Microarray analysis of global changes in gene expression during cardiac myocyte differentiation

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Peng, Chang-Fu, Yi Wei, Jeffrey M. Levsky, Thomas V. McDonald, Geoffrey Childs, and Richard N. Kitss. Microarray analysis of global changes in gene expression during cardiac myocyte differentiation. Physiol Genomics 9: 145–155, 2002. First published April 16, 2002; 10.1152/physiogenomics.00027.2002.—Significant progress has been made in defining pathways that mediate the formation of the mammalian heart. Little is known, however, about the genetic program that directs the differentiation of cardiac myocytes from their precursor cells. A major hindrance to this kind of investigation has been the absence of an appropriate cell culture model of cardiac myocyte differentiation. Recently, a subline of P19 cells (P19CL6) was derived that, following dimethyl sulfoxide (DMSO) treatment, differentiates efficiently over 10 days into spontaneously beating cardiac myocytes. We demonstrate that these cells are indeed cardiac myocytes as they express cell type-specific markers and exhibit electrophysiological properties indicative of cardiac myocytes. The requirement for DMSO stimulation in this paradigm was shown to be limited to the first 4 days, suggesting that critical events in the differentiation process occur over this interval. To uncover relationships among known genes and identify novel genes that mediate cardiac myocyte differentiation, a detailed time course of changes in global gene expression was carried out using cDNA microarrays. In addition to the activation of genes encoding cardiac transcription factors and structural proteins, increases were noted in the expression of multiple known genes and expressed sequence tags (ESTs). Analysis of the former suggested the involvement of a variety of signaling pathways in cardiac myocyte differentiation. The 16 ESTs whose expression was increased during the early phase of cardiac myocyte differentiation may be novel regulators of this process. Thus this first report of large-scale changes in gene expression during cardiac myocyte differentiation has delineated relationships among the expression patterns of known genes and identified a number of novel genes that merit further study.

DNA microarray; cardiac myocyte

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significant progress has been made over the past decade in identifying genes that play key roles in vertebrate heart formation. Among others, these genes include transcription factors from the Nkx, GATA, MEF2, HAND, and T-box families. Inactivation of most of these genes in the mouse results in striking abnormalities in cardiac morphogenesis. This is illustrated by the absence of right ventricle in MEF2C and DHAND nulls, and failure of the bilaterally symmetric cardiogenic areas to fuse into a heart tube (reflecting a more general defect in ventral morphogenesis) in the GATA-4 nulls (19, 27). Despite the importance of each of these genes in heart formation, however, none appears critical for cardiac myocyte differentiation. Specifically, in each of these knockout models, cardiac myocytes still arise, although alterations in gene expression have been noted. Although the combinatorial effects of loss of function mutations in known genes have not yet been completely defined, these data suggest that novel genes play important roles in cardiac myocyte differentiation.

Identification of the genes that mediate cardiac myocyte differentiation has been hindered by the lack of an appropriate cell culture model. Primary heart muscle cells and transformed cardiac myocyte cell lines are not useful in this regard because they have already irreversibly differentiated. Although embryonic stem (ES) cells (35) and bone marrow stromal cells (23) can be induced to differentiate into cardiac myocytes in vitro, they do not provide an optimal system for assessing changes in gene expression, because the resulting population is an admixture of cell types including undifferentiated cells, cardiac myocytes, and other cell types. P19 cells are a euploid, multipotent, mouse cell line derived from an embryonal carcinoma (26). When treated with retinoic acid, aggregates of these cells differentiate into neurons and glial cells. When treated with dimethyl sulfoxide (DMSO), on the other hand, aggregated P19 cells form cardiac and skeletal myocytes. As in the case of ES cells, however, the differentiation of P19 cells into cardiac myocytes is very inefficient and requires cellular aggregation, which is thought to induce endoderm as a co-stimulus (39).

Recently, a clonal derivative of P19 cells (P19CL6) was isolated following long-term culture of P19 clones that had been selected for the ability to spontaneously differentiate efficiently over 10 days into spontaneously beating cardiac myocytes. The 16 ESTs whose expression was increased during the early, stimulus-dependent phase of cardiac myocyte differentiation was shown to be limited to the first 4 days, suggesting that critical events in the differentiation process occur over this interval. To uncover relationships among known genes and identify novel genes that mediate cardiac myocyte differentiation, a detailed time course of changes in global gene expression was carried out using cDNA microarrays. In addition to the activation of genes encoding cardiac transcription factors and structural proteins, increases were noted in the expression of multiple known genes and expressed sequence tags (ESTs). Analysis of the former suggested the involvement of a variety of signaling pathways in cardiac myocyte differentiation. The 16 ESTs whose expression was increased during the early phase of cardiac myocyte differentiation may be novel regulators of this process. Thus this first report of large-scale changes in gene expression during cardiac myocyte differentiation has delineated relationships among the expression patterns of known genes and identified a number of novel genes that merit further study.

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contract without aggregation following treatment with DMSO (11). P19CL6 cells differ from the parental cell line in that they differentiate efficiently into cardiac myocytes upon treatment with DMSO. Spontaneous beating is evident by 10 days. In addition, there is no morphological evidence of skeletal myogenesis, and transcripts encoding MyoD and myogenin are absent (11). Bone morphogenetic protein (BMP) signaling through TAK1 and SMADs has been shown to be necessary, but not sufficient, for P19CL6 cells to differentiate into cardiac myocytes (29, 30). Specifically, a second yet-to-be-identified signal emanating from DMSO is required.

The present study uses DMSO-treated P19CL6 cells to assess global changes in gene expression during cardiac myocyte differentiation. We first demonstrate that these cells are indeed cardiac myocytes on the basis of their expression of cell type-specific proteins and electrophysiological properties. Second, the minimum time of DMSO stimulation required for differentiation to occur was mapped to 4 days. Last, a detailed time course of changes in gene expression was assessed using mouse cDNA microarrays. The results show that multiple changes in gene expression occur during the differentiation of these multipotent stem cells into cardiac myocytes. Interrelationships among the expression patterns of known genes in this group suggest the involvement of a variety of signaling pathways in this differentiation program. In addition, a relatively limited subset of novel genes was identified whose expression increases during the early, stimulus-dependent phase of differentiation. Investigation of these genes may provide mechanistic insights into the earliest events in cardiac myocyte differentiation.

MATERIALS AND METHODS

P19CL6 cell culture. P19CL6 cells were obtained from Dr. Issei Komuro (Chiba University, Japan; komuro-tyk@umin.ac.jp), who has maintained stocks of these cells since the death of Dr. A. Habara-Okubo, who derived this line (11). Requests for these cells should be sent to Dr. I. Komuro. For ease of shipping, however, cells may be sent from our laboratory upon his consent. P19CL6 cells were cultured in growth medium consisting of α-minimal essential medium (GIBCO-BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (GIBCO-BRL), 2.2 g/l of sodium bicarbonate (Sigma, St. Louis, MO), and 100 μM penicillin and 100 μg/ml streptomycin (GIBCO-BRL) at 37°C with 5% CO₂. To induce differentiation, 1% DMSO (Sigma) was added to the growth medium (differentiation medium).

Immunofluorescence. Following treatment in DMSO-containing or DMSO-free media for 5, 10, or 15 days, cells were washed twice with PBS, fixed for 5 min in 100% methanol at −20°C, dried at room temperature, and stored at 4°C. For immunocytochemistry, cells were washed twice with PBS, permeabilized with 0.1% IGEPAL-630 (Sigma) for 15 min, and then incubated with MF20 (Developmental Studies Hybridoma Bank, University of Iowa) or ventricular myosin light chain 2 (MLC2v) (generous gift from Dr. Ken Chien, University of California, San Diego) antibodies for 1 h at a concentration of 1:200 and 1:50, respectively. Following three PBS washes, the cells were incubated for 1 h with FITC-conjugated anti-mouse or anti-rabbit IgG, respectively. The cells were then washed six times with PBS before microscopic examination. Images were acquired with a digital camera using IP Lab (Scanalytics, Fairfax, VA), and the fluorescence was displayed using Adobe Photoshop 6.0.

Electrophysiology. After treatment with DMSO for 10 days, cells were washed twice with PBS and then detached with 0.2 M EDTA, pH 8.0. The detached cells were plated sparsely and grown on glass coverslips in 35-mm tissue culture dishes. The coverslips were taken directly from the cell culture incubator and placed in an acrylic/polystyrene perfusion chamber (Warner Instruments, Hamden, CT) for immediate electrophysiological measurements as previously described (16). Extracellular solution was 150 mM NaCl, 1.8 mM CaCl₂, 4 mM KCl, 1 mM MgCl₂, 5 mM glucose, 10 mM HEPES buffer, pH 7.4 at room temperature. Intracellular pipette solution was 126 mM KCl, 4 mM Mg-ATP, 2 mM MgSO₄, 5 mM EGTA, 0.5 mM CaCl₂, and 25 mM HEPES buffer, pH 7.2, at room temperature. Osmolarity was adjusted to maintain the internal solution 20–30 mOsm less than the external solution. The whole cell patch clamp configuration was used. Patch pipettes were pulled and polished to obtain a tip resistance of 2–3 MΩ in the above solutions. An Axonpatch-1D patch clamp amplifier (Axon Instruments, Union City, CA) was used for voltage clamp measurements, and voltage protocols were controlled via PC using pClamp6 acquisition and analysis software (Axon Instruments).

Northern blotting. Ten micrograms of total RNA from cells treated with DMSO for 2, 4, 6, 8, 10, 12, and 14 days and untreated controls were size fractionated on 1% agarose gels with formaldehyde in MOPS buffer, following which the RNA was transferred to a nylon membrane (Millipore, Bedford, MA) and cross-linked. Probes for Northern hybridization were the full-length coding regions of mouse GATA-4 and MEF2C. The probe was labeled by the random primer method using [α-32P]dCTP and purified using Nick columns G-50 (Roche, Nutley, NJ). Prehybridization and hybridization were performed using Quickhyb (Stratagene, La Jolla, CA) for 15 and 60 min, respectively. The membrane was washed in 2× SSC/0.1% SDS at room temperature for 20 min and then in 0.2× SSC/0.1% SDS at 65°C for 20 min. Autoradiography was performed at −80°C for 4 h.

Microarray preparation and hybridization. Mouse cDNA microarrays were generated by the shared Microarray Facility of the Albert Einstein College of Medicine. A set of sequence verified mouse GEMarray (Incyte/Genome Systems, Palo Alto, CA) expressed sequence tags (ESTs) plus 212 selected mouse IMAGE collection clones were PCR amplified and spotted onto polylysine-coated microscope slides using a custom-designed robot (3). The microarrays contain 8,956 genes of known sequence. These were hybridized for 18–24 h at 54°C as described (25).

Microarray probe preparation. Total RNA was harvested from cells cultured in DMSO-containing media for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days using the guanidine isothiocyanate method from the RNaseasy Maxi kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. One hundred micrograms of total RNA was reverse transcribed for 1 h at 42°C in a 40-μl reaction containing 1 μg of oligo dT primers (GIBCO-BRL), 2.5 mM each of dATP, dGTP, and dCTP, 1 mM dTTP, 10 mM DTT, 1 μl RNasin (Promega, Madison, WI), 4 μl of Cy5- or Cy3-labeled dUTP (Amersham, Piscataway, NJ), and 2 μl of Superscript II reverse transcriptase (GIBCO-BRL) followed by the addition of another 2 μl of the reverse transcriptase for an additional hour of incubation. The reaction was stopped by heating at 94°C for 2 min and brought to a volume of 100 μl with water; the RNA was digested with 2 μl of RNase ONE (Promega) at 37°C for 15 min. Both probe
solutions were combined in a Microcon YM50 spin column and centrifuged at 12,000 g for 10 min. After three washes with water, the combined probe was eluted from the column.

Microarray data collection and analysis. Slides were scanned and signal intensities for each element were calculated using GenePixPro software (Axon Instruments). Throughout the experiments, any element’s signal present in areas containing excessive noise, printing artifacts, or high regional areas of background was eliminated from further calculations. Also, any elements with a net total intensity less than 250 in either channel, which is less than twofold the localized noise around negative spots on the microarray, were likewise not considered, because of the higher amount of variability in replicate arrays. Each channel (Cy3 and Cy5) was normalized by using the average spot signal intensity across the whole array. To reduce interarray variability, we performed repeat arrays and selected expression level cutoffs. After the data had been preprocessed and entered into an Access database, queries were run to produce dnasets containing genes meeting reproducibility, signal ratio, and net intensity thresholds. Only genes with relative ratios >2 at three consecutive time points were considered to exhibit induced expression. The selected genes were analyzed using the hierarchical algorithms in Cluster from Stanford University. The ratio values were transformed into log2 for analysis using Jackknife Correlation Coefficient (14). The data presented are from one complete set of microarray experiments. Similar results were obtained in a second set of experiments in which RNA from an independent set of cell cultures was analyzed.

RESULTS

DMSO stimulates multipotent P19CL6 cells to differentiate efficiently into cardiac myocytes. To test the efficiency with which P19CL6 cells differentiate upon DMSO stimulation and to more completely characterize the phenotype of the resulting cardiac myocytes, the cells were treated with 1% DMSO for various durations and then scored for spontaneous contractions, the expression of muscle-specific proteins, and electrophysiological properties characteristic of cardiac myocytes. Because only confluent cultures (>70%) were able to differentiate with DMSO, it was not possible to determine the percentage of individual cells that were positive for these parameters. Therefore, the percentage of the field that stained positive was quantitated as a surrogate. No spontaneous contractions were evident in cultures maintained in DMSO-free media for 5, 10, or 15 days or in DMSO-containing media for 5 days. In contrast, after 10 days of DMSO stimulation, ~60% of the field initiated spontaneous contractions, a characteristic of cardiac myocytes, and this percentage approached 100% following 15 days of DMSO treatment.

Consistent with these macroscopic observations, most of the field stained positively with MF20, an antibody that recognizes all vertebrate sarcomeric myosins, following 10 days (not shown) and 15 days (Fig. 1B) of DMSO treatment. In contrast, MF20 staining was absent in cultures treated with DMSO for only 5 days (not shown) and in those maintained in DMSO-free media at all time points including 15 days (Fig. 1A). These data demonstrate that DMSO-differentiated P19CL6 cells are striated muscle cells.

To confirm these results, immunostaining was performed for MLC2v, a second striated muscle-specific marker. MLC2v staining was observed in cultures treated with DMSO for 10 days (not shown) or 15 days (Fig. 1E) but not 5 days (not shown), whereas staining was absent in cultures maintained in DMSO-free media for all of these times including 15 days (Fig. 1D). These results indicate that DMSO-differentiated P19CL6 cells are striated muscle cells. Since MLC2v expression begins at 8.0 days postconception in the mouse embryo (33) and continues through adulthood, however, the presence of this marker does not allow us to determine the developmental stage of these cardiac myocytes.

To assess the identity of DMSO-treated P19CL6 cells using another approach, electrophysiological properties were characterized with patch clamp techniques (16). This analysis revealed action potentials with a shoulder (Fig. 1G), which are indicative of cardiac myocytes. In addition, broad calcium transients were observed (Fig. 1K), which are characteristic of this cell type. Taken together, these electrophysiological measurements, spontaneous contractions, and expression of striated muscle-specific markers indicate that DMSO-treated P19CL6 cells are cardiac myocytes.

Defining the temporal requirement for DMSO stimulation in the differentiation of P19CL6 cells to cardiac myocytes. The previous experiments demonstrated that 10 days of DMSO treatment results in spontaneous contractions in most P19CL6 cells. In contrast, cultures treated with DMSO for only 5 days did not exhibit spontaneous contractions at the 5-day time point. These observations suggest that the differentiation process requires either a longer period of DMSO stimulation, a longer time in culture, or both. To determine the time requirement for DMSO, P19CL6 cells were treated with DMSO-containing media for 0, 2, 4, 6, 8, or 10 days (Fig. 2). In each case, the media was subsequently changed to DMSO-free media for the balance of the experiment (10 or 12 days). This experimental design allowed the time of DMSO treatment to be varied while holding the total time in culture constant. The presence of DMSO during only the first 2 days was inadequate for spontaneous contractions to be detected at the end of a total of 10 or 12 days. In contrast, DMSO treatment during the initial 4 days was sufficient for spontaneous contractions to be observed at 12 days, although not 10 days. Stimulation with DMSO for the first 6 days resulted in spontaneous contractions at both 10 and 12 days. Thus the minimal window for DMSO treatment is approximately during the first 4 days.

Analysis of global changes in gene expression during differentiation of P19CL6 cells into cardiac myocytes. To begin to examine changes in gene expression during DMSO-induced differentiation of P19CL6 cells into cardiac myocytes, we used Northern blotting to assess the temporal expression patterns of two genes that play important roles in cardiogenesis: GATA-4 and MEF2C, which are first expressed in the precardiac
mesoderm between embryonic (E) days 7.0–7.5 and 7.75, respectively (8, 13). The expression of GATA-4 was faintly detectable at day 2 and became maximal after 10 days (Fig. 3, top). In contrast, the expression of MEF2C was first detectable at 8 days and peaked after 10 days (Fig. 3, bottom). Thus DMSO-induced differentiation of P19CL6 cells is accompanied by induction of genes with established roles in heart development.

To extend this analysis to multiple genes, changes in expression were analyzed using a microarray contain-
ing 8,956 mouse cDNAs. Each array was hybridized with Cy3-labeled fluorescent cDNA prepared from undifferentiated P19CL6 cell RNA. We compared this standard reference sample on each array with Cy5-labeled fluorescent probe prepared from P19CL6 cells at different times following induction of differentiation with DMSO. Again, as expected, the abundance of transcripts encoding transcription factors that play important roles in cardiogenesis increased over the time course of DMSO-induced P19CL6 differentiation. For example, the expression of GATA-6 was increased 3-fold at day 2 and 40-fold at day 10 (not shown), that of GATA-4 was increased 4-fold at day 3 and 58-fold at day 10 (not shown), and that of MEF2C was increased 4-fold at day 7 and 10-fold at day 10 (Fig. 4C). These results demonstrate that GATA factors get activated before MEF2C in this model and that our microarray results are consistent with data obtained independently from Northern blotting experiments.

Since the inactivation of multiple genes critical for cardiac morphogenesis, including Nkx2.5 (22, 41), GATA-4 (19, 27), and MEF2C (21), fails to disrupt the differentiation of cardiac myocytes from their precursors, one objective of assessing global changes in gene expression is to identify potential novel regulators of this differentiation process. Another objective is to explore interrelationships among key signaling pathways. To accomplish these goals, the microarray data were analyzed both by temporal and functional clustering. Overall changes in expression will be described here. Interrelationships among the expression patterns of individual genes will be considered in the DISCUSSION.

During the 10-day time course of DMSO-induced P19CL6 differentiation, the expression of 1,293 genes on the microarray (565 known and 728 ESTs) increased and 1,928 (822 known and 1,106 ESTs) decreased more than twofold at one or more time points. If one considers only those genes whose expression was altered at three or more consecutive time points, then these numbers become 541 (224 known and 317 ESTs) increased and 469 (234 known and 235 ESTs) de-

**Fig. 2.** Temporal requirement for DMSO treatment in the differentiation of P19CL6 cells into cardiac myocytes. P19CL6 cells were treated with DMSO-containing media for 0, 2, 4, 6, 8, or 10 days. In each case, the media was subsequently changed to DMSO-free media for the remainder of 10 or 12 days. Cultures were then scored for spontaneous contractions as a differentiation marker. Four days of initial DMSO treatment was the minimum sufficient for spontaneous contractions.

**Fig. 3.** Northern blot analysis of GATA-4 (top) and MEF2C (bottom) expression in P19CL6 cells treated with DMSO for 0, 2, 4, 6, 8, 10, 12, and 14 days. The 28S band on each membrane is shown as loading control.
increased. Our analysis, will focus primarily on increases in gene expression at three or more consecutive time points.

Our studies showing that 4 days, but not 2 days, of DMSO treatment is sufficient for cultures to exhibit spontaneous contractions 12 days later (Fig. 2) suggest that critical events in the differentiation process occur over the first 4 days and provide a biological marker around which to analyze temporal patterns of gene expression (Fig. 4). Accordingly, changes in gene expression were grouped into those beginning before day 4, when differentiation becomes independent of DMSO (group I); from day 4 onward (group II); and from day 8 onward (group III).

Group I includes genes whose expression is increased more than twofold on all of days 1–3, regardless of whether these increases are transient or maintained. Surprisingly, this group comprises only 13 known genes and 16 ESTs (Fig. 4A). Known genes include cellular retinoic acid binding protein 1; the secreted ligand SLIT1; and lymphoid enhancer binding protein 1 (LEF1), a downstream effector of Wnt signaling. The 16 ESTs provide candidate regulators of early events in cardiac myocyte differentiation that merit further investigation.

Group II comprises those genes whose expression is increased more than twofold on all of days 4–10 and not increased more than twofold on all of days 1–3. This group contains 11 known genes and 15 ESTs (Fig. 5B). Known genes include cellular retinoic acid binding protein II; and the basic helix-loop-helix transcription factor eHAND, which is expressed initially in the precardiac mesoderm and later in cells fated to become conotruncus and left ventricle (6, 40).

Group III comprises those genes whose expression is increased more than twofold on all of days 8–10 and not increased more than twofold on days 1–5. Group III contains 58 known genes and 90 ESTs. Known genes include BMP-1, an extracellular ligand in the TGFβ family known to mediate cardiac myocyte differentiation (38); calcineurin catalytic subunit, part of a pathway that mediates cardiac myocyte hypertrophy (28); MEF2C; and, as expected, terminal differentiation markers such as atrial myosin light chain 2.

In addition to analyzing changes in gene expression in relationship to a biological marker, such as when differentiation becomes stimulus-independent, it is often informative to group these changes according to the functions of the proteins they encode. Accordingly, we employed a modified version of the hierarchical clustering method used in the Stanford Gene Cluster Software (9) to place the 541 genes activated at three or more consecutive time points over the 10-day differentiation scheme into 15 functional groups that exhibit 6 distinct temporal expression patterns (Supplemental Table 1, published online at the Physiological Genomics web site; and Fig. 5). The functional groups include extracellular matrix, extracellular and intracellular signaling molecules, and transcription factors, among others. Potential roles of selected genes encoding extracellular ligands, signaling molecules, and transcription factors in cardiac myocyte differentiation will be considered in the DISCUSSION. In addition, a supplement to this paper (Supplemental Table 3) containing the complete set of increases and decreases in expression of all 8,956 genes on the microarray at all 10 time points is posted at the Physiological Genomics web site. The data on the web site will allow investigators to analyze expression patterns for genes and pathways not discussed here and, in so doing, facilitate the generation of additional hypotheses to be tested.

DISCUSSION

We have demonstrated that P19CL6 cells can be induced by DMSO to differentiate efficiently into cardiac myocytes and that this cell culture model provides an excellent system for delineating global changes in gene expression during this process. Descriptions of large scale changes in gene expression are not intended to provide mechanistic information but rather the rationale and basis for future hypothesis-driven studies. Toward this end, this screen revealed that relatively few genes are activated during the initial, stimulus-dependent stages of cardiac myocyte differentiation. In addition, as will be discussed below, correlation of the multiple changes in gene expression that occur over the entire 10-day course of differentiation suggests the involvement of a variety of signaling pathways in driving these polypotent cells toward this specialized phenotype.


Fig. 4. Changes in global gene expression during DMSO-induced differentiation of P19CL6 cells into cardiac myocytes relative to when the process becomes stimulus independent (day 4). Mouse cDNA microarrays were used to compare total RNA from cells treated with DMSO for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days with RNA from untreated controls. Genes are listed vertically, and time points are shown horizontally. Red and green indicate increases and decreases in expression respectively. Known genes and expressed sequence tags (ESTs) are listed according to the following criteria. A: expression increased >2.0-fold on all of days 1–3 without regard to expression at other times. B: expression increased >2.0-fold on all of days 4–10 with expression not increased >2.0-fold on all of days 1–3. C: expression increased >2.0-fold on all of days 8–10 with expression not increased >2.0-fold on days 1–5 and without regard to expression at other times. Graphs to the left of schematically illustrate the selection criteria for A–C; arrows on the abscissa of each graph correspond to day 4, the time at which differentiation to cardiac myocytes is no longer dependent on the presence of DMSO. The accession numbers for each of the known genes and ESTs shown here can be found in Supplemental Table 2, published online at the Physiological Genomics web site.
DMSO-stimulated P19CL6 cells: a cell culture model of cardiac myocyte differentiation. DMSO-stimulated P19CL6 cells may presently be the best cell culture paradigm with which to study global changes in gene expression during cardiac myocyte differentiation. The main advantage of this system over ES cells (17) and bone marrow stromal cells (23) is the apparent efficiency with which DMSO-stimulated P19CL6 cells differentiate into cardiac myocytes. Because only confluent cultures (>70%) are able to differentiate, it has not been possible to measure this efficiency on a cell-by-cell basis. Using, as a surrogate, the percentage of the field that exhibits a cardiac myocyte-specific marker, however, high efficiencies of differentiation have been measured in this and previous studies (11). This approach, however, does not exclude the possibility of contaminating cell types. In fact, preliminary electron-microscopic studies suggest the presence of cells other than cardiac myocytes (unpublished data Hayakawa Y and Kitsis R). Although the identities of these cells are not known, it would not be surprising if DMSO-stimulated P19CL6 cultures were contaminated by endoderm, an inducer of cardiac myocyte differentiation (38), as is thought to be the case with the parental P19 cells (39). This caveat notwithstanding, P19CL6 cells provide a powerful system with which to screen for genes involved in the regulation of cardiac myocyte differentiation as long as it is recognized that the expression of genes chosen for further study must be verified to occur within cardiac myocytes.

Fig. 5. A: temporal cluster analysis of changes in global gene expression during DMSO-stimulated differentiation of P19CL6 cells into cardiac myocytes. Genes are listed vertically and time points horizontally according to each of 6 temporal expression patterns. Only increases involving 3 or more consecutive time points are included. B: temporal expression patterns in A are displayed graphically, showing averages over all of the genes in that group. Identities of individual genes and their temporal clusters are provided in Supplemental Table 1, published online at the Physiological Genomics web site.
Identification of the early activated genes following DMSO stimulation of P19CL6 cells. The initiators of differentiation within cardiogenic precursors have remained elusive. We therefore chose to examine the early-activated genes following DMSO stimulation of P19CL6 cells. Although there were multiple increases in gene expression (1,293 increases more than twofold at one or more time point) over the entire 10-day time course, the expression of only 29 (out of 8,956) genes was increased persistently during the initial DMSO-dependent phase of cardiac myocyte differentiation. Of these, 13 are known genes and will be discussed below. The remaining 16, which are ESTs, are among the most interesting data provided by this screen as some could encode novel regulators of the earliest cell autonomous events in cardiac myocyte differentiation. It is practical to undertake further analysis of this relatively small number of novel genes. If one extrapolates our results with ~9,000 genes to a genome-wide screen, then perhaps 50–80 novel candidate regulators of cardiac myocyte differentiation would be expected to emerge. Even this number of genes might be amenable to individual study. One potential limitation of our gene selection approach is the inclusion criteria of expression at three simultaneous time points (days 1–3 in this case). Although this was adopted to exclude false-positives, its stringency might exclude potential regulators that are expressed over a brief window of time. This concern may be mitigated to some extent by observations in better understood differentiation systems such as skeletal muscle where, for example, Myf-5, the earliest skeletal muscle determination factor in the mouse, is expressed at high levels from embryonic days 8.0–11.5 (34).

Another caveat with regard to the identification of genes that mediate the earliest events in cardiac myocyte differentiation is that the preceding discussion deals only with increases in gene expression. In fact, cardiogenesis is known to be negatively regulated by a variety of signals (36, 42). In addition, in heterokaryon experiments, the cardiac myocyte phenotype is not dominant (10). For these reasons, it is likely that genes whose expression decreases during DMSO-induced differentiation of P19CL6 cells may also be involved in the regulation of early differentiation.

Alterations in expression of known genes provide insights into signaling pathways that mediate cardiac myocyte differentiation. Functional clustering of genes identified by high-throughput screening techniques such as cDNA microarrays provides a more general window into signaling pathways that may be operating during cardiac myocyte differentiation. Our analysis grouped genes functioning in many diverse cellular processes/settings such as apoptosis, cell surface molecules, cytoskeleton, extracellular matrix, extracellular signaling, intracellular signaling, metabolism, transcription factors and others (Supplemental Table 1). We will discuss some examples of changes in gene expression that may define known and potentially novel regulatory pathways.

The expression of extracellular ligands BMP-1 and BMP-5 are induced gradually during differentiation of P19CL6 cells (Supplemental Table 1 and Fig. 5B, clusters 3 and 4). Members of the Delta-like and Jagged family, membrane-associated ligands, bind the receptor Notch, resulting in proteolytic events that release the Notch intracellular domain, which translocates to the nucleus and binds the transcription factor RBP-J, converting it from a repressor to an activator (1). Target genes of RBP-J include Hairy/Enhancer of Split basic helix-loop-helix transcriptional repressors that inhibit cellular differentiation. Three novel Hairy-related transcription factors (HRT-1, -2, and, -3) have been identified in the developing heart, blood vessels, pharyngeal arches, and other locations of epithelial-mesenchymal interactions (4, 18, 20, 32), and Notch signaling has been demonstrated to induce the transcription of these genes (31). This signaling pathway also appears to be operating during differentiation of P19CL6 cells into cardiac myocytes, as transcripts encoding HRT-1 and HRT-3 increase starting at 4 and 7 days of differentiation, respectively. The roles of these proteins in cardiac myocyte differentiation remain to be defined.

Since P19CL6 cells are also capable of differentiating into neurons upon stimulation with retinoic acid (11), it is reasonable to hypothesize that inhibition of the neural pathway may be important for these cells to assume a cardiac fate. For example, expression of the transcription factor Tbx-6, a repressor of neural differentiation (2), is activated (Fig. 4B) during DMSO-induced P19CL6 differentiation. Consistent with this putative role, Tbx-6 is not activated during retinoic acid-induced differentiation of P19 cells into neurons (unpublished data, Wei Y and Childs G). Repressing alternative differentiation pathways may be important for pluripotent cells to become a specific lineage. Such a scenario may permit more general signaling systems...
such as retinoids, which function throughout the embryo, to positively modulate cardiac myocyte differentiation without ectopically activating other cell-type-specific developmental programs (e.g., neural). In fact, the expressions of two cellular retinoid binding proteins increase during DMSO-induced differentiation of P19CL6 cells into cardiac myocytes (Fig. 4, A and B).

Wnt ligands signal through receptors of the Frizzled family, resulting in specific transcriptional responses (15). The latter are brought about when DNA binding proteins of the TCF/LEF (T cell factor/lymphocyte enhancer factor) family, which lack transactivation capabilities, interact with catenins, which cannot bind DNA but can interact with the p300/CBP (p300/CREB binding protein) transcriptional coactivators (7). Signaling downstream of Frizzled increases in the abundance of catenins by inhibiting glycogen synthase kinase-3, which phosphorylates catenins targeting them for degradation in the proteosome. The Wnt pathway has been implicated in the suppression of cardiogenesis, and Wnt antagonists such as Dkk-1 and Crescent appear to be important in promoting cardiogenesis (24, 36, 42). Counterintuitively, we see increases in the expression of components of the Wnt pathway during the differentiation of P19CL6 cells into cardiac myocytes. The expression of Frizzled-4 is increased two- to threefold between days 6 and 8 (Supplemental Table 1 and data not shown), whereas that of LEF-1 is increased two- to fivefold between days 1 and 8 (Fig. 4A and data not shown). Transcripts encoding Dkk-3 are detectable at similar levels throughout the time course (Dkk-1 and Crescent not on the microarray). The interpretation of these observations is not clear at the present time and may require a more complete understanding of Wnt target genes in P19CL6 cells.

The expression of Snail and Twist, two transcription factors involved in mesoderm determination, increase during the differentiation of P19CL6 cells into cardiac myocytes (Supplemental Table 1 and Fig. 5B, clusters 2 and 5, respectively). Twist is a direct activator of MEF2 in Drosophila (5), whereas it inhibits transactivation by MyoD in mammalian cells (12). Interestingly, the time course of increases in Twist expression in DMSO-treated P19CL6 cells (days 5–10) parallels and slightly precedes that of MEF2C (days 7–10). We hypothesize that Twist enforces the cardiac myocyte differentiation program in these cells while repressing the skeletal muscle program that is observed late in the course of DMSO stimulation in the parental P19 cells (26).

The expression of a variety of other transcription factors is also induced during the differentiation of P19CL6 differentiation. Among these are members of the Hox, ETS, and ELK families, caudal type homeobox 2, core binding factor-β and others (Supplemental Table 1). Differences in the kinetics of activation of these genes suggest that their encoded proteins play specific roles in the differentiation process. Elucidation of the target genes of these factors will be an important component of understanding the “wiring diagram” of cardiac myocyte differentiation. The ability to generate stable P19CL6 lines that express specific transcription factors inducibly should make it possible to define causal, as well as temporal, relationships among the expression patterns of multiple genes using microarrays.

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