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Review Article

Specific differentiation of mesenchymal stem cells by small molecules

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Abstract: Mesenchymal stem cells (MSCs) are multipotent, self-renewing cells harboring multi-lineage differentiation potential and immunosuppressive properties that make them an attractive candidate for biological cell-based regenerative medicine. In addition to its undoubted clinical interest, controlling the fate and behaviors of MSCs is a crucial prerequisite for their therapeutic applications in regenerative medicine. Stem cell differentiation and modulation of functional activities are generally controlled by "cocktails" of growth factors, signaling molecules, and/or genetic manipulations. However, these approaches have several limiting factors, such as undefined conditions leading to heterogeneous populations of cells and unexpected risks of virus-mediated genetic modifications. Small molecules targeting specific signaling pathways have been shown to be key modulators in controlling stem cells' fate and function. Small molecules are also important tools for understanding mechanistic and developmental processes. Furthermore, the precise mode of action of small molecules for controlling MSCs fate is still under study. However, Wnt, GSK, and other protein kinases signaling pathways are likely to be involved. These target-based manipulations of stem cells fate by small molecules provide new insights into stem cell biology, and facilitate the development of regenerative medicine using stem cells. Here, we review the recent progress in controlling MSCs fate and functional activities by small molecules.

Keywords: Mesenchymal stem cells, cell fate, differentiation, small molecules

Introduction

Stem and progenitor cells are less specialized cells that have both the ability for self-renewal and the potential to differentiate into specialized cells in response to specific signals [1]. Mesenchymal stem cells (MSCs) are derived from a population of stromal cells present in bone marrow and various tissues, which also can differentiate into different tissue lineages [2-4]. In the last decade, it has been well established that MSCs have the ability to differentiate various cell types encompassing osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, adipocytes, and other mesenchymal phenotypes [5], suggesting their use as a source of cells for various application of regenerative medicine. In addition to their potentials, understanding the fundamental mechanisms to control cell fate and function is a critical step to translational application of MSCs. In many studies, stem cell fate and function have been generally controlled by "cocktails" of growth factors, signaling molecules, and/or genetic manipulations [6, 7]. However, the composition and/or conditions of most of these cocktails are not exactly defined and they do not specifically regulate stem cell fate, resulting in heterogeneous population of cells. Because cell-based therapies may require large quantities of stem cells for clinical uses, it would be advantageous to isolate specific small molecules that either maintain self-renewal or drive tissue specific fates of stem cells [8]. In addition, virus-mediated genetic manipulation introduces unacceptable risks of permanent transgene integration to the genome. The resulting genomic alteration and possible reactivation

of viral transgenes pose serious clinical concerns [9].

Small molecules have shown to be useful tools for modulating cell fate and function by targeting specific signals and mechanisms [10-12]. In addition, they provide some distinct advantages over other techniques, such as gene manipulation, preconditioning, and pretreatment with effectors including growth factors and cytokines that are fast, reversible, and precise temporal regulators of protein function [10]. Indeed, various cell permeable small molecules have been proven to be clearly useful for inducing the differentiation of various stem cells including MSCs. Moreover, the immense potential of orally delivered small molecules as regenerative therapies are now widely recognized [13]. In this review, we focus on recent advances in the use of small molecules to control MSCs fates such as proliferation, differentiation, and functional activity.

The control of cell fate by small molecules

Small molecules

Small molecules have been synthesized and tested in phenotypic analysis to screen biologically useful molecules for over 100 years [14]. Although one compound was tested at a time in the earliest era, modern libraries contain thousands and even millions of small molecules that are synthesized through combinatorial chemistry and tested by high-throughput screening techniques [14]. The complementary approach to classical genetics, where small molecules are exploited to probe biological functions, is termed "chemical genetics" [15, 16]. While classical genetics establishes genotype-phenotype relationships through means of genetic manipulation, chemical genetics uses small molecules that intervene in signaling pathways through direct interaction with proteins to unravel relationships between proteins and phenotypes. By analogy to classical genetics, small compounds in chemical genetics are equivalent to mutations in classical genetics [17]. In fact, given that chemicals can be removed from cells by simple washing, they are analogues to conditional mutations. Although small molecules allow reversible temporal and dose-dependent control of protein function, one of their major limitations is that a single compound often affects multiple proteins and subsequently multiple pathways [18, 19]. Thus, treatment with a single small molecule can be analogous to simultaneous modulations in several genes.

The method of using small molecules to modulate and control the stem cell fate has a long history. Indeed, *all-trans*-retinoic acid has been used for over 30 years to induce differentiation of both mouse and human embryonal carcinoma cells [20, 21]. Hexamethylenebisacetamide and DMSO also have been used to promote differentiation of embryonal carcinoma cells and erythroleukemia cells, respectively [22, 23]. In addition, stem cells share many properties with tumor cells and these similarities can provide insights to control and direct cell behavior. Indeed, small molecules are already standard chemotherapeutics in the treatment for cancer [8]. A number of small molecules have been examined for anti-cancer effects (especially induction of apoptosis), and recently, for stem cell self-renewal and specific differentiation in potential approaches to regenerative medicine. Indeed, both natural and synthetic small molecules have been shown to be useful chemical tools for manipulating the fates of cells [8].

Modulation of stem cell fate by small molecules

As mentioned above, small molecules have been used to modulate and/or control cell fate, and behavior, especially stem cells, for a long time. In fact, most of the studies that are currently being conducted and/or are ongoing in this field focus on the revealing the mechanisms of self-renewal and differentiation of embryonic stem cells (ESCs) and the generation of induced pluripotent stem cells (iPSCs) [8, 24]. Indeed, various small molecules have been identified to regulate the differentiation and self-renewal of ESCs. For example, Pluripotin, CHIR99021, and A83-01 can support self-renewal of ESCs through targeting the RasGAP, GSK-3, and ALK5 signaling pathways, respectively [25-27]. (-) Indolactam V can enhance the pancreatic differentiation of ESCs through PKC pathway, and stauprimide and SB431542 induces differentiation of ESCs through NME2 and ALK5 pathways [28, 29]. In addition, several small molecules were revealed to affects somatic cell reprogramming. BIX-01294, RG108, and parnate can promote MEFs reprogramming through regulating G9a HMTase, DNA

MTase, and lysine-specific demethylase, respectively [10, 30, 31]. Interestingly, a small molecule, Reversine, can induce dedifferentiation of muscle and fibroblast cells into a more primitive multipotent state [32-34].

Regulation of differentiation in MSCs by small molecules

Mesenchymal stem cells: A brief overview

MSCs are a heterogeneous subset of stromal cells that are classically derived from bone marrow [2]. But they have been isolated from most connective tissues including adipose tissue [35], periosteum [36], and synovial membrane [11]. MSCs have apparent advantages, such as easy availability, few ethical concerns, and low immunogenicity, compared to embryonic stem cells and other tissue-specific stem cells. Indeed, because MSCs can be easily isolated and expanded in culture, they can be administered immediately instead of waiting weeks and/or months for adequate numbers of cells to be achieved by cell culture. MSCs retain their growth and multilineage potential over several passages, although they are moral [37, 38]. In addition to their multilineage transdifferentiation potential, one particularly useful characteristic of MSCs is their apparent immunoprivilege. They display local immunosuppressive properties that permit successful transplanting in an allogenic setting. Indeed, experiments in non-human primates have shown that allogenic MSCs were not rejected but showed similar effects on the autologous MSCs and were detected nine months after transplantation in the recipient [39-41].Overall, these studies have suggested MSCs as an attractive candidate cell type for tissue engineering, regenerative medicine, and autoimmune disease treatment.

Specific differentiation of MSCs by small molecules

For practical use of stem cells for regeneration therapy, there are at least three prerequisites: (*i*) the directed differentiation of stem cells to specific cell types, (*ii*) achievement of high survival rate of the cells after transplantation, and (*iii*) prevention of undifferentiated stem cells that are prone to form teratomas and/or cancers [42]. Amongst them, the *ex vivo* directed differentiation of stem cells to specific cell types for treating target diseases may provide better clinical results. Classically, osteogenic differentiation of human MSCs requires incubation in fetal bovine serum (FBS)-containing medium supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone, resulting in an increase in alkaline phosphatase activity and calcium deposition [43, 44]. The chondrogenic differentiation is performed with a high cell-density pellet or a micromass culture treated with transforming growth factor (TGF)-β in a serum-free medium; this results in the production of cartilage-specific, highly sulfated proteoglycans and type II collagen [45]. For adipogenic differentiation, MSCs are treated with FBS containing medium supplemented with dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin. The differentiation is detected bythe oil red O staining for lipid vacuoles [45]. In addition to these reports, many studies have shown that a number of small molecules can be used to control the fate of MSCs for a variety of applications. For example, 5-azacytidine (5-aza-C) can induce murine and human bone marrow stromal cells into cardiomyocytes [46-48], and also induce mouse mesenchymal progenitor cell line, C3H10T1/2 cells, into myoblast, osteoblasts, adipocytes, and chondrocytes (**Table 1**) [43]. It has been revealed that 5-aza-C does not directly induce or activate the specific differentiation pathways of MSCs, but rather convert the cells into a competent spontaneous differentiation state [49]. However, recent reports showed that 5-aza-C-treated human mesenchymal stem/progenitor cells derived from umbilical cord, cord blood and bone marrow do not generate cardiomyocytes in vitro at high frequencies [50, 51]. This suggests that the induction of specific differentiation by small molecules might be dependent on cell types. *All-trans* retinoic acid is one of the prominent molecules that has long been in control of cell fate. Recently, it was reported that retinoic acid may induce chondrogenesis, osteoblastogenesis, and neuronal differentiation of MSCs [52-54]. Peroxisome proliferator activated receptor γ (PPARγ) agonist and antagonists are used as adipogenesis modulators [55, 56]. Dexamethasone, ascorbic acid, and β-glycerophosphate can also be used to induce osteogenesis or adipogenesis of MSCs under defined conditions (**Table 1**) [43, 57].

To identify small molecules that selectively differentiate MSCs into defined-lineages, we

screened a small library (41 compounds) of characterized and commercially available inhibitors of six major subfamilies of protein kinases that are known to inhibit various cellular processes: TK (tyrosine kinase families), TKL (tyrosine kinase-like families), CMGC (CDK, MAPK, GSK3, CLK families), CAMK (Ca/calmodulin dependent protein kinase), AGC (PKA, PKG, and PKC families), and CKI (Casein kinase family) [58]. Because such important cellular processes are likely to be controlled by a complicated orchestration of many signaling pathways including many undiscovered ones, common signal modulators, such as protein kinases, are likely to play an important role in balancing multiple signals affecting several aspects of the above prerequisites [42]. Protein kinases belong to one of the largest protein families in the human genome, and they play critical roles in signaling pathways implicated in the development, differentiation, proliferation, and death of cells. Several signaling pathways have been known to induce or suppress differentiation of stem cells, such as the pathways involving mitogen-activated kinases, glycogen synthase kinase-3, PI3-kinase, and others [24, 44, 59-61]. A glycogen synthase kinase 3β(GSK-3β) inhibitor synthesized by combinatorial chemistry has been shown to induce neurogenesis [24]. In this study, among several candidates that drive rat MSCs into specific cell types, H-89, an inhibitor of protein kinase A, was found to be potentially implicated in chondrogenesis of the MSCs, which is a derivative of isoquinolinesulfonamide, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl [42]. We also found that H-1152, a Rho kinase inhibitor, was the best inducer of differentiation of ESCs into tyrosine hydroxylase (TH), a catecholaminergic neuronal marker, -positive neurons. H-1152 is another cell-permeable isoquinoline sulfonamide derivative that acts as a highly specific, potent, and ATP-competitive inhibitor of G protein ROCK (*K*i=1.6 nM) [42]. Based on the above results, the enhanced differentiation of rat MSCs to specific lineages support the notion that, although cell differentiation may be the result of a complex orchestration of many signals from multiple signaling pathways, even a single chemical reagent is enough to alter the relative balance of many signals to enhance differentiation of stem cells to particular cell types. They also suggest that the process may be optimized by a ''mixture'' of various multiple kinase inhibitors as well as other kinds of small molecules. Because these compounds may interact with ''off-target'' kinases as well as other unknown proteins, our observation should be considered as a practical approach to finding chemical reagents for inducing stem cell differentiation to a specific cell type even when most of the signaling pathways are unknown [42]. Thus, the approach described or some variation of it may be applied to other stem cells, including human stem cells, to find chemical molecules that can trigger initiation, inhibition, or even reversion of the differentiation process of stem cells or progenitor cells.

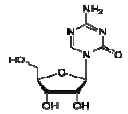
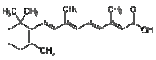
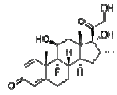
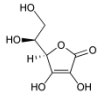
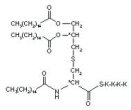
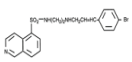
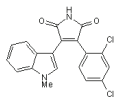
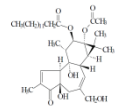
In recent studies, we found that phorbol myristate acetate (PMA), a PKC activator, and can upregulate cardiogenic properties from MSCs and subsequently chemically activated cardiogenic MSCs preventing sudden deaths after engraftment onto infarcted rats by electromechanically synchronizing with the host myocardium [62]. These suggest that small molecules may regulate the stem cell functions as well as the fate. In addition, we also found that a kind of glycogen synthase kinase-3 (GSK-3) inhibitor may induce the endothelial differentiation of MSCs in recent studies, which might be an important implication for treating vascular diseases (unpublished).

Wu et al. also identified a small molecule that selectively differentiates MSCs into osteogenic lineages through screening a combinatorial heterocyclic compound library in the mouse mesenchymal progenitor cells [63]. Purmorphamine, 2,6,9-trisubstituted purine compound, was identified as a potent osteoblast differentiation inducing agent that can activate Cbfa1/Runx2 (a key regulator of bone development) as well as upregulate other bone specific markers, such as osteopontin and collagen I. In addition, it was reported that decalpenic acid, a novel small molecule from *Penicilliumverruculosum* CR37010, induces early osteoblastic markers in mouse C3H10T1/2 cells [64]. Pevsner-Fischer et al. reported that toll-like receptors are associated with MSCs function and their inhibitor, Pam3Cys, increases MSC proliferation and inhibits MSC differentiation into chondrocytes, osteocytes, and adipocytes (**Table 1**) [65].

Concluding remarks

Although substantial growth arose in recent years, stem cell research and development are

Table 1. Small molecules in controlling the fate of mesenchymal stem cells

Structure	Name	Target pathway	Effects	Results
	5-azacytidine		DNA methyltransferase inhibition	Promotes cardiomyogenic, adipogenic differentiation [46, 48, 67]
	*all-trans* retinoic acid	Smad/p38	Regulation of DNA transcription	Induces chondrogenesis , osteoblastogenesis, and neuronal differentiation [52-54]
	dexamethasone		Increases alkaline phosphatase activity and enhances calcium deposition [68]	Increases the effect of 5-aza and induces differentiation program [57]
	ascorbic acid		Increases alkaline phosphatase activity	Induces adipogenesis or osteogenesis [43] and enhance proliferative activity [69]
	rosiglitazone		PPARγ activation	Adipogenesis modulation [55, 56]
	Pam3cys	NF- κB	TLR inhibition	Increases MSCs proliferation and inhibits MSCs differentiation into chondrocytes, osteocytes, and adipocytes [65]
	purmorphamine	Hedgehog	Smo inhibition	Induces osteogenesis [70]
	H-89		Protein kinase A inhibition	Induces chondrogenesis [42]
	SB216763	Wnt	GSK-3β inhibition	Induces endothelial differentiation
	phorbol myristate acetate		Protein kinase C activation	Induces cardiomyogenic differentiation [62]

still in an early stage. The control of stem cell fate and function is an important step for clinical applications of stem cells in regenerative medicine. In addition to the focus on pluripotent stem cells including ESCs and iPSCs, understanding and controlling the fate and function of adult stem cells such as, MSCs, *in vitro* and *in vivo* might be a significant challenge for developing better therapeutic approaches to regenerative medicine. Among various approaches in controlling stem cell fate, small molecules are considered as the best tool in the next era, be-

cause small molecules can target signaling transduction pathways (for example, tyrosine kinase receptors) and affect various cellular events, such as, DNA replication, differentiation, proliferation, and apoptosis. Obviously the field is interdisciplinary and involves chemists, a range of biologists, and bioinformaticians. Indeed, chemists have to build small molecule libraries based on the structural motifs of known and/or potential regulators in specific pathways, and stem cell biologists may have to study to better understand the molecular interactions between small molecules and specific behaviors of stem cells. Small molecules are also important tools for understanding mechanistic and developmental processes because they can be added or removed at any point during development while mutations tend to persist throughout the organism's life [8]. Such temporal control is critical in understanding the specific timing of developmental processes [66]. Because of the current challenges in stem cell biology to control stem cell fate and function, small molecules might emerge as a powerful strategy to identify drugs that may regulate stem cell activity and may ultimately be useful to *in vivo* stem cell biology and therapy.

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