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Small Molecules that Induce Cardiomyogenesis in Embryonic Stem Cells

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Stem cells are multipotent cells with the ability to self-renew and differentiate into specialized cells in response to appropriate signals.[1] Most tissues have endogenous stem/progenitor cells which, upon injury to the organ, can proliferate and differentiate at the damaged site. The adult heart is composed mainly of postmitotic and terminally differentiated cells. Although a subpopulation of myocardial cells with cardiac stem cell character was identified recently, their limited availability hinders therapeutic applications.[2] Stem cells derived from other tissues, such as bone marrow, have been shown to be capable of repairing heart damage in animal models,[3] but inefficient differentiation and possible fusion with somatic cells limit their use in cardiac repair.[4] Pluripotent embryonic stem (ES) cells represent a possible unlimited source of functional cardiomyocytes. However, the in vitro differentiation of ES cells into cardiomyocytes involves a poorly defined, inefficient, and relatively nonselective process.[5] Consequently, the development of new approaches for the directed differentiation of ES cells into cardiomyocytes will likely facilitate therapeutic application of ES cells in heart disease, as well as provide important tools for probing the molecular mechanism of cardiomyocyte differentiation and heart development.

Recently, several small organic molecules identified in phenotypic cellular screens of combinatorial libraries of "privileged" heterocycles[6] were shown to be capable of inducing the selective in vitro differentiation of both adult stem cells and embryonic stem cells into osteoblasts and neurons.[7,8] We therefore decided to screen this same library for molecules that would selectively and efficiently induce the differentiation of murine ESCs to cardiomyocytes. The mouse embryonic carcinoma (EC) cell line P19 was initially chosen for high throughput screening. P19 cells, like ESCs, are pluripotent and can differentiate into cardiomyocytes after aggregation, formation of embryoid bodies (EBs), and treatment with 1% DMSO.[9] Moreover, P19 cells are easy to culture, amenable to genetic manipulation, and have a low frequency of spontaneous cardiac differentiation.

To develop an appropriate reporter assay, the promoter region (~700 kb) of the rat atrial natriuretic factor (ANF) gene[10] was cloned and inserted upstream of the luciferase gene in the PGL3-BV reporter plasmid.[11] ANF is a polypeptide hormone that is synthesized primarily in cardiac myocytes and is a downstream target of several cardiomyogenesis transcriptional factors; it is considered a specific cardiomyocyte "marker" gene.[12] A stable P19 clone harboring the reporter plasmid afforded a 5- to 7-fold increase (Figure 1) in luciferase signal upon standard cardiomyogenesis differentiation conditions for P19 cells (EB formation and treatment with 1% DMSO). This cell line was used to screen a 100 000 compound heterocycle library in a monolayer format, and approximately 80 compounds were identified that up-regulated luciferase activity >4-fold in the absence of EBs. Sarcomeric myosin heavy chain (MHC) is one of the essential motor proteins responsible for cardiac muscle contractibility[5] and was used as a secondary assay for differentiation. Thirty-five of the 80 compounds also induced MHC expression in P19CL6 cells[13,14] as determined by immunostaining with anti-MHC antibody (MF20) (Figure 2F). Among the latter compounds, four diaminopyrimidines, cardiogenol A−D (Table 1), were the most potent in inducing MHC expression. These compounds share significant structural similarities. All have a 2-hydroxylamino substitution at the C-4 position, and all have bulky, hydrophobic groups at the C-2 position, suggesting that they function by a common mechanism.

To confirm that these compounds are general cardiomyogenesis inducing agents, we analyzed their effects on undifferentiated R1

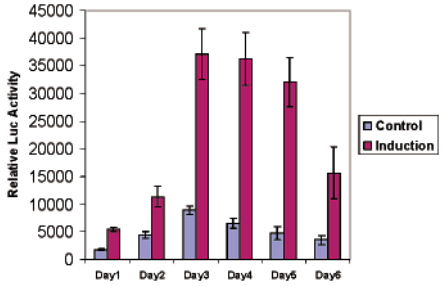

Figure 1. A high throughput screen for cardiomyogenesis using an ANF-promoter reporter assay.

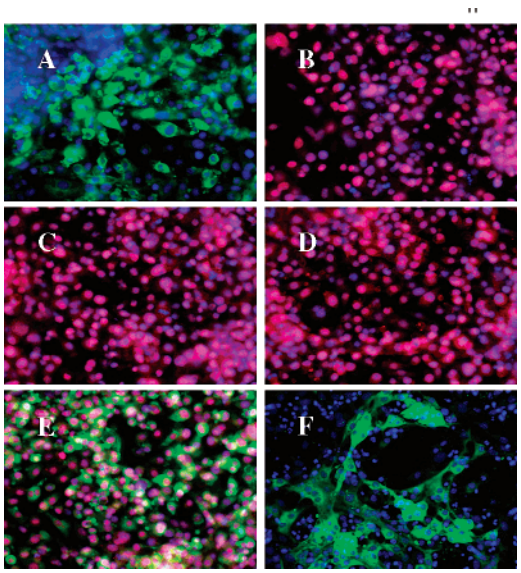

Figure 2. Immunostaining of cardiac muscle markers in ESCs (A−E) and P19CL6 cells (F) treated with 0.25 μM cardiogenol C: (A) and (F) myosin heavy chain (green); (B) GATA-4 (red); (C) MEF2 (red); (D) Nkx2.5 (red); and (E) myosin heavy chain (green) and MEF2 (red). Cell nuclei were stained with DAPI (blue).

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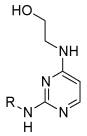
Table 1. Chemical Structures and Biological Activities of Cardiogenols

	R	EC_{50}	Optimal Activity
Cardiogenol A		1 μM	++
Cardiogenol B		0.5 μM	+++
Cardiogenol C		0.1 μM	++++
Cardiogenol D		0.1 μM	++++

++: 10-25% cells are positive for MHC after 7 days.
+++: 25-40% cells are positive for MHC after 7 days.
++++: 40-55% cells are positive for MHC after 7 days.



mouse ESCs, which can be maintained in a pluripotent state with the addition of leukemia inhibitory factor (LIF) in the culture medium. R1 cells were plated in a monolayer in gelatin-coated 384-well or 96-well plates in the absence of LIF and in the presence of 0.25 μM of compounds. After 7 days (3 days with compounds and then 4 days without compounds), the presence of beating cardiac muscle was visualized under microscope. In addition to the expression of MHC (Figure 2A), the cardiac specific gene, GATA-4, was detected by immunofluorescent staining using anti-GATA4 antibody (Figure 2B). GATA-4 is a transcription factor restricted to developing heart, and its overexpression enhances cardiomyogenesis in P19 cells.[15−17] Neither MHC nor GATA-4 is expressed in undifferentiated R1 mouse ESCs. It was also observed that compound treatment slowed cellular proliferation with no significant cell death, indicating that this process is not simply a selection for cardiac precursor cells with the death of cells in other lineages.

Cardiogenol C,[18] which has a *p*-methoxy aniline substituent at the pyrimidine C2 position, is the most potent compound with an EC_{50} of 0.1 μM for inducing the differentiation of MHC positive cardiomyocytes from ESCs. Cardiogenol C showed significant cellular toxicity only at concentrations greater than 25 μM; after treating R1 cells 0.25 μM compound for 3 days and further culturing in medium without compound for 4 days, more than 50% of the cells stained positive for MHC and more than 90% of the cells are positive for GATA-4, consistent with the previous observation that GATA-4 is expressed earlier than MHC.[5] Moreover, there were many beating areas in R1 cells treated with cardiogenol C, suggesting that these MHC positive cells can form functional cardiac muscle. These results indicate that the majority of the cell population was induced by cardiogenol C to differentiate into cardiac lineage (in the absence of aggregation and EB formation). This is in contrast to the current standard method of inducing cardiomyogenesis of ESCs by aggregation and formation of EBs, which results in only 5% of the cell population forming cardiomyocytes.[5]

To further characterize the activity of cardiogenol C, we examined the expression of the cardiac muscle cell specific transcription factors MEF2 and Nkx2.5 (Figure 2C and D). Members of the MEF2 family are essential for muscle development.[19,20] Nkx2.5 together with GATA-4 regulates the expression of multiple cardiac muscle specific genes[21,22] (e.g., myosin light chain 2V, atrial natriuretic factor, eHAND, and homeodomain transcription factor HOP); targeted interruption of Nkx2.5 gene is embryonic lethal and results in arrest of cardiac development.[23] Approximately 90% of cardiogenol C treated cells stain positive for MEF2 and Nkx2.5, further confirming that ESCs are differentiated into cardiac muscle by cardiogenol C.

We are currently carrying out biochemical and genomics experiments to identify the molecular targets of cardiogenol C. These experiments may reveal novel molecular mechanisms related to cardiomyogenesis, and ultimately facilitate the application of ESCs to the repair of damaged myocardium in acute heart diseases.

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Supporting Information Available: Detailed experimental procedures and compound characterization (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) Cardiogenol C. ^{1}H NMR (400 MHz, DMSO): δ (ppm) 3.42 (m, 2H), 3.55(m, 2H), 3.72 (s, 3H), 6.21 (d, 1H, J = 7.2), 6.97 (d, 2H, J = 8.9), 7.41 (d, 2H, J = 8.4), 7.66 (d, 1H, J = 7.1), 8.93 (s, 1H), 10.07 (s, 1H). High-resolution MS (MALDI-FTMS): Calculated [MH^+] ($C_{13}H_{17}N_4O_2$) 261.1346, found 261.1342. See Supporting Information for more details.
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