and/or developmentally restricted boundaries. This includes the de-differentiation of a mature cell into a more naive state, the trans-differentiation of one cell type across germ-layer restricted boundaries, and the complete epigenomic overhaul that occurs during reprogramming. As our understanding of the epigenetic processes controlling lineage specification and maintenance has matured, so has our scrutiny of a cell's identity. We can now map the methylation patterns of DNA,

characterize the modifications on histones, and determine gene expression profiles with much greater precision. In addition, advances in cell/tissue transplant, ES cell transgenics and lineage tracing have given us more rigorous means to characterize a cell's developmental potential and origin.

Traditionally, a cell's capacity for differentiation was characterized by cell surface-marker expression and/or morphology following in vitro differentiation. While this is a critical first step in establishing potential, it is the least stringent assay for a cell in culture and can give rise to in vitro artifacts. For instance, in vitro growth and/or differentiation can result in stress-induced aberrations in gene expression due to sub-optimal culture conditions (e.g., when a fibroblast is cultured in serum free media or upon chemical stress).[190] Further, stress can also lead to cytoskeletal alterations in the biochemical properties of filamentous proteins (e.g., Nestin and GFAP), which are often used to mark a given lineage. As a result one cell type may mimic another morphologically, by aberrant gene expression or by possessing properties of both the original cells and the de-/trans-differentiated cells; a phenomenon known as mosaicism or cellular mimicry. Additionally, errors can arise from the contamination of multipotent cells in primary isolates. Collectively, any one these artifacts can lead to misinterpretation of results.[191]

Nevertheless, these sorts of artifacts can generally be ruled out by assessing several cell-type specific markers in the de-/trans-differentiated cells; by clonal analysis of differentiation potential; or by comparing the expression profile and morphology of the resultant cells to genuine controls (Figure 9). If the de-/trans-differentiated cells meet these criteria, more rigorous assays can be applied including functional assessment of the differentiated cells (e.g., action potential in neurons or insulin secretion in pancreatic β-cells). Further, engraftment potential and the ability to contribute to normal tissue/organ replacement, repair or development can also be assessed (although cell fusion-related artifacts have been reported).[192]

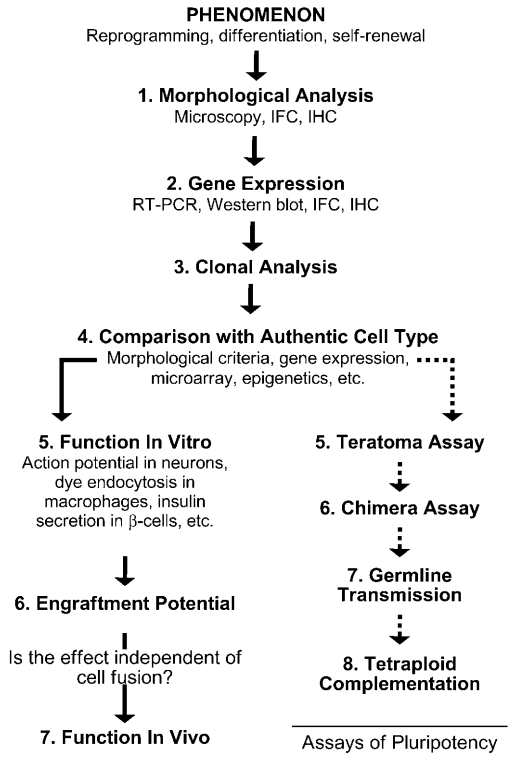


Figure 9. Flow chart of the methods used to analyze developmental potential and/or cell fate. Strategies used exclusively for pluripotent cells are displayed on the right under dashed arrows.

While these methods can be used for cells with any degree of developmental potential, they are more commonly used to demonstrate multipotency or lineage identity. When establishing pluripotency, additional analyses (Figure 9) include 1) the ability to generate tumors that express cell types representative of all the primary germinal layers (germ cell tumors or teratomas); 2) injection of cells into a developing blastocyst where the resulting organism consists of both host and donor cells (referred to as a chimera); and 3) the ability to contribute to the germ line of subsequent generations.[7a] The most stringent of all tests for developmental potential is the injection of cells into tetraploid blastocysts, which results in an organism formed entirely from the donor cells (tetraploid embryo complementation).[17]

In the following section, a number of chemicals that manipulate developmental potential (Table 4) and their validation using the aforementioned assays will be described. For a more comprehensive view on methods to assess cellular plasticity and potential pitfalls/misinterpretations, several excellent reviews address various aspects of this topic.[7a,185,193]

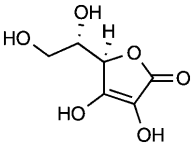
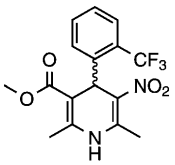
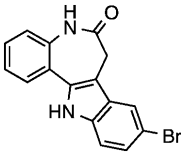
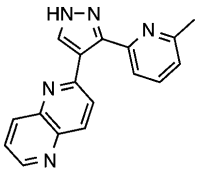
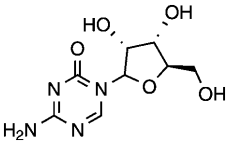
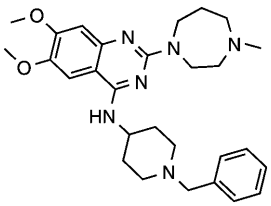
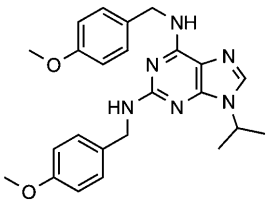
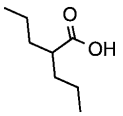
8.2. De-Differentiation of Lineage-Restricted Cells

8.2.1. Murine Myoblasts

During the formation of skeletal muscle, proliferating myoblasts stop dividing and fuse into multinucleated myotubes. Recent in vitro studies suggest that terminally differentiated murine myotubes can be induced to undergo de-differentiation into mesenchymal progenitor cells by the ectopic expression of Msx1[194] or by the addition of extracts from regenerating newt limb.[195] In order to identify small molecules that reverse the cell fate of terminally differentiated myotubes, we developed an assay based on the disassembly of multinucleated myotubes into individual myoblasts. This screen identified myoseverin, which induced the cleavage of multinucleated myotubes into myoblast-like cells that were capable of proliferating and re-forming myotubes.[196] Affinity chromatography of cell extracts and examination of cytoskeletal proteins revealed that myoseverin binds tubulin and disintegrates the highly ordered microtubule cytoskeleton within the myotube. Microtubule disruption thereby induced the reversion of terminally differentiated myotubes to precursors that were again responsive to both growth and differentiation conditions. While the effects of myoseverin likely result from cytoskeletal remodeling, and the resultant cells are still unipotent, this experiment demonstrated proof-of-principle that terminally differentiated states can be altered by a small molecule.

To identify small molecules that induce de-differentiation of myoblasts into multipotent progenitors, a new screen was designed in which C2C12 murine myoblasts were treated with

Table 4: Selected small-molecule modulators of cellular plasticity.

Compound	*Target* Function
	**L-Ascorbic acid** Increases the efficiency of direct reprogramming
	**Bay K8644 (BayK)** *L-type Ca^{2+} channels* With BIX, replaces Sox2 and c-Myc during direct reprogramming
	**Kenpaullone** *Unknown* Replaces Klf4 during direct reprogramming
	**RepSox** *Type I Receptor (ALKs 4, 5, 7)* Replaces Sox2 and c-Myc during direct reprogramming
	**5-Azacytidine (5-AzaC)** *DMNTs* Increases the efficiency of direct reprogramming
	**BIX 01294 (BIX)** *G9a* With BayK, replaces Sox2 and c-Myc during direct reprogramming
	**Myoseverin** *Microtubules* Reverts terminally differentiated myotubes into myoblasts
	**Valproic Acid (VPA)** *HDACs* Increases the efficiency of direct reprogramming ± c-Myc

compound, re-plated under osteogenesis-inducing conditions (which only affect mesenchymal progenitor cells) and assayed for their ability to differentiate into osteoblasts. A 2,6-disubstituted purine, reversine, was found which inhibits terminal myotube formation from myoblasts.[197] Instead the reversine-treated myoblasts differentiate into osteoblasts or adipocytes following exposure to appropriate differentiation conditions (Figure 8). Importantly, the de-differentiation effect observed with reversine is inductive rather than selective. That is, 1) cells expanded as single cell clones and treated with reversine exhibit multipotent differentiation potential; 2) trans-differentiation of myoblasts directly into osteoblasts or adipocytes is not observed under the conditions used to induce osteogenesis or adipogenesis; 3) in the absence of adipogenesis- or osteogenesis-inducing medium, continuous reversine treatment alone had no adipogenic or osteogenic activity; and 4) significant cell death is not observed at the reversine concentrations used (i.e., reversine is not simply enriching progenitor cells by selectively killing myoblasts).

More recently, reversine has been used as a de-differentiation agent in a number of different applications. For example, Anastasia et al. demonstrated that reversine treatment transformed primary murine and human dermal fibroblasts into myogenic-competent cells at high frequency both in vitro and in vivo.[198] Additionally, the treated fibroblasts could be converted into osteoblasts under appropriate conditions. Likewise, Saraiya et al. demonstrated that lineage committed cartilaginous cells isolated from rat vertebral columns could be de-differentiated with reversine.[199] Treated cells were re-endowed with multipotent mesenchymal potential and could differentiate into osteoblasts, adipocytes or back into cartilage under lineage-appropriate differentiation conditions. Of note, the de-differentiated cells showed a significantly enhanced potential to differentiate into chondrocytic cells. The de-differentiation activity of reversine, however, is not conserved across all cell lineages. In certain cell types, reversine acts as a potent differentiation-inducing molecule. For example, reversine promotes differentiation in a number of different cancer cell lines.[200] In another study, Kim et al. found that reversine treatment of the 3T3L1 pre-adipocyte line under adipocyte differentiation conditions induced a synergistic enhancement of adipocyte differentiation.[201]

Subsequent work in our group confirmed that reversine is active in multiple cell types, including 3T3E1 osteoblasts (treated cells demonstrate adipogenic potential) and human primary skeletal myoblasts (treated cells demonstrate osteogenic and adipogenic potential).[39] Further, we identified mitogen activated extra-cellular signal regulated kinase (MEK1), and nonmuscle myosin II heavy chain (NMMII) as the cellular targets of reversine using affinity-based methods. cDNA overexpression experiments coupled with siRNA and small molecule inhibition experiments indicated that inhibition of both MEK1 and NMMII are required for reversine's activity. By using a series of cell cycle stage-specific inhibitors, we found that the effects of reversine on the cell cycle (reversine induces an accumulation of cells in the G2M-phase) may also contribute to its de-differentiation activity. Indeed, work from the Cortese and co-workers had previously demonstrated that reversine inhibits the cell cycle regulatory Aurora kinases.[200b] Based on this notion, they applied other structurally dissimilar Aurora kinase inhibitors (VX-680 and Hesperadin) to C2C12 cells and found that these

compounds, like reversine, are able to de-differentiate the cells back to the multipotent state.[202] Significantly, they found that C2C12 cells expressing kinase inhibitor-resistant Aurora kinase B mutants are insensitive to the de-differentiation activity of reversine. Collectively, these results support a model whereby epigenetic changes resulting from Aurora kinase inhibition mediate chromatin remodeling, which in turn restores the multipotent state. Interestingly, this study converges ultimately on a very similar mechanism to that which was originally proposed. By blocking NMMII, reversine induces an accumulation of cells in the G2M-phase of the cell cycle and, at the same time, modulates acetylation of histone H3 by inhibiting MEK-dependent signaling.[39]

8.2.2. *Oligodendrocyte Precursor Cells*

OPCs are unipotent progenitors that give rise to oligodendrocytes—the cells that ensheath axons and allow for rapid signal transduction in the brain. OPCs constitute roughly 5–8% of the total cells in the adult brain and persist throughout the lifetime of an adult organism. They are also highly motile and respond to demyelinating insults.[173] As such, OPCs represent an attractive endogenous source of cells to de-differentiate for central nervous system repair.

OPCs removed from their niche and cultured in vitro become bipotent and can give rise to type 2 astrocytes, in addition to oligodendrocytes.[162,203] More strikingly, OPCs are competent for neuronal differentiation following exposure to BMP2 and expansion in NSC growth media (containing bFGF).[204] However, the mechanistic intricacies governing OPC plasticity have yet to be resolved. In an effort to provide a better understanding of this process, we developed a small-molecule screen to identify chemical modulators of OPC potential. Toward this end, primary rat OPCs were transfected with a Sox2 promoter-GFP reporter. Sox2 is a transcription factor that is expressed early in the developing brain and is essential for the maintenance of the multipotent state in NSCs.[205] Additionally, Sox2 is highly expressed in NSCs but not in lineage restricted precursors, such as OPCs.[204b,206] Therefore, it was assumed that an increase in the multipotency of OPCs would require Sox2 expression, consistent with the previous observation that the epigenetic reactivation of Sox2 was essential for the BMP2-induced conversion of OPCs to neural stem-like cells (NSLCs).[204b]

Chemical libraries were then screened to identify molecules that activate Sox2 expression in OPCs.[207] After which, hits were analyzed in a secondary assay to determine if they de-differentiated OPCs into a neurogenesis-competent state. The four compounds that passed this analysis (butyrate, TSA, MS-275, and apicidin; Figure 10a) were all known HDAC inhibitors. Clonal analysis indicated that HDAC inhibitor-treated OPCs exhibited much higher potential to form neurons than the DMSO-treated controls. These experiments demonstrate that inhibition of HDAC activity can expand the developmental potential of OPCs to include the neuronal lineage, as opposed to selective amplification of a residual multipotent side-population. Next, we showed that the de-differentiated OPC clones were indeed multipotent as analyzed by a variety of neuron, astrocyte and oligodendrocyte lineage-specific markers (Figure 10b). Concurrent with our finding, Liu et al. also demonstrated that HDAC-inhibitor treated OPCs regain multipotency. Furthermore, they demonstrated that such cells were able to engraft and form functional neurons in vivo.[208] From a mechanistic standpoint, we found that Sox2 is epigenetically reactivated during OPC reprogramming and that this reactivation is necessary (using siRNA-targeting methods) but not sufficient to reprogram OPCs. In addition, genome-wide transcription profiling showed that HDAC treated OPCs rapidly acquired several molecular characteristics of NSCs while simultaneously silencing a large group of oligodendrocyte lineage-specific genes.

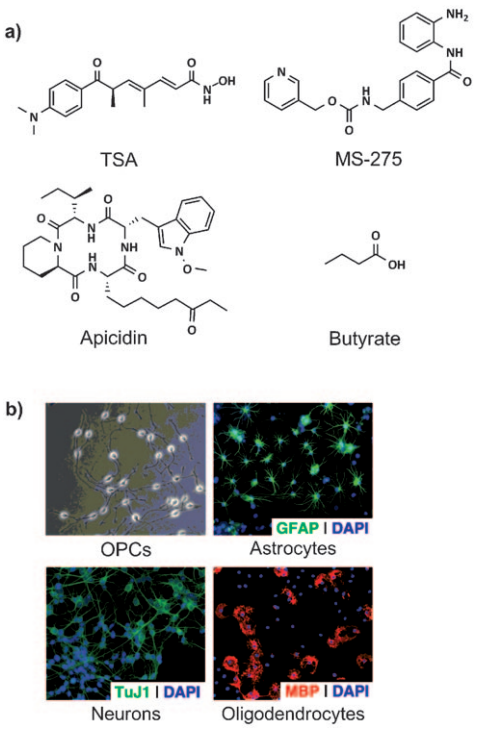


Figure 10. OPC reprogramming. a) Multiple structural classes of HDAC inhibitors can reprogram OPCs (hydroxamic acids, TSA; benzamides, MS-275; cyclic tetrapeptides, apicidin; and short-chain fatty acids, butyrate). b) Inhibition of HDAC activity affords OPCs tripotent differentiation potential. Images reprinted with permission from Ref. [207]; Copyright 2007, National Academy of Sciences, USA.

The observation that HDAC inhibition can expand developmental potential in stark contrast to the notion that HDAC inhibitors are routinely used as therapeutic agents to differentiate naive cancer cells.[209] Indeed, Breslow, Marks and colleagues described the role of HDAC inhibition in transformed cells more than 30 years ago following up on the observation that 2% DMSO induces growth arrest and terminal differentiation of erythroleukemia cells in culture.[210] Clearly, HDACs play a different, and context dependent, role in neural lineage specification (also discussed in Section 7.3.2). Specifically, when HDAC activity is low, neurogenesis occurs at the expense of glial differentiation, and when HDAC activity is high terminal glial differentiation can proceed. Consistent with this observation, HDAC inhib-

itors promote neurogenesis in NSCs,[169] promote dendrite outgrowth and synapse formation in mouse brains,[172] block terminal oligodendrocyte formation,[167] and impair myelination.[211] Our study extends this body of work thereby demonstrating that HDAC activity is not only responsible for glial lineage specification, it is also required to maintain OPC lineage identity. Collectively, global histone acetylation, induced by HDAC inhibition, can partially reverse the lineage restriction of OPCs thereby expanding their differentiation potential to include the neuronal lineage (Figure 10b).[207,208]

8.3. Reprogramming to the Pluripotent State

The demonstration of SCNT in mammals has given rise to the notion that banks of patient-specific nuclear transfer (nt)ES cell lines could be generated as a source of cells, tissues, and organs to replace those lost to old age or damaged by disease.[212] However, SCNT has not been reproduced in human cells, owing largely to the fact that human oocytes are difficult to obtain. This shortcoming has motivated the development of other techniques to reprogram adult cells back to the pluripotent state.[16,187–189,213] Among these techniques, direct reprogramming of somatic cells with transcription factor cocktails has led to renewed interest in the notion of creating patient-specific pluripotent cell banks because it both circumvents the sociopolitical issues surrounding the use of human ES and ntES cells and provides a tractable means to study the mechanistic underpinnings of this process. Furthermore, disease-specific pluripotent human cell lines can be readily created by direct reprogramming to study disease development and progression in the laboratory.[18]

The direct reprogramming of somatic cells to pluripotency was demonstrated in 2006, when Takahashi and Yamanaka converted adult mouse fibroblasts to iPS cells by overexpressing the transcription factors Oct4, Sox2, Klf4, and c-Myc (Figure 11a).[16b] In the short time since its description, numerous groups have extended the original work to a variety of other mammalian species, most notably humans.[16a,189,214] Despite the enormous potential of iPS cells, several limitations inherent to the process hinder their application in a clinical setting. Namely, viral-mediated delivery of reprogramming factors introduces unacceptable risks of permanent transgene integration into the genome. The resulting genomic alterations, and the possibility for viral transgene activation, have the potential to give rise to transforming events in subsequently derived cell populations.[215] Moreover, each of the primary reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) also have potent oncogenic activity under certain cellular contexts.[216] Direct reprogramming is also a slow and inefficient process (ca. 0.001–3% of cells are reprogrammed), which limits mechanistic dissection of the underlying biology.[7a,217]

Since Yamanaka's initial discovery, a surge of interest in reprogramming has made significant progress toward addressing the aforementioned shortcomings. For example, optimization of the original reprogramming method has facilitated the reprogramming of both mouse and human

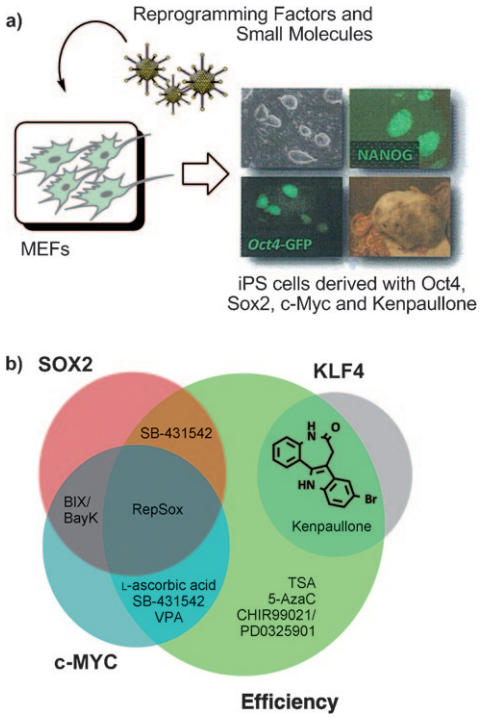


Figure 11. Murine fibroblast reprogramming. a) Ectopic expression of the transcription factors Oct4, Sox2 and c-Myc, when used with kenpaullone, can reprogram MEFs into iPS cells. Kenpaullone-derived iPS cells demonstrate characteristics of pluripotent ES cells, including tight colonies; expression of ES cell markers from endogenous loci (such as Oct4 and Nanog) and are capable of contributing to mouse development when injected into developing mouse embryos (chimera). Images reprinted with permission from ref. [233]; Copyright 2009, National Academy of Sciences, USA. b) Small molecules that increase the efficiency of direct reprogramming (green circle) or obviate the need for given transcription factors during the direct reprogramming of murine fibroblasts to iPS cells. Compounds that replace Sox2, Klf4, and/or c-Myc are contained within the red, grey, and blue circles, respectively. Selected chemical structures are illustrated in Tables 1 and 4.

somatic cells to iPS cells with the 3-factor combination of Oct4, Sox2, and Klf4 in the absence of c-Myc.[217] Other strategies to reduce the number of reprogramming factors have taken advantage of endogenously expressed transcription factors in certain cell types, thereby precluding the need for ectopic expression of these factors, such as Sox2.[50,218] More recently, iPS cells have also been generated using excisable vectors,[219] non-integrating vectors,[220] and transient transfection approaches.[221] Furthermore, the clinical potential of iPS cells was furthered by recent work demonstrating that human peripheral blood cells can be reprogrammed without the need for tissue biopsies.[222] Although these methods have solved some of the limitations of genomic disruption and those posed by the presence of multiple proviruses in the genome of iPS cells, they either remain inefficient or fail to further our understanding of the mechanistic details of epigenomic reprogramming. Small molecules offer a powerful alternative to genetic reprogramming methods. Specifically, it appears that small molecules

can be used in place of, or in combination with, genetically delivered transcription factors to reprogram cells (Table 4).

8.3.1. Direct Reprogramming with Known Target- and Pathway-Based Modulators

Research in mammalian cloning suggests that reorganization of chromatin architecture is a rate limiting step during the reprogramming of a somatic genome.[223] Consistent with this notion, the application of small molecules that affect chromatin structure improves the efficiency of SCNT (i.e., DNA methyltransferase inhibitors and/or HDAC inhibitors; Figure 12).[224] The removal of epigenetic marks is also a barrier during direct reprogramming. For example, treatment of MEFs with 5-AzaC, a small-molecule inhibitor of DNA methyltransferase, facilitates 4-factor reprogramming during a brief temporal window by removing methylation marks.[225] HDAC inhibitors and histone methyl transferase inhibitors have also been shown to increase the efficiency of reprogramming (Figure 11b).[226] In particular, the HDAC inhibitor VPA was shown to strongly increase 3-factor reprogramming efficiency in the absence of c-Myc in both mouse and human cells and to allow 2-factor reprogramming (Oct4 and Sox2) of human fibroblasts in the absence of Klf4 and c-Myc.[226a,b]

Molecularly defined conditions that maintain mouse ES cells in the pluripotent state[49] also have a strong effect on reprogramming efficiency.[50] Specifically, application of MEK/ERK (PD0325901) and GSK-3β (CHIR99021) inhibitors shorten the amount of time required to reprogram MEFs and neural precursors and increase the fraction of cells that become iPS cells (Figure 11b). These inhibitors also obviate the need for Sox2 and c-Myc in neural precursor reprogramming. More recently it was demonstrated that cellular senescence (both spontaneous and virus/oncogene-induced) is a major roadblock, credited in large part for the low efficiency of reprogramming.[227] This results in part from the accumulation of reactive oxygen species.[228] Based on this notion, Esteban et al. found that L-ascorbic acid (vitamin C) strongly increases the reprogramming efficiency of human and murine fibroblasts transduced with Oct4, Sox2, and Klf4 (Figure 11b).[229] Interestingly, however, this activity could not be recapitulated with other antioxidants suggesting that the activity of L-ascorbic acid is independent of its antioxidant properties.

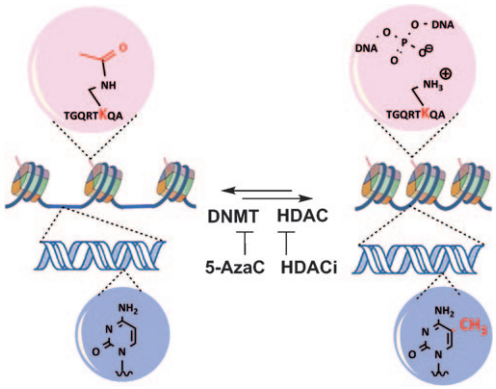


Figure 12. Epigenetic modifications. Open chromatin, which allows for active gene expression, is illustrated on the left. Closed chromatin, in which gene expression is repressed, is illustrated on the right. Histone acetyltransferases (HATs) open chromatin structure by transferring acetyl groups to lysines on histone proteins. DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) condense chromatin structure by methylating DNA and deacetylating histone proteins, respectively. Small-molecule inhibitors (HDACi and DNMTi) can interfere with these processes thereby preventing chromatin condensation and gene silencing. Chemical structures of epigenetic modifying small molecules are illustrated in Figure 10 (HDACi) and Table 4 (HDACi and DNMTi).

8.3.2. Identification and Characterization of Novel Modulators of Direct Reprogramming

The identification of novel small molecules that induce pluripotency should provide useful chemical tools to unravel and study the molecular mechanisms governing this process. Toward this end, we and others have developed unbiased, high-throughput screens to identify small molecules that can functionally replace reprogramming transcription factors. The induction of pluripotency in fibroblasts by the ectopic expression of Oct4, Sox2, Klf4, and c-Myc requires a minimum of 8–12 days and occurs at low frequency.[230] The slow kinetics and inefficiency of reprogramming impede the development of robust assays to screen large chemical libraries with a reliable readout that can be captured on a well-to-well basis in a miniaturized format. To overcome these limitations, we relied on the high sensitivity and quantitative readout capabilities of luciferase; the firefly luciferase gene was inserted into the *Nanog* locus by homologous recombination in mouse ES cells. *Nanog* was selected because it plays important roles in maintaining the undifferentiated state,[231] is completely inactivated in somatic lineages, and iPS cells selected for *Nanog* reactivation demonstrate complete developmental potential.[215,232] A Nanog-luciferase (NL) mouse strain was created from the knock-in ES cell line from which a therapeutically relevant cell type, skin fibroblasts, could be isolated for screening.

We first applied this screen to identify compounds that can replace Klf4.[233] NL-MEFs were transduced with a subset of reprogramming factors comprising Oct4, Sox2, and c-Myc (OSM) and screened against a large, chemically diverse small-molecule library.[234] From this assay, one molecule—kenpaullone—was found to facilitate the formation of iPS cell colonies that were indistinguishable from ES cells by morphological criteria and expressed pluripotency-associated markers from endogenous loci (Figure 11a). Furthermore, iPS cells created with kenpaullone were germline-competent. Kenpaullone is a sub-micromolar inhibitor of GSK-3β, CDK1/cyclin B, CDK2/cyclin A, and CDK5/p35 and exhibits inhibitory activity toward various other kinases at higher concentrations.[235] However, known inhibitors of these kinases were unable to reproduce the activity of kenpaullone in either the NL- or colony-forming assays. Similarly, Hanna et al. recently demonstrated that mouse EpiSCs could be reprogrammed back to the ICM-like state with kenpaullone and that this activity could not be recapitulated with GSK-3β specific inhibitors.[236] Collectively, these data suggest that kenpaullone's activity does not result from its well-docu-

mented role as a GSK-3β or cell cycle inhibitor, but rather from an as of yet known mechanism. More recently, application of this screening platform in Oct4, Klf4 and c-Myc transduced NL-MEFs has also led to the identification of chemical complements for Sox2.[237]

The Eggan and Rubin groups have also developed and implemented screens to identify small molecules that replace Sox2. Specifically, they transduced *Oct4*-GFP knock-in MEFs with Oct4, Klf4 and c-Myc (OKM), treated the cells with a small-molecule library consisting of 800 known pharmacological agents, and assayed for compounds that replace Sox2 based on ES cell morphology and the re-emergence of *Oct4*-GFP expression. They identified three compounds that obviated the need for Sox2.[67a] Among these were two inhibitors of the transforming growth factor receptor 1 (TGFBR1; E-616452 and E-616451) and a Src-family kinase inhibitor (EI-275). The authors demonstrated that E-616452 (referred to as RepSox) is able to function in place of Sox2 or both Sox2 and c-Myc simultaneously (Figure 11b). Indeed, RepSox treated and 3- or 2-factor (OKM or Oct4 and Klf4) transduced murine fibroblast-derived iPS cells were pluripotent as judged by a number of rigorous criteria (e.g., contribution to chimeric mice). Further, they demonstrated that inhibition of TGFβ-signaling was the operative mechanism for RepSox with other known chemical inhibitors of TGFβ-signaling or TGFβ-neutralizing antibodies.

RepSox has proven to be a very useful tool to study direct reprogramming, and its application serves to highlight several of the advantages of using small molecules to dissect this process. For example, the authors demonstrate that RepSox acts during a defined time window by exploiting the temporal control afforded by small molecules. That is, application of RepSox for any 24 h period during days 7 to 12 post-OKM viral transduction can functionally replace Sox2. This observation suggests that it may be possible to find molecules that work synergistically at different stages of the reprogramming process.

Further, RepSox is able to replace either Sox2 or c-Myc with an efficiency similar to viral transduction of either factor. This demonstrates that there need not always be a discrete, one-to-one mapping between the functions of the reprogramming factors and their chemical replacements. RepSox also—like kenpaullone[233]—does not act by directly activating transcription of the reprogramming factor it replaces. Instead, Ichida et al. provide convincing evidence that the pluripotency-inducing function of RepSox is mediated through activation of Nanog gene expression, where Nanog can functionally replace either RepSox or Sox2.

Maherali and Hochedlinger also found that disruption of TGFβ-signaling enhances reprogramming efficiency, and more specifically, that application of a chemical inhibitor of TGFβ-signaling can bypass the requirement for exogenous Sox2 or c-Myc.[67b] However, unlike the work by Icheda et al., this study suggests that inhibition of TGFβ signaling cooperates in the reprogramming of murine fibroblasts during the initiation phase of reprogramming by enabling faster and more efficient reprogramming. Specifically, they found that TGFβ inhibition with SB-431542 strongly increased 4-factor reprogramming efficiency (> 10-fold) and that it was able to replace Sox2 most effectively when applied during days 0 to 3 post-OKM transduction (Figure 11b). Despite these minor discrepancies, the results from both studies demonstrate the feasibility of replacing reprogramming transgenes with small molecules that modulate discrete cellular pathways (or processes) rather than by globally altering chromatin structure.

Significant challenges still face the therapeutic application of pluripotent human cells. For example, the majority of the work described above was carried out with murine cells. While some of these findings readily translate to human systems,[226a,229] others do not,[67a,226b,233] owing in part to aspects unique to human pluripotency and reprogramming. Specifically, human reprogramming takes even longer (ca. 4 weeks) and is more inefficient than in mouse (ca. 0.01%). To address these limitations, a chemical cocktail consisting of the TGFβ and MEK/ERK signaling inhibitors was recently shown to improve the reprogramming efficiency approximately 200-fold and to reduce the time in half.[238] Though this method is a significant improvement on previous methods, it also poses an interesting predicament—TGFβ and FGF signaling play a major role in maintaining the pluripotent state of human cells.[13] As such, the duration and timing of chemical treatment will require careful control in order to maximize the reprogramming activity of these compounds while minimizing their differentiation-inducing effects on newly arising pluripotent cells.

Another major issue limiting the application of human pluripotent cells (i.e., ES and/or iPS cells) is the development of techniques for their long term, karyotypically stable culture and robust methods to direct their differentiation toward specific lineages. Progress toward the latter was described above in Sections 6.2 and 6.3 for human ES cells and many of those methods are now being applied in human iPS cells as well. A notable example from Studer's group showed that neural lineages can be efficiently differentiated in high purity from human iPS cells by simultaneously inhibiting both the TGFβ and BMP signaling pathways (with SB-431542 and Noggin, respectively).[83]

9. Functional Proliferation of Mature Cells

Terminally differentiated, post-mitotic mammalian cells are thought to have little or no regenerative capacity, since they have exited the cell cycle and are already committed to their final specialized form and function. The ability to overcome this general loss of regenerative potential (i.e., to divide and replace damaged/lost tissue) would likely have a significant impact on heart disease (cardiomyocytes), liver cirrhosis (hepatocytes), type 1 diabetes mellitus (pancreatic β-cells), multiple sclerosis (oligodendrocytes), and the like. Encouragingly, there are instances in which organs restore lost mass without the involvement of stem cells. The cells in nearly all fetal organs possess varying degrees of regenerative potential,[239] and, in non-disease settings, certain adult cells can also proliferate. For example, cells in a healthy adult liver are capable of proliferating to replace damaged tissue[240] and pancreatic β-cell mass can expand to meet metabolic demands

during pregnancy.[241] Consequently, the development of methods that harness this proliferative potential or those that reversibly stimulate adult cells to re-enter the cell cycle may provide new therapeutic approaches without the need for stem cells.

9.1. Target- and Pathway-Based Approaches to Proliferate Cardiomyocytes

Ischemic heart disease is characterized by the progressive loss of cardiac myocytes and is a major cause of morbidity and mortality in the industrialized world.[149b,242] Cardiomyocytes are highly restricted in their ability to divide. As a result, the primary response of the mammalian heart to injury is scar formation, which prevents cardiac repair. Thus, the loss of cardiomyocytes after damage caused by events such as myocardial infarction generally results in a compensatory response that is inadequate to restore function. The identification of small molecules that promote the proliferation of cardiomyocytes may offer a novel means to treat heart disease.

It was recently shown that p38-MAPK signaling negatively regulates mammalian cardiomyocyte proliferation through the regulation of genes required for mitosis. Following up on this finding, Engel et al. demonstrated that a p38-MAPK inhibitor (SB203580) increased DNA synthesis and mitosis in both neonatal and adult cardiomyocytes.[239a] In another example, Keating and co-workers found that the GSK-3β inhibitor BIO promoted proliferation in mature adult cardiomyocytes.[51a] Consistent with its function as a GSK-3β inhibitor, BIO treatment elevated β-catenin activity suggesting that the increase in proliferative ability may be due, in part, to the activation of the canonical Wnt/β-catenin pathway. Together, these studies demonstrate that small molecules can restore proliferative potential to terminally differentiated cells.

9.2. Identification and Characterization of Compounds that Proliferate β-Cells

Type 1 diabetes results from the autoimmune destruction of the insulin-secreting β-cells in pancreatic islets.[243] As a result, an insufficient amount of insulin is secreted to maintain normal glucose metabolism. Daily administration of exogenous insulin is thus required to maintain glucose homeostasis in type 1 diabetes. Insulin therapy, however, does not fully recapitulate the strict control of blood glucose that is exerted by endogenous β-cells. As a consequence, diabetic patients undergoing insulin therapy eventually develop organ-threatening damage. Therefore a method to restore functional islets by regenerating endogenous β-cells would offer a significant advantage relative to conventional insulin therapy. Indeed, evidence suggests that β-cell mass is dynamic and can expand to meet metabolic demands, for example during pregnancy or after the pancreas is stressed (e.g., after a partial pancreatectomy).[241,244] Therefore, an alternative approach to current treatments (i.e., insulin and/or islet transplantation) may be the selective induction of β-cell regeneration by external stimuli. In fact, β-cell mass can be expanded in response to incretins and other hormones such as glucagon-like peptide-1 (GLP-1),[245] the GLP-1 receptor agonist exendin-4,[246] and hepatocyte growth factor in animal models.

Previous attempts, by us and others, to screen for molecules that proliferate primary β-cells have yielded unsatisfactory results. This is due in part to the limited availability of human β-cells, difficulties associated with obtaining large numbers from rodents, and the proliferative variability of cells from donor to donor. To overcome these limitations, we used a reversibly immortalized mouse β-cell line, which provides the quantities of homogeneous, functional β-cells required for large-scale cell-based screens. To this end, a mouse β-cell line was reversibly immortalized by placing the SV40 large T antigen (TAg) under the control of the tetracycline (Tet)-On system. These engineered β-cells proliferate when TAg is induced by doxycycline (Dox, a Tet analog), and undergo growth arrest upon withdrawal of Tet (Figure 13a).[247] Although not a true mimic of in vivo

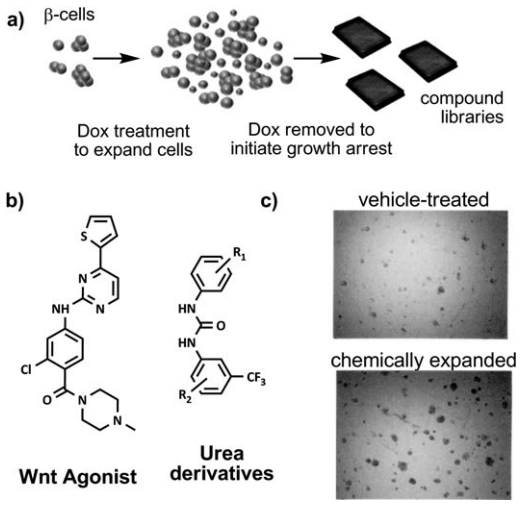


Figure 13. β-Cell expansion: a) Screening platform to identify compounds that proliferate β-cells. b) Compounds that expand β-cells. c) Dissociated rat islets treated for 4 days with compounds in (b) expand clonally and maintain a β-cell phenotype.

quiescent β-cells, this system readily allowed us to identify small molecules that induced the reversible proliferation of growth-arrested mouse β-cells.[51c] Among these were a variety of GSK-3β inhibitors (Wnt agonists; Figure 13b)—which confirmed the findings of a recent report that demonstrated that inhibition of GSK-3β stimulates replication of rat islets and alleviates the toxic effects of high concentrations of glucose and palmitate[51b]—and a group of dihydropyridine derivatives that reversibly induce β-cell replication by activating L-type calcium channels. An expanded screen has led to the discovery of a novel class of diaryl urea derivatives (Figure 13b) that are more potent and effective in murine β-cells than those previously identified, and demonstrate significant proliferative activity in human islets.[248] Moreover, the expanded islets stain for C-peptide (a

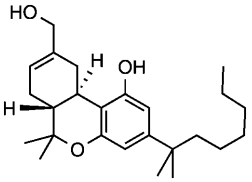
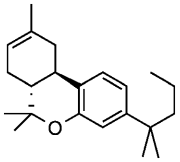
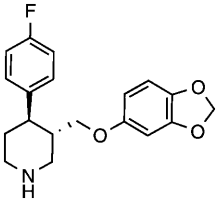
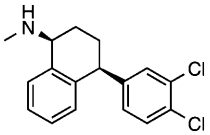
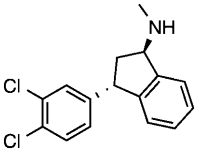
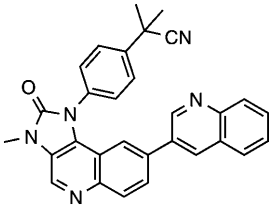
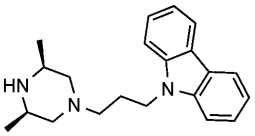
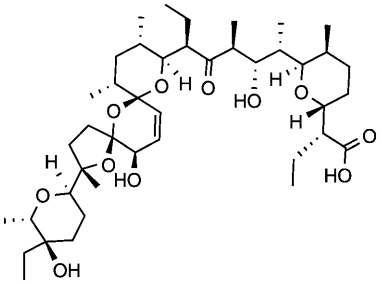
β-cell marker) and are glucose responsive. An analogue with acceptable systemic exposure has protective effects in β-cell stress assays in vitro and restores normal glucose levels when administered shortly after STZ treatment in a rodent type 1 diabetes model. This derivative also has reversible proliferative effects on retinal pigmented epithelial cells (RPEs). Importantly, the expanded cells appear to retain RPE character both in vitro and in vivo. Current and future efforts with these compounds are aimed at identifying the mechanism(s) of action and more fully exploring their activities in rodent models of type 1 diabetes and age-related macular degeneration. One challenge to the in vivo use of such molecules will be their ability to selectively proliferate a specific cell population. Thus the first likely application of these agents will be to expand donor cells ex vivo for transplantation. One must also be wary that the activity of proliferative molecules is reversible and does not promote oncogenic transformation.

10. Chemical Control of Cancer Stem Cell Fate

Tumors are composed of a heterogeneous mix of several types of differentiated and undifferentiated cells. The various stages of differentiation exhibited by cells in certain tumors (e.g., acute myelogenous leukemia) support a hierarchical model.[4a,b] At the apex of this hierarchy lie the least differentiated cells, which exhibit many properties of stem cells, including the ability to self-renew and differentiate. As such, these cells are now popularly referred to as CSCs. Furthermore, CSCs also possess characteristics of stem cells that make them more resistant to therapy (e.g., a long lifespan, relative quiescence, resistance to drugs and toxins through the expression of multi-drug resistance transporters, an active DNA-repair capacity, and a resistance to apoptosis). Therefore, some tumors appear to have a built-in population of drug-resistant multipotent cells that can survive chemotherapy.[249] Since most current chemotherapeutic regimens rely on nonselective cytotoxins that target rapidly dividing cells, it is conceivable that quiescent CSCs may survive treatment and are responsible for cancer recurrence.

The notion of CSCs as the tumor-maintaining population provides a basis to develop therapeutic strategies beyond traditional antiproliferative agents.[4a] For example, potential approaches to eradicate CSC could involve the inhibition of survival pathways unique to CSCs, inhibiting CSC self-renewal, or inducing differentiation to make naive CSCs behave more like the chemotherapy-sensitive and treatable tumor bulk. Clearly, a more detailed understanding of the biological properties of CSCs and tools that selectively target their unique properties will provide methods to address CSCs therapeutically. In the following section, we review molecules that are being used to control the fate of CSCs (Table 5).

Table 5: Selected small-molecule modulators of CSC fate.

Compound	*Target* Function
	**HU-210** *CB_1R and CB_2R* Inhibits the growth of GBM CSCs
	**JWH-133** *CB_2R* Inhibits the growth of GBM CSCs
	**Paroxetine** *Monoamine Transporters* Selselectively eliminates GBM CSCs
	**Sertraline** *Seratonin Transporter* Selectively eliminates GBM CSCs
	**Indatraline** *Monoamine Transporters* Selectively eliminates GBM CSCs
	**NVP-BEZ235** *PI3K and mTOR* Inhibits the growth of prostate cancer CSCs
	**Rimcazole** *Sigma Receptor & Dopamine Transporter* Selectively eliminates GBM CSCs
	**Salinomycin** *Unknown* Selectively targets breast CSCs

10.1. Targeting CSC Survival

Unbiased cell-based approaches to identify agents that specifically target CSCs are limited by the rarity of these cells within tumors and their relative instability in culture. To address the former shortcoming, Gupta et al. implemented a method to create large numbers of breast cancer cells with CSC characteristics.[250] The induction of an epithelial–mesenchymal transition (EMT) in normal or neoplastic mammary

epithelial cell populations has been shown to result in the enrichment of cells with stem-like properties.[251] With this in mind, they induced an EMT in normal breast cancer cells by knocking down E-cadherin expression with a short hairpin RNA.

These cells were then used in a high-throughput screen to identify compounds that are selectively toxic to breast CSCs. This was done by performing parallel screens of a chemical library using breast epithelial cells and breast CSCs, and then comparing the toxicity profile between the two lines. About 10% of the tested compounds reduced the viability of the breast CSCs. While the vast majority (98%) of this sub-set also reduced the viability of breast epithelial cells, a number of compounds including, etoposide, salinomycin, nigericin, and abamectin were selectively toxic in CSCs (with an IC_{50} 7- to 10-fold lower for CSCs than control cells). Importantly, these four compounds also preferentially killed cells that had undergone Twist-induced EMT, which suggests that their selectivity is independent of the mechanism used to induce EMT. Furthermore, in vivo analysis demonstrated that salinomycin treatment impaired the ability of breast CSCs to seed mammary tumors and their tumor growth. This study suggests that methods to specifically kill CSCs may provide a new avenue for the development of antitumor therapies by searching for agents that target specific states of cancer cell differentiation.

10.2. Targeting Pathways Involved in CSC Self-Renewal

The importance of specifically targeting CSCs while sparing normal stem cell function will be critical for a successful therapeutic strategy. In particular, an understanding of the similarities and differences between cancer and normal stem cells must be improved. One characteristic that is shared between CSCs and normal stem cells is the ability to self-renew.[252] Pathways such as PI3K/Akt,[74] Wnt/β-catenin,[253] Notch,[254] and Hh[255] have been found to be altered in human cancers,[256] and although these pathways are involved in normal stem cell self-renewal, more and more studies now suggest that they function differently in normal versus cancer cells and that such dissimilarities may be exploited.

PTEN is a tumor suppressor and modulator of several major signaling pathways that are commonly deleted or inactivated in multiple types of cancers.[257] It controls signaling through the PI3K-Akt-FoxO pathway, which triggers several downstream targets (e.g., mTOR) and elicits diverse cellular responses involved in proliferation, survival, and cell growth. In fact, it has recently been demonstrated that the PTEN-PI3K/Akt pathway is responsible for maintaining CSC self-renewal in diverse types of cancers.[74,258] By analyzing gene expression signatures in both prostate CSCs and in the tumor bulk, we found that prostate CSCs exhibit preferential activation of the PI3K/Akt pathway. Indeed, the tumorigenic potential of prostate cells was significantly enhanced upon activation of PI3K signaling (i.e., knock-down of PTEN or FoxO3a) in a murine xenograft model.[74] Consistent with these results, inhibition of PI3K activity by a dual PI3K/mTOR inhibitor (NVP-BEZ235) led to growth inhibition of prostate CSCs in vitro and in a mouse xenograft model. Furthermore, the combination of NVP-BEZ235, which targets prostate cancer progenitor populations, and the chemotherapeutic drug Taxotere, which targets the bulk tumor, is more effective in eradicating prostate tumors than either agent alone. Taken together this data strongly suggests that the PTEN-PI3K/Akt pathway is critical for prostate CSC maintenance and that targeting PI3K/Akt signaling may be beneficial in prostate cancer treatment by preventing the self-renewal of prostate CSCs.

Small-molecule modulators have also been used to probe differences in Notch signaling between normal and cancer stem cells. For example, blockade of Notch signaling with a γ-secretase inhibitor (GSI-18) abolished the potential for medulloblastoma cells to establish tumors in vivo, suggesting that the loss of tumor forming capacity was due to the depletion of CSCs.[259] Consistent with this result, Notch signaling levels were higher in the CSCs, providing a potential mechanism for their increased sensitivity to inhibition of this pathway. As such, medulloblastoma CSCs seem to be selectively vulnerable to agents inhibiting the Notch pathway.

10.3. Targeting Pathways Involved in CSC Differentiation

10.3.1. Differentiation of CSCs with Known Target- and Pathway-Based Modulators

CSCs, like normal stem cells, exhibit the ability to self-renew and differentiate into cells of the bulk tumor. Thus, in order to maintain a CSC pool, CSCs must retain the capacity to produce daughter cells with the same developmental potential. If all of the cells within the CSC pool differentiate, the pool will eventually be depleted. In fact, this notion—the induction of cancer cell differentiation—has been used successfully in a variety of cancers. For example, HDAC inhibitors and vitamin D analogues are used routinely to promote differentiation in several blood cancers that are driven by naive cells.[209,260] Acute promyelocytic leukemia cells, which often express a fusion protein composed of the promyelocytic leukemia gene and the RA receptor-α,[261] can be rapidly differentiated with RA.[262] Additionally, application of the Hh antagonist cyclopamine in medulloblastoma mouse models both efficiently blocks proliferation and induces changes in gene expression consistent with initiation of neuronal differentiation.[62a]

Glioblastoma multiforme (GBM), a very aggressive and deadly form of human brain cancer, may also be driven by CSCs. Similar to normal NSCs, glioblastoma CSCs can self-renew, are multipotent, and maintain the ability to respond to developmental stimuli.[263] For example, BMPs—which promote astroglial differentiation of adult NSCs[264]—also decrease proliferation and increase differentiation in GBM CSCs.[265] In addition, in vivo delivery of BMP4 effectively blocks tumor growth and mortality in 100% of mice after intra-cerebral grafting of human GBM cells. Endocannabinoid signaling is another pathway known to regulate cell proliferation and differentiation in normal NSCs.[266] Targeting this pathway with the cannabinoid agonists HU-210 and JWH-133 promoted glial differentiation of primary human

GBM CSCs, as illustrated by an increased number of S-100β- and GFAP-expressing cells.[267] Moreover, cannabinoid challenge decreased the efficiency of GBM CSCs to initiate glioma formation in vivo. TGFβ-signaling also plays diverse roles in NSCs[68a] and has been recently implicated by two independent groups as a factor mediating stemness in GBM CSCs. Ikushima et al. reported that inhibition of TGFβ signaling with SB-431542 drastically diminished tumorigenicity in GBM CSCs by promoting their differentiation. Consistent with this result, SB-431542-treated GBM CSCs exhibited less lethal potency in intracranial transplantation assays.[68b] Similarly, Penuelas et al. demonstrated that TGFβ enhanced the self-renewal capacity of GBM CSCs, though they suggest that this occurs through the SMAD-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway.[68c]

10.3.2. *Identification and Characterization of Novel Modulators of CSC Differentiation*

The identification of CSCs in GBM has presented the opportunity to develop therapies that specifically target the disease initiating and driving population of cells. While such techniques have seen preliminary success in animal models, they rely on perturbing pathways known to be operative in normal stem cell function (e.g., TGFβ or BMP signaling). This lack of selectivity for the disease causing cells will likely lead to varying degrees of toxicity. To address this limitation, we designed an unbiased screen to identify novel targets that specifically affect CSC function and not healthy brain stem cells (Figure 14a).[37] As discussed above, CSCs only make up a fraction of the entire tumor. In order to obtain enough cells to screen, we developed culture conditions that allow for the establishment and maintenance of GBM CSCs from primary human GBM tumors as adherent cultures. These conditions led to a selection for mitogen-responsive CSCs that could be expanded in sufficient quantity for screening.[37,263b] Such cells maintain undifferentiated characteristics (e.g., Nestin expression, a high degree of proliferative capacity; Figure 14b and d), the ability to respond to developmental stimuli (e.g., BMP-induced astroglial differentiation; Figure 14c) and can efficiently form intracranial xenograft tumors in mice (Figure 14f). Importantly, the histological features of these tumors are characteristic of high-grade brain tumors in human patients.[37]

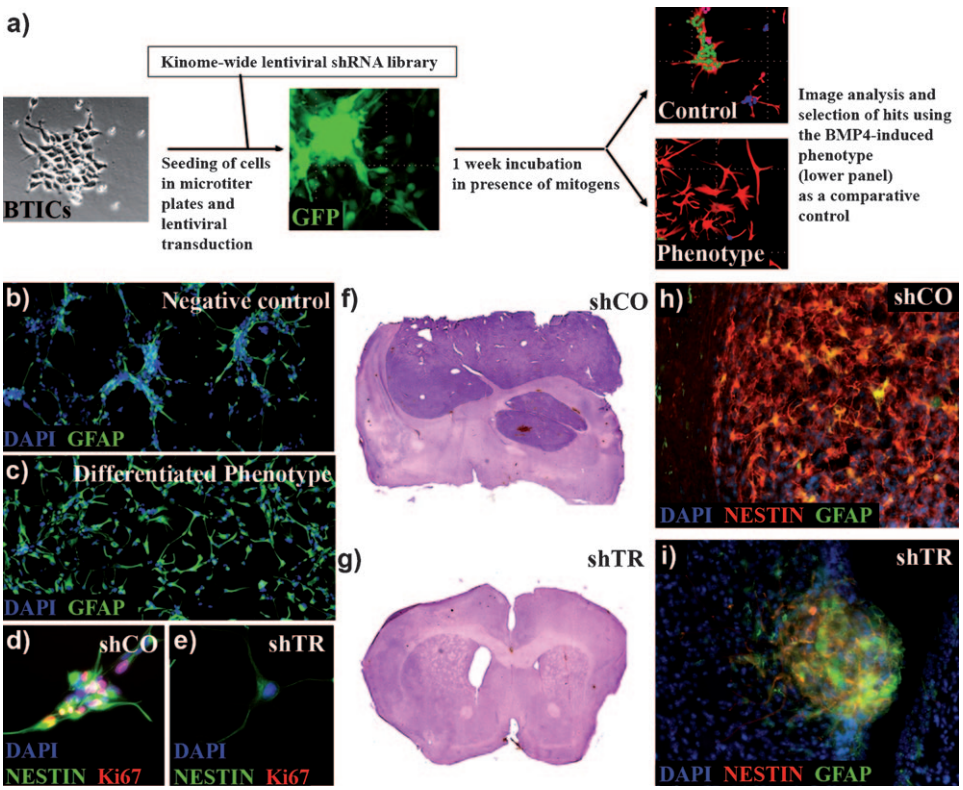


Figure 14. TRRAP is a regulator of GBM CSC fate. a) Scheme illustrating the shRNA-based screening strategy for forced GBM CSC differentiation. b,c) Overt and robust phenotypic differences were detected in untreated (b) versus differentiated (c) GBM CSCs in 384-well microtiter plates. d,e) GBM CSCs that were transduced with lentiviral control shRNA (shCO) formed proliferative colonies (d), whereas GBM CSCs undergoing forced differentiation, for example upon knockdown of the adapter protein TRRAP (shTR), resulted in a dispersion of colonies and increased immuno-positivity for GFAP (e). f) Control xenograft tumors showed massive growth and h) immuno-positivity for the stemness marker Nestin. g) Tumor burden was drastically decreased in TRAAP-deficient lesions. i) These tumors were also small and appeared to be more differentiated as indicated by a Nestin-negative, GFAP-positive phenotype. Figure adapted with permission from Ref. [37]; Copyright 2010, Elsevier.

These cells were transduced with an arrayed, kinome-targeted lentiviral shRNA library to identify kinases that control the differentiation of GBM CSCs, which can subsequently be targeted by small-molecule inhibitors. To minimize potential screening artifacts, several parameters were used to quantitatively represent GBM CSC differentiation based on BMP4-treated positive control wells; these include total cell number (to monitor potential cytotoxicity), colony number, and the mean number of cells per colony (GBM CSCs are able to form colonies from individual cells, whereas differentiated astrocyte-like cancer cells lack self-renewal potential, and rather appear as single cells; Figure 14b–e). Interestingly, only about 10% of the hits from the screen showed reproducibility across multiple patient-derived GBM CSC lines. This differential response is likely due to the genetic heterogeneity between the CSC lines. Since candidate gene knockdowns common to a number of different specimens are of greater interest, only these were fur-

ther characterized. The strongest differentiation phenotype was elicited by knockdown of transcription/transformation domain-associated protein (TRRAP), which is a large multidomain protein and a member of the PI3K-related family. Knockdown of TRRAP drastically suppressed the tumorigenicity of several different GBC CSC lines in vivo (Figure 14 f–i).[37] This study shows that unbiased screens can identify differentiation-based CSC-specific cancer targets that can be used to guide the development of small-molecule drugs.

More recently, we and others have used a similar approach to identify small molecules de novo that are either selectively toxic to GBM CSCs or induce their differentiation. For example, we have used a differentiation-based promoter–reporter strategy to rapidly screen small molecules in GBM CSC lines. Lead molecules that reproducibly activated the reporter were then counter-screened using the image-based conditions described above. Concurrently, those molecules that were toxic in the primary screen have been counter-screened with primary human astrocytes and human ES cell-derived NPCs. Collectively, these two strategies have led to a variety of novel molecules that selectively target GBM CSCs. Current efforts with these compounds are aimed at identifying the relevant biological targets and examining their effect and selectivity in vitro and in vivo.[268]

Pollard et al. have used comparable strategies to generate and expand GBM CSC lines[263b] which were then used to carry out image-based screens of a 450-member compound collection comprising known drugs that have been used in the clinic for various indications. Of the 23 drugs that killed all GBM CSC lines, 7 are known to modulate the monoamine signaling pathways. Among these, two monoamine re-uptake inhibitors (indatraline and paroxetine) had no effect on fibroblasts; a dopamine transporter/sigma receptor modulator (rimcazole) and serotonin-specific re-uptake inhibitor (sertraline) resulted in cell death for all tumor lines and fetal NS cells but had no effect on fibroblasts. The results from this assay extend previous findings which suggested that brain CSCs may be acutely sensitive to modulation of monoamine signaling and, in particular, the serotonin signaling pathway.[159]

11. Concluding Remarks and Perspective

Although cell-based small-molecule screens have been used for decades in drug discovery and cell biology, the value of such approaches in the stem cell field is just now being realized. In fact, chemical approaches have been employed with increasing frequency in stem cell biology because small molecules have characteristics that make them uniquely suited to regulate the complex signaling pathways that control self-renewal, pluripotency and the differentiation state. Indeed, such synthetic molecules can be readily used to 1) regulate stem cell fate; 2) revert lineage-restricted cells back to the multipotent or pluripotent state; 3) expand therapeutically desirable mature cell types; and 4) study tumor hierarchy and the role of cells with stem cell properties in cancer. These molecules are providing new insights into the complex mechanisms that govern developmental potential and are beginning to find application in therapeutic settings. Indeed, many of these advances are being driven by the productive collaboration between chemists and biologists in academic labs rather than pharma.

Clearly, there are many other exciting opportunities to be pursued—satellite cells in muscle-wasting diseases, cardiomyocytes in heart disease and even HSCs in blood pharming; as well as challenges to be understood and overcome including the role of fibrosis and the niche in determining cell fate. It is clear that our understanding of stem cells and the complex process that govern development have and will continue to be advanced by the application of chemical principles and small-molecule tools. Ultimately, the merging of these two disciplines will undoubtedly accelerate the promise of regenerative medicine as a routine therapeutic modality for many devastating and life-threatening diseases.

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