Down Syndrome Critical Region-1 Is a Transcriptional Target of Nuclear Factor of Activated T Cells-c1 within the Endocardium during Heart Development*

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Patients with Down syndrome have characteristic heart valve lesions resulting from endocardial cushion defects. The Down syndrome critical region 1 (DSCR1) gene, identified at the conserved trisomic 21 region in those patients, encodes a calcineurin inhibitor that inactivates nuclear factor of activated T cells (NFATc) activity. Here, we identify a regulatory sequence in the promoter region of human DSCR1 that dictates specific expression of a reporter gene in the endocardium, defined by the temporal and spatial expression of Nfatc1 during heart valve development. Activation of this evolutionarily conserved DSCR1 regulatory sequence requires calcineurin and NFATc1 signaling in the endocardium. NFATc1 proteins bind to the regulatory sequence and trigger its enhancer activity. NFATc1 is sufficient to induce the expression of Dscr1 in cells that normally have undetectable or minimal NFATc1 or DSCR1. Pharmacologic inhibition of calcineurin or genetic Nfatc1 null mutation in mice abolishes the endocardial activity of this DSCR1 enhancer. Furthermore, in mice lacking endocardial NFATc1, the endogenous Dscr1 expression is specifically inhibited in the endocardium but not in the myocardium. Thus, our studies indicate that the DSCR1 gene is a direct transcriptional target of NFATc1 proteins within the endocardium during a critical window of heart valve formation.

Down syndrome (DS),5 produced by trisomy of all or part of chromosome 21, occurs at a frequency of 1 in 43 spontaneous abortions and 1 in 750 live births (1). Most DS fetuses (up to 50–75%) die in utero (2, 3). Approximately 40–50% of the surviving DS patients have characteristic congenital heart disease (4). Indeed, DS is a major cause of congenital heart defects in humans (5). Endocardial cushion defect is the predominant cardiac abnormality in DS, leading to characteristic atrioventricular septal or heart valve lesions (4, 6). Previous work from our laboratory and others demonstrates that the calcineurin/nuclear factor of activated T cells (NFATc) signaling pathway is essential for the development of endocardial cushions and heart valves (7–9), and that perturbations of the pathway lead to many features of Down syndrome (7, 10, 11).

Calcineurin is a Ca2+/calmodulin-dependent phosphatase (12). Activated calcineurin dephosphorylates NFATc proteins, triggering NFATc translocation from the cytoplasm into the nucleus, where NFATc proteins form transcription complexes with cofactors to regulate target gene expression (13, 14). Two genes, DYRK1a and DSCR1, localized within the Down syndrome critical region (DSCR) of human chromosome 21, were shown to synergistically reduce the nuclear occupancy of the NFATc proteins (10, 15). DYRK1a is a kinase that promotes NFATc nuclear export (10, 15), and DSCR1 (RCAN1, calcipressin1, or MCIP1) encodes an inhibitor of calcineurin (16–19). Apparently a 1.5-fold overexpression of these two proteins leads to the dysregulation of calcineurin/NFAT signaling, resulting in a constellation of DS features that are almost perfectly reproduced in Nfatc mutant mice (10).

Although recent work has shown that the expression of DSCR1 in neurons and the heart is induced by the NFAT pathway (20, 21), it is not clear how DSCR1 interacts with NFATc1 within the endocardium during heart valve development. The role of calcineurin in regulating DSCR1 expression in the heart remains undefined. Nor is there unambiguous evidence that DSCR1 is a direct transcriptional target of NFATc1 in the heart. Here, we identify an enhancer of the human DSCR1 gene that contains all the elements required to direct endocardial DSCR1 expression that is entirely defined by calcineurin/NFATc1 activity in the heart. We present both in vivo and in vitro evidence that DSCR1 is a direct transcriptional target of NFATc1 in the heart, and that its expression is controlled by calcineurin/NFATc1 signaling.

PECAM, platelet endothelial cell adhesion molecule; RT, reverse transcription; NRE, NFAT-responsive element; GFP, green fluorescent protein; E, embryonic day; MEF, mouse embryonic fibroblast; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
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vitro and in vivo evidence that the DSCR1 gene is a direct downstream target of the NFATc1 transcription factor in the endocardium of a developing heart.

**EXPERIMENTAL PROCEDURES**

**Mouse Husbandry**—All mouse strains were maintained in outbred backgrounds at the Stanford University Research Animal Facility under the National Institutes of Health guidelines. The date of observation of a vaginal plug was set as day 0.5, which was confirmed by ultrasonography prior to sacrificing pregnant mice (22) and examination of embryo morphology and somite number. Nfatc1−c4 mutant mouse lines were described previously (7).

**Generation of DSCR1-lacZ Mice**—The plasmid used in transgenic mouse production was constructed by linking the NFAT-dependent human DSCR1 enhancer upstream of the basal hsp68 promoter and lacZ reporter gene (23). The DSCR1 enhancer contained 904 base pairs derived from sequences flanking exon4 (−874 to +30) with the first nucleotide of exon 4 designated as 1. After removal of the prokaryotic sequences, transgene fragments were introduced by pronuclei microinjection of fertilized oocytes of C57B6/C3H mice.

**Gel Mobility Shift Assay**—Binding of nuclear proteins to DNA oligonucleotide probes was done as described previously (24, 25). Nuclear proteins were extracted from embryonic hearts at embryonic day (E) 11.5 and E12.5. Anti-NFATc1 (clone 7A6, Santa Cruz Biotechnology) was used for antibody supershift experiments. Oligonucleotides used were: wild-type (wt)-NFAT-responsive element (NRE)-1, TCAGCTGTGTTTTTTCCATTCTC–CCCAGG; mutant-NRE1, TCAGCTCTGTTGTAGTTCTC–CCCAGG; wt-NRE2, AGCATAGGGAAAATGACTAAGG; mutant NRE2, AGCATAGCTGCTAGCATGACTAAGG; wt-NRE3, AGGTACCTTTCCTCAGTAGAAGATCTACAC; mutant NRE3, AGGTACCTTTCCTCAGTAGAAGATCTACAC (sequences underlined represent the intact or mutant NFAT core binding sites).

**Plasmids, Cell Culture, and Transfection**—Full-length mouse NFATc1 was cloned into pMES vector. The constitutively active calcineurin and DSCR1 luciferase reporter were described previously (26, 27). Nine copies of wt or mutant NRE3 were cloned into pGL3-promoter (Promega). Mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum and nonessential amino acids. Cells were transfected with Lipofectamine 2000 (Invitrogen).

**lacZ Staining, RNA in Situ Hybridization, and Immunostaining**—Whole-mount lacZ staining was performed as described previously (7). The mouse Dscr1 3′-untranslated region corresponding to exons 5, 6, and 7 was generated by reverse transcription (RT)-PCR and subcloned into pCRII vector. This probe was common to all DSCR1 isoforms. In situ hybridization was performed using a standard protocol. For platelet endothelial cell adhesion molecule (PECAM)-1 staining, paraffin-embedded tissue sections were trypsin-digested and incubated with rat anti-PECAM-1 antibody (Pharmingen). NFATc1 antibody (Santa Cruz Biotechnology) was applied to paraformaldehyde-fixed REN cells. Alexa fluorophore-conjugated secondary antibodies (Molecular Probes) were used to visualize antibody staining.

**Adenoviral Infection, cDNA Microarray Analysis, and RT-PCR**—The REN cell was infected with an adenoviral expression cassette consisting of a cytomegalovirus promoter, a constitutively activated NFATc1, and downstream green fluorescent protein (GFP) linked by internal ribosomal entry site (IRES). Cells were harvested for RNA isolation 2 days after infection. Human 10K cDNA chip was used for cDNA microarray analyses that were performed at Genomics/Microarray Facility, The Wistar Institute. The arrayed DNA (expressed sequence tag AA629707) was located at the 3′ end of DSCR1 for all four human transcript isoforms. For RT-PCR, total RNAs were isolated from independently infected REN cells. RT-PCR was performed using a standard protocol with the DSCR1 specific primers.

**RESULTS**

The DSCR1 Regulatory Promoter Region Is Evolutionally Conserved and Under Calcineurin/NFATc1 Regulation—A promoter element located upstream of exon 4 of the human DSCR1 gene was identified previously (27). The promoter showed an over 75% homology among rat, mouse, and human sequences, and the NREs within were perfectly conserved (Fig. 1A). By gel mobility shift analysis, we found that endogenous NFATc proteins present in the nuclear extracts of mouse embryonic hearts bound strongly to oligonucleotides containing the NRE1, -2, or -3 present in the promoter (Fig. 1B, lanes 1, 3, and 5). The presence of NFATc1 proteins in the binding complex was confirmed by disconnection of the protein-DNA complexes with anti-NFATc1 antibody (Fig. 1B, lanes 2, 4, and 6). Furthermore, mutations of the canonical NFAT binding sites of NREs from GGAA(A/T)TCTCC to CTAG(C/G)CTAG abolished the binding of endogenous NFATc1 proteins to NRE2 and -3 but not NRE1 (Fig. 1C, lanes 2, 4, and 6), suggesting that NFATc1 binds to canonical sites of NRE2 and -3, and a noncanonical site of NRE1, perhaps through GGAGA/TCTCC that is similar to the canonical site. Overall, these findings indicate that endogenous NFATc1 proteins bind to the NREs of the DSCR1 enhancer.

**FIGURE 1. NFATc proteins bind and transactivate a conserved enhancer derived from the DSCR1 gene.** A, alignment of part of the enhancer from human, mouse, and rat. The nucleotide immediately upstream of the start codon ATG of exon 4 is defined as position −1. Oligonucleotides (NREs) used in the gel shift assay are boxed. B, binding of NFATc proteins to the enhancer. (γ32P) ATP-labeled NRE oligonucleotides were incubated with nuclear extracts from mouse embryonic hearts. DNA-protein complex (arrow) was resolved by gel electrophoresis. For the NFAT, DNA complexes were disrupted after incubating nuclear protein extracts with anti-NFATc1 antibody. C, binding of NFATc proteins to intact and mutant NREs. The consensus NFAT binding sites (GGAA) were substituted with CTAG in the mutant NREs. For the formation of NFAT, DNA complexes (arrow) were disrupted for mutant NRE2 and -3. D, transactivation of the DSCR1 enhancer by calcineurin and NFATc1. CnA* and NFATc1 activated the enhancer in MEFs. E, NRE3 functions as an NFAT-dependent enhancer. Nine copies of intact or mutant NRE3 were cloned upstream of luciferase reporter. Transactivation of the mutant NRE3 (pGL3-Mut(NRE3)3) by calcineurin and NFATc1 is greatly reduced compared with intact NRE3 (pGL3-wt(NRE3)3).
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To study whether NFATc1 could transactivate the DSCR1 promoter, we performed a luciferase reporter assay. The regulatory sequence of DSCR1 gene was cloned upstream of the luciferase reporter gene (27). In the MEFs, we found that overexpression of NFATc1 and constitutively active calcineurin (CaNα) significantly enhanced the luciferase reporter activity (Fig. 1D). Overexpression of NFATc1 alone, however, had minimal activation of the reporter gene in MEFs because of the fact that NFATc proteins, whether overexpressed or endogenous, were located predominantly in the cytoplasm of MEFs in the absence of CaNα (data not shown). CaNα triggered the nuclear localization of endogenous or overexpressed NFATc proteins, leading to reporter activation (data not shown and Fig. 1D). Thus, the ability of NFATc1 proteins to bind to the enhancer and trigger the enhancer activity depends on their nuclear localization controlled by calcineurin. This indicates that NFATc proteins regulate the expression of DSCR1 at the transcriptional level. To examine whether NFATc1 could exert its transcriptional activity through the NREs of DSCR1 enhancer, we cloned the NRE3 element upstream of the luciferase reporter gene. We found that CaNα and NFATc1 strongly activated the luciferase expression of this reporter (Fig. 1E). Mutations of the NFAT binding site on NRE3 significantly reduced the transcriptional activity of NFATc1 on the reporter gene (Fig. 1F). Together, these studies indicate that NFATc1 proteins transcriptionally activate the DSCR1 enhancer through the NREs.

**NFATc1 Induces Endogenous DSCR1 Expression in Cells That Normally Have Undetectable or Minimal Expression of NFATc1 or DSCR1**—To test whether NFATc1 proteins were sufficient to trigger endogenous DSCR1 expression, we chose the human mesothelioma cell line (REN cell) that had no detectable expression of NFATc1 and had only minimal expression of DSCR1. These cells were infected with either adenovirus expressing constitutively nuclear NFATc1-IRES-GFP or with GFP and examined for the expression of DSCR1. The subcellular localization of the constitutively nuclear NFATc1 proteins was confirmed by immunostaining (Fig. 2A). Western blot analysis showed the expression of constitutive activated NFATc1 proteins in REN cells after infection with adenovirus (Fig. 2B). REN cells infected with control GFP-only virus did not express detectable endogenous NFATc1. Cells expressing NFATc1-IRES-GFP were sorted, cultured, and then subjected to transcript microarray analysis. Transcript analysis revealed that NFATc1 enhanced the expression of DSCR1 mRNA in REN cells by approximately 10-fold (Fig. 2C). The induction of DSCR1 mRNA expression was confirmed by semiquantitative RT-PCR in independently infected cells (Fig. 2D). These results indicate that NFATc1 is sufficient to activate endogenous DSCR1 expression.

**The DSCR1 Enhancer Activity in the Endocardium Requires Both Calcineurin and NFATc1—**Both the in vitro binding and cellular transactivation assays suggested that NFATc transcriptionally regulated the expression of DSCR1. To investigate whether the enhancer activity of DSCR1 gene depends on NFATc proteins in vivo, we generated a transgenic mouse reporter line (DSCR1-lacZ), where a β-galactosidase reporter gene was driven by the human DSCR1 enhancer. By whole-mount lacZ staining, we found that the DSCR1-lacZ activity was expressed in the heart and neural tube during a specific time window of development from E9.5 to E12.5 (Fig. 3A). This expression pattern partially recapitulated the endogenous DSCR1 expression in the heart (28) and corresponded to the time window of NFATc1 expression in the endocardium (7–9). Detailed histological analysis of the heart revealed a specific expression of this transgene in endocardial cells (Fig. 3B). At E9.5, the transgene was expressed throughout the endocardium. By E11.5, the expression became limited to the cushion endocardium of the cardiac outflow tract and atrioventricular canal. This unique expression pattern was identical to that of NFATc1 as the latter was expressed in all endocardial cells at E9.5, and its expression became restricted to the cushion endocardial cells by E11.5 (7–9).

Because the expression of DSCR1-lacZ colocalized with NFATc1 expression both temporally and spatially during heart development, we investigated whether the DSCR1 promoter activity was indeed regulated by NFATc1 in vivo. In Nfatc1 null embryos harboring the DSCR-lacZ reporter gene, we found that the expression of lacZ in the endocardium was abrogated (Fig. 3C), indicating that NFATc1 was required in vivo for...
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**Endogenous DSCR1 Gene Expression in the Endocardium Requires NFATc1**—To address whether endogenous DSCR1 expression required NFATc1 in the endocardium, we performed a RNA in situ hybridization in Nfatc1 null embryos with a probe specific for DSCR1. This probe was generated from the 3’-untranslated region corresponding to exons 5, 6, and 7 of mouse Dscr1 and detected all the common isoforms of Dscr1. We found that DSCR1 was expressed in both the endocardium and myocardium of wild-type mice at E12.5 (Fig. 4A). In mice lacking NFATc1, the expression of DSCR1 in the endocardium was significantly reduced or abolished (Fig. 4, B and D). In contrast, its expression was preserved in the myocardium that did not normally express NFATc1 (Fig. 4B). To rule out a global change of gene expression in the endocardium of Nfatc1−/− mice, we examined the expression of endocardial PECAM-1 in the mutant mice. By immunostaining, we found no significant changes of endocardial PECAM-1 expression in Nfatc1−/− embryos (Fig. 4F). These observations indicate that NFATc1 proteins specifically regulate the expression of endogenous DSCR1 in the endocardium and that the expression of DSCR1 in the myocardium is NFATc1-independent. Overall, our studies unequivocally demonstrate that the regulation of endocardial DSCR1 expression depends on calcineurin/NFATc1 signaling and that DSCR1 is a direct downstream target of NFATc1 proteins within the endocardium.

**DISCUSSION**

We have presented multiple lines of evidence indicating that the calcineurin inhibitor DSCR1 is a direct transcriptional target of NFATc1 during critical windows of heart valve development. Because the activity of endocardial NFATc1 itself depends on calcineurin (Fig. 3E), the regulation of DSCR1 by NFATc1 may provide a negative feedback control mechanism to maintain endocardial NFATc1 homeostasis during heart development (Fig. 4G). This working model suggests that reduced NFATc1 activity attenuates DSCR1 expression, boosting calcineurin activity and promoting further NFATc1 nuclear...
entry to restore NFATc1 level. Excessive NFATc1 activity, in contrast, may enhance the expression of DSCR1, which inhibits calcineurin and blocks the entry of additional NFATc1 molecules into the nuclei, and thus normalizes NFATc1 activity. This working model is further supported by previous observations that enhanced DSCR1 expression inhibits NFATc1 nuclear localization and transcriptional activity in the heart (10, 30). The negative feedback loop may serve to maintain endocardial NFATc1 protein function at a stable level during heart valve morphogenesis. However, mice lacking DSCR1 and/or DSCR1-like 1 survive to adulthood with no apparent severe defects in cardiac morphogenesis (31). These findings may suggest the presence of redundant pathways that compensate for the loss of function of DSCR1.

A mouse Dscr1 enhancer (~700 bp) was used previously to drive lacZ transgene expression in an embryonic heart. Weak transgene expression was detected in the endocardial cushion of the outflow tract at E9.5–E12.5 and in a small subset of NFATc1-expressing cells of the atrioventricular canal (21), suggesting that the enhancer did not contain all elements required to recapitulate endogenous DSCR1 expression in the endocardium defined by NFATc1 activity. In this study, the lacZ transgene is controlled by the homologous human enhancer (~900 bp). We have observed broader panendocardial expression of the transgene at E9.5. By E11.5, the expression is restricted to the cushion endocardium of the cardiac outflow tract and atrioventricular canal (Fig. 3). This unique spatial-temporal expression profile is identical to that of NFATc1 (7–9) and demonstrates a functional conservation of the DSCR1 enhancer between the human and mouse species. Indeed, the transgene expression is abolished in Nfatc1 null embryos (Fig. 3), indicating that endocardial DSCR1 expression is both spatially and temporally defined by NFATc1. Thus, the longer human enhancer used in our study contains a complete set of elements required to direct endocardial DSCR1 expression in a NFATc1-dependent manner. Additionally, we show that endogenous NFATc1 proteins physically bind to the enhancer through the NREs and that NFATc1 is both necessary and sufficient for the activation of DSCR1 expression (Figs. 1 and 2). Among NFATc signaling, NFATc1, but not NFATc2/3/4, is uniquely required for activating endocardial DSCR1 expression (Fig. 3). We further show that the DSCR1 expression requires calcineurin to trigger NFATc1 nuclear localization and activation (Figs. 1 and 3).

These observations provide direct evidence that the endogenous DSCR1 expression in the developing endocardium is controlled by calcineurin/NFATc1 signaling. NFATc1 transcriptionally activates DSCR1 expression through a conserved enhancer region. The regulation of DSCR1 and NFATc1 may provide a feedback mechanism to ensure proper maintenance of NFAT signaling strength during heart development.

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FIGURE 4. NFATc1 activates the expression of endogenous DSCR1 in the endocardium. A, the expression pattern of DSCR1 mRNA by in situ hybridization at E12.5. Dscr1 mRNA is expressed in both the myocardium (arrowheads) and cushion endocardium (arrows). Black dots, Dscr1 mRNA; blue, hematoxylin counterstain; *, valve leaflet. B, the expression of DSCR1 in the cushion endocardium (arrow) is abolished in the absence of NFATc1. DSCR1 expression in the myocardium (arrowhead) is preserved. *, valve leaflet. C, higher magnification of panel A showing abundant Dscr1 mRNA (black dots) expression in the cushion endocardium (arrows). D, higher magnification of panel B, showing minimal Dscr1 mRNA expression in the cushion endocardium of Nfatc1−/− embryos (arrow). E and F, PECAM-1 immunostaining of cushion endocardium of wild-type (E) and Nfatc1−/− embryos (F) at E12.5. Green, PECAM-1 proteins; blue, 4’,6-diamidino-2-phenylindole nuclear stain. G, a working model illustrating a negative feedback loop that controls NFATc1 activity and DSCR1 expression within the endocardium. DSCR1 inhibits calcineurin and NFATc1 signaling required for its own expression. NFATc1 proteins activate the expression of DSCR1, a calcineurin inhibitor, to regulate its nuclear localization and activity.
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