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Editorial

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Future Medicinal Chemistry

Chances and challenges of RXRγ targeting for regenerative multiple sclerosis treatment

"...data also strongly support a very promising future role of RXRγ in regenerative MS treatment but the development of a suitable CNS bioavailable RXRγ selective agent will be a challenging task."

Keywords: blood–brain barrier • multiple sclerosis • retinoid x receptor • RXR subtype selectivity

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Multiple sclerosis (MS), which currently affects 2.1 million people worldwide, typically arises in early adulthood, has a significant impact on quality of life and is among the most severe health burdens. Although recent years have seen a lot of progress in MS treatment with the approval of orally available therapies such as fingolimod and dimethyl fumarate there is still a great unmet medical need since none of the available therapeutic options can reverse the progress of the disease [1,2].

MS is characterized by an autoimmune destruction of the CNS. Through the activity of autoreactive T-lymphocytes myelin sheds are destroyed and autoantibodies and B-lymphocytes promote a concomitant inflammatory process in the CNS. Current MS treatment and experimental approaches in late-stage development tend to modify the inflammatory cascade and to repress inflammation. Consequently, they target inflammatory signaling cascades such as chemokine or sphingosin-1-phosphate signaling, the metabolism of proliferating immune cells as well as adhesion molecules. With the development of several potent and specific agents MS treatment has strongly improved and is expected to be further augmented by forthcoming agents. Still, current and succeeding therapies can only decelerate the disease progress and reduce relapse rates while failing to restore a healthy state in existing lesions. Hence, novel approaches are strongly required that can reverse MS progression and restore the function of neuronal tissue after damage. Recent *in vitro* and *in vivo* data suggest that the nuclear retinoid X receptors (RXRs) might have the potential to fulfill this need [1–3].

The RXRs existing in the three subtypes RXRα, RXRβ and RXRγ are highly important nuclear receptors by acting as partners for nuclear receptors that form heterodimers such as retinoic acid receptors, vitamin D receptor, peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs) and farnesoid X receptor. The resulting nuclear receptor heterodimers which act as ligand-activated transcription factors are mostly permissive indicating that they can be activated by an agonist of either monomer. All three RXRs can equally form heterodimers and due to their importance as heterodimer partner, every cell expresses at least one RXR subtype. However, RXRs have a distinct tissue distribution making tissue selectivity conceivable for selective RXR ligands. In addition the widespread role in nuclear receptor signaling as heterodimer partner, RXRs also have important functions as monomers and homodimers [4,5].

While knockout of RXRα or RXRβ is lethal, knockout of RXRγ in mice revealed that the receptor has profound functions for the homeostasis of the adult CNS [6,7]. Recent results suggest a role of RXRγ in CNS protection and remyelination. Huang *et al.* [8] discovered significantly increased expression of RXRγ in isolated

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CNS tissue after induced focal demyelination in rats. Moreover, RXRγ was found in the cytosol of oligodendrocyte precursor cells and the nuclei of oligodendrocytes isolated from lesions while it was not detectable in oligodendrocytes from nonlesioned tissue. This indicates that the receptor might have a crucial role in oligodendrocyte precursor cell differentiation to mature oligodendrocytes in lesions. Over time, the number of RXRγ expressing oligodendrocytes in lesions increased significantly. Furthermore, purified oligodendrocyte precursor cells *in vitro* predominantly displayed cytosolic RXRγ but after differentiation to myelinating oligodendrocytes, RXRγ was especially detectable in the nucleus. Finally, RXRγ was also found significantly upregulated in postmortem lesion tissue samples from human MS patients and the receptor revealed higher nuclear than cytosolic localization [8].

When RXRγ signaling was inhibited by knockout or by an antagonist, the differentiation of oligodendrocyte precursor cells to myelinating oligodendrocytes was impaired and the expression of myelin basic protein subsided. In contrast, the RXR agonist 9-*cis*-retinoic acid dose-dependently induced the expression of myelin basic protein [9] and promoted the differentiation of oligodendrocyte precursor cells to myelinating oligodendrocytes *in vitro* [8]. 9-*cis*-retinoic acid was also capable of increasing remyelination in *ex vivo* samples of demyelinated mouse cerebellar slice cultures but did not increase the number of myelinating oligodendrocytes. These effects of 9-*cis*-retinoic acid on oligodendrocyte differentiation and myelin formation were blockable with RXR antagonists. Moreover, when 9-*cis*-retinoic acid was applied to rats after toxin-induced demyelination, myelin regeneration was significantly improved compared with untreated animals and generated thicker axons [8]. Altogether, this data suggest that RXRγ signaling is crucial for remyelination by oligodendrocytes. With additional anti-inflammatory properties in the CNS [10–12], RXRγ activation therefore seems a very promising experimental approach for a regenerative MS treatment.

However, selective RXRγ activation in the CNS with a small molecule is a challenging task that demands several qualities of the agent. First, RXRs are abundantly present in virtually every tissue and fulfill crucial roles in development and differentiation. RXRα is expressed in liver, lung, kidney, intestine, skeletal muscle and skin and RXRβ is nearly ubiquitous while only RXRγ is limited to CNS, heart muscle cells and skeletal muscle [4]. The only marketed RXR agonist bexarotene was originally approved for second-line treatment of cutaneous T-cell lymphoma. However, congruent with the vast role of RXRs, bexarotene and other clinically investigated RXR agonists suffer from adverse effects such as elevated blood triglycerides, hepatomegaly and hypothyroidism [13]. Furthermore, the pharmacological effects of bexarotene are examined in diverse conditions including various forms of cancer [14], metabolic [5] and cardiovascular [15] disorders as well as Alzheimer's disease [16]. On one hand this further confirms the receptor's potential role in CNS protection but on the other hand indicates that RXR agonists may cause many desired and undesired pharmacological effects. Therefore, the widespread presence of RXRs holds potential for a variety of side effects when RXRs are pharmacologically activated and makes selective RXR agonists necessary [5,13].

Second, although the three RXRs are encoded by three distinct genes all subtypes display high similarity and so far, sufficiently subtype selective ligands are lacking. The amino acid residues forming the ligand binding site are highly conserved over all three RXR subtypes suggesting that subtype selectivity could be driven only by secondary conformational changes in the ligand binding domains. Structure activity relationship (SAR) studies and structural optimization programs have discovered some RXR agonists with moderate preference for single subtypes but no actual selectivity has been claimed yet. In contrast, more success has even been made with RXR partial agonists and RXR ligands with selectivity for a certain heterodimer including RXR–LXR and RXR–PPAR selective agents. Although the common requirements for RXR agonists such as an L-shape, a carboxylic acid head group and a lipophilic backbone are defined, the SAR for subtype selectivity remains unknown [5,13].

Third, for targeting RXRγ in oligodendrocyte precursor cells and oligodendrocytes, CNS bioavailable compounds are necessary. However, the blood–brain barrier constitutes a significant hurdle for small-molecule agents. Median properties of marketed CNS drugs embrace a clogP value of 2.8, a low molecular weight of 305 Da and, most important, a pK_a value of 8.4 [17]. These characteristics are poorly compatible with the requirements on nuclear receptor modulators since ligands of nuclear receptors usually and of RXR [5] especially comprise an acidic function for a crucial neutralizing interaction with residues of the activation function 2 in helix 12 of the ligand binding domain. However, most carboxylic acids fail to cross the blood–brain barrier and additionally often are substrates of P-glycoprotein which hinders their use in CNS drugs [17,18].

RXRs are gaining recognition in medicinal chemistry and clinical research for their manifold physiologi-

cal and pathophysiological properties. Recent *in vitro* and *in vivo* data also strongly support a very promising future role of RXRγ in regenerative MS treatment but the development of a suitable CNS bioavailable RXRγ selective agent will be a challenging task. With the discovery of such an agent a very valuable avenue to a novel therapeutic approach with regenerative effects might be accessible, however, and its success might be worth the challenges.

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Therapeutic Delivery

A discussion on adult mesenchymal stem cells for drug delivery: pros and cons

Mesenchymal stem cells (MSCs) are emerging as candidates for drug delivery to treat numerous diseases. Their ease of isolation, expansion and reduced ethical concern, coupled with their 'plastic' immune functions and homing abilities make MSCs an appealing choice as cellular vehicle for drug delivery, including the delivery of RNA. However, while MSCs are currently listed for thousands of clinical trials, there are many confounding factors that have yet to be elucidated. In this review, we address many of the benefits of MSCs as therapeutic agents, and discuss confounding factors that require further scientific exploration.

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In the ever-evolving field of drug design, one issue that remains constant is the challenge to efficiently deliver drugs. More importantly, the challenge is to deliver drugs precisely at an anatomical region. The use of biologics and recombinant proteins, such as cytokines and perhaps small molecules, are limited because they are often unstable and short lived, with indiscriminate targeting at various organs. Due to the desire to develop more efficient delivery methods, there is a growing interest to use nanoparticles and cell delivery systems such as stem cells [1–3]. Cell delivery systems are beneficial in that the drug of interest can be synthesized by the cell for release within a specific microenvironment. Such a system can be termed '*in situ* biologic'.

Although several stem cells, such as neural stem cells, are currently investigated as vehicles for drug delivery [2,4], this review discusses the potential advantages for mesenchymal stem cells (MSCs). Initially investigated as a tool for regenerating connective tissues, MSCs are now being used in various diseases with thousands of registered clinical studies. To get insights into the total number of clinical trials using MSCs, we searched the ClinicalTrials database since all clinical trials are required to register in this public database. Using the search terms, 'mesenchymal stem cells', 'MSC' or 'mesenchymal stromal cells', the results showed 4683 trials registered on clinicaltrials.gov as of 31 July 2015. We however acknowledge that this could be an underestimation since successful trials in other parts of the work may not be represented in this database. Also, it is possible that our search terms did not 'capture' all of the clinical trials with MSC.

Despite the large number of human adult stem cells currently in clinical trials, the fate as well as the biology of the injected MSCs within their new microenvironment is poorly understood [5]. There are several issues that might account for the current problems to efficiently deliver stem cells. These include, but are not limited to, the lack of a consensus method to isolate and culture MSCs, the use of different methods to inject MSCs in animal models and patients for the same disease, and more importantly, the source of MSCs for use in the same disease model. Despite these deficiencies, MSCs continue to show promising results in both experimental and clinical trials. This study discusses the biology of

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Key terms

Differentiation: The capacity by which a stem cell matures into a specialized cell.

Veto-property: The ability of third party mesenchymal stem cells (MSCs) to suppress the activated T-cell responses from two different parties, such as would occur in mixed lymphocyte reaction or in graft versus host responses.

License: The process by which factors within an inflammatory microenvironment interact with MSCs, resulting in the stem cells transitioning into immune suppressor cells.

MSCs and introduces topics on the advantages and disadvantages of these stem cells as a cellular vehicle for drug delivery methods. We provide solutions to the 'black boxes' in the field to address how the current deficiencies in the field could be addressed.

Mesenchymal stem cells

MSCs are a class of adult stem cells predominantly found in the bone marrow and adipose tissues. The location of MSCs is ubiquitous, and have been reported in organs such as the first trimester fetal blood, fetal lung and liver, fetal membrane, placenta, umbilical cord blood, menstrual blood, peripheral blood, ear, nasal mucosa, dental pulp and the trabecula meshwork of the eye [6–16]. While found in multiple tissues, these MSCs are generally thought to share many properties such as phenotype and functions. However, it should be noted that robust research studies are needed to determine if the subtle differences among MSCs from various tissues could hinder their efficient use for particular diseases.

First described as colony-forming unit fibroblasts by Dr Alexander J Friedenstein in 1970 [17], MSCs have now been identified by various research groups that provided these cells with alternative designations. These terms include stromal stem cells, bone marrow stromal stem cells, skeletal stem cells, multipotent mesenchymal stromal cells, mesenchymal progenitor cells and pericytes among others. Despite the use of multiple names, there are common characteristics such as shared surface markers and the ability of these cells to undergo lineage differentiation. The commonly used functional differentiation generally uses defined methods to differentiate MSCs into adipocytes, chondrocytes, osteocytes, and, to a lesser extent, fibroblasts [6,18]. The ability to form fibroblasts is important because these cells are part of the bone marrow stromal compartment. This brings up one of the issues in the field, and that is to determine if MSCs can easily differentiate into a particular cell type based on memory of its tissue of origin. There is a need to compare the different sources of MSCs to determine if the ease of forming other cell types is linked to memory. However, *in vitro* studies have determined that MSCs can form cells of all germ layers, including the generation of functional neurons [19].

In the bone marrow, MSCs seem to function in a 'gatekeeper' role [20]. Anatomically, within the bone marrow, MSCs are positioned in a manner so that they are among the first cell type to contact other cells entering the cavity. In this regard, MSCs are among the last cell type to contact the hematopoietic cells exiting the bone marrow. Since MSCs have been shown to be the pericytes, they are expected around all blood vessels [21–23]. Thus, it is possible that MSCs could be the 'gatekeeper' cells in all organs and tissues.

The ability of MSCs to exert veto-property (suppressing stimulatory T-cell responses) provides these stem cells with the ability to be transplanted across allogeneic barriers [24]. However, the immune-suppressive properties of MSCs depend on the microenvironment in which the stem cells are present. Research studies over the past decade have overwhelmingly shown that MSCs can exert either immune-suppressive or immune-enhancing functions, based on the microenvironment (reviewed in [25,26]).

MSCs can be induced to release cytokines to regulate the microenvironment through paracrine stimulation as well as to autoregulate themselves through specific cytokine receptors [27]. Similarly, MSCs, through the expression of cytokine receptors and Toll-like receptors, can also interact with specific molecules within the microenvironment [28–32].

The vast number of studies, and perhaps the ease by which MSCs can be licensed to become immune suppressor cells, allow these cells to transition to the clinic for treatment of inflammatory diseases. Although similar functions contributed to their application in regenerative medicine and drug delivery, there are other advantages to these cells for patients. The ensuing sections of this review article discuss the advantages of MSCs as cellular vehicles for drug delivery.

Mesenchymal stem cells: harvesting, isolation & expansion

Unlike other stem cells, such as neural and embryonic stem cells, MSCs can be isolated with minimal safety or ethical concerns. MSCs can be harvested from numerous tissues, including the bone marrow, adipose tissue, Wharton's jelly and placenta. More importantly, MSCs can be expanded with ease *in vitro*. Thus far, there is no clear report that expanded MSCs have undergone transformation.

Human bone marrow samples can be obtained by an aspiration from the posterior iliac crest of healthy donors and immediately placed in media containing

preservative-free heparin [24]. Human adipose tissue can be obtained by elective liposuction of subcutaneous tissue or by en bloc resection of skin and subcutaneous tissue following an abdominoplasty, panniculectomy or resection of other areas with excess tissue [33]. Placenta and Wharton's jelly can be obtained from discarded placenta and umbilical cord following birth [34,35]. As this review article is not a method paper, we have just briefly discussed the different sources of MSCs.

Since the tissue sources of MSCs contain other cell types, the key is to expand MSCs without contamination from other cells. For example, bone marrow aspirates contain hematopoietic and nonhematopoietic cells. The latter includes adipocytes, endothelial cells and fibroblasts [36]. To isolate the MSCs, the lipoaspirate, placenta and umbilical cord are enzymatically digested, and each of the samples is cultured to select for the adherent cell population. After three passages, the remaining cells are MSCs, which can be identified by surface marker expression positive for CD73, CD90, CD105 and negative for CD34, CD45 and HLA-DR expression; and capable of multiple lineage generation (i.e., adipogenic, chondrogenic, osteogenic) [37]. Crucially, the MSCs can be passaged and expanded *in vitro* for several generations, permitting generation of large numbers of cells from a single donor. Thus far, there is no report of these cells transforming in culture [38,39].

Mesenchymal stem cells as immune modulator

This section briefly discusses the immune modulatory properties of MSCs. The molecular mechanisms by which MSCs regulate immune functions could result in targets so that MSCs can be engineered in drug delivery methods to regulate the immune microenvironment.

MSCs emerged as immune-related cells when it was observed that MSCs inhibited T-cell proliferation *in vitro* [40,41]. This observation was validated *in vivo*, when MSCs were coadministered with hematopoietic stem cells to reduce the incidence of graft versus host disease (GvHD) [42]. Although the exact mechanism is unknown, MSCs induce cell cycle arrest in the G0/G1 phase in both $CD4^+$ (T_H) and $CD8^+$ (T_C) T cells, possibly through the indoleamine-pyrrole 2,3-dioxygenase dioxygenase, prostaglandin E2 or inhibitory molecule PD1 pathways [43,44]. Alternatively, MSCs may inactivate T cells via major histocompatibility complex (MHC) molecule expression; in this fashion, MSCs can act as antigen-presenting cells. However, MSCs lack the costimulatory molecules necessary to fully stimulate the T cells, resulting in T-cell anergy [45].

Additionally, MSCs secrete various cytokines which can influence T-cell maturation and polarization [46,47]. Although the mechanisms are yet unknown, MSCs secrete HGF, IL-6, IL-10 and TGF-β1, which have been implicated in the T-cell maturation and polarization process [47]. Specifically, MSCs suppress the polarization into T_H1 and T_H17 cells – needed for T_C-cell activation – and enhance T_H2 and regulatory T-cell polarization [46].

The immune-suppressor functions of MSCs have led to further research studies to broadly understand the immune-modulatory properties of these stem cells. In cases of early and/or chronic inflammation, when IFN-γ is low, MSCs can act as antigen-presenting cells capable of activating a T-cell response [20,45,48,49]. Thus, paradoxically, MSCs can be licensed to immunosuppression during acute inflammation [41,50,51]. However, it is unclear what happens to the MSCs when inflammation subsides.

Mesenchymal stem cell homing: implications for drug delivery

MSCs are capable of homing to sites of inflammation [52]. The information is mixed regarding the ability of MSCs to cross the blood–brain barrier (BBB). While some have reported an ease of these stem cells crossing the BBB, others have shown little evidence of such movement [2,53]. The experimental evidence indicates that MSCs can enter the inflamed brain where they can be licensed as immune suppressor cells [41]. Based on this information, MSCs can be candidates for stem cell treatments to reduce inflammation, as well as to deliver drugs, to areas of inflammation, including the brain (Figure 1). While the exact mechanism of MSC homing is poorly understood (reviewed in [54]), the CXCR4 gradient appears to be involved [28]. Part of the difficulty in understanding MSC homing is due to their heterogeneity. MSCs from differing microenvironments – or even from within the same microenvironment – can express different chemokine receptors, which could influence MSCs homing capacity [55].

Despite several experimental and clinical trials, there is little evidence to support the integration of transplanted MSCs as replacement cells [56]. Despite the lack of lingering MSCs in the transplanted organs, there are reports of benefit, indicating that MSCs, through unidentified methods, can induce tissue repair [57,58]. It should be noted that while some reports have identified MSCs at the site of physical injury [58], other studies have reported beneficial clinical outcomes in the absence of engraftment [56]. At this time, there is no clear explanation for the varied results across the globe. We speculate that the varied data might be due to the source of MSCs and, more importantly, the culture methods. Regardless, the ability of MSCs to cross allogeneic barriers, home to

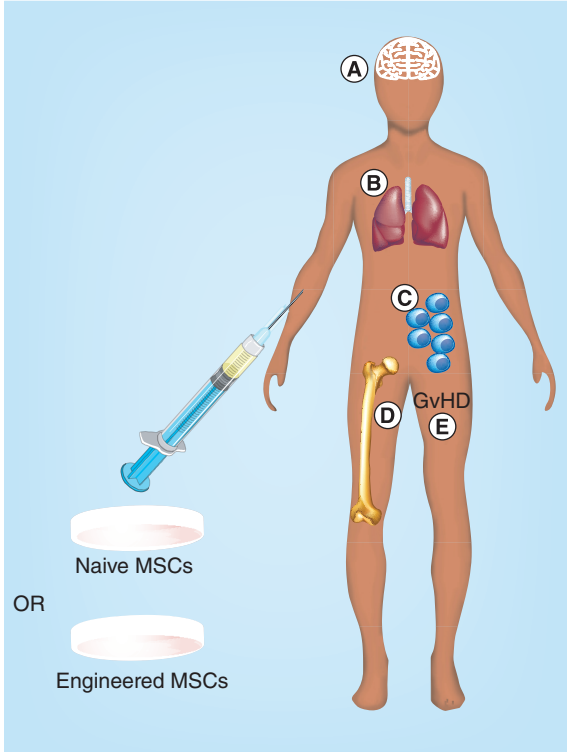

Figure 1. Naive or engineered mesenchymal stem cells can be administered to a patient intravenously.
The transplanted MSCs are shown homing to various tissues to exert functional changes, depending on the microenvironment. MSCs can migrate to the brain **(A)**, where they have been shown to deliver drugs to target glioblastoma [2], they can target organs such as the lung **(B)** or home to sites of diagnosed or nondiagnosed tumors **(C)**, to the bone marrow **(D)**, or can act peripherally in treating GvHD **(E)**.
GvHD: Graft versus host disease; MSC: Mesenchymal stem cell.

sites of inflammation and secrete soluble factors have provided the impetus for current interest to develop engineered MSCs for drug delivery.

Mesenchymal stem cell: cellular communication

MSCs release a variety of microvesicles (MVs) such as exosome [57,59–60]. These MV contain RNA and proteins that can be shuttled between MSCs and other cell types. The role of the MSC-derived exosomes is a subject of intense investigation; it is clear that MSCs can use the exosomes for intercellular communication [57,60]. Once the exosomes are transferred from MSCs into another cell type, the contents, such as the mRNA and noncoding RNA, can influence the cells' functions [61–63]. MSCs can also receive MV from microenvironmental cells to change their behavior. The ability of exosomes to mediate cellular communication is being applied as a method to treat diseases. Indeed, MSC-derived exosomes are currently being examined as a treatment for acute myocardial infarction [57].

Another method of intercellular communication is gap junctions, whereby direct passage is formed between the cytoplasm of two cells. Gap junctional intercellular communication permits passage of small molecules, including miRNA, between cells to effect target cell function [64].

MSC-mediated intercellular communication can also be accomplished by cytokine release. Paracrine effects can be generated either by mounting cytokines onto the MSCs' cell surface or by releasing the cytokines into the extracellular space [29,65]. The released cytokines can trigger various cell processes, including VEGF-mediated vascularization and angiogenesis during wound healing [66]. It is thus possible that MSCs, either engineered to overexpress a cytokine or nonengineered, could be administered to a patient for the benefit of the MSC-derived cytokines at the site of interest.

Mesenchymal stem cells as vehicles for drug delivery

MSCs have shown promise as a vehicle for drug delivery [2]. MSCs have been used to deliver cytokines, prodrugs, apoptosis-inducing proteins and antiangiogenic agents to tumors and other areas of inflammation [67–70]. These findings are being used in numerous clinical trials to treat primary and metastatic tumors, as well as inflammatory conditions (ClinicalTrials.gov website) [71,72]. Outside of their use in drug delivery, the anti-inflammatory roles of MSCs, as discussed above, could be the key advantage of MSC as a vehicle for the desired drugs. This property of MSCs will provide them with the ability to be used as an off-the-shelf source.

The use of MSCs to deliver drugs to areas of inflammation would depend on a nonrandom process since MSCs express chemotactic receptors allowing them to migrate to sites of tissue injury. This will allow for a relatively more efficient delivery system to target the treatment in precise regions. MSCs are advantageous for drug delivery because, in addition to being able to home to the regions of tumor growth, these cells are fully capable of transcriptional, translational and post-translational expression of large amounts of genetic information allowing them to secrete therapeutic substances into the tumor microenvironment.

Autologous adipose tissue-derived MSCs (AT-MSCs) implanted in fistulas of patients with Crohn's disease showed no adverse effects and confirmed safety of the therapy [73]. Further, a clinical trial using insulin-producing AT-MSCs transfused with unfractionated cultured bone marrow in Type 1 diabetes mellitus patients showed promising results [74].

The intrinsic tropism of MSCs for brain injury and brain tumors and their ability to breach the BBB gives these stem cells great potential for treatment of brain disorders and cancers [2,75]. AT-MSCs genetically engineered to express carboxyl esterase and a secreted form of TNF-related apoptosis-inducing ligand (TRAIL) expression vector showed significant therapeutic effects against brainstem gliomas, indicating the clinical applicability of nonviral gene transfer in treatment of brainstem gliomas [76]. MSCs engineered to produce bone morphogenic protein (BMP4) had a suppressive effect on glioblastoma, decreasing their proliferation [77]. Further, MSCs transfected with anti-miRNA-9 conferred chemosensitivity to human glioblastoma cells [2].

Another approach to treating malignant tumors involves the use of oncolytic viruses. AT-MSCs have shown to be capable to deliver myxoma virus to infect and kill human glioblastoma cells *in vitro* and *in vivo* [3]. MSCs have the ability to support multiple rounds of myxoma virus replication, allowing long-term viral replication, potentially maximizing the amount of time and virus available for delivery into brain tumors. Josia *et al.* showed that there was a significant increase in animal survival when human glioma U-87 cells were coinjected with myxoma virus-infected MSCs, and survival was also significantly increased in animals bearing U-87 orthotopic xenografts that received a single intracranial injection of myxoma virus-infected MSCs [3].

There are intense research studies on the application of MSCs in RNA therapeutics. RNA-based therapies have traditionally been hindered by the instability of RNA and targeted delivery. These issues among others contributed to the difficulty to translate RNA delivery to patients. MSCs can be engineered to deliver specific RNAi, including siRNA, shRNA and miRNA. Since MSCs can be available as off-the-shelf stem cell source, these engineered cells would be easily available for application to patients, and to particular tissues [78]. Experimentally, *in vitro* and *in vivo*, the use of MSCs to deliver RNA seems to be feasible and effective [2,79,80]. The challenge will be to ensure that the MSCs home to the specific tissue to deliver their 'cargo'. An improved method will allow the RNAi to rapidly deliver the RNA to its specific location.

Despite these advantages, the efficiency of MSC homing to the brain has been limited. A recent study showed that by pre-exposing MSC to glioma-conditioned media and the extracellular matrix proteins fibronectin and laminin, there were significant enhancements of the individual homing steps [81]. Similarly, preculturing MSCs with nonmalignant cerebrospinal fluid resulted in increased migratory speed and distance traveled [82].

Key terms

Engineered MSCs: The use of bioengineering or the inserting of RNA or drugs into MSCs; sometimes known as second generation MSCs.

Immune-privilege and immune-evasive: These terms describe the process by which MSCs exert a weak allogeneic response, providing the MSCs with sufficient time to evade the immune system *in vivo*.

Alternative methods in drug delivery with mesenchymal stem cells

Several clinical studies have indicated safety of MSCs, as well as promise for many diseases and disorders, including inflammation and tissue repair. There is no evidence that MSCs linger in patients, nor evidence of transformation [38,39,83]. Often times, high doses of MSCs are required; however, intravenous injection of high-dose MSCs is well tolerated in large mammals [84–86].

The delivery of MSCs to an area of interest can be challenging. For example, developing a strategy to maintain MSCs within the poorly defined borders of an autologous skin graft [87]. Similarly, studying the responses of MSCs in monolayer cell culture might not recapitulate when the MSCs are placed in an *in vivo* microenvironment that would be 3D [88–90]. Thus, bioengineering such as the use of biomaterial scaffolds have been gaining attention in cell delivery [87–89]. These engineered studies could lead to the MSCs being manufactured into bioelectrosprays for clinical application as scaffolds of MSCs. Briefly, the cells are resuspended in a charged medium, which is discharged into an electrical field. Upon reaching their target plate, the MSCs can form a cellular scaffold [91,92]. Similarly, the MSCs can be grown on a scaffold of other materials, which can then be used *in vivo* [93]. These electrospinning methods, including biospinning, do not appear to have an adverse effect on the cells [88,93,94].

MSCs exhibit low levels of MHC-II [95]. This property, combined with the MSC's ability to be immune-suppressive are partly responsible for these cells being available as off-the-shelf source. This permits them to be used across allogeneic barrier [24,95]. The ability of MSCs to be easily available makes these stem cells particularly attractive for clinical applications. Scientists vary with regards to how they view the functions of MSCs. While many consider MSCs to be immune privileged, others suggest that the MSCs are actually immune evasive via immune suppression, as discussed above [96]. Regardless of the mechanism,

the MSCs can effectively evade the immune system, minimizing the risk of rejection.

Possible confounds & unmet areas of investigation

MSC-based treatments require a large number of cells, with over 9×10^6 MSCs/kg being administered to some patients with GvHD [86]. This volume of cells will likely require expansion *in vitro*. Extended culture of MSCs *in vitro* may result in accumulation of mutations, which could result in the cells becoming malignant in patients.

Additionally, the infused MSCs could have damning effects on undiagnosed tumors, which could exist for years prior to diagnosis [97]. Specifically, MSCs have been reported to support and protect cancer cells [98,99]. Thus, if the patent has an undiagnosed tumor, or the injected MSCs contain undiagnosed tumor cells, there is likely to be an effect. This effect might depend on the subset of the cancer cells and the microenvironment. Since MSCs are immune suppressor cells, the MSCs could protect the tumor [100,101]. Conversely, MSCs could also support dormancy [102,103]. In this regard, the MSCs could prevent clinical progression of the cancer. This scenario needs to be forefront when designing treatment with MSCs. In the absence of such consideration, it is likely that in treating one condition with MSCs another unknown clinical condition could be exacerbated. Here, we propose that an extension of stem cell translation to patients should include the oncologists. One of the limitations in screening patients is that even with extensive patient screening, undiagnosed tumors can be missed. We therefore propose that patients treated with MSCs should be followed since the discussed risks cannot be ignored.

There is little information on the movement of MSCs following transplantation in humans. Although tracking studies were performed in animals, it is unclear how the findings can be extrapolated to humans. Safety studies in animals generally use healthy subjects [83]. These 'clean' models are different from clinical situations, where there could be an insult such as tissue injury. As discussed above, MSCs express receptors for several cytokines and chemokines and can therefore home and interact with their ligands within the microenvironment.

Healthy mice injected with MSCs showed undetectable cells after 48 h [83]. It is important to know if any of the injected MSCs can be found at a single site because this could lead to distinct biological effect. If there are MSCs lingering, even in small amounts, the capacity of MSCs to express MHC-II must be considered. Although MSCs normally express low levels of MHC-II, its expression can be upregulated by IFN-γ, which could occur in the case of viral infection [24,95,104]. If this occurs, localized GvHD could result. Additionally, other chronic types of inflammation, including those that could occur in obese individuals or the aging, may be sufficient to upregulate MHC-II. One could argue that low levels of MHC-II might be an advantage to induce tolerance to the MSCs. This however, has not been shown in experimental studies. The development of chimera in bone marrow transplantation has been well studied and understood. Scientists and physicians involved in regenerative medicine with stem cells could benefit from existing clinical knowledge.

The tissue from which the MSCs originate must also be considered. While the adipose and placenta are attractive tissue sources due to reduced ethical concerns, there are variations in the microenvironment that could affect the functions of the stem cells. In general, when adipose tissues are taken from an individual, this could be from individuals with high BMIs. In these cases, the MSCs are likely to be derived from an environment of inflammation. In this case, if the MSCs are used without expansion, their function could be different from MSCs coming from an individual who may be lean. Thus, when evaluating clinical trials with MSCs from adipose tissue, it is important to analyze the outcome in the context of sex of the donor, as well as BMI and age, among other parameters.

In the case of MSCs being expanded, it cannot be assumed that the cells will return to baseline functions. Although this could be the case, there is a lack of robust studies to prove if the licensed primary MSCs can revert to baseline activity. In addition, it is unclear if the microenvironment of the MSCs affects long-term multipotency [50,51]. Although placenta-derived cells are among the MSCs in clinical trial [105,106], there is additional caution with this source of cells since they are isolated from the placenta where the formation of syncytia or fusing is a normal occurrence. This is especially concerning given that placenta-derived exosomes contain syncytin-1 and syncytin-2, which are normally involved in trophoblast cell fusion for formation of the syncytium during placenta morphogenesis [107,108]. As far as we are aware, there is no study to address this issue, which is important to assure the safety of placenta-derived MSCs. It is important to note that recent clinical trials have not found negative effects of placenta-derived MSCs [105,106].

The most important issue for MSCs in drug delivery is their homing. Although there are studies using imaging to track MSCs, the scientific data remain unclear if all MSCs, regardless of the source, home to all organs. This is an important issue because if the engineered MSCs containing the desired drug are used to treat metastatic tumors, it would be important for the MSCs to home to the target organs. If the MSCs have a memory for the source organ, this could be a serious issue

because it might not be feasible to engineer different sources of MSCs for one patient. To be specific, if several sources of MSCs are needed, it is highly unlikely that MSCs from the bone marrow and adipose tissues will be from the same donors. This would result in the patient receiving MSCs with two different MHC types.

Conclusion

While there may be some yet unknown risks to MSC-based treatments, the clinical trials continue to show the benefit and safety of MSCs. Unmodified and engineered MSCs are nontumorigenic, and MSCs used to deliver anti-miRNA to tumors have been effective at treating glioblastoma, among other tumors, in animal models with specificity and minimal off-site toxicity [2]. Their unique properties of crossing allogeneic barriers and homing to specific regions – including the brain and areas of inflammation – make MSCs appealing candidates for drug delivery to selective regions. Further research is needed to elucidate which subpopulations of MSCs, based on chemokine receptors and/or tissue of origin, may be best for specific applications.

In the event that MSCs can show efficacy in delivering a drug with precise homing to the site of tissue insult, the question remains what happens to the MSCs after the drug is delivered. The fate of MSCs *in situ* is discussed above. These issues are needed to ensure that the MSCs do not linger for prolonged period. On the other hand, it might be desired to prolong the multipotent state for the MSCs to avoid reinjection of another set of MSCs. This question could be answered with more robust studies.

As MSCs are engineered to deliver drugs, these cells are moving within varied microenvironments *in vivo*. The MSCs, which are functionally plastic cells, could begin to respond to the microenvironment to produce other factors and small microvesicles. Thus, if a trial to deliver drugs seems to have negative results, the idea should not be abandoned; rather, it would be necessary to investigate the process experimentally to determine if the MSCs are induced to provide confounding factors. This would require additional engineering of the MSCs, perhaps in a third or fourth generation cell type.

Future perspective

The transplantation of MSC-based treatments will continue to grow in the clinical arena. It is expected that there will be successes and failures. Together, scientists will learn and use the information to improve how MSCs will efficiently be used in to treat patients. It is expected that the next decade will be focused on developing MSCs to deliver drugs and also use their function to continue to regenerate tissues.

As the science progresses in understanding the fate of transplanted stem cell, studies will be developed to determine if specific sources of MSCs would be needed for a particular treatment. As an example, presently, it is unclear if all sources of MSCs can be used for any specific disease. Future studies will need to answer this fundamental question. Specifically, what source of stem cell would be the most efficient for the particular application, for example, bone marrow- versus adipose-derived MSCs? The differentiation stage of the MSCs is important. In some cases, it might be important to

Executive summary

Mesenchymal stem cells

- Mesenchymal stem cells (MSCs) have an attractive property to be used as 'off-the-shelf' stem cells, making them available on demand. In addition, there are reduced ethical issues with MSCs, and relatively simple to expand. The 'plastic' immune properties of MSCs (immunostimulatory and immunosuppressive) provides them with a wide range of application. In addition to the above, MSCs can migrate to sites of tissue insult due to their ability to migrate to chemoattractants. At different tissues, MSCs could interact with other cells through the secretion of small vesicles and direct intercellular communication.

Mesenchymal stem cells as vehicles for drug delivery

- MSCs can deliver cytokine, protein, RNA and prodrug cargo to different organs, including sites of inflammation. The ease by which MSCs cross the blood–brain barrier makes it easy to deliver drugs for brain disorders, in particular brain tumors such as glioblastoma.

Alternative method of drug delivery

- Transplanted MSCs do not appear to linger in the patient, nor are they tumorigenic. Since *in vivo*, MSCs are in a microenvironment of three dimensions, bioengineering techniques need to be employed for efficient drug delivery.

Possible confounds & unmet areas of investigation

- Large number of MSCs are needed to treat patients, requiring prolonged manipulation *in vitro*. Although the current literature does not show prolonged lingering of MSCs in patients, this remains a concern since major histocompatibility complex class II could be expressed from allogeneic MSCs. A major issue is the ability of MSCs to support and protect cancer cells. This is of particular interest to undiagnosed cancer. A major question that needs to be answered is whether all sources of MSCs can home equally to a particular tissue.

use multipotent MSCs to deliver the drugs. In other cases, it might be desired to have the MSCs partly differentiated so that they can undergo senescence shortly after the drug is delivered. There is still the issue of what methods should be used to introduce the stem cells for the most efficient and safe outcomes.

As the use of stem cell progress into the clinic there would be an increased need to screen the recipients and perhaps the donors for cancer. This is particularly important because MSCs can interact with cancer cells. The screenings will be necessary to monitor MSC recipients for early signs of tumors before and after treatment: with proper monitoring before and while the MSCs are active, the risk of the MSCs supporting undiagnosed tumors can be minimized.

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Review
Special Focus Issue: Chemical Biology

Future Medicinal Chemistry

Chemical genetics and regeneration

Regeneration involves interactions between multiple signaling pathways acting in a spatially and temporally complex manner. As signaling pathways are highly conserved, understanding how regeneration is controlled in animal models exhibiting robust regenerative capacities should aid efforts to stimulate repair in humans. One way to discover molecular regulators of regeneration is to alter gene/protein function and quantify effect(s) on the regenerative process: dedifferentiation/reprograming, stem/progenitor proliferation, migration/remodeling, progenitor cell differentiation and resolution. A powerful approach for applying this strategy to regenerative biology is chemical genetics, the use of small-molecule modulators of specific targets or signaling pathways. Here, we review advances that have been made using chemical genetics for hypothesis-focused and discovery-driven studies aimed at furthering understanding of how regeneration is controlled.

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Regenerative biology explores how lost body parts, appendages, tissues or cells are replaced. Interest in regenerative processes extends to Aristotle's time; yet despite establishing experimental biology as a disciplined practice [1], regenerative biology has largely been limited to descriptive dissertations throughout much of its history. Today, with new genetic and imaging methodologies applicable to a wide variety of regenerative model species, the field abounds with fresh insights into the cellular and molecular mechanisms controlling regeneration. In addition, the advent of embryonic and induced pluripotent stem cells (ESC and iPSC, respectively) has spawned a new field, regenerative medicine, emphasizing the development of therapeutic strategies for reversing the course of degenerative diseases in humans.

Currently, there are two main approaches applied to regenerative therapeutics: first, transplantation of cells derived from differentiated stem cell cultures and; second, stimulation of the regenerative potential of endogenous stem cells to repair damaged tissues or replace lost cells. Within the field of chemical biology, testing and screening small-molecule modulators of molecular targets and signaling pathways have the potential to bridge these two approaches by revealing common mechanisms for controlling stem cells; in other words, pathways for regulating reprogramming/dedifferentiation, proliferation and differentiation of stem cell cultures and within the context discrete stem cell niches *in vivo*. Due to the comparative ease of *in vitro* testing, the vast majority of insights into stem cell biology using small molecules have come from efforts to increase reprogramming efficiency, maintain pluripotency or direct differentiation of ESC and iPSC cultures. Accordingly, many exceptional reviews have covered these topics [2–6] as well as concomitant advances in small-molecule chemistry [7,8]. In this review, we focus on contributions that chemical biology has made to classical regenerative biology, within the context of whole-organism screening in model species exhibiting robust reparative mechanisms. We will cover hypothesis-driven studies ('reverse' chemical genetics) and discovery-oriented screens ('forward' chemical genetics), emphasizing common paradigms and representative

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Key terms

Regeneration: The process of replacing lost tissues/cells.

Dedifferentiation: The process by which somatic cells can convert to a stem cell-like state, characterized by the upregulation of genes associated with an earlier stem/progenitor state and serve as a source of new cells during regeneration.

Differentiation: The process of cell fate acquisition in which a cell exits the cell cycle and expresses genes delineating a specific lineage and/or cell type.

Chemical genetics: The use of the chemical modulators to investigate the role of molecules and molecular signaling pathways in biological processes of interest.

studies for each strategy (Figure 1). In addition, we will discuss how the scale of injury likely affects the nature of the regenerative process and impacts model amenability to large-scale assay platforms. Finally, we will integrate how insights from this work could aid the development of regenerative therapeutics.

Chemicals & biology

The use of chemicals to modulate universal biological processes – such as mitosis (colchicine), transcription (actinomycin D) and translation (cyclohexemide) – has a long and productive history. Similarly, application of chemical modulators of more discrete molecular targets has been a common practice in multiple biological disciplines for decades (e.g., neurotransmitter inhibitors). However, systematic use of small molecules to probe gene function or to pursue large-scale drug discovery, in other words, 'pharmacological' [9] or 'chemical' genetics [10] necessitated the development of combinatorial chemistry. The ability to synthesize large libraries of chemical variants availed targeting of individual gene products with high specificity. Interestingly, an initial reductionist emphasis (one drug, one target) has recently evolved to embrace the reality of polypharmacology (one drug, multiple targets) as both a complication to overcome [11] and a potential advantage to leverage [12,13] in high-throughput screening (HTS).

Compared with genetic manipulations, chemical modulators provide several significant advantages: first, temporal control – the ability to limit compound exposures to specific stages or reverse effects upon 'washout'; second, graded responses – titrations can be used to elicit dose-dependent effects, inducing phenotypes akin to an allelic series of genetic mutants and third, refractory to redundancy or genetic compensation – modulators acting on homologs, common downstream signaling molecules, or even entire gene families can circumvent issues arising with single gene manipulations [14]. When applied as a platform for discovery, large-scale chemical screens can reveal new insights into almost any biological process of interest. However, one of the confounding factors associated with chemical-based approaches is nonspecificity due to: first, effects on multiple members of a protein family or, second, modulations of pathways other than the intended target. The first issue can actually be viewed as a strength, circumventing genetic compensation/redundancy issues by using a pan-family modulator to target an entire class of proteins. As a test for specificity of observed effects, both issues may be addressed by either testing multiple modulators of the implicated protein/pathway or using dose-response strategies and titrating to a level that promotes specificity of binding. Nevertheless, questions of specificity may cloud interpretations of chemical biology assays and efforts to allay this concern should be pursued vigorously when this methodology is a central component of a study.

As mentioned above, the vast majority of chemical biology screens have utilized cell culture systems. This has the advantages of straightforward treatment regimens and reduced toxicity compared with *in vivo* systems. However, despite their simplified nature, reductionist approaches have not been particularly successful for drug discovery [15]. Conversely, serendipitous discovery of compounds eliciting specific phenotypic effects – in other words, phenotypic screening has played a long and storied role in drug development [16]. To adapt this approach to chemical genetics, several groups have begun to perform large-scale drug screens directly in living animal models [17–22] (see Table 1 for a list of the research discussed below and additional studies). Here, we focus on recent applications of chemical genetics to regenerative biology spanning organismal, appendage, organ and cellular replacement paradigms.

Regenerative biology & chemical genetics

It is often said that 'regeneration recapitulates development'. Indeed, regenerative paradigms involve developmental signaling pathways regulating the proliferation, differentiation and patterning of stem cells and their progeny [23]. Classical genetic approaches to studying regeneration are somewhat stymied therefore by the need to implement conditional approaches, such as temperature-sensitive screens, to circumvent lethal or altered morphology phenotypes. Thus, a key advantage of applying chemical versus classical genetics to regenerative biology is that it more readily facilitates temporal dissection of the roles played by developmental signaling pathways. However, the particular cellular mechanisms used to replace lost tissue can show remarkable context specificity, both across species and between different organs within the same species, some of which are not typically associated with development (Table 2). Moreover, environmental factors that shaped developmental

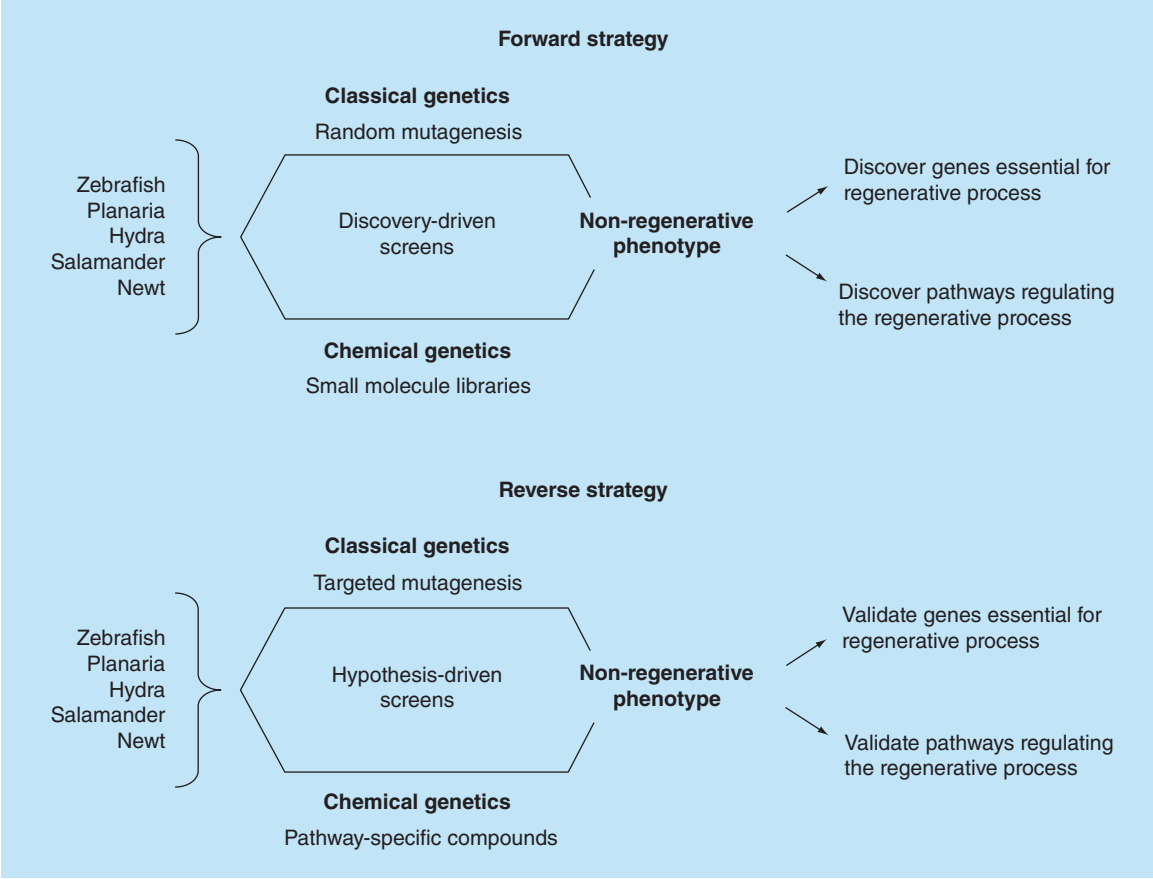

Figure 1. The forward strategy is a discovery-driven screening approach whereby genes/chemicals are randomly tested for effects on a phenotype of interest – the emphasis is on disrupting the phenotype first, then determining which genes/pathways are involved. The reverse strategy is a hypothesis-driven approach that uses prior knowledge to select a given gene/pathway to investigate regarding a regenerative paradigm of interest – the emphasis is on disrupting a gene/pathway first (either by knocking it down or inhibiting function using small-molecule inhibitors) and determining phenotypic effects secondarily.

events are unlikely to be maintained at more mature stages. Thus, it is important to keep in mind that much remains to be discovered; in other words, cellular and molecular mechanisms at play during regenerative processes may be discrete from developmental pathways. A broader understanding of the combinatorial interactions among components of discrete stem cell niches and between implicated signaling pathways should help to define ways to stimulate endogenous repair mechanisms in humans. In the following sections, we will highlight studies that have applied chemical genetics in regenerative model species to reveal molecular factors that impinge upon regenerative processes. We begin by discussing studies that have used hypothesis-driven reverse chemical genetics to explore molecular mechanisms controlling regeneration.

Reverse chemical genetics

Organismal regeneration: planaria

A handful of remarkable species are able to regenerate completely after being transected. For instance, planaria can completely regenerate from small fragments containing stem cells known as neoblasts [24]. Moreover, transplanting a single neoblast cell, the clonogenic neoblast, can rescue a lethally irradiated host [25]. Following injury (e.g., bisection), neoblasts respond by proliferating and migrating toward the wound site, giving rise to progeny that form the regenerative blastema [26], a group of dedifferentiating mesenchymal cells that aggregate beneath the injury site following wound healing. Surviving cells also undergo remodeling to integrate with the newly generated cells. The molecular signaling events regulating this process have yet to be fully characterized. Early key mechanistic hypotheses were developed in planaria by applying anesthetics and inhibitors of respiration, mitosis or protein synthesis, demonstrating the value of chemical biology to regenerative paradigms [27]. More recently, studies using long-term RNAi have implicated classical developmental signaling pathways (e.g., BMP, Hedgehog, Wnt) in regulating patterning during regeneration and numerous genes in modulating neoblast proliferation [28–30].

Table 1. Forward genetic studies.

Tissue	*In vivo* or *in vitro*	Model system	Number of compound	Identified targets	Ref.
Bone	*In vitro*	Immortalized murine osteoblast cell line	30,000	Statins	[127]
	In vitro	Myoblast with BMP2 treatment	5405	Rapamycin and FK-506	[128]
	In vitro	Preosteoblastic MC3T3E1 cells by expressing GFP	2500	Glabrisoflavone (GI)	[156]
	In vitro	Mesenchymal stem cells	1280	Raf–MEK–ERK pathway targeting osteogenic factors	[130]
Fin	*In vivo*	Wild-type larval zebrafish	2000	Glucocorticoids	[125]
	In vivo	Transgenic larval zebrafish	520	Imidazoline receptor antagonist	[126]
Heart	*In vitro*	Pluripotent mouse stem cell line (P19CL6)	147,000	Sulfonylhydrazones	[132]
	In vitro	Mouse embryonic stem cell line	550	Wnt inhibitor	[134]
	In vitro	Mouse embryonic stem cell-derived cardiomyocyte	280	Inhibitors of glycogen synthase kinase-3, p38 mitogen-activated protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II and activators of extracellular signal-regulated kinase	[133]
Hair cell	*In vivo*	Multiple larval zebrafish transgenic line	1680	Topoisomerase activity and cell cycle	[135]
	In vivo	Wild-type and multiple larval zebrafish transgenic lines	470	Fucoidan	[136]
Pancreas	*In vivo*	Zebrafish transgenic line	7186	Adenosine pathway	[145]
	In vivo	Zebrafish transgenic line	3131	Retinoic acid and GTP	[150]
	In vivo	Zebrafish transgenic line	833	Retinoic acid, serotonergic signaling, glucocorticoids	[151]
	In vivo	Zebrafish transgenic line	Over 500,000	NF-κB pathway	[152]
	In vitro	Primary rodent and porcine islet β cells	850	Adenosine	[148]
Muscle	*In vivo*	Zebrafish sapje and sapje-like fish	1120	Aminophylline	[143]
	In vivo	Zebrafish sapje	640	Fluoxetine	[142]

Pharmacological inhibitors of candidate signaling pathways have been used to complement RNAi studies. For instance, Tasaki *et al.* used the MAPK/ERK kinase (MEK) inhibitor U0126 to demonstrate that reductions in ERK signaling maintained blastemal cells in a proliferative state, thus blocking differentiation. This effect could be rescued by knocking down expression of a MAPK phosphatase (*mkpA*) with RNAi [31], presumably by enhancing residual ERK activity. The same group has shown that ERK activity specifically promotes 'head' differentiation during regeneration, acting in opposition to posterior Wnt signals [32]. MEK inhibition also demonstrated that crosstalk between ERK and Wnt signaling is necessary for regeneration of the pharyngeal apparatus – in other words, the area between the head and tail regions. Interestingly, using a different MEK inhibitor (PD 98059), Ermakov *et al.*, found that proliferation outside the blastema is actually reduced [33], but saw similar disruptions in head differentiation.

Furthermore, chemical genetics experiments in planaria have revealed a role for gap junctions during regeneration. Gap junction (GJ) proteins are specialized channel proteins located in the plasma membrane and essential for cell–cell communication. In planaria, the innexin family of GJ proteins (invertebrate

homologs of connexins) consists of at least a dozen members expressed in semioverlapping domains [34]. Thus, to completely disrupt GJ function during regeneration would require the coordinated action of multiple RNAi oligos. Alternatively, a single pan-innexin chemical inhibitor that disrupts the entire innexin family, such as heptanol, can suffice to block all GJ communication. Nogi and Levin applied this method to explore the role of GJ communication in planarian regeneration [35]. Transient exposures to heptanol during the first 2 days of regeneration following amputation resulted in 'anteriorization' of the posterior blastema, characterized by a lack of tail regeneration or the appearance of second head in the posterior segment. This study not only discovered a critical role for innexins in anterior–posterior (AP) polarization during regeneration but also demonstrates an important advantage of chemical biology, and one that is typically seen as a complication to be surmounted: nonspecificity. Here, a single chemical reagent was used to disrupt an entire protein family to achieve the desired effect on signaling, an outcome that would have been difficult to achieve with genetic manipulations due to redundancy, compensation and/or combinatorial applications of gene knockdown toolsets. This study was followed up by Oviedo *et al.* to explore how anteriorized regenerative structures reacted to subsequent amputations [36]. To induce anteriorization, they used timed exposures to an optimized dose of another GJ inhibitor, octanol, which blocked only a subset GJ types but allowed normal neoblast function. When ectopic heads were reamputated up to 6 weeks later, thus in the absence of any molecular manipulation, regenerated structures retained the respecified head morphology. This study thus demonstrated that a brief disruption of GJ communication is sufficient to induce persistent physiological alterations in patterning of regenerated structures without alteration of the genome, a remarkable observation with far-reaching implications.

Similar studies have examined the role of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMP) in extracellular matrix (ECM) remodeling during regeneration [37]. Balestini *et al.* found that the natural alkaloid berberine could disrupt planarian head regeneration, resulting in malformation of visual system, while tail regeneration proceeded normally [34]. BrdU-labeling and anti-pH3 staining indicated the berberine effects were not through alterations in cell proliferation, instead berberine significantly reduced expression of *Dj-mmp1*, *Dj-mt-mmpa*, *Dj-ast4* and *Dj-timp*. This finding provided direct evidence that MMPs and TIMPs are important to the regenerative process in planaria.

In addition to enabling studies on biochemical signaling, chemical genetics also facilitates investigation of biophysical signaling events that coordinate regeneration. A recent study by Beane *et al.* explored the effect of ionic gradients on axial polarity during planarian regeneration using ion transport inhibitors to modulate membrane voltage [38]. Exposure to the compound SCH-28080 specifically inhibited H,K-ATPases (ion transporters responsible for depolarizing the anterior blastema during regeneration), induced hyperpolarization and resulted in a headless regener-

Table 2. List of animal models with regenerative ability.

Animal model	Regenerative capacity	Adaptable for HTS	Ref.
Invertebrates			
Hydra	All tissues	Yes	[157]
Planaria	All tissues	Yes	[158]
Drosophila	Imaginal discs	Yes	[159]
Cockroach	Leg	Yes	[160]
Vertebrates			
Newts	Limbs, tail, heart, lens, spinal cord, brain, jaw, retina, hair cells of the inner ear	No	[161]
Frogs	Premetamorphic limbs, tail, retina, lens, hair cells of the inner ear	No	[162]
Zebrafish	Fins, tail, heart, liver, spinal cord, hair cells of inner ear, lateral line	Yes	[163]
Chicks	Hair cell of the inner ear	No	[164]
Mice	Liver, digit tips	No	[165,166]
Deer	Antler	No	[167]

HTS: High-throughput screening.

ate. The researchers hypothesized that the effects of membrane voltage manipulations might be mediated via changes in calcium flux. Indeed, inhibition of L-type voltage-gated calcium channels (using nicardipine) also resulted in reductions in head regeneration. Conversely, modulation of chloride flux (using ivermectin to keep glutamate-gated chloride channels open), or activating voltage-gated calcium channels (using praziquantel), resulted in a two-headed phenotype [39]. In addition, H,K-ATPase was shown to regulate left–right asymmetrical patterning [40] and apoptosis, thus disrupting remodeling in the head [41]. Chemical modulators leading to headless or two-headed phenotypes induced corresponding changes in the expression of anterior/posterior transcriptional factors in the blastema. Collectively, the data suggest that membrane voltage reiteratively comes into play during planarian regeneration to regulate several key steps including: specifying polarity, apoptosis, remodeling, proliferation and transcription.

Epimorphic regeneration: appendage replacement

Appendage regeneration (e.g., limbs, fins, digits, etc.) proceeds through fundamental stages of wound healing, blastema formation and patterning [42]. Lineage-tracing studies have generated insights into blastema formation. Interestingly, the blastema retains spatial memory, establishing a proximal/distal axis early on and maintaining it throughout the regenerative process [42,43]. Multiple studies have used chemical modulators to reveal key developmental signaling pathways, such as Wnt and Fgf [44], that are important for blastema formation and proximal/distal axis maintenance.

The Levin group has utilized chemical genetics to explore the role of ion currents in tail regeneration in *Xenopus laevis* utilizing an inhibitor of voltage-gated sodium channels (called tricaine or MS222) [45]. Sodium channel blockade inhibited tail regeneration, revealing that sodium ion influx was critical for a successful regenerative response. Their study further demonstrated that sodium influx was important during initial stages of repair as exposure in the first few hours after injury was sufficient to prevent regeneration. A screen for the presence or absence of known signaling pathways markers further demonstrated that inhibition of sodium ion influx impaired regeneration by modulating Notch and Msx1 induction, thus correlating biophysical signaling with biochemical signaling during appendage replacement. Moreover, transient activation of sodium ion influx during the wound-healing stage using the chemical activator monensin resulted in enhanced regeneration during a refractory period where regeneration does not normally occur. This study demonstrated that stage-specific modulations are important for identifying enhancers of regeneration as well as to decipher sequences of signaling events during regenerative processes. It also highlights a key advantage to using chemical as opposed to genetic modulators: improved temporal control over pathway manipulations.

Another attractive platform to study regeneration is the zebrafish caudal fin. In 1995, Johnson and Weston described an ENU-directed genetic screen for mutations that inhibit adult tail fin regeneration [46], demonstrating the advantages of applying forward screening strategies to regeneration in zebrafish (see below for further discussion). Since then, multiple studies have utilized transgenic/mutant fish and chemical modulators to reveal specific roles for signaling pathways such as Wnt [47], FGF [48] and Notch [49].

Both adult and larval zebrafish are capable of regenerating fins. Many markers for each phase of regeneration, such as *dlx5* for wound epithelium and *msxE* for blastema, are also conserved between different appendage regeneration models [50,51]. In addition, *raldh2*-mediated retinoic acid (RA) signaling regulates several appendage/tissue regeneration paradigms [52,53]. A chemical genetics study used the timing of *raldh2* expression in the blastema of the regenerating larval fin to investigate a panel of molecular regulators of appendage regeneration. However, not only does chemical genetics identify signaling pathways involved during regeneration but it can also reveal the hierarchy of signaling interactions across pathways. For example, inhibitors of FGF (SU5402) and ERK (UO126) block larval fin regeneration. However, RA coexposure rescued the effects of these pharmacological inhibitors, suggesting that RA signaling acts downstream of FGF and ERK during regeneration [54].

A more recent chemical genetics study sought to reveal additional signaling pathways involved in cell proliferation and migration responses postinjury [55]. Inhibitors of different oncogenic pathways were tested in the larval fin regeneration model by looking for any change in the proliferative response following amputation. Interestingly, p38 and MEK1 inhibition resulted in an increase in proliferation while PI3K and ErbB inhibition caused a decrease in proliferation within the wound epithelium and blastema; these effects were additive when fish were treated concurrently with inhibitors to both pathways. In addition, ErbB/PI3K inhibition could also abolish migration of cells into the blastemal region. Finally, ErbB impairment arrested regeneration when fish were exposed at later stages, suggesting the ErbB is required for proliferation throughout of the regenerative process. Together these

data imply that ErbB and PI3K interact functionally to impact cell proliferation and cell migration during regeneration. These and a host of similar studies have demonstrated the usefulness of chemical genetics for revealing new insights into signaling pathways involved in epimorphic regeneration.

Tissue regeneration

Appendage regeneration involves the replacement of complex multitissue structures. It is therefore reasonable to assume that epimorphic regeneration largely follows a developmental program. Conversely, tissue regeneration is restricted to a more discrete landscape, sometimes involving a single stem cell niche. Accordingly, it is less clear to what extent tissue regeneration 'recapitulates development' or whether pathways specific to the regenerative process are called into play as well. Below we will examine how chemical genetics has determined some of the key signaling mechanisms involved in two important tissue regeneration models.

Bone

Bone loss is a common health problem incurred as a result of injuries, aging and disease. Human bones can regenerate after injury following a well-characterized healing and remodeling process [56,57]. However, when large quantities of bone mass are lost, our regenerative capacities can be outstripped. Better understandings of the mechanisms regulating bone regeneration are thus needed to facilitate more effective bone repair.

It is known that crosstalk between osteoblast and osteoclast cells maintains bone homeostasis [58]. Many transcription factors (e.g., *sox9*, *runx2*, *osx*, *atf4*, *ap1*) and signaling pathways (Hh, Wnt, Notch, BMP, FGF) are critical for osteoblast differentiation and thus may be useful therapeutically [59–61]. For instance, recombinant BMP proteins have been used to treat bone disease [62]. However, recombinant proteins have multiple limitations, which restrict their application [63]. Accordingly, small molecules are being used to target pathways regulating bone regeneration, such as osteoblast differentiation [63,64].

Osteogenesis in zebrafish scales and mammalian bone utilizes similar signaling mechanisms [65]. Based on previous studies implicating Wnt/Sp7 interactions in osteoblast differentiation [66], De Vrieze *et al.* screened a small library of Wnt modulators in an *ex vivo* zebrafish scale culture model [67]. For this, they developed a transgenic line in which luciferase was driven by the Sp7/osterix promoter, enabling screens for factors promoting osteoblast differentiation. In their proof-of-principle study, they accurately predicted the effects of 70% of characterized Wnt modulators and identified riluzole, genistein and niclosamide as having strong osteogenic activity [67]. It will be extremely interesting to learn how well these findings 'translate' to mammalian model systems as this particular system is well-suited to large-scale forward discovery screens (see below).

Heart

Amphibians [68], fish [69–71] and neonatal mice [72] have the ability to regenerate heart tissue after injury; however, human cardiomyocytes have an extremely limited capacity to regenerate following injury or disease [73]. Thus, finding ways to enhance this ability in humans has garnered a great deal of attention. Importantly, zebrafish heart regeneration also involves proliferation of cardiomyocytes postinjury, thus providing a model to reveal pertinent signaling pathways. Using a fluorescent ubiquitination-based cell cycle indicator (FUCCI) system, Choi *et al.* identified several compounds that modulate cardiomyocyte proliferation in zebrafish embryos. In particular, they found that the Hh, Igf and Tgfβ pathways all stimulate cardiomyocyte proliferation during development [74]. They further demonstrated that these compounds also have similar effects on cardiomyocyte proliferation during heart regeneration. Similarly, Huang *et al.* and Chablais and Jazwinska found roles for Igf and Tgfβ, respectively, during heart regeneration.

Using NVP-AEW-541, a pharmacological inhibitor of the Igf1 receptor, Huang *et al.* demonstrated that inhibiting Igf signaling impairs cardiac regeneration by inhibiting proliferation of cardiomyocytes [75]. Specifically, Igf signaling appears to play a critical role in regulating proliferation of a subpopulation of gata4:GFP-labeled subepicardial cardiomyocytes postinjury. This subpopulation migrates to the wound site and proliferates, and is believed to be a primary source for new cardiomyocytes during heart regeneration [76]. Thus, Igf signaling is implicated in controlling the regenerative potential of the heart by modulation of a subset of cells that act as cardiomyocyte stem cells.

In addition, the Tgfβ pathway regulates three discrete aspects of heart regeneration. Using the compound SB431542 to block signaling from Tgfβ-type I receptors, Chablais and Jazwinska found cardiac regeneration was disrupted following cryoinjury. To dissect the function of Tgfβ signaling at different stages of the reparative and regenerative processes, they limited exposure to SB431542 to three discrete windows of time [77]. This strategy demonstrated that Tgfβ signaling was essential: initial scar formation – revealing this temporary collagenous tissue at the early-stage postdamage (14 dpci: 14 days postcryoinjury). Besides for collagen, it was also required for the deposition of other ECM, such as fibronectin and tenascins, which

are essential for ECM remodeling. A short-period Tgfβ inhibitor exposure after a 7-day recovery showed a significant reduction of proliferating myocytes in the boundary of the injury, which indicated Tgfβ had stimulating role in cardiomyocyte proliferation.

While individual signaling pathways may have discrete effects on regenerative processes, it is important to understand how multiple pathways integrate following injury as well. Based on previous studies reporting that either FGF1 treatments or p38MAK inhibition can decrease cardiomyocyte apoptosis in ischemic heart disease [78,79], Engel *et al.* investigated the result of combining p38MAPK inhibition with exogenous FGF1 [80]. Their study revealed that in an acute myocardial injury, combining FGF1 and p38MAPK inhibitor treatments increased cardiomyocyte proliferation as well as improved and extended cardiac function compared with administration of FGF1 or p38MAPK inhibitors alone.

Cellular regeneration

While tissue and appendage regeneration paradigms have clear clinical significance, the majority of diseases associated with the promise of stem cell biology are degenerative or autoimmune disorders involving the progressive loss of specific cell types (e.g., Parkinson's disease). The study of cellular regeneration, as a distinct regenerative biology paradigm, will therefore be important for defining cell-specific stem cell niches and for discovering mechanisms that regulate endogenous stem cell responses to selective cell death.

Hair cells

Hair cells are the primary sensory neurons of the auditory system and later line organ (in fish). In mammals, it has been assumed that lost hair cells are not replaced – thus, deafness due to hair cell loss is currently considered irreversible in humans. Intriguingly, a limited amount of regeneration has been seen in recent studies in mice [81,82]. In some nonmammalian systems, such as birds and fish, lost hair cells are readily replaced by surrounding cells called supporting cells [83–85]. Hair cells can be replaced by two distinct mechanisms: first, nonproliferative – direct transdifferentiation of support cells into hair cells [86] and/or, second, proliferative – mitotic expansion of support/progenitor cell pools and subsequent differentiation of progeny into hair cells [87,88]. Zebrafish larvae regenerate lateral line hair cells rapidly after damage, with almost all hair cells being replaced after 72 h [89]. Thus, zebrafish larvae are an excellent model system for applying chemical genetics to hair cell regeneration [90]. For instance, to explore cellular mechanisms involved in hair cell regeneration, Mackenzie and Raible inhibited cell division using a small-molecule inhibitor of microtubule assembly [83,91]. They demonstrated that blocking proliferation inhibited regeneration, in turn revealing that transdifferentiation could not compensate for disrupted support cell proliferation. Similarly, another group investigated if support cells underwent chromatin remodeling when transitioning from a quiescent to proliferative state [92]. Application of valproic acid and trichostatin A (TSA) showed that inhibition of histone deacetylase (HDAC) activity suppressed support cell proliferation, demonstrating the importance of HDAC in hair cell regeneration.

Small-molecule screens are particularly useful for exploring the role of developmental signaling pathways in regenerative processes. Several groups have utilized this approach to investigate the role of the Notch and Wnt pathways in hair cell regeneration [89,93]. For instance, following neomycin-induced hair cell ablation, pharmacological inhibition of Notch signaling (using the γ-secretase inhibitor DAPT) promoted SC proliferation and resulted in a concomitant increase in the number of regenerated hair cells [89]. The authors went on to show that DAPT acted specifically on a subpopulation of 'internal' support cells suggesting the existence of functionally distinct subtypes of support cells. In a similar study, Head *et al.* utilized a GSK3β inhibitor, 1-azakenpaullone (Az), to ask if Wnt activation could stimulate SC proliferation during hair cell regeneration. Following neomycin treatment, Az exposure led to elevated proliferation of support cells and an increase in the numbers of differentiating hair cells [93]. Collectively, these and related studies have demonstrated the power of chemical genetics for reveal critical insights into the regulation of regenerative process, such as hair cell regeneration.

Retinal cells

The retina, being an extension of the CNS, displays a woefully limited capacity for regeneration in mammals. Thus, the primary aim of cell-based retinal therapies is to provide the eye with functional replacements for cell types lost to disease or injury. This could be achieved either by transplantation of retinal neurons obtained by *in vitro* differentiation of stem cells, or by stimulating endogenous repair mechanisms. Although mammals do not have persistent retinal neurogenic sources, this capacity has been preserved in amphibians, chicks and fish. Four retinal stem cell niches have been described: first, the ciliary marginal zone (CMZ), a region at the circumferential perimeter of the retina that is responsible for annular growth but which normally does not contribute substantially to the regenerate; second, the retinal pigment epithelium (RPE), which in the birds and amphibians has been shown to undergo transdif-

ferentiation to give rise to new retinal cells; third, rod-committed progenitors, localized in the outer nuclear layer and believed to be committed to the rod photoreceptor lineage and fourth, Müller glia (MG), the primary glial cell type of the retina which responds to injury and currently represents a potentially conserved retinal stem cell across vertebrate organisms [94,95]. Another intriguing possibility for restoring vision, particularly relevant to chemical biology, involves a novel approach using light-activated photoswitch chemicals to convert retinal ganglion cells (RGCs) into transducers of light. This strategy has recently been applied to restore visual responses in blind rd1 mice lacking photoreceptors [96,97].

Intriguingly, although mammalian MG do not normally enter the cell cycle after retinal injury, in cell culture – or when treated with certain growth factors *in vivo* – (MG) appear to retain the potential for repair [98]. Primary human MG cells grown in defined culture conditions have been shown to differentiate into both photoreceptor cells and RGCs. Moreover, transplantation of rod photoreceptor precursors and RGC precursors derived from human MG cell cultures can successfully integrate into the host retina, restoring function in P23H rats exhibiting slow rod degeneration and in Lister hooded rats where RGCs were damaged by NMDA injection, respectively [99,100]. Immortalized MG cell lines derived from the adult human retina can also differentiate into retinal neurons [101]. On transplantation, these cells showed better migration in the neonatal retina of Lister hooded rats than into the dystrophic retina of the RCS rat indicating developmental cues may be critical for integration. These studies clearly indicate that human MG retains the capacity to replace lost retinal cells. Therefore, understanding how the regenerative potential of MG cells is regulated will be key to developing therapies seeking to stimulate endogenous repair mechanisms in the human eye.

Teleosts (ray-finned fishes) display a robust capacity to replace lost retinal neurons following a range of injury paradigms such as acute light lesion [102], surgical lesion [103] or cell-specific ablation [104–107]. Initially, progenitor cells in the outer nuclear layer were thought to be the only source of regenerating cells in teleosts. However, studies utilizing transgenic fish with GFP-labeled MG revealed that the primary injury-responsive retinal stem cell in the zebrafish was the MG [108–110]. Moreover, it was revealed that MG gives rise to outer nuclear layer progenitors, which are thought to be restricted to the rod cell lineage. Although MG are normally quiescent, responsible predominantly for maintaining general homeostasis, they can be induced upon injury to dedifferentiate to a stem-like state, reenter the cell cycle, and give rise to progenitor cells which differentiate to replace lost neurons. Unfortunately, in mammals, MG normally responds to injury by entering reactive gliosis [111]. Thus, retinal regeneration researchers are focused on delineating how dedifferentiated stem cell activation and reactive gliosis differ; to define mechanisms that stimulate beneficial versus deleterious MG responses to retinal injury. A series of excellent recent reviews have covered the current state of understanding of the MG stem cell niche [94,112,113]. Here, we will focus on how the use of chemical genetics has revealed important clues into how the regenerative potential of MG cells is controlled.

As Wnt signaling is central to numerous biological processes, Ramachandran *et al.*, investigated the role of β-catenin, the central signaling molecule in the Wnt signaling pathway, during retinal regeneration [114]. They observed that following a retinal stab wound, β-catenin, accumulated in the nucleus of MG and MG-derived progenitors. Using chemical genetics – pyrvinium (a casein kinase 1-α activator) or XAV939 (a tankyrase inhibitor) – to block β-catenin accumulation, resulted in a reduction in proliferation of MG-derived progenitors [115]. This suggested that β-catenin was required for the proliferation of retinal progenitors during regeneration. They further tested the role of Wnt/β-catenin by enhancing signaling using lithium chloride (LiCl), a GSK-3β inhibitor that prevents β-catenin degradation. In response to LiCl injection, the number of proliferating cells increased. Remarkably, LiCl injection stimulated proliferation in both the injured and uninjured retina, and progenitors in the uninjured retina gave rise to multiple retinal neuron subtypes.

Meyers *et al.*, further explored the role of Wnt/β-catenin in the CMZ during development and in MG cells during regeneration using an intense light lesion paradigm that limits cell loss to photoreceptors [116]. Using timed administration of a GSK-3β inhibitor (Az) during retinogenesis, they found that when Wnt activation was initiated at 36 h postfertilization (hpf), neuronal differentiation was blocked and progenitors were maintained in a proliferative state. Consistent with this, inhibition of Wnt (XAV939) at 3 days postfertilization (dpf) resulted in a loss of progenitor cell markers and a decrease in proliferation in the CMZ. However, exposure to Az at 6 dpf was not sufficient to induce proliferation in the central retina of larval fish, in contrast to injection of LiCl in adults. Finally, upon light lesion, treatment with Az from 1–5 or 3–5 days postlesion (dpl) led to a selective decrease in the number of proliferating MG cells (outer nuclear layer progenitor proliferation was unchanged). However, no effects were seen when treatments were limited to 0–3 dpl. This suggested that Wnt signaling is not required for MG activation but does

Key term

Sheddases: Enzymes that can cleave extracellular components of transmembrane proteins resulting in release of the ectodomain.

alter subsequent proliferation patterns. The authors went on to show that Wnt activation blocked asymmetric, self-renewing, divisions of MG following a light lesion, instead driving all daughter cells toward a progenitor fate and diminishing the number of MG cells. Together with Ramachandran *et al.*, the data suggest that Wnt/β-catenin plays a central role in controlling either dedifferentiation and/or proliferation of MG cells in zebrafish. In contrast, Zhu *et al.* recently showed that, in the chick, loss of nuclear β-catenin is correlated with proliferation of ciliary margin cells and RPE-derived progenitors following retinectomy [117]. Collectively, these studies highlight how differences between species, across injury paradigms, among alternative stem cell niches, and/or regarding the compounds used (with respect to chemical genetics), may have a profound impact on interpretations of the role specific signaling pathways play in regenerative processes.

To further characterize factors involved in retinal regeneration, Wan *et al.* hypothesized that MG secrete factors that stimulate their own dedifferentiation through activation of genes such as *ascl1a* [118]. To identify such factors, they screened for EGFR ligands that were upregulated following stab wounds. HB-EGF (heparin-binding epidermal-like growth factor) was the only ligand highly induced as early as 1 h postinjury. Knockdown of this gene led to a reduction in proliferating MG-derived progenitors while intravitreal injection of HB-EGF led to increased numbers of progenitors in the injured and uninjured retina, respectively. HB-EGF is released by ectodomain shedding, thus inhibition of sheddases by GM6001 (a pan metalloproteinase inhibitor) was used to further test the role of HB-EGF. GM6001 prevented proliferation of MG-derived progenitors following injury, suggesting that HB-EGF (or other factors activated by ectodomain shedding) was required for dedifferentiation. Since EGFR activation is associated with MAPK signaling [119], the authors investigated the role of EGFR signaling using pharmacological inhibitors of EGFR (PD153035), MAPK (ERK1 and 2 inhibitors, PD98059 and SL327). Their results demonstrated that inhibition of the EGFR signaling by any of these reagents reduced the number of proliferating progenitors by as much as 75%. This study highlights one of the potential drawbacks of chemical genetics; the lack of discrete downstream signaling molecules (e.g., the MAPK cascade and receptor tyrosine kinases) can cause ambiguity when using modulators of these factors. Nevertheless, applying multiple modulators, as exemplified by Wan *et al.*, can largely circumvent this issue.

As Notch signaling is an important regulator of retinoblasts during development, several groups have investigated whether Notch also impacts retinal regeneration. Following a stab wound injury, exposure to DAPT (an inhibitor of Notch/γ-secretase activity) led to an increase in proliferation at the injury site [118]. Similar observations were made using an improved γ-secretase inhibitor (RO4929097) following light lesioning of photoreceptors [120]. In addition, injection of RO4929097 to the uninjured eye was sufficient to stimulate MG proliferation (in contrast to DAPT) [118]. This is in keeping, however, with a study showing that sustained Notch activation is necessary to maintain glial fates in early postnatal MG cells in the developing mouse retina [121]. Thus, in fish, perhaps MG cells are predisposed to dedifferentiation with sustained Notch signaling being necessary to maintain their glial identity. In the stab wound paradigm, the effect of inhibiting Notch signaling could be negated by a loss in EGFR signaling, as MAPK or EGFR inhibition suppressed the effect of DAPT. In further studies, Ramachandran *et al.* demonstrated that *insm1a* was expressed in MG-derived progenitor cells and its suppression also resulted in an increase in the number of progenitors [122]. Therefore, the authors explored interactions between *insm1a* and Notch signaling. Using DAPT, they demonstrated that Notch was upstream of *insm1a*; exposure to DAPT abolished *insm1a* expression upregulation during regeneration. The authors argued that inhibition of HB-EGF expression by Notch-dependent *insm1a* upregulation may serve as a feedback mechanism to limit the zone of dedifferentiating MG cells. To test this, they inhibited EGFR signaling (PD158780) in an *imsm1a*-depleted retina, and showed that this prevented the expansion of MG-derived progenitor cells. These results suggest that interactions between HB-EGF, Notch and Insm1a define the zone of responsive MG cells at the site of stab wound retinal injuries.

The Jak/Stat (Janus kinase/Signal transducers and activators of transcription) signaling pathway is a transduction cascade for many growth factors and cytokines. Stat3 is expressed in MG cells following injury and, combined with Ascl1a immunolabeling, has been used to delineate three distinct populations of MG: Stat3-expressing Ascl1a-negative quiescent cells, Stat3-positive Ascli1a-positive proliferating cells and Stat3-negative Alsc1a-positive proliferating cells [123]. More recently, Zhao *et al.* utilized the stab wound model to explore roles for Jak/Stat signaling in MG activation during retinal regeneration. Chemical inhibitors of Jak/Stat signaling (JSI-124 and P6) sup-

pressed the generation of MG-derived progenitors in the injured retina by preventing induction of *ascl1a* expression. Timed exposures to inhibitors at 0–2 dpi and 2–4 dpi revealed that Jak/Stat signaling was not only critical for formation of progenitors but also for their expansion later on, demonstrated by a lack of BrdU-positive cells in injured fish retinas. These studies suggest that Jak/Stat signaling regulates MG activation and progenitor cell expansion during retinal regeneration.

Wan *et al.*, recently explored the role of insulin, IGF-1 and FGF signaling components in inducing MG cell dedifferentiation [103]. Insulin expression is increased in proliferating MG-derived progenitors following a stab wound injury, and knockdown caused a reduction in the number of proliferating MG-derived progenitors. Injection of insulin into the uninjured eye resulted in a dose-dependent increase in proliferation. Interestingly, synergistic effects were seen when ineffective concentrations of insulin and HB-EGF (or IGF-1 and FGF2) were injected as a pair. Based on the fact that knockdown of Igf signaling components (Igfra or Igfbp3) also resulted in a decrease progenitor cells, the authors explored the role of downstream signaling, PI3/Akt, using the pharmacological inhibitors LY294002 and PI-103. Both inhibitors reduced the number of proliferating progenitor cells postinjury, and when HB-EGF, insulin, or IGF-1/FGF-2 were injected into the uninjured eye – as did inhibition of MAPK (UO126) or β-catenin (pyrvinium). This last result is unexpected, as the molecules used to stimulate MG activation are not predicted to act through all four of the signaling cascades being pharmacologically inhibited. Moreover, it suggests that regeneration is dependent on coordinated interactions between all of the implicated pathways, thus rather than any one being sufficient for MG activation, crosstalk between them must be initiated when any singular path is exogenously stimulated. Nevertheless, this study exemplifies the power (and caveats) of applying chemical genetics to a complex multifactorial questions.

Besides responding to exogenous stimuli, MG also phagocytose dead cells. Bailey *et al.* explored the role of this particular function of MG in retinal regeneration using a light lesion model [124]. The authors used a chemical inhibitor of microglial phagocytosis called L-SOP and demonstrated that inhibition of phagocytosis reduced the number of proliferating MG cells and the number of regenerated cone photoreceptors. Since L-SOP is also an agonist of the metabotropic glutamate receptor (mGluR), they investigated if the effect on proliferation was mediated by mGluR using different agonists and antagonists. Their results indicated that the effect of L-SOP was not mediated by mGluR. L-SOP exposure did not affect Ascl1a or Stat3 expression in MG, suggesting that this chemical was acting either in parallel or downstream of Ascl1a activation. This work implicated a unique role for phagocytosis in regulating the response to retinal cell loss. One caveat of this study is whether phagocytic microglia also plays a role in modulating retinal regeneration, a possibility the authors felt was less likely based on normal distribution and morphology of microglia in L-SOP-treated retinas.

As described in the above examples, testing the role of suspected signaling pathways directly has been a powerful approach to increasing our understanding of regenerative biology. However, a major limitation is that it builds upon prior knowledge, thus biasing studies toward known targets and leaving less characterized pathways untested. A more general, nonbiased, approach can be achieved using forward chemical screens. This discovery-based strategy can provide valuable insights into any pharmacologically targeted factor impacting regenerative processes and thereby aid the development of therapeutic strategies as well.

Forward chemical genetics

Forward screening strategies provide an opportunity to discover novel effectors of biological processes of interest. Unlike reverse screens, no prior knowledge of underlying molecular mechanisms is necessary. Rather, such approaches are limited only by the availability of robust phenotypic assays amenable to miniaturization and/or compatible with large-scale screening strategies. The forward screening paradigm has therefore been essential for expanding our understanding of unknowns; critical for unveiling functional insight into factors and pathways that had either previously gone uncharacterized or unappreciated with respect to the biology of interest. Below we review some of the contributions that forward chemical screening has made to our understanding of regenerative biology.

Organismal regeneration

To our knowledge, although forward genetic screens have been applied to planarian regeneration, forward chemical screening has yet to be applied to organismal regeneration.

Appendage replacement: epimorphic regeneration

Taking advantage of the fact that the key players of regeneration are conserved between the adult and larval fin regeneration model, Mathew *et al.* performed a forward chemical genetics screen for modulators of caudal fin regeneration [125]. The ability to per-

form the assay in larval zebrafish enabled the use of microtiter culture (96-well plates); akin to common HTS assay formats. Accordingly, this was the first large-scale chemical screen involving a tissue regeneration paradigm. The compound library screened was comprised of 2000 bioactives and the US FDA-approved small molecules (MicroSource Discovery Systems). Libraries of existing drugs have several benefits: first, existing drugs are well characterized with regard to mechanisms of action (MoA), facilitating follow-up validations to identify target pathways; second, associated toxicities are known, focusing off-site analyses on organ systems likely to be impacted and; third, as a 'drug re-purposing' strategy it provides a potential fast track to clinical trials. After screening 32,000 amputated larval fish, 17 hits were implicated that inhibited fin regeneration. Interestingly, five of the hit compounds were glucocorticoids. This was the first evidence that activation of glucocorticoid signaling pathway can inhibit regeneration, demonstrating the potential of forward chemical genetics in tissue regeneration. Taking advantage of the ability to limit compound exposures to discrete time windows, Mathew *et al.* went on to demonstrate that a particularly potent glucocorticoid, beclomethasone dipropionate, was effective only when applied during the first 4 h after injury. This analysis revealed that glucocorticoid activation was targeting pathways critical for the formation of the wound epithelium and blastema – in other words, the initiation of a regenerative response – and not later stages involving stem/progenitor proliferation and differentiation.

In a related screen, Oppedal and Goldsmith screened for chemical inhibitors of caudal fin regeneration in adult zebrafish [126]. A total of 520 compounds were tested (a subset of the LOPAC 1280 library from Sigma), of which 13 were implicated in the primary screen and 2 were validated. Follow-up assays focused on an imidazoline receptor antagonist, AGN192403, as the implicated pathway was a potential novel modulator of regeneration. Tests with like-compounds confirmed the imidazoline receptor as the target and further analysis revealed that AGN192403 prevented blastema formation and proliferative outgrowth, but had no effect on initial stages of wound healing. Interestingly, the authors went on to show that prolonged exposure to AGN192403 (≥5 days postamputation) led to sustained disruptions in fin regeneration, even up to 2 weeks after washout. These studies reveal the potential of forward genetics to identify novel regulators of appendage regeneration, and reiterate the importance of chemical genetics for defining phase-specific roles for targeted factors/pathways in regenerative processes.

Tissue regeneration

Bone

To our knowledge, an *in vivo* model of bone regeneration compatible with large-scale chemical screening has not yet been described. However, small-molecules screens carried out in cultured cells with osteogenic potential have shown promising results. For example, Mundy *et al.* screened 30,000 compounds in a genetically modified immortalized murine osteoblast cell line in which BMP2 expression could be measured by luciferase assay [127]. They found statin, a drug known to lower cholesterol levels, could enhance osteoblast differentiation. This effect was further verified by increasing new bone formation in an *ex vivo* model, neonatal calvarial bone culture. In another example, Darcy *et al.* screened 5405 chemical compounds and found 45 that enhanced BMP2-induced osteoblast differentiation of myoblast cells [128]. Among them, two known anticancer immunosuppresants, rapamycin and FK-506, were further investigated. Both stimulated preosteoblast cells to differentiate into osteoblasts with or without BMP induction. Moreover, rapamycin countered the inhibitory effect of TGFβ1 on osteoblastogenesis. Both studies exemplify how existing drugs may have additional therapeutic benefits beyond original indications.

Naturally, discovering new drugs is also of great interest. In another cell study, Hojo *et al.* created a transgenic preosteoblast MC3T3E1 cell line expressing GFP under the regulation of a collagen type-1 promoter fragment; thus, GFP expression was correlated with osteogenesis. Using this tool, they screened 2500 natural and synthetic compounds, identifying an isoflavone derivative, glabrisoflavone, as an inducer of osteoblast differentiation independent of BMP, Runx2 and Wnt signaling. Glabrisoflavone is extracted from leaves of the licorice plant, *Glycyrrhiza glabra* [129]. Although extracts from *G. glabra* have been used in traditional medicines for many years, the specific biological function and molecular target(s) of glabrisoflavone remain unknown. Nevertheless, this study suggests a new application of this compound, and/or isoflavones in general, a finding clearly worthy of further investigation.

Monitoring stem cell commitment to the osteoblast lineage is another way to discover compounds contributing to osteogenesis. Alves *et al.* screened 1280 pharmacologically active compounds in mesenchymal stem cells and found five osteogenic factors targeting either Raf-MEK-ERK or the cAMP signaling pathways [130]. Another study similarly screened 1040 small molecules using an alkaline phosphatase assay to indicate osteogenesis. Thirty-six molecules were found to promote osteogenesis, while 20 compounds inhibit by increasing and decreasing the ALP activ-

ity [131]. Collectively, these screening studies highlight the advantages of applying forward chemical screening to the issue of bone repair, providing new applications for known drugs and discovering novel inroads into promoting osteogenesis.

Heart

Effective heart repair will require a deeper understanding of the regulation of myocardial differentiation, in particular with regard to the use of stem cell transplants. To discover new factors promoting myocardial fates, Sadek *et al.* screened 147,000 compounds using a pluripotent mouse stem cell line (P19CL6), leveraging the expression of the *Nkx2.5* gene as a marker of cardiovascular progenitor cells [132]. They identified a class of molecules, sulfonyl-hydrazone, competent for upregulating *nkx2.5* and other cardiac markers in a variety of stem cell types. Importantly, sulfonyl-hydrazone treatment of human mobilized peripheral blood mononuclear cells (M-PBMCs) induced increased expression of cardiac marker genes. Human M-PBMCs represent perhaps the most promising stem cell source for cardiac repair, thus showing pharmacological conservation of sulfonyl-hydrazone effects in this cell line enhances the potential for therapeutic benefit. Together, the data support sulfonyl-hydrazone small molecules as promising drugs for promoting cardiac repair.

Using an *in vitro* system, Uosaki *et al.* screened 280 kinase inhibitors on mouse embryonic stem cell-derived cardiomyocytes [133]. The initial screening identified nine chemicals that impacted cardiomyocyte proliferation, modulating four corresponding kinase signaling pathways: inhibitors of GSK-3, MAPK or CaMKII and activators of ERK. In another *in vitro* screen, Wnt was identified as a key regulator of heart regeneration when fluorescently labeled human embryonic stems cells were treated with 550 modulators of known pathways and analyzed using high-throughput imaging [134]. It should be noted that although these studies only examined small molecules targeting known pathways, these tools have potential to be expanded to the large-scale screening to search for wide range of chemicals.

As discussed above, degenerative and autoimmune disorders are often linked to the promise of stem cells. A better understanding of how the regenerative potential of endogenous stem cell niches is regulated at the molecular level will aid efforts to develop reparative therapies for these conditions. Unfortunately, most cellular regeneration paradigms represent the proverbial 'black box' in this respect. Forward screens therefore provide perhaps the best option for bringing cellular regeneration – the replacement of specific disease-relevant cell types – to light.

Cellular regeneration

Hair cells

Recent studies have revealed supporting cells as the source of regenerating hair cells and reverse chemical genetics has been useful for delineating roles of canonical development signaling pathways, such as Wnt and Notch (see above). Namdaran *et al.* adopted an unbiased forward screening strategy to discover novel small-molecule modulators of support cell proliferation following neomycin-induced hair cell loss [135]. Screening 1680 FDA-approved drugs resulted in the identification of both inhibitors and enhancers of hair cell regeneration. This study implicated glucocorticoids, such as dexamethasone and prednisolone, as enhancers of hair cell regeneration; this is in contrast to previous fin regeneration studies in which glucocorticoid agonists had an inhibitory effect [125]. In addition, glucocorticoid treatment, in the absence of hair cell loss, also stimulated an increase in hair cell numbers. Furthermore, as other anti-inflammatory compound classes failed to have an effect on hair cell regeneration, their data suggested glucocorticoids may be acting via mechanisms other than immunosuppression – perhaps as direct neuroprotectants. Lastly, this study also discovered novel inhibitors of hair cell regeneration, such as topotecan (an inhibitor of topoisomerase activity), which acted by preventing support cell proliferation, and estrogen receptor antagonists which delayed hair cell regeneration by reducing support cell proliferation.

In a similar study, Moon *et al.* screened 470 compounds for modulation of hair cell regeneration in zebrafish larvae [136]. Using several transgenic lines to facilitate screening via confocal microscopy, they identified 20 compounds that enhanced regeneration by more than 25%. Their screen identified LMWF (low-molecular-weight fucoidan), a natural product present in brown seaweed as an enhancer of regeneration. Based on the fact that Notch and FGF signaling also impact hair cell development and regeneration, Moon *et al.* utilized pharmacological inhibitors of Notch and FGF to test the role of these signaling pathways and to determine if coexposure with LMWF could alter their capacity to influence regeneration [89,136,137]. Their studies showed that LMWF did not improve the capacity of DAPT to enhance regeneration, indicating that the effect of LMWF is not synergistic with Notch signaling. However, LMWF could rescue the delayed regeneration phenotype induced by FGF inhibition, suggesting that LMWF targets are downstream or dominant over FGF. Further studies are required to characterize the molecular mechanism of action of the LMWF.

Skeletal muscle

Although skeletal muscle has an inherent ability to regenerate, regenerative capacity decreases with age [138] and with muscular dystrophy. Muscular dystrophy is a genetic disease caused by mutations in muscle proteins resulting in muscle degeneration [139]. The most common form of this disease is Duchenne muscular dystrophy (DMD), caused by mutations in the gene dystrophin [140]. In DMD patients, muscle stem cells known as satellite cells are depleted with age, possibly due to sustained activation and impaired renewal, resulting in increased muscle degeneration [141]. In zebrafish, disrupted skeletal muscle structure can be easily examined using a birefringence assay, measuring the degree to which light is skewed when going through the normally arrayed structure of the sarcomeres. To identify potential therapeutics for DMD, Waugh *et al.* performed a forward screen for chemicals that can restore normal birefringence in a mutant zebrafish model of DMD [142]. Screening 640 largely FDA-approved compounds resulted in the identification of six hits, three of which were classified as monoamine agonists. A secondary screen of additional monoamine agonists (and serotonin) determined that fluoxetine, a selective serotonin reuptake inhibitor, provided the strongest rescue phenotype (other than serotonin itself). Microarray expression studies suggested that fluoxetine may act by sustaining calcium homeostasis, and disruptions in this pathway have been previously implicated in DMD.

A similar study, by Kawahara and Kunkel was conducted in *sapje* and *sapje-like* mutants [143]. They screened a library of 1120 compounds, the majority being FDA-approved. Using the same birefringence assay, they identified seven hits that restored a normal muscle phenotype. Out of the seven identified hits, aminophylline, a nonselective phosphodiesterase inhibitor, had the strongest effect. Aminophylline was known to increase intracellular cAMP levels leading to activation of cAMP-dependent protein kinase (PKA), and increased activation of PKA was detected in treated fish. These studies exemplify how combining genetic mutants with forward chemical screens can be used to derive novel insights into stem cell biology and to further the development of reparative therapeutics for patients.

Pancreatic β-cells

The target of β-cell regeneration studies is to increase β-cell mass as a potential therapy to treat diabetes. Molecular regulators of β-cell proliferation and differentiation have not been fully characterized, although human β-cell replication has been clearly observed in response to metabolic demand, such as in obesity or during pregnancy [144]. Therefore, identification of chemicals that increase β cell production will enhance our understanding of molecular mechanisms that represent potential therapeutic solutions for diabetes.

In order to identify modulators of β-cell regeneration, Andersson *et al.* screened 7186 compounds (including FDA-approved drugs, natural products and uncharacterized entities) in a transgenic zebrafish model enabling selective ablation of β cells [145–147]. After screening ~100,000 larvae, five compounds were identified that doubled the number of regenerating β cells. Remarkably, four of the five hit compounds were predicted to act by enhancing the adenosine signaling. Critically, adenosine signaling was validated in a mouse model of diabetes, promoting β-cell proliferation. Equally important, the activity of the most effective adenosine agonist, NECA, faded when normal glucose levels were achieved. Hence, large-scale *in vivo* chemical screening identified adenosine signaling as a novel pathway for promoting increased β-cell mass. Annes *et al.* also identified adenosine kinase inhibitors (ADK-Is) as promoters of β-cell replication in mammalian tissue culture [148]. They screened ~850 compounds for increased numbers of PDX1/Ki67-positive β cells in primary rat islet cultures. They identified two hits and determined that effect of one, ADK-Is, was mediated by the mTOR pathway and, moreover, was specific to β cells. The adenosine pathway is known to provide cytoprotective effects as an anti-inflammatory agent and can promote repair in a variety of tissues [149]. Taken together, these data suggest that inflammation may function to inhibit β-cell proliferation, and provides a promising new therapeutic target for diabetes. In summary, whole-organism and *in vitro* screens converged on the adenosine signaling pathway as a promising therapeutic target for restoring β-cell mass in diabetic patients.

The screen performed by Andersson *et al.* was not biased toward any specific cellular mechanism for increasing β-cell numbers and hence allowed discovery of a broad range of hit compounds [145]. A more targeted screen for modulators of β-cell differentiation was performed by Rovira *et al.*, using transgenic lines to identify small molecules that induced premature secondary islet formation [150]. They screened a library of 3131 compounds consisting mainly clinically approved drugs (the Johns Hopkins Drug Library, JHDL) and identified six compounds promoting endocrine cell differentiation. Follow-up MoA studies on the compounds, mycophenolic acid and tetraethylthiuram disulfide, revealed two novel targets involved in β-cell differentiation, GTP and RA, respectively. Using a series of alternative modulators, they showed that inhibition of GTP or RA synthesis was sufficient to induce secondary islet formation. Investigating another

means of increasing β-cell mass, Tsuji *et al.* performed a high content screen in transgenic larval zebrafish to identify compounds stimulating β-cell replication [151]. Screening a library of 833 compounds resulted in the identification of 20 hits. Interestingly, all hits fell into one of three categories: stimulators of retinoid acid signaling, enhancers of serotonergic signaling or glucocorticoid receptor ligands. The effect of glucocorticoids were found to be indirect (i.e., increased glucose levels), while serotonin and RA signaling appeared to have direct effects (i.e., no effect on glucose levels) – albeit these results were based on a single representative compound from each category. Representative hits were further tested for effects on β-cell regeneration using a Type I diabetic model involving selective β-cell ablation [146,147]. Both RA and prednisolone enhanced β-cell regeneration, while the serotonin reuptake inhibitor, trazodone, had no effect. Since all three serotonergic compounds implicated in the original assay induced only a relatively mild amount of β-cell proliferation, the authors concluded that compounds promoting robust proliferation can also lead to enhanced regeneration.

In an effort to more comprehensively screen for chemicals that increase β-cell mass, we recently completed the first quantitative high-throughput screen (qHTS) in a vertebrate model organism [152]. We adopted existing HTS instrumentation (a microtiter plate reader) to reporter-based assays in larval zebrafish; a methodology termed ARQ*iv* (Automated Reporter Quantification *in vivo*) which we developed to harness the full potential of zebrafish for whole-organism drug discovery [99]. ARQ*iv* can screen fish at true high-throughput rates, in turn, allowing us to apply HTS best practices, such as qHTS; in other words, titrating all compounds across a six- to eightfold dilution series in the primary screen to reduce false hit-call rates [153]. Using a robotized iteration of the ARQ*iv* platform and the JHDL for qHTS, we screened over 500,000 larval zebrafish, implicating a total of 177 drugs as stimulators of β-cell differentiation and/or proliferation. To date, 24 of 39 drugs rescreened have been validated, and MoA follow-ups have revealed another novel regulator of β-cell differentiation, the NF-κB signaling pathway. The data further suggest that serotonergic signaling stimulates β-cell proliferation selectively, without altering the proliferation of other endocrine cell subtypes, and also stimulates β-cell replication in mice. These findings have important therapeutic implications as increasing β-cell mass in a cell-type specific manner could have significant benefits for diabetic patients (e.g., reduced side effects).

In summary, forward chemical screens have identified several novel pathways for promoting β-cell differentiation or to stimulate β-cell proliferation. Both end points are useful, for instance, compounds that induce endocrine cell fate could be used to guide cell fate in stem cell cultures. More intriguingly, recent findings suggest that existing β cells proliferate to maintain homeostasis and during regeneration in mammalian models, thus compounds that stimulate β-cell proliferation represent a promising new therapeutic strategy for diabetic patients. The screens discussed above, and many others which we were unable to summarize in detail (see Table 1), provide examples of the power of forward genetics for revealing new insights into regenerative biology and for aiding the development of regenerative therapies.

Conclusion & future perspective

In this review, we have discussed how chemical genetics can reveal key molecular components and/or pathways that regulate regeneration. Reverse genetic strategies can resolve the role of known molecular pathways, while forward screens represent a more exploratory approach for revealing novel targets. Within the context of chemical genetics, both methods facilitate the understanding of regenerative mechanism, thus assist the design of new therapeutics. Supported by recent advances in computer-assisted automation and robotics, as well as robust quantitative assays, whole-organism high-throughput drug screening is well-positioned to drive the discovery of novel insight into regenerative processes. Several rapidly developing technologies have the potential to enhance the power of whole-organism chemical screening for regenerative biology. First, CRISPR-based gene editing enables the development-sophisticated disease models in regenerative species useful for both reverse and forward chemical genetics. Second, microfluidics and femtosecond laser surgery [154] will provide a powerful platform for adapting more regenerative animal models to HTS. Third, automated microscopy facilitates image-based 'high content' screening of animal models in multiwell and/or microfluidic formats. Finally, recent advances in the synthesis of tagged libraries facilitate compound pooling and/or targeting of more than one pathway [155]. In summary, chemical genetics has made significant contribution in moving the field of regeneration biology forward. We expect that the combination of recent technological breakthroughs with small-model species enabling *in vivo* HTS will accelerate progress in regenerative biology research.

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Executive summary

- Chemicals targeting biochemical and biophysical signaling can provide valuable insights into regeneration at organismal, tissue and cellular levels.
- Chemical genetics has the power to dissect out the stage-specific roles for molecular pathways during regenerative processes.
- Several strengths of chemical genetics: temporal control, graded responses and the ability to circumvent genetic redundancy and/or compensation, are extremely useful for studying highly dynamic processes such as regeneration.
- While reverse genetics helps to define/validate roles of known molecules, forward genetics can implicate the unknown and therefore has a greater potential to provide insight into novel regulators of regenerative biology.
- Whole-organism quantitative high-throughput screening (qHTS) for enhancers and repressors of regeneration can speed the pace of discovery in the field of regeneration.

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