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Myocyte Enhancer Factor 2C and Nkx2–5 Up-regulate Each Other's Expression and Initiate Cardiomyogenesis in P19 Cells*

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The Nkx2–5 homeodomain protein plays a key role in cardiomyogenesis. Ectopic expression in frog and zebrafish embryos results in an enlarged myocardium; however, expression of Nkx2–5 in fibroblasts was not able to trigger the development of beating cardiac muscle. In order to examine the ability of Nkx2–5 to modulate endogenous cardiac specific gene expression in cells undergoing early stages of differentiation, P19 cell lines overexpressing Nkx2–5 were differentiated in the absence of Me_2SO. Nkx2–5 expression induced cardiomyogenesis in these cultures aggregated without Me_2SO. During differentiation into cardiac muscle, Nkx2–5 expression resulted in the activation of myocyte enhancer factor 2C (MEF2C), but not MEF2A, -B, or -D. In order to compare the abilities of Nkx2–5 and MEF2C to induce cellular differentiation, P19 cells overexpressing MEF2C were aggregated in the absence of Me_2SO. Similar to Nkx2–5, MEF2C expression initiated cardiomyogenesis, resulting in the up-regulation of Brachyury T, bone morphogenetic protein-4, Nkx2–5, GATA-4, cardiac α-actin, and myosin heavy chain expression. These findings indicate the presence of a positive regulatory network between Nkx2–5 and MEF2C and show that both factors can direct early stages of cell differentiation into a cardiomyogenic pathway.

The NK-2 class homeobox gene product Nkx2–5/Csx plays a key role in cardiac muscle development (1–3). Nkx2–5 is the mouse homologue of the *Drosophila* gene *tinman* (4, 5), which is essential for specification of heart muscle progenitors in the fly (6). Both *tinman* and Nkx2–5 are expressed in the heart lineage as a result of signals from decapentaplegic and bone morphogenetic protein (BMP)[1] signaling, which are members of the TGFβ superfamily (2, 7, 8). Mice lacking Nkx2–5 still form a beating linear heart tube, in which most myogenic genes are expressed, but the mice die due to defective heart looping (9–11). Ectopic expression of Nkx2–5 in frog and zebrafish embryos results in an enlarged myocardium, suggesting that Nkx2–5 recruits additional cells into the heart from the heart morphogenetic field (12, 13). However, expression of Nkx2–5 in fibroblasts was not able to trigger the development of beating cardiac muscle (12, 13).

The MEF2 family of transcription factors has been shown to play a critical role in the cell type-specific transcription of genes in cardiac, skeletal, and smooth muscle cells (14, 15) as well as in brain and neuronal cells (16–21). There are four vertebrate MEF2 family members, MEF2A, -B, -C, and -D (14). They contain a conserved MADS box/MEF2 domain at their N termini, which is both necessary and sufficient for dimerization and DNA binding to an AT-rich MEF2 binding site. *Drosophila* lacking the single *D-mef2* gene are deficient in cardiac, skeletal, and smooth muscle development, indicating an essential role for D-MEF2 in the development of these tissues (22–24). However, mice lacking MEF2B showed no phenotype, whereas mice lacking MEF2C were deficient in cardiac looping, dying around embryonic day 10 (25). Consequently, the role of MEF2 family members in murine muscle development is more difficult to assess due to possible functional redundancy.

Promoter analysis has shown that MEF2 sites mediate the expression of several muscle-specific genes in cardiac muscle, including cardiac myosin light chain 2, cardiac troponin T, muscle creatine kinase, and α-myosin heavy chain (26–29). The first MEF2 family member to be expressed during mouse development is MEF2C, which is found on embryonic day 7.5 in cells of the cardiac mesoderm (30). Mice lacking MEF2C were deficient in the expression of a subset of cardiac specific genes, including atrial natriuretic factor, cardiac α-actin, α-myosin heavy chain, and the basic helix-loop-helix factor dHAND. However, other cardiac muscle genes such as *MLC2v* and *MLC2a* were expressed normally (25). Since MEF2B was up-regulated in MEF2C mutant mice, it is likely that MEF2B may partially substitute for MEF2C activity. The ability of MEF2 family members to induce muscle development in tissue culture is controversial. One study documented that MEF2A initiates skeletal myogenesis in fibroblasts (31), but these results were not confirmed by others (32).

The zinc finger transcription factor GATA-4 also plays a key role in cardiac muscle development (3, 33, 34). GATA-4 is expressed in the precardiac mesoderm at 7.5 days postcoitum and in the endocardial and myocardial layers of the heart tube (35). GATA-4 can regulate a number of cardiac structural genes, such as α-myosin heavy chain, cardiac troponin-C, atrial natriuretic factor, and brain natriuretic peptide (36–40). Mice lacking GATA-4 develop cardiomyocytes, which express cardiac muscle-specific genes. However, these mice die early due to defective morphogenetic movements required for the formation of the linear cardiac tube (41, 42).

We have analyzed the ability of both Nkx2–5 and MEF2C to activate endogenous cardiac muscle-specific gene expression in murine P19 embryonal carcinoma cells. The differentiation of

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[1] The abbreviations used are: BMP, bone morphogenetic protein; MEF2, myocyte enhancer factor 2; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; kb, kilobase pair(s).

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these pluripotent stem cells is initiated by cellular aggregation in the presence of differentiating agents and emulates the biochemical and morphological processes that occur during early embryonic development (43, 44). Aggregation of P19 cells in the absence of differentiating agents activates the expression of the mesoderm marker, Brachyury T (45), but few of the cells continue to differentiate. P19 cells treated with retinoic acid differentiate into various neuroectodermal derivatives, including neurons, astrocytes, and glia (44, 46). P19-derived neurons express the neurogenic basic helix-loop-helix transcription factor MASH1 (47) and MEF2C (16), which can physically interact to synergistically activate transcription.

P19 cells aggregated in the presence of Me_2SO may differentiate into cardiac and skeletal muscle along with other mesodermal and endodermal cell types (48). The resulting cardiomyocytes are embryonic in nature and first appear at day 6 following Me_2SO treatment. Semiquantitative RT-PCR analysis has shown that GATA-4 is first expressed on day 3 of differentiation and is followed by Nkx2–5 on day 4 of differentiation (49). MEF2C appears to be expressed constitutively but is up-regulated at day 6.

The rationale for examining the ability of stem cells to differentiate in the presence of exogenous transcription factors is to provide the factor of interest with an environment similar to that of the developing embryo. Therefore, the presence or absence of tissue-restricted components within the host cells does not limit the function of the transcription factor. In addition, sufficient material can be obtained for subsequent analysis. For example, P19 cells overexpressing either GATA-4 (49) or MyoD (50) differentiate into cardiac muscle or skeletal muscle, respectively, when aggregated in the absence of Me_2SO. Using this model system, we report that both Nkx2–5 and MEF2C initiated the development of cardiac muscle when overexpressed in cells aggregated in the absence of Me_2SO.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The DNA construct PGK-MEF2C contains the phosphoglycerate kinase (*pgk-1*) promoter (51) driving the coding region of human MEF2C (20). This isoform of MEF2C binds DNA and activates transcription. The construct PGK-Nkx2–5 contains the *pgk-1* promoter driving a 1.6-kb *Eco*RI fragment containing the complete open reading frame of mouse Nkx2–5 cDNA (5). The construct PGK-Puro contains the *pgk*-1 promoter driving the gene encoding puromycin resistance, as described (50). The construct PGK-LacZ contains the *pgk-1* promoter driving the gene encoding β-galactosidase. PGK-vector DNA is a plasmid containing the *pgk-1* promoter alone.

Tissue Culture—P19 embryonal carcinoma cells were cultured as described (44) with the modification that 5% Cosmic calf serum (HyClone, Logan, Utah) and 5% fetal bovine serum (CanSera, Rexdale, Ontario, Canada) were used to supplement the α-minimal essential medium. Stable cell lines expressing Nkx2–5 (5) were isolated following transfection of P19 cells with 8 μg of PGK-Nkx2–5, 2.5 μg of B17 (52), 1 μg of PGK-LacZ, and 1 μg of PGK-Puro, as described previously (50). Control P19 cells were isolated by transfection with the same complement of plasmids except that the 8 μg of PGK-Nkx2–5 plasmid was replaced by PGK-vector DNA. Cells were selected for 1 week in 2 μg/ml puromycin. Three cell lines that expressed high levels of Nkx2–5 were termed P19(Nkx2–5) cells. Transfected cells expressing low levels of Nkx2–5 behaved similarly to P19 cells and were not pursued further. Stable cell lines expressing MEF2C were isolated in a similar fashion to P19(Nkx2–5) cells. P19 cells were transfected with 6.5 μg of a plasmid containing PGK-MEF2C or 6.5 μg of PGK-vector alone, 2.5 μg of B17, 1 μg of PGK-LacZ, and 1 μg of PGK-Puro. All experiments reported were performed at least twice with at least two cell lines, with similar results. Fig. 1 shows immunofluorescence from P19(Nkx2–5) clone 1, and Figs. 3*B*, 4, 5, and 6 show data from P19(MEF2C) clone 1.

Differentiation was initiated by plating 5×10^5 cells into 60-mm bacterial dishes in the presence or absence of 0.8% Me_2SO. Cells were cultured as aggregates for 4 days and then plated in tissue culture dishes and harvested for RNA or fixed for immunofluorescence at the time indicated.

Immunofluorescence—P19, P19(Nkx2–5), and P19(MEF2C) cells were plated on day 4 of differentiation onto gelatin coated coverslips. For identifying myosin heavy chain (53), cells were fixed in methanol at −20 °C and reacted with antibody as described (50). Immunofluorescence was visualized with a Zeiss Axioskop microscope. Images were captured with a Sony 3CCD color video camera; processed using Northern Exposure, Adobe photoshop, and Corel Draw software; and printed with a dye sublimation phaser 450 Tektronic printer.

Northern Analysis—Total RNA was isolated by the lithium chloride/urea extraction method, and 6 μg were examined by Northern blot analysis as described previously (50). The probes used were a 600-base pair *Pst*I fragment from the human cardiac α-actin last exon (54), a 1.5-kb *Hin*dIII/*Xba*I fragment of MEF2C cDNA (55), a 1.55-kb *Xho*I/*Bam*HI fragment of MEF2A cDNA (56), a 1.55-kb *Xho*I/*Bam*HI fragment of MEF2B cDNA (57), a 1.5-kb *Xho*I/*Bam*HI fragment of MEF2D cDNA (58), a 1-kb *Hin*dIII/*Bam*HI fragment of mouse BMP-4, a 2.4-kb *Xba*I fragment of GATA-4 (36), a 1.6-kb *Eco*RI fragment of Nkx2–5 cDNA (5), and a 1.6-kb *Eco*RI/*Bam*HI fragment of the mouse Brachyury T cDNA (59). Northern blots were visualized with a Molecular Dynamics PhosphorImager SI and quantitated with ImageQuant software. Averages and S.E. values were calculated and reported.

Reverse Transcription Polymerase Chain Reaction—Total cellular RNA was extracted with TRIzol Reagent according to the manufacturer (Life Technologies, Burlington, Ontario, Canada) and treated with DNase I, amplification grade, at a concentration of 1 unit/μg RNA. The first strand cDNA synthesis was performed using Superscript II RNase H^- reverse transcriptase according to the manufacturer (Life Technologies) with 1 μg of total RNA. Platinum *Taq* DNA Polymerase (Life Technologies) was used to perform the PCR with 25 cycles: 94 °C for 1 min, 55–72 °C for 2 min, depending on the melting temperature of the primers, and 72 °C for 2 min. The amount of first strand reaction added to the PCR was titrated for each set of primers, and the quantity used was chosen in the middle of the linear range. Products were detected by Southern blot analysis with a probe from the corresponding cDNA. Negative controls performed with all RT-PCR experiments included a water control for the PCR and a water control for the complete RT-PCR. RNA was examined for genomic contamination after the DNase treatment by performing the RT-PCR in the absence of Superscript II RNase H^- reverse transcriptase. The following pairs of primers were used: 5′-tccatccacgtcggccaggct-3′ and 5′-gtagggctcaaccacagcagt-3′ for tubulin, with an annealing temperature of 61 °C; 5′-actctggaggcgagatggg-3′ and 5′-ctcggcattacgacgccacag-3′ for GATA-4 (41), with an annealing temperature of 72 °C; and 5′-cctctagagcagagctgcgcgcggagatg-3′ and 5′-ggtggcttccgtcgccgccgtgc-3′ for Nkx2–5 (5), with an annealing temperature of 72 °C.

RESULTS

P19(Nkx2–5) Cells Differentiate into Cardiac Muscle in the Absence of Me_2SO—In order to examine the activity of Nkx2–5 in the context of differentiating stem cells, three P19 cell lines that stably express high levels of Nkx2–5 were isolated and termed P19(Nkx2–5) cells. These cells continued to express the stem cell marker Oct-3 (60) when grown as monolayer cultures, indicating that they retain a stem cell phenotype (data not shown) similar to that observed for P19(MyoD) cells (50).

To examine whether expression of Nkx2–5 in P19 cells can induce differentiation into cardiac muscle, P19 and P19(Nkx2–5) cells were aggregated for 4 days without Me_2SO and fixed on day 6. The amount of cardiac muscle was quantitated by counting cardiomyocytes in cultures that were stained by immunofluorescence with the anti-myosin heavy chain antibody, MF20 (53). Aggregation of P19(Nkx2–5) cells in the absence of Me_2SO resulted in the differentiation of abundant cardiac muscle (30–60% of total cells, Table I). This is demonstrated by the presence of cardiomyocytes expressing myosin heavy chain in P19(Nkx2–5) cells (Fig. 1*D*), compared with control cell cultures (Fig. 1*B*).

Total mRNA was isolated from P19 and P19(Nkx2–5) cultures on days 0 and 6 of differentiation in the absence of Me_2SO and subjected to Northern blot analysis. High levels of Nkx2–5 were expressed on both day 0 and day 6 in P19(Nkx2–5) cultures (Fig. 2*A*, *lanes 3–8*) but not in P19 control cultures (Fig. 2*A*, *lanes 1* and *2*). The formation of abundant Nkx2–5-induced cardiac muscle is indicated by high levels of cardiac α-actin

TABLE I
Comparison of the cell types produced after aggregation of various P19 cell lines

Cell line and source	Differentiated cell types produced	Drug requirement	Extent of differentiated cell type	Time required for differentiation
			%	*days*
P19(MEF2C) (this report)	Cardiac	None	1.5–3[a]	6
P19(Nkx2–5) (this report)	Cardiac	None	30–60[a]	6
P19(MyoD) (50)	Skeletal	None	~30	6
P19(GATA-4) (49)	Cardiac	None	10–25	6
P19 (44)	Cardiac	Me_2SO	~15	6
	Skeletal	Me_2SO	<5	9

[a] The percentage of differentiated cells was calculated by dividing the total number of cardiomyocytes counted in 15 representative fields by the total number of cells, estimated from counting Hoechst-stained nuclei, for at least two cell lines in at least two experiments.

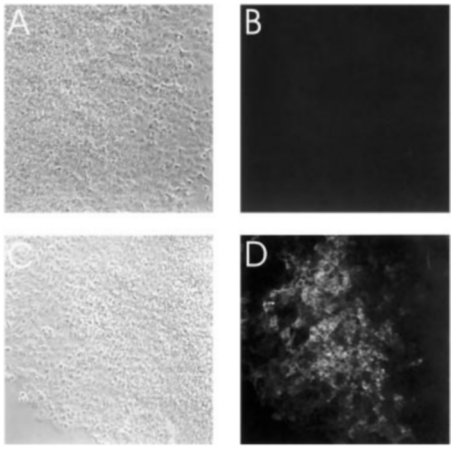

FIG. 1. **Nkx2–5 induces cardiomyogenesis in P19 cells aggregated in the absence of Me_2SO.** P19 (*A* and *B*) and P19(Nkx2–5) (*C* and *D*) cells were aggregated without Me_2SO. Cells were fixed in methanol on day 6, stained with MF20, and visualized by phase contrast microscopy (*A* and *C*) or fluorescence microscopy (*B* and *D*; magnification, × 16).

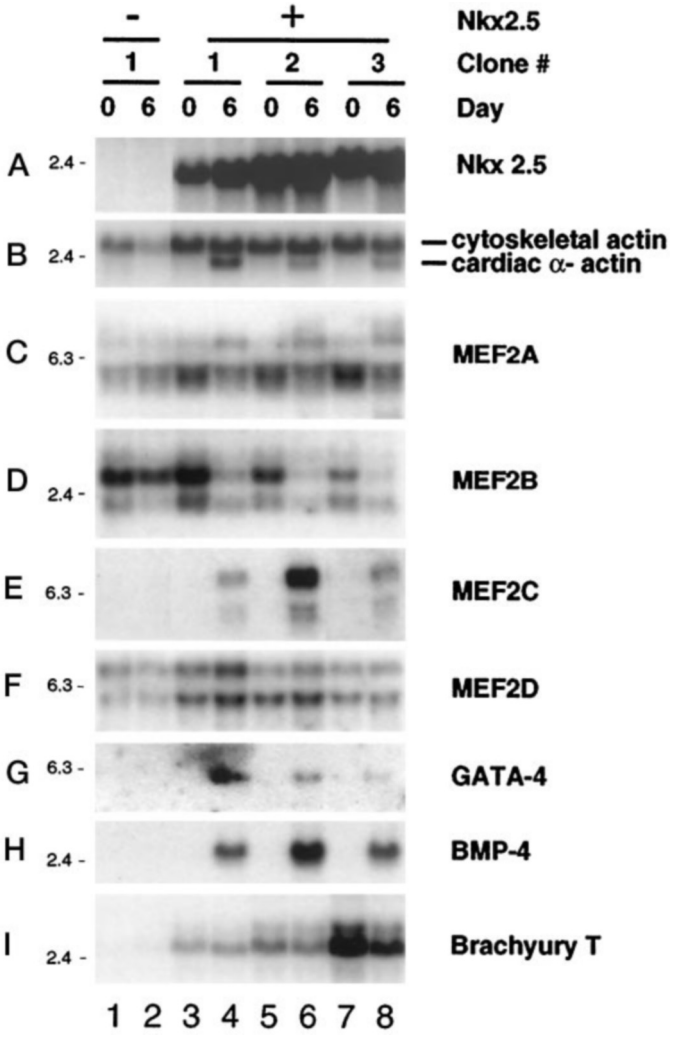

FIG. 2. **Nkx2–5 expression results in the activation of MEF2C, GATA-4, BMP-4, and Brachyury T expression.** P19 and P19(Nkx2–5) cells were differentiated without Me_2SO, and total RNA was harvested on days 0 and 6 of differentiation. Northern analysis was performed on 6 μg of RNA with probes for Nkx2–5 (*A*), cardiac α-actin (*B*), MEF2A (*C*), MEF2B (*D*), MEF2C (*E*), MEF2D (*F*), GATA-4 (*G*), BMP-4 (*H*), and Brachyury T (*I*). The cytoskeletal actin transcript in *B* serves as a loading control. The approximate sizes of the bands are indicated relative to the position of 18 or 28 S rRNA, measured as 6.3 or 2.4 kb, respectively.

expression present on day 6 of differentiation in P19(Nkx2–5) cultures (Fig. 2*B*, *lanes 4*, *6*, *8*) but not in P19 control cultures (Fig. 2*B*, *lane 2*).

Nkx2–5-induced Differentiation Up-regulates the Expression of Factors Involved in Cardiomyogenesis—The MEF2 family of transcription factors has been shown to bind MEF2 sites present in muscle-specific genes and activate their expression (14). The expression patterns of the four MEF2 family members were examined in P19 and P19(Nkx2–5) cultures aggregated in the absence of Me_2SO. Of the four factors, only MEF2C demonstrated a 9 ± 3-fold increase in expression ($n = 3$) in P19(Nkx2–5) cultures on day 6 (Fig. 2*E*, *lanes 4*, *6*, and *8*), compared with P19 control cultures (Fig. 2*E*, *lane 2*). MEF2A showed a slight 0.32 ± 0.07-fold increase ($n = 3$) in the expression of the upper transcript on day 6 compared with day 0 in P19(Nkx2–5) cultures (Fig. 2*C*, *lanes 4*, *6*, and *8*). MEF2D levels did not change appreciably with differentiation in either P19 (Fig. 2*F*, *lanes 1* and *2*) or P19(Nkx2–5) (Fig. 2*F*, *lanes 3–8*) cultures. MEF2B levels decreased by 0.80 ± 0.04-fold ($n = 3$) during differentiation of P19(Nkx2–5) cultures (Fig. 2*D*, *lanes 4*, *6*, and *8*), but not of P19 cultures (Fig. 2*D*, *lane 2*). Thus, MEF2C is the only MEF2 factor whose expression is up-regulated to very high levels in differentiated P19(Nkx2–5) cultures.

The expression level of GATA-4 was also examined in P19 and P19(Nkx2–5) cultures. GATA-4 transcript levels were increased 7 ± 1-fold ($n = 3$), in P19(Nkx2–5) cultures on day 6 (Fig. 2*G*, *lanes 4*, *6*, and *8*), when compared with day 0 P19(Nkx2–5) cultures (Fig. 2*G*, *lanes 3*, *5*, and *7*) or to control P19 cultures (Fig. 2*G*, *lanes 1* and *2*).

BMP-4 is a member of the tumor growth factor-β superfamily of signaling molecules (61) and plays an important role in cardiac muscle development (8). BMP-4 transcript levels were increased 10 ± 2-fold ($n = 3$) in P19(Nkx2–5) cultures on day 6 (Fig. 2*H*, *lanes 4*, *6*, and *8*), when compared with day 0 P19(Nkx2–5) cultures (Fig. 2*H*, *lanes 3*, *5*, and *7*) or with control P19 cultures (Fig. 2*H*, *lanes 1* and *2*).

Brachyury T is a member of the T-box family of transcription factors (62). It is expressed in the primitive streak at the onset of gastrulation and is a marker of mesoderm formation (63).

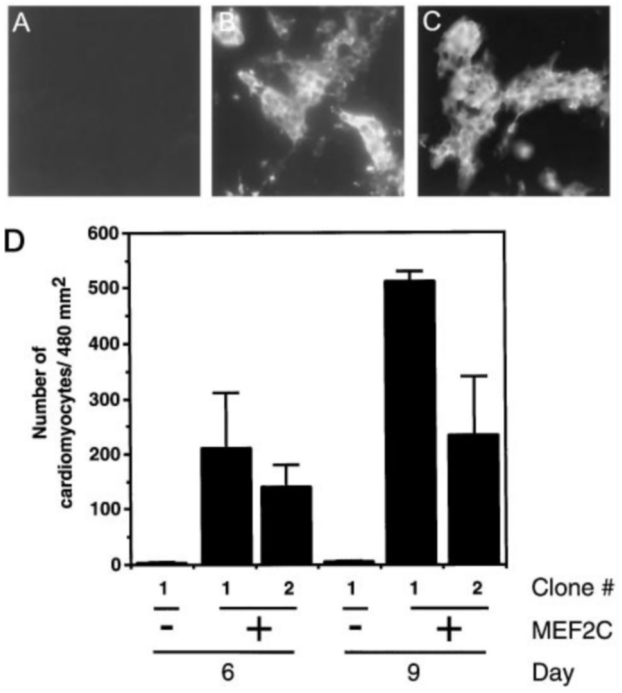

FIG. 3. **MEF2C induces cardiac muscle development in cells aggregated without Me_2SO.** P19 (*A* and *C*) and P19(MEF2C) (*B*) cells were aggregated with (*C*) and without (*A* and *B*) Me_2SO. Cells were fixed in methanol on day 9 and stained with MF20 (magnification, × 40). The total number of cardiomyocytes was counted from each condition, averaged, and depicted graphically (*D*). *Error bars* represent S.E. (n = 3–5).

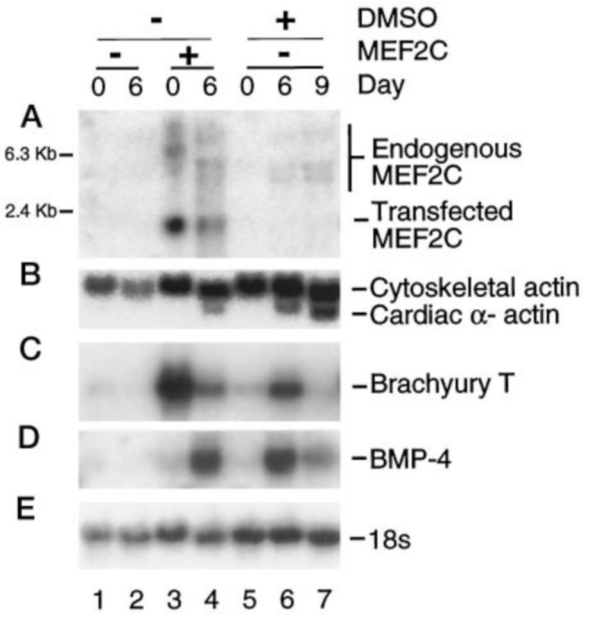

FIG. 4. **MEF2C up-regulates the expression of cardiac α-actin and mesoderm-patterning factors.** P19 and P19(MEF2C) cells were differentiated with and without Me_2SO (*DMSO*), and total RNA was harvested on day 0, 6, and 9 of differentiation. Northern analysis was performed on 6 μg of RNA with probes for MEF2C (*A*), cardiac α-actin (*B*), Brachyury T (*C*), BMP-4 (*D*), and 18 S (*E*, loading standard). The approximate sizes of the bands are indicated relative to the position of 18 or 28 S rRNA, measured as 6.3 or 2.4 kb, respectively.

During P19 cell differentiation either with or without Me_2SO, Brachyury T expression peaks on day 2 of differentiation (45). P19(Nkx2–5) cells grown in monolayer expressed 13 ± 4-fold (n = 5) higher levels of Brachyury T than control P19 cells grown in monolayer (Fig. 2*I*, compare *lanes 3*, *5*, and *7* with *lane 1*). These results indicate that Nkx2–5 can direct early stages of P19 cell differentiation into a cardiac muscle lineage.

In summary, Nkx2–5 expression resulted in the induction of cardiac muscle development in P19 cells aggregated in the absence of Me_2SO. During the process of cardiomyogenesis, the expression levels of Brachyury T, BMP-4, MEF2C, and GATA-4 were up-regulated, while the levels of MEF2B were inhibited.

MEF2C Expression Results in Cardiomyogenesis in P19 Cells When Aggregated in the Absence of Me_2SO—To compare the developmental potential of P19 cells that overexpress MEF2C with that observed for P19(Nkx2–5) cells, three P19 cell lines were isolated that stably express high levels of MEF2C, termed P19(MEF2C) cells. Similar to P19(MyoD) cells (50) and P19(Nkx2–5) cells, monolayers of these cells did not differentiate and continued to express the stem cell marker Oct-3 (60), indicating that they retain a stem cell phenotype (data not shown).

In order to examine whether or not MEF2C can modulate the endogenous P19 cell differentiation pathway into cardiac muscle, P19 and P19(MEF2C) cells were aggregated for 4 days with and without Me_2SO and stained on day 9 by immunofluorescence with the anti-myosin heavy chain antibody, MF20 (53). For cultures aggregated without Me_2SO, cardiac muscle was observed in P19(MEF2C) cells (Fig. 3*B*) but not in P19 cells (Fig. 3*A*). Thus, MEF2C induced the development of cardiac muscle in P19 cells aggregated without Me_2SO. P19 cells aggregated with Me_2SO differentiated into cardiac muscle in both the presence and absence of MEF2C expression (data not shown), indicating that MEF2C did not affect the ability of P19 cells to differentiate into cardiac muscle when aggregated with Me_2SO. The MEF2C-induced cardiac muscle (Fig. 3*B*) is morphologically indistinguishable from the Me_2SO-induced cardiac muscle in the control P19 cells (Fig. 3*C*) but is in much less abundance (Table I). The amount of cardiac muscle present in P19 and P19(MEF2C) cultures aggregated without Me_2SO was quantitated by counting cardiomyocytes that reacted with the anti-myosin heavy chain antibody, MF20. Cardiomyocytes represented 1.5–3% of the total cells in MEF2C (Table I) cultures but were negligible in P19 cultures (Fig. 3*D*). Therefore, MEF2C induces the development of cardiac muscle in differentiating P19 cells without the requirement for Me_2SO.

MEF2C Up-regulates Cardiac α-Actin, Nkx2–5, and GATA-4 Expression—To confirm the observations obtained by immunofluorescence, Northern blot analysis was used to examine the expression of muscle-specific genes. The presence of cardiac α-actin transcripts indicates the development of muscle in cultures. Total mRNA from P19 and P19(MEF2C) cultures was harvested on day 6 after aggregation without Me_2SO. In addition, RNA was harvested from P19 cultures on days 6 and 9 after aggregation with Me_2SO. The latter control was included to compare MEF2C-induced and Me_2SO-induced cardiomyogenesis. The results are shown in Fig. 4, *A* and *B*. Transfected MEF2C transcripts were expressed in P19(MEF2C) cells (Fig. 4*A*, *lanes 3* and *4*) and not in P19 control cells (Fig. 4*A*, *lanes 1*, *2*, *5*, *6*, and *7*). Transfected MEF2C transcripts could be differentiated from endogenous transcripts by size. Several bands appear for the endogenous MEF2C transcripts due to alternative splicing (64). Interestingly, the expression of exogenous MEF2C transcripts seemed to result in the activation of endogenous MEF2C expression. The expression levels of MEF2A, MEF2B, and MEF2D did not appear to change in any of the conditions examined (data not shown). Similar levels of MEF2C expression, both endogenous and exogenous, were found in the other clone examined, P19(MEF2C) clone 2 (data not shown).

MEF2C expression induced the formation of cardiac muscle in cells aggregated without Me_2SO. This is demonstrated by

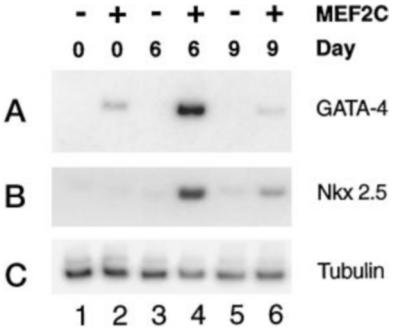

FIG. 5. **MEF2C up-regulates the expression of Nkx2–5 and GATA-4.** Semiquantitative RT-PCR was used to examine the level of expression of GATA-4 (*A*), Nkx2–5 (*B*), and tubulin (*C*), in P19 and P19(MEF2C) cultures aggregated without Me_2SO on days 0, 6, and 9 of differentiation.

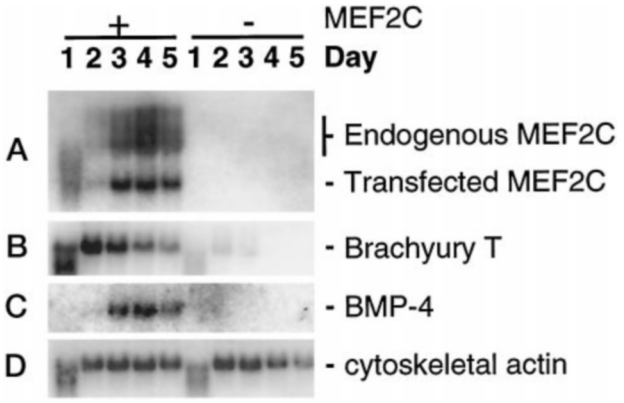

FIG. 6. **MEF2C directs early stages of P19 cell differentiation into a cardiomyogenic pathway.** P19 and P19(MEF2C) cells were differentiated without Me_2SO and total RNA was harvested during a time course of differentiation. Northern analysis was performed on 6 μg of RNA with probes from MEF2C (*A*), Brachyury T (*B*), BMP-4 (*C*), and cardiac α-actin (*D*, loading standard).

the expression of cardiac α-actin in P19(MEF2C) cells when compared with P19 cells (Fig. 4*B*, *lanes 2* and *4*). P19 cells aggregated with Me_2SO expressed cardiac α-actin at higher levels than P19(MEF2C) cells aggregated without Me_2SO (Fig. 4*B*, compare *lane 6* with *lane 4*). These Me_2SO-treated P19 cultures also express endogenous MEF2C (Fig. 4*A*, *lanes 6* and *7*), indicating a role for MEF2C in the endogenous P19 cell differentiation into cardiac muscle.

Nkx2–5 and GATA-4 levels could not be detected in P19(MEF2C) cardiomyocytes or in Me_2SO-induced cardiomyocytes by Northern blot analysis (data not shown) and so were examined by semiquantitative RT-PCR. A 45 ± 32-fold activation of GATA-4 was observed in P19(MEF2C) cultures on day 0, compared with control P19 cells (Fig. 5*A*, compare *lane 1* with *lane 2*), indicating an activation of GATA-4 expression when grown in monolayer in these cells. Activation of GATA-4 expression increased in P19(MEF2C) cultures on day 6 of differentiation without Me_2SO to 97 ± 61-fold ($n = 2$) (Fig. 5*A*, compare *lane 4* with *lane 3*). By day 9 of differentiation without Me_2SO, the activation of GATA-4 had decreased (Fig. 5*A*, *lane 6*).

A 6 ± 2-fold up-regulation of Nkx2–5 expression ($n = 3$) was observed in P19(MEF2C) cultures on day 6 of differentiation without Me_2SO, compared with control P19 cultures (Fig. 5*B*, compare *lane 4* with *lane 3*). By day 9 of differentiation without Me_2SO, the activation of Nkx2–5 had decreased (Fig. 5*B*, *lane 6*). Thus, in agreement with the immunofluorescent staining, the Northern blot and RT-PCR analysis results support the observation that MEF2C induces cardiac muscle development in the absence of Me_2SO.

MEF2C Directs Early Stages of P19 Cell Differentiation into a Cardiomyogenic Pathway—In order to gain a better understanding of the pathway used by MEF2C to induce cardiogenesis, the expression patterns of factors involved in mesoderm patterning were examined by Northern blot analysis (Fig. 4, *C* and *D*). Brachyury T was expressed 16 ± 8-fold higher ($n = 3$) in P19(MEF2C) cells grown in monolayer when compared with control P19 cells (Fig. 4*C*, compare *lane 3* with *lanes 1* and *5*). This indicates that MEF2C expression can result in the regulation of Brachyury T expression in monolayer cultures.

P19 cells expressed very low levels of BMP-4 when grown in monolayer or on day 6 after cellular aggregation in the absence of Me_2SO (Fig. 4*D*; *lanes 1* and *2*). Overexpression of MEF2C resulted in a 60 ± 30-fold enhancement of BMP-4 expression ($n = 3$) after cellular aggregation in the absence of Me_2SO (Fig. 4*D*; compare *lanes 4* and *2*). Me_2SO-induced cardiac muscle also expressed BMP-4 (Fig. 4*D*, *lanes 6* and *7*).

A time course of differentiation was examined in order to determine the stage at which MEF2C modulates the expression of mesoderm-patterning factors (Fig. 6). Northern blots were performed on RNA harvested at various times during the differentiation of P19 and P19(MEF2C) cells cultured without Me_2SO. P19(MEF2C) cells express both exogenous and endogenous MEF2C at high levels compared with control P19 cells (Fig. 6*A*). The increase in the expression of exogenous MEF2C during the time course is most likely due to regulatory regions at the site of insertion of the transfected DNA. A survey of 14 cell lines overexpressing either MyoD or myogenin has shown that 60% of the clones increase the expression of the transfected gene during aggregation, 30% stay the same, and 10% decrease the expression.[2] These differences had no effect on the ability of these cells to undergo myogenesis, and they seem to be a general phenomenon of stable transfection in P19 cells.

P19(MEF2C) cultures expressed considerably higher levels of Brachyury T at all time points examined, when compared with control P19 cells (Fig. 6*B*). BMP-4 levels were not substantially enhanced in P19(MEF2C) cultures until day 3 of differentiation, compared with P19 control cultures (Fig. 6*C*). Consequently, Brachyury T is the earliest endogenous factor whose expression is enhanced, directly or indirectly, by MEF2C activity in monolayer cultures and is followed on day 3 of differentiation by the up-regulation of BMP-4. Therefore, it would appear that MEF2C can direct early stages of P19 cell differentiation into a cardiomyogenic pathway.

DISCUSSION

MEF2C and Nkx2–5 up-regulate each other's expression and induce cardiomyogenesis in aggregated P19 cells. These findings are consistent with their expression pattern and activity. Both Nkx2–5 and MEF2C are expressed in the heart lineage (2, 14). In transient transfection experiments, Nkx2–5 and MEF2C activate the promoters of cardiac muscle-specific genes, which contain Nkx and MEF2 binding sites in their promoters, respectively (14, 40, 65). Overexpression of Nkx2–5 in *Xenopus* and zebrafish embryos leads to enlarged hearts (12, 13), indicating that given the right environment, Nkx2–5 can recruit cells to the cardiac lineage. However, no experiment to date has demonstrated the ability of either Nkx2–5 or MEF2C to initiate the development of beating cardiomyocytes in mammalian tissue culture systems (12, 13, 31, 32). The analysis of mice lacking Nkx2–5 or MEF2C is complicated by genetic redundancy resulting in possible compensatory mechanisms (9,

[2] A. G. Ridgeway, H. Petropoulos, S. Wilton, and I. S. Skerjanc, manuscript in preparation.

25). Therefore, our finding that MEF2C and Nkx2–5 induce cardiomyogenesis in P19 cells is consistent with and extends our understanding of the function of these factors.

The ability of Nkx2–5 and MEF2C to initiate the development of cardiac muscle in differentiating P19 cells can be compared with the function of other transcription factors in P19 cells (Table I). In cells aggregated in the absence of Me_2SO, GATA-4 also initiates cardiomyogenesis (49), whereas MyoD triggers skeletal myogenesis (50). Therefore, Nkx2–5, MEF2C, GATA-4, and MyoD are all capable of inducing myogenesis in P19 cells aggregated in the absence of Me_2SO. The results from overexpression of these factors in P19 cells can be compared with their function in fibroblasts. While MyoD initiates skeletal myogenesis in fibroblasts (66), neither Nkx2–5 nor MEF2 family members have been shown to initiate cardiomyogenesis in fibroblasts (12, 13, 31, 32). The difference between the results obtained by transfection of Nkx2–5 and MEF2C into fibroblasts compared with transfection into P19 cells may be due to the presence of positive regulatory factors and/or the absence of negative regulatory factors in aggregated P19 cells. The expression of these factors may be regulated by P19 cell aggregation in a Me_2SO-independent fashion.

In addition, the regulation of MEF2C and Nkx2–5 could involve a change in the chromatin structure of the target DNA. A recent analysis of the cardiac β-myosin heavy chain gene identified DNase I-hypersensitive sites present in neonatal hamster heart but not in adult (67). These results suggest that chromatin structure may participate actively in cardiac gene expression. Furthermore, MEF2C has been shown to interact with p300 and CBP, which are transcriptional adapter molecules that display histone acetyltransferase activity (68). The role of chromatin structure in regulating MEF2C and Nkx2–5 function in P19 cells remains to be determined.

Nkx2–5 and MyoD trigger myogenesis in a large proportion (>30%) of the culture, whereas MEF2C induces cardiomyogenesis in a small percentage (<3%) of the total culture. The difference in the extent of differentiation induced by MEF2C and Nkx2–5 could be due to the pattern of expression of factors available to regulate their activity. In the case of Nkx2–5, positive regulatory factors may be present in a large proportion of cells in the culture, whereas for MEF2C, they may be expressed in a small fraction of the culture. Alternatively, MEF2C may require more than one regulatory factor, and only a small subset of cells may contain the correct combination. These regulatory factors could include other transcription factors, adapter molecules, kinases, or phosphatases. Therefore, the ability of MEF2C and Nkx2–5 to induce cardiogenesis is dependent upon the array of regulatory factors provided by the specific cellular environment.

MEF2 factors have been shown to interact and cooperate with MyoD (31, 32, 69) and the neurogenic basic helix-loop-helix protein MASH1 (16, 19). In addition, MEF2C may be regulated by phosphorylation events due to interactions with kinases such as casein kinase-II (70) and mitogen-activated protein kinase (71, 72). Nkx2–5 interacts and synergizes with GATA-4 and a MADS box factor (serum response factor) (65, 73, 74). However, it is unclear whether or not MEF2C can synergize or interact with Nkx2–5. In summary, protein/protein interactions and posttranslational modifications are important for regulating MEF2C and Nkx2–5 activity, and they most likely play a role in regulating MEF2C and Nkx2–5 function during the aggregation of P19 cells.

MEF2C and Nkx2–5 appear to direct early stages of P19 cell differentiation into a cardiomyogenic pathway. Both MEF2C and Nkx2–5 expression result in the enhancement of Brachyury T expression, as early as day 0, and BMP-4, as early as day 3. This result indicates that MEF2C and Nkx2–5 do not cause a "jump" in phenotype from stem cells into cardiomyocytes. Instead, these cultures appear to be directed through the various mesodermal stages that would occur in Me_2SO-induced cultures. The exact subset of cells expressing Brachyury T and BMP-4 is undetermined at present, due to the heterogeneous nature of P19 cell aggregates. However, each cell destined to become a cardiomyocyte may be exposed to signals resulting from the expression of either factor. Therefore, Nkx2–5 and MEF2C progressively guide P19 cells into a cardiomyogenic pathway, and the mechanism by which this occurs remains to be elucidated.

Since Nkx2–5 and MEF2C are known to bind to cardiac muscle-specific promoters and activate their expression, it is logical to assume that these factors are functioning in a similar fashion in P19 cells. Nevertheless, it is possible that the observed change in cell type is due to an indirect effect of overexpression of Nkx2–5 or MEF2C. These factors, when overexpressed, could be functioning by a "squelching" mechanism by binding a cofactor or derepressing gene expression. They could also be activating the expression of another transcription factor that activates cardiac muscle-specific gene expression. Finally, the up-regulation of Brachyury T and BMP in P19(MEF2C) cultures could be enhancing spontaneous cardiac muscle development to the levels detected in these cultures.

It has been shown previously that the expression of Brachyury T in P19 cell monolayers treated with BMP-4 is not sufficient to initiate differentiation (45). However, we have found that BMP-4 expression was activated in all cultures destined to undergo cardiomyogenesis.[3] These results, taken together with the finding that BMP-4 activates cardiomyogenesis in anterior mesoderm cultures (8), suggest that BMP-4 signaling pathways regulate MEF2C and Nkx2–5 cardiomyogenic activities in aggregated P19 cells.

Nkx2–5 was expressed in differentiated P19(MEF2C) cultures, and MEF2C was expressed in differentiated P19(Nkx2–5) cultures. The observation that MEF2C, but not MEF2A, -B, or -D, was expressed at high levels in differentiated P19(Nkx2–5) cultures suggests that MEF2C is specifically involved in early stages of cardiac muscle development. This finding is consistent with the order of appearance of the MEF2 factors during murine cardiomyogenesis, in which MEF2C is expressed half a day earlier than the other three family members (30, 57). MEF2B is the next MEF2 factor to be expressed, and its up-regulation in MEF2C −/− mice (25) is thought to compensate for the loss of MEF2C activity. However, in P19(Nkx2–5) cultures producing abundant cardiac muscle, MEF2B levels are considerably down-regulated, in agreement with previous studies in P19 cells (75). The finding that P19(Nkx2–5) cultures up-regulate the expression of MEF2C parallels observations in *Drosophila* in which Tinman regulates D-MEF2 (76). Our findings, that MEF2C and Nkx2–5 up-regulated each other's expression and the expression of GATA-4, and the finding that GATA-4 activates the expression of Nkx2–5 in P19 cells (49) suggest the presence of a positive regulatory network between Nkx2–5, MEF2C, and GATA-4. This network would amplify and maintain cardiomyogenesis in a similar fashion to the amplification and maintenance of myogenesis by MEF2 and the myogenic regulatory factors.

In summary, the P19 cell system provides a powerful tool for examining the ability of transcription factors to initiate cellular differentiation and for subsequent analysis of the mechanism(s) involved. Since P19 stem cells can be programmed to differentiate into a wide variety of cell types, the function of

[3] I. S. Skerjanc and S. Wilton, unpublished results.

any individual protein is not limited by the presence or absence of tissue-specific factors and/or tissue-specific chromatin structure, which may be required for the biological activity of that protein. Using this system, we have shown that MEF2C and Nkx2–5 are capable of inducing the development of cardiac muscle.

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