PROTEINS AND SMALL MOLECULES FOR CELLULAR REGENERATIVE MEDICINE

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I. INTRODUCTION

By replenishing lost functional tissues and cells, regenerative medicine could change the treatment paradigm for a broad range of degenerative and ischemic diseases such as stroke, heart attack, and diabetes (9, 42, 77). Although this goal of generating functional replacement for diseased tissues holds great promise, regenerative medicine has thus far found only limited successes. The concept of organ regeneration has been proven in clinical practice for 50 years in the field of bone marrow transplantation, in which patients receive donor hematopoietic stem cells that replenish their entire repertoire of immune cells (70). However, gaps in our understanding of pluripotent cells and their maturation into differentiated tissues have presented substantial barriers to regeneration of other organs.

To provide cellular therapy in organ systems with complex three-dimensional structure requires comprehensive control of multipotent cells—difficultion into a target cell type, delivery to a desired tissue, and integration into a durable functional structure. At each step of this process, proteins and small molecules provide essential signals and, in some cases, may themselves act as effective therapies. Identifying these signals is thus a fundamental goal of regenerative medicine. In this review we discuss current progress using proteins and small molecules to regulate tissue regeneration, both in combination with cellular therapies and as monotherapy.

II. MAINTENANCE AND EXPANSION OF PLURIPOTENT CELLS

Pluripotent cells have the ability to regenerate all of an organism’s mature tissue types. A variety of pluripotent cells have been used as sources for differentiated cells. These
include embryonic stem cells, hematopoietic stem cells, and mesenchymal stem cells. To characterize these cells and to begin exploring their therapeutic application requires the isolation of pure cell populations in large quantities under conditions that preserve their undifferentiated state. Careful tissue culture studies and phenotypic chemical screens have identified proteins and small molecules that maintain the multipotency of these cells.

A. Hematopoietic Stem Cells

Hematopoietic stem cells are a population of progenitor cells found primarily in the bone marrow that can replenish all mature blood lineages and are established sources for marrow replacement in patients with hematologic malignancies. In addition, hematopoietic stem cells have been implicated, either through paracrine signaling or perhaps transdifferentiation, in regeneration of myriad tissues (17). However, hematopoietic stem cells are a rare population in the marrow (and the placenta), and strategies for isolating and expanding pure hematopoietic stem cell populations are of great interest.

Robust techniques for expanding hematopoietic stem cells may allow more widespread use of the placenta as a hematopoietic stem cell source. Umbilical cord blood, purified from the placenta and umbilical vessels after birth, can be an alternative source of hematopoietic stem cells. It is more accessible than bone marrow but is limited by low numbers of progenitor cells. Umbilical cord blood is currently used for ~20% of transplants in the United States and 50% in Japan (17). Hematopoietic stem cells from umbilical cord blood are relatively immature, so the need for immunologic matching is less stringent. They provide an option for treating patients without a match in the marrow registry. However, a major limitation for this source is the low volume of hematopoietic stem cells, which results in delayed engraftment and immune reconstitution.

Efforts to enhance the number of hematopoietic stem cells from umbilical cord blood could substantially increase the availability of cells for marrow transplant. This has focused interest on signaling pathways that regulate the proliferation of hematopoietic stem cells. Because they mediate cell-fate decisions in multiple cell types, proteins of the Notch family were hypothesized to play a role in hematopoietic stem cell proliferation. Notch proteins are single-pass transmembrane receptors that interact with membrane-bound ligand expressed on adjacent cells (2). In response to binding, an intracellular domain of Notch translocates to the nucleus to modify gene expression. Hematopoietic precursors express a Notch homolog (44). Treatment of mouse
and human hematopoietic cells with immobilized recombinant version of Notch ligands, Jagged and Delta, leads to expansion of hematopoietic stem cells (18, 21, 82). Notch ligand has been tested in a phase I clinical trial for patients undergoing myeloablative umbilical cord blood transplant (51).

Hematopoietic stem cell growth responds with great sensitivity to the extracellular milieu. For example, hematopoietic stem cell proliferation varies with extracellular copper levels. Chelation of extracellular copper promotes replication of cultured hematopoietic stem cells and inhibits their differentiation (53). Treatment of hematopoietic precursors with the copper chelator tetraethylenepentamine increases hematopoietic stem cell number and promotes engraftment in mice (54). A phase I/II clinical trial tested the addition of tetraethylenepentamine to umbilical cord blood and found it to be safe, although no improvement in engraftment was observed (20). A phase II/III trial is currently underway.

Chemical screening has identified additional compounds with the ability to expand hematopoietic stem cell populations. A cellular imaging screen for compounds that maintained the expression of characteristic hematopoietic stem cell-surface proteins CD34 and CD133 identified a molecule termed StemReginin1 (4). Treatment of umbilical cord blood samples with SR1 resulted in increased hematopoietic stem cell number and engraftment in mice. This compound acts as an antagonist at the aryl hydrocarbon receptor. The aryl hydrocarbon receptor has been implicated in several pathways regulating hematopoiesis, but its precise role is not well understood. This chemical screen thus identified a potential lead compound for improving hematopoietic stem cell engraftment and a novel pathway important in stem cell biology.

Chemical screens using whole animals rather than dissociated cells have also identified novel compounds that regulate hematopoietic stem cell behavior. In a zebrafish model, North et al. (47) screened a chemical library for compounds that could induce stem cell formation. Very few of the compounds tested had any effect on the number of hematopoietic stem cells, but a significant portion of the active compounds modulated the prostaglandin pathways. The main effector prostanoid in fish is prostaglandin E2, and treatment of fish with a stabilized dimethyl-prostaglandin E2 analog increased the number of hematopoietic stem cells produced during development and accelerated marrow recovery from irradiation. Similarly, mouse bone marrow treated ex vivo with prostaglandin E2 produces more hematopoietic stem cells. Further experiments suggest that prostaglandin E2 interacts with the Wnt signaling cascade by activating cAMP/PKA-mediated phosphorylation of β-catenin and thus promoting hematopoietic stem cell proliferation. This live-animal chemical screen thus implicated a central developmental signaling pathway in hematopoietic stem cell proliferation and identified a potential chemical modulator of the pathway.

An additional limitation to hematopoietic stem cell transplantation is the surveillance of the recipient immune system. Allogeneic hematopoietic stem cells can repopulate the recipient immune system, but their use is limited by immune rejection. The risk of rejection can be mitigated by treatment with aggressive immunosuppressive regimens, which make patients susceptible to infection, or by inclusion of donor T lymphocytes, which can precipitate graft-versus-host disease.

Thus, in addition to treatments that can expand hematopoietic stem cells, it would be desirable to find treatments that can lessen their immunogenicity. Zhang et al. (100) developed a set of culture conditions including stem cell factor, thrombospondin, fibroblast growth factor-1, insulin growth factor binding protein 2, and angiopoietin-like proteins that promotes the efficient expansion of mouse and human hematopoietic stem cells. At least in mice, expansion under these conditions allows for evasion of immune rejection (101). These culture conditions upregulate the cell surface protein CD274, which may inhibit immune activation. If extended to humans, these findings have the potential to address the limitations imposed by immune rejection and graft-versus-host disease. More broadly, these findings suggest that the immunogenicity of transplanted cells may be modified ex vivo and not require immunosuppressant therapy.

B. Mesenchymal Stem Cells

The bone marrow contains, in addition to hematopoietic stem cells, a second population of multipotent progenitor cells usually called mesenchymal stem cells (80). These progenitors give rise to mesenchymal tissues such as osteoblasts, chondrocytes, and adipocytes. In addition, mesenchymal stem cells may promote regeneration of other tissues perhaps by modulating the immune response and providing necessary paracrine factors (12).

Specification of mesenchymal stem cell fate can be altered by protein or small molecule treatments. Activation of either bone morphogenetic protein signaling with the protein bone morphogenetic protein-4, or hedgehog signaling with the synthetic agonist purmorphamide, induces osteoblast formation from mesenchymal progenitors (92, 94). In contrast, an agonist of PPAR-γ, the drug rosiglitazone, can generate adipocytes from mesenchymal stem cells (as well as human embryonic stem cells) (97). Despite increasing data on the paracrine signaling roles of mesenchymal stem cells, the specific factors produced by mesenchymal stem cells have not been identified. Isolating these molecules represents an important goal for stem cell biology and an exciting set of potential candidates for small molecule and protein regenerative therapies.
III. DIFFERENTIATION OF PLURIPOTENT CELLS

Pluripotent cells can in principle be given as therapies while still undifferentiated, relying on signals from the in vivo microenvironment to specify their fate. Although this approach has worked in bone marrow transplant, it has had limited translational success in other organs. An alternative approach with likely less neoplastic potential is therapeutic delivery of differentiated cells previously generated from pluripotent cells in vitro. The blueprint for creating differentiated cells in vitro comes largely from the programs that produce each cell type during development. Unbiased chemical screens have also identified novel compounds and pathways important for cell fate specification. Here we will discuss several key cell types that have been generated with these strategies.

A. Cardiomyocytes

The loss of heart cells is a major cause of heart failure, so the generation of heart cells in vitro has been an important goal of stem cell science. A combination of insights gained from cardiovascular development and unbiased chemical screens have identified several compounds that promote cardiomyogenesis. For example, developmental studies found that loss of retinoic acid activity in a mouse is associated with ventricular hypoplasia or severely abnormal heart formation (24, 73). This suggested a role for retinoic acid in differentiation of pluripotent cells into cardiomyocytes. Indeed, treatment of murine embryonic stem cells with retinoic acid enhanced cardiomyogenesis and development of ventricular cardiomyocytes (91).

Similarly, mouse knockouts of several bone morphogenetic protein family members resulted in nonviable embryos with disrupted heart formation. However, simple treatment of embryonic stem cells with these bone morphogenetic proteins did not induce cardiac formation. Characterization of normally developing embryos showed that the bone morphogenetic protein inhibitor noggin is expressed during a narrow developmental window in the primary heart field of the mouse. It is known from experiments in Xenopus that treatment of embryos with the anti-helminthic suramin alters the fate of the Spemann organizer (the primitive knot that organizes gastrulation in vertebrates) and induces cardiac differentiation. This led to the hypothesis that suramin could induce cardiac differentiation of embryonic stem cells. Indeed, treatment of mouse embryonic stem cells with suramin did promote cardiac differentiation (28). Surprisingly, suramin treatment of mouse embryonic stem cells specifically generated cardiomyocytes with sinus node properties (90).

Inhibition of bone morphogenetic protein signaling with a chemical inhibitor leads to a similar increase in cardiomyocyte formation. A chemical screen in zebrafish for molecules disrupting dorsoventral patterning identified the compound dorsomorphin. Characterization of the molecule and its cellular actions found that it inhibits bone morphogenetic protein signaling (FIGURE 2). Dorsomorphin treatment of mouse embryonic stem cells ex vivo during the first day of differentiation produces a 20- to 30-fold increase in beating cardiomyocytes after 12 days of differentiation (29).

Chemical screening has also identified molecules from novel pathways that induce differentiation of pluripotent cells into cardiomyocytes. Wu et al. (93) performed a cell-based screen in a pluripotent mouse embryonic carcinoma cell line searching for compounds that could activate a reporter for the rat atrial natriuretic factor, a marker of cardiac differentiation. As a secondary screen, these compounds were tested for the ability to increase expression of sarcomeric myosin heavy chain, an essential component of the cardiac contractile machinery. These screens identified a set of four heterocyclic compounds, termed cardiogenols, that were most potent. Further characterization of their role in promoting differentiation of human pluripotent cells will be necessary.

Modulation of calcium signaling pathways can also promote cardiomyogenesis. Developmental studies have established that electrical activity and calcium signaling play a key role in the differentiation of myocytes. This suggests that compounds modulating calcium signaling could regulate cardiomyogenesis in vitro. Indeed, a screen for compounds activating a fluorescent reporter for α-myosin heavy chain identified the L-type calcium channel blocker verapamil and the calcineurin inhibitor cyclosporine as promoters of cardiomyogenesis (60). These findings imply that cell physiology is essential for cell fate determination.

Once conditions are established to robustly generate cardiomyocytes, a more complex objective is the selective production of precise subtypes of cardiac cells. Small molecules have already been identified that address this goal in the mouse. It is known from experiments in Xenopus that treatment of embryos with the anti-helminthic suramin alters the fate of the Spemann organizer (the primitive knot that organizes gastrulation in vertebrates) and induces cardiac differentiation. This led to the hypothesis that suramin could induce cardiac differentiation of embryonic stem cells. Indeed, treatment of mouse embryonic stem cells with suramin did promote cardiac differentiation (28). Surprisingly, suramin treatment of mouse embryonic stem cells specifically generated cardiomyocytes with sinus node properties (90).

Targeted efforts to produce subtypes of cardiomyocytes have been based on known differences in protein expression within the heart. For example, small-conductance calcium-activated potassium channels are primarily expressed on myocytes in the atrium, atrioventricular node, and pulmonary veins. The small molecule EBIO selectively increases conductance through these channels. In turn, EBIO treatment induces formation of cardiac pacemaker cells (33). Understanding differences in protein expression between...
cell types in the heart may inform strategies to selectively generate each cardiac cell type.

**B. Endothelial Precursor Cells**

Diseases of inadequate blood flow and tissue ischemia, such as stroke and myocardial infarction, are longstanding targets for regenerative medicine. One strategy for treating these disorders is to stimulate the formation of new blood vessels by recruiting endothelial precursor cells to areas of injury and stimulating their proliferation.

HMG-CoA reductase inhibitors of the statin class improve outcomes in patients with atherosclerotic heart disease, and this benefit may be mediated in part by stimulation of endothelial precursor cells. The best understood mechanism of action for statins is the lowering of lipids to reduce the burden of atherosclerotic plaque and stabilize existing plaques (84). However, statins have been postulated to benefit patients independently of effects on cholesterol levels (19). In rats with normal cholesterol levels, treatment with simvastatin promotes angiogenesis by an Akt-dependent mechanism. Serine phosphorylation of Akt induces phos-
phosphorylation of endothelial nitric oxide synthase and nitric oxide production, which enhances survival of endothelial precursor cells (35, 36). Treatment of humans with stable coronary artery disease with statins increases the number and migratory capacity of peripheral endothelial precursor cells within 1 wk (17, 83). This strategy may be particularly effective in patients with coronary artery disease because of their decreased nitric oxide bioavailability. The resounding clinical success of statins may, in part, be due to some unanticipated effects on vascular progenitors.

C. Endoderm/Pancreas

Type I diabetes results from the autoimmune destruction of insulin-producing pancreatic beta cells, and the current treatment is subcutaneous administration of insulin to replace the function of missing beta cells. Many cell-based approaches to directly replace destroyed beta cells have been proposed. To develop a source of beta cells, techniques must be developed first to differentiate pluripotent cells into the endoderm lineage, and then from endoderm into pancreatic beta cells. Unbiased chemical screens have isolated several molecules that can promote differentiation into endoderm and pancreatic beta cells.

During development, activin A and nodal proteins induce formation of definitive endoderm in mice and humans. Borowiak et al. (5) performed a chemical library screen to identify compounds that could induce endoderm in the absence of these factors. Mouse embryonic stem cells containing a fluorescent reporter for Sox17, an endoderm marker, were treated with a library of 4,000 compounds. From these two compounds, induce definitive endoderm-1 (IDE-1) and induce definitive endoderm-2 (IDE-2) were identified that strongly activated Sox17 and other markers of endoderm (5). Activin A and nodal induce endoderm formation during development by activation of transforming growth factor (TGF)-β signaling and Smad phosphorylation, and the IDE compounds also act through this pathway (FIGURE 2).

An alternative screening strategy is to search for compounds that enhance endoderm formation in the presence of known endoderm-promoting factors. Such compounds could be useful either in vitro to more robustly generate endoderm or in vivo to increase native endoderm differentiation. In mouse embryonic stem cells incubated with activin A, simultaneous treatment with the kinase inhibitor staurosporine enhanced endoderm formation (17, 103). However, staurosporine was not sufficient to induce endoderm. These data suggest the existence of multiple interconnected pathways contributing to lineage specification, only some of which are sufficient to determine fate on their own.

After definitive endoderm is generated in vitro, a separate set of conditions can be used to promote formation of pancreatic progenitors. Chen et al. (16) treated endoderm cells with a library of small molecules to determine which could induce pancreatic progenitors. The screen identified indolactam V as a molecule capable of directing differentiation of pancreatic progenitors from endoderm (16, 44). Treatment with IDE followed by indolactam V is a two-step protocol for production of pancreatic progenitors that utilizes only small molecules. Thus creation of functional beta cells with serial targeted small molecule and protein exposures appears feasible.

D. Neurons

Increasing knowledge of the developmental pathways of neurogenesis has enabled efforts to recreate neural specification from pluripotent cells in vitro. Initial strategies relied on coculture with stromal feeding layers and required embryoid body formation as an intermediate, which resulted in ill-defined culture conditions, low yields, and prolonged differentiation time. Classical neurodevelopment studies identified inhibitors of bone morphogenetic proteins as crucial factors for neural fate induction. Chambers and coworkers demonstrated that treatment of human embryonic stem cells and induced pluripotent stem cells with noggin (an endogenous bone morphogenetic protein inhibitor) and a small molecule inhibitor of Smad (a downstream effector of TGF-β) promoted efficient conversion to neuronal cells (13, 18, 21, 82). Subsequent chemical screens have identified inhibitors of glycogen synthase kinase 3β (GSK3β) as promoters of neuronal differentiation (22, 51).

Once neural precursors have been generated, further manipulations can selectively promote the formation of different neuronal cell types. These protocols can be inspired by the sequence of factors present in vivo during neurodevelopment. The generation of spinal motor neurons in vivo is a multistep process. Ectodermal cells receive signals through the bone morphogenetic protein, fibroblast growth factor, and Wnt pathways to acquire rostral neural identity. These neurons then acquire a spinal identity in response to retinoic acid. Finally, the motor neuron identity is acquired in response to sonic hedgehog. Following the same logic, treatment of mouse embryonic stem cells in vitro with retinoic acid followed by a synthetic hedgehog agonist did generate cells with the characteristics of motor neurons. When reintroduced into the spinal cord of developing mouse embryos, these cells were able to form functionally integrated spinal motor neurons. Thus motor neurons can be generated from embryonic stem cells in vitro under conditions similar to those during development, and the resultant cells are capable of forming functional nerve cells in vivo (53, 89).

Neural precursors can also be directed to generate cortical neurons by mimicking developmental signals. Sonic hedgehog signaling during development is necessary to create the ventral identity of the forebrain. Treatment of neural precursors in culture with the sonic hedgehog inhibitor cyclo-
pamin enhances differentiation into cerebral cortex. The resultant neurons exhibit the neurotransmitter profile, electrophysiological behavior, and morphology of pyramidal neurons of the cerebral cortex. When grafted into developing brains, they form appropriate projections (27, 54). Treatment with a single chemical compound is sufficient to direct neural precursor cells into cortical neurons.

IV. CELLULAR REPROGRAMMING

Generation of pluripotent cells from human embryos is constrained by the limited number of available embryos and ethical challenges. In 2006, Takahashi and Yamanaka (75) demonstrated that adult somatic cells could be reprogrammed to pluripotency by the genetic introduction of four transcription factors, generating induced pluripotent stem cells (20, 74). Building on this finding, genetic techniques have been developed to generate a variety of cell types, including neurons, cardiomyocytes, and pancreatic beta cells directly from adult somatic cells (4, 31, 32, 85). These techniques were first developed for cultured cells but have now been employed successfully in vivo (47, 57, 71, 102). Despite the technical challenges of in vivo gene transduction, there is some evidence that cells produced in this way more closely resemble native differentiated cells, perhaps because of the endogenous microenvironment (57).

Separately, progress has been made replacing some or all of the transcription factors necessary for reprogramming to pluripotency with small molecules or peptides delivered across the cell membrane (12, 66, 87). Although currently direct reprogramming is feasible only through genetic manipulations, the convergence of these lines of research offers the prospect of new somatic cells derived from other resident cell types through treatment with proteins and small molecules. This prospect is particularly appealing in clinical scenarios such as myocardial ischemia, where a ready population of fibroblasts provides an ideal starting material for reprogramming into new cardiomyocytes.

V. PROLIFERATION OF SOMATIC CELLS

Regeneration of tissues can be accomplished by differentiation of pluripotent cells or by promoting proliferation of already differentiated cells. It was traditionally thought that highly differentiated organs such as the heart and the brain were incapable of cell division in the adult mammal. However, recent evidence suggests that even in these tissues it may be possible to stimulate mitosis. This strategy has the advantage of avoiding the technical and regulatory challenges associated with pluripotent cells.

A. Cardiomyocytes

Mammalian heart growth during embryonic development depends on cardiomyocyte proliferation. Repair of injured hearts in newts and zebrafish also relies on cardiomyocyte proliferation, whereas in humans there is not a robust proliferative response to injury (48, 92, 94). Mammalian cardiomyocytes exit the cell cycle soon after birth and show minimal evidence of myocyte DNA synthesis and proliferation (72, 97). Although elements of the cell cycle machinery are activated, the process is aborted prior to cell division, suggesting the presence of negative regulators (24, 58, 73). This raises the possibility that by targeting and blocking these inhibitory pathways, the proliferative response to injury can be unmasked.

Proliferation of adult cardiomyocytes requires that these cells reenter the cell cycle. The p38 MAP kinase pathway regulates expression of genes important for cell cycle entry including cyclin A and cyclin B. In fetal cardiomyocytes, overexpression of p38 reduces proliferation, whereas p38 knockout increases myocyte cell division. Adult cardiomyocytes in culture do not proliferate, but treatment with an inhibitor of p38 MAP kinase induces cell division (25, 91). In rats with acute myocardial injury, treatment with p38 MAP kinase inhibitor and fibroblast growth factor-1 (to induce angiogenesis) reduces scarring and improves cardiac function (1). By allowing reentry into the cell cycle, inhibitors of p38 MAP kinase may provide an avenue to proliferation of adult myocytes.

Additional insights into cardiomyocyte replication have come from experiments studying proliferation of pluripotent cells. Although these are disparate cell types, they share common pathways of proliferation. As described above, inhibition of the Wnt pathway promotes pluripotency and replication in mouse and human embryonic stem cells. Interestingly, treatment of rat neonatal or adult cardiomyocytes with the GSK3β inhibitor 6-bromoindirubin-3′-oxime (BIO) induces entry into the S phase of mitosis and cell division. Cardiomyocytes treated with BIO have higher levels of nuclear B-catenin, suggesting that BIO activity in cardiomyocytes stimulates the Wnt pathway (78, 98). This example demonstrates a shared proliferation pathway and a shared small molecule between undifferentiated pluripotent cells and highly differentiated cardiomyocytes.

Another strategy to reactivate the cell cycle in adult cardiomyocytes has been to introduce molecules present during development with the hope of restoring fetal properties to the adult cell. Periostin is a component of the extracellular matrix associated with epithelial-mesenchymal transition during cardiac development that can induce proliferation in adult cardiomyocytes (10, 29, 38). Adult rat cardiomyocytes treated with recombinant periostin dramatically increase DNA synthesis with an increase in cell division. Injection of recombinant periostin into rat myocardium stimulates DNA synthesis and cell division in vivo. Following myocardial infarction, mice treated with an epicardial
The activity of 

\[ \text{GSK3} \]

inhibits \( \beta \)-catenin activity by promoting its degradation. The small molecule 6-bromoindirubin-3'-oxime (BIO) inhibits GSK3\( \beta \) and thus increases the activity of \( \beta \)-catenin.

Exenatide (Byetta) is a synthetic analog of glucagon-like peptide-1, an intestinal hormone that stimulates insulin production in response to glucose. Treatment of mice with glucagon-like peptide-1 increases insulin production by increasing beta cell mass. Exenatide validates the targeting of beta cell proliferation in diabetes.

The connection between insulin signaling and beta cell proliferation suggests that drugs affecting insulin signaling may also regulate beta cell mass. For example, inhibition of GSK3 activity could induce beta cell proliferation. Treatment of cultured rat islet cells with structurally diverse GSK3 inhibitors increases the rate of replication to threefold both in an insulinoma cell line and isolated rat islets. Thus alteration of the insulin-signaling pathway provides a target for increasing beta cell mass.

To identify additional compounds that could induce proliferation of a mouse beta cell line, Schultz and colleagues performed an unbiased chemical screen. This screen found several candidates that could induce beta cell proliferation, including molecules active in the Wnt and protein kinase C pathways. They also found that dihydropyridine agonists of the L-type calcium channel induced beta cell proliferation.

This agrees with previous studies that demonstrated a role for calcium signaling in beta cell proliferation and diabetes. For example, genome-wide association studies have identified calcium channel genes. In addition, \( \alpha_1d \) L-type calcium channel knockout mouse demonstrated reduced postnatal beta cell proliferation resulting in hypoinsulinemia and impaired glucose tolerance. In experiments to characterize the effect of treatment with calcium channel agonists, investigators found that primary rat beta cells treated with a calcium channel agonist showed a 2.5-fold increase in proliferation, and a similar effect was seen in isolated human beta cells. Based on mRNA expression data, they hypothesize that influx of calcium through L-type calcium channels activates RAS signaling to trigger cell cycle activation and beta cell proliferation.

### B. Pancreatic Beta Cells

Conditions of increased insulin demand such as pregnancy, obesity, or partial pancreatectomy have been demonstrated to produce increased beta cell proliferation, raising the possibility that beta cells can be induced to proliferate with pharmaceutical treatment. Identifying proteins or small molecules that can mimic this effect has been a longstanding goal of therapeutic research in diabetes.

Exenatide (Byetta) is an existing diabetes treatment that functions, at least in part, by promoting beta cell proliferation. The drug is a synthetic analog of glucagon-like peptide-1, an intestinal hormone that stimulates insulin production in response to glucose. Treatment of mice with glucagon-like peptide-1 increases insulin production by increasing beta cell mass up to twofold. Glucagon-like peptide-1 increases beta cell mass by three mechanisms: increased proliferation, decreased apoptosis, and differentiation of stem cells into islet cells.

The cellular mechanism of action for many neuropsychiatric drugs remains uncertain. For example, valproic acid is an established drug for the treatment of epilepsy and mood disorders, but its precise mechanism of action in these disorders is not clear. One molecular mechanism of action for
valproic acid is the inhibition of histone deacetylase activity. Treatment of neural progenitors in culture with valproic acid enhances neuronal differentiation while inhibiting astrocyte and oligodendrocyte differentiation; a similar effect was seen with two unrelated histone deacetylase inhibitors. Rats treated with valproic acid exhibited a decrease in proliferation of neural precursors with increased differentiation into mature neurons (30). By modifying chromatin accessibility, valproic acid likely alters transcription factor binding leading to changes in cell fate. Thus promotion of neurogenesis is not only feasible but may explain the mechanism of action for an established psychiatric medication.

The role of neurogenesis in the pathogenesis of depression is unclear, but several studies have implicated neurogenesis in antidepressant action. Chronic treatment with antidepressants from the selective serotonin reuptake inhibitors, selective norepinephrine reuptake inhibitors, and tricyclic antidepressant classes has been shown to increase neurogenesis in rat hippocampus and results in the incorporation of new, mature neurons (39, 86). In a mouse model of depression, irradiation of the subgranular zone prior to antidepressant treatment blocked neurogenesis and eliminated behavioral improvement in response to fluoxetine (62). These experiments have been extended to nonhuman primates. In adult bonnets, depression was associated with decreased hippocampal neurogenesis. Treatment with fluoxetine stimulated neurogenesis and improved depressive behaviors, but this effect was abrogated by brain irradiation, suggesting a requirement for neurogenesis in the antidepressant effects of fluoxetine (103).

The realization that several classes of existing neuropsychiatric medications alter neurogenesis suggests that novel compounds that affect neurogenesis may also have clinical use. A chemical library screen for compounds that promote differentiation of cultured rat hippocampal neuronal precursors identified several molecules termed neuropathiazols (95). An optimized version of these compounds, KHS101, significantly increases neuronal differentiation in rat hippocampus. Cross-linking experiments identified binding of the compound to TACC3, a protein thought to play a role in regulating progenitor expansion as a component of the centrosome and mitotic spindle apparatus. These studies identify a novel pathway by which small molecules may regulate neurogenesis.

**VI. MOBILIZATION**

Pluripotent or multipotent cells can be obtained either from embryos, by reprogramming of differentiated cells or by mobilization from stores in the adult. The use of adult stem cells involves their mobilization from stores into the peripheral bloodstream. Once mobilized, they may be harvested for ex vivo treatments or targeted to remote areas of injury. Mobilization of adult hematopoietic stem cells is performed clinically to promote reconstitution of the immune system following myeloablative chemotherapy or to obtain cells for bone marrow transplant. Current protocols for mobilization already incorporate treatment with proteins, and ongoing investigations seek to identify compounds to increase the efficacy of mobilization from the bone marrow or other sources in the adult.

The primary agent used clinically for hematopoietic stem cell mobilization from the bone marrow is granulocyte colony-stimulating factor. This growth factor stimulates the proliferation and differentiation of neutrophils and their migration out of the bone marrow. To improve the performance of granulocyte colony-stimulating factor, PEGylated and glycolylated versions have been engineered with longer duration of action and superior pharmacokinetics. Despite their widespread use, these agents have a failure rate that one study estimates at nearly 30% (56). This has generated interest in the discovery of additional agents to aid in mobilization of refractory bone marrow.

Insights into the molecular determinants of stem cell retention in the bone marrow have enabled the discovery of additional agents that promote mobilization. The interaction between the C-X-C chemokine receptor 4 (CXCR4) on the surface of progenitor cells and Stromal cell-derived factor-1 on the surface of stromal cells promotes the retention of progenitor cells in the bone marrow. Granulocyte colony-stimulating factor interferes with this interaction by reducing the expression of both of these proteins on the surface of cells. This molecular insight enabled the development of a specific CXCR4 antagonist, AMD3100 (Plerixafor), that acts more rapidly than granulocyte colony-stimulating factor to mobilize hematopoietic stem cells from the bone marrow. The combination of granulocyte colony-stimulating factor and AMD3100 synergistically improves hematopoietic stem cell mobilization from the bone marrow in mice and humans (7). The combination has shown promising results in clinical trials for bone marrow transplant (56). Stem cell factor is an endogenous ligand for the tyrosine kinase receptor c-kit, which is expressed on hematopoietic stem cells. Recombinant stem cell factor has been shown to act in synergy with granulocyte colony-stimulating factor in mobilization of bone marrow hematopoietic stem cells (41).

The bone marrow harbors multiple distinct progenitor cells that give rise to different cell lineages, including hematopoietic stem cells, mesenchymal stem cells, stromal progenitor cells, and endothelial progenitor cells. Pitchford et al. (55) devised a set of conditions that selectively mobilize different populations of stem cells. Treatment with CXCR4 antagonist effectively mobilizes hematopoietic stem cells, but not endothelial progenitor cells or stromal progenitor cells. However, in mice pretreated with vascular endothelial growth factor, treatment with CXCR4 antagonist did induce endothelial precursor and stromal progenitor cell mo-
bilitation, whereas hematopoietic stem cell mobility was reduced. These data suggest multiple intersecting signaling pathways that regulate the expansion and migration of stem cells from the bone marrow.

The ability to mobilize hematopoietic stem cells from marrow, and the multipotency of these cells, presents an opportunity to regenerate other tissues by reprogramming of these cells. This strategy has been proposed to regenerate infarcted myocardium. Building on experiments demonstrating that hematopoietic stem cells delivered into the myocardium surrounding an infarct could regenerate functional myocytes and coronary vessels (49), Orlic et al. (50) hypothesized that mobilization of sufficient quantities of hematopoietic stem cells into the bloodstream in the presence of myocardial necrosis could also facilitate myocardial regeneration. Mice treated with stem cell factor and granulocyte colony-stimulating factor increased circulating stem cells 250-fold (50). These mice also had improved hemodynamics and overall survival. Although the use of bone marrow cells for cardiac regeneration remains controversial, this approach demonstrates a paradigm for regenerative medicine that employs protein therapeutics to mobilize precursor cells that then rely on endogenous injury and repair signals to regenerate tissue.

VII. PROMOTING ENGRAFTMENT AND CELL HOMING

The delivery of pluripotent cells to a target tissue may involve direct injection of cells or infusion into the bloodstream. Although direct injection may be feasible for some organs, it is less desirable due to its invasiveness. Direct infusion of cells into the bloodstream requires a means to direct these cells to the tissue of interest and often to a specific compartment within that tissue. In some settings, endogenous endocrine factors can recruit cells to an injured tissue, but ex vivo treatments to selectively direct transplanted cells to a desired organ may provide greater control and efficacy for cell targeting. Indeed, the low efficiency of homing and retention of delivered cells in the target tissue is currently a major limitation to cell therapies.

As cells travel through the bloodstream, proteins expressed on their cell surface interact with molecules displayed in the surrounding cells to determine their localization. This process is best understood for immune cells, which circulate in the bloodstream before migrating into areas of infection (67). Several classes of adhesion molecules contribute to the homing of immune cells to sites of inflammation. The process of homing involves an initial rolling adhesive interaction between circulating cells and the vascular endothelium (mediated by selectins) followed by direct migration into the extracellular matrix (mediated by integrins). As injured tissues display a unique complement of selectins and integrins, they are able to selectively recruit immune cells. By understanding the molecular mechanism of this recruitment, investigators have engineered pluripotent cells to be recruited to these same sites of injury.

Selectins mediate the rolling of cells along the vascular endothelium that initiates their targeting to injured tissue. Both P- and E-selectins are receptors present on the surface of vascular endothelium in injured tissue that promote rolling of cells at these sites (69). Leukocytes, as well as hematopoietic stem cells, express selectin ligands that aid in trafficking of these cells to sites of injury. Selectin ligands are not expressed on other progenitor cells, such as mesenchymal stem cells or umbilical cord stem cells. Multiple strategies have been developed to use this system to selectively target such progenitor cells to tissues of interest.

For example, carbohydrates of the sialyl Lewis family are the active site of P-selectin glycoprotein ligand, which is found on the surface of hematopoietic stem cells and leukocytes. To transfer this moiety to other progenitor cells, Sarkar et al. (63) conjugated streptavidin-linked P-selectin ligands to biotinylated cells and demonstrated recruitment of these cells to sites of injury. This technique allows for the targeting of any cell type of interest to sites of injury after ex vivo modification.

The cell surface protein E-selectin is expressed on marrow endothelial cells where it recognizes E-selectin ligands primarily on hematopoietic stem cells. This interaction is crucial for directing these cells to bone. Umbilical cord blood hematopoietic stem cells do not express E- or P-selectin ligands. It is known that selectin ligands must be α-1–2-fucosylated to bind their targets. Treatment of cord blood hematopoietic stem cells with fucosyltransferase and a fucose donor greatly increased binding to P- and E-selectin and enhanced the engraftment of human cord blood stem cells in mouse bone marrow (96). Similarly, mesenchymal stem cells also lack the surface carbohydrate modifications that promote binding to E-selectin ligands and therefore do not traffic to bone. Ex vivo treatment of mesenchymal stem cells with glycosyltransferase can also generate an E-selectin ligand. These cells bind tightly to E-selectin and home to the bone marrow (61).

Binding of integrins to the extracellular matrix replaces the low-affinity interaction provided by selectins with high-affinity adhesion. β-2 Integrin family members interact with ligands on the endothelium that are expressed in response to inflammation or injury. Activation of integrins ex vivo by either synthetic antibodies or endogenous ligands has shown promise for increasing adhesion of transplanted progenitor cells (14). Each treatment activates a distinct complement of endogenous integrins that direct cells to a specific location. For example, the nuclear protein high-mobility group protein B1 activates an integrin expression pattern that attracts cells to areas of necrosis (15), while the adipose
tissue secreted hormone leptin activates a set of integrins that targets cells to the vascular intima (64). Better understanding of integrin biology will yield a more comprehensive integrin address book.

Another strategy for homing of cells takes advantage of epitopes expressed selectively by injured tissue. For example, injured myocardium exposes proteins that differentiate it from surrounding normal tissue, such as the normally intracellular myosin light chain. By crosslinking two antibodies, one recognizing the CD45 protein on marrow progenitor cells and the other directed against the myosin light chain protein, Lee et al. (37) generated a bispecific antibody that could tether progenitor cells to injured myocardium. Hematopoietic stem cells treated cx vivo with the bispecific antibody were infused into rats following myocardial infarction. Antibody treatment significantly increased the number of progenitor cells that collected in the peri-infarct region. Infusion of antibody-treated cells significantly improved ejection fraction in rats postinfarct (37). These designer antibodies could target hematopoietic stem cells to any tissue by identifying unique epitopes expressed there.

Understanding the endogenous systems of progenitor cell recruitment provides a model for engineering progenitor cell targeting. The recruitment of progenitor cells to areas of injury depends on the secretion of chemoattractant cytokines. Stromal cell-derived factor-1 is secreted during acute ischemia and is necessary for neovascularization. Stromal cell-derived factor-1 binds to the CXCR4 receptor on endothelial precursor cells and hematopoietic stem cells and recruits these cells to sites of injury (FIGURE 4). However, chronic ischemia lowers stromal cell-derived factor-1 levels below that of control subjects (81). Transplantation of fibroblasts expressing stromal cell-derived factor-1 into the peri-infarct zone after myocardial infarction did improve homing of progenitor cells to the area of injury (3).

By chemically modifying endogenous recruitment signals, researchers have generated more effective chemoattractants. For example, one impediment to the delivery of stromal cell-derived factor-1 protein is its inactivation by cleav-ants. For example, one impediment to the delivery of stroma-neural signals is its inactivation by peptidases. By chemically modifying endogenous recruitment signals, researchers have generated more effective chemoattractants. For example, one impediment to the delivery of stromal cell-derived factor-1 protein is its inactivation by cleav-

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By chemically modifying endogenous recruitment signals, researchers have generated more effective chemoattractants. For example, one impediment to the delivery of stromal cell-derived factor-1 protein is its inactivation by cleavage (FIGURE 4). In response to tissue injury, matrix metalloproteinase-2 cleaves stromal cell-derived factor-1 into a neurotoxic byproduct. To overcome this, our lab engineered a protease-resistant version of stromal cell-derived factor-1 and tethered it to self-assembling peptides to form nanofibers. When delivered to rat myocardium following myocardial infarction, this peptide recruited endothelial precursor cells and increased capillary density. Rats treated with protease-resistant protein nanofibers improved cardiac function (65).

Alternatively, stromal cell-derived factor-1 degradation can be limited by inhibition of its natural peptidase. The peptidase dipeptidyl peptidase IV is bound to the membranes of many cell types and is also found as a soluble plasma protein. Inhibition of dipeptidyl peptidase IV in mice with the small molecule diprotin A increases the concentration of stromal cell-derived factor-1 in the heart following myocardial infarction (FIGURE 4). This leads to increased progenitor cell recruitment to the ischemic myocardium and improved neovascularization and ventricular function (99). These experiments illustrate the importance of endogenous promoters of regeneration and the endogenous inhibitors pathways that may limit their effectiveness. Targeting of these inhibitors represents an effective strategy for regenerative medicine.

**FIGURE 4.** Stromal cell-derived factor (SDF-1) recruits stem cells to sites of injury and is regulated by degradation. SDF-1 binds to the cell-surface receptor CXCR4 (C-X-C chemokine receptor 4) on embryonic and hematopoietic stem cells to recruit these cells to sites of injury. SDF-1 is degraded by extracellular peptidases such as dipeptidyl peptidase IV (DPPIV). Inhibition of DPPIV with diprotin A decreases SDF-1 degradation and increases SDF-1 activity. Similarly, engineering of SDF-1 to be resistant to protease activity (SDF-1*) increases its activity. SDF-1 plays an important role in homing of stem cells, and its activity is increased by pharmacological and protein engineering strategies to reduce its degradation.

**VIII. TARGETING THE STEM CELL NICHE**

Adult stem cells reside within a microenvironment that provides mechanical support and paracrine factors, the “stem cell niche.” Targeting components of the stem cell niche provides an alternative strategy for promoting regeneration. The preceding sections describe the wide array of factors that must be applied at the appropriate time and place to support proliferation, differentiation, and activity of pluripotent cells. Targeting members of the stem cell niche may simplify this complexity by providing the necessary factors to support pluripotent cell activity.
Prostaglandin E2 has a direct effect on hematopoietic stem injury, prostaglandin E2 is generated in the bone marrow. The activity of osteoblasts and osteoclasts is regulated by prostaglandins. In response to bone marrow injury, prostaglandin E2 is generated in the bone marrow. Prostaglandin E2 has a direct effect on hematopoietic stem cells but also acts indirectly by stimulating osteoclasts. The bones of mice treated with prostaglandin E2 showed significant increase in the spacing of bone trabeculae. These changes provided space for greater hematopoietic stem cell proliferation. Together with direct proliferative signals for hematopoietic stem cells, these changes contribute to an expansion of the hematopoietic stem cell population.

Bone marrow stromal cells display additional paracrine factors that are important for hematopoiesis, such as hyaluronic acid. Treatment with hyaluronidase depletes cell-surface hyaluronic acid and inhibits hematopoiesis. Similarly, animals treated with the chemotherapeutic 5-fluorouracil have decreased hyaluronic acid levels in the bone marrow and reduced hematopoiesis. Treatment with exogenous hyaluronic acid increased its concentration in the bone marrow and rescued the inhibition of hematopoiesis associated with 5-fluorouracil treatment.

In addition to the chemical environment, members of the stem cell niche craft the physical microenvironment of pluripotent cells. In the bone marrow, osteoblasts and osteoclasts sculpt the marrow architecture placing and removing bone. The activity of osteoblasts and osteoclasts is regulated by prostaglandins. In response to bone marrow injury, prostaglandin E2 is generated in the bone marrow. Prostaglandin E2 has a direct effect on hematopoietic stem cells but also acts indirectly by stimulating osteoclasts. The bones of mice treated with prostaglandin E2 showed significant increase in the spacing of bone trabeculae. These changes provided space for greater hematopoietic stem cell proliferation. Together with direct proliferative signals for hematopoietic stem cells, these changes contribute to an expansion of the hematopoietic stem cell population.

Additional candidates for regenerative therapeutics continue to emerge as the physiochemical composition of the hematopoietic stem cell niche is better defined. One intriguing feature of the hematopoietic stem cell niche is its relatively low oxygen tension. An oxygen gradient exists within the bone marrow with these cells found in the most hypoxic regions. This environmental feature allows for treatments that specifically target hematopoietic stem cells without affecting other cells in the bone marrow. For example, the small molecule tirapazamine reacts with DNA to form double-stranded breaks and cell death in the absence of oxygen. Treatment of mice with this compound selectively depletes hematopoietic stem cells from the bone marrow.


