Erratum

Disruption of MEF2 activity in cardiomyoblasts inhibits cardiomyogenesis

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There was an error in the first e-press version of the article published in *J. Cell Sci.* **119**, 4315-4321.

The first e-press version of this article wrongly gave the title as: Disruption of MEF2 activity HDAC in cardiomyoblasts inhibits cardiomyogenesis.

We apologise for this mistake.

Disruption of MEF2 activity in cardiomyoblasts inhibits cardiomyogenesis

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Summary

Myocyte enhancer factors (MEF2s) bind to muscle-specific promoters and activate transcription. *Drosophila Mef2* is essential for *Drosophila* heart development, however, neither MEF2C nor MEF2B are essential for the early stages of murine cardiomyogenesis. Although *Mef2c*-null mice were defective in the later stages of heart morphogenesis, differentiation of cardiomyocytes still occurred. Since there are four isoforms of MEF2 factors (MEF2A, MEF2B, MEF2C and MEF2D), the ability of cells to differentiate may have been confounded by genetic redundancy. To eliminate this variable, the effect of a dominant-negative MEF2 mutant (MEF2C/EnR) during cardiomyogenesis was examined in transgenic mice and P19 cells. Targeting the expression of MEF2C/EnR to cardiomyoblasts using an *Nkx2-5* enhancer in the P19 system resulted in the loss of both cardiomyocyte development and the expression of *GATA4*, *BMP4*, *Nkx2-5* and *MEF2C*. In transiently transgenic mice, MEF2C/EnR expression resulted in embryos that lacked heart structures and exhibited defective differentiation. Our results show that MEF2C, or genes containing MEF2 DNA-binding sites, is required for the efficient differentiation of cardiomyoblasts into cardiomyocytes, suggesting conservation in the role of MEF2 from *Drosophila* to mammals.

Key words: Cardiomyogenesis, MEF2C, Transgenic mice

Introduction

Myocyte enhancer factors 2 (MEF2s) belong to the MADS-box family of transcription factors. Members of this family contain a conserved MADS-box domain and a MEF2 domain at the N-terminus of the protein, which are involved in DNA-binding and dimerization, and a C-terminal transactivation domain involved in the regulation of transcriptional activity. The four MEF2 proteins in vertebrates (MEF2A, MEF2B, MEF2C and MEF2D) bind A-T-rich MEF2 sites as homo- and heterodimers, and are expressed in precursors of the three muscle lineages, as well as in neurons (reviewed in Black and Olson, 1998). MEF2C is the first member of the family to be expressed in the mouse, with transcripts appearing in the precardiac mesoderm at embryonic day 7.5 (E7.5) (Edmondson et al., 1994). Promoter analysis has shown that MEF2 sites are essential for the expression of muscle-specific genes in cardiac muscle and skeletal muscle (Amacher et al., 1993; Black and Olson, 1998).

The strongest indication of an essential role for MEF2 in muscle development comes from studies in *Drosophila*. Loss-of-function mutations of the single *Mef2* gene resulted in a block in the development of all muscle cell types in the embryo (Lilly et al., 1995). Although the cardiac muscle contractile proteins were not expressed in these mutants, a dorsal vessel formed normally and expressed tinman, a homeobox transcription factor. Together with the finding that *Mef2* contains essential tinman-binding sites (Cripps et al., 1999; Gajewski et al., 1999), these data suggest that *Mef2* controls the conversion of cardiomyoblasts into cardiomyocytes.

In contrast to studies in *Drosophila*, cardiomyocyte development still occurs in *Mef2c*-null mice. *Mef2c*-null mice died at E10.5 due to a failure of the heart to undergo looping morphogenesis (Lin et al., 1997; Vong et al., 2005). Although the right ventricular region did not form and a subset of cardiac muscle-specific genes were downregulated to background levels, cardiomyocytes still formed. Since levels of MEF2B protein was increased more than sevenfold, it may partially compensate for the loss of MEF2C in these mice. That MEF2C shares functions with MEF2B is also supported by the normal development of *Mef2b*-null mice (Lin et al., 1997).

During cardiac muscle development, genes encoding the transcription factors Nkx2-5, GATA4, and MEF2C are first expressed in cardiac precursor cells. Several studies have shown that they act in combination with additional cofactors to activate the expression of several cardiac-specific genes (Braun et al., 1989; Chen et al., 1996; Chen and Schwartz, 1996; Grepin et al., 1994; Sepulveda et al., 1998). The overexpression of each of these factors initiates cardiomyogenesis in P19 cells and can also activate the expression of each other (Dodou et al., 2004; Grepin et al., 1997; Jamali et al., 2001; Reecy et al., 1999; Searcy et al., 1998; Skerjanc et al., 1998). Mice lacking Nkx2-5, and *Drosophila* lacking the Nkx2-5 homologue *tinman* or the GATA4 homologue *pannier*, show a downregulation of MEF2

(Gajewski et al., 1999; Gajewski et al., 1997; Nguyen et al., 1994; Tanaka et al., 1999). Furthermore, essential GATA sites are present in enhancers of MEF2C and Nkx2-5 (Dodou et al., 2004; Lien et al., 1999; Searcy et al., 1998). Therefore, whereas the genetic hierarchy for Nkx2-5, GATA4 and MEF2C is unclear in mammals, there exists a positive regulatory loop that probably supports and maintains cardiomyogenesis.

Experiments using dominant-negative MEF2A have shown that MEF2A is essential for skeletal myogenesis in myoblast cell lines (Ornatsky et al., 1997). However, an essential role for MEF2 factors in mammalian cardiomyogenesis has not been determined. More specifically, we were interested in examining the importance of MEF2 factors during cardiomyoblast differentiation by using a dominant-negative MEF2 mutant (MEF2C/EnR), a strategy used in other studies to examine MEF2 activity (Karamboulas et al., 2006). MEF2C/EnR was created by replacing the C-terminal activation domain with the engrailed repressor domain. To study the role of MEF2 in cardiac-destined cells, the expression of MEF2C/EnR was driven by an *Nkx2-5* enhancer to direct expression in cardiomyoblasts. The targeted expression of MEF2C/EnR resulted in the loss of cardiomyogenesis in both P19 cells and transgenic mouse embryos. Therefore, MEF2, or genes containing MEF2 DNA-binding sites, are essential for the proper maintenance and differentiation of cardiomyoblasts.

Results

The expression of MEF2C/EnR in cardiomyoblasts inhibits cardiomyogenesis in P19 cells

To determine whether the expression of MEF2C/EnR inhibits the differentiation of cells destined to the cardiac lineage, we used the *Nkx2-5* enhancer (Lien et al., 1999) to drive expression of MEF2C/EnR in cardiomyoblasts. Stable cell lines, termed P19(Nkx-MEF2C/EnR), were aggregated with DMSO, and the cultures were fixed and stained with the anti-MyHC antibody MF20 after 6 days in culture. Myosin-heavy-chain-positive cells with the morphology of cardiomyocytes were observed in P19(control) but not in P19(Nkx-MEF2C/EnR) cultures (Fig. 1). Thus, targeting the expression of MEF2C/EnR to cardiomyoblasts inhibited their differentiation into cardiomyocytes.

The loss of cardiac muscle in the Nkx-MEF2C/EnR cultures was confirmed by examining the expression of cardiac muscle markers by northern blot analysis. Cardiac α-actin, *GATA4*, and *BMP4* (Fig. 2A-C) were not expressed in P19(Nkx-MEF2C/EnR) cell lines compared with control cells grown under cardiac-muscle-inducing conditions. The mesoderm markers Brachyury T and *Wnt5b* (Fig. 2D,E) were expressed in both P19(Nkx-MEF2C/EnR) and P19(control) cell lines, indicating that mesoderm induction occurred normally. The decrease in Brachyury T levels in P19(control) but

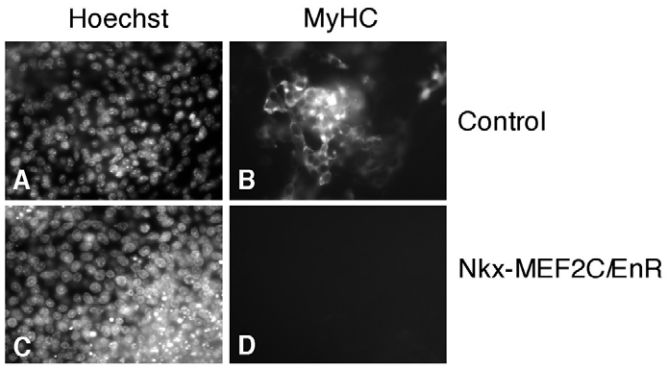

Fig. 1. (A-D) The expression of MEF2C/EnR in cardiomyoblasts inhibits the development of cardiac muscle. P19(Nkx-MEF2C/EnR) cells (C,D) and P19(control) cells (A,B) were aggregated with DMSO and fixed for immunofluorescence on day 6 of differentiation to examine the presence of cardiac muscle. Cells were stained with the anti-MyHC antibody MF20 (B,D), and nuclei were counter-stained with Hoechst dye 33258 (A,C) (Magnification 400×).

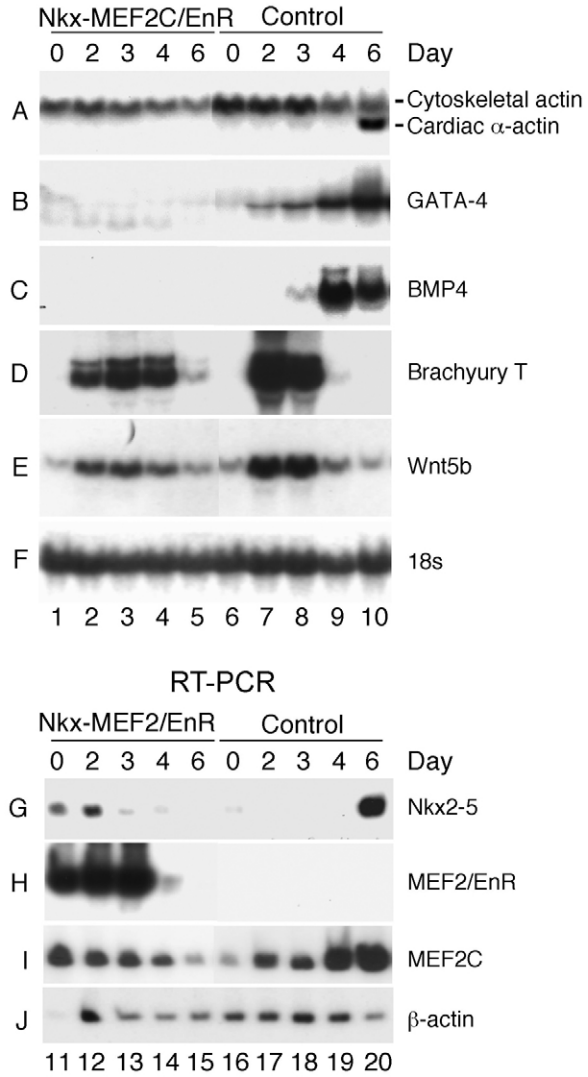

Fig. 2. MEF2C/EnR expression in cardiomyoblasts initially enhances Nkx2-5 and MEF2C transcript levels and subsequently downregulates cardiac α-actin, *Gata4*, *Bmp4*, *Nkx2-5* and *Mef2c* transcripts. (A-J) P19(Nkx-MEF2C/EnR) and P19(control) cells were differentiated in the presence of DMSO. 12 μg of total RNA that was harvested on days 0, 2, 3, 4 and 6 were northern blotted and probed as indicated (A-E). *Nkx2-5*, MEF2C/EnR, *Mef2c*, and β-actin expression were detected using RT-PCR (G-I). A 750 bp *Eco*RI fragment of rabbit 18S cDNA (F) or a 520 bp PCR product of mouse β-actin (J) were used as loading standards.

not P19(Nkx-MEF2C/EnR) cells is consistent with the differentiation of control, but not P19(Nkx-MEF2C/EnR), cells into cardiomyocytes. Therefore, the expression of MEF2C/EnR disrupted cardiomyogenesis after mesoderm induction.

The expression of MEF2C/EnR, endogenous MEF2C, and Nkx2-5 was not detected efficiently by northern blot analysis and therefore was examined by reverse transcriptase (RT)-PCR. *Nkx2-5* and endogenous *MEF2C* expression were downregulated on day 6 of differentiation in P19(Nkx-MEF2C/EnR) cells compared with control cells (Fig. 2G,I, lanes 15 and 20), further confirming the loss of cardiomyogenesis at this time point.

Low levels of *Nkx2-5* were detected in control P19 cells on day 0 (Fig. 2G, lane 16) as shown previously (Gianakopoulos and Skerjanc, 2005). In P19(Nkx-MEF2C/EnR) cells, *Nkx2-5*, MEF2C/EnR, *MEF2C* and *GATA4* (not shown) expression was upregulated between day 0 and 2 compared with control cells (Fig. 2G-J, respectively, lanes 11-13). MEF2C/EnR was expressed in a pattern similar to endogenous *Nkx2-5* in P19(Nkx-MEF2C/EnR) cells (Fig. 2H compared with G), indicating that the exogenous *Nkx2-5* enhancer was functioning in a similar fashion to endogenous *Nkx2-5*. Although P19(control) cells generally differentiate into cardiomyocytes that represent 15-20% of the cells, P19(Nkx-MEF2C/EnR) cells show an early enhancement of cardiomyoblast markers but a subsequent downregulation and inhibition of differentiation. This early enhancement of cardiomyoblast formation is consistent with our previous results showing a potential relief of class II histone deacetylases (HDACs) inhibition by MEF2C/EnR, because MEF2C/EnR can bind to HDAC4 (Courey and Jia, 2001; Karamboulas et al., 2006; Lu et al., 2000; Tolkunova et al., 1998). In summary, the expression of MEF2C/EnR by an Nkx-enhancer results in the overall inhibition of cardiomyogenesis with the downregulation of *Nkx2-5*, *GATA4*, *MEF2C* and *BMP4* expression.

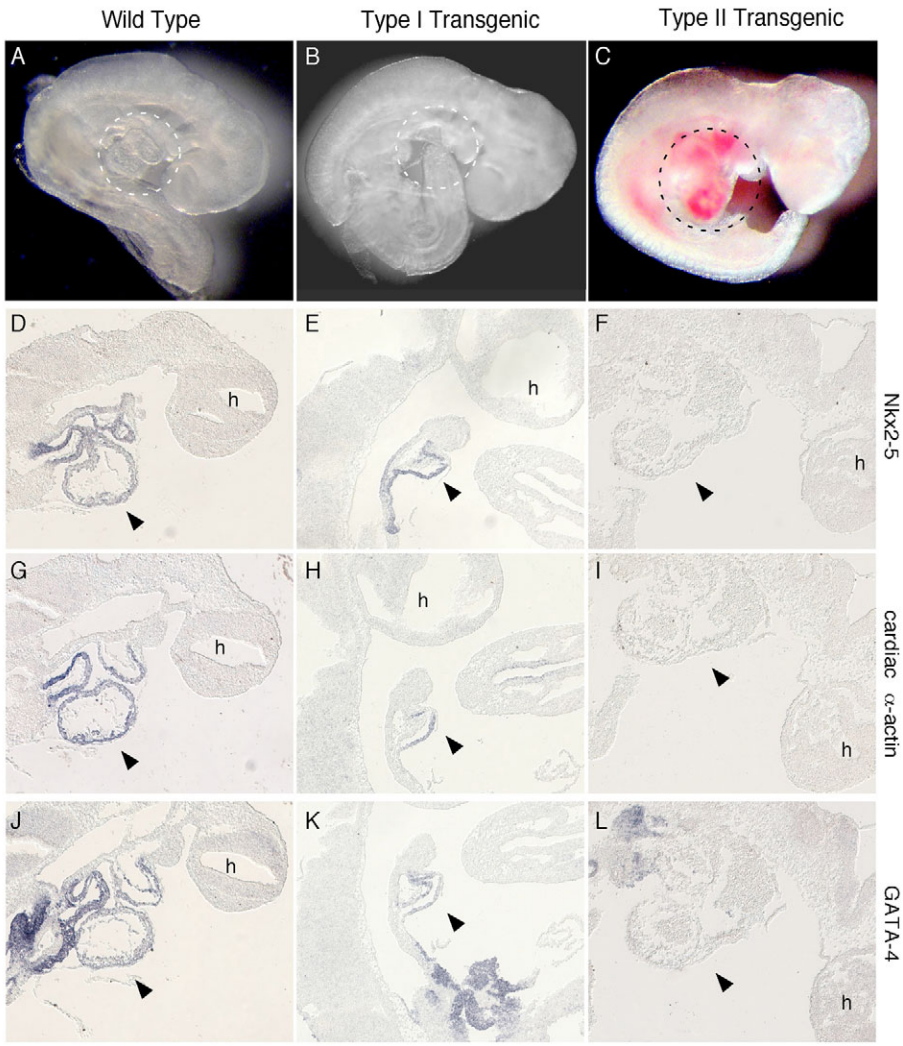

Fig. 3. MEF2C/EnR expressed via an *Nkx2-5* enhancer abrogated heart muscle development in type-I transgenic mouse embryos. Wild-type (A,D,G,J), type-I (B,E,H,K) and type-II (C,F,I,L) embryos were harvested on E9.5 and photographed under a dissecting microscope (A-C). The heart area is indicated by a circle. Cryosections were prepared and in situ hybridization was performed with probes against *Nkx2-5* (D-F), cardiac α-actin (G-I), and *Gata4* (J-L). The heart region (arrowhead) and head (h) are indicated.

The expression of MEF2C/EnR in cardiomyoblasts inhibits cardiomyogenesis in transiently transgenic embryos

To determine whether MEF2C/EnR can inhibit cardiomyogenesis during murine embryogenesis, transiently transgenic mouse embryos were generated by injecting the Nkx-MEF2C/EnR DNA into one-cell zygotes. Embryos were harvested between E7.5 and E10.5 and genotyped by PCR analysis of yolk sac DNA. A total of 46 transgenic embryos was identified in this manner. Of these, 26 were either non-viable or were harvested too early (<E6.5) owing to the difficulty of correctly timing the rate of development of transiently transgenic embryos. Two distinct phenotypes were observed for the viable transgenic embryos. Three transgenic embryos were obtained that exhibited a severe loss of heart structure formation (type-I embryos). Six transgenic embryos displayed enlarged and thin-walled hearts (type-II embryos). Eleven transgenic embryos were wild type in appearance, presumably due to the lack or low levels of expression of the transgene.

Of the three type-I embryos, two showed complete absence of cardiac muscle (data not shown), and one embryo contained a small heart remnant (Fig. 3B) compared with wild-type heart (Fig. 3A). A representative type-II embryo is shown in Fig. 3C. Blood was pooling in the heart, which was larger than that of a corresponding wild-type heart.

Embryos were cryosectioned and in situ hybridization was performed to detect *Nkx2-5* (Fig. 3D-F), *cardiac α-actin* (Fig. 3G-I), and *GATA4* (Fig. 3J-L) transcripts. In the type-I transgenic embryos, structures resembling the atrial and ventricular chambers were absent compared with wild-type littermates. In the type-I transgenic embryo that had the most cardiac muscle, a small region next to the pharynx expressed *Nkx2-5*, *GATA4*, and *cardiac α-actin* transcripts (Fig. 3E,H,K compared with D,G,J), possibly indicating incomplete penetrance of transgene expression. All three type-I embryos exhibited severe defects in cardiomyoblast differentiation, proliferation and heart chamber maturation. Expression of the transgene could not be detected after E9 by in situ hybridization with an engrailed ribonucleotide probe, although this probe could detect endogenous *En2* expression in the brain, and heart-muscle-specific expression of *En2* in E8.5 transgenic embryos (data not shown). This is probably due to the downregulation of the enhancer *Nkx2-5* itself by expression of MEF2C/EnR, as shown in vitro between day 4 and 6 in differentiating P19 cells (Fig. 2H, lanes 14 and 15), resulting in the subsequent loss of MEF2C/EnR expression.

In the type-II transgenic embryos, cryosectioning revealed a large area of heart tissue that did not stain by in situ hybridization, presumably because of cell death and degradation of the RNA transcripts (Fig. 3F,I,L). The thinness of the heart wall in type-II transgenic embryos was indicated by immunofluorescence with an anti-MyHC antibody, MF20 (Fig. 4). MyHC staining indicates multiple layers of cardiomyocytes in wild-type embryos (Fig. 4D) compared with the heart in type-II transgenic mice that is frequently only a single layer of cells (Fig. 4F). In one type-I transgenic embryo, a small region of MyHC staining was detected (Fig. 4E), the other two did not express MyHC (data not shown).

In summary, the disruption of MEF2 function in the developing embryonic heart leads to two major phenotypes. The type-I phenotype displays a severe loss of heart formation and the type-II phenotype shows a relatively normal heart morphology but with a thin-walled myocardium, leading to cardiac insufficiency.

Discussion

Targeting MEF2C/EnR to cardiomyoblasts with an *Nkx2-5* enhancer resulted in the loss of cardiomyogenesis and the downregulation of *Gata4*, *Nkx2-5*, *Bmp4* and cardiac α-actin in P19 cells. Mesoderm induction, indicated by expression of Brachyury T and *Wnt5b*, was unaffected. Therefore, disruption of MEF2 function at the cardiomyoblast stage abrogated P19 cell differentiation into cardiomyocytes. Furthermore, type-I transgenic mice expressing MEF2C/EnR driven by an *Nkx2-5* enhancer displayed a severe lack of heart structure formation, indicating differentiation, proliferation and heart-chamber maturation defects. These results support the presence of a regulatory loop between GATA4, Nkx2-5 and MEF2C, which is disrupted by MEF2C/EnR leading to a loss of cardiomyoblast maintenance and differentiation (Fig. 5).

The strategy of using a dominant-negative MEF2, fused to the EN2 repressor domain, has produced a more severely impaired heart phenotype than the loss of a single MEF2 family member. Mice lacking MEF2B or MEF2C developed heart muscle but, in the latter case, the mice died between E9.5 and E10.5, exhibited defective looping morphogenesis, and a subset of cardiac muscle-specific genes were downregulated (Lin et al., 1997). In addition, mice lacking MEF2C displayed severe vascular abnormalities, similar to mice lacking the vascular-specific endothelial-cell growth factor VEGF or its receptor Flt-1 (Bi et al., 1999; Lin et al., 1998). Interestingly, late-targeted deletion of MEF2C in hearts after looping morphogenesis resulted in viable mice, indicating that MEF2C does not have an essential role for cardiomyogenesis at later stages (Vong et al., 2005). In the targeted dominant-negative approach used here, the role of MEF2 factors in the heart has been examined in the absence of vascular defects. Furthermore, the functional redundancies and compensatory mechanisms occurring in the gene deletion studies are bypassed by the active dominant-negative approach. However, we cannot conclude which MEF2 family member may be involved and do not know what proportion of total gene transcription is regulated by endogenous MEF2 as opposed to the other cardiac muscle transcription factors, because of the remodeling of chromatin structure by EnR. Furthermore, we cannot rule out

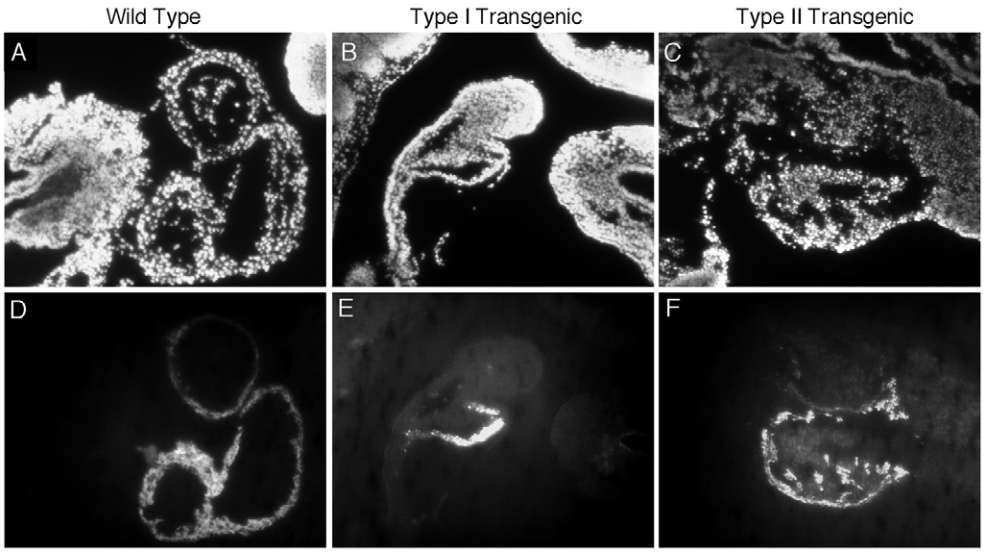

Fig. 4. MEF2C/EnR expressed via an *Nkx2-5* enhancer resulted in a thin-walled myocardium in type-II founder transgenic mouse embryos. Immunofluorescence was performed with anti-MyHC antibody MF20 (D,E,F) to indicate the cardiomyocytes in sections from wild-type (A,D), type-I (B,E) and type-II (C,F) transgenic embryos. Sections were stained with Hoechst dye 33258 to indicate nuclei (A-C).

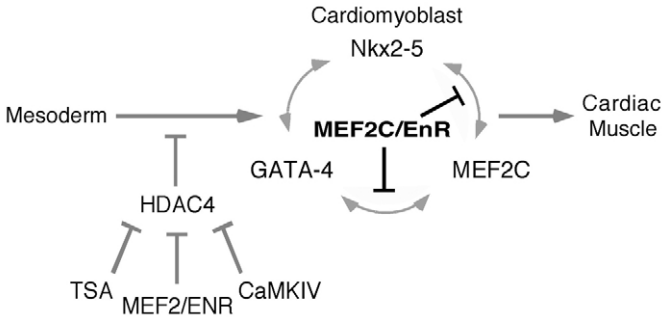

Fig. 5. Mechanistic model for the effect of MEF2 disruption on the differentiation of cells into cardiac muscle. In the cardiomyoblast, MEF2C, GATA4 and Nkx2-5 are important for the maintenance of the cardiomyoblast phenotype and for subsequent differentiation into cardiomyocytes (Dodou et al., 2004; Grepin et al., 1997; Jamali et al., 2001; Reecy et al., 1999; Searcy et al., 1998; Skerjanc et al., 1998). The presence of MEF2C/EnR downregulated the expression of genes encoding Nkx2-5, MEF2C and GATA4, and inhibited the progression of cardiomyoblasts into cardiomyocytes. An initial enhancement of Nkx2-5 and MEF2C in the stem/mesoderm cell confirmed previous results showing an increase in *Nkx2-5* and *Gata4* expression, probably due to the relief of HDAC inhibition of cardiomyoblast formation by MEF2C/EnR (Karamboulas et al., 2006).

that non-specific dominant-negative effects have contributed to the observed phenotype. However, our previous experiments with dominant-negative fusions to the engrailed repressor have each identified a specific phenotype, which appeared logical for the transcription factor examined (Petropoulos et al., 2002; Ridgeway and Skerjanc, 2001; Petropoulos et al., 2004; Jamali et al., 2001).

It is unlikely that the observed effects were due to the expression of the engrailed repressor domain itself. We had previously overexpressed the engrailed region alone, driven by the *Pgk1* promoter, in P19 cells and had seen no effect on cardiomyogenesis (see Jamali et al., 2001). Others have shown that injecting the engrailed repressor domain into one cell of two-cell-stage *Xenopus* embryos had little effect on heart development, compared with injecting Nkx2.3 or Nkx2.5-engrailed repressor domain fusion constructs (Fu et al., 1998). Furthermore, the expression of GATA4-engrailed, but not the engrailed repressor alone, blocked GATA4- and GATA6-directed transcriptional responses and agonist-induced cardiomyocyte hypertrophy in cultured rat primary cardiomyocytes (Liang et al., 2001). Finally, ectopic expression of an engrailed repressor fused to *Pitx2c*, but not *Pitx2a*, to the left lateral plate mesoderm randomized heart looping in chick embryos, indicating that the engrailed repressor domain was probably not responsible for the phenotype observed (Yu et al., 2001). Therefore, it is unlikely that our results are owing to the expression of engrailed alone.

Three type-I transgenic embryos were obtained with a substantial loss of heart structures, including atria and ventricles. Of the three, only one embryo showed some cardiac α-actin or Nkx2-5 expression by in situ hybridization (Fig. 3). This small variability in phenotype is probably caused by differences in transgene expression due to copy number and position effects at the site of transgene insertion. Six type-II transgenic embryos with a thin-walled myocardium were also obtained. Presumably, the transgene expression was weak or delayed, allowing heart formation to occur, but subsequent expression of MEF2C/EnR greatly decreased the extent of myogenesis in the myocardium. Numerous other cardiac mutant embryos display a similar thin-walled myocardium, including mice lacking HOP (Chen et al., 2002; Shin et al., 2002), Nkx2-5 (Lyons et al., 1995), dHAND (Srivastava and Olson, 1997), and TEF-1 (Chen et al., 1994).

Nkx2-5 expression is first initiated at E7.5 (Lien et al., 1999). Transgene expression could be observed in embryos between E7.5 and E8 (data not shown), the earliest time points examined. However, transgene expression was not observed in later-stage embryos (data not shown) although the *Nkx2-5* enhancer should be still active. The lack of transgene expression in the type-1 transgenic embryos may be explained by the downregulation of the *Nkx2-5* enhancer by MEF2C/EnR, as seen in the P19(Nkx-MEF2C/EnR) cell lines (Fig. 2H). This downregulation is probably reinforced by the inhibition of the positive regulatory loop between Nkx2-5, GATA4 and MEF2C (Dodou et al., 2004; Grepin et al., 1997; Jamali et al., 2001; Lien et al., 1999; Searcy et al., 1998; Skerjanc et al., 1998).

Our results, showing a loss of cardiomyogenesis in P19(Nkx-MEF2C/EnR) cells, parallel the results in transgenic embryos expressing *Nkx2-5*-promoter-driven MEF2C/EnR. Previous studies have indicated similar results when comparing these two systems. For example, mice lacking both *Meox1* and *Meox2* did not express *Pax3*, *Myf-5* and myogenin, resulting in defective skeletal myogenesis (Mankoo et al., 2003). A similar phenotype was observed in P19 cells expressing a mutant Meox-EnR fusion protein (Petropoulos et al., 2004). In addition, Wnt11 can induce cardiomyogenesis in *Xenopus* embryonic explants and also in P19 cells (Pandur et al., 2002), indicating that developmental pathways can be conserved from lower vertebrates to murine embryonal carcinoma cells.

We have demonstrated that MEF2C/EnR can inhibit cardiomyogenesis when targeted to cardiomyoblasts. The early increase in expression of Nkx2-5, GATA4, MEF2C, and MEF2C/EnR in the P19(Nkx-MEF/EnR) cell lines was initially unexpected. However, a low level of Nkx2-5 expression was seen in P19(Control) cells on day 0 (Fig. 2) and in previous studies (Gianakopoulos and Skerjanc, 2005). Furthermore, another article by us published in this issue (Karamboulas et al., 2006) suggests that MEF2C/EnR can relieve the inhibition of HDAC on the mesoderm to enhance the formation of cardiomyoblasts. Therefore, the results presented here further support a repressive role for HDAC in cardiac muscle specification.

In summary, our results show that MEF2, or genes containing MEF2 DNA-binding sites, are essential for the maintenance and differentiation of cardiomyoblasts. This is probably due to the formation of a positive regulatory loop between MEF2C, Nkx2-5 and GATA4. Since flies lacking the single *Mef2* gene also showed defective differentiation of cardiomyoblasts (Lilly et al., 1995), our results suggest a conservation of MEF2 function from the fly to mammals.

Materials and Methods

Plasmid constructs

The dominant-negative MEF2C/EnR mutant was created by fusing the MEF2 C-terminus, containing the DNA-binding domain, to the mouse engrailed (EN2)

protein repressor domain as previously described (Karamboulas et al., 2006). The Nkx-MEF2C/EnR construct was designed by replacing the *Pgk1* promoter of the MEF2C/EnR-2 construct (*Eco*RI-*Bam*HI) (Karamboulas et al., 2006), with a 2.2 kb *Eco*RI-*Kpn*I fragment containing the 500-bp long and the 1.7-kb long *Nkx2.5* enhancer regions (Lien et al., 1999).

Cell culture and DNA transfections

P19 cells were cultured in αD-minimum essential media (α-MEM) supplemented with 5% cosmic calf serum (Hyclone, Logan, Utah) and 5% fetal bovine serum (CanSera, Rexdale, Ontario), as described (Wilton and Skerjanc, 1999). Stable cell lines were isolated 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 Nkx-MEF2C/EnR, transfections were performed using the FuGENE^{TM} 6 transfection system according to the manufacturer's protocol (Roche Molecular Biochemicals, Laval, QC, Canada) with 2.04 μg Nkx-MEF2C/EnR, 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 vector construct.

Stable P19(Nkx-MEF2C/EnR) clonal populations were chosen by their low expression of MEF2C/EnR transcripts and their high content of MEF2C/EnR DNA. All experiments were performed three times, with at least two clonal populations for each cell line.

Aggregation of P19 cells in the presence of DMSO induces differentiation of cardiomyocytes by day 6 in culture (Skerjanc, 1999). P19(Control) and P19(Nkx-MEF2C/EnR) stable cell lines were aggregated in the presence of 0.8% DMSO for 4 days and then plated into tissue culture dishes in the absence of drug (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). RNA was harvested on the days indicated and cells were fixed and examined by immunofluorescence on day 6.

Immunofluorescence and northern blot analysis

Cells were plated onto gelatin-coated coverslips and on day 6 were fixed in –20°C methanol for 5 min and then rehydrated with PBS for 15 min at room temperature. The mouse anti-MyHC monoclonal antibody supernatant, MF20 (Bader et al., 1982) was used to detect the expression of myosin heavy chain as described previously (Ridgeway and Skerjanc, 2001; Ridgeway et al., 2000b). Briefly, 100 μl of anti-MyHC/PBS (1:1) was incubated for 1 hour at room temperature. Cells were washed with PBS and then 100 μl of a goat anti-mouse IgG(H+L) Cy3-linked antibody, anti-Cy3/PBS (1:100) (Jackson Immunoresearch Laboratories, PA) was incubated for 1 hour at room temperature. After washes with PBS, coverslips were mounted and immunoflourescence was visualized using a Zeiss Axioskop microscope as described previously (Ridgeway et al., 2000a; Ridgeway and Skerjanc, 2001).

For Northern blot analysis, total RNA was isolated from differentiated P19(Control) or P19(Nkx-MEF2C/EnR) cultures at the times indicated and analyzed as described previously (Ridgeway et al., 2000a). Total RNA (12 μg) was separated on a 1% agarose-formaldehyde gel, transferred to Hybond-N (Amersham Pharmacia Biotech), and crosslinked by UV irradiation. The blots were hybridized with DNA probes labeled with (α-^{32}P)dCTP using a multiprime labeling kit (Amersham Pharmacia Biotech).

The DNA probes cardiac α-actin, GATA4, MEF2C, Nkx2-5, Brachyury T, BMP4, Wnt 5b, β-actin and 18s (for standardization of loading) have been previously described (Gianakopoulos and Skerjanc, 2005; Ridgeway and Skerjanc, 2001). In addition, a 597 bp PCR product of MEF/EnR using the following oligonucleotides: 5′ primer taatggatgagcgtaacagacagg; 3′ primer acatcgcatcaccaacttcttcat., and a 520 bp PCR product of β-actin using the primers described below.

Reverse transcription PCR

The conditions used for reverse transcription polymerase chain reaction were described previously (Karamboulas et al., 2006). For PCR amplification, the conditions and pairs of primers used for *Nkx2-5*, *GATA4*, *MEF2C* and *β-actin* were described previously (Karamboulas et al., 2006). In addition, oligonucleotides and conditions used for *MEF2C/EnR* include: 5′ primer taatggatgagcgtaacagacagg and 3′ primer atgaagaagttggtgatgcgatgt with an annealing temperature at 60°C (2 min), 26 cycles.

Generation and genotyping of transiently transgenic founder embryos

The Nkx-MEF/EnR fragment was isolated by digestion with *Eco*RI-*Bgl*II followed by 1% agarose gel electrophoresis and QIAEX gel extraction kit (Qiagen Inc., Canada) purification. The purified DNA was microinjected at a concentration of 1.5 ng/μl into the pronucleus of fertilized eggs derived from matings of C57BL/6-C3H F1 mice. After injection, the eggs were cultured to the two-cell stage and transferred into the oviduct of pseudo-pregnant CD-1 female mice. Mice were sacrificed from 8.5d to 10.5d and the embryos were harvested, photographed, fixed and cryosectioned for in situ hybridization. Yolk sacs were frozen for genotyping. Stable founder mice were not obtained because the phenotype was predicted to be lethal (Lin et al., 1997; Vong et al., 2005).

DNA was isolated from the yolk sac by proteinase K digestion. PCR to amplify MEF2C/EnR and β-actin was performed with the primers described above. PCR products were subjected to agarose gel electrophoresis, transferred to Hybond-N and hybridized to a radioactively labeled MEF2C/EnR or β-actin fragment, using protocols described above. DNA from yolk sacs was subjected to PCR three times for each sample.

In situ hybridization

Embryos were fixed overnight in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and subjected to an increasing gradient of sucrose-PBS (10-30%). Embryos were embedded in sucrose-OCT (Tissue-Tek), stored at –80°C, and cryosectioned (10-12 mm) on the day of the experiment. In situ hybridization was performed according to Wallace and Raff (Wallace and Raff, 1999). Briefly, the sections were air-dried for at least 2 hours before overnight hybridization in a moist chamber with a specific ribonucleotide probe (diluted 1:1000) at 65°C. Following the usual stringency washes, slides were treated with an alkaline-phosphatase-conjugated antidigoxigenin antibody and stained in Nitro Blue tetrazolium/5-bromo-4-chloro-3-indoylphosphate. Templates of *Nkx2-5*, *En2*, *GATA4* and cardiac α-actin were in vitro transcribed to generate the respective digoxigenin-labeled antisense riboprobes.

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