Cardiac hypertrophy and dilation are mediated by neuroendocrine factors and/or mitogens as well as through internal stretch- and stress-sensitive signaling pathways, which in turn transduce alterations in cardiac gene expression through specific signaling pathways. The transcription factor family known as myocyte enhancer factor 2 (MEF2) has been implicated as a signal-responsive mediator of the cardiac transcriptional program. For example, known hypertrophic signaling pathways that utilize calcineurin, calmodulin-dependent protein kinase, and MAPKs can each affect MEF2 activity. Here we demonstrate that MEF2 transcription factors induced dilated cardiomyopathy and lengthening of myocytes. Specifically, multiple transgenic mouse lines with cardiac-specific overexpression of MEF2A or MEF2C presented with cardiomyopathy at base line or were predisposed to more fulminant disease following pressure overload stimulation. The cardiomyopathic response associated with MEF2A and MEF2C was not further altered by activated calcineurin, suggesting that MEF2 functions independently of calcineurin in this response. In cultured cardiomyocytes, MEF2A, MEF2C, and MEF2-VP16 overexpression induced sarcomeric disorganization and focal elongation. Mechanistically, MEF2A and MEF2C each programmed similar profiles of altered gene expression in the heart that included extracellular matrix remodeling, ion handling, and metabolic genes. Indeed, adenoviral transfection of cultured cardiomyocytes with MEF2A or of myocytes from the hearts of MEF2A transgenic adult mice showed reduced transient outward K⁺ currents, consistent with the alterations in gene expression observed in transgenic mice and partially suggesting a proximal mechanism underlying MEF2-dependent cardiomyopathy.

Myocyte enhancer factor 2 (MEF2) was originally identified as a muscle-enriched DNA binding activity from differentiated myotubes, although it is now recognized to be widely distributed in most tissues. MEF2 DNA binding activity consists of homo- and heterodimers of four separate gene products in mammals, referred to as Mef2a–d (2, 3). MEF2 dimers bind to the consensus sequence CTA(A/T)₄TAG present in the 5’-transcriptional regulatory regions of most skeletal and cardiac muscle structural genes characterized to date (2, 3). In general, Mef2a–d genes are widely expressed in the adult vertebrate organism, although a number of specific regulatory functions have been identified in immune, skeletal muscle, cardiac muscle, and neuronal cells (4–7).

MEF2 factors are related to another MADS box-containing transcription factor known as serum response factor (SRF) (8). Similar to SRF, members of the MEF2 family have been implicated in regulating inducible gene expression in response to mitogen and/or stress stimulation. In the heart, myocytes undergo developmental and pathophysiological hypertrophy in response to neuroendocrine, mitogen, and stress stimulation. Such stimuli activate intracellular signal transduction cascades, resulting in the modification of transcription factor activity and the reprogramming of cardiac gene expression. A number of lines of evidence suggest that MEF2 factors might regulate inducible gene expression in response to stimuli that underlie the cardiac hypertrophic response. For example, MEF2 DNA binding activity in the heart was shown to be up-regulated 2–3-fold by both pressure and volume overload hypertrophy (9). MEF2 DNA binding activity was also shown to be enhanced in myopathic hearts from mdx:myoD−/− mice (10). Mef2c null mice have altered cardiac gene expression and die during early embryonic development with arrested heart tube morphogenesis, suggesting a critical role in developmental growth (7). Mice expressing a dominant-negative mutant of MEF2C in the heart also die during postnatal development with attenuated ventricular growth (10). Finally, a portion of Mef2a null mice die suddenly during the perinatal period with dilated right ventricles, myofibrillar disorganization, and mitochondrial structural abnormalities (11).

Hypertrophic stimulation of the adult heart is associated with activation of a number of intracellular signaling pathways, including mitogen-activated protein kinase (MAPK), calcineurin, protein kinase C, calmodulin-dependent protein kinase, insulin-like growth factor 1 pathway constituents, and altered intracellular Ca²⁺ handling (12, 13). Consistent with the activation of these discrete intracellular signaling pathways, MEF2 factors can be activated by Ca²⁺ (14–18), calcineurin (14, 19–21), p38 MAPK (22–26), big MAPK-1 (BMK1) (26, 27), and calmodulin-dependent protein kinase (28). More provocatively, MEF2 factors are also regulated through association with class factor; MAPK; mitogen-activated protein kinase; BMK1, big MAPK-1; HDACs, histone deacetylases; TAC, transverse aortic constriction; CnA, calcineurin A subunit; TRITC, tetramethylrhodamine isothiocyanate; RT, reverse transcription; Ad, adenovirus; GFP, green fluorescent protein; L², transient outward current; MKK6, MAPK kinase 6; NFAT, nuclear factor of activated T-cells; MEKS, MAPK kinase 5; H/R ratio, thickness/radius ratio.

**Myocyte Enhancer Factors 2A and 2C Induce Dilated Cardiomyopathy in Transgenic Mice**

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Received for publication, September 16, 2005, and in revised form, January 27, 2006 Published, JBC Papers in Press, February 9, 2006, DOI 10.1074/jbc.M502172000
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Western Blotting and Immunocytochemistry—Western blotting and immunocytochemistry were performed as described previously (40, 41). The following antibodies were used: rabbit anti-MEF2C polyclonal antibody (1:500, Cell Signaling Technology); rabbit anti-MEF2A polyclonal antibody (1:1000) and anti-α-tubulin monoclonal antibody (1:200) (Santa Cruz Biotechnology, Inc.); anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (1:5000; Research Diagnostics, Inc.); anti-α-actinin (1:300) and anti-myosin (1:250) monoclonal antibodies (Sigma), anti-focal adhesion kinase (1:400) and anti-phospho-Tyr397 focal adhesion kinase (1:400) antibodies (Upstate Biotechnologies, Inc.).

Cardiomyocyte Cultures and Recombinant Adenovirus—All in vitro experiments were performed in neonatal ventricular myocytes isolated from 1–2-day-old rats as described previously (40). cDNAs encoding MEF2A (human), MEF2B (mouse), MEF2C (mouse), MEF2D (mouse), MEF2C (amino acids 1–143) fused to the VP16 transcriptional activation domain (MEF2-VP16), and the dominant-negative MEF2C(R3T) mutant were used to generate recombinant adenovirus. These cDNAs were subcloned into the pShuttle vector for the Adeno-X system (Clontech) to generate replication-deficient adenoviruses. A MEF2-containing adenovirus (Ad), Ad-β-galactosidase (control), or Ad-green fluorescent protein (GFP; control) was used to infect cultured cardiomyocytes at an approximate multiplicity of infection of 50 for a period of 2 h, followed by analysis 24, 36, or 48 h afterward (40). Under these conditions, >98% of the cells showed expression of the viral gene insert. Assessment of cultured neonatal rat cardiomyocyte cell-surface area (hypertrophy) was performed as described previously (from at least 200 cells in three separate experiments each) (42).

Affymetrix Gene Expression Profiling and Bioinformatics—Total RNA samples were prepared from individual high expressing MEF2A transgenic hearts at 2 weeks of age and compared with two wild-type hearts at 2 weeks of age. Alternatively, a separate array was performed on cardiac RNA collected from line 2 MEF2C transgenic mice and wild-type controls at 4 weeks of age. Biotin-labeled target cRNA was prepared from T7-transcribed cDNA made from 10 μg of the total RNA using the recommended Affymetrix protocol (43, 44) and hybridized for expression analysis to the Affymetrix GeneChip U74Av2 set using antibody-based fluorescence signal amplification. GeneChips were scanned in the Affymetrix 425S scanner using Affymetrix Microarray Suite Version 5.0. Intensity data were scaled to a target of 1500, and the results were analyzed using both Microarray Suite Version 5.0 and GeneSpring Version 5.0.3 (Silicon Genetics, Inc., Redwood City, CA). Data values used for filtering and clustering were “Signal,” “Signal Confidence,” “Absolute Call” (Absent/ Present), and “Change” ( Increase, Decrease, Unchanged) as implemented in Microarray Suite 5.0. Data were normalized as follows. The 50th percentile of all measurements was used as a positive control for each array. Measurements for each gene were divided by this synthetic positive control, assuming that this was at least 10. The bottom 10th percentile signal level was used as a test for correct background subtraction. The measurement for each gene in each sample was divided by the average of the corresponding value in the two wild-type samples, assuming that the value was at least 1.0. Genes regulated consistently between the replicates were identified by data filtering using Student’s t test (p < 0.01) among genes that were called “Present” in the MEF2 transgenic samples. Gene category information was based on all publicly available gene ontology information from the Gene Ontology Consortium (available http://www.geneontology.org/) as harvested from Swiss-Prot, GeneCards, Compugen, LocusLink, and GenBankTM as well as exhaustive MEDLINE literature searches.
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Electrophysiological Recordings in Neonatal Cardiomyocytes—Neonatal rat cardiomyocytes were isolated and cultured as described previously (45). For patch-clamp recording experiments, \(1.5 \times 10^6\) myocytes were plated on laminin-coated coverslips in 35-mm culture dishes. After 24 h in culture, the medium was replaced with serum-free medium, and viral infections were performed (multiplicity of infection of 10 for AdGFP and AdMEF2A). Typically, >95% of the myocytes showed AdGFP expression 36 h after infection. Whole cell voltage-clamp recordings were done as described previously (45, 46) at room temperature to measure transient outward \(I_{\text{to}}\) and inward rectifier \((\text{IK}_1)\) \(K^+\) currents, at least 36 h after serum withdrawal and infection with AdMEF2A. Myocytes were perfused for at least 15 min before measurements were performed with a solution containing 140 mmol/liter NaCl, 4 mmol/liter KCl, 2 mmol/liter CaCl\(_2\), 1 mmol/liter MgCl\(_2\), 0.5 mmol/liter CdCl\(_2\), 10 mmol/liter HEPES, and 10 mmol/liter glucose (pH 7.4). The intracellular solution contained 140 mmol/liter KCl, 1 mmol/liter MgCl\(_2\), 10 mmol/liter EGTA, 10 mmol/liter HEPES, and 5 mmol/liter MgATP (pH 7.25). Whole cell currents were filtered at 2 kHz (Axon 200A amplifier).

Electrophysiological Recordings in Cardiomyocytes from Adult Mouse Whole Hearts—Single ventricular myocytes were obtained from both ventricles of adult mice (3 months old) of both sexes. The cell isolation technique used in these experiments has been described previously (47). All current recordings were obtained in the whole cell voltage-clamp configuration of the patch-clamp technique by using 1.60 outer diameter borosilicate glass electrodes (Garner Glass Co.). Cell capacitance was measured using voltage ramps of 1 V/s from a holding potential of 0 mV. Series resistance was within the range of 2–11 M\(\Omega\). Most of the data presented in these studies were obtained with electrodes with a resistance of 0.5–3 M\(\Omega\)s. Whole cell \(Ca^{2+}\)-independent transient outward \(K^+\) currents were evoked by a series of depolarizing voltage steps (680 ms) from –40 to +80 mV in 10-mV increments from a holding potential of –40 mV at a frequency of 0.5 Hz. Ventricular cardiomyocytes were perfused with normal Tyrode’s solution containing 138 mmol/liter NaCl, 4 mmol/liter KCl, 2 mmol/liter CaCl\(_2\), 1 mmol/liter MgCl\(_2\), 10 mmol/liter glucose, 10 mmol/liter HEPES, and 0.33 mmol/liter NaH\(_2\)PO\(_4\) (adjusted to pH 7.4 with NaOH). \(I_{\text{to}}\) was largely eliminated by 0.3 mM CdCl\(_2\) included in the recording solution. The pipette solution contained 120 mmol/liter potassium glutamate, 10 mmol/liter KCl, 2 mmol/liter CaCl\(_2\), 10 mmol/liter MgCl\(_2\), 10 mmol/liter HEPES, 5 mmol/liter EGTA, and 2 mmol/liter MgATP (adjusted to pH 7.2 with KOH). Cell capacitance was estimated by integrating the area under an uncompensated capacity transient elicited by a 25-mV hyperpolarizing test pulse (25 ms) from a holding potential of 0 mV. \(I_{\text{to}}\) was defined as the difference between the peak transient current and the steady-state current at the end of a 500-ms voltage-clamp pulse. All experiments were carried out at room temperature (20–22 °C). Whole cell currents were analyzed with Clampfit Version 6.03 software (Axon Instruments). Pooled data are expressed as means ± S.E. All current amplitudes were normalized to the cell capacitance and expressed as densities (picoamperes/picofarad).

Measurement of Adult Cardiomyocyte Length and Width—Wild-type and MEF2A transgenic adult mouse hearts (2 months old) were dissected, washed in ice-cold cannulation buffer (10 mm 2,3-butanedione monoxime and 10 \(\mu\)M CaCl\(_2\)). Cells were collected by centrifugation and resuspended in 4% paraformaldehyde for fixation. Isolated cardiomyocytes were photographed, and the length and width of ~200 cells from each mouse were measured using NIH Image software, and the length/width ratios were calculated.

Statistical Analysis—Data are expressed as means ± S.E. Differences between experimental groups were evaluated for statistical significance using Student’s \(t\) test or one- or two-way analysis of variance. \(p\) values <0.050 were considered to be statistically significant.

RESULTS

Characterization of MEF2A and MEF2C Transgenic Mice—Although a number of reports have suggested a role for MEF2 in regulating the hypertrophic growth of the adult myocardium, it has yet to be formally evaluated. To directly investigate the ability of MEF2 to induce the cardiac hypertrophic response, we generated a series of cardiac-specific transgenic mice using the \(\alpha\)-myosin heavy chain promoter. cDNAs encoding MEF2A and MEF2C were selected for overexpression because they have been proposed to be the predominant MEF2 isoforms expressed in the postnatal mouse heart.
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Echocardiography in wild-type and medium and high expressing MEF2A transgenic mice

All measurements are means ± S.E. and each animal was measured three separate times. Septal and left ventricular (LV) wall thickness was assessed in systole and is shown in millimeters. WT, wild-type mice; TG-Med, medium expressing MEF2A transgenic mice; TG-High, high expressing MEF2A transgenic mice; LVED, left ventricular end-diastolic dimension (mm); LVES, left ventricular end-systolic dimension (mm); FS, fractional shortening.

|                | 1 month old | 2 months old | 3 months old | 3 weeks old |
|----------------|-------------|--------------|--------------|-------------|
|                | WT          | TG-Med       | WT           | TG-Med      | WT           | TG-Med       | WT           | TG-High     |
| LVED (mm)      | 3.39 ± 0.10 | 3.38 ± 0.05  | 3.66 ± 0.16  | 4.19 ± 0.08*| 3.88 ± 0.12  | 4.43 ± 0.11* | 2.53 ± 0.24  | 3.71 ± 0.16*|
| LVES (mm)      | 2.21 ± 0.11 | 2.17 ± 0.09  | 2.48 ± 0.23  | 3.42 ± 0.19*| 2.43 ± 0.06  | 3.55 ± 0.11* | 1.40 ± 0.11  | 2.96 ± 0.09*|
| Septum (mm)    | 0.96 ± 0.06 | 1.08 ± 0.06  | 1.07 ± 0.09  | 1.03 ± 0.19*| 1.33 ± 0.09  | 1.10 ± 0.06  | 0.79 ± 0.02  | 0.78 ± 0.09*|
| LV (mm)        | 0.87 ± 0.05 | 1.00 ± 0.07  | 1.20 ± 0.12  | 0.83 ± 0.13 | 1.27 ± 0.02  | 1.03 ± 0.06* | 0.69 ± 0.06  | 0.64 ± 0.03*|
| FS (%)         | 34.65 ± 2.23| 35.95 ± 2.28 | 32.58 ± 3.00 | 18.61 ± 2.86*| 37.11 ± 0.97*| 18.89 ± 0.92*| 14.14 ± 2.35 | 20.16 ± 0.91*|

p < 0.05, MEF2A transgenic mice versus age-matched control mice. Four to six mice were analyzed in each group.

![Figure 2. Characterization of MEF2A transgenic mice following pressure overload stimulation.](image)

A, heart weight normalized to tibia length (HW/TL) in wild-type (WT) and low (TG-low) and medium (TG-med) expressing MEF2A transgenic mice at 2 months of age following a sham or TAC surgical procedure for 2 weeks (n = five to eight mice/group). *, p < 0.05 versus sham of the same genotype; #, p < 0.05 versus wild-type TAC. B, lung weight normalized to tibia length (LW/TL) as an indication of heart failure in wild-type and low and medium expressing MEF2A transgenic mice at 2 months of age following a sham or TAC surgical procedure for 2 weeks (n = five to eight mice/group). *, p < 0.05 versus sham of the same genotype; #, p < 0.05 versus wild-type TAC. C, cardiomyocyte surface area in hearts from wild-type and low and medium expressing MEF2A transgenic mice following a sham or TAC surgical procedure for 2 weeks (n = five hearts/group). *, p < 0.05 versus sham of the same genotype; #, p < 0.05 versus wild-type TAC. D, cardiac fractional shortening (FS) in wild-type and low and medium expressing MEF2A transgenic mice at 2 months of age following a sham or TAC surgical procedure for 2 weeks (n = five to eight mice/group) #, p < 0.05 versus wild-type TAC. E, hematocrit/ eosin-stained histological cross-sections of hearts from wild-type and medium expressing MEF2A transgenic mice at 2 months of age following a sham or TAC surgical procedure for 5 weeks. F, echocardiography-measured H/R ratio in sham mice and after TAC stimulation in the indicated groups. The H/R ratio indicates dilation versus concentric hypertrophy and is the ratio of the average of the left ventricular wall and septal thickness divided by one-half of the left ventricular end-diastolic dimension.

heart (10). Three individual MEF2A lines were initially generated and characterized by 1.9-, 3.5-, and 4.2-fold more MEF2A protein expression in the heart when normalized to glyceroldehyde-3-phosphate dehydrogenase (Fig. 1A). Overexpression of MEF2A resulted in three different migrating species of MEF2A that collapsed upon calf intestinal alkaline phosphatase treatment, suggesting different phosphorylation isoforms (data not shown). The highest expressing line demonstrated neonatal lethality between 2 and 4 weeks of age with significant elevations in heart weight normalized to body weight, whereas the low and medium expressing MEF2A transgenic lines showed no lethality and had normal heart weights up to 3 months of age (Fig. 1B). High expressing MEF2A transgenic mice showed severe impairment in ventricular performance at 3 weeks of age as assessed by echocardiography (Fig. 1C). Low expressing MEF2A transgenic mice had essentially normal ventricular performance (data not shown), whereas medium expressing transgenic mice showed a functional deficit at 2 and 3 months of age, as well as ventricular chamber dilation (Fig. 1C and Table 1). It is interesting to note that medium expressing MEF2A transgenic mice showed dramatic reductions in cardiac functional performance and dilation before increases in heart weight were present, suggesting that any manifestation or propensity toward heart weight increase could be secondary to the reduction in ventricular function. High expressing MEF2A transgenic mice at 3 weeks of age also manifested a severe reduction in fractional shortening and dramatic dilation of the left ventricles (Table 1). In association with this dosage-dependent profile of cardiomyopathy, high expressing transgenic mice showed increased expression of hypertrophy/stress-associated genes such as atrial natriuretic factor and skeletal α-actin at 3 weeks of age (Fig. 1D). Medium expressing transgenic mice also eventually showed increased expression of these stress marker genes as they aged and developed hypertrophy as a secondary consequence of reduced functional performance (data not shown).

Low and medium expressing lines had normal heart weights up to 3 months of age, despite a progressive deterioration in ventricular performance. To more carefully evaluate the hypertrophic program associated with MEF2A-mediated cardiomyopathy, 8-week-old low and medium expressing transgenic mice were subjected to TAC for 2 weeks to induce cardiac pressure overload. Wild-type mice showed a 33% increase in heart weight normalized to tibia length, whereas low and medium expressing MEF2A transgenic mice showed 44 and 91% increases, respectively (p < 0.05) (Fig. 2A). Moreover, medium expressing MEF2A transgenic mice showed significantly greater pulmonary congestion measured by lung weight to tibia length (p < 0.05) (Fig. 2B), greater cellular hypertrophy in the heart measured by direct assessment of myofiber diameters (p < 0.05) (Fig. 2C), and greater decompensation in ventricular performance as assessed by echocardiography (p < 0.05) (Fig. 2D). Histological assessment also demonstrated greater cardiac enlargement and ventricular wall dilation in medium expressing MEF2A transgenic mice compared with wild-type controls (Fig. 2E). Echocardiography showed a greater dilation in the left ventricular...
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by an increase in ventricular weight normalized to body weight or tibia fractional shortening and ventricular chamber dilation, followed thereafter MEF2C transgenic mice first showed a progressive base-line decrease in the lethality observed in MEF2A transgenic mice with 4.2-fold overexpression (lines 1 and 3) (Fig. 3). The only viable remaining line (line 2) showed only 1.6-fold overexpression of MEF2C in the heart by Western blotting (Fig. 3B), suggesting that MEF2C overexpression is not well tolerated, similar to the lethality observed in MEF2A transgenic mice with 4.2-fold overexpression. Indeed, MEF2C transgenic mice presented with a similar cardiomyopathic phenotype as characterized in MEF2A transgenic mice. Specifically, MEF2C transgenic mice first showed a progressive base-line decrease in fractional shortening and ventricular chamber dilation, followed thereafter by an increase in ventricular weight normalized to body weight or tibia length (Fig. 3, C and D, and Table 2). Induction of hypertrophy/stress-associated marker genes such as atrial natriuretic factor, B-type natriuretic peptide, β-myosin heavy chain, and skeletal α-actin was also observed at 1 month of age (Fig. 3E). Ventricular weight was used to assess hypertrophy over total heart weight (as used in MEF2A transgenic mice) because MEF2C transgenic mice had large atrial clots, consistent with a heart failure phenotype and poor ventricular function (Fig. 3A). MEF2C transgenic mice were also subjected to TAC stimulation to assess enhancement of hypertrophic enlargement as described in MEF2A transgenic mice. However, such attempts failed due to extreme lethality in MEF2C mice at only 1–3 days following pressure overload stimulation, consistent with the hypothesis that MEF2C overexpression induces fulminant cardiomyopathy that renders the mice intolerant to additional insults (data not shown). In conclusion, MEF2A and MEF2C each appear to induce a similar profile of cardiomyopathy in the mouse heart, with a functional deficit significantly preceding hypertrophic enlargement.

MEF2A and MEF2C Do Not Phenotypically Interact with Calcineurin in the Heart—A number of reports have suggested that calcineurin can directly activate MEF2 transcriptional and/or DNA binding activity (14, 19–21). To evaluate the potential importance of calcineurin as an upstream activator of MEF2-induced cardiac hypertrophy, MEF2A and MEF2C transgenic mice were both crossed with transgenic mice expressing activated calcineurin (39). Previously, the degree of cardiac hypertrophy induced by the same activated calcineurin transgene was shown to be dramatically enhanced by intercrossing into the Htdalcago7−/− genetic background (35). However, the degree of cardiac hypertrophy induced by activated calcineurin was not increased in either MEF2A or MEF2C transgenic mice at 7 and 9 weeks of age as assessed by gravimetry and echocardiography (Fig. 4A–C), and myocyte cellular areas were not increased more than seen with just the activated calcineurin transgene (data not shown). Ventricular weight divided by body weight was measured in MEF2C crossed mice given large atrial clots not seen in MEF2A transgenic mice. The relative degree of functional decompensation associated with the MEF2C transgene was not enhanced by the presence of the activated calcineurin transgene (Fig. 4D).

Thus, on a phenotypic level, calcineurin does not appear to function upstream of MEF2A or MEF2C in regulating the hypertrophic or myopathic response of the heart.

Phenotypic Assessment of MEF2 Overexpression in Cultured Cardiomyocytes and Adult Hearts—To further investigate the potential mechanisms whereby MEF2A or MEF2C overexpression might induce dilated cardiomyopathy in transgenic mice, a reductionist approach was employed in cultured neonatal rat cardiomyocytes using recombinant adenoviruses for MEF2A, MEF2B, MEF2C, MEF2D, and MEF2-VP16. We first investigated whether MEF2A, MEF2C, or MEF2-VP16 overexpression alone would induce hypertrophy over 24 or 48 h. However, none of the MEF2-expressing recombinant adenoviruses used here induced substantial cardiomyocyte hypertrophy, whereas control experiments with adenoviruses expressing activated MAPK kinase-6 (Mkk6), GATA4, activated calcineurin, or SRF showed pronounced hypertrophy (data not shown). Moreover, neither AdMkk6 nor AdΔCtnA showed greater hypertrophy when co-infected with AdMEF2A, AdMEF2C, or AdMEF2-VP16, supporting the contention that MEF2...
**TABLE 2**

Echocardiography in wild-type and MEF2C transgenic mice

All measurements are means ± S.E., and each animal was measured three separate times. Septal and left ventricular (LV) wall thickness was assessed in systole and is shown as millimeters. WT, wild-type mice; TG, MEF2C transgenic mice; LVED, left ventricular end-diastolic dimension (mm); LVES, left ventricular end-systolic dimension (mm); FS, fractional shortening.

|            | 1 month old     | 2 months old    | 3 months old   |
|------------|-----------------|-----------------|---------------|
|            | WT              | TG              | WT            | TG              |
| LVED (mm)  | 2.88 ± 0.23     | 3.55 ± 0.62     | 3.54 ± 0.25   | 4.73 ± 0.56a    |
| LVES (mm)  | 1.72 ± 0.08     | 2.54 ± 0.79     | 2.28 ± 0.31   | 3.83 ± 0.75a    |
| Septum (mm)| 0.92 ± 0.03     | 0.82 ± 0.11     | 1.07 ± 0.10   | 0.78 ± 0.20a    |
| LV (mm)    | 0.83 ± 0.15     | 0.76 ± 0.12     | 1.02 ± 0.08   | 0.76 ± 0.08a    |
| FS (%)     | 39.90 ± 7.48    | 29.65 ± 9.94    | 35.92 ± 5.53  | 19.61 ± 7.60a   |
|            |                 |                 | 40.93 ± 5.53  | 15.31 ± 4.47a   |

* * * p < 0.05, MEF2C transgenic mice versus age-matched control mice. Five or more mice were analyzed in each group.

**FIGURE 4.** Crossing MEF2A and MEF2C transgenic mice with activated calcineurin transgenic mice. A, heart weight normalized to body weight (HW/BW) in wild-type (WT) mice, medium expressing MEF2A transgenic mice, activated calcineurin transgenic mice (ΔCnA), and double transgenic (Double TG) mice at 9 weeks of age (n = four to five mice/group). *, p < 0.05 versus wild-type mice. B, ventricular weight normalized to body weight (VW/BW) in wild-type mice, line 2 MEF2C transgenic mice, activated calcineurin transgenic mice (ΔCnA), and double transgenic mice at 7 weeks of age (n = four to five mice/group). *, p < 0.05 versus wild-type mice. C, echocardiography-measured left ventricular (LV) wall thickness in wild-type mice, line 2 MEF2C transgenic mice, activated calcineurin transgenic mice (ΔCnA), and double transgenic mice at 7 weeks of age (n = four to five mice/group). *, p < 0.05 versus wild-type mice. D, echocardiography-measured fractional shortening (FS) in wild-type mice, line 2 MEF2C transgenic mice, activated calcineurin transgenic mice (ΔCnA), and double transgenic mice at 7 weeks of age (n = four to five mice/group).

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Global Assessment of Altered Gene Expression in MEF2A and MEF2C Hearts—Although MEF2A and MEF2C overexpression induced cardiomyopathy, the potential downstream transcriptional targets or pathways that mediate this phenotype are unknown. Here we generated RNA from the hearts of two wild-type and two MEF2C transgenic mice (line 2) at 4 weeks of age, as well as two wild-type and two high expressing MEF2A transgenic mice at 2 weeks of age for analysis of total gene expression alterations using the Affymetrix GeneChip mouse U74Av2.
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FIGURE 5. Confocal immunocytochemistry of neonatal cardiomyocyte architecture and sarcomeric organization following Ad-β-galactosidase, AdMEF2A, AdMEF2C, AdMEF2-VP16, and AdSRF infection. MEF2A, MEF2C, and MEF2-VP16 led to disorganization in the myosin heavy chain and α-actinin, yet the intermediate filament network as assessed using anti-α-tubulin antibody was not altered. AdSRF (control) overexpression did not induce the same phenotypic disorganization in sarcomeres or cellular elongation. Identical results were observed in three independent experiments. Adβgal, Ad-β-galactosidase.

FIGURE 6. Assessment of indexes of ventricular remodeling. A, Western blot of phosphorylated focal adhesion kinase (P-FAK) and total focal adhesion kinase (FAK) in neonatal rat cardiomyocyte cultures infected with the indicated adenoviruses for 24 h. Adβgal, Ad-β-galactosidase. B, RT-PCR with pro-type X collagen α1 (Type-X Col; rat) in neonatal rat cardiomyocyte cultures infected with the indicated adenoviruses for 24 h. C, measurement of myocyte length/width ratios in MEF2A transgenic adult mice at 2 months of age. Three wild-type (WT) and five transgenic (MEF2A TG) hearts were dissociated, and ~200 myocytes were measured in each. *, p < 0.05 versus wild-type mice. L7, ribosomal protein L7.

set containing 36,000 genes. Approximately 1.2 and 1.8% of all genes were altered in expression by 2-fold or more in MEF2C and MEF2A transgenic hearts, respectively. However, we were most interested in profiles of genes that might suggest common pathway alterations due to MEF2 overexpression. Three unique subsets of genes were observed as being altered in MEF2 transgenic hearts, including genes involved in the extracellular matrix and remodeling (supplemental Table 2), genes involved in ion handling (supplemental Table 3), and genes involved in metabolism (supplemental Table 4). Because the microarray analyses with MEF2A and MEF2C transgenic mice were performed at different ages (2 versus 4 weeks), the data are not listed together. These early time points were selected for RNA analysis because they precede fulminant heart disease and might suggest more proximal mechanisms.

The first subset of gene alterations included proteins involved in the extracellular matrix or its regulation. The most interesting of these genes were selected for confirmation by RT-PCR, including β5 integrin, α5 integrin, biglycan, lumican, peristin, matrix G1α protein, connective tissue growth factor, and fibulin, all of which were significantly up-regulated in the hearts of MEF2C transgenic mice (Fig. 7A). Most of these gene alterations were also observed in MEF2A transgenic hearts (data not shown). These alterations in extracellular matrix-associated genes and cell attachment genes are especially interesting, as they suggest a role in ventricular dilation and remodeling.

A significant subset of metabolic genes was also significantly altered in both MEF2A and MEF2C transgenic hearts, consistent with a previous assertion that MEF2 can regulate or participate in controlling the expression of genes involved in mitochondrial energy production and general metabolism (11, 20, 21). However, cardiomyopathy in general is known to be associated with similar alterations in metabolic genes, as observed here, characterized by decreased expression of fatty acid-related metabolic genes and increased expression of glycolytic genes (see “Discussion”).

Finally, a unique profile of altered ion-handling genes was also identified in MEF2A and MEF2C transgenic hearts. Specifically, high expressing MEF2A transgenic mice showed increased expression of genes such as Pkd2l2, Kcnj3, Kcnk4, Kcnj4, Mgf29, and many others (supplemental Table 3). Many of these same genes were also significantly altered in MEF2C transgenic mice. For example, Kcnk2, Kcnk3, and Cacna2d2 were down-regulated 3.6-, 1.9-, and 2.1-fold in MEF2C hearts, respectively, and Kcnk1 and Fxyd6 were up-regulated 2.6- and 2.0-fold, respectively. The MEF2A array data were confirmed by RT-PCR with low, medium, and high expressing MEF2A transgenic mice (Fig. 7B). In all cases, RT-PCR with high expressing MEF2A transgenic mice confirmed the array data. However, only some of these changes were observed in medium expressing MEF2A transgenic mice, whereas no alterations were observed in low
expressing MEF2A transgenic mice (Fig. 7B). Such alterations in ion-handling genes are especially interesting given that Mef2a null mice die from sudden death, suggesting predisposition to arrhythmia (see “Discussion”) (11).

To explore the potential biologic relevance of one of the three gene profiles described above, an assessment of correlative ionic currents was performed in cultured cardiomyocytes infected with AdMEF2A + AdGFP or with AdGFP alone as a control. Whole cell voltage-clamp recordings were performed at room temperature (see “Materials and Methods”). Although no significant changes in inward rectifying currents were observed (data not shown), MEF2A-overexpressing myocytes displayed a very prominent reduction in transient outward K\(^+\) currents (Fig. 8, A and B). Consistent with the adenoviral overexpression data on neonatal cardiomyocytes, myocytes isolated from MEF2A transgenic adult mice showed a nearly identical profile of reduced transient outward K\(^+\) currents (\(p < 0.05\)) (Fig. 8C). This reduction in \(I_{\text{to}}\) is consistent with the pronounced decrease in Kcnjd2 gene expression (Fig. 7B), which is the predominant pore-forming \(\alpha\)-subunit of the transient outward current in rodent cardiomyocytes. Alterations in Kv4.2 gene expression and \(I_{\text{to}}\) current have been shown to modulate the cardiac action potential profile and calcium entry and to promote cardiac hypertrophy/myopathy (see “Discussion”). In conclusion, these data suggest at least one regulatory paradigm whereby MEF2 regulates expression of a subset of genes that have a direct influence on cardiomyocyte physiology and the potential to induce a cardiomyopathic phenotype in vivo.

**DISCUSSION**

**Evidence for MEF2 as a Hypertrophic Mediator**—Here we presented the first experimental evidence that MEF2 transcription factors are capable of inducing cardiomyopathy in vivo. Although previous lines of evidence have indirectly suggested a potential role for MEF2 factors in regulating cardiac hypertrophy, they did not directly address the ability of MEF2 to program such a response. An inference to MEF2 as a hypertrophic mediator can also be made based on known similarities between SRF and MEF2, both of which are MADS box-containing DNA-binding factors that respond to stress, developmental, and mitogen stimulation. Transgenic mice overexpressing SRF in the heart were previously shown to have a phenotype of hypertrophic cardiomyopathy, reminiscent of MEF2A and MEF2C transgenic mice (48). It is interesting that SRF overexpression in cultured neonatal cardiomyocytes induced a
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phenotype that was reminiscent of hypertrophy, including increased sarcomeric organization and increased cell-surface area, yet MEF2 overexpression did not (Fig. 5).

MEF2A and MEF2C are also directly phosphorylated by p38 MAPK, resulting in enhanced transcriptional activity (5, 22–26). Furthermore, a p38-docking domain that is necessary for efficient p38-mediated phosphorylation was recently identified in MEF2A, MEF2C, and MEF2D (24). These studies suggested a role for MEF2 in regulating cardiac hypertrophy given the data that p38 itself is a modifier of this response (49). However, although p38 prominently regulates the hypertrophic growth of neonatal cardiomyocytes in culture, recent studies in transgenic and gene-targeted mouse models suggest that this kinase is not a positive mediator of the hypertrophic response and may, in fact, even inhibit it (50–52). Thus, MEF2 is unlikely to function as a hypertrophic mediator downstream of signals (phosphorylation) from p38, consistent with the inability of AdMKK6 to generate enhanced hypertrophic growth when co-infected with AdMEF2A or AdMEF2C (data not shown). However, chronic activation of p38 in the hearts of transgenic mice results in profound myopathy and dilation, suggesting that MEF2 could mediate pathological responses, such as dilation, downstream of p38 in the heart (53).

Ca\(^{2+}\) acting through the Ca\(^{2+}\)-sensitive phosphatase calcineurin can also activate MEF2 transcriptional and/or DNA binding activity, possibly through direct dephosphorylation (14, 19–21). For example, MEF2 reporter transgenic mice show enhanced activity in the extensor digitorum longus when crossed with transgenic mice expressing activated calcineurin in their skeletal muscle (19). The expression of the MEF2-lacZ reporter is also reduced by the calcineurin inhibitor cyclosporin A or by a transgene expressing the calcineurin regulatory protein known as modulatory calcineurin-interacting protein, collectively suggesting that calcineurin regulates the MEF2-lacZ reporter transgene in skeletal muscle (54). However, these results depend on the specificity of the MEF2-dependent reporter, which utilizes concatamers of an AT-rich element from the desmin regulatory region placed upstream of a minimal heat shock promoter (36). It is interesting that this AT-rich element can also bind the muscle enriched transcription enhancer factor 1 family of DNA-binding proteins (55). Transcription enhancer factor 1 was also shown to directly mediate hypertrophy-responsive gene expression in cardiac myocytes (56). Thus, the conclusion that calcineurin functions dominantly upstream of MEF2 in striated muscle requires further investigation. We did not observe an increase in cardiac hypertrophy or the degree of functional decompensation between MEF2A and MEF2C transgenic mice crossed with activated calcineurin transgenic mice. In contrast, a synergistic increase in hypertrophy was observed when calcineurin transgenic mice were crossed with mice lacking Hdac9, suggesting that the degree of calcineurin-regulated hypertrophy could still be dramatically augmented with a specific pathway interaction (35). In conclusion, although calcineurin is known to function as a potent regulator of the cardiac hypertrophic response, partially through activation of NFAT transcription factors (39), MEF2 is unlikely to be a significant downstream hypertrophic effector of calcineurin in the heart, although it could still participate in maladaptive responses downstream of calcineurin.

More recently, MEF2 factors have been indirectly implicated in mediating cardiac hypertrophy through their interaction with class II HDAC transcriptional repressors (17, 29–31). Olson and co-workers (17, 32–35) demonstrated a fundamental paradigm whereby class II HDACs shuttle between the cytoplasm and nucleus in a signal-dependent manner to regulate the transcriptional activity of MEF2 factors. The extrusion of HDAC4 and HDAC5 from the nucleus, which permits MEF2 transcriptional activity, is mediated by direct phosphorylation of HDAC4 and HDAC5 through calmodulin-dependent protein kinase and protein kinases C and D (17, 32–34, 57). Given this paradigm, loss of specific class II HDACs should activate MEF2, potentially leading to cardiac hypertrophy. Indeed, Hdac9 null mice develop exaggerated hypertrophy following pressure overload or when crossed with the calcineurin transgene (35). However, class II HDACs interact with a large array of transcription factors other than MEF2. For example, we have observed an indirect interaction between class II HDACs and NFAT transcription factors, suggesting a role as downstream effector of HDAC (58). Despite the likelihood that HDACs function through other transcription factors to control hypertrophy, it is also possible that MEF2 overexpression is without a primary influence on hypertrophy because it is completely inhibited by endogenous class II HDACs. However, the ability of MEF2 to promote cardiomyopathy and dilation would then have to be independent of HDAC regulation.

Loss-of-function approaches have also suggested a role for MEF2 in regulating cardiac hypertrophy/cardiomyopathy. For example, mice expressing a dominant-negative mutant of MEF2C in the heart using the α-myosin heavy chain promoter die during postnatal development, presumably due to a phenotype of ventricular dilation and attenuated ventricular growth (10). These results suggest that MEF2 transcriptional activity is required for postnatal maturation of the heart, also referred to as developmental hypertrophy, but do not address the role of MEF2 in mediating the pathological hypertrophic response of the adult myocardium. Another point to consider is that Mef2a null mice die suddenly during the perinatal period with dilated right ventricles, myofibrillar disorganization, and mitochondrial structural abnormalities, consistent with the hypothesis that MEF2 activity is necessary for efficient developmental hypertrophy (11).

The data presented here show a dosage-dependent cardiomyopathic phenotype and a progressive reduction in ventricular performance associated with MEF2A or MEF2C overexpression in the heart. Such reductions in cardiac function can promote a secondary neuroendocrine-stimulated hypertrophy as the heart attempts to compensate. Such an indirect influence could also enhance TAC-induced cardiac hypertrophy, as observed here. Thus, some increase in heart weight could result as a secondary consequence of decreased ventricular performance that leads to a secondary neuroendocrine-driven response, whereas the remaining increase in heart weight could result from dilation itself and addition of sarcomeres in series. For example, myocytes from MEF2A transgenic mice were noticeably altered in their length/width ratios, consistent with a dilated phenotype or addition of sarcomeres in series (and a loss of cross-sectional area). Addition of sarcomeres in series can lead to overall increases in heart weight, thus being suggestive of hypertrophy at the whole organ level, although at the cellular level, MEF2A does not appear to regulate the more classically defined index of hypertrophy associated with increased cross-sectional area. Indeed, MEF2 overexpression in cultured neonatal cardiomyocytes did not promote definitive hypertrophy. However, overexpression of MEF2 in neonatal myocytes did promote noticeable sarcomeric disorganization and focal elongation, consistent with its ability to induce dilated cardiomyopathy with compromised ventricular performance in vivo (see below).

The phenotype of MEF2-overexpressing transgenic mice and adenovirus-infected neonatal cardiomyocytes is reminiscent of transgenic mice expressing an activated MEK5 mutant in the heart or in adenovirus-infected myocytes (59). It is interesting that MEF2 is directly phosphorylated by BMK1, which is directly activated by MEK5 (26, 27). Expression of activated MEK5 induces elongation of cardiac myocytes in culture, whereas activated MEK5 transgenic mice show addition of
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sarcomeres in series with a loss of myocyte cross-sectional area (59). Activated MEK5 transgenic mice also show profound ventricular dilation, reduced fractional shortening, and activation of hypertrophic gene expression. This overall phenotype is remarkably similar in nearly every respect to our observations in MEF2A and MEF2C transgenic mice and AdMEF2A- and AdMEF2C-infected neonatal myocytes, which is particularly relevant given the known ability of MEK5-BMK1 to directly activate MEF2 by phosphorylation (26, 27).

A final issue that should be discussed relates to the relevance of MEF2 overexpression as a means to understand its functional role. The degree of MEF2A or MEF2C protein overexpression that produced viable lines overexpression as a means to understand its functional role. The degree of MEF2A or MEF2C protein overexpression that produced viable lines was rather mild and arguably within a “physiological” range, suggesting of MEF2A or MEF2C protein overexpression that produced viable lines.

Non-hypertrophic Functions of MEF2—MEF2 has been implicated as a mediator of apoptosis in a cell type-dependent manner. For example, MEF2 has been implicated as a necessary regulator of cell death in T-lymphocytes or T-cell hybridomas in response to calcium signals (15, 60). In contrast, enhanced MEF2 activity is associated with the survival of cultured primary neurons or neuron-like cell lines (61–64). For example, inhibition of MEF2 in neuronal cultures with a dominant-negative mutant of MEF2 or with specific kinases that inactivate MEF2 enhances cell death (62–64). Here we also investigated the ability of MEF2 to alter the cell death of neonatal cardiomyocytes in culture to determine whether it functions in a prosurvival or pro-apoptotic manner. Cardiomyocytes were infected with recombiant adenoviruses encoding MEF2A, MEF2B, MEF2C, MEF2D, or the dominant-negative MEF2C(R3T) mutant. Although overexpression of wild-type MEF2A–D had no effect on base-line DNA laddering, expression of the dominant-negative MEF2C(R3T) mutant increased DNA laddering, suggesting that MEF2 activity is protective against apoptosis in cardiomyocytes, similar to neurons (data not shown).

MEF2 was originally named myocyte-specific enhancer-binding factor 2 based on its induction in differentiated skeletal muscle cells and based on its ability to regulate expression of numerous muscle-specific genes. Indeed, MEF2-binding sites have been identified within the promoters of most skeletal and cardiac muscle structural genes examined to date (2, 3). Moreover, loss of Mef2c in gene-targeted mice results in loss or down-regulation of multiple cardiac structural genes in the developing heart (7). Thus, MEF2 has been proposed to function as a mediator of contractile gene expression in striated muscle. This assertion is indirectly supported by our analysis of MEF2 overexpression in cultured cardiomyocytes, where sarcomeric disorganization was prominent (Fig. 5). Such alterations in sarcomeric organization could be attributed to mismatches in gene expression for structural and sarcomeric proteins, which would further promote cardiomyopathy in vivo. Although this hypothesis is attractive, it is unlikely because few consistent alterations were observed in the expression levels of structural and sarcomeric genes in MEF2A or MEF2C mice or even in adenovirus-infected neonatal cardiomyocytes overexpressing very high levels of MEF2C also subjected to array analysis (data not shown). Thus, MEF2A and MEF2C do not appear to induce cardiomyopathy or sarcomeric disorganization through a mechanism involving direct imbalances in expression of sarcomeric genes. However, these results do not mean that loss of MEF2 activity has no impact on expression of cardiac structural genes, as described previously (7).

In contrast to the lack of alterations in structural genes associated with increased MEF2 activity in cardiomyocytes, MEF2 overexpression promoted dramatic alterations in a subset of genes encoding ion-handling proteins or genes that indirectly modulate ion handling. One of these genes, Kcnd2, encodes the pore-forming Kv4.2 α-subunit of cardiac \( l_{\text{io}} \) and thereby controls \( l_{\text{io}} \) density in the rodent myocardium (65).

The physiological significance of this observed MEF2-dependent alteration in Kcnd2 expression was verified by patch clamping in cultured cardiomyocytes following acute AdMEF2A infection and in myocytes isolated from MEF2A transgenic adult mice. Reductions in \( l_{\text{io}} \) density are the major cause of action potential duration prolongation (65) in heart disease and have been linked to elevated Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels, enhanced contractility, and promotion of cardiac hypertrophy via calcium-in-dependent pathways as well as delayed repolarization affecting the synchrony of inward Ca\(^{2+}\) fluxing (66). Because MEF2 is itself directly regulated by Ca\(^{2+}\) concentration, the observed effects of MEF2 on \( l_{\text{io}} \) suggest a complex and dynamic feedback network for the regulation of cardiac function (electrical and contractile properties), hypertrophy, and altered gene expression. It is interesting to note that calmodulin-dependent protein kinase has been shown to also regulate \( l_{\text{io}} \) in atrial cardiac myocytes (67), especially interesting given the known relationship whereby calmodulin-dependent protein kinase can regulate MEF2 activity through HDAC4 and HDAC5 nuclear translocation (33, 34).

That MEF2 factors might specifically regulate a subset of ion-handling genes is supported by the observation that MEF2 proteins are expressed most prominently in excitable tissues, such as heart, skeletal muscle, and brain. Indeed, Mef2a deletion promotes a phenotype of sudden death and cardiomyopathy in mice (11). However, hearts from Mef2a null mice were reported to have no alterations in expression of the arrhythmia-promoting genes Kvlqt1, minK, merG, and Scn5a (11). None of these genes were altered in our MEF2A- or MEF2C-overexpressing hearts, excluding this specific subset of ion-handling genes (supplemental Table 3). Thus, a more selected subset of genes likely contributes to the cardiomyopathic phenotype observed in MEF2A and MEF2C transgenic hearts. Indeed, alterations in a subset of ion-handling genes, such as Kcnd2, have already been reported to induce cardiac hypertrophy and myopathy (68, 69) and to modulate hypertrophy induced by α-adrenergic stimulation (46) or pressure overload (70).

Thus, we propose that the alterations in ion-handling genes might function as a primary disease-inducing lesion partially underlying the MEF2-mediated cardiomyopathy. Although this prediction would be difficult to prove directly, it is nonetheless consistent with the relatively rapid profile of current alterations that occurred in AdMEF2A-infected cultured cardiomyocytes (within 36 h). Moreover, consensus MEF2 DNA-binding sites are present in the promoters of the Kcnj3, Kcnk2, and Kcnj3 genes (data not shown). The observed changes in a subset of metabolic genes may have some direct regulatory relationships or could also represent early secondary changes associated with the impending cardiomyopathic phenotype in MEF2A and MEF2C transgenic hearts. In contrast, the observed alterations in extracellular matrix- and cell attachment-associated genes may represent a more primary disease mechanism underlying the propensity toward ventricular dilation associated with MEF2 overexpression in vivo or toward the sarcomeric disorganization and focal elongation observed in culture. In conclusion, we have provided the first proof of principle that MEF2 can dominantly drive dilated cardiomyopathy in vivo, potentially in association with a primary alteration in a subset of ion-handling genes and extracellular matrix-associated genes.

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