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. 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. Stem cells derived from other tissues, such as bone marrow, have been shown to be capable of repairing heart damage in animal models, but inefficient differentiation and possible fusion with somatic cells limit their use in cardiac repair. 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. 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 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. 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. 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 was cloned and inserted upstream of the luciferase gene in the PGL3-BV reporter plasmid. 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. A stable P19 clone harboring the reporter plasmid afforded a 5- to 7-fold increase 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 and was used as a secondary assay for differentiation. Thirty-five of the 80 compounds also induced MHC expression in P19CL6 cells as determined by immunostaining with anti-MHC antibody (MF20). 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...
of multiple cardiac muscle specific genes\textsuperscript{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.\textsuperscript{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.

References

13. The P19Cl6 mouse cell line is a subclone of P19 EC cells with higher potential for cardiomyogenesis.
18. Cardiogenol C. \textsuperscript{1}H NMR (400 MHz, DMSO): \( \delta \) (ppm) 3.42 (m, 2H), 3.55(m, 2H), 3.72 (s, 3H), 6.21 (d, \( J = 7.2 \)), \( J = 6.97 \) (d, \( J = 8.9 \)), 7.41 (d, \( J = 8.4 \)), 7.66 (d, \( J = 7.1 \)), 8.93 (s, 1H), 10.07 (s, 1H). High-resolution MS (MALDI-FTMS): Calculated (MH\textsuperscript{+}) \( (C_{19}H_{12}O_{8}\text{Na})_2 \) 7631.1346, found 7631.1342. See Supporting Information for more details.

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