



Axenfeld-Rieger syndrome-associated mutants of the transcription factor FOXC1 abnormally regulate NKX2-5 in model zebrafish embryos

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FOXC1 is a member of the forkhead family of transcription factors, and whose function is poorly understood. A variety of FOXC1 mutants have been identified in patients diagnosed with the autosomal dominant disease Axenfeld-Rieger syndrome, which is mainly characterized by abnormal development of the eyes, particularly those who also have accompanying congenital heart defects (CHD). However, the role of FOXC1 in CHD, and how these mutations might impact FOXC1 function, remains elusive. Our previous work provided one clue to possible function, demonstrating that zebrafish *foxc1a*, an orthologue of human FOXC1 essential for heart development, directly regulates the expression of *nkx2.5*, encoding a transcriptional regulator of cardiac progenitor cells. Abnormal expression of *Nkx2-5* leads to CHD in mice and is also associated with CHD patients. Whether this link extends to the human system, however, requires investigation. In this study, we demonstrate that FOXC1 does regulate human NKX2-5 expression in a dose-dependent manner via direct binding to its proximal promoter. A comparison of FOXC1 mutant function in the rat cardiac cell line H9c2 and zebrafish embryos suggested that the zebrafish embryos might serve as a more representative model system than the H9c2 cells. Finally, we noted that three of the Axenfeld-Rieger syndrome FOXC1 mutations tested increased, whereas a fourth repressed the expression of NKX2-5. These results imply that mutant FOXC1s might play etiological roles in CHD by abnormally regulating NKX2-5 in the patients. And zebrafish embryos can serve as a useful *in vivo* platform for rapidly evaluating disease-causing roles of mutated genes.

Axenfeld-Rieger syndrome (ARS) is an autosomal dominant disease, mainly characterized by abnormal development of the anterior segment of the eyes. Other than eye defects, ARS patients are also accompanied by other symptoms including facial, dental, umbilical defects, hearing loss, skeletal abnormalities, and/or congenital heart defects (CHD) that are characterized by atrial septal defects, mitral valve dysplasia, and heart failure (1). The ARS patients are genetically heterogeneous.

About 40% of ARS patients are reported to carry mutations in FOXC1 or PITX2 (2–6). Other mutations in several chromosomal loci, including PAX6, 13q14, and 16q24 have also been implicated in ARS (4, 7). Among the patients, the ones who carry FOXC1 mutations are more likely to have CHD than those carrying mutant PITX2 (1, 8). The facts suggest that FOXC1 mutants might be the pathogenic genes of CHD.

Foxc1, a dual transcription factor, is known to play crucial roles in vertebrate embryogenesis. Heterozygous mice carrying spontaneous mutation of *Foxc1* (*Foxc1^{chl/+}*) display ocular abnormalities (9). The homozygous mice (*Foxc1^{chl}*) die prenatally or perinatally with hydrocephalus, multiple skeletal, ocular, genitourinary, and cardiovascular defects (10), the similar lethal phenotypes occurring in *Foxc1* knockout mice (*Foxc1^{lacZ}*) (11). In zebrafish, embryos carrying knockout alleles of *foxc1a*, the orthologous gene of mammalian *Foxc1*, die at 9–10 days post-fertilization with severe heart defects, disrupted somites, and smaller eyes (12, 13). These results reveal that the abnormal phenotypes of the mice and zebrafish carrying *Foxc1* null alleles phenocopy the syndrome of ARS patients with CHD carrying FOXC1 mutants. However, the types of FOXC1 mutants carried by ARS patients with CHD are different from those carried by *Foxc1* knockout model animals. First, the patients each carries one copy of mutated FOXC1 (4–6). Second, except FOXC1 (c.210_210delG/p.Q70Hfs*8) that has a premature stop codon and is likely to be a null allele (14), the other 7 of the 8 FOXC1 mutants identified from ARS patients with CHD including FOXC1(c.245G>C/p.S82T), FOXC1(c.255-256GC>TT/p.L86F), FOXC1(c.335T>C/p.F112S), FOXC1(c.253G>C/p.A85P), FOXC1(c.380T>G/p.R127L), FOXC1(c.508C>T/p.R170W), and FOXC1(c.446G>A/p.G149D) hold missense mutations and are not likely to be null alleles (14–21). Third, some patients that carry FOXC1 mutants do not have the syndrome of CHD (4, 5, 8). Together with the facts that *Foxc1^{+/-}* heterozygous mice and zebrafish have normal heart development (9, 12, 22), it is reasonable to conclude that *Foxc1* or *foxc1a* knockout model animals should not explain the malfunctioned consequences of the human FOXC1 mutants and the roles of the FOXC1 mutants in CHD remains to be answered.

Previously, we reported that *Foxc1a* controls zebrafish cardiac development by directly regulating the expression of

This article contains supporting information.

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nkx2.5, encoding a transcriptional regulator of cardiac progenitor cells (12). On the other hand, *NKX2-5* mutations are reported to underlie a variety of CHD in humans, mainly including septal defects and atrioventricular conduction defects (23–26). *Nkx2-5* heterozygous knockout mice only have half the normal number of atrioventricular node cells (27) and about 40% of *Nkx2-5* heterozygous knockout mice in the inbred C57BL/6 strain background have atrial septal defect (ASD) and/or ventricular septal defect (VSD) (27–31), the similar cardiac abnormalities to those occurred in human CHD patients. Moreover, some mutations in the *NKX2-5* promoter identified from the CHD patients are responsible for enhancing the expression of *NKX2-5* (32, 33). These results imply either abnormally increased or abnormally reduced expressions of *NKX2-5* should be involved in CHD. Therefore, we hypothesize that the *FOXC1* mutants identified from ARS patients with CHD might be the etiology of their CHD by abnormally altering the expression level of *NKX2-5* in the patients. In this study, we first confirm that the mechanism of *Foxc1* controlling the expression of *Nkx2-5* is conserved in mammals. By evaluating the activity of the *FOXC1* mutants in regulating the expression of *NKX2-5* using rat cardiac cell line H9c2 and zebrafish embryos, we then demonstrate that the malfunctions of the *FOXC1* mutants displaying in zebrafish embryos but not H9c2 cells are consistent with their roles in regulating the expression of *NKX2-5* in CHD patients and *FOXC1*(c.255-256GC>TT/p.L86F), *FOXC1*(c.335T>C/p.F112S), and *FOXC1*(c.446G>A/p.G149D) might be involved in CHD by abnormally increasing the expression of *NKX2-5*, whereas *FOXC1*(c.508C>T/p.R170W) might do it by abnormally repressing the expression of *NKX2-5* in the heart of patients.

Results

FOXC1 regulates *NKX2-5* expression in a dose-dependent manner via direct binding to the proximal promoter of *NKX2-5* in H9c2 cells

Previously, we reported that zebrafish *Foxc1a* regulated *nkx2.5* expression via direct binding to its promoter during zebrafish heart development. To explore whether mammalian *Foxc1* plays the same role in regulating the expression of *Nkx2-5*, we first performed a bioinformatic assay on evolutionary conserved regions (ECR) between the promoters of zebrafish *nkx2.5* and mammalian *Nkx2-5* using ECR Browser (RRID:SCR_001052). The results showed that no ECR was found between the promoters of zebrafish and mammals although the ECR is present among the mammalian *Nkx2-5* promoters of human, monkey, mouse, and rat (Fig. S1A). However, we found that there are six canonical *FOXC1*-binding sites with the core sequence of (A/C)AA(T/C)A in the human *NKX2-5* promoter (Fig. 1A) although the sequences of the candidate-binding sites are not 100% identical to those of zebrafish *nkx2.5* promoter (12) by searching the JASPAR database (RRID:SCR_003030). To test whether the promoter containing the presumptive binding sites are directly responsive to mammalian *Foxc1*, we then examined the expression of *Nkx2-5* in the rat cardiomyocytes cell line H9c2 overexpressed or knocked down rat *Foxc1*, respectively. Western blotting analyses revealed that overexpression of *Foxc1* increased the expression (Fig. S2, A, B, and E),

whereas knockdown of *Foxc1* decreased the expression of *Nkx2-5* (Fig. S2, C, D, F, and G). The results suggest that the expression of mammalian *Nkx2-5* is regulated by *Foxc1*.

To investigate whether the regulation is direct, we performed Dual-Luciferase Reporter Assay on the human *NKX2-5* promoter. The results showed that the relative firefly luciferase activity driven by the 1791-bp flanking sequence upstream of the *NKX2-5* translation start site (referred as “promoter” thereafter) was increased by human *FOXC1* overexpression in a dose-dependent manner (Fig. 1B). The truncated promoter with different lengths (1149 and 629 bp) also showed more activity induced by WT *FOXC1* than by the functional null mutant *FOXC1*(p.Q70Hfs*8), although the 629-bp promoter had weaker activity than the 1149-bp promoter, and the 1791-bp promoter had similar activity to the 629-bp promoter (Fig. 1C). The results imply that there are at least two binding sites of *FOXC1* in the 1149-bp promoter of *NKX2-5*.

To identify the binding sites of *FOXC1* in the *NKX2-5* promoter, we performed a ChIP PCR assay on the chromatin isolated from H9c2 cells co-transfected with human *NKX2-5* promoter and the expression plasmid of human *FOXC1*. The results showed that *FOXC1* was significantly enriched in the S5 region (−779 to −979 bp) of human *NKX2-5* promoter (Fig. 1D), which is consistent with the bioinformatical results that three putative *FOXC1*-binding sites lie in the S5 of the promoter (Fig. 1A). Dual-Luciferase Reporter Assay on the mutant promoters carrying mutated core sequences of the putative *FOXC1*-binding sites individually or simultaneously showed that mutations of the three putative *FOXC1*-binding sites in the S5 were responsible for the reduced activity of the mutant promoters regulated by *FOXC1* (Fig. 1, E and F). Taken together, our results demonstrate that the molecular mechanism of *FOXC1* directly regulating the *NKX2-5* expression is conserved in mammals.

The *FOXC1* mutants identified from ARS patients with CHD exhibit different activities of regulating the *NKX2-5* expression in H9c2 cells

Because *FOXC1* directly controls the expression of *NKX2-5*, we evaluated the functions of the eight *FOXC1* mutants (Fig. S3, A and B) identified from ARS patients with CHD (14–21) on regulating the transcriptional activity of *NKX2-5* in H9c2 cells to uncover their roles in CHD by overexpressing the same amount of their expression vectors that produced the same level proteins of the *FOXC1* mutants in H9c2 cells except for *FOXC1*(c.210_210delG), which is presumably encoded in the N-terminal part with 70 aa of *FOXC1* that would not be recognized by the polyclonal antibody in the western blotting (Fig. S3, B and C). The results of the Dual-Luciferase Reporter Assay showed that 8 mutants fell into 4 different categories based on their functions. *FOXC1*(p.Q70Hfs*8) and *FOXC1*(p.S82T) displayed significantly reduced, although still having residual, transcriptional activity of regulating the *NKX2-5* promoter compared with the WT *FOXC1* (54.17 and 54.08% activities of WT, respectively) (Fig. 2, A and B). On the contrary, *FOXC1*(p.A85P) and *FOXC1*(p.R127L) had similar transcriptional activity of regulating the *NKX2-5* promoter to the WT *FOXC1* (Fig. 2, C and D). On the other hand, *FOXC1*(p.L86F) and

FOXC1 mutants abnormally regulate NKX2-5 expression

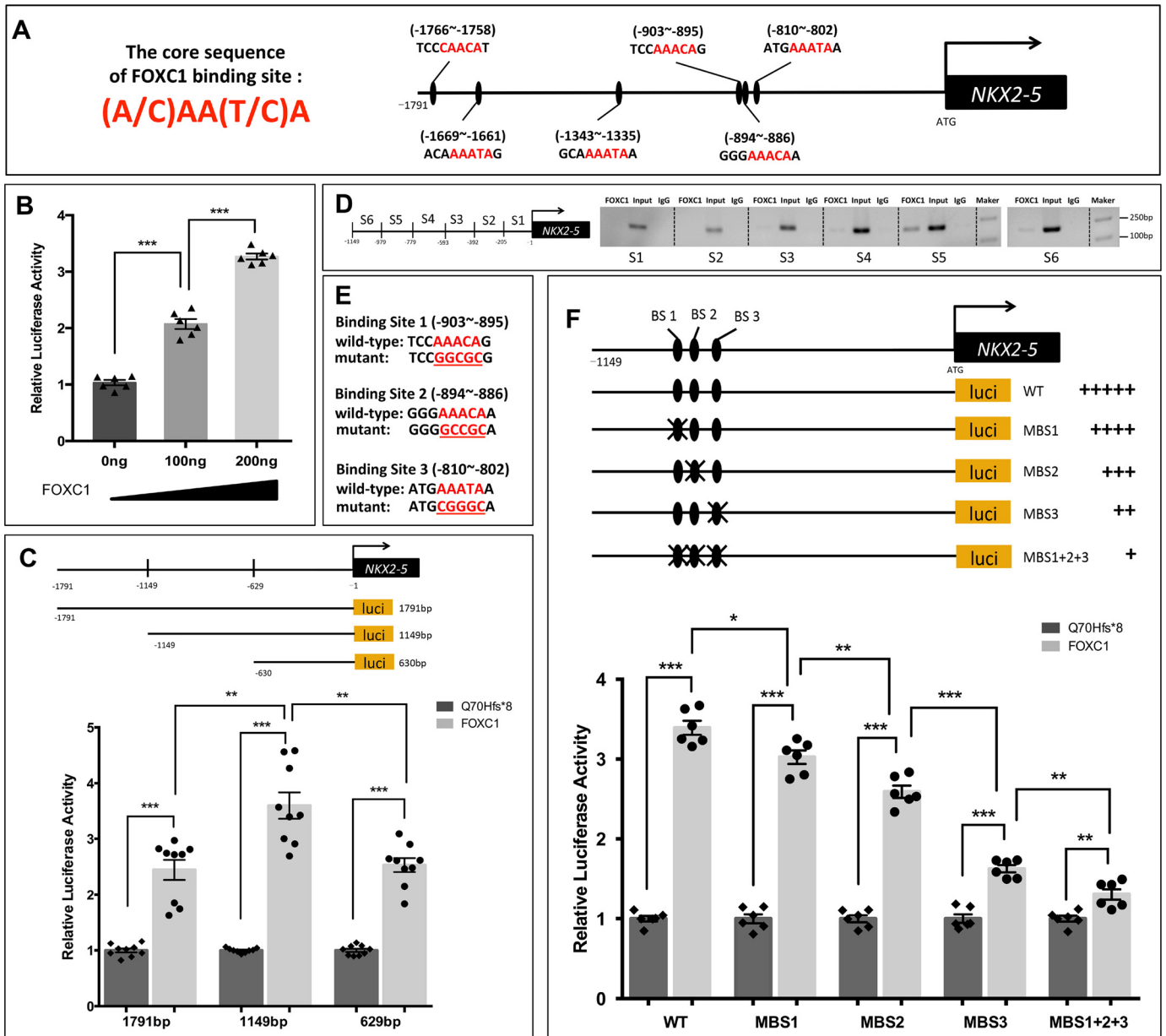


Figure 1. FOXC1 directly regulates the expression of NKX2-5 by binding to its proximal promoter in H9c2 cells. *A*, schematic showing putative FOXC1 transcription factor-binding sites in a 1791-bp 5'-flanking sequence upstream of the NKX2-5 translation start site (ATG). *B*, results of a Dual-Luciferase Reporter Assay showing the responses of the NKX2-5 promoter to different doses of FOXC1. *C*, schematic (top) showing the firefly luciferase reporter expression constructs comprising the different lengths of upstream regulatory sequences of NKX2-5, namely 1791, 1149, or 630 bp, and the coding sequences of NKX2-5 or firefly luciferase, and the results (below) of the Dual-Luciferase Reporter Assay on the three expression constructs. *D*, schematic (left) showing the dissection of the 1149-bp regulatory sequences of NKX2-5 into S1–S6 regions and the results of the ChIP-PCR assay (right) indicating that S5 contains FOXC1-binding sites. *E*, the WT sequences and location of FOXC1-binding sites (BS) in S5 of the NKX2-5 regulatory sequence (top), and the mutant FOXC1-binding sites (MBS) with changed core sequence. *F*, schematic (top) showing the reporter expression constructs carrying WT BS or MBS of FOXC1, and the results (below) of Dual-Luciferase Reporter Assay on the five expression constructs. x axis (*B*, *C*, and *F*), the amount of overexpressed FOXC1 (*B*), the reporter expression constructs with different lengths of regulatory sequences (*C*), or the reporter expression constructs carrying WT BS or MBS of FOXC1 (*F*). Light gray columns (*C* and *F*), transfected with WT FOXC1; dark gray columns (*C* and *F*), transfected with the same amount of functional null mutated FOXC1 (p.Q70Hfs*8) as control. y axis (*B*, *C*, and *F*), relative activity of firefly luciferase reporter. The relative luciferase activity in the control group (dark gray columns) was normalized as 1.0 and the relative firefly luciferase activity in the experimental group (light gray columns) was shown relative to the control group. ATG, start codon.

FOXC1(p.F112S) exhibited more transcriptional activity of regulating the NKX2-5 promoter than the WT (126.21 and 148.85% activities of WT, respectively) (Fig. 2, *E* and *F*), whereas FOXC1(p.G149D) and FOXC1(p.R170W) significantly inhibited the transcriptional activity of regulating the NKX2-5 promoter (65.57 and 37.81% activities of control group without overexpression of WT FOXC1) (Fig. 2, *G* and *H*).

The FOXC1 mutants identified from ARS patients with CHD display different activities of regulating the *nkx2.5* expression in zebrafish embryos

To further evaluate the functions of FOXC1 mutants on regulating the transcriptional activity of endogenous NKX2-5, we examined the expression of *nkx2.5* in the 14-h post-fertilization (hpf) zebrafish embryos overexpressed with WT FOXC1 or

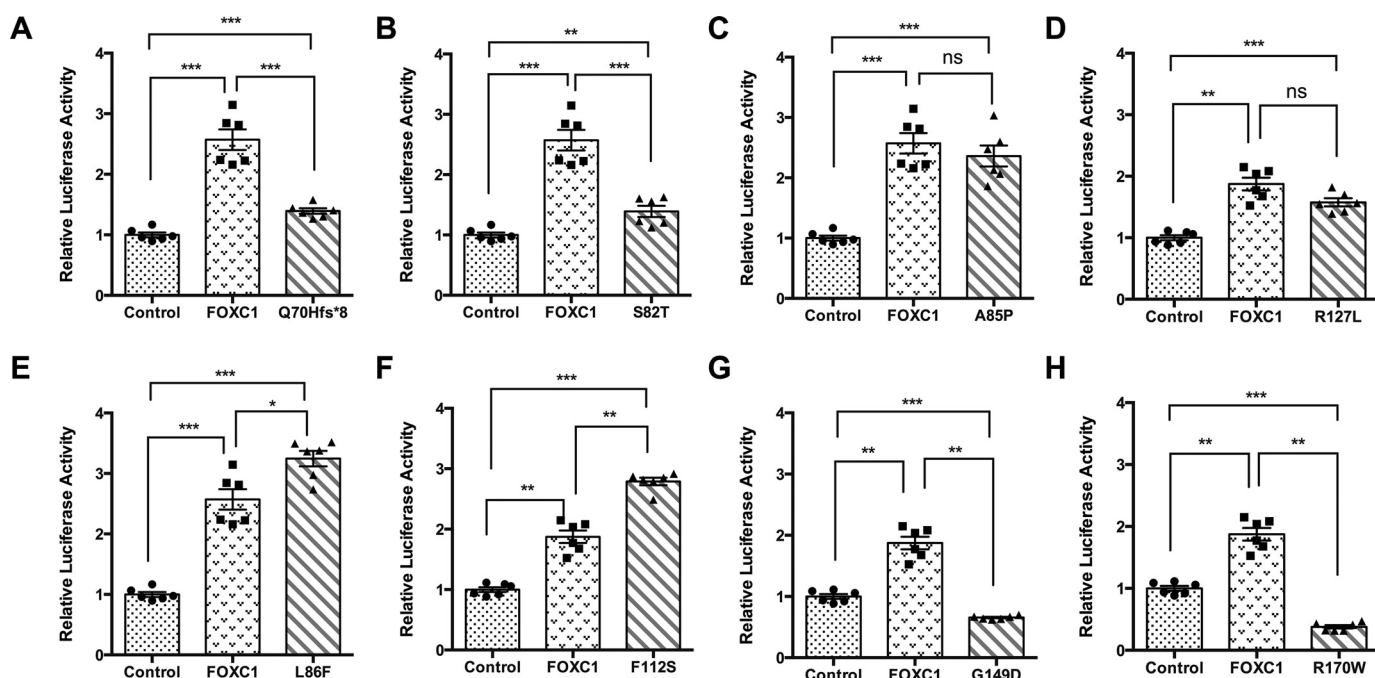


Figure 2. FOXC1 mutants display different abilities of transcriptionally regulating the expression of *NKX2-5* in H9c2 cell line. Dual-Luciferase Reporter Assays were carried out in H9c2 cells to examine the FOXC1 mutants' abilities of transcriptionally regulating the 1149-bp *NKX2-5* promoter. *x* axis, different expression plasmids overexpressed in H9c2 cells. *Control*, the control group only transfected with empty expression plasmid pCMV5. *Other columns*, the experimental group transfected with the expression plasmid of FOXC1 mutants. *y* axis, the relative expression level of firefly luciferase driven by the 1149-bp promoter of *NKX2-5*. The relative firefly luciferase activity in the control group (*Control*) was normalized as 1.0 and the relative expression levels of firefly luciferase in the mutants or WT FOXC1 group was shown relative to the control group.

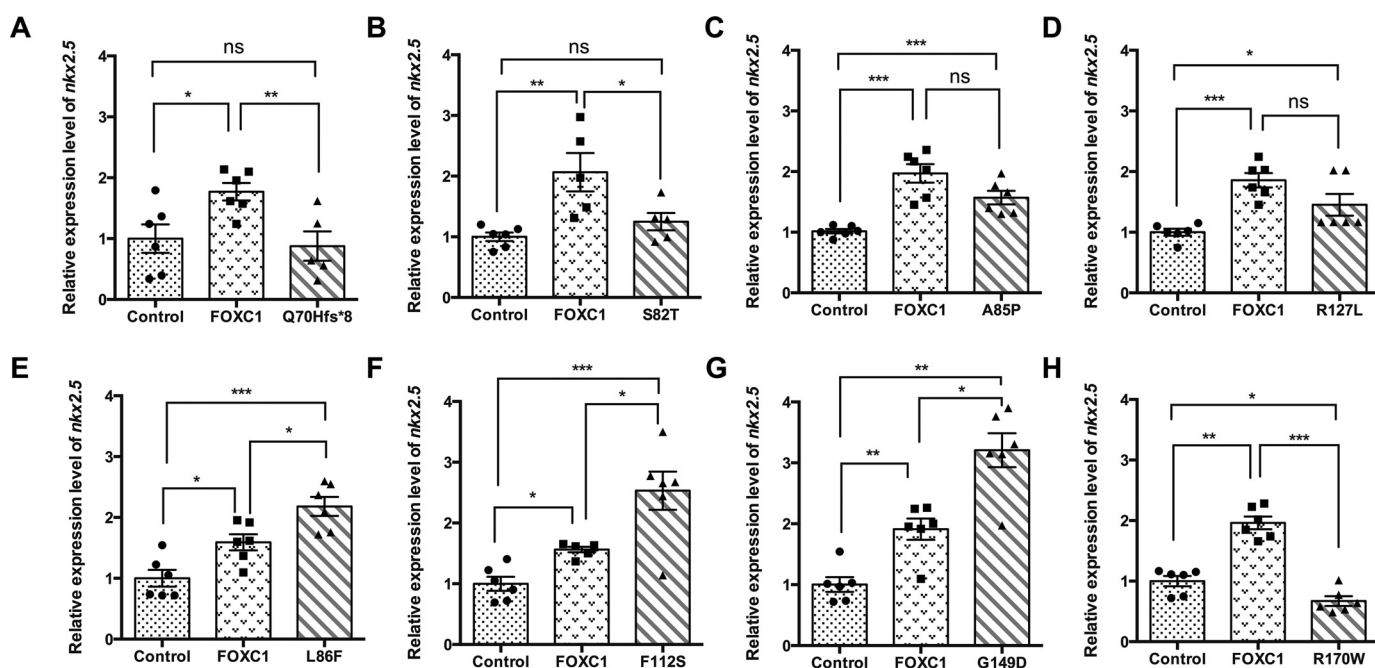


Figure 3. FOXC1 mutants display different abilities of transcriptionally regulating the expression of endogenous *nkx2.5* in zebrafish embryos. qRT-PCR results showing the expressions of endogenous *nkx2.5* in zebrafish embryos microinjected with WT FOXC1, FOXC1 mutants, and negative control GFP mRNA. *x* axis, different mRNAs microinjected. *Control* denotes the GFP mRNA microinjected. *y* axis, the relative expression level of endogenous *nkx2.5*. The relative expression level of *nkx2.5* in the GFP-microinjected group was normalized as 1.0 and the relative expression levels of *nkx2.5* in the mutant or WT FOXC1 group was shown relative to the GFP-microinjected group.

FOXC1 mutants by quantitative RT-PCR analysis. The results showed that the expression level of endogenous *nkx2.5* was significantly increased about two times in the embryos overexpressed with WT FOXC1 compared with the control embryos

overexpressed with GFP (Fig. 3). Unlike having residual activities of regulating the *NKX2-5* expression in H9c2 cells, FOXC1 (p.Q70Hfs*8) and FOXC1 (p.S82T) did not up-regulate the expression of endogenous *nkx2.5* in the zebrafish embryos at all

FOXC1 mutants abnormally regulate NKX2-5 expression

(Fig. 3, A and B). Being consistent with their having normal activity of regulating the *NKX2-5* expression in H9c2 cells, FOXC1(p.A85P) and FOXC1(p.R127L) up-regulated the expression of *nkx2.5* in the zebrafish embryos in a way similar to WT FOXC1 (Fig. 3, C and D). Similar to their enhanced activities of regulating the *NKX2-5* expression in H9c2 cells, FOXC1(p.F112S) and FOXC1(p.L86F) exhibited significantly stronger activity of up-regulating the expression of endogenous *nkx2.5* in zebrafish embryos than WT FOXC1 (Fig. 3, E and F). The expression levels of *nkx2.5* in the zebrafish embryos overexpressed with FOXC1(p.F112S) or FOXC1(p.L86F) were 162 or 137% of the embryos overexpressed with WT FOXC1 (Fig. 3, E and F). Similar to its repressive activity of regulating *NKX2-5* expression in H9c2 cells, FOXC1(p.R170W) inhibited the expression of endogenous *nkx2.5* in zebrafish embryos (Fig. 3H). The expression level of *nkx2.5* in the 14-hpf embryos overexpressed with FOXC1(p.R170W) was 67.07% of those overexpressed with GFP (Fig. 3H). However, opposite to its repressive activity of regulating the *NKX2-5* expression in H9c2 cells, FOXC1(p.G149D) abnormally up-regulated the expression of endogenous *nkx2.5* in zebrafish embryos (Fig. 3G). The expression level of *nkx2.5* in the embryos overexpressed with FOXC1(p.G149D) was 167% of those overexpressed with WT FOXC1 (Fig. 3G).

The FOXC1 mutants play consistent roles in rescuing the decreased ventricle minor axis shortening fraction of *foxc1a*^{nju18} embryos with their activities of regulating the *nkx2.5* expression evaluated by zebrafish embryos

Previously, we reported that overexpression of *nkx2.5* well-rescued the disrupted heart ventricular contraction function of *foxc1a*-null zebrafish embryos that had reduced expression of the endogenous *nkx2.5* (12). Consistently, both the expression level of *nkx2.5* in the 14-hpf embryos (Fig. S4B) and the ventricle minor axis shortening fraction (SF) in 50-hpf embryos (Fig. S4C) were significantly reduced in the zebrafish embryos that the promoter activity of *nkx2.5* were knocked down by CRISPRi (Fig. S4A) (34). Taken together, the results suggest the expression changes of *nkx2.5* directly regulated by *Foxc1a* plays an essential role in zebrafish heart function.

Now that the 8 FOXC1 mutants were determined to have different activities on regulating the expression of *nkx2.5*, they should play different roles in the heart function. To test this hypothesis, we examined the abilities of the FOXC1 mutants in rescuing abnormal heart function of *foxc1a* null zebrafish embryos by microinjecting the WT or FOXC1 mutants' mRNAs into the embryos at the one-cell stage derived from Tg (*cmcl2:eGFP*)^{m225};*foxc1a*^{nju18/+} zebrafish parents and then measuring their SF at 50 hpf, respectively. The results showed that WT FOXC1 well-rescued the decreased SF of *foxc1a*^{nju18} embryos (Fig. 4, A and B, A–B', and G–J) but did not change the SF of their WT and *foxc1a*^{nju18/+} siblings (Fig. S5A). Being consistent with their having no activities of regulating the expression of endogenous *nkx2.5* in the zebrafish embryos (Fig. 3), FOXC1(p.Q70Hfs*8) and FOXC1(p.S82T) did not rescue the decreased SF of *foxc1a*^{nju18} embryos at all (Fig. 4, A–C, A'–C', and G, Fig. S5C). Similarly, FOXC1(p.R127L), FOXC1

(p.A85P), FOXC1(p.L86F), FOXC1(p.F112S), and FOXC1(p.G149D) well-rescued the decreased SF of *foxc1a*^{nju18} embryos (Fig. 4, A–B, A'–B', D, D', F, F', H, and J, Fig. S5, B and C), whereas FOXC1(p.R170W) further reduced the SF of *foxc1a*^{nju18} embryos (Fig. 4, A–B, A'–B', E, E', and I). The results are consistent with the unaltered activity of FOXC1(p.R127L) and FOXC1(p.A85P) in up-regulating the expression of *nkx2.5*, the enhancing activity of FOXC1(p.L86F), FOXC1(p.F112S), and FOXC1(p.G149D) in up-regulating the expression of *nkx2.5*, and the inhibitory activity of FOXC1(p.R170W) in repressing the expression of *nkx2.5* in zebrafish embryos, respectively.

The activity of the polymorphic FOXC1 alleles identified from the aborted fetus with CHD in regulating the expression of *nkx2.5* evaluated by zebrafish embryos but not H9c2 cells is consistent with their roles in the CHD patients

To explore whether FOXC1 mutants are the genetic basis for sporadic CHD, we genotyped 48 aborted fetuses with CHD and 13 non-CHD aborted fetuses using their heart tissues. Three FOXC1 alleles of FOXC1(c.1140_1141insGGC/p.G380_A381insG), FOXC1(c.1359_1360insGGC/p.G456_Q457insG), and FOXC1(c.1140_1141insGGC;1359_1360insGGC/p.G380_A381insG;G456_Q457insG) were identified in both CHD and non-CHD fetuses (Fig. S6, A and B). The results indicate these alleles are polymorphic but not mutant, as described before (35). To evaluate their functions on regulating the expression of *NKX2-5*, we co-transfected H9c2 cells with different combinations of the expression plasmids of FOXC1 alleles and the WT FOXC1 and then performed Dual-Luciferase Reporter Assays. Compared with WT FOXC1, FOXC1(p.G380_A381insG), FOXC1(p.G456_Q457insG), FOXC1(p.G380_A381insG;G456_Q457insG), FOXC1(p.G456_Q457insG) plus FOXC1(p.G380_A381insG), FOXC1(p.G380_A381insG) plus FOXC1, FOXC1(p.G456_Q457insG) plus FOXC1, and FOXC1(p.G380_A381insG;G456_Q457insG) plus FOXC1 all significantly increased more transcriptional activity of the *NKX2-5* promoter than WT FOXC1 (27.75, 44.67, 60.71, 59.38, 47.66, 22.18, and 33.27% more, respectively) (Fig. 5A).

To confirm the activity of the polymorphic alleles of FOXC1 in regulating the expression of *NKX2-5*, we microinjected the mRNAs of the three FOXC1 alleles into zebrafish embryos at the 1-cell stage and then examined the endogenous expression of *nkx2.5*. The results showed that FOXC1(p.G380_A381insG), FOXC1(p.G456_Q457insG), and FOXC1(p.G380_A381insG;G456_Q457insG) had similar activity to the WT FOXC1 in regulating expression of endogenous *nkx2.5* in zebrafish embryos (Fig. 5B).

To examine whether the functions evaluated by zebrafish embryos or the H9c2 cell model represent the regulatory effect of FOXC1 on the expression of *NKX2-5* in the heart of the aborted fetuses, we examined the *NKX2-5* expression level in the heart tissue samples isolated from 48 CHD aborted fetuses and the 12 non-CHD aborted fetuses, respectively. qRT-PCR analysis revealed that the expression level of *NKX2-5* in the heart tissue samples was not significantly different among the CHD or non-CHD fetuses with different genotypes of FOXC1

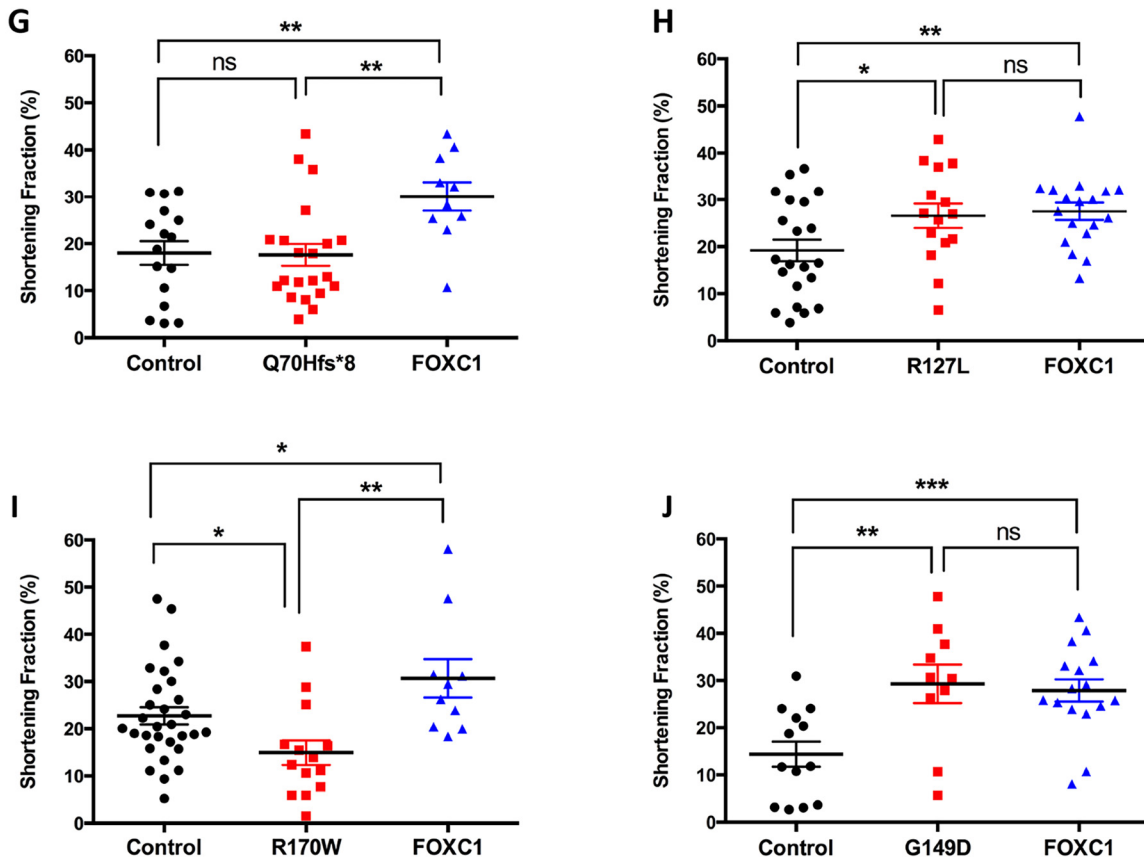
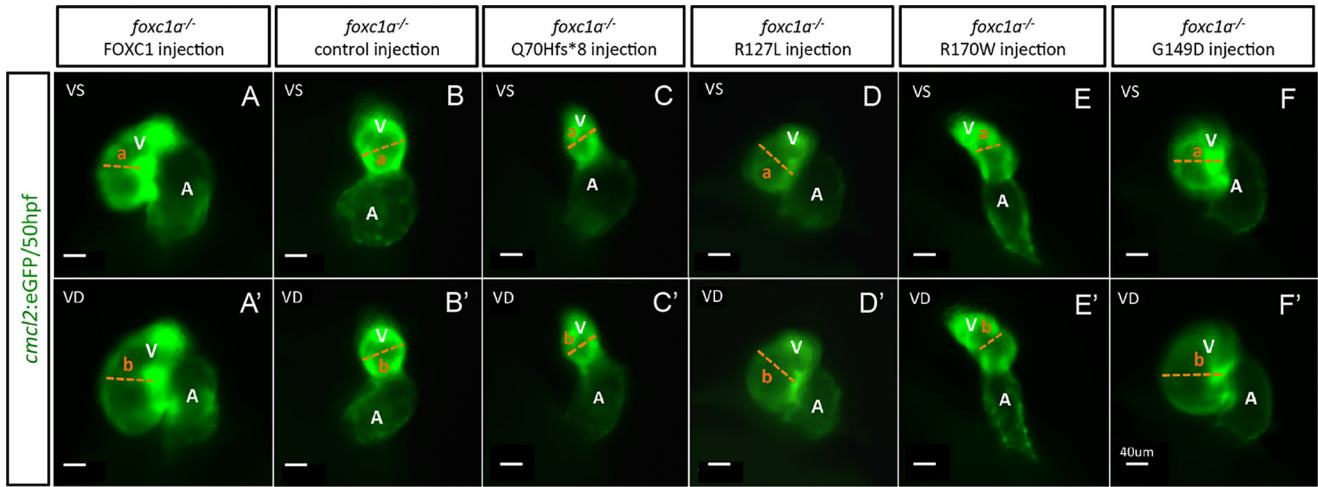


Figure 4. Heart ventricular contraction functions of *foxc1a*-null embryos are altered diversely by overexpression of different FOXC1 mutants. A-F and A'-F', ventral view of the AS phase (A-F) and VS phase (A'-F') heart of Tg(*cmcl2:eGFP*)^{m225};*foxc1a*^{-/-} embryos microinjected with different mRNAs under fluorescence microscopy at 50 hpf. A, atrium; V, ventricle. The orange dotted line indicates the ventricle minor axis. a, ventricle minor axis at the VS phase; b, ventricle minor axis at the VD phase. G-J, scatter plots showing the SF of ventricle (y axis) in the Tg(*cmcl2:eGFP*)^{m225};*foxc1a*^{-/-} embryos microinjected with control mRNA (black dots), WT FOXC1 mRNA (blue triangles), and mutated FOXC1 mRNA (red squares) at 50 hpf (x axis).

alleles (Fig. 5C, Fig. S6, B and C). In other words, the fetus that carried FOXC1(p.G380_A381insG), FOXC1(p.G456_Q457insG), or FOXC1(p.G380_A381insG;G456_Q457insG) had similar expression levels of *NKX2-5* to the ones carrying WT *FOXC1* in heart tissues. The results suggest that the functions of the FOXC1 mutants on heart development evaluated by zebrafish embryos but not H9c2 cells are consistent with their roles in human subjects. Consistently, FOXC1 repressed the expression

of *NKX2-5* in HEK293T cells in a dose-dependent way (Fig. S7), which is totally opposite to its transcriptionally regulating activity in H9c2 cells (Fig. 1B).

Discussion

Although more than 75,000 known genetic variants are reported to be associated with human diseases (36), the roles of

FOXC1 mutants abnormally regulate NKX2-5 expression

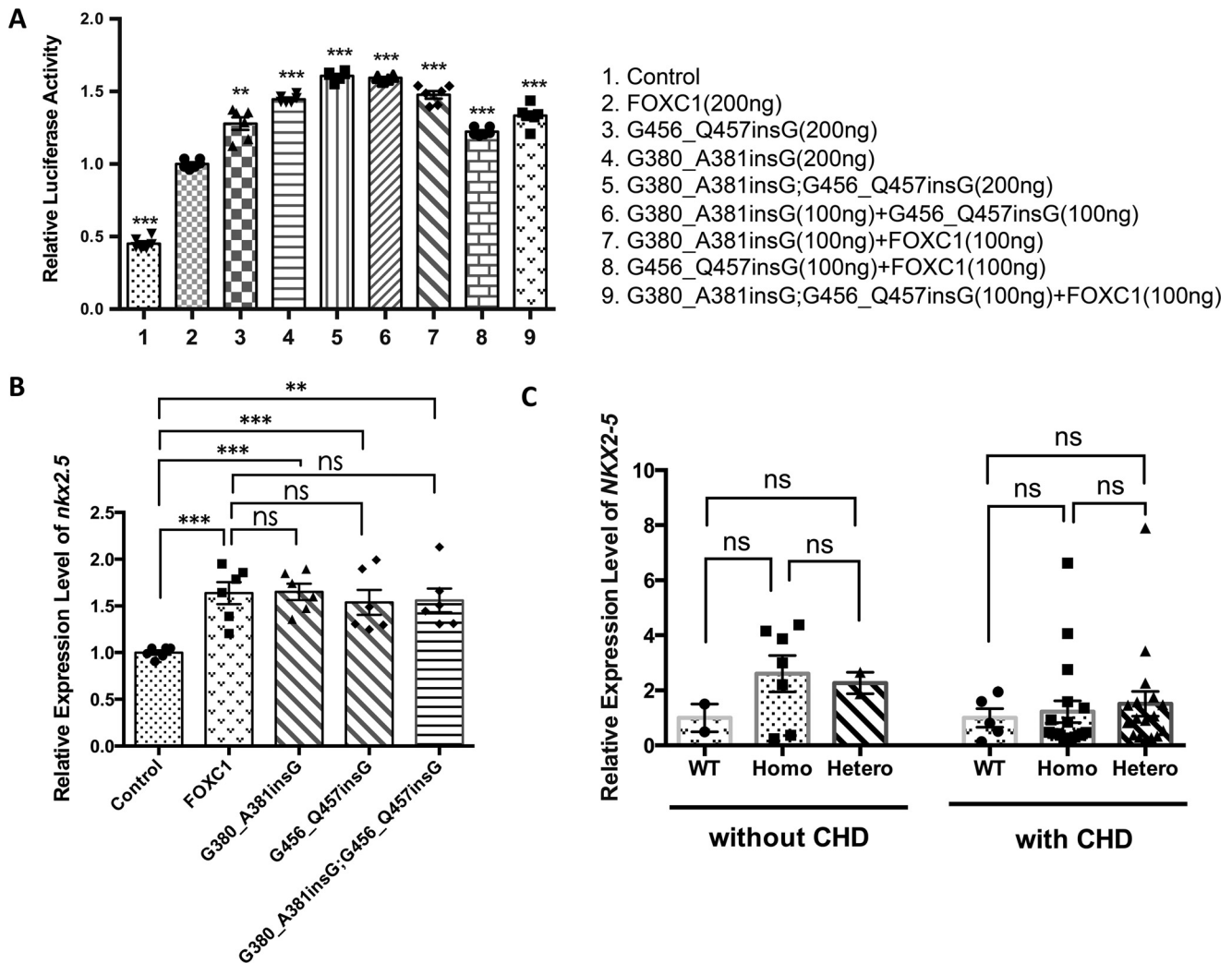


Figure 5. FOXC1(p.G380_A381insG), FOXC1(p.G456_Q457insG), and FOXC1(p.G380_A381insG;G456_Q457insG) carried by sporadic patients with CHD have the same activity of regulating the expression of endogenous *nkx2-5* as WT FOXC1 in zebrafish embryos at 50 hpf. **A**, Dual-Luciferase Reporter Assays were carried out in H9c2 cells to examine the FOXC1 mutants' abilities of transcriptionally regulating the 1149-bp *NKX2-5* promoter. *x* axis, different expression plasmids overexpressed in H9c2 cells. *Control*, the control group only transfected with empty expression plasmid pCMV5. *Other columns*, the experimental group transfected with the expression plasmid of FOXC1 mutants. *y* axis, the relative activity of firefly luciferase driven by the 1149-bp promoter of *NKX2-5*. The relative firefly luciferase activity in the WT group (Group #2: FOXC1) was normalized as 1.0 and the relative expression levels of firefly luciferase in the mutants or control group were shown relative to the WT FOXC1 group. **B**, qRT-PCR results show the expressions of endogenous *nkx2.5* in the zebrafish embryos microinjected with different mRNAs. *x* axis, different mRNAs microinjected. *Control* denotes the GFP mRNA-microinjected. *y* axis, the relative expression level of endogenous *nkx2.5*. The relative expression level of *nkx2.5* in the GFP-microinjected group was normalized as 1.0 and the relative expression levels of *nkx2.5* in WT FOXC1, G380_A381insG, G456_Q457insG, and G380_A381insG;G456_Q457insG groups were shown relative to the GFP-microinjected group. **C**, qRT-PCR results show the relative expression level of *NKX2-5* in the heart tissues isolated from the aborted fetuses with CHD (*left*) and without CHD (*right*). *x* axis, the genotypes of fetuses. *WT*, WT FOXC1; *homo*, the fetus carrying two same or different polymorphic alleles; *hetero*, the fetus carrying one WT allele and one polymorphic allele. The detailed expression of *NKX2-5* in each patient with a different genotype is shown in Fig. S5. *y* axis, the relative expression level of *NKX2-5* in the heart tissues of patients carrying WT FOXC1 was normalized as 1.0 and the relative expression level of *NKX2-5* in the heart tissues carrying FOXC1 polymorphic allele(s) was shown relative to the WT group.

most of them in the diseases are not yet determined. At least 8 FOXC1 mutants have been identified in the ARS patients with CHD and were considered as the likely etiology of the CHD (14–21). To understand their roles in CHD, we first demonstrated in this study that the molecular mechanism of Foxc1 directly regulating the expression of *nkx2.5* is conserved in mammals (Fig. 1). By evaluating the functions of different FOXC1 mutants on regulating the expression of *NKX2-5* using the rat cardiac cell line H9c2 and zebrafish embryos, we found that FOXC1(p.G149D) displayed a totally opposite activity, although the other 7 FOXC1 mutants exhibited somehow similar activities (Figs. 2G and 3G) in the two different model

systems. Compared with WT FOXC1, FOXC1(p.G149D) repressed the transcriptional activity of the *NKX2-5* promoter in H9c2 cells (Fig. 2G), whereas it abnormally increased the expression of *nkx2.5* in zebrafish embryos (Fig. 3G). Similarly, FOXC1(p.G380_A381insG), FOXC1(p.G456_Q457insG), and FOXC1(p.G380_A381insG;G456_Q457insG) identified from aborted fetus abnormally enhanced the transcriptional activity of regulating the *NKX2-5* promoter in H9c2 cells (Fig. 5A), whereas they had similar activities in regulating the expression of *nkx2.5* in zebrafish embryos to WT FOXC1 (Fig. 5B). Importantly, the enhancing activity of FOXC1(p.G149D) in abnormally up-regulating the expression of zebrafish *nkx2.5* was

further confirmed by its capacity of rescuing the reduced SF of *foxc1^{nju18}* embryos. The normal activity of FOXC1(p.G380_A381insG), FOXC1(p.G456_Q457insG), and FOXC1(p.G380_A381insG;G456_Q457insG) in up-regulating the expression of zebrafish *nkx2.5* was consistent with the normal expression of *NKX2-5* in the sporadic patients with CHD carrying the three polymorphic alleles of FOXC1 (Fig. 5C). Consistently, WT FOXC1 were found to inhibit the expression of *NKX2-5* in HEK293T cells in a dose-dependent way (Fig. S7), which is the opposite effect of FOXC1 examined in H9c2 cells (Fig. 1). Additionally, previous studies revealed that most of the FOXC1 mutants displayed functional loss in HeLa, COS-7, or HEK293T cell lines (37–40). Taken together, these results suggest that the different activities of FOXC1 mutants' regulating the expression of *NKX2-5* in different cells are ascribed to the different environments where FOXC1 mutants are able to serve as either repressors or activators, depending on the different targeted gene, co-factor, cell contexts, or cell types and the functions of FOXC1 mutants evaluated by zebrafish embryos but not H9c2 cells be more likely to represent their roles in patients.

FOXC1(c.245G>C/p.S82T), a missense mutation occurring in the forkhead domain (FHD), was identified from the patients with ARS with ASD (14). Previously, it was demonstrated that FOXC1(p.S82T) had reduced DNA binding capacity, nuclear localization, and transcriptional activation ability (37, 38). Consistent with this, our results in this study showed it exhibited complete loss of activity in regulating the expression of *nkx2.5* in zebrafish embryos (Fig. 3). FOXC1(c.210_210delG/p.Q70Hfs*8) was identified in a three-generation family with six members. The proband had congenital glaucoma with ASD. But her mother, also carrying the mutated gene, had congenital glaucoma without CHD (14). In this study, we demonstrated that it displayed no activity of up-regulating the expression of *nkx2.5* in zebrafish embryos at all (Fig. 4). The result is consistent with FOXC1(p.Q70Hfs*8) being a null allele with a premature stop codon that presumptively encodes a 70-aa N-terminal part without FHD, the DNA-binding domain (Fig. S3B). Together with the fact that heterozygous mice carrying a nonsense mutation of *Foxc1* (*Foxc1^{ch/+}*) and heterozygous *Foxc1* knockout mouse and zebrafish do not have CHD (9), we concluded that FOXC1(p.Q70Hfs*8) and FOXC1(p.S82T) might not be involved in CHD, which is consistent with “the mother” having no CHD in the pedigree carried FOXC1(p.Q70Hfs*8). The CHD of proband should be ascribed to other genetic or epigenetic reasons, at least not due to abnormally regulating the expression of *NKX2-5* by FOXC1(p.Q70Hfs*8) or FOXC1(p.S82T).

FOXC1(c.253G>C/p.A85P) was identified in the proband who had congenital glaucoma accompanying CHD. The sister of the proband, carrying the mutant gene, died due to severity of CHD, whereas the father of the proband had only the glaucoma phenotype without CHD (17). FOXC1(c.380T>G/p.R127L) was identified in the proband who had severe glaucoma with CHD. The father of the proband, also carrying the mutated allele, has glaucoma but no cardiac defects (18). In this study, we demonstrated that both FOXC1(p.R127L) and FOXC1(p.A85P) normally regulate the expression of *nkx2.5* in

zebrafish embryos like WT FOXC1. Therefore, we concluded that neither FOXC1(p.R127L) nor FOXC1(p.A85P) is involved in CHD, or at least not by altering the expression of *NKX2-5* in the patients, which is consistent with the ARS patients carrying these mutations having no CHD. The cardiac defects found in the family are likely due to other genetic or epigenetic reasons.

FOXC1(c.335T>C/p.F112S) was found in a four-generation family with 14 members. It was detected in all family members with ARS, but not in normal members. In addition to glaucoma, two of the affected patients had CHD (15). The same FOXC1(p.F112S) mutation was also found in another family, that 5 individuals with the mutation were diagnosed with ARS, and three of them had CHD (14). FOXC1(c.255-256GC>TT/p.L86F) was identified in an ARS patient who had myocardial infarction that occurred at age 41. The mother of this proband carrying the mutation suffered from ARS but no CHD (20). Although both FOXC1(p.F112S) and FOXC1(p.L86F) displayed near-normal DNA-binding and nuclear localization ability in COS-7 cells, yet had severely impaired transcriptional activation capacity in HeLa cells (20, 37). Because previous research revealed that p32 significantly inhibited FOXC1-mediated transcription activation in a dose-dependent manner, whereas FOXC1(p.F112S) displayed an abolished interaction with P32 (41), it is reasonable to hypothesize that FOXC1(p.F112S) might have defective activity in protein–protein interactions and therefore exhibited a reduced capacity of regulating the expression of *NKX2-5* significantly. However, being inconsistent with the previous report, our results in this study demonstrated that FOXC1(p.F112S) and FOXC1(p.L86F) abnormally increased activity of regulating the expression of *nkx2.5* in zebrafish embryos (Fig. 3, E and F) and well-rescued the decreased SF of *foxc1a^{nju18}* embryos (Fig. S5, B and C). Combining that abnormally increased expression of *NKX2-5* is associated with patients with CHD (32, 33), our results suggest that FOXC1(p.F112S) and FOXC1(p.L86F) might be involved in CHD by abnormally increasing the expression of *NKX2-5* and the incomplete genetic penetration of the mutated gene might be the reason for the affected patients having no cardiac defects.

FOXC1(c.446G>A/p.G149D) was found in an ARS patient with a heart defect (19). FOXC1(c.508C>T/p.R170W) was identified in the proband who had congenital glaucoma with CHD. Her mother had glaucoma, but no cardiac phenotype, and her maternal grandmother had glaucoma with ASD (16). The mutation occurs in Wing 2 of the FHD domain, which is a necessary FOXC1 for normal DNA-binding and transactivation functions (42). Previous research revealed that both FOXC2 and FOXC1 had some of the same disease-causing mutations because their FHDs share 98% sequence identity (43). The mutations G143D and R164W in FOXC2 correspond to G149D and R170W in FOXC1. Results from electrophoretic mobility shift assay experiments showed that FOXC2(p.G143D) and FOXC2(p.R164W) showed slightly reduced DNA-binding activity (43). Inconsistent with previous studies, we demonstrated in this study that FOXC1(p.R170W) inhibited the transcription of *nkx2.5* in zebrafish embryos (Fig. 3H) and could not rescue the decreased SF of *foxc1a^{nju18}* embryos (Fig. 4I), whereas FOXC1(p.G149D) greatly enhanced the

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expression of *nkx2.5* in zebrafish embryos (Fig. 3G) and well-rescued the decreased SF of *foxc1a^{nju18}* embryos (Fig. 4J). Combining the facts that reduced expression of *nkx2.5* caused the abnormal heart function (Fig. S4C) and abnormally increased the expression of *NKX2-5* is associated with the patients with CHD (32, 33), our results suggest that FOXC1(p.R170W) might be involved in CHD by abnormally reducing the expression of *NKX2-5* in the ARS patients, whereas FOXC1(p.G149D) might do it by abnormally increasing the expression of *NKX2-5*. Because not all the heterozygous *Nkx2-5* mice suffer from CHD (28–31), it is reasonable to explain why the family member (the mother) carrying FOXC1(p.R170W) did not have CHD (16).

FOXC1(c.1359_1360insGGC/p.G456_Q457insG), FOXC1(c.1140_1141insGGC/p.G380_A381insG), and FOXC1(c.1359_1360insGGC;1140_1141insGGC/p.G456_Q457insG-G380;A381insG) were first reported in a pedigree with ARS in which two affected individuals of this pedigree were also detected with another pathogenic gene *PITX2* mutation (34). The two mutation sites of FOXC1 are not in the FHD domain and referred to as benign/likely benign mutations (p.G456_Q457insG: rs398123612; p.G380_A381insG: rs76840944). In this study, we found the three alleles also occurred in the normal fetus without any diseases (Fig. S6, A and B). Therefore, they should not belong to mutated genes but polymorphic alleles of WT FOXC1. Additionally, the expression levels of *NKX2-5* had no significant differences among the heart tissues of the CHD and non-CHD aborted fetus. Together with the fact that they had similar activity of regulating the expression of *nkx2.5* in zebrafish embryos, we conclude that none of the three alleles are involved in CHD.

Taken together, our results reveal that the functions of mutant genes evaluated by zebrafish embryos might be more likely to represent their roles in the patients carrying the mutants and FOXC1(c.335T>C/p.F112S), FOXC1(c.255-256GC>TT/p.L86F), and FOXC1(c.446G>A/p.G149D) might be involved in CHD by abnormally increasing the expression of *NKX2-5*, whereas FOXC1(c.508C>T/p.R170W) might do it by abnormally repressing the expression of *NKX2-5* in the patients due to their altered capacity of protein–protein interactions. It will be very meaningful to identify the co-factors of FOXC1 that regulate expression of *NKX2-5* in future studies.

Materials and methods

Ethics statement

The breeding and experimental protocols involved in using zebrafish were approved by the IACUC of the Model Animal Research Center, Nanjing University. All methods were performed in accordance with the relevant guidelines and regulations.

Heart tissue samples isolated from 48 CHD aborted fetuses (gestational age range: from 17 weeks + 6 days to 31 weeks) and the 13 non-CHD control aborted fetuses (gestational age range: from 19 to 28 weeks) were collected from Nanjing Maternity and Child Health Care Hospital, China. The diagnosis of the CHD fetuses was based on prenatal fetal system ultrasonography and echocardiography. The protocol of collecting and using human heart tissue samples from aborted fetuses was

approved by The Human Ethics Committee of Nanjing Maternity and Child Health Care Hospital and was therefore performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Informed consents were obtained from the fetus's parents.

Zebrafish husbandry

Zebrafish are raised in circulating water system (ESEN, China) with a room temperature of 28.5 °C, fixed light/dark for 14 h and 10 h, respectively. Zebrafish are fed shrimps (Salt Creek, USA) once every morning and afternoon. The WT Tubingen strain (TU), Tg(*cmcl2:eGFP*)^{m225}, *foxc1a^{nju18/+}* and Tg(*cmcl2:eGFP*)^{m225};*foxc1a^{nju18/+}* mutant lines were used in this study.

Cell culture

H9c2 and HEK293T cell lines (ATCC, USA) were grown in high-glucose Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (PAN, German) and 1% penicillin/streptomycin (Gibco, USA) under 5% carbon dioxide (CO₂) at 37 °C. Cells were transfected with plasmids using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol.

Construction of expression plasmids

The coding sequence (CDS) of WT *FOXC1* (NM_001453.2) was cloned into EcoRI/HindIII sites of pCMV5 plasmid by PCR using primer pairs FOXC1-F and FOXC1-R (Table S1) and genomic template isolated from HEK293T. The PCR was performed under 98 °C, 3 min; 21 × (95 °C, 15 s; 72 °C, 30 s, (–0.5 °C/cycle); 72 °C, 2 min); 15 × (95 °C, 15 s; 58 °C, 30 s; 72 °C, 2 min); 72 °C, 7 min. The coding sequences of the *FOXC1* mutants were subcloned by overlapping PCR using the pCMV5-*FOXC1* plasmid as the template and the point mutations were introduced by overlapping PCR primers listed in Table S1. The overlapping PCR conditions were the same as that of the cloned CDS of WT *FOXC1*. The coding sequences of the *FOXC1* mutants were finally recombined into EcoRI–HindIII sites of the pCMV5 plasmid by PCR using primer pairs of FOXC1-F and FOXC1-R (Table S1). Subclones were confirmed by direct sequencing from both ends.

Cloning of NKX2-5 promoter and Dual-Luciferase Reporter Assay

Human *NKX2-5* promoters with different lengths were individually cloned and then recombined into NcoI-XhoI sites of pGL3 basic vector (Promega, USA) by PCR using HEK293T genomic DNA as template and the different PCR primers listed in Table S1. The 1149-bp promoters of human *NKX2-5* with different mutant-binding sites were subcloned by overlapping PCR using the pGL3 basic vector containing the WT 1149-bp promoter of human *NKX2-5* as the template and the mutant-binding sites were introduced by overlapping PCR primers listed in Table S1. The promoters with different mutant-binding sites were finally recombined into NcoI-XhoI sites of pGL3

basic vector by PCR using the primer pairs listed in Table S1, respectively.

The H9c2 cell line was incubated in a 24-well–culture plate for testing the promoter activity of *NKX2-5* regulated by FOXC1. Each well was transfected with 100 ng of pGL3 basic vectors in which the expression of firefly luciferase was driven by different types of human *NKX2-5* promoters, 1 ng of *Renilla* luciferase expression vectors (inner control), and 200 ng of WT FOXC1 expression vectors or 200 ng of mutant FOXC1 expression vectors. Transfected cells were grown for 24 h. The relative activity of firefly luciferase was measured using the Dual-Luciferase Reporter Assay kit according to the manufacturer's protocol (Promega, USA). Reactions were replicated a minimum of three times.

ChIP-PCR

H9c2 cells were incubated in 100-mm culture plate for ChIP assay. The cultured cells were transfected with 4 μ g of pGL3 basic vectors containing 1149-bp human *NKX2-5* promoters, and 8 μ g of WT FOXC1 expression vectors (pCMV5-FOXC1). 72 h after transfection, the cells were harvested. The ChIP-PCR assay was performed using the EZ-ChIP Chromatin immunoprecipitation kit (Millipore, USA) following the manufacturer's instructions. 5 μ g of FOXC1 antibody (ab5079, Abcam) was used to immunoprecipitate all DNA crossed with FOXC1 protein, and 1 μ g of mouse IgG was used as negative control. The semiquantitative PCR was performed under the condition of 95 °C, 5 min; 25 \times (95 °C 30 s; 52 °C, 30 s; 72 °C, 15 s); 72 °C, 7 min. The PCR products were then subjected to separation by 2% agarose electrophoresis. The primers used for ChIP-PCR are listed in Table S1.

In vitro synthesis of mRNA

To obtain the *in vitro* synthesis templates of mRNA, we first amplified the full-length CDS of WT FOXC1, FOXC1 mutants, or GFP from their expression plasmids by PCR using Phanta Super-Fidelity DNA Polymerase (Vazyme, China). T7 promoter and Kozak sequences were introduced in the forward primers listed in Table S1. Then, the PCR amplification products were purified using the GeneJET PCR Purification Kit (Thermo, USA). Next, the capped mRNAs were synthesized using the purified PCR amplification products as template by the mMESSAGE mMACHINE T7 Kit. The capped mRNA was further tailed using a Poly(A) Tailing Kit. The capped and tailed mRNAs were purified with the MEGAclean Transcription Clean-Up Kit (Invitrogen, USA).

Microinjection of mRNAs into zebrafish embryos

mRNAs of WT FOXC1, FOXC1 mutants, and GFP (control) were diluted to 30 ng/ μ l by RNase-free nanopure water and microinjected into the zebrafish embryos at the 1-cell stage. The amount of microinjection was 1 nl of diluted mRNA solution per embryo. The microinjected embryos were raised at 28 °C for further analysis. At least 30 live embryos were used for each experiment.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed to examine the relative expression levels of *nkx2-5* and *NKX2-5* by using ABI Prism 7300 sequence detector (PE Biosystems, USA) with FastStart Universal SYBR Green Master (ABI). Total RNAs of whole zebrafish embryos or H9c2 cells were extracted using Direct-zol RNA MiniPrep (Zymo Research, USA). Total RNAs were reverse-transcribed into cDNA using a commercial kit (Vazyme, China). The cDNA was then used as template to perform qRT-PCR following the manufacturer's protocol using primers listed in Table S1. The relative mRNA level of zebrafish *nkx2-5* was normalized to the *hprt1* mRNA level, and the relative mRNA level of human *NKX2-5* was normalized to the *GAPDH* mRNA level.

Live imaging and quantification of the shortening fraction of ventricular zebrafish embryonic hearts

To calculate the SF of the ventricular, we anesthetized zebrafish embryos with ethyl 3-aminobenzoate methanesulfonate (30 mg/liter) for 5 min at room temperature, and then mounted with 4% methyl cellulose (Sigma, USA). 10-s movies of 50-hpf zebrafish embryonic hearts were taken using Olympus DP71 digital camera (Olympus, Japan) under a fluorescent dissecting microscope (Leica, Germany) as reported previously (12). Then, pictures of maximum ventricular systole (VS) and maximum ventricular diastole (VD) phases were obtained using Photoshop software from heart movies. The SF was calculated by the equation, $SF = [(width\ of\ ventricle\ at\ VD - width\ of\ ventricle\ at\ VS) / width\ of\ ventricle\ at\ VD] \times 100$ (44, 45).

DNA extraction and genotyping of aborted fetus

Genomic DNA was extracted from the heart tissue samples by a FastPure Cell/Tissue DNA Isolation Mini Kit (Vazyme, China). The CDS of FOXC1 was amplified by PCR with Phanta Super-Fidelity DNA Polymerase (Vazyme, China) using the isolated genomic DNA as template and primers listed in Table S1. The PCR products were bidirectionally sequenced.

Genotyping of zebrafish embryos

The embryos derived from *foxc1a^{nju18/+}* parents were genotyped using the RT-PCR method as described previously (12). Total RNAs were extracted from a single embryo using TRIzol reagent (Invitrogen) for each assay. RNA was reverse-transcribed (Vazyme, China) into cDNA. Some of the cDNA was then used as template to genotype *foxc1a* alleles and the other was used for qRT-PCR. The primers for genotyping zebrafish embryos are listed in Table S1. The PCR conditions were 94 °C, 2 min; 35 \times (94 °C, 30 s; 60 °C, 30s; 72 °C, 30s); 72 °C, 7 min. PCR products were then subjected to 1% agarose gel electrophoresis separation.

Statistics

Experiments were performed independently two or three times. Data are shown as mean \pm S.E. Statistical analysis was carried out with GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Data were first tested for normality using the

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Kolmogorov–Smirnov’s test. If data sets exhibit normal distribution, we employed Student’s *t* test for equal variances or Welch’s *t* test for unequal variances. If data sets were found not to exhibit normal distribution, a Mann-Whitney test was applied. A value of $p < 0.05$ (*) was considered statistically significant, and $p < 0.01$ (**) plus $p < 0.001$ (***) were considered statistically very significant.

Data availability

All the data described are located within the manuscript and the [supporting information](#).

Author contributions—Q. Zhang, Y. Y., L. H., N. L., and D. J. data curation; Q. Zhang and Y. Y. formal analysis; Q. Zhang writing-original draft; D. L. and P. H. resources; D. L. visualization; D. L. and Q. Zhao writing-review and editing; Q. Zhao supervision; Q. Zhao funding acquisition.

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Abbreviations—The abbreviations used are: ARS, Axenfeld–Rieger syndrome; CHD, congenital heart defects; ASD, atrial septal defect; ECR, evolutionary conserved region; hpf, hours post-fertilization; SF, shortening fraction; qRT, quantitative RT; FHD, forkhead domain; CDS, coding sequence; VS, ventricular systole; VD, ventricular diastole.

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