PITX2 Loss-of-Function Mutation Contributes to Congenital Endocardial Cushion Defect and Axenfeld-Rieger Syndrome

Cui-Mei Zhao¹,²*, Lu-Ying Peng²*, Li Li², Xing-Yuan Liu³, Juan Wang¹, Xian-Ling Zhang⁴, Fang Yuan⁵, Ruo-Gu Li⁵, Xing-Biao Qiu⁶, Yi-Qing Yang⁵,⁶,⁷*

¹ Department of Cardiology, Tongji Hospital, Tongji University School of Medicine, Shanghai, China, ² Division of Medical Genetics, Tongji University School of Medicine, Shanghai, China, ³ Department of Pediatrics, Tongji Hospital, Tongji University School of Medicine, Shanghai, China, ⁴ Department of Cardiology, Shanghai Tenth People’s Hospital, Tongji University School of Medicine, Shanghai, China, ⁵ Department of Cardiology, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China, ⁶ Department of Cardiovascular Research Laboratory, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China, ⁷ Department of Central Laboratory, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China

* cuimeizhao@sina.com (C-MZ); luying_peng@163.com (L-YP); dryyq@tongji.edu.cn (Y-QY)

Abstract

Congenital heart disease (CHD), the most common type of birth defect, is still the leading non-infectious cause of infant morbidity and mortality in humans. Aggregating evidence demonstrates that genetic defects are involved in the pathogenesis of CHD. However, CHD is genetically heterogeneous and the genetic components underpinning CHD in an overwhelming majority of patients remain unclear. In the present study, the coding exons and flanking introns of the PITX2 gene, which encodes a paired-like homeodomain transcription factor essential for cardiovascular morphogenesis as well as maxillary facial development, was sequenced in 196 unrelated patients with CHD and subsequently in the mutation carrier’s family members available. As a result, a novel heterozygous PITX2 mutation, p.Q102X for PITX2a, or p.Q148X for PITX2b, or p.Q155X for PITX2c, was identified in a family with endocardial cushion defect (ECD) and Axenfeld-Rieger syndrome (ARS). Genetic analysis of the pedigree showed that the nonsense mutation co-segregated with ECD and ARS transmitted in an autosomal dominant pattern with complete penetrance. The mutation was absent in 800 control chromosomes from an ethnically matched population. Functional analysis by using a dual-luciferase reporter assay system revealed that the mutant PITX2 had no transcriptional activity and that the mutation eliminated synergistic transcriptional activation between PITX2 and NKX2.5, another transcription factor pivotal for cardiogenesis. To our knowledge, this is the first report on the association of PITX2 loss-of-function mutation with increased susceptibility to ECD and ARS. The findings provide novel insight into the molecular mechanisms underpinning ECD and ARS, suggesting the potential implications for the antenatal prophylaxis and personalized treatment of CHD and ARS.
Introduction

Congenital heart disease (CHD) is the most prevalent type of birth defect in humans, with an estimated prevalence of 1% among living neonates, and is the most common non-infectious cause of infant morbidity and mortality, accounting for roughly 30% of neonatal demises caused by miscellaneous developmental malformations [1]. Traditionally, various CHDs are categorized as at least 21 distinct entities with specific anatomic lesions, including ventricular septal defect, atrial septal defect, tetralogy of Fallot, endocardial cushion defect (ECD), double outlet right ventricular, patent ductus arteriosus, and transposition of the great vessels [1]. Distinct forms of CHDs can occur separately or in combination, leading to reduced exercise performance, degraded quality of life, delayed brain development or brain injury, thromboembolic stroke, pulmonary hypertension, impaired pulmonary function, metabolic disorders, muscle dysfunction, abnormal autonomic nervous activity, infective endocarditis, cardiac enlargement or congestive heart failure, arrhythmias, and sudden cardiac death [2–13]. Obviously, CHD has imposed an enormous economic burden on patients and health care systems, and the socioeconomic burden is anticipated to increase in the future with increasing CHD adults [14,15]. Despite the pronounced clinical importance, the molecular mechanisms underpinning CHD remain poorly understood.

In vertebrates, the heart is the first organ that develops to function. Cardiovascular morphogenesis is a complex, dynamic biological process that requires the orchestration of cardiac cell commitment, differentiation, proliferation and migration, and both environmental and genetic risk factors may interrupt this accurate temporal and spatial cooperation, yielding a wide range of CHD [16–38]. There is increasing evidence that highlights the pivotal role of cardiac transcription factors in embryonic cardiogenesis, and a long list of mutations in the cardiac transcription factor genes, including NK and GATA families, have been implicated in the pathogenesis of CHD [39–65]. However, CHD is of striking genetic heterogeneity and the genetic components predisposing to CHD in an overwhelming majority of patients remain to be identified.

Recently, there is increasing evidence demonstrating that the transcription factor PITX2, a member of the bicoid-like homeodomain family of transcription factors, plays a crucial role in cardiovascular morphogenesis and maxillary facial development. The PITX2 gene was originally identified as a causative gene for the human Axenfeld-Rieger’s syndrome (ARS), which is characterized by eye, teeth, craniofacial and umbilical abnormalities as well as heart defects [66–68]. To date, four different isoforms of PITX2 transcripts, which are generated by differential mRNA splicing and alternative promoter usage, have been identified, of which PITX2a, PITX2b and PITX2c differ only in their amino-termini and exist in human, mouse, chick, zebrafish and xenopus, while the fourth isoform, PITX2d, which lacks most homeodomain along with the entire amino-terminal domain, is detected only in humans. Notably, PITX2c is the predominant transcript in the embryonic and adult hearts of the mouse and human, mainly responsible for cardiogenesis [69–78]. In Xenopus embryos, partial depletion of PITX2c mRNA using chemically modified antisense oligonucleotides resulted in cardiac dysmorphology, including abnormalities of outflow tract, atrial septation and relative atrial-ventricular chamber positioning as well as restriction of ventricular development [79]. In mice, targeted disruption of PITX2c resulted in embryonic lethality with different kinds of congenital cardiovascular malformations, including ECD, atrial isomerism, double-outlet right ventricle, transposition of the great artery and abnormal aortic arch [80,81]. In humans, PITX2cmutations have been causatively associated with isolated congenital heart diseases [82–84]. These findings justified screening PITX2 as a preferred candidate gene for CHD in other cohorts of patients.
Materials and Methods

Study participants

In this study, 196 unrelated CHD patients and 400 unrelated individuals with no cardiac structural aberrations were enrolled from the Chinese Han population. The available relatives of an index patient with an identified PITX2 mutation were also included. All participants underwent detailed clinical evaluation, which included individual and familial histories, comprehensive physical examination, and echocardiography with color flow Doppler. The patients also underwent chest X-ray, electrocardiogram or cardiac catheterization examination when there was a strong clinical indication. Medical records of the deceased or unavailable relatives of a mutation carrier were also reviewed. The patients with known chromosomal abnormalities were excluded from the study. Peripheral venous blood samples were taken from all participants. This study conformed to the ethical guidelines of the Declaration of Helsinki. The study protocol was reviewed and approved by the ethics committee of Tongji Hospital, Tongji University (the ethical approval number for cases and controls: LL(H)-09-07; the date for the approval: July 27, 2009). Written informed consent was signed by participants or their guardians prior to study.

Genetic analysis of human *PITX2*

Genomic DNA was isolated from peripheral blood leukocytes using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The coding regions and splice junction sites of the *PITX2* gene was sequenced initially in 196 unrelated patients with CHD, and genotyping *PITX2* was performed subsequently in the available relatives of a mutation carrier and 400 unrelated control individuals. The referential genomic DNA sequence of *PITX2* was derived from GenBank (accession no. NC_000004), which was at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). The primer pairs used to amplify the coding exons and intron–exon boundaries of *PITX2* by polymerase chain reaction (PCR) were shown in Table 1. The PCR was performed and the PCR product was sequenced as previously described [82]. A sequence variation was verified by re-sequencing an independent PCR-amplified product from the same subject. Additionally, for an identified sequence variant, the Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS) and NCBI’s single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) databases were queried to confirm its novelty.

Alignment of multiple *PITX2* protein sequences across species

Multiple amino acid sequences of the PITX2 proteins from various species were aligned using the online MUSCLE program, version 3.6 (http://www.ncbi.nlm.nih.gov/).

| Table 1. The primers to amplify the coding exons and flanking introns of *PITX2*. |
|---|---|---|---|
| Exon | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) | Amplicon (bp) |
| 2 | GAGCTTAGCTGAGAGATGCT | CCACTGGCCGATTGTTCTTG | 385 |
| 3 | TGTCTCTTTTGTTCCCTTTTC | CCAGAGGGGAGATGTTCAAG | 399 |
| 4 | CAGCTTGGCTGAGAACTCG | TGACTTCCCTGGGGCAGAG | 442 |
| 5 | CAGCTTCCACGGCTCTCGT | GTGCTCTCACCACATTCTCTC | 387 |
| 6 | AATCTTCACCTGCCCATCTCG | AGTCTTTCAGGCCGAGTT | 677 |

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Plasmids and site-directed mutagenesis

The expression plasmid PITX2c-pcDNA4 was a kind gift from Georges Christé at Physiopathologie des Troubles du Rythme Cardiaque, Faculté de Pharmacie de Lyon, Université Lyon 1, France. The recombinant expression plasmid NKX2.5-pEFSA and the atrial natriuretic factor (ANF)-luciferase reporter plasmid (ANF-luc), which contains the 2600-bp 5'-flanking region of the ANF gene and expresses Firefly luciferase, were kindly provided by Dr. Ichiro Shiojima, from the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chuo-ku, Chiba, Japan. The procollagen lysyl hydroxylase (PLOD1) promoter plasmid PLOD1-luc, which contains the nucleotides from -60 to -3180 of the PLOD1 gene, was constructed as described previously [85]. The PITX2a and PITX2b isoforms were PCR-amplified from cDNA clones as described previously [86] and inserted into the pcDNA4 plasmid (Invitrogen, Carlsbad, CA, USA), respectively. The identified mutation Q102X, or Q148X, or Q155X was introduced into the wild-type PITX2a, or PITX2b, or PITX2c, respectively, by using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with a complementary pair of primers. Each of the mutants was sequenced to confirm the desired mutation and to exclude any other sequence variations.

 Luciferase reporter gene assays

Chinese hamster ovary (CHO) cells were seeded in 12-well plates and cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. Cell transfections were performed 24 h after plating, with Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. The ANF-luc construct and an internal control reporter plasmid pGL4.75 (hRLuc/CMV, Promega), which expresses Renilla luciferase, were used in transient transfection assays. CHO cells were transfected with 2 μg of wild-type PITX2–pcDNA4 or mutant PITX2–pcDNA4 or empty vector pcDNA4, 2.0 μg of ANF-luc reporter construct, and 0.04 μg of pGL4.75 control reporter vector. For co-transfection experiments, 1 μg of wild-type PITX2–pcDNA4, 1 μg of mutant PITX2–pcDNA4, 2.0 μg of ANF-luc, and 0.04 μg of pGL4.75 were used. Transfected cells were harvested 24 h after transfection, then lysed and assayed for reporter activities. Firefly luciferase and Renilla luciferase activities were measured with the Dual-Glo luciferase assay system (Promega). The activity of the ANF promoter was presented as fold activation of Firefly luciferase relative to Renilla luciferase. Three independent experiments were conducted in triplicate for wild-type and mutant PITX2a, or PITX2b, or PITX2c, and results are representative of three separate experiments.

For the analysis of the synergistic transcriptional activation between PITX2 and NKX2.5 [87], another transcription factor crucial for normal cardiovascular development [40–48], CHO cells were grown and transfected with 2μg of wild-type or mutant PITX2–pcDNA4, alone or together with 2μg of wild-type NKX2.5-pEFSA, 5μg of PLOD1-luc, and 0.04 μg of pGL4.75 using Lipofectamine 2000 Transfection Reagent (Invitrogen).

Statistical analysis

The significance of differences in luciferase activity was analyzed using the unpaired Student’s t test. A two-tailed P value less than 0.05 was considered to be statistically significant.
Results
Baseline characteristics of the study subjects

A cohort of 196 unrelated patients with CHD was clinically investigated in contrast to a total of 400 ethnically-matched unrelated controls. All the participants had no established environmental risk factors for CHD, such as maternal illness and drug use in the first trimester of pregnancy, parental smoking and long-term exposure to toxicants as well as ionizing radiation. The control individuals had no evidence of organic cardiac diseases, and their echocardiographic results were normal. The baseline clinical characteristics of the 196 CHD patients are summarized in Table 2.

Table 2. Baseline clinical characteristics of the 196 unrelated patients with congenital heart disease.

| Variable                        | Statistic |
|---------------------------------|-----------|
| Male gender (%)                 | 102 (52.0) |
| Age (years)                     | 5.2 ± 2.4 |
| Positive family history (%)     | 36 (18.4) |
| Prevalence of different types of CHD |           |
| Isolated CHD (%)                | 105 (53.6) |
| VSD (%)                         | 32 (16.3)  |
| ASD (%)                         | 27 (13.8)  |
| PDA (%)                         | 20 (10.2)  |
| ECD (%)                         | 6 (3.1)    |
| AS (%)                          | 5 (2.6)    |
| PA (%)                          | 5 (2.6)    |
| CoA (%)                         | 4 (2.0)    |
| PS (%)                          | 3 (1.5)    |
| TA                              | 2 (1.0)    |
| HLHS (%)                        | 1 (0.5)    |
| Complex CHD (%)                 | 72 (36.7)  |
| TOF (%)                         | 28 (14.3)  |
| DORV + VSD (%)                  | 17 (8.7)   |
| ECD + TGA (%)                   | 14 (7.1)   |
| TA + VSD (%)                    | 9 (4.6)    |
| TGA + VSD (%)                   | 4 (2.0)    |
| Others (%)                      | 19 (9.7)   |
| Incidence of arrhythmia         |           |
| Atrial fibrillation (%)         | 16 (8.2)   |
| Atroventricular block (%)       | 8 (4.1)    |
| Treatment                       |           |
| Surgical repair (%)             | 118 (60.2) |
| Catheter-based closure (%)      | 57 (29.1)  |
| Follow-up (%)                   | 21 (10.7)  |

CHD, congenital heart disease; VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; ECD, endocardial cushion defect; AS, aortic stenosis; PA, pulmonary atresia; CoA, coarctation of the aorta; PS, pulmonary stenosis; TA, truncus arteriosus; HLHS, hypoplastic left heart syndrome; TOF, tetralogy of Fallot; DORV, double outlet of right ventricle; TGA, transposition of great arteries.

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Identification of a novel PITX2 mutation

By sequencing of PITX2 in the 196 patients, a heterozygous sequence variation was identified in one patient, with a mutational prevalence of about 0.51%. Specifically, a substitution of thymine for cytosine at the first nucleotide of codon 102 of PITX2a (c.304C>T), or codon 148 of PITX2b (c.442C>T), or codon 155 of PITX2c (c.463C>T), predicting the transition of glutamine-encoding codon to a stop codon at amino acid 102 for PITX2a (p.Q102X), or 148 for PITX2b (p.Q148X), or 155 for PITX2c (p.Q155X), was identified in an ECD patient with positive family history. The sequence electropherograms showing the identified nonsense PITX2 variation compared with the corresponding control sequence are shown in Fig 1A. The schematic diagrams showing the structural domains of the wild-type and mutant PITX2 proteins are presented in Fig 1B. The variation was neither observed in 800 control chromosomes nor reported in the EVS's and NCBI's SNP databases, which were consulted again on September 1, 2014. Genetic screening of the mutation carrier’s family members demonstrated that the variation was present in all affected family members available, but absent in unaffected family members examined. Analysis of the pedigree showed that in the family the mutation co-segregated with ECD transmitted as an autosomal dominant trait with complete penetrance. The pedigree structure of the family is illustrated in Fig 1C. Besides, the proband (III-3) had also transposition of the great arteries, and her father (II-5) and uncle (II-1) had also mitral valve cleft and right aortic arch. Interestingly, all the mutation carriers had also oligodontia, maxillary hypoplasia and iris hypoplasia, and the proband (III-3) and her father (II-5) had also congenital umbilical hernia, a phenotype of Axenfeld-Rieger syndrome (ARS). The phenotypic characteristics and results of genetic screening of the affected pedigree members are listed in Table 3.

Multiple alignments of PITX2 protein sequences across species

A cross-species alignment of PITX2 protein sequences displayed that the altered amino acid, p. Q102 for PITX2a, or p.Q148 for PITX2b, or p.Q155 for PITX2c, was completely conserved evolutionarily among all vertebrates (Fig 2).

Transactivational activity of the mutant PITX2

As shown in Fig 3, the wild-type PITX2a, PITX2b and PITX2c activated the ANF promoter by ~29-fold, ~14-fold and ~11-fold, respectively; whereas the same amount (2 μg) of mutant PITX2a, PITX2b or PITX2c activated the ANF promoter by ~1-fold. When the same amount of wild-type PITX2 (1 μg) was cotransfected with mutant PITX2 (1 μg), the induced activation of the ANF promoter was ~14-fold for PITX2a, ~6-fold for PITX2b and ~4-fold for PITX2c. These results suggest that the mutant PITX2 has no transactivational activity when compared with its wild-type counterpart.

Synergistic transcriptional activity between mutant PITX2 and NKX2.5

As shown in Fig 4, in the presence of 2μg of wild-type NKX2.5, 2μg of wild-type PITX2a, PITX2b and PITX2c activated the PLOD1 promoter by ~11-fold, ~5-fold and ~32-fold, respectively; while the same amount (2μg) of Q102X-mutant PITX2a, or Q148X-mutant PITX2b or Q155X-mutant PITX2c activated the PLOD1 promoter by ~2-fold, indicating that the mutation blocks the synergistic transactivational activity between PITX2 and NKX2.5.

Discussion

In the current study, a novel heterozygous mutation in the PITX2 gene, p.Q102X for PITX2a, p.Q148X for PITX2b, or p.Q155X for PITX2c, was identified in a family with congenital ECD
and ARS. Genetic analysis of the pedigree showed that the nonsense mutation was transmitted in an autosomal dominant pattern with complete penetrance. The mutation, which was absent in the 800 reference chromosomes, altered the amino acid highly conserved evolutionarily among vertebrates. Functional assays unveiled that each isoform of the mutant PITX2 lost the ability to transactivate the \textit{ANF} and \textit{PLOD1} promoters and that the mutation eliminated the

![Fig 1. PITX2 mutation associated with endocardial cushion defect and Axenfeld-Rieger syndrome. (A) Sequence electropherograms showing the heterozygous PITX2 mutation compared with its control. The arrow indicates the heterozygous nucleotides of C/T in the proband (mutant) or the homozygous nucleotides of C/C in the corresponding control individual (wild-type). The rectangle signifies the nucleotides comprising a codon of PITX2. (B) Schematic diagrams showing the structural domains of wild-type and mutant PITX2 proteins with the disease related mutation indicated. The mutation found in patients with endocardial cushion defect and Axenfeld-Rieger syndrome is shown above the structural domains of the mutant PITX2 proteins. NH2 denotes amino-terminus; TAD1, transcriptional activation domain 1; HD, homeodomain; NLS, nuclear localization signal; TAD1, transcriptional inhibitory domain 1; TAD2, transcriptional activation domain 2; TID2, transcriptional inhibitory domain 2; COOH, carboxyl-terminus. (C) Pedigree structure of the family with endocardial cushion defect and Axenfeld-Rieger syndrome. Family members are identified by generations and numbers. Square indicates male family member; circle, female member; symbol with a slash, the deceased member; closed symbol, affected member; open symbol, unaffected member; arrow, proband; “+”, carrier of the heterozygous mutation; “-”, non-carrier.

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and ARS. Genetic analysis of the pedigree showed that the nonsense mutation was transmitted in an autosomal dominant pattern with complete penetrance. The mutation, which was absent in the 800 reference chromosomes, altered the amino acid highly conserved evolutionarily among vertebrates. Functional assays unveiled that each isoform of the mutant PITX2 lost the ability to transactivate the \textit{ANF} and \textit{PLOD1} promoters and that the mutation eliminated the
synergistic transcriptional activation between PITX2 and NKX2.5. Hence, it is very likely that genetically defective PITX2 confers enhanced susceptibility to ECD and ARS in these mutation carriers.

It has been revealed that PITX2 is abundantly expressed in the developing hearts, craniofacial organs, and abdominal wall, especially in myocardium related to endocardial cushions of the atrioventricular canal, and functions to mediate multiple target genes that are amply expressed during embryogenesis, including ANF and PLOD1 [66–74]. Therefore, the transcriptional effect of a mutant PITX2 may be characterized by using the ANF and PLOD1 promoters. In this study, functional analyses demonstrated that the mutation identified in

Table 3. Phenotypic characteristics and status of PITX2 mutation of the affected pedigree members.

| Identity | Gender | Age (years) | Cardiac defects | Extracardiac defects | Genotype |
|----------|--------|-------------|-----------------|----------------------|----------|
| I-1      | M      | 50\(^a\)    | ECD             | OD, MH, IH           | NA       |
| II-1     | M      | 31          | ECD, RAA, MVC   | OD, MH, IH           | +/-      |
| II-5     | M      | 26          | ECD, RAA, MVC   | OD, MH, IH, UH       | +/-      |
| III-3    | F      | 1           | ECD, TGA        | OD, MH, IH, UH       | +/-      |

\(^a\)Age at death.

M, male; F, female; ECD, endocardial cushion defect; MVC, mitral valve cleft; RAA, right aortic arch; TGA, transposition of the great arteries; OD, oligodontia; MH, maxillary hypoplasia; IH, iris hypoplasia; UH, umbilical hernia; NA, not available; +/-, heterozygote.

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Fig 2. Alignment of multiple PITX2 amino acid sequences among species. The altered amino acid of p.Q102 for PITX2a, or p.Q148 for PITX2b, or p.Q155 for PITX2c is completely conserved evolutionarily among vertebrates.

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patients with ECD and ARS abolished the transcriptional activation of ANF- or PLOD1-driven luciferase reporter by PITX2 and eliminated the transcriptionally synergistic activation between PITX2 and NKX2.5, indicating that functionally impaired PITX2 is potentially an alternative molecular mechanism underpinning CHD and ARS.

**Fig 3. Transactivational defects caused by PITX2 mutation.** Transcriptional activation of atrial natriuretic factor promoter driven luciferase reporter in CHO cells by wild-type or mutant PITX2, alone or in combination, showed that the mutant PITX2 did not transactivate gene expression. Data are derived from three independent experiments repeated in triplicate. Mean fold activation and standard deviations are shown. ** and * represent $P<0.001$ and $P<0.01$, respectively, when compared with wild-type PITX2.

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**Fig 4. No synergistic transcriptional activation between NKX2.5 and mutant PITX2.** The synergistic transactivation of the PLOD1 promoter in CHO cells by NKX2.5 and mutant PITX2 was eliminated by the mutation. All data are derived from three independent experiments repeated in triplicate. Mean fold activation and standard deviations are shown. ** represents $P<0.001$, when compared with NKX2.5 plus wild-type PITX2.

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Previous studies have established that multiple important genes are transcriptionally regulated by PITX2c during cardiovascular development [87], and mutations in several target genes, such as NKX2.5 and GATA4, have been causally implicated in CHD including ECD [40–48,51–58]. Therefore, mutated PITX2c may increase the vulnerability to CHD by altering the expressions of such cardiac-specific target genes.

In humans, PITX2c mutations have been implicated in the pathogenesis of other CHDs. Wang and co-workers [82] screened PITX2c in 382 unrelated patients with CHDs and found two heterozygous mutations, p.W147X and p.N153D, in two patients with CHD, respectively, including a one-year-old male patient with double outlet right ventricle in combination with ventricular septal defect and a four-year-old female patient with isolated ventricular septal defect. Yuan et al. [83] scanned PITX2c in 150 unrelated patients with CHDs and identified two novel heterozygous PITX2c mutations, p.H98Q and p.M119T, in two patients with atrial septal defects, respectively. Wei and colleagues [84] also sequenced PITX2c in 170 unrelated neonates with CHDs and detected two novel heterozygous PITX2c mutations, p.R91Q and p.T129S, in two unrelated newborns with transposition of the great arteries and ventricular septal defect, respectively. Functional analysis demonstrated that all the above-mentioned PITX2c mutations were consistently associated with significantly diminished transcriptional activity [82–84]. In this study, a novel PITX2 loss-of-function mutation is identified in patients with ECD and ARS, thus expanding the phenotypic spectrum linked to PITX2 mutation.

Association of genetically compromised PITX2 with enhanced susceptibility to ECD has been demonstrated in animal models [79–81]. In mice, PITX2 deficiency results in complicated cardiac defects, including atrial septal defect, ventricular septal defect, ECD, hypoplasia of the right ventricle, and failure to form normal cardiac valves [81]. Further studies shows that ablation of PITX2 results in distortion, rather than loss, of muscle anlagen, suggesting that its function becomes critical during the colonization of, and/or fiber assembly in, the anlagen. In addition, myogenic cells lacking PITX2 are smaller and more symmetrical with decreased motility, which may prevent proper assembly of higher-order fibers within anlagen [88]. Nevertheless, PITX2c expression in mesenchymal cushion cells remains a controversial topic. Furtado and colleagues [70] reported that in mice PITX2c was expressed in trabecular and septal, as well as non-trabecular, myocardium, and had a strong expression bias in myocardium associated with individual endocardial cushions of the ativoventricular canal and outflow tract, which are essential for cardiac septation. Two other groups [80,89] also reported the expression of PITX2c in these structures. Fate-mapping studies using a PITX2 cre recombinase knock-in allele showed that daughters of PITX2-expressing cells populated the right and left ventricles, atrioventricular cushions and valves and pulmonary veins. In PITX2 mutant embryos, descendants of PITX2-expressing cells failed to contribute to the atrioventricular cushions and valves and the pulmonary vein, resulting in abnormal morphogenesis of these structures [80]. However, lineage-tracing studies in mice showed that myocardium did not transform into mesenchyme in cushions [90]. In humans, PITX2c was expressed predominantly in left atria, with lower levels in right atrium and left and right ventricles [72]. Due to pronounced spatial and temporal difference in gene expression even for the same species, further work will be necessary to clarify this issue, especially for all isoforms of PITX2 in human heart.

Up to now, in humans mutated PITX2 has been linked to type 1 ARS [66–68], type 2 iridogoniodygenesis [91], Peters’ anomaly [92], ring dermoid of cornea [93], various congenital heart diseases [16,82–84], and atrial fibrillation [94–97]. In this study, a novel PITX2 mutation was linked to atypical ARS with ECD being the main phenotype. The remarkable phenotypic diversities caused by PITX2 mutations may be explained as follows. Firstly, different genetic backgrounds, including possibly common SNPs altering disease susceptibility, contribute to the variable phenotypes. Secondly, distinct epigenetic modifiers may account for the significant
phenotypic heterogeneity among these mutation carriers. Thirdly, delayed penetrance or incomplete penetration may also be responsible for the discrepant clinical expressivity. Finally, mutations as found in this study may be merely a genetic risk factor predisposing to a disease, rather than a direct cause, and environmental risk factors may be required for the onset of the disease [98].

Conclusions

In conclusion, this study firstly links PITX2 loss-of-function mutation to ECD and ARS, which provides novel insight into the molecular mechanisms of CHD and ARS, implying potential implications in antenatal prophylaxis and personalized treatment of CHD and ARS.

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Author Contributions

Conceived and designed the experiments: CMZ LYP YQY. Performed the experiments: CMZ LL XYL JW XLZ FY RGL XBQ YQY. Analyzed the data: CMZ LYP YQY. Contributed reagents/materials/analysis tools: CMZ LL XYL XLZ YQY. Wrote the paper: CMZ LYP YQY. Funding: YQY LL LYP.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. American Heart Association Statistics Committee and Stroke Statistics Subcommittee (2014) Heart disease and stroke statistics—2014 update: a report from the American Heart Association. Circulation 129: e28–e292. doi: 10.1161/01.cir.000044139.02102.80 PMID: 24352519

2. Müller J, Hess J, Hager A (2012) Minor symptoms of depression in patients with congenital heart disease have a larger impact on quality of life than limited exercise capacity. Int J Cardiol 154: 265–269. doi: 10.1016/j.ijcard.2010.09.029 PMID: 20926144

3. Mulkey SB, Swearingen CJ, Melguizo MS, Schmitz ML, Ou X, Ramakrishnaiah RH, et al. (2013) Multi-tiered analysis of brain injury in neonates with congenital heart disease. Pediatr Cardiol 34: 1772–1784. doi: 10.1007/s00246-013-0712-6 PMID: 23652966

4. Hoffmann A, Chockalingam P, Balint OH, Dadashev A, Dimopoulos K, Engel R, et al. (2010) Cerebrovascular accidents in adult patients with congenital heart disease. Heart 96: 1223–1226. doi: 10.1136/hrt.2010.196147 PMID: 20692328

5. Dimopoulos K, Wort SJ, Gatzoulis MA (2014) Pulmonary hypertension related to congenital heart disease: a call for action. Eur Heart J 35: 691–700. doi: 10.1093/eurheartj/ehu437 PMID: 24168793

6. Alonso-Gonzalez R, Borgia F, Diller GP, Inuzuka R, Kempny A, Martinez-Naharro A, et al. (2013) Abnormal lung function in adults with congenital heart disease: prevalence, relation to cardiac anatomy, and association with survival. Circulation 127: 882–890. doi: 10.1161/CIRCULATIONAHA.112.126755 PMID: 23382015

7. Martinez-Quintana E, Rodríguez-González F, Nieto-Lago V (2013) Subclinical hypothyroidism in grown-up congenital heart disease patients. Pediatr Cardiol 34: 912–917. doi: 10.1007/s00246-012-0571-6 PMID: 23143351

8. Ohuch H, Miyamoto Y, Yamamoto M, Ishihara H, Takata H, Miyazaki A, et al. (2009) High prevalence of abnormal glucose metabolism in young adult patients with complex congenital heart disease. Am Heart J 158: 30–39. doi: 10.1016/j.ahj.2009.04.021 PMID: 19540399

9. Kröönström LA, Johansson L, Zetterström AK, Dellborg M, Eriksson P, Cider Å (2014) Muscle function in adults with congenital heart disease. Int J Cardiol 170: 358–363. doi: 10.1016/j.ijcard.2013.11.014 PMID: 24295997

10. Moutafi AC, Manis G, Dellos C, Tousoulis D, Davos CH (2014) Cardiac autonomic nervous activity in adults with coarctation of the aorta late after repair. Int J Cardiol 173: 566–568. doi: 10.1016/j.ijcard.2014.03.120 PMID: 24684999
11. Rushani D, Kaufman JS, Ionescu-Ittu R, Mackie AS, Pilote L, Therrien J, et al. (2013) Infective endocarditis in children with congenital heart disease: cumulative incidence and predictors. Circulation 128: 1412–1419. doi: 10.1161/CIRCULATIONAHA.113.001827 PMID: 24060942

12. Fahed AC, Roberts AE, Mital S, Lakdawala NK (2014) Heart failure in congenital heart disease: a confluence of acquired and congenital. Heart Fail Clin 10: 219–227. doi: 10.1016/j.hfc.2013.09.017 PMID: 24275306

13. Perry JC (2012) Sudden cardiac death and malignant arrhythmias: the scope of the problem in adult congenital heart patients. Pediatr Cardiol 33: 484–490. doi: 10.1007/s00246-012-0171-5 PMID: 22318852

14. Tutarel O, Kempny A, Alonso-Gonzalez R, Jabbour R, Li W, Uebing A, et al. (2014) Congenital heart disease beyond the age of 60: emergence of a new population with high resource utilization, high morbidity, and high mortality. Eur Heart J 35: 725–732. doi: 10.1093/eurheartj/ehu257 PMID: 23882067

15. Hunter RM, Isaac M, Frigiola A, Blundell D, Brown K, Bull K (2013) Lifetime costs and outcomes of repair of Tetralogy of Fallot compared to natural progression of the disease: Great Ormond Street Hospital cohort. PLoS One 8: e59734. doi: 10.1371/journal.pone.0059734 PMID: 23533645

16. Zaidi S, Choi M, Wakimoto H, Ma L, Jiang J, Overton JD, et al. (2013) De novo mutations in histone-modifying genes in congenital heart disease. Nature 498: 220–223. doi: 10.1038/nature12141 PMID: 23665959

17. Wang W, Wang Y, Gong F, Zhu W, Fu S (2013) MTHFR C677T polymorphism and risk of congenital heart defects: evidence from 29 case-control and TDT studies. PLoS One 8: e58041. doi: 10.1371/journal.pone.0058041 PMID: 23536781

18. Wang C, Xie L, Zhou K, Zhan Y, Li Y, Li H, et al. (2013) Increased risk for congenital heart defects in children carrying the ABCB1 Gene C3435T polymorphism and maternal periconceptional toxicants exposure. PLoS One 8: e68807. doi: 10.1371/journal.pone.0068807 PMID: 23874772

19. Chang SW, Misliankar M, Misra C, Huang N, Dajusta DG, Harrison SM, et al. (2013) Genetic abnormalities in FOXP1 are associated with congenital heart defects. Hum Mutat 34: 1226–1230. doi: 10.1002/humu.22366 PMID: 23766104

20. Sanchez-Castro M, Gordon CT, Petit F, Nord AS, Callier P, Andrieux J, et al. (2013) Congenital heart defects in patients with deletions upstream of SOX9. Hum Mutat 34: 1628–1631. doi: 10.1002/humu.24115316

21. Lukasz A, Beutel G, Kümpers P, Denecke A, Westhoff-Bleck M, Schieffer B, et al. (2013) Angiopoietin-2 in adults with congenital heart disease and heart failure. PLoS One 8: e66861. doi: 10.1371/journal.pone.0066861 PMID: 23826161

22. Long F, Wang X, Fang S, Xu Y, Sun K, Chen S, et al. (2013) A potential relationship among beta-defensins haplotype, SOX7 duplication and cardiac defects. PLoS One 8: e672515. doi: 10.1371/journal.pone.0072515 PMID: 24009689

23. Lahm H, Deutsch MA, Dreßen M, Doppler S, Werner A, Hörer J, et al. (2013) Mutational analysis of the human MESP1 gene in patients with congenital heart disease reveals a highly variable sequence in exon 1. Eur J Med Genet 56: 591–598. doi: 10.1016/j.ejmg.2013.09.001 PMID: 24056064

24. Wang E, Jin W, Duan W, Qiao B, Sun S, Huang G, et al. (2013) Association of two variants in SMAD7 with the risk of congenital heart disease in the Han Chinese population. PLoS One 8: e72423. doi: 10.1371/journal.pone.0072423 PMID: 24039762

25. Chen D, Qiao Y, Meng H, Pang S, Huang W, Zhang H, et al. (2013) Genetic analysis of the TBX3 gene promoter in ventricular septal defects. Gene 512: 185–188. doi: 10.1016/j.gene.2012.10.066 PMID: 23116943

26. Wu M, Li Y, He X, Shao X, Wang Y, Zhao M, et al. (2013) Mutational and functional analysis of the BVES gene coding region in Chinese patients with non-syndromic tetralogy of Fallot. Int J Mol Med 31: 899–903. doi: 10.3892/ijmm.2013.1275 PMID: 23403794

27. Gong X, Wu X, Ma X, Wu D, Zhang T, He L, et al. (2013) Microdeletion and microduplication analysis of chesneoconotruncal defects patients with targeted array comparative genomic hybridization. PLoS One 8: e76314. doi: 10.1371/journal.pone.0076314 PMID: 24098474

28. Al Turki S, Manickaraj AK, Mercer CL, Gerety SS, Hitz MP, Lindsay S, et al. (2014) Rare variants in NR2F2 cause congenital heart defects in humans. Am J Hum Genet 94: 574–585. doi: 10.1016/j.ajhg.2014.03.007 PMID: 24702954

29. Bansal V, Dorn C, Grunert M, Klaassen S, Hetzer R, Berger F, et al. (2014) Outlier-based identification of copy number variations using targeted resequencing in a small cohort of patients with Tetralogy of Fallot. PLoS One 9: e85375. doi: 10.1371/journal.pone.0085375 PMID: 24400131
30. Zhu X, Deng X, Huang G, Wang J, Yang J, Chen S, et al. (2014) A novel mutation of Hyaluronan synthase 2 gene in Chinese children with ventricular septal defect. PLoS One 9: e87437. doi:10.1371/journal.pone.0087437 PMID: 24558368

31. Liu AP, Chow PC, Lee PP, Mok GT, Tang WF, Lau ET, et al. (2014) Under-recognition of 22q11.2 deletion in adult Chinese patients with conotruncal anomalies: implications in transitional care. Eur J Med Genet 57: 306–311. doi: 10.1016/j.ejmg.2014.03.014 PMID: 24721633

32. Lin B, Wang Y, Wang Z, Tan H, Kong X, Shu Y, et al. (2014) Uncovering the rare variants of DLC1 isoform 1 and their functional effects in a Chinese sporadic congenital heart disease cohort. PLoS One 9: e90215. doi:10.1371/journal.pone.0090215 PMID: 24587289

33. Xu J, Lin Y, Si L, Jin G, Dai J, Wang C, et al. (2014) Genetic variants at 10p11 confer risk of Tetralogy of Fallot in Chinese of Nanjing. PLoS One 9: e89636. doi:10.1371/journal.pone.0089636 PMID: 24594544

34. Cowan J, Tariq M, Ware SM (2014) Genetic and functional analyses of ZIC3 variants in congenital heart disease. Hum Mutat 35: 66–75. PMID: 24123890

35. Cai B, Zhang T, Zhong R, Zou L, Zhu B, Chen W, et al. (2014) Genetic variant in MTRR, but not MTR, is associated with risk of congenital heart disease: an integrated meta-analysis. PLoS One 9: e89609. doi: 10.1371/journal.pone.0089609 PMID: 24595101

36. Liu Y, Wang F, Wu Y, Tan S, Wen Q, Wang J, et al. (2014) Variations of CITED2 are associated with congenital heart disease (CHD) in Chinese population. PLoS One 9: e98157. doi:10.1371/journal.pone.0098157 PMID: 24848765

37. Lozić B, Krželj V, Kuzmić-Prusac I, Kuzmanić-Šamija R, Capkun V, Lasan R, et al. (2014) The OSR1 rs12329305 Polymorphism Contributes to the Development of Congenital Malformations in Cases of Stillborn/Neonatal Death. Med SciMonit 20: 1531–1538. doi:10.12659/MSM.890916 PMID: 25164089

38. Lalani SR, Belmont JW (2014) Genetic basis of congenital cardiovascular malformations. Eur J Med Genet 57: 402–413. doi:10.1016/j.ejmg.2014.04.010 PMID: 24793338

39. Töpf A, Griffin HR, Glen E, Soemedi R, Brown DL, Hall D, et al. (2014) Functionally significant, rare transcription factor variants in tetralogy of fallot. PLoS One 9: e95453. doi:10.1371/journal.pone.0095453 PMID: 25093829

40. Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, et al. (1998) Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281: 108–111. PMID: 9651244

41. Reamon-Buettner SM, Borlik J (2010) NKX2-5: an update on this hypermutable homeodomain protein and its role in human congenital heart disease (CHD). Hum Mutat 31: 1185–1194. doi: 10.1002/humu.21345 PMID: 20725931

42. Huang W, Meng H, Qiao Y, Pang S, Chen D, Yan B (2013) Two novel and functional DNA sequence variants within an upstream enhancer of the human NKX2-5 gene in ventricular septal defects. Gene 524: 152–155. doi:10.1016/j.gene.2013.04.043 PMID: 23644027

43. Wang Z, Zou L, Zhong R, Zhu B, Chen W, Shen N, et al. (2013) Associations between two genetic variants in NKX2-5 and risk of congenital heart disease in Chinese population: a meta-analysis. PLoS One 8: e70979. doi:10.1371/journal.pone.0070979 PMID: 23936479

44. Reamon-Buettner SM, Sattlegger E, Ciribilli Y, Inga A, Wessel A, Borlak J (2013) Transcriptional defect of an inherited NKX2-5 haplotype comprising a SNP, a nonsynonymous and a synonymous mutation, associated with human congenital heart disease. PLoS One 8: e83295. doi:10.1371/journal.pone.0083295 PMID: 24376681

45. Costa MW, Guo G, Wolstein O, Vale M, Castro ML, Wang L, et al. (2013) Functional characterization of a novel mutation in NKX2-5 associated with congenital heart disease and adult-onset cardiomyopathy. CircCardiovasc Genet 6: 238–247. doi: 10.1161/CIRCGENETICS.113.000057 PMID: 23661673

46. Huong W, Meng H, Qiao Y, Pang S, Chen D, Yan B (2013) Two novel and functional DNA sequence variants within an upstream enhancer of the human NKX2-5 gene in ventricular septal defects. Gene 524: 152–155. doi: 10.1016/j.gene.2013.04.043 PMID: 23644027

47. Wang Z, Zou L, Zhong R, Zhu B, Chen W, Shen N, et al. (2013) Associations between two genetic variants in NKX2-5 and risk of congenital heart disease in Chinese population: a meta-analysis. PLoS One 8: e70979. doi:10.1371/journal.pone.0070979 PMID: 23936479

48. Reamon-Buettner SM, Sattlegger E, Ciribilli Y, Inga A, Wessel A, Borlak J (2013) Transcriptional defect of an inherited NKX2-5 haplotype comprising a SNP, a nonsynonymous and a synonymous mutation, associated with human congenital heart disease. PLoS One 8: e83295. doi:10.1371/journal.pone.0083295 PMID: 24376681

49. Costa MW, Guo G, Wolstein O, Vale M, Castro ML, Wang L, et al. (2013) Functional characterization of a novel mutation in NKX2-5 associated with congenital heart disease and adult-onset cardiomyopathy. CircCardiovasc Genet 6: 238–247. doi: 10.1161/CIRCGENETICS.113.000057 PMID: 23661673

50. Huong W, Meng H, Qiao Y, Pang S, Chen D, Yan B (2013) Two novel and functional DNA sequence variants within an upstream enhancer of the human NKX2-5 gene in ventricular septal defects. Gene 524: 152–155. doi: 10.1016/j.gene.2013.04.043 PMID: 23644027

51. Wang Z, Zou L, Zhong R, Zhu B, Chen W, Shen N, et al. (2013) Associations between two genetic variants in NKX2-5 and risk of congenital heart disease in Chinese population: a meta-analysis. PLoS One 8: e70979. doi:10.1371/journal.pone.0070979 PMID: 23936479

52. Reamon-Buettner SM, Sattlegger E, Ciribilli Y, Inga A, Wessel A, Borlak J (2013) Transcriptional defect of an inherited NKX2-5 haplotype comprising a SNP, a nonsynonymous and a synonymous mutation, associated with human congenital heart disease. PLoS One 8: e83295. doi:10.1371/journal.pone.0083295 PMID: 24376681
49. Zhao L, Ni SH, Liu XY, Wei D, Yuan F, Xu L, et al. (2014) Prevalence and spectrum of Nkx2.6 mutations in patients with congenital heart disease. Eur J Med Genet 57: 579–586. doi: 10.1016/j.ejmg.2014.08.005 PMID: 25195019

50. Wang J, Zhang DF, Sun YM, Li RG, Qiu XB, Qu XK, et al. (2014) NKKX2-6 mutation predisposes to familial atrial fibrillation. Int J Mol Med 34: 1581–1590. doi: 10.3892/ijmm.2014.1971 PMID: 25319568

51. Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA, et al. (2003) GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424: 443–447. PMID: 12845333

52. Rajagopal SK, Ma Q, Obler D, Shen J, Manichaikul A, Tomita-Mitchell A, et al. (2007) Spectrum of heart disease associated with murine and human GATA4 mutation. J Mol Cell Cardiol 43: 443–447. PMID: 17643447

53. Yang YQ, Gharibeh L, Li RG, Xin YF, Wang J, Liu ZM, et al. (2013) GATA4 loss-of-function mutations underlie familial tetralogy of fallot. Hum Mutat 34: 1662–1671. doi: 10.1002/humu.22434 PMID: 24000169

54. Wang E, Sun S, Qiao B, Duan W, Huang G, An Y, et al. (2013) Identification of functional mutations in GATA4 in patients with congenital heart disease. PLoS One 8: e62138. doi: 10.1371/journal.pone.0062138 PMID: 23626780

55. Yang YQ, Wang J, Liu XY, Chen XZ, Zhang W, Wang XZ (2013) Spectrum of GATA4 associated with congenital atrial septal defects. Arch Med Sci 9: 976–983. doi: 10.5114/aoms.2013.39788 PMID: 24482639

56. Li RG, Li L, Qiu XB, Yuan F, Xu L, Li X, et al. (2013) GATA4 loss-of-function mutation underlies familial dilated cardiomyopathy. BiochemBiophys Res Commun 439: 591–596. doi: 10.1016/j.bbrc.2013.09.023 PMID: 24041700

57. Xiang R, Fan LL, Huang H, Cao BB, Li XP, Peng DQ, et al. (2014) A novel mutation of GATA4 (K319E) is responsible for familial atrial septal defect and pulmonary valve stenosis. Gene 534: 320–323. PMID: 24498650

58. Li J, Liu WD, Yang ZL, Yuan F, Xu L, Li RG, et al. (2014) Prevalence and spectrum of GATA4 mutations associated with sporadic dilated cardiomyopathy. Gene 548: 174–181. doi: 10.1016/j.gene.2014.07.022 PMID: 25017055

59. Jiang JQ, Li RG, Wang J, Liu XY, Xu YJ, Fang WY, et al. (2013) Prevalence and spectrum of GATA5 mutations associated with congenital heart disease. Int J Cardiol 165: 570–573. doi: 10.1016/j.ijcard.2012.09.039 PMID: 23031282

60. Huang RT, Xue S, Xu YJ, Zhou M, Yang YQ (2014) Somatic GATA5 mutations in sporadic tetralogy of Fallot. Int J Mol Med 33: 1227–1235. doi: 10.3892/ijmm.2014.1674 PMID: 24573614

61. Shi LM, Tao JW, Qiu XB, Wang J, Yuan F, Xu L, et al. (2014) GATA5 loss-of-function mutations associated with congenital bicuspid aortic valve. Int J Mol Med 33: 1219–1226. doi: 10.3892/ijmm.2014.1700 PMID: 24638895

62. Huang RT, Xue S, Xu YJ, Yang YQ (2013) Somatic mutations in the GATA6 gene underlie sporadic tetralogy of Fallot. Int J Mol Med 31: 51–58. doi: 10.3892/ijmm.2012.1188 PMID: 23175051

63. Xu L, Zhao L, Yuan F, Jiang WF, Liu H, Li RG, et al. (2014) GATA6 loss-of-function mutations contribute to familial dilated cardiomyopathy. Int J Mol Med 34: 1315–1322. doi: 10.3892/ijmm.2014.1896 PMID: 25119427

64. Liang D, Zhen L, Yuan T, Huang J, Deng F, Wu YH, et al. (2014) miR-10a regulates proliferation of human cardiomyocyte progenitor cells by targeting GATA6. PLoS One 9: e103097. doi: 10.1371/journal.pone.0103097 PMID: 25068583

65. McCulley DJ, Black BL (2012) Transcription factor pathways and congenital heart disease. Curr Top Dev Biol 100: 253–277. doi: 10.1016/B978-0-12-387786-4.00008-7 PMID: 22449847

66. Semina EV, Reiter R, Leysens NJ, Alward WL, Small KW, Datson NA, et al. (1996) Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. Nat Genet 14: 392–399. PMID: 8944018

67. Hjalt TA, Semina EV (2005) Current molecular understanding of Axenfeld-Rieger syndrome. Expert Rev Mol Med 7: 1–17.

68. Tumer Z, Bach-Holm D (2009) Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. Eur J Hum Genet 17: 1527–1539. doi: 10.1038/ejhg.2009.93 PMID: 19513095

69. Simard A, Di Giorgio L, Amen M, Westwood A, Amendt BA, Ryan AK (2009) The Pitx2c N-terminal domain is a critical interaction domain required for asymmetric morphogenesis. Dev Dyn 238: 2459–2470. doi: 10.1002/dvdy.22062 PMID: 19681163
70. Furtado MB, Biben C, Shiratori H, Hamada H, Harvey RP (2011) Characterization of Pitx2c expression in the mouse heart using a reporter transgene. Dev Dyn 240:195–203. doi: 10.1002/dvdy.22492 PMID: 21089073

71. Kahr PC, Piccini I, Fabritz L, Greber B, Scholer H, Scheld HH, et al. (2011) Systematic analysis of gene expression differences between left and right atria in different mouse strains and in human atrial tissue. PLoS One 6: e26389. doi: 10.1371/journal.pone.0026389 PMID: 22039477

72. Kirchhof P, Kahr PC, Kaese S, Piccini I, Vokshi I, Scheld HH, et al. (2011) PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression. Circ Cardiovasc Genet 4: 123–133. doi: 10.1161/CIRCGENETICS.110.958058 PMID: 21282332

73. Hsu J, Hanna P, Van Wagoner DR, Barnard J, Serre D, Chung MK, et al. (2012) Whole genome expression differences in human left and right atria ascertained by RNA sequencing. Circ Cardiovasc Genet 5: 327–335. doi: 10.1161/CIRCGENETICS.111.961631 PMID: 22474228

74. Torrado M, Franco D, Hernández-Torres F, Crespo-Leiro MG, Iglesias-Gil C, Castro-Beiras A, et al. (2014) Pitx2c is reactivated in the failing myocardium and stimulates myf5 expression in cultured cardiomyocytes. PLoS One 9: e90561. doi: 10.1371/journal.pone.0090561 PMID: 24595098

75. Schweickert A, Campione1 M, Steinbeisser H, Blum M (2000) Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left-right asymmetry. Mech Dev 90: 41–51. PMID: 10585561

76. Liu C, Liu W, Lu MF, Brown NA, Martin JF (2001) Regulation of left-right asymmetry by thresholds of Pitx2c activity. Development 128: 2039–2048. PMID: 11493526

77. Galli D, Domínguez JN, Zaffran S, Munk A, Brown NA, Buckingham ME (2008) Atrial myocardium derives from the posterior region of the second heart field, which acquires left-right identity as Pitx2c is expressed. Development 135: 1157–1167. doi: 10.1242/dev.014563 PMID: 18272591

78. Lozano-Velasco E, Chinchilla A, Martínez-Fernández S, Hernández-Torres F, Navarro F, Lyons GE, et al. (2011) Pitx2c modulates cardiac-specific transcription factors networks in differentiating cardiomyocytes from murine embryonic stem cells. Cells Tissues Organs 194: 349–362. doi: 10.1159/000323533 PMID: 21389672

79. Dagle JM, Sabel JL, Littig JL, Sutherland LB, Kolker SJ, Weeks DL (2003) Pitx2c attenuation results in cardiac defects and abnormalities of intestinal orientation in developing Xenopuslaevis. Dev Biol 262: 268–281. PMID: 14550790

80. Liu C, Liu W, Palie J, Lu MF, Brown NA, Martin JF (2002) Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions. Development 129: 5081–5091. PMID: 12397115

81. Kitamura K, Miura H, Miyagawa-Tomita S, Yanazawa M, Katoh-Fukui Y, Suzuki R, et al. (1999) Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. Development 126: 5749–5758. PMID: 10572050

82. Wang J, Xin YF, Xu WJ, Liu ZM, Qiu XB, Qu XK, et al. (2013) Prevalence and spectrum of PITX2c mutations associated with congenital heart disease. DNA Cell Biol 32: 708–716. doi: 10.1089/dna.2013.2185 PMID: 24083357

83. Yuan F, Zhao L, Wang J, Zhang W, Li X, Qiu XB, et al. (2013) PITX2c loss-of-function mutations responsible for congenital atrial septal defects. Int J Med Sci 10: 1422–1429. doi: 10.7150/ijms.6809 PMID: 23983605

84. Wei D, Gong XH, Qiu G, Wang J, Yang YQ (2014) Novel PITX2c loss-of-function mutations associated with complex congenital heart disease. Int J Mol Med 33: 1201–1208. doi: 10.3892/ijmm.2014.1689 PMID: 24604414

85. Hjalt TA, Amendt BA, Murray JC (2001) PITX2 regulates procollagen lysyl hydroxylase (PLOD) gene expression: implications for the pathology of Rieger syndrome. J Cell Biol 152: 545–552. PMID: 11157981

86. Cox CJ, Espinoza HM, McWilliams B, Chappell K, Morton L, Hjalt TA, et al. (2002) Differential regulation of gene expression by PITX2 isoforms. J Biol Chem 277: 25001–25010. PMID: 11948188

87. Ganga M, Espinoza HM, Cox CJ, Morton L, Hjalt TA, Lee Y, et al. (2003) PITX2 isoform-specific regulation of atrial natriuretic factor expression: synergism and repression with Nkx2.5. J Biol Chem 278: 22437–22445. PMID: 12692125

88. Campbell AL, Shih HP, Xu J, Gross MK, Kioussi C (2012) Regulation of motility of myogenic cells in filling limb muscle anlagen by Pitx2. PLoS One 7:e35822. doi: 10.1371/journal.pone.0035822 PMID: 22558231

89. Ai D, Liu W, Ma L, Dong F, Lu MF, Wang D, et al. (2006) Pitx2 regulates cardiac left-right asymmetry by patterning second cardiac lineage-derived myocardium. Dev Biol 296:437–449. PMID: 16836994
90. de Lange FJ, Moorman AF, Anderson RH, Männer J, Soufan AT, de Gier-de Vries C, et al. (2004) Lineage and morphogenetic analysis of the cardiac valves. Circ Res 95: 645–654. PMID: 15297379
91. Banerjee-Basu S, Baxevanis AD (1999) Threading analysis of the Pitx2 homeodomain: predicted structural effects of mutations causing Rieger syndrome and iridogoniodysgenesis. Hum Mutat 14: 312–319. PMID: 10502778
92. Doward W, Perveen R, Lloyd IC, Ridgway AE, Wilson L, Black GC (1999) A mutation in the Rieg1 gene associated with Peters’ anomaly. J Med Genet 36: 152–155. PMID: 10051017
93. Xia K, Wu L, Liu X, Xi X, Liang D, Zheng D, et al. (2004) Mutation in PITX2 is associated with ring dermoid of the cornea. J Med Gene 41: e129. PMID: 15591271
94. Yang YQ, Xu YJ, Li RG, Qu XK, Fang WY, Liu X (2013) Prevalence and spectrum of PITX2c mutations associated with familial atrial fibrillation. Int J Cardiol 168: 2873–2876. doi: 10.1016/j.ijcard.2013.03.141 PMID: 23611745
95. Zhou YM, Zheng PX, Yang YQ, Ge ZM, Kang WQ (2013) A novel PITX2c loss-of-function mutation underlies lone atrial fibrillation. Int J Mol Med 32: 827–834. doi: 10.3892/ijmm.2013.1463 PMID: 23913021
96. Wang J, Zhang DF, Sun YM, Yang YQ (2014) A novel PITX2c loss-of-function mutation associated with familial atrial fibrillation. Eur J Med Genet 57: 25–31. doi: 10.1016/j.ejmg.2013.11.004 PMID: 24333117
97. Qiu XB, Xu YJ, Li RG, Xu L, Liu X, Fang WY, et al. (2014) PITX2C loss-of-function mutations responsible for idiopathic atrial fibrillation. Clinics (Sao Paulo) 69: 15–22. doi: 10.6061/clinics/2014(01)03 PMID: 24473555
98. Yu H, Xu JH, Song HM, Zhao L, Xu WJ, Wang J, et al. (2014) Mutational spectrum of the NKX2-5 gene in patients with lone atrial fibrillation. Int J Med Sci 11: 554–563. doi: 10.7150/ijms.8407 PMID: 24782644