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NEXN inhibits GATA4 and leads to atrial septal defects in mice and humans

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Aims Cardiac structural genes have been implicated as causative factors for congenital heart diseases (CHDs). NEXN is an F-actin binding protein and previously identified as a disease gene causing cardiomyopathies. Whether NEXN contributes to CHDs aetiologically remains unknown. Here, we explored the function of NEXN in cardiac development.

Methods and results First, we determine the role of NEXN in cardiac differentiation using mouse P19cl6 *in vitro* model; we demonstrated that NEXN inhibited cardiac contractile markers, serving as a negative regulator. Interestingly, we found this effect was mediated by GATA4, a crucial transcription factor that controls cardiac development by knockdown, overexpression, and rescue experiment, respectively. We then generated transgenic mouse models and surprisingly, we discovered cardiac-selective expression of the *NEXN* gene caused atrial septal defects (ASDs). Next, to search for the mutations in *NEXN* gene in patients suffering from ASDs, we sequenced the exon and exon–intron joint regions of the *NEXN* gene in 150 probands with isolated ASDs and identified three mutations in the conserved region of *NEXN* (c.-52-78C>A, K199E, and L227S), which were not found in 500 healthy controls. Finally, we characterize the related mechanisms and found all mutations inhibited GATA4 expression.

Conclusion We identify *NEXN* as a novel gene for ASD and its function to inhibit GATA4 established a critical regulation of an F-actin binding protein on a transcription factor in cardiac development.

Keywords Atrial septal defect • NEXN • GATA4 • Actin • Mutation

1. Introduction

Congenital heart diseases (CHDs) are the leading cause of birth defects and genetic factors play important roles in their development. For a long time, it has been known that gene defects in cardiac transcriptional factors (TFs) including GATA4, NKX2.5, and TBX5 cause non-syndromic cardiac malformations.[1,2] Later research revealed cardiac structural genes to be a novel category of CHD candidates.[3–5] Mutant sarcomeric proteins such as MYH6 and ACTC1, were previously documented as major causes of familial cardiomyopathies for they impair the function of force generation in heart.[6] Novel mutations of MYH6 and ACTC1 were also found in CHD patients with or without signs of cardiomyopathy.[3,5] Moreover, reduced expression of MYH6 or ACTC1 affects atrial septation in embryonic chicken hearts, indicating a critical role of the

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structural proteins in heart development. Nevertheless, the known causative genes account for only a small proportion of CHDs, novel genetic factors need to be identified for their diagnosis and prediction.

The actin cytoskeleton plays a central role in cellular morphogenesis, migration, division, and cell communication. Actin-dependent events have been implicated in cell differentiation and embryonic development.[5,7,8] NEXN is an F-actin binding protein at cell–matrix junctions.[9] We previously reported it is highly expressed in muscle.[10] Recent findings showed that NEXN functions to stabilize the Z-disc for force generation in adult cardiomyocytes, loss-of-function mutations in *NEXN*, were identified in both dilated cardiomyopathy and familial hypertrophic cardiomyopathy patients. Mutant NEXN demonstrated a reduced actin binding ability or destabilization of Z-disc in patients.[11,12] Of note, the expression of NEXN is detectable at an early stage of cardiac development,[11] but whether it plays a role in CHDs is unknown.

In this study, we identified *NEXN* as a novel gene involved in ASDs using engineered mouse models and human genetic approaches. Moreover, we revealed its function to inhibit GATA4, a transcription factor crucial for genesis of septal defects in cardiac development.

2. Methods

2.1 Cell differentiation, transfection, and establishment of cell lines

P19cl6 cells were used for cardiomyocyte differentiation as described previously.[13] In brief, P19cl6 cells were cultured in growth medium (a-MEM with 5% serum) and transferred at ~80% confluence to differentiation medium [growth medium containing 1% DMSO (Sigma, USA)]. The first day of DMSO treatment was designated as Day 0 of differentiation.

Cells were transfected with plasmids or siRNAs (see Supplementary material online, *Table S1*) with Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. siRNA transfection efficiency was assessed by a Cy5 labelling method.[14] To establish stable cell lines, pIRES2-*NEXN* and control plasmid were transfected into P19cl6 cells, followed by 800 μg/mL G418 (Gibco, USA) selection. Cell lines from independent clones were maintained for further experiments.

2.2 Quantitative PCR and *NEXN* cloning

Total RNA was isolated from cells or tissues with TriZol reagent (Invitrogen, USA) and reverse-transcribed to cDNA with random primers and M-MLV reverse transcriptase (TransGen, China) as described. Quantitative PCR (qPCR) was carried out using an ABI 7300 Real Time PCR system. Triplicate cDNA aliquots were amplified using sequence-specific primers (see Supplementary material online, *Table S2*) with PowerSYBR (Applied Biosystems, USA). 18S rRNA was used as an internal control to normalize cDNA levels. Each experiment was performed independently at least three times. Methods for cloning *NEXN* coding region are described in Supplementary material online.

2.3 Immunoblotting

Immunoblotting was performed as previously described.[15] Primary antibodies to NEXN (BD Biosciences, CA, USA), GATA4 (Millipore, Germany), GATA6, and GAPDH (Santa Cruz, USA) were diluted in 5% BSA, followed by the secondary antibodies IRDye 680CW-conjugated goat anti-mouse IgG or IRDye 800CW-conjugated goat anti-rabbit IgG (LI-COR, USA). The blots were visualized using Odyssey (LI-COR, USA).

2.4 Immunofluorescence and F-actin staining

MF20 antibody is purchased from DSHB. F-actin was stained with phalloidin-tetramethylrhodamine B, the density of F-actin bundles was measured by Image J. For details, refer to Supplementary material online.

2.5 Generation and identification of *NEXN* transgenic mice

A 9.6 kb purified enzyme fragment was obtained from a TG construct containing a 2 kb complete *NEXN* cDNA under the control of the mouse beta-MHC (β-MHC) promoter. The fragment was microinjected into the pronuclei of fertilized oocytes derived from C57BL/6J × CBA mice. These mice were bred to C57BL/6J mice. Microinjection of pronuclei was carried out at the Transgenic Core Facility of Peking University. All protocols were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Peking University accredited by AAALAC international (IACUC No: IMM-Tian XL-02).

Genomic DNA from tail biopsies was used to identify the *NEXN* by PCR with the following transgene-specific primers: forward 5′-AGCACCTG CATTCTTACCAT-3′ and reverse 5′-TCCTGAGTGCTGAGCACAGT-3′; the LPL gene was amplified as an internal control with the forward primer 5′-GGATGGACGGTAAGAGTGATTC-3′ and the reverse primer 5′-A TCCAAGGGTAGCAGACAGGT-3′.

2.6 Histological analysis and echocardiography

The depth of anaesthesia was confirmed by lack of tail pinch response. Before isolation of tissues, mice were anaesthetized with ketamine 80 mg/kg and xylazine 16 mg/kg, ip. The adequacy of anaesthesia was monitored by testing respiration rate, mucous membrane colour, and tactile stimulus response. Whole hearts from E17.5, neonatal, and adult transgenic mice were excised, fixed, sectioned at 5 μm, and stained with haematoxylin/eosin by standard methods. To assess heart function, adult transgenic and wild-type control mice were anaesthetized with pentobarbital (50 mg/kg body weight). A heart rate of >500 beats/min was considered appropriate. Echocardiography was performed with ultrasonography (VisualSonics high-resolution Vevo 770 system, Canada). The heart was imaged using M-mode and two-dimensional measurements were made at the level of the papillary muscles in triplicate.

2.7 Study populations

We recruited 150 unrelated ASD patients and 150 age- and ethnically matched healthy controls from three medical centres in China. Patients were ascertained as having secundum ASD from their clinical files. Patients with other congenital or acquired heart diseases were excluded. To assess allele frequency, another 350 unrelated healthy individuals were tested. Samples of peripheral blood (2–3 mL) were collected from all participants. Written informed consent was obtained from all participants in the human population study which conformed to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board, Institute of Molecular Medicine at Peking University.

2.8 Mutation detection

DNA mutations from patients were detected by direct sequencing as we previously described.[16] Thirteen genomic fragments, including all coding exons and adjacent intronic regions of *NEXN*, were amplified and sequenced with an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA). For primers of *NEXN* for mutation analysis, see Supplementary material online, *Table S3*. The sequences generated were first compared with their wild-type sequences deposited in GenBank (NG_016625.1) and sequences from the control population in this study. Once a sequence variation was found, the original genomic DNA was then re-amplified and sequenced in both directions to confirm the change.

A mutation was defined by the following criteria: (i) it was found in an ASD-affected individual but not in 500 healthy controls and (ii) it occurred in an evolutionarily conserved region or apparently changed an amino-acid or caused an abnormal open reading frame, termination, splicing or binding site for transcription. The mutations were validated by functional

experiments. The minor allele frequency of any novel SNP detected in this study was calculated based on the genotyping data from the 500 controls by direct DNA sequencing.

2.9 Luciferase analysis

The 153 bp DNA fragment (from c.-52-122 to c.-22) from an ASD patient containing the c.-52-78C>A mutation or a wild-type fragment from control individuals was amplified by PCR. The fragments were cloned in a pGL3-luciferase vector. A 1.6 kb NEXN promoter was amplified by PCR using the forward primer 5′ −AAACTCGAGCCTTCACAGCCCGTTTG TAT-3′ and the reverse prime r 5′-AAATAAGCTTGCATCGAGCATG CATAAGAA-3′. The promoter was inserted upstream of the intron fragment. Lysates were collected after the constructs were transfected into C2C12 cells for 48 h, and luciferase activity was measured by the ratio of light units of firefly to *Renilla* with a dual-luciferase reporter assay kit (Promega, USA).

2.10 Statistical analysis

All data are shown as mean ± SEM. Statistical analysis was performed via a *t*-test to evaluate single-factor differences between two sets of data, or via ANOVA followed by the Bonferroni *post-hoc* test for multiple comparisons. The ASD ratio of TG mice was analysed by Pearson's χ^2 test or Fisher's exact test. '*n*' value refers to the number of independent experiments. $P < 0.05$ was considered statistically significant.

3. Results

3.1 NEXN deficiency promotes expression of cardiac contractile markers and transcription factor GATA4

To investigate the role of NEXN in cardiac development, we first explored its function in cardiac differentiation. P19cl6, a clone of the

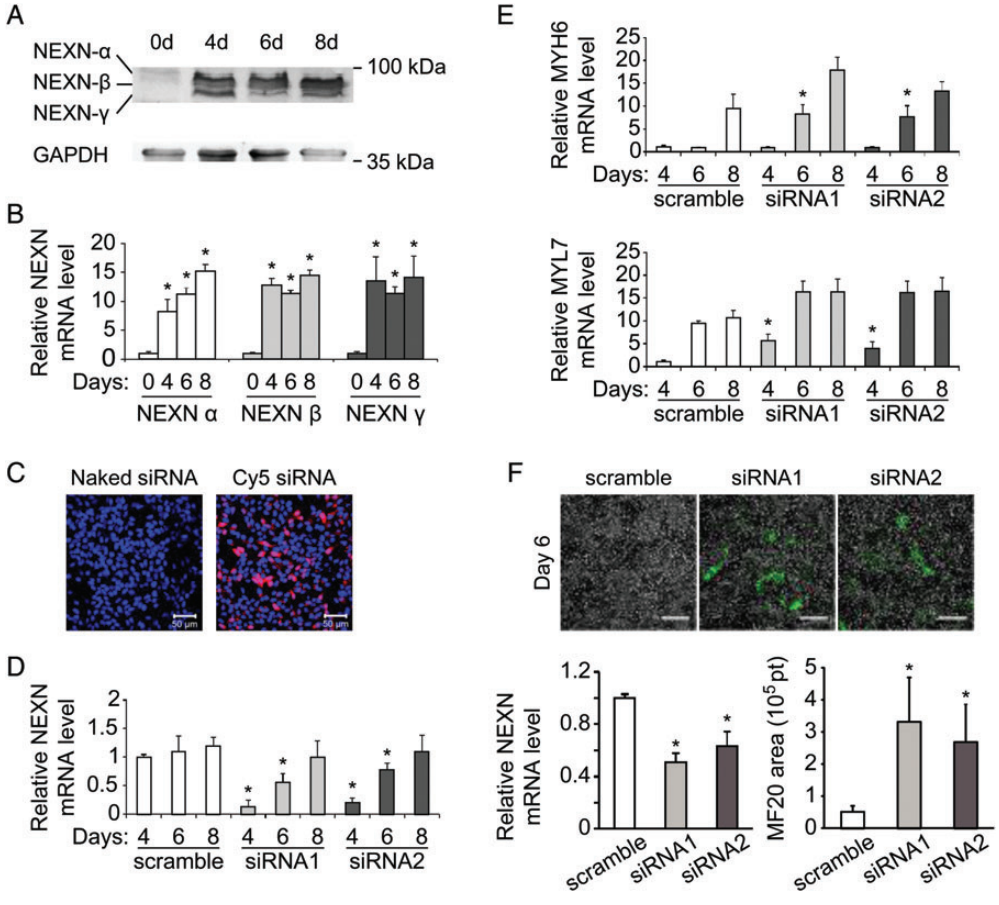

Figure 1 Knockdown of NEXN promotes the expression of cardiac contractile markers and GATA4. (*A*) Induced NEXN expression in cardiac differentiation by western blot with GAPDH as loading control. Three NEXN isoforms were shown. (*B*) Induced expression of NEXN isoforms in P19cl6 differentiation were detected by qPCR, fold change compared with control on Day 0 was shown (*$P < 0.05$ vs. Day 0), 18S as an internal control. (One-way ANOVA) $n = 5$. (*C*) Transfection efficiency of NEXN siRNA in p19cl6, labelled by Cy5. $n = 3$. (*D*) *NEXN* was knocked down on Day 4 and Day 6 by transient siRNA transfection on Day 2 in P19cl6 cells by qPCR, fold change compared with scramble control on Day 4 was shown (*$P < 0.05$ vs. scramble control at corresponding time point), 18S as an internal control. (One-way ANOVA) $n = 5$. (*E*) Induced MYH6, MLY7 expression was decreased during differentiation after *NEXN* siRNA transfection, fold change compared with scramble control on Day 4 was determined by qPCR. (*$P < 0.05$ vs. scramble control at corresponding time point), 18S as an internal control. (One-way ANOVA) $n = 5$. (*F*) Cells were stained for anti-sarcomeric MHC antibody (MF20, green) on Day 6 of differentiation after *NEXN* siRNA transient transfection in P19cl6 on Day 2. Knockdown efficiency of *NEXN* siRNA on Day 6 was determined by qPCR, fold change compared with scramble control was shown. Fluorescent density for MF20 of differentiated cells were quantitated by Image J. Scale bars, 250 μm (*$P < 0.05$ vs. scramble control). (One-way ANOVA) $n = 3$.

P19 mouse embryonic cell line, was chosen as the cell model for its extensive use. When P19CL6 cells were treated with dimethyl sulfoxide they began differentiation which followed the normal developmental program *in vivo*; cardiac transcription factors (TF) within 4 days and later contractile protein markers within 8–12 days was sequentially induced. It has been implicated that events occurred at an early stage (Days 0–4) of the differentiation program are crucial for determining cell fate.[17] To assess the NEXN expression in cardiac differentiation, we detected NEXN using PCR with primers according to the GenBank sequences. Three mouse *NEXN* isoforms, *NEXNα, NEXNβ,* and *NEXNγ* (GenBank No: HQ179984, HQ179982 and HQ179983, respectively) were identified. Both the RNA and protein levels of *NEXN* isoforms were induced as early as Day 4 in cardiac differentiation (*Figure 1A* and *B*). We then assessed the requirement for NEXN in differentiation by an siRNA-mediated knockdown experiment. Two effective siRNAs were transiently transfected into p19cl6 cells on Day 2. About 70% cells were transfected (*Figure 1C*). Quantitative PCR demonstrated that *NEXN* expression was decreased on Day 4 and Day 6 (*Figure 1D*). Interestingly, we found expression of the contractile markers for differentiated cardiomyocytes, MYH6 and MYL7, was increased in NEXN-deficient cells than that in controls (*Figure 1E*). Consistently, MF-20 staining revealed more differentiated cells in NEXN deficiency cells as early as Day 6 (*Figure 1F*). The findings that NEXN deficiency enhances expression of the contractile markers suggested induced NEXN provide a negative signal in regulating cardiac differentiation.

3.2 NEXN overexpression inhibits cardiac differentiation

We speculated that gain-of-function of NEXN has an inhibitory effect on cardiac differentiation. To test this, we established stable cell lines overexpressing *NEXNα*, the full-length and dominant *NEXN* isoform in cardiomyocytes. NEXNα overexpression was determined using isoform-specific qRT–PCR (*Figure 2A*). It was found that the expression levels of induced MYH6 and MYL7 in cell differentiation were markedly repressed by NEXN (*Figure 2B*) and differentiated cells were greatly reduced as well (*Figure 2C*), which confirmed our speculations.

3.3 NEXN inhibits expression of transcription factor GATA4 in cardiac differentiation

We then asked how NEXN regulates MYH6 and MYL7. It is known the transcriptional expression of these markers is directly controlled by cardiac transcription factors. We therefore examined expression of GATA4, TBX5, MEF2C, and NKX2.5, the most essential TFs for cardiac differentiation[18,19] using qPCR in the *NEXN* siRNA experiment. Surprisingly, we found only GATA4 was elevated in response to the NEXN insufficiency (*Figure 3A*) among the TFs. No significant changes were found in the other factors. Consistent with this, GATA4 was dramatically inhibited by NEXN overexpression, but the other TFs were not affected (*Figure 3B*).

GATA4 is a critical regulator controlling cardiac differentiation and embryonic heart development. Reduced function of GATA4 results in diverse CHDs.[20–22] Our data indicate that GATA4 is the responsible factor for NEXN dysfunction. To further confirm this, the specific effect of NEXN on GATA4 was determined by a rescue experiment. We transiently expressed GATA4 in the NEXN cell lines by plasmid transfection on Day 2, it was found the expression of MYH6 and MYL7 was reactivated (*Figure 3C*). In addition, other putative transcriptional target genes of GATA4, such as ANP, cTnC, andNCX1 were also detected in the differentiation process. We found the GATA4 targets were markedly suppressed by NEXN overexpression. In contrast, SCN5A, CaV1.2, RYR2, and MYL2, the markers whose expression is independent of GATA4, were normally induced (see Supplementary material online, *Figure S1*). Collectively, we concluded that NEXN inhibits GATA4, and then disrupts the cardiac differentiation program.

3.4 Transgenic mice with cardiac-selective overexpression of *NEXN* developed an atrial septal defect phenotype

To examine the effects of gain-of-function of NEXN on cardiac development *in vivo*, we generated cardiac-specific transgenic mice (TGM) expressing full length *NEXNα* under the control of a βMHC promoter,

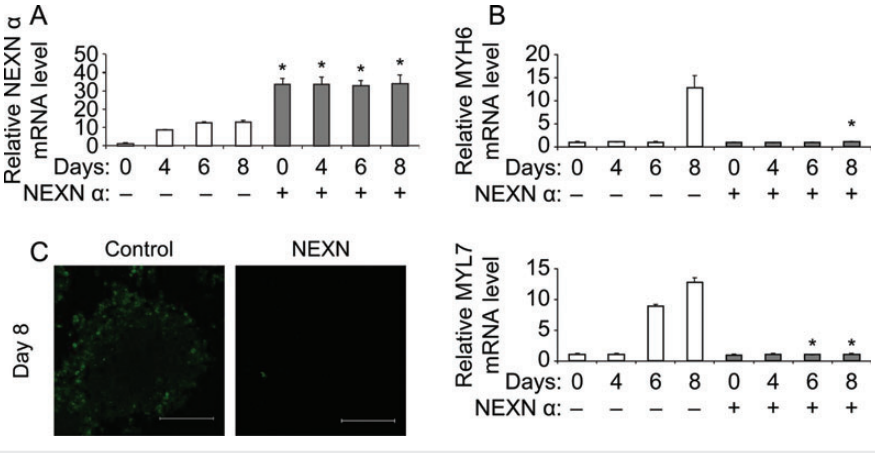

Figure 2 Overexpression of NEXN inhibits cardiac differentiation. (*A*) Overexpression of NEXNα in P19cl6 stable cell line by qPCR, fold change compared with non-transgene control on Day 0 was shown (*$P < 0.05$ vs. control at corresponding time point), 18S as an internal control. (One-way ANOVA) $n = 4$. (*B*) Expression of cardiac contractile markers in differentiation of P19cl6 cells stably overexpressing NEXNα by qPCR, fold change compared with non-transgene control on Day 0 was shown (*$P < 0.05$ vs. control at corresponding time point), 18S as an internal control. (One-way ANOVA) $n = 5$. (*C*) MF-20 staining on Day 8 in differentiation of P19cl6 cells stably overexpressing NEXNα. Scale bars, 100 μm. $n = 3$.

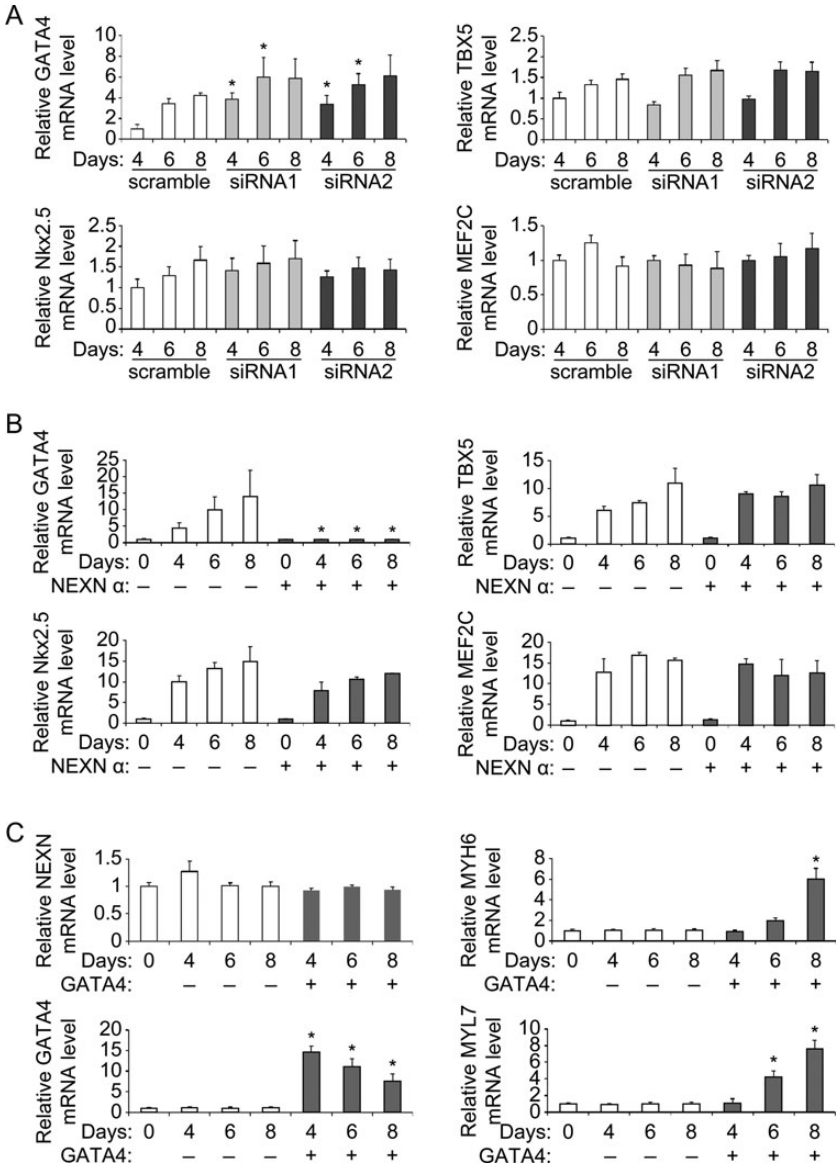

Figure 3 NEXN inhibits expression of GATA4 in cardiac differentiation. (*A*) Expression of cardiac transcription factors after *NEXN* siRNA transient transfection in differentiation of P19cl6 cells by qPCR, fold change compared with control on Day 0 was shown (*$P < 0.05$ vs. scramble control at corresponding time point), 18S as an internal control. (One-way ANOVA) $n = 5$. (*B*) Expression of cardiac transcription factors in differentiation of P19cl6 cells stably overexpressing NEXNα by qPCR, fold change compared with non-transgene control on Day 0 was shown (*$P < 0.05$ vs. control at corresponding time point), 18S as an internal control. (One-way ANOVA) $n = 4$. (*C*) GATA4 overexpression by transient transfection on Day 2 reactivated expression of MYH6 and MYL7 in differentiation of P19cl6 cells stably overexpressing NEXN using qPCR, fold change compared with vector control on Day 0 was shown (*$P < 0.05$ vs. control at corresponding time point), 18S as an internal control. (One-way ANOVA) $n = 4$.

which drives the expression of the transgenes in the embryonic heart. A typical genotyping result of TG and NTG mice by RT–PCR was demonstrated (*Figure 4A*).

Two mouse transgenic lines *TGM(βMHC-NEXN)L10* (line 10) and *TGM(βMHC-NEXN)L40* (line 40) were obtained and analysed. Their copy numbers of transgenes were 24 and 14, estimated by real-time PCR. We examined protein levels of NEXN in transgenic hearts at E17.5, when the βMHC promoter activity is high. For line 10, NEXN was shown a 4.5-fold increase compared with the non-transgenic (NTG) littermate controls. Not surprisingly, an inverse association between GATA4 and NEXN expression was demonstrated *(Figure 4B)*. The localization of transgene was shown by immunofluorescence using NEXN primary antibody and FITC-labelled secondary antibody. More fluorescence was observed in TG heart including ventricle and atrium, compared with control under the same experimental condition (see Supplementary material online, *Figure S2*). Next, morphological examination in line 10 was performed by analysing serial histological sections of the transgenic hearts. Unexpectedly, in the interatrial septum, we noticed clear openings with a high frequency in TGM at E17.5. In humans, an atrial–septal defect (ASD) appears at the foetal stage and usually closes on its own soon after birth. But if it persists, a pathological ASD occurs. In serial sections of neonatal hearts, we still found a much higher frequency

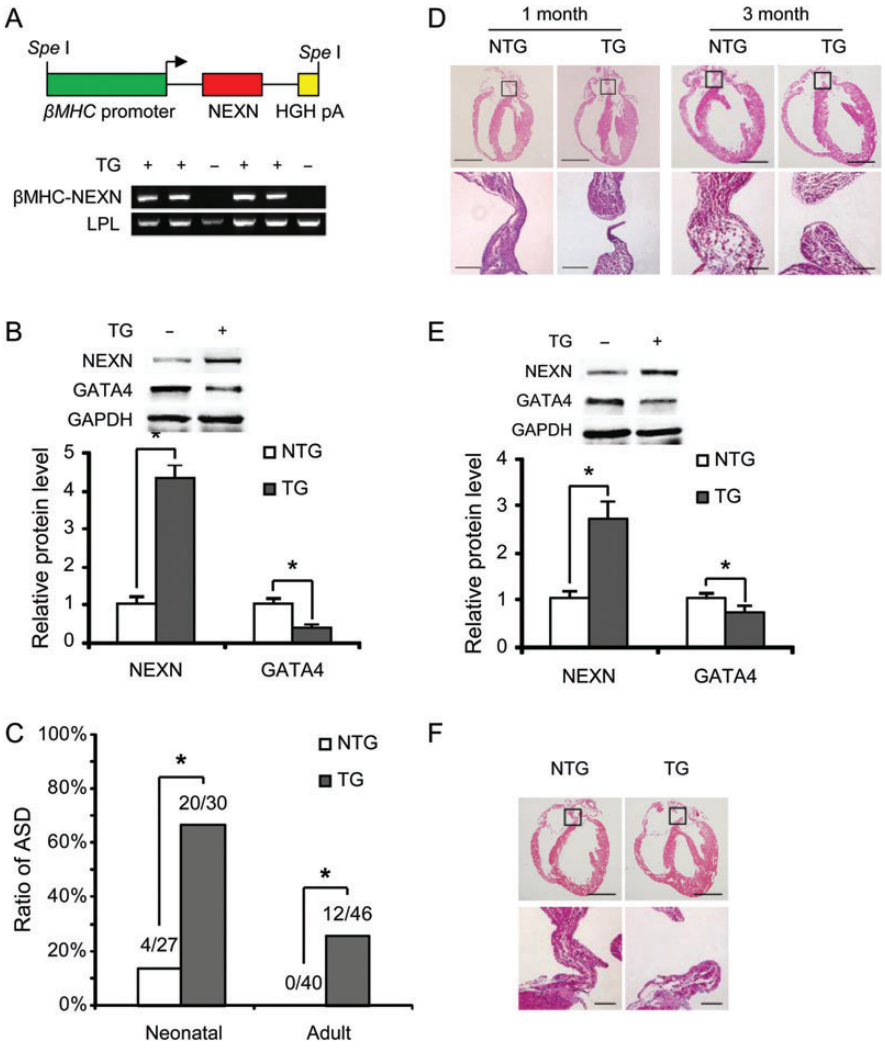

Figure 4 NEXN induces ASD in transgenic mice. (*A*) Schematic map of the *TGM (βMHC-NEXN)* construct and a genotyping result of TG and NTG mice by PCR with genomic DNA as template. (*B*) Protein levels of NEXN and GATA4 at E17.5 in transgenic hearts of *TGM (βMHC-NEXN) L10* by western blot (upper panel). Quantitative analysis of NEXN and GATA4 protein levels in TG and NTG groups (lower panel), fold change compared with NTG control was shown (*$P < 0.05$ vs. control, One-way ANOVA). Animal number for each experiment is 5, $n = 3$. (*C*) Ratio of ASD in postnatal and adult TG and NTG mice from line 10. Numbers of ASD mice and total mice for each group are shown. (*$P < 0.05$ by Fisher's exact test). (*D*) Morphologies of HE-stained heart sections from adult mice (1 month and 3 month) of line 10. Scale bars, 2 mm (upper panels) and 200 μm (lower panels). (*E*) Protein levels of NEXN and GATA4 at E17.5 in transgenic hearts of *TGM (βMHC-NEXN) L40* by western blot (upper panel). Quantitative analysis of NEXN and GATA4 protein levels in TG and NTG groups (lower panel), fold change compared with NTG control was shown (*$P < 0.05$ vs. control, One-way ANOVA). Animal number for each experiment is 3–4, $n = 3$. (*F*) Morphology of HE-stained heart sections from 3-month mice of line 40. Scale bars, 2 mm (upper panels) and 200 μm (lower panels).

of atrial–septal openings in the TG than that in the NTG group, which suggests a pathological morphology (*Figure 4C*). To confirm this, atrial–septal in adult TG and NTG littermate control mice were examined, and ASD was only found in the TGM group (*Figure 4C*). The transgenic hearts manifested an ostium secundum type of ASD (*Figure 4D*). Moreover, we verified the ASD phenotype and GATA4 expression in line 40 with a lower copy numbers of transgenes (*Figure 4E*). Atrial–septal openings in adult transgenic hearts could be found as well (*Figure 4F*). Besides, heart function in adult TG/NTG mice measured by ultrasonography (M-mode) showed no significant differences in heart rate, ejection fraction, or ventricular wall thickness (see Supplementary material online, *Table S4*). In short, we generated *TGM (βMHC-NEXN)* mice which demonstrated a definite ASD phenotype.

3.5 Identification of *NEXN* mutations in ASD patients

ASDs are among the most common types of congenital heart defects and result in a left-to-right shunting of oxygenated blood. The aetiology of ASD remains largely unknown. To evaluate the relevance of our finding of NEXN in mice to humans, *NEXN* mutations were screened in 150 sporadic secundum ASD patients and 150 healthy controls by direct sequencing. As a result, we identified five novel variants, which were not reported in the NCBI dbSNP database (see Supplementary material online, *Table S5*). Three variants were found only in ASD patients (*Figure 5A*). We then tested frequencies of the three novel variants in an additional ethnically matched 350 healthy controls by DNA

sequencing; none of the variants were identified. Next, six known ASD genes (*GATA4, GATA6, NKX2.5, TBX5, αMHC*, and *ACTC1*) were sequenced and they were not found in the *NEXN* variant carriers.

The variant c.-52-78C>A is located at the boundary of intron 1 adjacent to exon 2. A heterozygous (c.595A>G, p.K199E) variant and a homozygous (c.680T>C, p.L227S) variant that were evolutionally conserved resulted in amino-acid changes. K199E and L227S reside in exon7, the region adjacent to a coiled-coil domain. The conservation analysis and locations of the ASD mutations were shown in *Figure 5B*.

The clinical data showed that the three mutation carriers were diagnosed as having secundum ASDs with no clinical manifestations of cardiomyopathy or other self-reported inherited diseases (*Table 1*). We could not determine whether other family members of the carriers had the same mutations because we were unable to obtain their informed consent.

In addition to the mutations, we also identified two known SNPs, the frequency of each having no significant difference between ASD cases and controls.

3.6 *NEXN* mutations in ASD patients inhibit GATA4

To determine whether the novel *NEXN* mutations in ASD are functional, mutated sequences were cloned into plasmids and compared with their wild-type (WT) controls. It was reported that intron 1 contains cis-regulatory sequences for certain gene expression.[23] For the variant c.-52-78C>A located in first intron of NEXN, we amplified an intronic fragment (from c.-52-122 to c.-22) containing c.-52-78C>A from the ASD patient or a wild-type fragment from a control individual. The fragments were subcloned into pGL3 vector with NEXN promoter. The regulatory effect of c.-52-78C>A on gene expression was assessed by a well-established luciferase assay. We found that c.-52-78C>A increased the promoter transcriptional activity compared with the WT sequence (*Figure 6A*). Thus, the mutation may cause GATA4 inhibition by increasing NEXN yield.

For the other two ASD mutations in the *NEXN* coding region, K199E and L227S, we first investigated their effects on GATA4 in P19cl6 cells by transient transfection. Q131E, a known loss-of-function mutation of *NEXN* in familial hypertrophic cardiomyopathy, was taken as a control.[12] We found effects of Mutant (MT)-NEXNs and WT-NEXN on GATA4 expression is different. The ASD mutants, both K199E and L227S, showed specific and stronger inhibitory effects on GATA4 than that of WT-NEXN, in contrast, Q131E showed no inhibition (*Figure 6B*). These results were further validated by real-time PCR (*Figure 6C*). Moreover, effects of WT- or MT-NEXN on F-actin bundle formation in C2C12 were determined, it was shown K199E and L227S, but not Q131E, enhanced F-actin bundling (*Figure 6D*). Thus, we demonstrated that all three *NEXN* mutations from ASD patients produced gain-of-function, similar to overexpression of NEXN. GATA4 was inhibited as a result. These consistent data in mice and humans suggested that NEXN interferes with cardiac development due to GATA4 inhibition.

4. Discussion

The most important findings in this study are as follows: (i) *NEXN* is identified as a novel gene for ASD and (ii) we found GATA4 is the downstream molecule that is functionally linked with NEXN associated ASD. We have provided the following evidences: (i) NEXN overexpression specifically inhibits expression of GATA4 and its downstream targets *in vitro*; (ii) NEXN produced ASD phenotype in transgenic heart of mice; (iii) three *NEXN* mutations were found in 150 ASD patients; and (iv) functional analysis showed the ASD mutations inhibited

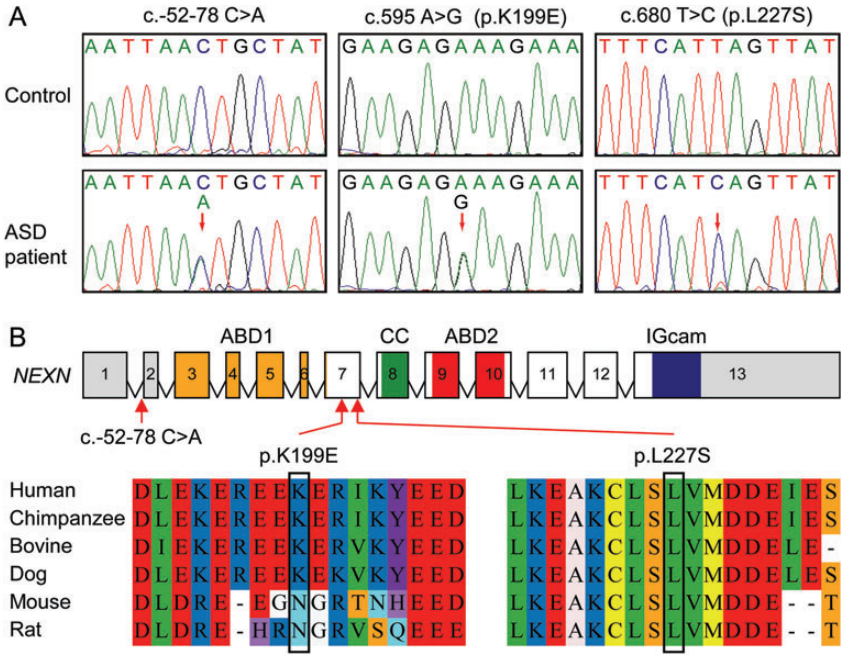

Figure 5 *NEXN* mutations in ASD patients. (*A*) Sequences of WT- (upper) and MT-*NEXN* (lower) in ASD and control individuals. (*B*) Conservation analysis and locations of novel *NEXN* mutations in ASD patients. Squares denote exons; ∨ denote introns; UTR regions and functional domains are indicated by different colours. ABD, actin-binding domain; CC, coiled-coil; IGcam, immunoglobulin superfamily class.

Table 1 Phenotype of patients with NEXN mutations

Nucleotide change	Amino acid change	Gender	Age	Anatomic size (mm)	RV pressure (mmHg)	PA pressure (mmHg)	LAD (mm)	LVDd (mm)	FS (%)	EF (%)	ECG
c.-52-78C>A	–	M	16	20	48/0	48/20	29	38	35	68	RBBB
c.595A>G	p. K199E	F	22	26	40/0	40/9	31	39	36	73	RBBB
c.680 T>C	p. L227S	M	8	11	25/5–13	25/12–17	29	40	35	65	–

RV, right ventricle; PA, pulmonary artery; LAD, left-atrial dimension; LVDd, left-ventricular end-diastolic dimension; FS, fractional shortening; EF, ejection fraction; ECG, electrocardiogram; M, male; F, female; RBBB, right bundle-branch block.

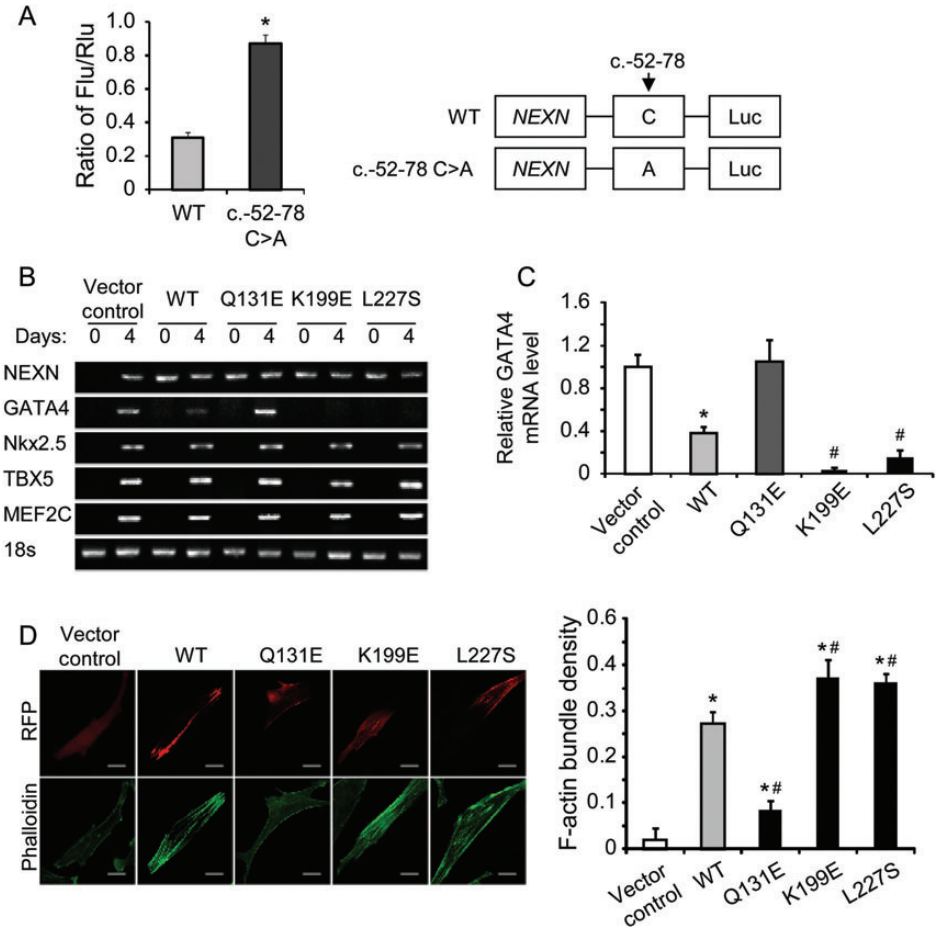

Figure 6 *NEXN* mutations in ASD patients inhibit GATA4. (*A*) Left: c.-52-78C>A increased *NEXN* promoter activity as determined by luciferase analysis. Right: Schematic map of the pGL3-luciferase construct design for mutation analysis. Flu, firefly luciferase; Rlu, *Renilla* luciferase. (*$P < 0.05$ vs. WT control) (Students *t*-test) $n = 5$. (*B*) GATA4 and NEXN expression by semi-quantative RT–PCR in P19cl6 cells treated with wt- or mt-NEXN transfection followed by differentiation. 18 s as an internal control. $n = 5$. (*C*) As described in (*B*), GATA4 expression was quantified by qPCR in independent experiments, fold change compared with vector control was shown (*$P < 0.05$ vs. vector control; #$P < 0.05$ vs. WT *NEXN*), (One-way ANOVA) $n = 4$. (*D*) Gain-of-function of NEXN enhances F-actin bundling. Left: Effects of WT- or MT-NEXN on F-actin bundle formation after their transfection in C2C12. F-actin was stained with phalloidin (green). NEXN-RFP (red) and RFP vector control are illustrated. Scale bars, 10 μm. Right: Quantification of F-actin bundles by Image J. (*$P < 0.05$ vs. vector control; #$P < 0.05$ vs. WT *NEXN*). (One-way ANOVA) $n = 3$.

GATA4 expression. Taken together, we concluded gain-of-function in NEXN inhibits GATA4 and cardiac development.

Actin-dependent events in differentiation have been described in various cell types. Lack of ACTC1, the only actin in embryonic heart, disrupts cardiac differentiation and produces delayed looping and reduced atrial septation in chick.[5] In combination with our present data of an F-actin regulator caused an ASD phenotype, we considered that proper actin cytoskeleton is critical for the formation of the atrial septum.

How structural disorders impair heart morphogenesis was not clear. Mutant ACTC1 and MYH6 exhibited reduced function to prevent

normal septal development as downstream targets directed by transcription factors. Unlike ACTC1 and MYH6, the ASD phenotype by NEXN was generated by increased expression or enhanced function, suggesting a different mechanism. An important finding of this study is that NEXN exhibited a specific inhibition effect on GATA4, which contributes, at least partially, to the pathological process of ASD. Thus, a novel molecular connection from NEXN to a transcription factor is established.

GATA4 is a critical regulator of cardiac gene expression where it controls cardiomyocyte differentiation, embryonic development, and stress responsiveness of the adult heart. However, transcriptional regulation of GATA4 is largely unknown. We presented the first evidence that GATA4 is regulated by an actin regulator, suggesting a new regulatory interaction between morphogenesis and gene expression.

Unlike other TFs, GATA4 possesses a function in cell morphology in regulating the actin cytoskeleton, serving as a nuclear mediator of the Rho/rock pathway.[24] Overexpression of GATA4 induces F-actin bundle formation and sarcomere reorganization in the adult heart.[24,25] We speculated that, as positive regulators of F-actin bundling in cardiomyocytes, NEXN and GATA4 may have negative feedback regulation in between. Ounzain *et al*.[26] reported that F-actin binding protein STARS is repressed by GATA4 in the embryonic and adult heart. In our case, endogenous NEXN was down-regulated by GATA4 overexpression (see Supplementary material online, *Figure S3*). On the other hand, we provide evidence in this study that gain-of-function of NEXN enhanced F-actin bundling, as a consequence, GATA4 was negatively regulated. NEXN may not be a direct regulator interacting with GATA4 for the following reasons: (i) NEXN resides in cytoplasm while GATA4 is nuclear-localized; (ii) no translocation of NEXN caused by its overexpression was found in our experiments. Therefore, we presumed there exits one or more mediators involved in the NEXN-GATA4 pathway. For example, c-Abl, which regulates actin dynamics in the cytoplasm and shuttles between the nucleus and the cytoplasm, is a potential candidate. Although the underlying molecular processes of how NEXN inhibits GATA4 remains to be clarified, our findings indicate that regulatory interactions between structural proteins and TFs during cardiac development are bidirectional.

GATA6 is another critical GATA member in cardiac development and disorders, particularly in regulating cardiac hypertrophy.[27] It has been reported that GATA4 and GATA6 act cooperatively and they have functional redundancy in heart.[28] To determine the role of GATA6 in the context of NEXN overexpression, we have measured GATA6 expression *in vitro* and *in vivo*. It was found GATA6 expression was decreased, although the alteration was not as dramatic as that of GATA4 (see Supplementary material online, *Figure S4*). To further determine the role of GATA6 in the pathogenesis of ASD induced by NEXN, we detected effect of NEXN mutations, K199E and L227S, on GATA6 expression by real time PCR. It was found that the ASD mutations did not cause significant GATA6 inhibition (see Supplementary material online, *Figure S5*). In our opinion, the response of GATA6 to NEXN is not as sensitive as that of GATA4 in cardiomyocytes. However, GATA6 may play a more important role in vascular smooth muscle cells.[29]

In this study, we generated a mammalian model displaying the ASD phenotype in *NEXN* transgenic heart under the control of the βMHC promoter. We had expected a spectrum of heart defects, especially ventricular phenotypes, to be produced in this transgenic model. We presumed the specific ASD phenotype was a consequence of insufficient expression of GATA4 occurred at an early developmental stage. As GATA4 is a dosage-sensitive and stage-dependent regulator,[30,31] deficiency of GATA4 in mice leads to various heart abnormalities including septal defects depending on the context.[22] In humans, loss-of-function mutations of GATA4 particularly result in ASDs.

NEXN is a causal factor of cardiomyopathies. Here we report NEXN produced morphological defects. Three NEXN mutations have been identified from 150 ASD patients in our study, we thereby estimate the frequency of NEXN mutations in sporadic ASD patients was about 2%, similar to that of GATA4. Hence, systematic screening for this gene should be considered to further elucidate its impact on cardiac development. Overall, we have identified *NEXN* as a new gene involved in ASDs and have established a connection between a cardiac structural protein and a transcription factor, which will provide new insights into the pathogenesis and the genetic basis of congenital heart diseases.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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