Requirement of transcription factor NFAT in developing atrial myocardium

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Nuclear factor of activated T cell (NFAT) is a ubiquitous regulator involved in multiple biological processes. Here, we demonstrate that NFAT is temporally required in the developing atrial myocardium between embryonic day 14 and P0 (birth). Inhibition of NFAT activity by conditional expression of dominant-negative NFAT causes thinning of the atrial myocardium. The thin myocardium exhibits severe sarcomere disorganization and reduced expression of cardiac troponin-I (cTnI) and cardiac troponin-T (cTnT). Promoter analysis indicates that NFAT binds to and regulates transcription of the cTnI and the cTnT genes. Thus, regulation of cytoskeletal protein gene expression by NFAT may be important for the structural architecture of the developing atrial myocardium.

Introduction

The heart is the first organ to develop after completion of gastrulation, which occurs at around embryonic day (E)* 7.5 (Kaufman, 1992; Harvey and Rosenthal, 1999; Kaufman and Bard, 1999). During early morphogenesis, endocardium and myocardium, in conjunction with a middle layer of “cardiac jelly,” form a simple heart tube. Subsequent folding, remodeling, and septation turn the linear heart tube into a four-chambered heart. These processes involve multiple transcription factors and signaling pathways (Sucov, 1998; MacLellan and Schneider, 2000; Srivastava, 2001). Later cell proliferation, wall thickening, and trabeculation of the myocardium lead to a mature, functional heart. However, molecular processes that are involved in late morphogenesis of heart maturation remain to be identified.

The transcription factor, nuclear factor of activated T cells (NFAT), was first identified as an important regulator for interleukin (IL)-2 gene expression (Durand et al., 1988). Subsequent reports indicate that NFAT is involved in multiple biological processes, including cardiac and skeletal muscle hypertrophy, adipocyte differentiation, and memory and plasticity (Ho et al., 1998; Molkentin et al., 1998; Graef et al., 1999; Yang et al., 2002). To understand NFAT functions, dominant-negative NFAT (dnNFAT) inhibitors have been generated (Northrop et al., 1994; Aramburu et al., 1998; Chow et al., 1999; Miskin et al., 2000; Saneyoshi et al., 2002). The dnNFAT interferes with targeting of calcineurin without affecting the phosphatase catalytic activity. Transgenic expression of dnNFAT in the thymus blocks endogenous NFAT functions, such as IL-2 expression (Chow et al., 1999). Therefore, dnNFAT allows examination of the consequences of reduced NFAT activity in physiologically relevant models.

Previous papers have shown that targeted disruption of NFATc1 impairs heart valve and septum formation, and hence causes embryonic lethality (de la Pompa et al., 1998; Ranger et al., 1998). Heart valves are originated from endocardial cushion tissue (Harvey and Rosenthal, 1999; Kaufman and Bard, 1999), and immunofluorescence analysis has shown that NFATc1 is expressed transiently in this tissue. These reports indicate a specific spatial and temporal requirement for NFATc1 function. Although these reports are relevant for understanding one role of NFAT in heart development,
functions of NFAT in other cell types that are also important for heart function remain elusive. For example, cardiac myocytes are critical for maintaining the structural integrity of the heart and generating mechanical force for circulation. In diseased hearts, cardiac myocytes respond to an increased demand for output and become hypertrophic; a common early sign of heart failure. NFAT has been demonstrated to play an important role in cardiac hypertrophy (Molkentin et al., 1998; Sussman et al., 1998; Rothermel et al., 2001; Zou et al., 2001). Thus, the role of NFAT in myocardium must be thoroughly examined because it may provide knowledge and insights into new drugs and therapies for conditions associated with hypertrophy and cardiac dysfunction.

The purpose of this paper is to examine the role of NFAT in developing cardiac myocytes. Here, we report that there is a temporal requirement of NFAT in the developing myocardium. Inhibition of NFAT causes a severe defect in the cytoskeletal structure and reduced expression of cardiac troponin-I (cTnI) and cardiac troponin-T (cTnT). Thus, NFAT is required for proper cardiac myocyte development.

Results
Subcellular distribution of NFAT in developing myocardium

To investigate the role of NFAT in the developing myocardium, we examined the subcellular distribution of NFAT (Fig. 1 A). Immunofluorescence analysis revealed that NFAT was located in the cytoplasm and was not detected in the nucleus of the developing cardiomyocytes at E14. At E16, NFAT was detected in both the nucleus and the cytoplasm. At E18, staining of nuclear NFAT was diminished, suggesting nuclear export has begun. At P0, NFAT was not detected in the nucleus. Quantitation of nuclear intensity also revealed temporal NFAT nuclear localization (Fig. 1 B). These data indicate that NFAT is translocated into the nucleus after E14 and exported by P0.

Expression of dnNFAT blocks NFAT nuclear localization

To examine whether NFAT regulates gene expression in developing cardiomyocytes, we have generated heart-specific, tetracycline-inducible dnNFAT transgenic mice (Fig. 2 A). The α-myosin heavy chain (MHCα) promoter was used to express the reverse tetracycline regulator (a tet-on system) because the spatial and temporal expression of MHCα in the developing and adult heart is well characterized (Lyons et al., 1990; Ng et al., 1991; Subramaniam et al., 1991; Buckingham et al., 1992). The MHCα promoter drives strong expression of the transgene in the developing atria and, to a lesser extent, in the developing ventricles. The tet-on inducible system was chosen because the kinetics for transgene induction is more responsive as compared with the tet-off scheme (Kistner et al., 1996; Gingrich and Roder, 1998). Thus, the temporal requirement of NFAT in developing atrial myocardium would be closely represented.

To induce expression of dnNFAT, doxycycline (Dox; a potent analogue of tetracycline)-treated water was given to transgenic-positive (Tg+) mice and wild-type (Wt) non-transgenic littermate controls. Administration of Dox induced expression of dnNFAT in Tg+, but not Wt, mice (Fig. 2 A). Importantly, dnNFAT was detected only in the presence of Dox. Immunofluorescence analysis further revealed that administration of Dox induced dnNFAT expression and blocked NFAT nuclear accumulation in the developing atria (Fig. 2, B and C). However, low expression of dnNFAT in the developing ventricles was not sufficient to block NFAT nuclear localization. These data indicate that conditional expression of dnNFAT provides an effective means to examine the temporal requirement of NFAT in the developing atrial myocardium.
Figure 2. Expression of dnNFAT blocks NFAT nuclear localization. (A) Schematic representation of constructs for the transgene creation. Expression of tetracycline regulator (rtTA) is driven by the heart-specific α-MHC promoter. Expression of FLAG epitope-tagged dnNFAT is regulated by the Tet-responsive promoter. Co-injection of both constructs generates heart-specific, tet-inducible expression of dnNFAT. Expression of dnNFAT in Wt and Tg hearts, in the presence (+) or absence (−) of Dox, was examined by immunoblotting analysis using the M2 mAb that recognizes the FLAG epitope. (B and C) Subcellular distribution of NFAT in Wt and Tg+ E16 heart was examined by immunofluorescence analysis. Expression of dnNFAT (blue) in Wt and Tg+ atria (A), in the presence (+) or absence (−) of Dox, was examined using the M2 mAb. Co-localization (yellow) of NFAT in the nuclei (arrowheads) was observed in Wt atria (B). Expression of dnNFAT blocks nuclear translocation, and thus separated staining of NFAT (green) and the nuclei (red) was observed in Dox-treated Tg+ atria. Localization of NFAT and the expression of dnNFAT in the ventricles (V) were also shown. The relative intensity of nuclear NFAT in Wt and Tg+ atria are presented (C). Bars, 30 μm.
Inhibition of NFAT causes thinning in the developing myocardium

To examine the role of NFAT in the developing myocardium, timed-pregnant mice were given Dox-treated or untreated plain water starting on E0.5, continuing until the embryos were harvested. Histological analysis of E16 atria revealed thinning in the atrial walls of Tg embryos as compared with their Wt littermates (Fig. 3A). Dox-treated Tg atria exhibited thinner atrial walls as compared with their Wt littermates. Enlarged images to illustrate thinning of atrial walls were shown (B). RA, right atrium; LA, left atrium. Bars: low magnification, 200 μm; high magnification, 60 μm. Administration of cyclosporin A (CsA) disrupts myocardium development. Wt pregnant mice were given CsA daily starting on E14. Control and CsA-treated embryos were harvested on day of birth (P0). Sagittal sections of developing atria and ventricles were shown. (D and E) Expression of calcineurin (Cn) attenuates thinning of the atrial walls in dnNFAT mice. Heterozygous Tg male mice expressing dnNFAT were crossed with Tg female mice expressing calcineurin. Dox-treated embryos were harvested on E16 and day of birth (P0). Immunofluorescence analysis indicated localization of NFAT in the nuclei (yellow) in calcineurin-expressing hearts (D). Histological analysis revealed that expression of calcineurin reduced thinning of myocardium in dnNFAT-expressing hearts (E). Bars: E16 hearts, 40 μm; P0 hearts, 75 μm.

The inhibitory effect of dnNFAT is mediated by suppression of calcineurin-mediated nuclear translocation of NFAT (Aramburu et al., 1998; Chow et al., 1999; Miskin et al., 2000). Previous reports established that expression of calcineurin causes cardiac hypertrophy and sudden death (Molkentin et al., 1998). We tested whether expression of calcineurin rescues thinning of the myocardium in dnNFAT embryos. Heterozygous Tg male mice expressing dnNFAT were crossed with Tg female mice expressing calcineurin. Immunofluorescence analysis indicated that expression of calcineurin in dnNFAT hearts reduced thinning of myocardium (Fig. 3D). Expression of calcineurin rescued thinning of the myocardium in dnNFAT hearts. We further examined the role of NFAT in the developing myocardium by determining the atrial wall thickness of Dox-treated, Tg hearts that were harvested at various embryonic stages (Fig. 4A). Tg hearts harvested at E14 exhibited similar wall thickness and appearance as their Wt littermates. However, Tg hearts harvested at E16...
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showed reduced atrial wall thickness. Tg+ hearts harvested at E18 or P0 also exhibited reduced atrial wall thickness. In the absence of Dox, E16 Tg+ and Wt hearts exhibited similar wall thickness and appearance. Morphometric analysis to determine the atrial wall thickness also indicated a decrease in the Dox-treated Tg+ atria as compared with their Wt littermates (Fig. 4 B). Unlike the atria, Tg+ and Wt ventricles exhibited similar wall thickness (Fig. 4 C). Together, these data demonstrate that there is a reduction in the myocardium in hearts expressing dnNFAT after E14.

We further examined the temporal requirement for NFAT at the later stages of the developing myocardium (Fig. 5 A). Pregnant mice were exposed to Dox from E0 to E8 and then given untreated, plain water from E8 to E16. Tg+ and Wt hearts, which were harvested on E16, exhibited similar atrial wall thickness and appearance. However, E16 Tg+ hearts that were exposed to Dox from E8 to E16 exhibited thinner atrial walls as compared with their Wt littermates. When Dox was withdrawn at E14 (i.e., Dox treatment from E8 to E14), thinning of atrial walls was not observed on Tg+ hearts harvested on E16. These data indicate that NFAT is required at the later stages of myocardium development.

Next, we determined the requirement of NFAT between E14 and P0 (Fig. 5 B). We examined whether administration of Dox after E14, when NFAT has already translocated into the nucleus (Fig. 1), causes thin myocardium. Tg+ embryos exposed to Dox from E16 to P0 or from E16 to E18, exhibited thinner atrial walls as compared with their Wt littermates. These data indicate that NFAT inhibition also causes thin myocardium after E16.

We also determined whether thinning of the myocardium is reversible. When Tg+ embryos were exposed to Dox for two days from E14 to E16 and harvested at E18, their atrial walls were thinner than the E18 Wt littermates (Fig. 5 B). Newborn (P0) Tg+ pups that were exposed to Dox from E16 to E18 exhibited thinner atrial walls as well. Therefore, thinning of the myocardium is not reversible. Together, these data demonstrate that NFAT is required continuously between E14 and P0 for myocardium development.

Cell viability of the thin myocardium

Next, we examined whether apoptosis plays a role in the thinning of the myocardium (Fig. 6 A). TUNEL assays were performed to detect apoptotic cells from Dox-treated, E14 and E16, Tg+ and Wt hearts. No apoptotic nuclei could be detected in either Tg+ or Wt atria at E14 or E16. Control showed an intense staining of nuclei with fragmented DNA. In addition, the number of nuclei found in Tg+ and Wt atria was similar (Fig. 6 B). Thus, thinning of the myocardium in Tg+ heart is not due to the loss of cardiac myocytes.

Cytoarchitecture of the thin myocardium

Thinning of the myocardium may be due to defects in cytoskeletal and sarcomeric structure. Ultrastructural studies revealed classic sarcomeric pattern with layers of mitochondria between bands of connected myofilaments in the Wt myocytes (Fig. 7). Periodic patterning of M and Z lines generated from overlapping thick and thin filaments was also observed. However, the cytoarchitecture of Tg+ cardiac myocytes was disrupted, in addition to the reduction in cell thickness. There were varying degrees of sarcomere disorganization, ranging from misalignment of fiber bundles and random orientation of sarcomeres to complete loss of sarcomere and mitochondrial patterning and disappearance of Z lines. In the most severe case, only scattered individual filaments were observed. These data indicate that the structural components of the myocardium are severely disrupted in dnNFAT hearts.

Expression of cytoskeletal and sarcomeric proteins in Tg+ and Wt hearts

Disruption of sarcomere formation indicated that the structural components of cardiac myocytes are altered. We examined whether the expression level of cytoskeletal and sarcomeric proteins were affected in Tg+ hearts (Fig. 8). Hearts from multiple litters of Dox-treated embryos were harvested...
at P0. Isolated hearts were further micro-dissected to separate the atria from the ventricles. Whole-cell extracts generated from the pooled samples were subjected to immunoblotting analysis. The expression level of vinculin, desmin, α-actinin, actin, α-actinomyosin, and caveolin-3 was similar in Wt and Tg atria (Fig. 8 A). The expression level of these proteins in ventricles was also similar. Thus, these proteins do not account for the disruption of sarcomere formation.

Next, we examined the expression level of the members of the troponin complex (Fig. 8 B). Immunoblot analysis indicated that tropomyosin and troponin-C expression levels were similar in Tg+ and Wt atria and ventricles. The expression of slow skeletal troponin-I (sSTnI) and slow skeletal troponin-T (sSTnT) were also similar. However, the expression level of cTnI and cTnT was reduced in the Tg+ atria as compared with the Wt control. The expression level of cTnI and cTnT was similar in Tg+ and Wt ventricles. These data indicate that reduced expression of specific members of the troponin complex may, in part, account for the disruption of myocytes cytoarchitecture.

NFAT binding elements are present in the cTnI and the cTnT genes

Reduced cTnI and cTnT protein expression suggested that NFAT might directly regulate transcription of these genes. Sequence analysis indicated that there are putative NFAT binding elements in the regulatory regions of the cTnI and cTnT promoters (Fig. 9 A). The cTnI NFAT site is located 300 bp upstream of the transcriptional start site. The cTnT NFAT sites are present in the first intron, which is located between the 5′-untranslated region (exon 1) and the ATG translational start site (exon 2). We performed electrophoretic mobility shift assays to test whether these putative binding elements interact with NFAT. Electrophoretic mobility shift assays revealed formation of specific NFAT–DNA complexes (Fig. 9 B). Incubation with NFAT anti-
body either abolished formation or caused supershift of the NFAT–DNA complexes. These data indicate that NFAT binds to the cTnI and cTnT gene promoters.

Further, we performed competition analysis to examine NFAT binding on cTnT and cTnI NFAT elements. Excess amount of the Wt (but not the mutated) NFAT binding elements abolished formation of NFAT–DNA complexes (Fig. 9 C). Canonical NFAT binding elements from the IL-2 and the peroxisome proliferator–activated receptor γ2 (PPARγ2; proximal and distal) genes also reduced, although to different extents, formation of cTnT or cTnI NFAT–DNA complexes (Fig. 9 D). Previous reports indicated that the canonical NFAT element from the IL-2 gene binds NFAT and AP-1 (Fos and Jun) while C/EBP is present in the PPARγ2 proximal NFAT site. The different degree of competition on the cTnT site 1, cTnT site 2, and cTnI NFAT elements suggested formation of distinctive NFAT complexes. Thus, different NFAT complexes may bind to the cTnT and cTnI genes.

We also examined NFAT regulation of the cTnI gene in different species. Sequence alignment indicated that similar NFAT-binding elements are located in the human, rat, and mouse cTnI genes (Ausoni et al., 1994; Bhavsar et al., 1996, 2000; Murphy et al., 1997; Di Lisi et al., 1998; Fig. 9 A). Importantly, the sequence similarity extends to the adjacent nucleotides, suggesting interaction with a comparable NFAT partner for regulating cTnI gene expression. Electrophoretic mobility shift assays revealed cross competition among the different cTnI NFAT elements (Fig. 9 E). Competition with other canonical NFAT elements also reduced formation of the mouse cTnI NFAT complex to different degrees. These data indicate similar regulation of the cTnI gene transcription by NFAT.

NFAT regulates the cTnI and the cTnT gene promoters

Next, we performed luciferase reporter gene assays to examine whether NFAT regulates transcription of the cTnI and the cTnT genes (Fig. 10 A). The cTnI promoter (−1 to
was subcloned upstream of a luciferase reporter gene. The cTnI luciferase reporter was cotransfected with either constitutive active calcineurin (ΔCn) or constitutive nuclear NFATc4 (cnNFATc4; Fig. 10 B). Coexpression of either ΔCn or cnNFATc4 increased cTnI reporter gene activity. Importantly, deletion to −283, to remove the cTnI NFAT binding site, or mutation at the NFAT binding sequence, abolished NFAT-mediated gene transcription (Fig. 10 C). A luciferase reporter plasmid containing a triple repeat of the cTnT site 1 NFAT element was also examined (Fig. 10 D). Coexpression of either ΔCn or cnNFATc4 increased cTnT reporter gene activity. Together, these data indicate that NFAT binds to and regulates cTnI and cTnT gene expression. Regulation of cTnI and cTnT gene expression by NFAT may, in part, account for the structural architecture of the developing myocardium.

Discussion

Requirement for NFAT in the heart

The transcription factor NFAT plays an important role in cytokine gene expression (Rao et al., 1997; Crabtree, 1999). NFAT was once thought as being a specific regulator in immune cells. However, recent papers have proposed that NFAT is a ubiquitous regulator for cell growth, differentiation, and apoptosis (Horsley and Pavlath, 2002). In this report, we demonstrate that NFAT regulates transcription of cTnI and cTnT genes and plays a critical role in the structural architecture of the developing myocardium. Thus, our data provide new evidence to expand the repertoire of functions of NFAT.

In this paper, we show that NFAT activity is required in the myocardium between E14 and P0. Previous gene-targeting studies indicated that NFATc1 is required in the endocardium between E7.5 to E13.5 for heart valve development (de la Pompa et al., 1998; Ranger et al., 1998), whereas NFATc3 and NFATc4 are important in endothelial cells for vasculature formation between E7.5 and E8.5 (Graef et al., 2001). Administration of CsA at the early stage (before E10.5) of cardiac morphogenesis also affects vasculature formation. In this report, we show that late administration of CsA (starting on E14) causes thin myocardium and myofiber disorganization. Thus, the role of NFAT in heart development is spatially and temporally regulated. In addition, different NFAT members seem to differentially regulate these functions. Future studies to examine the temporal interval of activation and the expression profile of different members will be critical to understand the biological functions mediated by NFAT.

In this report, we demonstrate that inhibition of NFAT activity by expression of dnNFAT causes thinning of atrial walls. Kinetics of dnNFAT induction and disinhibition is critical to reveal when NFAT activity is required. Our data

Figure 6. Cell viability of the developing mouse atria. (A) TUNEL assays were performed to examine cell viability in Dox-treated developing mouse atria. As compared with control, which show an intense staining of fragmented DNA in apoptotic cells, TUNEL-positive cells were not detected at E14 and E16 Wt littermate and Tg+ atria, Bar, 50 μm. (B) The number of myocyte nuclei is similar. The number of intact nuclei in the Wt and the Tg+ atria was counted every 0.3 mm along the atrial wall and illustrated. RA, right atria; LA, left atria.
indicated that a 2-d administration of Dox, between E14 and E16, to induce dnNFAT is sufficient to cause thin myocardium. Because there is an uncertainty of time of conception, ranging from 8–12 h (Kaufman, 1992; Kaufman and Bard, 1999), and a lag time for transcription, translation, and subsequent accumulation of rtTA and dnNFAT, which is at least 8 h (Kistner et al., 1996; Akagi et al., 2001; Gunther et al., 2002; Ohno-Matsui et al., 2002; Perl et al., 2002), we believe that the examination of hearts at a 2-d interval should closely represent the temporal requirement of NFAT during development.

Conversely, withdrawal of Dox on E14, after 6 d of Dox treatment, before harvesting hearts on E16 (i.e., 2 d without Dox before examination of hearts) does not produce thinner atria. These data suggest a rapid loss of the inhibitory effect. The loss of inhibition may be due to a high turnover of dnNFAT, rtTA, or Dox. Alternatively, a substantial expression of endogenous calcineurin may also contribute to the efficient disinhibition. The inhibitory effect of dnNFAT is to act as a “sink” to interact with calcineurin, and hence, blocks endogenous NFAT functions (Northrop et al., 1994; Aramburu et al., 1998; Chow et al., 1999; Miskin et al., 2000; Saneyoshi et al., 2002). If there were insufficient accumulation of dnNFAT, calcineurin-mediated NFAT functions would remain active, which may account for the lack of effect of dnNFAT in developing ventricles. However, treatment with CsA to inhibit calcineurin receptor-dependent NFAT activation would rescue the phenotype.

Figure 7. Cytoarchitecture of the developing mouse atria. Ultrastructure of P0 atria was examined by transmission electron microscopy. Electron micrographs revealed characteristic banding pattern of sarcomere in Dox-treated Wt littermate control. Mitochondria layered between myofilaments were also observed. However, Dox-treated Tg+ atria displayed severely perturbed sarcomere and mitochondria. Varying degrees of sarcomeric disorganization were also observed. Cross-sectional cell thickness of Wt and Tg+ atria was shown (A). Low (A) and high (B) magnifications of Wt and Tg+ atria are presented. Bar, 1 μm.

Figure 8. Expression of cytoskeletal and sarcomeric proteins in Tg+ and Wt hearts. Dox-treated embryos were harvested on P0, micro-dissected to separate atria and ventricles, and genotyped. 20 Wt or Tg+ atria (or 10 ventricles) were collected from multiple litters, homogenized, and extracts obtained were separated on SDS-PAGE. Immunoblots were performed with vinculin, desmin, α-actinin, actin, myosin, and caveolin-3 antibodies (A). Expression of tropomyosin, troponin-C, ssTnl, ssTnT, cTnI, and cTnT were also examined (B).
NFAT activity causes myofiber disarray in addition to the thinning of the atrial wall, supporting a critical role of NFAT in the developing ventricles. Thus, a threshold for dnNFAT inhibition is a balance of expression between dn-NFAT, rtTA, and calcineurin.

Previous papers indicated that NFAT plays an important role in the calcineurin signaling pathway in cardiac hypertrophy (for reviews see Molkentin and Dorn, 2001; Sugden, 2001; Frey and Olson, 2003). Cardiac hypertrophy is an adaptive response to increase workload on physiological and pathological demand. Molecular analysis indicates reactivation of a fetal gene expression program during cardiac hypertrophy; for example, increased expression of atrial natriuretic factor in hypertrophic ventricular muscle. Thus, calcineurin activation during cardiac hypertrophy may correlate with the role of NFAT in developing myocardium to regulate gene expression. Expression profiling studies to determine common molecular targets of NFAT in developing myocardium and in hypertrophic hearts will shed new light on the role of calcineurin signaling in heart development and function.

NFAT-mediated gene transcription in the heart

Different isoforms of various sarcomeric proteins are differentially expressed in the developing and adult heart (Perry, 1998, 1999). In early embryonic stages, ssTnI is expressed, but eventually is replaced by cTnl, which is the predominant isoform in neonates and adult mice (Saggin et al., 1989; Gorza et al., 1993). Similarly, ssTnT is expressed in the embryonic heart, but is replaced by cTnT at later stages (Saggin et al., 1988; Wang et al., 2001). A critical time for the switch over of these TnT and TnI isoforms would be at or around birth, in order to prepare for the increased workload in neonates when all the major organs are fully developed and the circulation system becomes independent. Reduced structural integrity by NFAT inhibition and increased hemodynamics at the later developing stages may account for the abrupt thinning of the myocardium. Thus, NFAT may play a critical role in the switch over of troponin isoforms for the preparation of heart maturation.

Genomic analyses indicate that ssTnT and cTnl genes are located in the same gene locus (Barton et al., 1999). Interestingly, the cTnT and ssTnI genes are also closely linked.
Chromatin remodeling and activation of the gene loci (e.g., histone acetylation and increase DNase hypersensitivity) are likely involved in the switch over from ssTnT to cTnT or sTnI to cTnT during heart maturation. Previous reports indicated that NFAT binds to DNase hypersensitive sites and regulates the expression of several cytokine and PPARγ genes (Duncilffe et al., 1997; Agarwal et al., 2000; Ren et al., 2002). Because induction of dnNFAT reduced cTnI and cTnT (but not sTnI and ssTnI) expression, it is possible that temporally activated NFAT, in conjunction with different NFAT partners, binds to the sTnI/cTnT and the cTnI/ssTnI gene loci to facilitate the switch over and allow subsequent induction of cTnT and cTnT at or around birth.

NFAT was first identified as an important regulator in IL-2 gene expression (Jain et al., 1993; Northrop et al., 1993, 1994). Cooperative interaction between NFAT and AP-1 (Fos and Jun) is critical for expression of many cytokine genes in immune cells. Analogous to the NFAT–AP-1 complex, we have recently demonstrated that NFAT interacts with C/EBP to mediate expression of the PPARγ gene in adipocytes (Yang and Chow, 2003). Similarly, NFAT–C/EBP composite enhancer complex is found in the upstream regulatory region of the angiotensin-converting enzyme homologue gene (ACEH/ACE2), which is induced at E15 and becomes highly expressed at E17 in the mouse embryo (Crackower et al., 2002). Recent gene-targeting studies indicate that ACE2-null mice exhibit thin myocardium, whereas ACE2 mutant flies display heart defects (Crackower et al., 2002). In addition, a balance of ACE2 and ACE activity is proposed to regulate hemodynamics through the renin–angiotensin system (Eriksson et al., 2002; Turner and Hooper, 2002). Thus, it is tempting to speculate that besides structural proteins, NFAT regulates ACE2 expression during embryonic development and in pathophysiological hearts to modulate myocardium formation, and mediates compensatory response during hypertension and/or hypertrophy, respectively.

In addition to AP-1 and C/EBP, GATA and MEF2 transcription factors also interact with NFAT to mediate gene expression (Molkentin et al., 1998; Musaro et al., 1999; Blauser et al., 2000; Wu et al., 2000). Thus, NFAT forms a distinctive composite enhancer complex with different NFAT partners to mediate gene transcription. In this report, we show that NFAT binds to the cTnT and cTnT promoters. Importantly, the NFAT element is located in the mouse minimal cTnT promoter, which directs transgene expression in the developing cardiac atrioventricular canal (Di Lisi et al., 1998, 2000), supporting a role of NFAT in cardiac development. However, competition analysis indicates that different NFAT–DNA complexes are formed. These different NFAT complexes may represent additional regulation by NFAT (and its partners) in mediating multiple biological functions. Further identification of these NFAT partners will be important to understand the molecular basis of NFAT-mediated gene transcription.

In conclusion, we have demonstrated that NFAT is temporally and spatially required for the developing myocardium. Identifying the role of NFAT in the developing myocardium expands the repertoire of functions of NFAT.
of pseudo-fertilized eggs to generate transgenic mice. Two inducible Tg+ founders (line #3 and 8) were established and backcrossed onto C57BL/6 mice (The Jackson Laboratory). Genotyping analysis indicated that both transgenes were cotegrated and cosegregated together. Littermates from line #3 were used for further analysis.

Timed-pregnant mice were injected intraperitoneally with 25 mg/kg CsA daily starting at E14. Control mice received DMSO. Embryos were harvested on P0 and hearts were prepared. Representative sections were hematoxylin- and eosin-stained and were examined under a microscope (model IX70; Olympus).

**Immunofluorescence analysis**

Paraffin-embedded sections from E14 and E16 Tg+ and Wt hearts were deparaffinized and rehydrated through a series of graded alcohol and PBS washes. Rehydrated sections were incubated in blocking solution (2% goat serum and 0.1% Triton X-100 in PBS) for 20 min. The sections were then washed twice in wash buffer (0.1% Triton X-100 in PBS) and incubated with indicated primary antibody (diluted 1:100 in the blocking solution) overnight at 4°C. After washing three times in wash buffer (10 min each), the sections were incubated with the appropriate FITC-conjugated secondary antibody (diluted 1:500 in the blocking solution) for 45 min. The sections were then washed for 30 min, and counterstained with 10 μg/ml of bis-benzimide (Sigma-Aldrich) for 15 min to detect DNA. After a final wash for 20 min, a coverslip was mounted on the slides with ProLong® Antifade (Molecular Probes, Inc.). Sections were analyzed and images were generated using an inverted microscope (model IX70; Olympus) and a 12-bit cooled CCD camera (SensiCam; Photometrics). Images of antibody staining and nuclei counterstaining were deconvolved using I.P. Lab Spectrum software and merged into one image using Adobe Photoshop®. Intensity of NFAT nuclear staining was determined by using Adobe Photoshop® and NIH Image software.

**Embryonic heart preparation**

Embryonic hearts were harvested at various times during gestation as indicated. The date after conception was registered as E0.5. On the day of harvest, the embryos were decapitated and the hind limbs were removed for genotyping. The remaining torso was fixed in 10% buffered formalin overnight before isolation of the hearts through a dissecting microscope (model SV11; Carl Zeiss Microimaging, Inc.). Isolated hearts were then rinsed in PBS and dehydrated through a graded series of alcohol washes. After washing three times in xylene (10 min each), isolated hearts were immersed in paraffin (three times, 15 min each), oriented, and embedded. Embedded hearts were serially sectioned sagittally at a thickness of 7 μm using a microtome (Microm; Baxter Scientific), and were placed on super-frost plus slides (Fisher Scientific). Representative sections were hematoxylin- and eosin-stained and examined under a microscope (model IX70; Olympus).

**Expression of dnNFAT**

4-wk-old double Tg+ or Wt nontransgenic littermate mice were given either untreated, plain water or Dox-treated water (2 mg/ml) for 1 wk before isolation of their hearts. The hearts were removed, homogenized, and isolated cell extracts were separated on an SDS-PAGE (12%). Expression of dnNFAT was examined by immunoblotting analysis using the M2 mAb that recognizes the FLAG epitope.

**Morphometric analysis**

To determine atrial and ventricular wall thickness, digital images of Tg+ and Wt hearts harvested on different dates of gestation (E14, E16, E18, and P0) were generated. Nine individual fields from each heart were taken as separate images. Using NIH Image software (National Institutes of Health, Bethesda, MD), wall thickness was measured at eight different locations from each image and an average was taken from six embryos, which were derived from three different litters. The averages (i.e., 432 data points per condition) were then input into Sigma Plot and graphed.

To determine myocyte nuclei count, image photos were randomly taken from E14 and E16 Tg+ and Wt hearts (40x magnification). Nine photos, from three different hearts, of each condition were produced and the number of myocyte nuclei in the atrial walls was counted. The averages were then input into SigmaPlot® (SPSS, Inc.) and graphed. To determine individual cell thicknesses, 40 low magnification electron micrographs, with multiple cells per micrograph, were generated. Cell width at multiple points of each cell, from Wt or Tg+ atria, was measured. The averages for each condition were then input into SigmaPlot® and graphed.

**TUNEL stainings**

Paraffin sections were deparaffinized as described earlier in Materials and methods, and TUNEL staining was performed with the TACS™ 2 kit as recommended by the manufacturer (Trevigen, Inc.). Sections were examined using a microscope (Axioskop; Carl Zeiss Microimaging, Inc.) and photographed.

**Electron microscopy**

Newborn pups (P0) were killed by decapitation, and their hearts were removed. Hind limbs from the embryos were also removed for genotyping. 20 Tg+ or Wt atria (or 10 ventricles) were collected from multiple litters, homogenized in 1 ml × LDS sample buffer (140 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 0.1 M DTT, and 30 μM bromophenol blue), and boiled. Sample was cleared by centrifugation, and the amount of protein was determined by Ponceau-S staining. An equal amount of protein from Wt and Tg+ atria and ventricle was resolved on an SDS-PAGE (8% or 12%), and was electrotransferred to nitrocellulose membranes (Millipore). Immunoblotting analysis was performed to determine the expression level of various cytoskeletal or sarcomeric proteins.

**Gel mobility shift assays**

Double-stranded oligonucleotides encoding NFAT-binding sites from the cTnI gene promoter (5′-AGT CGG AGA AAG GGG GCT GGG-3′) and the cTnF gene promoter (site 1, 5′-AGA TGG ATC GAA TCG AAG AAG TCG TGC ACA AAG GGA-3′; and site 2, 5′-TGG CTT AGG AAA ATC CCA TTC GAG TGC TGC-3′) were labeled with [32P]dCTP and used as probes. Nuclear extracts prepared from NFAT+-transfected COS cells were used. Gel mobility shift assays were performed as described previously (Yang and Chow, 2003). For competition analysis, 10 pmol of unlabeled oligonucleotides (5′-2 NFAT, 5′-AGA AAG GAG GAA AAA CTG TTG CAT ACA GAA GG-3′; PPARα proximal NFAT, 5′-ATTACAGGAAAATATTGCCA-CACGTGTC-3′; PPARγ distal NFAT, 5′-AGA AGG AGA TTT AGG TTT TCC ATT TAA GAA-3′; mouse cTnI, 5′-CTC AAA CTT AGG AAA ACC CGG AGG AAG GTC-3′; and rat cTnI, 5′-TTG AAT CTC ACC GAG AAA ACC CAG TAG CAA GGT-3′) were incubated with the labeled probe before addition of nuclear extract.

**Luciferase assays**

The cTnI and cTnF promoter luciferase reporter constructs were cotransfected into BHK cells with either ΔcN or a cNcFAT construct. pRSV β-galactosidase plasmid was also cotransfected as a control. Luciferase assays were performed as described previously (Yang et al., 2002). The data are presented as relative luciferase activity, calculated as the ratio of the luciferase activity to the activity of β-galactosidase (mean ± SD; n = 4).

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