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CITED2 mutation links congenital heart defects to dysregulation of the cardiac gene VEGF and PITX2C expression

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ABSTRACT

CITED2, a cardiac transcription factor, plays an important role in cardiac development. *CITED2* mutations lead to a constellation of cardiac defects, which include tetralogy of Fallot and outflow tract malformations. However, the mechanisms underlying these mutations are poorly understood. We investigated the function and mechanism of two missense mutations, G184S and S192G, responsible for tetralogy of Fallot and aortic stenosis, respectively. We found that *CITED2* variants decreased its ability to mediate the expression of vascular endothelial growth factor (VEGF) and the expression of the paired-like homeodomain transcription factor 2-gamma (PITX2C), both of which are closely related to cardiac development. Luciferase reporter and mammalian two-hybrid assays showed that G184S and S192G in *CITED2* restored the expression of *VEGF*, which was due to a reduction in its competitiveness with hypoxia inducible factor 1-alpha (HIF1-α) for binding to CBP/p300. In addition, we found that the G184S and S192G mutant decreased cooperation between *CITED2* and transcription factor AP2-gamma (TFAP2C) in the transactivation of the *PITX2C* gene. These results provide important evidence that the mutation of *CITED2* may play a role in the development of congenital heart disease (CHD) in humans.

1. Introduction

Congenital heart disease (CHD) is responsible for the majority of deaths in infancy and is the leading cause of noninfectious infant mortality, with an incidence of approximately 1% [1,2]. As a result, CHD has become an important part of the overall disease burden faced by government health agencies. Transcription factors are known to play a fundamental role in all levels of heart development, including cardiac lineage determination, chamber formation, valvulogenesis, and septation [3,4]. The heterogeneity of CHD associated with single-gene defects, as demonstrated for *NKX2.5* or *TBX5* mutations [5,6], makes mechanistic understanding of gene function challenging and suggests a complex genetic network with modifier genes, genetic polymorphism, and an effect of environmental factors.

CITED2 is a cAMP-responsive element-binding protein (CBP)/p300-transactivator, with an ED(Glu/Asp)-rich C-terminal domain that functions as an important modulator in the development of the heart [7,8]. *CITED2*, which lacks a typical DNA-binding domain, has been proposed to be a negative regulator of HIF-1α through its competitive binding to the CH1 domain of CBP/p300, where HIF1-α also binds. The competitive binding leads to inhibit HIF-1 mediated signaling and to a decrease in HIF-1-responsive genes such as *VEGF* [9,10]. The mRNA levels of several HIF-1-responsive genes are increased in Cited$^{-/-}$ hearts [11]. As reported elsewhere, defects in Cited2$^{-/-}$ embryos closely resemble major defects observed in *VEGF* transgenic embryos [12]. *CITED2* is also a co-activator of the transcriptional activity of the *TFAP2* isoform, which is an extensively expressed nuclear protein with functions in embryogenesis, inflammation, and stress responses. *CITED2* and the *TFAP2* isoforms have been detected together at the Pitx2c promoter in embryonic mouse hearts [13,14]. This suggests that *CITED2* plays a role in left–right patterning through the Nodal-PITX2C pathway.

In mice, knock-out of Cited2 is lethal during embryogenesis and induces abnormalities as a consequence of cardiac malformations and left–right axis formation [15]. Cited2 plays an important role in the development of the heart, adrenal glands, and nervous system, as well as in fibroblast proliferation [14,16]. Some Cited2-null embryos express heterotaxia, which is associated with cardiac malformations [17]. Three mutations (S170-G178del, G178-S179ins9, and S198-G199del) leading to alterations of the amino acid sequences, were reported in German patients with CHD [18]. Two missense mutations (G184S and S192G) were also recently found in Chinese patients with CHD [19], but the mechanisms underlying these mutations are poorly understood.

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In this study, we investigated the effect of the two missense mutations, which are the result of substitution of highly conserved glycine residues at the 184th and 192nd positions in *CITED2*. Using a luciferase reporter assay, we showed that G184S in *CITED2* decreases the cooperation between *CITED2* and *TFAP2C* in the transactivation of the *PITX2C* gene. We also showed that the G184S and S192G in *CITED2* result in the loss of the ability to mediate the expression of *VEGF* by disabling competitive binding with HIF1-α to CBP/p300. Therefore, G184S and S192G mutations affect the transcriptional regulatory properties of *PITX2C* and *VEGF*, both of which are closely related to cardiac development. These data provide important evidence that the mutation of *CITED2* has a potential causative role in the development of CHD in humans.

2. Materials and methods

2.1. Site-directed mutagenesis and plasmid construction

Human CITED2, TFAP2C, and HIF1-α cDNA were obtained from OriGene True-Clone. The G184S and S192G mutations were constructed by polymerase chain reaction (PCR)-mediated mutagenesis with appropriate primers. The open reading frames of wild-type and mutant *CITED2*, as well as *TFAP2C* were amplified by PCR from cDNA, respectively, and inserted into the pEGFP-N1 vector (BD Biosciences, Palo Alto, CA, USA). An 870-bp fragment of the *VEGF* promoter and a 1300-bp fragment of the *PITX2C* promoter amplified by PCR from human genomic DNA were cloned into a KpnI and a XhoI digested luciferase reporter PLG3-basic vector construct, respectively. The primers for all of the PCRs were listed in Table 1.

The TK promoter pGL3-basic vector with 4× GAL4 DNA-binding sites, the Gal4-pCMX vector containing Gal4DBD, and the VP16-pCMX vector with the potent transactivating domain of HSV VP16 were provided by Dr. Ronald M. Evans (Salk Institute for Biological Studies, USA).

The pcDNA3.1-p300 and pCMX-VP16-p300CH1 plasmids were provided by Huang Baiqu (Institute of Genetics and Cytology, Northeast Normal University, China). Gal4-HIF1-α was obtained by cloning the DNA segments into the Gal4-pCMX vector at the NheI sites.

2.2. Cell culture, transfection, and transcriptional assays

The cell lines 293T and MCF-7 were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. Transfection was carried out using a standard calcium phosphate method or Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA).

Table 1
Primers used for PCR.

Name	Primer pair	
pGL3 basic-PITX2C Promoter	F	GGGGTACCGGGGACAAAAGGACTTTC
	R	CCGCTCGAGCCCTGTTGGCCTAACATC
pGL3 basic-VEGF Promoter	F	GGGGTACCTTTGGGTTTTGCCAGACT
	R	CCGCTCGAGAGGAGGGAGCAGGAAAGT
pC3.1(+)-TFAP2C	F	CGGGGTACCACGCCGGACGCCATGTTG
	R	TGCTCTAGACTCTCCTAACCTTTCTTCGTTCC
pCMX-VP16-p300CH1	F	CGCGGATCCTATGGCCGAGAATGTGGTGGAAC
	R	CTAGCTAGCCCAACGGGTGCTCCAGTCAAA
pCMX-GAL4-HIF1-α	F	CATGCTAGCGACCGATTCACCATGGAGG
	R	CATGCTAGCCTCTCCTAACCTTTCTTCGTTCC
Cited2-pEGFP	F	ACGCAAGCTTATGGCAGACCATATGATGGC
	R	ATTTGGTACCGTACAGCTCACTCTGCTGGG
Primers for G184S	F	CTGGCAGCAGCTCGAGCGGCGGCG
	R	GCCCGCGCCGCCGCTCGAGCTGCT
Primers for S192G	F	AGCAACGGCGGCGGCGGCAGCGGCAG
	R	GCCGCCGCCGTTGCTGCTGCCCGCG

2.3. Western blotting

Total proteins were extracted from the 293T cells transfected with CITED2-wild-type, CITED2-G184S, and CITED2-S192G expression plasmids. After electrophoresis, a polyvinylidene fluoride membrane was incubated with anti-β-actin antibody (Sigma, London, UK) and anti-GFP (Abcam, Cambridge, UK). The signals were visualized using the chemiluminescent substrate method with the SuperSignal West Pico kit provided by Pierce Co. (Rockford, USA).

2.4. Subcellular localization

The MCF-7 cells were seeded in six-well tissue culture plates 24 h prior to transfection at approximately 60% confluency. CITED2-GFP expression constructs containing wild-type or mutant were transfected using Lipofectamine 2000, according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were fixed and permeabilized in 4% paraformaldehyde for 10 min and 0.1% Triton X-100 for 20 min, and the DNA was stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 3 min at room temperature. The cells were then analyzed by fluorescence microscopy.

2.5. Mammalian two-hybrid assay

The 293T cells were cotransfected with equimolar amounts of pCMX-VP16-p300-CH1 (wild-type or mutant) or with the pCMX-VP16 vector alone, the pCMX-Gal4-HIF1-α, the TK promoter reporter plasmid, and the Renilla luciferase control plasmid pREP7-RLu. 100 μM Desferrioxamine (DFO) (Sigma, London, UK) was added 6 h after transfection; 30 h post-transfection, the cells were washed and lysed in passive lysis buffer. The transfection efficiency was then normalized to the paired Renilla luciferase activity using the Dual Luciferase Reporter Assay System according to the manufacturer's instructions. Values represent the means of three independent experiments performed in triplicate. The Student's *t*-test was used to determine the statistical significance of unpaired samples.

3. Results

3.1. Detection of expression levels of wild-type and mutant CITED2 by Western blot analysis

The amount of each of the in vitro translated wild-type and mutant G184S and S192G proteins was detected by Western blot

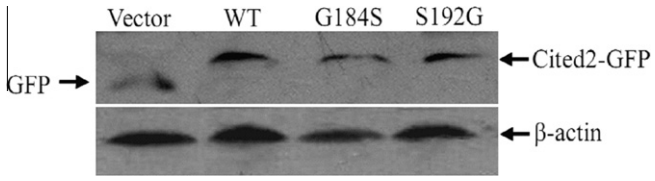

Fig. 1. Western blotting of wild type and mutant CITED2. The 293T cells were transfected with CITED2-wild type, CITED2-G184S, and CITED2-S192G expression plasmids, Western blotting was performed with GFP antibody.

analysis with anti-Cited2 antibody (Fig. 1). The wild-type and mutant proteins were expressed at similar levels in the Western blot analysis of the cell lysates, indicating that the mutation did not result in the expression or instability of the protein.

3.2. Effect of CITED2 variants on the transcriptional activation of HIF-1α to its target gene VEGF

The dual luciferase assay showed that CITED2-wild type efficiently repressed the promoter activity of *VEGF* under normoxic and hypoxia conditions (Fig. 2A). The CITED2-mutants G184S and S192G partly restored the CITED2-wild type mediated-transrepression of VEGF (Fig. 2B). Moreover, we also found that S192G altered CITED2-mediated HIF1-α repression more significantly than G184S.

The mammalian two-hybrid assay showed that CITED2's competitive activity with *HIF-1* α for binding to p300-CH1 was not so pronounced in the G184S mutant, with the G184S mutation only able to repress HIF-1 α by approximately 30% efficiency compared with CITED2-wild type. S192G significantly affected the activity of *CITED2*, resulting in approximately 40% repressive activity compared with CITED2-wild type (Fig. 2C). Thus, G184S and S192G in *CITED2* restored the expression of *VEGF* due to the reduced competitive activity of *CITED2* with *HIF-1* α for binding to p300-CH1.

3.3. Effect of CITED2 mutants on the cooperation between CITED2 and TFAP2C in the transactivation of the PITX2C

We found that CITED2-wild type co-activated the TFAP2C-mediated stimulation of the *PITX2C* reporter construct, which is a TFAP2-responsive gene (Fig. 3A). The CITED2-mutant G184S and S192G partly reduced the cooperative ability with *TFAP2C* to co-stimulate *PITX2C* expression (Fig. 3B).

3.4. Effect of CITED2 mutations on subcellular localization

Subcellular localization was determined using C-terminal GFP fusion constructs of mutant and wild-type CITED2, followed by fluorescence microscopy. The GFP control was located in the nucleus and in the cytoplasm, and CITED2-wild type was detected only in the nucleus of MCF-7 cells (Fig. 4). Mutant CITED2 were also located in the nucleus, indicating that the mutation of CITED2 did not alter its nuclear localization pattern.

4. Discussion

CITED2 encodes a CBP/EP300 interacting transcriptional modulator that functions as an important modulator in cardiac development. Mice lacking Cited2 die in utero, and they show various cardiac malformations including atrial and ventricular septal defects. Mutations of *CITED2* lead to a constellation of cardiac defects, which include tetralogy of Fallot and outflow tract malformations. Two mutations (G184S and S192G) were recently found in Chinese patients, but the mechanism underlying these is still not completely understood. Both the two mutations are located at highly

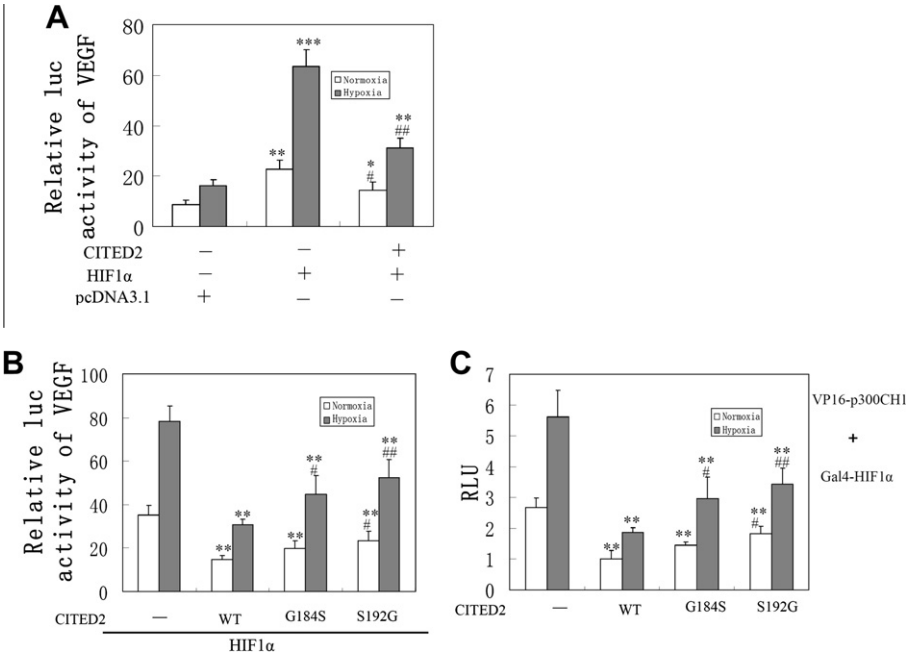

Fig. 2. Effect of CITED2 variants on the transcriptional activation of HIF-1 α to its target gene VEGF. (A) Effect of CITED2-wild type on the transcriptional activation of VEGF by regulating the transcriptional activity of HIF-1 α. The 293T cells were transfected with the VEGF reporter plasmid, together with the expression vector for HIF-1 α, CITED2, or pcDNA3.1. The luciferase activity was normalized to Renilla activity. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the untreated group ($n = 3$). (B) Effect of CITED2-mutant on the transcriptional activation of VEGF compared with the Effect of CITED2-wild type. Cotransfection with CITED2-wild type or with the mutant constructs was shown. $^{*}p < 0.05$, $^{**}p < 0.01$ versus pcDNA3.1 empty vector; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus wild-type. (C) Effect of the G184S and S192G of CITED2 on p300CH1-HIF-1 α interactions assessed by a mammalian two-hybrid assay. The 293T cells were cotransfected with pCMX-Gal4-HIF-1 α, pCMX-VP16-p300CH1, a TK promoter reporter plasmid, and a Renilla luciferase internal control plasmid, along with CITED2-wild type or mutant constructs as indicated. Data are shown as mean values from three independent experiments, with the bars indicating the S.D. The significance of differences was calculated using the independent-samples *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ versus empty vector; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus wild-type.

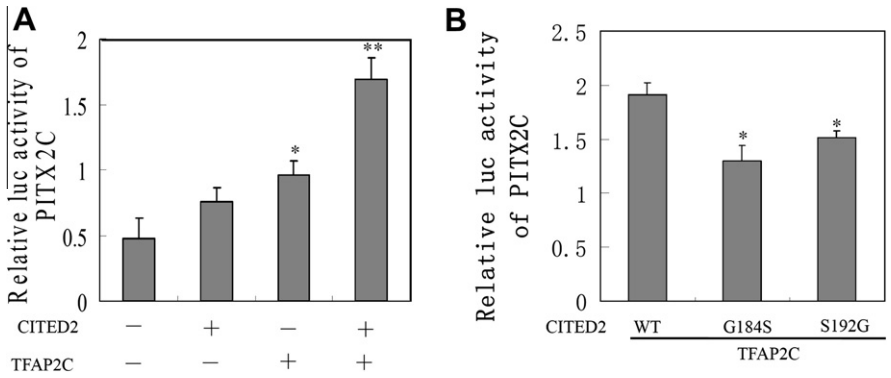

Fig. 3. Effect of CITED2 mutants on the cooperation between CITED2 and TFAP2C in the transactivation of the PITX2C. (A) The cooperation between CITED2-wild type and TFAP2C in the transactivation of the PITX2C gene. The 293T cells were transfected with the PITX2C reporter plasmid, together with the expression vector for CITED2, TFAP2C, or pcDNA3.1 alone. Luciferase activity was normalized to Renilla activity. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the untreated group ($n = 3$). (B) Effect of mutant CITED2 on the cooperation between CITED2 and TFAP2C in the transactivation of the PITX2C gene. Cotransfection with CITED2-wild type or with the mutant constructs is shown. $^{*}p < 0.05$, $^{**}p < 0.01$ versus pcDNA3.1 empty vector; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus wild-type.

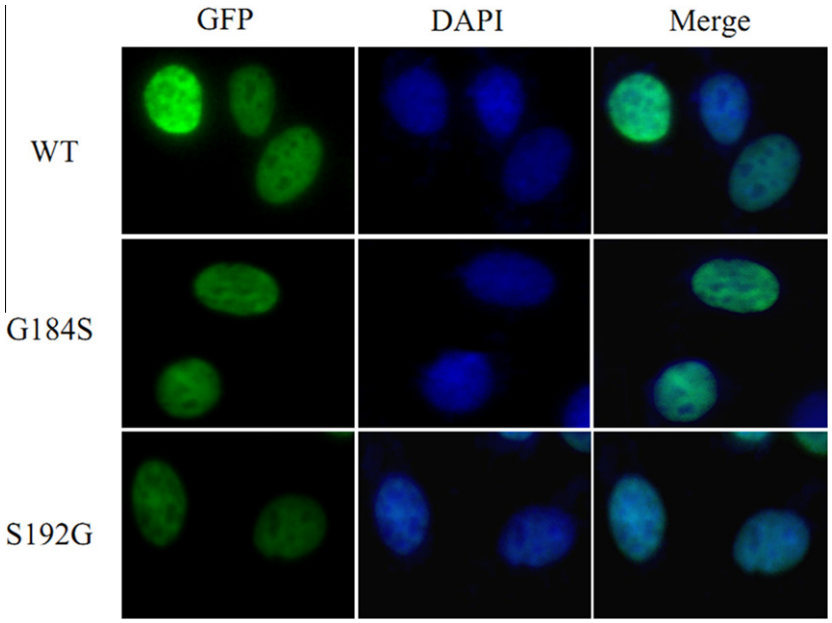

Fig. 4. Subcellular localization of CITED2 variants. The GFP-CITED2 expression constructs containing wild-type or mutant were transfected into MCF-7 cells using Lipofectamine 2000. Localization of wild-type and mutant CITED2 GFP-fusion protein in transfected MCF-7 cells was viewed by a fluorescent microscope. DAPI was used to stain the nucleus.

conserved regions of *CITED2* among many species (humans, mice, rats, cattle, and chickens) by bioinformatics analysis. In the current study, we found that G184S and S192G mutations affected the transcriptional regulatory properties of *PITX2C* and *VEGF*, both of which are closely related to cardiac development. Our results reveal the mechanism of mutant *CITED2* in CHD, indicating that mutation of *CITED2* has a potential causative role in the development of CHD in humans.

The *CITED2* protein has an important functional region rich in serine–glycine called the serine-rich junction (SGJ,S161-G199). Three known mutations (S170-G178del, G178-S179ins9, and S198-G199del) in German patients were located in the SGJ region. Two mutations (G184S and S192G) in Chinese people are also found in the SGJ region, which indicating that this region might be a mutation hot spot in CHD patients. The dual luciferase and mammalian two-hybrid assays showed that both the G184S and S192G mutants disrupt its repressive effect on the promoter activity of *VEGF*, which is upregulated by *HIF-1* α due to the disabling of the competitive binding of *CITED2* with *HIF-1* α for binding to CBP/p300. We also showed that G184S and S192G in *CITED2* decreased the cooperation between *CITED2* and *TFAP2C* in the transactivation of the *PITX2C* gene. In addition, the subcellular localization and Western blotting analysis showed that *CITED2* mutants in the SGJ did not cause the incorrect location and instability of the protein. Based on these observations, we consider that the SGJ region is required for *CITED2* to regulate the expression of cardiac genes in the development of the heart.

The *CITED2* protein has a CBP/p300 binding motif in the C-terminal 32 amino acids (D224-F255), which is required for competing with HIF-1 α in binding to CBP/p300 to inhibit HIF-1 mediated signaling. Although the G184S and S192G mutations were not located in the EP300-binding motif, we still found that mutations in the SGJ region decreased CITED2's ability to compete with HIF-1 α in binding to CBP/p300. We speculate that mutations in the SGJ region might change the protein structure and affect its ability to compete with HIF-1 α in binding to CBP/p300.

In conclusion, the function and mechanism of two missense *CITED2* mutations (G184S and S192G) in Chinese patients were investigated in this study. We found that G184S and S192G mutants affected the transcriptional regulatory properties of *PITX2C* and *VEGF*, both of which are closely related to cardiac development. Further studies showed that the two *CITED2* mutants restored the expression of *VEGF* due to the loss of its competitiveness with *HIF-1* α for binding to p300-CH1. Additionally, the mutations G184S and S192G decreased the cooperation between *CITED2* and *TFAP2C* in transactivation of the *PITX2C* gene. Our study provides important evidence that *CITED2* is a disease-causing gene for congenital heart malformations and that mutation of *CITED2* has a potential causative role in the development of CHD in humans.

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