HDAC activity regulates entry of mesoderm cells into the cardiac muscle lineage

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Accepted 25 July 2006
Journal of Cell Science 119, 4305-4314 Published by The Company of Biologists 2006
doi:10.1242/jcs.03185

Summary

Class II histone deacetylases (HDAC4, HDAC5, HDAC7 and HDAC9) have been shown to interact with myocyte enhancer factors 2 (MEF2s) and play an important role in the repression of cardiac hypertrophy. We examined the role of HDACs during the differentiation of P19 embryonic carcinoma stem cells into cardiomyoctyes. Treatment of aggregated P19 cells with the HDAC inhibitor trichostatin A induced the entry of mesodermal cells into the cardiac muscle lineage, shown by the upregulation of transcripts *Nkx2-5*, *MEF2C*, *GATA4* and cardiac α-actin. Furthermore, the overexpression of HDAC4 inhibited cardiomyogenesis, shown by the downregulation of cardiac muscle gene expression. Class II HDAC activity is inhibited through phosphorylation by Ca^{2+}/calmodulin-dependent kinase (CaMK). Expression of an activated CaMKIV in P19 cells upregulated the expression of *Nkx2-5*, *GATA4* and *MEF2C*, enhanced cardiac muscle development, and activated a MEF2-responsive promoter. Moreover, inhibition of CaMK signaling downregulated GATA4 expression. Finally, P19 cells constitutively expressing a dominant-negative form of MEF2C, capable of binding class II HDACs, underwent cardiomyogenesis more efficiently than control cells, implying the relief of an inhibitor. Our results suggest that HDAC activity regulates the specification of mesoderm cells into cardiomyoblasts by inhibiting the expression of *GATA4* and *Nkx2-5* in a stem cell model system.

Key words: Cardiomyogenesis, Gene expression, Stem cells

Introduction

The first stage of cardiomyogenesis involves the formation of cardiomyoblasts, which express a distinct subset of transcription factors, including the MADS box transcription enhancer factor 2 polypeptide C (MEF2C), the homeobox transcription factor Nkx2-5 and the zinc-finger protein GATA binding protein 4 (GATA4) (Zaffran and Frasch, 2002). They are involved in activating the expression of cardiac-muscle-specific genes to form the differentiated cardiomyocyte, including cardiac α-actin, myosin heavy chain and atrial natriuretic factor (Durocher et al., 1997; Lee et al., 1998; Morin et al., 2000; Sepulveda et al., 1998). Mice lacking Nkx2-5 or the MEF2 family member MEF2C display defective heart morphogenesis, with development arrested at the heart looping stage (Lin et al., 1997; Lyons et al., 1995). Mice deficient in GATA4 do not develop a heart tube owing to the failure of the bilateral primordial to fuse (Kuo et al., 1997; Molkentin et al., 1997). The phenotype of mice lacking any one of these factors is not as severe as the phenotype in *Drosophila*, suggesting a role for genetic redundancy in mice (Bodmer, 1993; Gajewski et al., 1999; Lilly et al., 1994). Nkx2-5, GATA4 and MEF2C independently induce cardiomyogenesis in a P19 stem cell model system (Grepin et al., 1997; Jamali et al., 2001; Skerjanc et al., 1998) and activate each other's expression, suggesting the formation of a positive regulatory loop (Dodou et al., 2004; Searcy et al., 1998).

Histone acetyl transferases (HATs) and histone deacetylases (HDACs) loosen or compact chromatin structure, respectively, and play an important role in global gene expression (reviewed in Grozinger and Schreiber, 2002; Kuo and Allis, 1998). In general, HDACs repress transcription and have been reported to be involved in cell-cycle regulation, cell proliferation and differentiation (Cress and Seto, 2000; Grozinger and Schreiber, 2002; Verdin et al., 2003; Yang and Seto, 2003). In mammalian cells, there are three classes of HDACs, class I, II and III, based on sequence homology to yeast HDACs (Gray and Ekstrom, 2001). Class II HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) contain an N-terminal extension important for protein-protein interactions and are highly expressed in skeletal muscle, heart and brain (reviewed in Fischle et al., 2001; Sengupta and Seto, 2004). The restricted expression of class II HDACs suggests an important role for their activity in these tissues. Although the hearts of HDAC5$^{-/-}$ and HDAC9$^{-/-}$ mice develop normally, they are hypersensitive to stress-induced signals (Chang et al., 2004; Zhang et al., 2002). Furthermore, most HDAC5-HDAC9 double knockout mice died owing to heart defects, suggesting a role for class II HDACs in heart development (Chang et al., 2004). However, the earliest stage during cardiomyogenesis regulated by class II HDACs remains uncharacterized.

The activity of class II HDACs is primarily regulated by its sub-cellular localization in the cell. Phosphorylation of HDACs by Ca^{2+}/calmodulin-dependent kinase (CaMK) at conserved serine residues promotes the binding of 14-3-3 protein to

HDACs exporting them out of the nucleus. Class II HDACs directly bind and repress MEF2 function in adult cardiomyocytes and in skeletal myoblasts (Dressel et al., 2001; Han et al., 2005; Lu et al., 2000a; McKinsey et al., 2000b). The hearts of transgenic mice overexpressing an activated CaMKIV undergo hypertrophy with the upregulation of embryonic transcripts such as atrial natriuretic factor (ANF) and a dramatic enhancement of MEF2C activity (Passier et al., 2000). Class II HDACs also bind to the MADS-box family member serum response factor (SRF) (Davis et al., 2003). Counteracting the effect of HDACs is the transcriptional co-activator p300 that contains intrinsic HAT activity. p300 competes with class II HDACs for binding transcription factors and has been shown to be required for the activation of MEF2 and SRF (Sartorelli et al., 1997; Slepak et al., 2001). Furthermore, p300 interacts with GATA4, and acetylation of GATA4 itself has been implicated in the regulation of cardiomyogenesis in embryonic stem cells (Dai and Markham, 2001; Kakita et al., 1999; Kawamura et al., 2005). In skeletal muscle, class II HDACs inhibit the differentiation of skeletal myoblast cell lines into skeletal myocytes, and inhibit the activity of MEF2 factors in promoter studies performed in fibroblasts (Frey et al., 2000; Lu et al., 2000a; Miska et al., 1999). Therefore, although extensive studies have examined the role for HDAC in cardiac hypertrophy and skeletal myogenesis, little work has been carried out to examine the role of HDAC in cardiomyogenesis.

In this study, we used mouse embryonic carcinoma stem cells, P19 cells (Edwards et al., 1983; McBurney and Rogers, 1982), to examine the mechanism of HDAC function and regulation during the differentiation of stem cells into cardiac muscle. P19 cells aggregated in the presence of the HDAC inhibitor trichostatin A (TSA) exhibited a dose-dependent induction of cardiomyogenesis, shown by the upregulated expression of the cardiomyoblast transcription factors *GATA4*, *Nkx2-5*, *MEF2C* and the cardiac structural gene cardiac α-actin. An activated form of CaMKIV also enhanced cardiomyogenesis, with the upregulation of *GATA4*, *Nkx2-5* and *MEF2C* expression whereas the inhibition of CaMK signaling reduced the level of *GATA4* transcripts. Furthermore, overexpression of HDAC4 inhibited cardiomyoblast formation and downregulated the expression of *GATA4* and *Nkx2-5*. Altogether, our results show that HDACs play a repressive role during the entry of mesoderm cells into the cardiac-muscle lineage and that blocking HDAC activity is sufficient to enhance this early stage of cardiomyogenesis.

Results

P19 cells aggregated in the presence of TSA induce cardiomyogenesis

To define the role of HDACs during cardiomyogenesis, we used the HDAC inhibitor TSA, which can promote growth arrest, differentiation and apoptosis (Kijima et al., 1993). TSA promotes the acetylation of histones in several cell types (Minucci et al., 1997; Yoshida et al., 1990; Minucci et al., 1997). P19 cells were aggregated in the presence of increasing concentrations of TSA (2 nM, 5 nM or 10 nM) in the absence of dimethyl sulfoxide (DMSO) and compared with DMSO-treated cells. Using an anti-myosin heavy-chain antibody (anti-MyHC), cardiomyocytes were detected in P19 cells that were aggregated in the presence of DMSO or TSA (5 nM is shown)

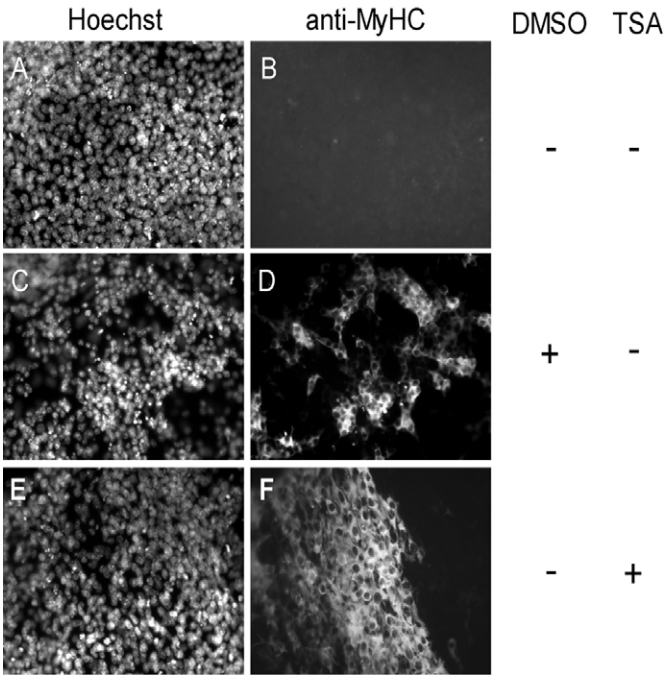

Fig. 1. TSA induces cardiomyogenesis. P19 cells were aggregated in the (A,B) absence of DMSO, (C,D) with 0.8% DMSO or (E,F) with 5 nM TSA. After 6 days, cells were stained with an anti-myosin heavy-chain antibody (anti-MyHC) and examined by immunofluorescence (B,D,F). Hoechst dye 33258 was used to visualize nuclei (A,C,E). Magnification 400×.

(Fig. 1D and F, respectively) but not in untreated cells (Fig. 1B). DMSO and TSA both induced cardiomyogenesis to similar levels; however, compared with DMSO, TSA-induced cardiomyocytes appeared more frequently in larger clumps. Therefore, TSA was sufficient to induce the differentiation of P19 cells into cardiac muscle.

The temporal expression pattern of cardiac muscle genes during TSA-induced cardiomyogenesis was examined. P19 cells aggregated in the presence of TSA exhibited an upregulation in the expression of the structural gene, cardiac α-actin on day 6 of differentiation in a dose-dependent manner (Fig. 2I; A lanes 9, 13 and 16). Similarly, the transcription factor *GATA4* and the signaling molecule *BMP4* were upregulated compared with cells not treated with TSA (Fig. 2I, B and C). A decrease of Brachyury T gene transcripts was observed for cells treated with 10 nM (Fig. 2I, D). The expression of *Nkx2-5* and *Mef2c*, determined by semi-quantitative reverse transcriptase (RT)-PCR, was also upregulated following TSA treatment compared with untreated cells (Fig. 2II, F and G). For comparison, RNA was harvested from P19 cells aggregated in the presence of DMSO. The upregulation of cardiac α-actin, *Gata4* and *Bmp4* was also observed (Fig. 2I, lanes 17-20) and appeared similar to 5 nM or 10 nM TSA treatment.

These results were quantified using densitometry for cultures treated with 5 nM TSA and the temporal pattern of enhancement of gene expression is depicted in Fig. 2III and IV. Upregulation of *Gata4* transcription levels was observed as early as day 2, and by day 6 there was an overall enhancement of six- to sevenfold for both *Gata4* and cardiac α-actin (Fig.

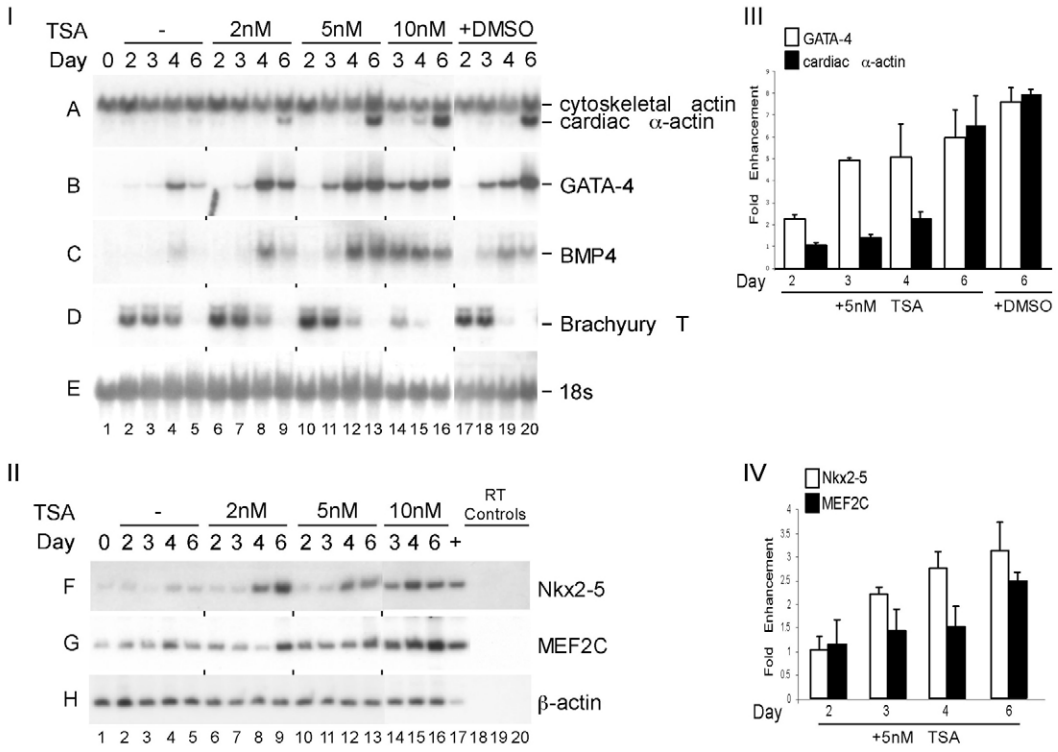

Fig. 2. TSA treatment of P19 cells induces the expression of cardiac α-actin, *Gata4*, *Bmp4*, *Mef2c* and *Nkx2-5*. P19 cells were aggregated and treated with 0, 2 nM, 5 nM or 10 nM TSA, or DMSO each day (day 0 to day 4). Total RNA was harvested on days 0, 2, 3, 4 and 6. (I) Northern blot analysis was used to detect the transcripts for cardiac α-actin, *Gata4*, *Bmp4* and Brachyury T from 12 μg of total RNA on the days indicated. (II) RT-PCR was performed on the RNA harvested on days 0, 2, 3, 4 and 6, and Southern blot analysis was used to detect for the expression of *Nkx2-5* and *Mef2c*. Lane 17 (+) shows a positive control with RNA from DMSO-induced cardiomyocytes on day 6. RT-PCR controls include the positive control without reverse transcriptase (lane 18), with control RNA (lane 19) and with PCR-H_2O (lane 20). (III, IV) Densitometric quantification was performed for the expression of (III) *Gata4* and cardiac α-actin by northern blot analysis and (IV) *Nxk2-5* and *Mef2c* by RT-PCR for cells treated with 5 nM TSA. Results are shown as the fold enhancement of transcript levels in TSA-treated cells compared with untreated cells on days 2, 3, 4 and 6. Results are also shown for the fold enhancement of *Gata4* and cardiac α-actin transcripts on day 6 in cells treated with DMSO compared with untreated cells. Error bars represent the standard error (+ s.e.) of three separate experiments.

2III). These results are comparable with DMSO-induced cardiomyogenesis, which exhibited an overall eightfold enhancement of *Gata4* and cardiac α-actin on day 6 (Fig. 2III). The upregulation of *Nkx2-5* in cells aggregated with TSA, was first observed on day 3, leading to an overall threefold enhancement compared with untreated cells (Fig. 2IV). *Mef2c* was upregulated 2.5-fold by day 6 (Fig. 2IV). Thus, TSA induced the differentiation of P19 cells into cardiomyocytes, to similar levels as DMSO.

HDAC4 overexpression inhibits cardiomyogenesis

Our finding that TSA enhances cardiomyogenesis implies an inhibitory role of HDACs. Since class II HDACs have been implicated in regulating the expression of cardiac genes during hypertrophy, we sought to determine whether HDAC4 overexpression was sufficient to inhibit the differentiation of P19 cells. Cell lines stably overexpressing HDAC4 [P19(HDAC4)] were generated and differentiated under cardiac-muscle-inducing conditions. Three stable clones overexpressing HDAC4 were isolated (Fig. 3I; A, lanes 4-12). The lower level of exogenous HDAC4 expression on day 0, compared with that on days 3 and 6, is most probably due to silencing of the *pgk* promoter in an undifferentiated stem cell, because of a higher level of compacted chromatin or the presence of differentiation-specific enhancers near the DNA insertion site. Endogenous HDAC4 expression appeared to decrease during the differentiation of P19 control cells into cardiomyocytes (Fig. 3A, lanes 1-3). Interestingly, endogenous HDAC4 levels appeared constant in P19(HDAC4) cells (Fig. 3A, lanes 4-12). On day 3, following P19(HDAC4) cell differentiation, an increase of Brachyury T gene expression was detected compared with control cells (13.0- ±0.5-fold, n=3) (Fig. 3I; B, lanes 5, 8 and 11, compared with lane 2). On day 6, a dramatic reduction in both GATA4 and cardiac α-actin transcripts was observed (10- ±2-fold and 3.0- ±0.5-fold, respectively, n=3) (Fig. 3I, C and D, respectively, lanes 6, 9 and 12, compared with lane 3). Expression of *Nkx2-5* was also decreased by 3.0- ±0.3-fold (n=3) in P19(HDAC4) cells, compared with control cells (Fig. 3II, F). MEF2C expression was also downregulated by day 6 in P19(HDAC4) cells lines (data not shown). These results indicate that, HDAC4 inhibits cardiomyogenesis of P19 cells at a stage subsequent to mesoderm induction, shown by the enhancement of Brachyury T gene expression, but prior to cardiomyoblast formation, shown by the downregulation of cardiac muscle transcripts such as *Gata4*, *Nkx2-5* and *Mef2c*.

Inhibition of CaMK signaling diminishes the specification of P19 cells into cardiomyoblasts

CaMK signaling can relieve the repressive effect of class II HDACs (McKinsey et al., 2000a; McKinsey et al., 2000b). An inhibitor of CaMK signaling, KN-93, was used to examine the role of CaMK in P19 cells. P19 cells were differentiated in the presence of DMSO and treated with 10 μM or 7.5 μM of KN-93 or KN-92 (a non-functional KN-93 analogue) (Fig. 4I). Treatment with the KN-93 inhibitor resulted in a 2- ±0.2-fold (n=3) decrease in *Gata4* transcripts on day 3 (Fig. 4I; A, lanes 6 and 7) compared with cells treated with KN-92 (Fig. 4I; A lanes 4 and 5) or with DMSO alone (Fig. 4I; A, lane 3). Brachyury T gene expression levels were not greatly affected by KN-93 treatment (Fig. 4I, B). P19 cells aggregated in the presence of increasing concentrations of KN-93 showed a dose-dependent decrease in *Gata4* expression (Fig. 4II). We were unable to look at day 6 of differentiation because of the toxicity of prolonged exposure to the compounds. These results show that inhibition of CaMK signaling reduces the expression of GATA4 and thus appears to inhibit cardiomyocyte specification.

Activated CaMKIV enhances cardiomyogenesis

To determine whether the expression of a constitutively activated form of CaMKIV (CaMKIV*) is sufficient to enhance the expression of cardiac muscle genes, P19 cells grown in monolayer cultures were transfected with CaMKIV* or with empty vector, and the expression of *Nkx2-5*, *Mef2c* and *Gata4* was analyzed by RT-PCR (Fig. 5I). These cells were also co-transfected with a PGK-GFP vector, and exhibited a transfection efficiency of ~40-50%. CaMKIV* expression was sufficient to upregulate the levels of *Nkx2-5* transcripts by 5- ±2-fold (n=8) after 1 day of transfection (Fig. 5I, A). *Mef2c* and *Gata4* transcripts were also upregulated by 2.0- ±0.1-fold (n=3) compared with cells not transfected with CaMKIV* (Fig. 5I, B and C).

To determine whether levels of upregulated *Nkx2-5* correlated with enhanced cardiomyogenesis in cells transfected with CaMKIV*, the number of cells stained with anti-MyHC antibodies was quantitated 4 days after transfection. Co-transfection of the GFP expression vector in these experiments showed a transfection efficiency of ~40-50%. Cultures transfected with CaMKIV* contained 2.5 times ±0.2 times

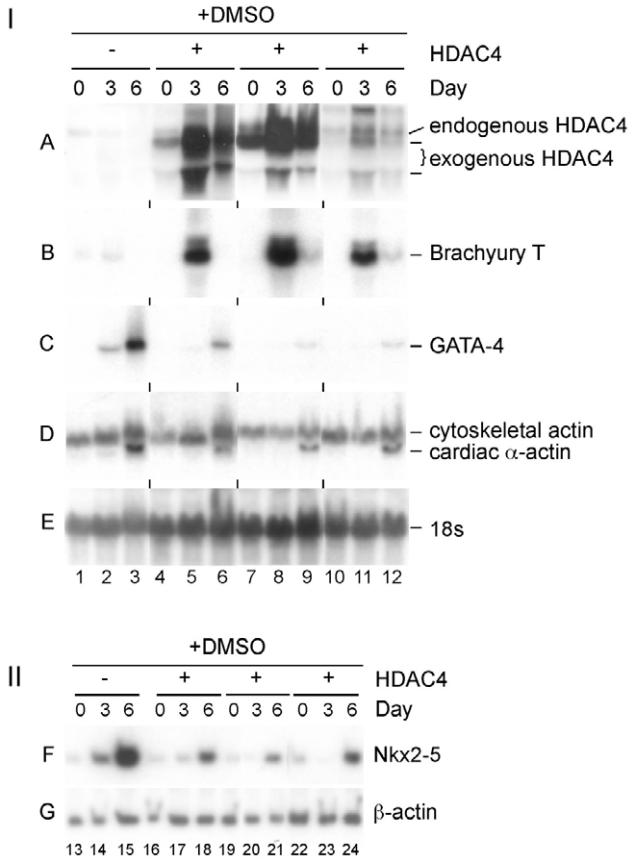

Fig. 3. Overexpression of HDAC4 inhibits cardiomyogenesis. P19(control) and P19(HDAC4) cell lines were aggregated in the presence of DMSO and total RNA was harvested on days 0, 3 and 6. (I) Northern blot analyses of 12 μg of total RNA from P19(control) and P19(HDAC4) cells probed as indicated on the right. (II) Total RNA was reverse-transcribed and PCR products for Nkx2-5 were detected by Southern blot analysis. Lanes were spliced from the same autoradiogram.

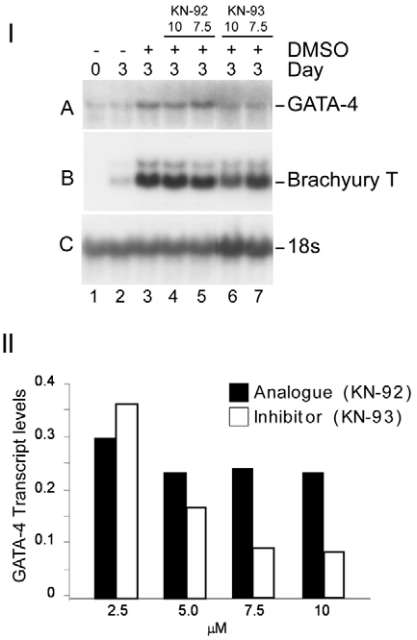

Fig. 4. Inhibition of CaMK activity diminishes the specification of P19 cells to cardiomyoblasts. P19 cells were aggregated with and without DMSO in the presence or absence of the CaMK inhibitor KN-93 or its non-functional analogue KN-92. (I) Total RNA was harvested on day 0 (lane 1); day 3 without DMSO (lane 2), day 3 with DMSO (lane 3), day 3 with DMSO and in the presence of KN-92 at 10 μM or 7.5 μM (lanes 4 or 5, respectively), and day 3 with DMSO and in the presence of KN-93 at 10 μM or 7.5 μM (lanes 6 or 7, respectively). Northern blot analysis was performed with 12 μg of total RNA to detect expression of the transcripts indicated on the right. (II) P19 cells were treated with 2.5 μM, 5.0 μM, 7.5μM and 10 μM of KN-93 or KN-92 and the level of *Gata4* expression was quantified by densitometry. Bars represent the densitometric measurement of *Gata4* transcripts from one representative experiment relative to rabbit 18S cDNA.

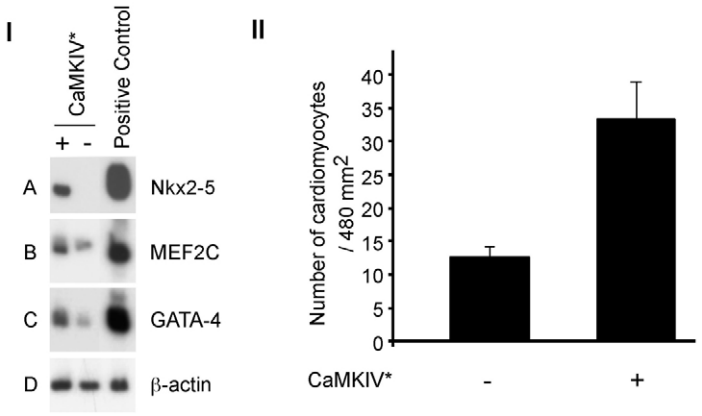

Fig. 5. CaMKIV* enhances cardiomyogenesis in P19 cells grown in monolayer. P19 cells were transfected with or without CaMKIV* (1.6 μg). (I) Total RNA was isolated from transfected cells after 1 day of transfection and reverse transcribed. PCR products from the amplification of *Nkx2-5*, *Mef2c*, *Gata4* and β-actin were detected using Southern blot analysis. (II) P19 cells were fixed after 4 days of transfection, stained with anti-myosin heavy-chain (MyHC) antibody, and examined by immunofluorescence. The number of MyHC-positive cells under each condition was counted. Error bars represent the standard error (+ s.e.); *n*=3.

(*n*=3) more MyHC-positive cells compared with control cultures transfected with vector alone (Fig. 5II). Thus, ectopic expression of CaMKIV* enhanced cardiomyogenesis in P19 monolayer cultures.

CaMKIV signaling activates a MEF2-responsive promoter

P19 cells were transiently transfected with HDAC4, MEF2C, and/or CaMKIV* to determine their ability to activate a MEF2-responsive promoter (2XMEF2-CAT). As demonstrated previously (Lu et al., 2000a), the ability of MEF2C to activate this reporter was attenuated in the presence of HDAC4 (Fig. 6). Furthermore, expression of CaMKIV* alone in P19 cells was sufficient to induce the activity of a MEF2-responsive reporter, similar to previous results showing that CaMKIV can activate MEF2 function in adult hearts (Passier et al., 2000). The ability of CaMKIV* to activate the MEF2-responsive promoter in P19 cells was attenuated by the expression of HDAC4. Co-transfection of CaMKIV* and MEF2C resulted in an additive enhancement of MEF2-reporter activity, which was also inhibited by HDAC4 overexpression. Finally, both MEF2C and CaMKIV* were unable to activate a reporter with mutated MEF2 sites (PE102-CATmut), indicating that the activation was dependent upon intact MEF2-binding sites in the promoter. These results show that CaMKIV* expression is sufficient to activate a MEF2-responsive promoter and that this activation can be inhibited by excess HDAC4.

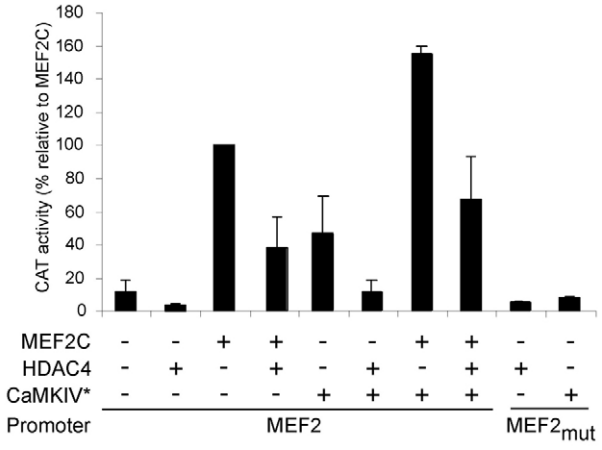

Fig. 6. CaMKIV* alone can activate a MEF2-responsive promoter, in a fashion that depends on the MEF2 sites. P19 cells were co-transfected with 0.8 μg of HDAC4, MEF2C and CaMKIV* expression vectors individually and in combination, and with either a wild-type or mutated MEF2-responsive promoter (1.4 μg) as indicated. Cells were harvested 24 hours after transfection and CAT assays were used to measure the activity from the MEF2-responsive promoter. Error bars represent the standard error (+ s.e.); *n*=2-13.

Stable overexpression of MEF2C/EnR enhances cardiomyogenesis

The finding that CaMKIV* can activate MEF2 expression and activity implies that HDACs control MEF2 function during cardiomyogenesis. Furthermore, the role of MEF2 factors in mammalian cardiomyogenesis has not been fully defined because of redundancy within the four MEF2-family transcription factors (Lin et al., 1997). To further understand the contribution of MEF2 factors in cardiomyogenesis, we used two different dominant-negative MEF2C constructs. One dominant-negative MEF2C was created by removing the C-terminal activation domain, termed MEF2CΔ (Kolodziejczyk et al., 1999). We created the other dominant-negative MEF2C by replacing the C-terminal activation domain with the engrailed repressor domain, termed MEF2C/EnR. To ascertain whether expression of the dominant-negative MEF2C mutants could affect cardiomyogenesis, stable P19 clonal populations expressing MEF2C/EnR [P19(MEF2C/EnR)], or MEF2CΔ [P19(MEF2CΔ)], driven by the *pgk* promoter were generated. These cell lines were aggregated in the presence of DMSO to determine the effect of the two dominant-negative MEF2 proteins on cardiac muscle development. Cells were fixed on day 6 or day 9 and examined by immunofluorescence with an anti-MyHC antibody. Unexpectedly, P19(MEF2C/EnR) cultures contained 8- ±2-fold more MF20-positive cardiomyocytes (Fig. 7I, B; *n*=3) compared with control P19 cells (Fig. 7I, A). A similar increase in cardiomyogenesis was observed with P19(MEF2CΔ) cells (data not shown), but not with the EnR domain alone (Jamali et al., 2001).

The immunofluorescence results were further confirmed by the examination of molecular markers by northern blot analysis. P19(MEF2C/EnR) cultures expressed the transfected MEF2C/EnR transcript, whereas control cultures did not (Fig. 7II, C). Interestingly, endogenous *Mef2c* was upregulated in P19(MEF2C/EnR) cultures (Fig. 7II, C). *Mef2c* transcripts are generally upregulated by day 6 during the differentiation of

P19 cells into cardiomyocytes, whereas *Mef2a*, *Mef2b* and *Mef2d* are ubiquitous (Skerjanc et al., 1998). The presence of cardiac muscle was greatly enhanced in cultures expressing MEF2C/EnR, as indicated by the increased expression of *Gata4* (Fig. 7II, D) and cardiac α-actin (Fig. 7II, E). Densitometric analysis of *Gata4* transcripts from several experiments indicated that cardiomyogenesis was enhanced by an average of 7- ±2-fold (n=5) in cultures expressing MEF2C/EnR, compared with control cultures. To further substantiate a cardiomyocyte phenotype, *Nkx2-5* expression was analyzed by RT-PCR (Fig. 7III) and found to be upregulated on days 6 and 9 in three clonal populations expressing MEF2C/EnR, compared with control cells. The enhancement of cardiomyogenesis was unlikely to be caused by selective cell death of non-cardiomyocytes, because the total RNA isolated from control and P19(MEF2C/EnR) cultures did not differ by more than 30% on average. Furthermore, P19(MEF2CΔ) cells exhibited similar levels of enhancement of cardiomyogenesis compared with P19(MEF2C/EnR) cells (data not shown). Therefore, cardiac myogenesis is enhanced in P19 cell cultures that constitutively express dominant-negative MEF2.

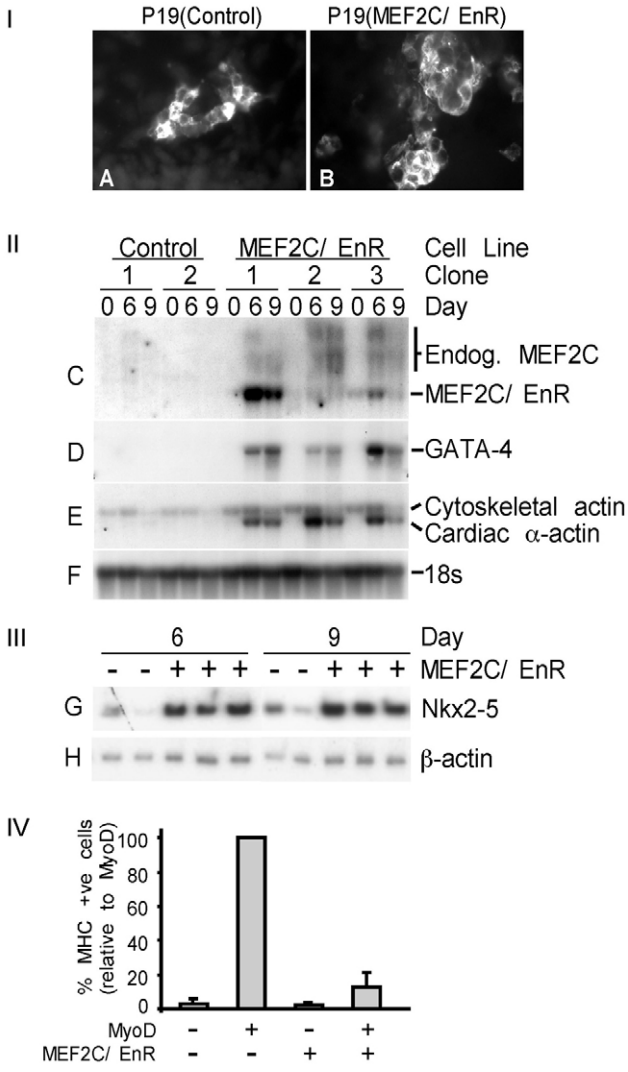

Fig. 7. Overexpression of dominant-negative MEF2 (MEF2C/EnR) enhances the development of cardiac muscle. P19(MEF2C/EnR) and P19(control) cells were differentiated in the presence of DMSO. (I), (A) P19(control) and (B) P19 (MEF2C/EnR) cells were fixed and immunostained on day 6 of differentiation with anti-MyHC antibody to detect cardiomyocytes (magnification 400×). (II) RNA was harvested on days 0, 6 and 9. Northern blots containing 6 μg of total RNA were probed as indicated on the right. (III) RNA from day 6 and 9 were reverse-transcribed and Nkx2-5 PCR products were detected using Southern blot analysis. (IV) 10T1/2 fibroblasts were transfected with and without MyoD and MEF2C/EnR. After 6 days in differentiation medium, MyHC-positive cells were counted and normalized for transfection efficiency with GFP and standardized to MyoD conversion levels. Error bars represent the standard error (+ s.e.); n=3.

The ability of these modified MEF2 proteins to function in a dominant-negative fashion was confirmed by assessing their effect on MyoD-mediated conversion of 10T1/2 fibroblasts into skeletal muscle. As expected, MEF2C/EnR reduced MyoD-induced myogenesis eightfold (Fig. 7IV). Similar results were obtained with MEF2CΔ (data not shown). Therefore, the dominant-negative MEF2 mutants used in these studies can repress transcription in the context of a myogenic conversion assay in fibroblasts (Fig. 7IV). Furthermore, expression of MEF2C/EnR in cardiomyoblasts also inhibited differentiation of P19 cells into cardiomyocytes (Karamboulas et al., 2006). In addition, expression of MEF2C/EnR was sufficient to inhibit MEF2 activity on a MEF2-responsive promoter (data not shown) and on a collagen X promoter (Stanton et al., 2004). Finally, we have found that the presence of MEF2C/EnR can enhance the amount of HDAC4 co-purified with a His-tagged MEF2C by two- to fourfold (data not shown), indicating that MEF2C/EnR can heterodimerize with His-tagged MEF2C and bind to HDAC4. Indeed, several publications show that the MADS/MEF2 domain (Lu et al., 2000b) and the EnR domain can recruit HDACs (Chen et al., 1999; Courey and Jia, 2001; Tolkunova et al., 1998). Given our finding that HDAC inhibits entry into the cardiac muscle lineage, and that MEF2ΔC or MEF2C/EnR can bind HDAC4, the enhancement of cardiomyogenesis by dominant-negative MEF2 could be explained by the binding and sequestering of class II HDACs from cardiac muscle transcription factors, resulting in enhanced transcription.

Discussion

In the present study, we used P19 cells to examine the role of HDACs during cardiomyogenesis. Conditions that inhibited HDAC activity, such as TSA treatment, activation of CaMK signaling pathways and overexpression of a dominant-negative MEF2, resulted in the induction of cardiomyogenesis in P19 cells. This was shown by upregulation of the cardiomyoblast transcription factors *Nkx2-5*, *Gata4* and *Mef2c* as well as upregulation of the structural genes cardiac α-actin and myosin heavy chain. Furthermore, conditions that could promote HDAC activity, such as overexpression of HDAC4 or inhibition of CaMK signaling, repressed cardiomyogenesis. Altogether these results indicate a role for HDAC activity during the specification of mesoderm cells into cardiomyoblasts in a stem cell model system. A mechanistic model is proposed in Fig. 8.

The present study is the first to show that aggregation of P19 cells in the presence of TSA was sufficient to induce cardiomyogenesis in the absence of DMSO. Previous studies

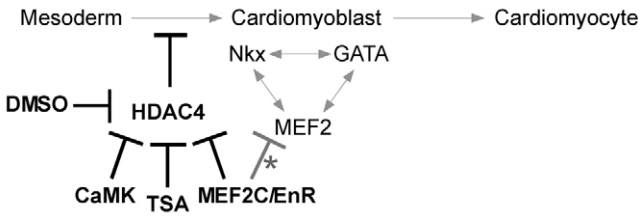

Fig. 8. Mechanistic model. HDAC activity negatively regulates the entry of mesoderm cells into the cardiac-muscle lineage. Relief of HDAC inhibition by TSA, CaMK or, possibly, DMSO enhances the differentiation of P19 stem cells into cardiomyocytes, whereas the overexpression of HDAC4 inhibits differentiation (bold, black). Thus, HDAC activity inhibits mesoderm cells from entering the cardiac lineage and upregulating the expression of *Nkx2-5*, *Gata4* and *Mef2c*. Furthermore, the overexpression of the dominant-negative MEF2C/EnR enhances cardiomyogenesis, by possibly disrupting HDAC activity, allowing more cells into the cardiac lineage. Similarly, MEF2C/EnR expressed in cardiomyoblasts displays an initial enhancement of the cardiac factors; however, MEF2C/EnR in these cells (asterisk) results in the subsequent downregulation of this positive loop and inhibition of cardiomyogenesis (Karamboulas et al., 2006).

found that, TSA added to aggregated embryonic stem (ES) cells at a later stage (day 7) promoted differentiation by activating GATA4 by acetylation, but not by increasing *Gata4* expression levels (Kawamura et al., 2005). However, here we show that P19 cells that had been aggregated in the presence of TSA enhanced the entry of mesoderm cells into the cardiac lineage by increasing the expression levels of *Gata4*. By contrast, one previous study showed that ES cells treated with TSA could not form aggregates (Lee et al., 2004). The rapid decrease in the levels of the mesoderm marker Brachyury T in cells treated with increasing amounts of TSA (up to 10 nM) was consistent with the early upregulation of the cardiomyoblast transcription factors and the cardiac-muscle-marker cardiac α-actin. Our results imply that DMSO may function in part by blocking HDAC activity to induce cardiomyogenesis. However, DMSO and TSA synergistically enhanced cardiomyogenesis (data not shown), suggesting they do not play completely redundant roles in this process. Furthermore, our results are consistent with the requirement for HAT activity during heart morphogenesis and for the activation of the transcription factors SRF, GATA4 and MEF2 (Dai and Markham, 2001; Kakita et al., 1999; Kawamura et al., 2005; Sartorelli et al., 1997; Shikama et al., 2003; Slepak et al., 2001; Yanazume et al., 2003).

We show that the overexpression of HDAC4 decreased the level of cardiac muscle development in P19 cells due to a significant decrease in *GATA4* and *Nkx2-5* transcript levels. Interestingly, *HDAC4* expression resulted in higher levels of Brachyury T transcripts, suggesting that HDAC4 prevents mesodermal cells from progressing into the cardiac lineage and supporting a role for HDAC in regulating the transition from mesoderm to cardiomyoblast. Furthermore, P19 cells that displayed a decrease in *HDAC4* expression during DMSO-induced differentiation exhibited enhanced cardiomyogenesis. The prevention of HDAC activity, at the transcriptional or post-translational level, appears to be essential for the progression of mesoderm cells into the cardiac lineage.

HDAC4 overexpression did not result in the complete loss of cardiac-muscle development. It is possible that HDAC4 is not the sole factor involved because there are several other class II HDACs. Indeed, the more dramatic effect observed by inhibiting all HDACs with TSA compared with the effect of overexpressing HDAC4 alone, is consistent with the evidence for redundant roles of HDACs found in the phenotype of knockout mice (Chang et al., 2004; Zhang et al., 2002). Furthermore, if DMSO functions in part by blocking HDAC activity, the extent of HDAC4 overexpression may not have been sufficient to override the blockade by DMSO. For example, HDAC4 may not be properly translocated to the nucleus under the conditions of these experiments, reducing its effectiveness.

We found that CaMKIV* was able to activate a MEF2-responsive promoter, suggesting that CaMKIV* can relieve the inhibition of endogenous MEF2 function by class II HDACs. These results are consistent with the ectopic expression of activated CaMKIV in the heart, which led to a more than 100-fold increase in transcriptional activity without changing MEF2-DNA binding (Passier et al., 2000). Since we found that overexpression of MEF2C/EnR does not efficiently and consistently enhance *Nkx2-5* expression after 24 hours in monolayer culture, it is possible the CaMKIV* is also activating other pathways. Previous studies have shown that CaMKIV* can activate the function of CREB, ATF-1, SRF, MEF2D, RORα, RORγ and COUP-TF1 (Blaeser et al., 2000; Kane and Means, 2000; Miranti et al., 1995; Sun et al., 1996; Tokumitsu et al., 1995). Furthermore, class II HDACs might repress gene expression mediated by SRF and GATA4 (Davis et al., 2003; McKinsey and Olson, 2004). Similarly, the inhibition by class II HDACs might be relieved by CaMK-independent mechanisms during cardiomyogenesis (Zhang et al., 2002). The importance of other signaling pathways in this process is also shown by our incomplete inhibition of GATA4 expression upon treatment with the CaMK inhibitor KN-93. Interestingly, our results suggest that the enhancement of cardiomyogensis by DMSO occurs, in part, by increasing intracellular Ca^{2+} (Morley and Whitfield, 1993), resulting in the activation of CaMK and the inhibition of HDAC activity.

In contrast to our previous results using dominant-negative approaches by fusing the EnR domain to other transcription factors, such as β-catenin, Pax3, Meox1 and Gli2 (Petropoulos et al., 2004; Petropoulos and Skerjanc, 2002; Ridgeway and Skerjanc, 2001), we show here that cells ubiquitously expressing either form of dominant-negative MEF2C displayed enhanced levels of differentiation into cardiac muscle. The enhancement of cardiomyogenesis by MEF2C/EnR might be due to the relief of a repressor activity in cells not yet committed to the cardiac lineage. Both MEF2CΔ and MEF2C/EnR contain the MADS/MEF2 domain, and the MEF2 dimer interface that binds to class II HDACs (Han et al., 2005; Lu et al., 2000a); and both act in a dominant-negative fashion to inhibit wild-type MEF2 activity (Fig. 7IV). Since endogenous MEF2 can bind to DNA and recruit repressors (Youn et al., 2000), MEF2C/EnR might bind class II HDACs and remove them from the inactive chromatin found in stem and/or mesoderm cells, allowing the chromatin structure to open, enhancing the transcription of cardiac muscle determination genes. This relief of HDAC repression on cardiac-muscle transcription factors would result in the

observed enhancement of cardiomyogenesis. Our model assumes that the chromatin structure of genes controlling cardiomyogenesis is initially closed in stem cells – in agreement with a variety of studies – and would not permit MEF2C/EnR binding to DNA (Berkes et al., 2004; Chambeyron and Bickmore, 2004; Tagoh et al., 2002).

The overall enhancement of cardiomyogenesis observed in P19(MEF2C/EnR) cell lines is probably due to an increase in cardiomyoblast specification at a level greater than the inhibition of cardiomyoblast differentiation in these cells. Further evidence for such an enhancement was observed in separate experiments (Karamboulas et al., 2006). Expression of MEF2C/EnR from an Nkx2-5 enhancer resulted in an initial enhancement of *Nkx2-5*, *Gata4* and *Mef2c* expression, due to low levels of *Nkx2-5* expression in P19 stem cells (day 0-2). This enhancement was then followed by the downregulation of cardiomyoblast transcripts and the inhibition of cardiac muscle differentiation (day 5-6). Therefore, whether MEF/EnR is an activator or a repressor depends on the timing and the characteristics of the cell it is expressed in. Transcription factors that can play both a positive and a negative role have been observed in other systems (Gaines and Berliner, 2003; Huo and Zhang, 2005).

In summary, we show that HDAC regulates the transition from mesoderm to cardiomyoblast during cardiomyogenesis in P19 cells. This is the first study to show that HDAC regulates this early step of cardiomyogenesis in a stem cell model system and may have an important therapeutic impact for future stem-cell-based therapies.

Materials and Methods

Plasmid constructs

The phosphoglycerate kinase 1 (*pgk-1*) promoter was used to drive the expression of various cDNAs. The DNA constructs PGK-Puro and PGK-LacZ have been previously described (Ridgeway et al., 2000a). Two dominant-negative MEF2 mutants (MEF2C/EnR-1 and MEF2C/EnR-2) were created, in which the engrailed repressor domain was fused to the MEF2 C-terminus. Both constructs behaved identically. MEF2C/EnR-1 contained the 198 amino acid N-terminal repression domain of the mouse EN-2 protein fused to the DNA-binding domain of MEF2C (the first 142 amino acids). PCR amplification of mouse MEF2C cDNA (Martin et al., 1994) was performed using the primers 5′-AAGGATCCGGGACGAGAGAG-AGAAGA-3′ (forward) and 3′-AACTCGAG**CTA**GATATCCTCAAAGCTGGGA-GGTGG-5′ (reverse). PCR amplification of the EN-2 cDNA (GenBank accession number NM 010134) was performed using the previously described primers 5′-AAGATATCGAGGAGAAGGATTCCAAGCCC-3′ (forward) and 3′-AAGAT-ATCCTACCCAGAGTGGCGCTGGCT-5′ (reverse) (Ridgeway and Skerjanc, 2001) that used a stop codon in the *pgk-1* promoter vector. MEF2C/EnR-2 was designed to verify the initial results and used the engineered stop codon (bold in the reverse MEF2C primer). To make use of this stop codon, EN-2 was amplified using the forward primer as above and the reverse primer 3′-AAGAT-ATCCTAGCCCAGAGTGGCGCTGGC-5′. The 594 bp *Eco*RV digestion product of the EN-2 PCR product was cloned into the engineered *Eco*RV site (underlined reverse MEF2C primer) at the 3′ end of the MEF2C PCR product. In addition, a C-terminal deletion of MEF2C (Kolodziejczyk et al., 1999), termed MEF2CΔ, was used containing only the first 142 aa of MEF2C (a generous gift from L. Megeney, Ottawa Health Research Institute, Ottawa, ON, Canada). PGK-vector DNA is a plasmid containing the *pgk-1* promoter alone. A constitutively activated CaMKIV construct (Kane and Means, 2000), CaMKIV*, which includes the amino acids 1-317 and lacks an auto-inhibitor domain, was isolated as a *Bam*HI-*Bgl*II fragment, blunted and cloned into the *Sma*I site of the PGK-vector. The pcDNA-HDAC4 construct has been previously described (Lemercier et al., 2002).

Cell culture and DNA transfections

P19 embryonic carcinoma cells were cultured in αD-minimum essential medium containing 5% Cosmic calf serum (Hyclone, Logan, Utah) and 5% fetal bovine serum (CanSera, Rexdale, Ontario), as described (Rudnicki and McBurney, 1987; Wilton and Skerjanc, 1999) and stable cell lines were generated as described previously (Ridgeway et al., 1999; Ridgeway et al., 2000a; Ridgeway and Skerjanc, 2001; Ridgeway et al., 2000b; Skerjanc et al., 1998; Skerjanc et al., 1994; Skerjanc and Wilton, 2000). For MEF2CΔ, transfections were performed using the calcium phosphate method with 8 μg of PGK-MEF2CΔ, 0.7 μg of PGK-Puro, 1 μg of PGK-LacZ and 2.5 μg of B17. For MEF2C/EnR, transfections were performed using the FuGENE^{TM} transfection system according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN) with 1.9 μg MEF2C/EnR, 0.68 μg B17, 0.24 μg PGK-LacZ, and 0.09 μg PGK-Puro. For HDAC4, transfections were performed using the FuGENE transfection system with 2.04 μg of HDAC4, 0.77 μg B17, 0.17 μg PGK-LacZ, and 0.09 μg PGK-Puro. P19 control cells were isolated from cultures transfected with the empty PGK or pcDNA-vector plasmid.

Stable P19(MEF2CΔ), P19(MEF2C/EnR) or P19(HDAC4) clonal populations were chosen by their expression of high levels of MEF2CΔ, MEF2C/EnR or HDAC4 transcripts in monolayer cultures by slot-blot analysis. All experiments were performed at least twice with at least two clonal populations for each cell line.

Differentiation was induced by aggregating 5×10^5 P19(control), P19(MEF2CΔ), P19(MEF2C/EnR), or P19(HDAC4) cells in Petri dishes with serum-supplemented medium containing 0.8% DMSO. Cells were aggregated for 4 days and then plated into tissue culture dishes without drug. The presence of DMSO induces P19 cells to differentiate into cardiac muscle cells by day 6 of differentiation (Skerjanc, 1999). Sub-optimal serum conditions were used to minimize the extent of cardiac muscle development in the control cultures for PGK-MEF2C/EnR and PGK-MEF2CΔ (Wilton and Skerjanc, 1999). At the time indicated, total RNA was harvested or cells were fixed for immunofluorescence.

To determine the effect of CaMKIV*, 0.25×10^5 P19 cells were seeded onto gelatin-coated coverslips in 35-mm tissue culture dishes, transfected after 24 hours with 1.6 μg of PGK-CaMKIV* or PGK-vector and 0.6 μg PGK-GFP (to determine transfection efficiency) and grown for an additional 4 days. The cells were fixed and stained with anti-myosin heavy-chain antibody (MF20) as mentioned above. The number of positively stained cells were counted and the average number is depicted in the graph (n=3). The number of GFP-positive cells were counted for each experiment and found to represent 40-50% of the total cells. To examine the expression of endogenous *Nkx2.5*, *GatA4* and *Mef2c*, total RNA was isolated and RT-PCR performed is described below.

To determine the effect of CaMKIV* on a MEF2 promoter, cells were transfected with 1.4 μg of PE102-CAT (2XMEF2-CAT) or PE102-CAT$_{mut}$ (2XMEF2$_{mut}$-CAT) (Ornatsky and McDermott, 1996) and 0.4 μg of PGK-LacZ and/or 0.8 μg of PGK-CaMKIV*, 0.8 μg of PGK-MEF2C, and/or 0.8 μg of CMV-HDAC4 using FuGENE 6 transfection system according to the manufacturer's protocol. Total DNA was brought up to a total of 4.2 μg of DNA with PGK-vector for each transfection. Cells were harvested 24 hours after transfection. β-Galactosidase and CAT assays were performed as described previously (Norton and Coffin, 1985; Sleigh and Lockett, 1986). Background CAT activity measured in cells transfected with the PGK-vector was subtracted from the CAT activity of each sample, which was then normalized for transfection efficiency with β-galactosidase activity from the transfected culture. Results were compared to the extent of promoter activation by MEF2C alone, which was set at 100%.

Co-purification of His-tagged MEF2C with HDAC4

P19 cells were transfected with 2 μg of His-tagged MEF2C, 2 μg of HDAC4, and with either 4 μg of empty vector control or 4 μg of MEF2C/EnR as described above. After 24 hours, His-tagged MEF2C was purified on a Ni-NTA agarose column according to the manufacturer's instruction (Qiagen). Samples were subjected to SDS-PAGE and western blotted with an anti-HDAC rabbit polyclonal antibody (Cell Signaling Technology). The experiment was repeated with the following amounts of His-tagged MEF2C, HDAC4 and MEF2C/EnR: 1.5 μg, 1.5 μg, 5 μg or 1 μg, 1 μg, 6 μg, respectively, with similar results.

Inhibitor experiments

To determine the effect of trichostatin A (TSA) (Sigma, St Louis, MO), P19 cells were aggregated in the presence of TSA for 4 days (without DMSO) at concentrations of 2 nM, 5 nM or 10 nM and compared with untreated cells. As a positive control for differentiation, P19 cells were aggregated for 4 days in the presence of DMSO (0.8%). Total RNA was harvested on the days indicated.

To determine the effect of CaMK signaling on the differentiation of P19 cells, the CaMK inhibitor KN-93 and the non-functional KN-93 analogue KN-92 (Calbiochem, La Jolla, CA) were added at 2.5 μM, 5.0 μM, 7.5 μM and 10 μM on day 1 and 2. Cells were harvested on day 3 and total RNA was harvested and examined by northern blot analysis.

Myogenic conversion of 10T1/2 fibroblasts

10T1/2 fibroblasts were routinely cultured in 10% 1:1 cosmic calf-fetal bovine serum in α-minimum Eagle's medium. Cells were transfected on gelatin-coated coverslips using the FuGENE 6 transfection reagent. Conversion was driven by 0.7 μg PGK-MyoD in the absence or presence of 0.3 μg CMV-HDAC4, 2.5 μg PGK-MEF2C/EnR or 2.5 μg PGK-MEF2CΔ, and 0.5 μg of pEGFP-N1 (Clontech Laboratories, Inc. Palo Alto, CA). Total DNA in each transfection was brought up to 4 μg with PGK vector plasmid. After 24 hours, cells were transferred to differentiation medium, containing 2% horse serum, for 6 days. Transfection efficiency for each culture was scored by analysing GFP fluorescence and myogenic

conversion was quantified by counting cells expressing myosin heavy chain (MyHC), as described below.

Immunofluorescence and northern blot analysis

For immunofluorescent labeling, cells were fixed in methanol at –20°C and incubated first with mouse anti-MyHC monoclonal antibody supernatant MF20, followed by goat anti-mouse IgG(H+L) Cy3-linked antibody (Jackson Immunoresearch Laboratories), as described previously (Ridgeway et al., 2000a; Ridgeway and Skerjanc, 2001; Ridgeway et al., 2000b). For northern blot analysis, total RNA was isolated from differentiated P19(control), P19(MEF2CΔ), P19(MEF2C/EnR), or P19(HDAC4) cultures at the times indicated and analyzed as described previously (Ridgeway et al., 2000a).

The DNA probes used were: a 600 bp *Pst*I fragment from last exon of the human cardiac α-actin gene (Rudnicki et al., 1990), a 2.4 kb *Xba*I fragment of *Gata4* cDNA (Grepin et al., 1994), a 1.5 kb *Hin*dIII/*Xba*I fragment of *Mef2c* cDNA (Martin et al., 1993), a 1.6 kb *Eco*RI fragment of *Nkx2-5* cDNA (Lints et al., 1993), a 1.6 kb *Eco*RI/*Bam*HI fragment of mouse Brachyury T cDNA (Herrmann et al., 1990), a 1.0 kb *Hin*dIII/*Bam*HI fragment of mouse *Bmp4* cDNA (Winnier et al., 1995) and a 52 bp PCR product of β-actin using the oligonucleotide primers described below. All blots were standardized using a 750 bp *Eco*RI fragment of rabbit 18S cDNA.

RT-PCR

Total RNA was cleaned using RNeasy Kit (Qiagen, Mississauga, Ontario, Canada), and treated with DNase I (amplification grade) at a concentration of 1 unit/μg RNA. The superscript II RNase H⁻ reverse transcriptase (Invitrogen Life Technologies) was used to synthesize the first cDNA strand from 5 μg total RNA. Platinum *Taq* DNA polymerase (Invitrogen Life Technologies) was used to perform the PCR. Southern blots were performed to detect products by using a probe from the corresponding cDNA. Negative controls performed with all RT-PCR experiments included a water control for the PCR reaction, a water control for the complete RT-PCR reaction and a control in the absence of reverse transcriptase. The following conditions and pairs of primers were used. β-actin, 5′-tcgtcgacaacggctccggcatgt-3′ (forward) and 3′-ccagccaggtccagacgcaggat-5′ (reverse) with 45 seconds at 94°C (denaturation), 45 seconds at 56°C (annealing), 1 minute at 72°C (extension), for 19 cycles. Nkx2-5, 5′-cctctagagcagagctgcgcgcggagatg-3′ (forward) and 3′-ggtggcttccgtcgccgccgtgc-5′ (reverse), with 1 minute 94°C (denaturation), 2 minutes 72°C (annealing), and 2 minutes 72°C (extension), for 26 cycles. GATA4, 5′-actctggaggcgagatggg-3′ (forward) and 3′-ctcggcattacgacgccacag-5′ (reverse) with annealing temperature of 72°C, 2 minutes for 25 cycles. Primers and conditions for MEF2C have been described previously (Gianakopoulos and Skerjanc, 2005).

We thank Josée Savage for critically reading the manuscript. HDAC4 was a generous gift from S. Khochbin via J. Torchia and T. M. Underhill. CaMKIV* and MEF2CΔ were kindly provided by Anthony R. Means and L. Megeney, respectively. We thank Greg Gloor for generously sharing his PCR facility. C.K. was supported by a Canadian Institutes of Health Research Doctoral Award and a Premier's Research Excellence Award in partnership with the Foundation for Gene and Cell Therapy. A.S.A.-M. was supported by an Ontario Women's Health Council/CIHR Institute of Gender and Health Fellowship. I.S.S. was supported by an Investigator Award from the Canadian Institute of Aging. This work was supported by a grant (MOP-53277) to I.S.S. from the Canadian Institutes of Health Research.

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