Evaluating the role of connexin43 in congenital heart disease: Screening for mutations in patients with outflow tract anomalies and the analysis of knock-in mouse models

Guo-Ying Huang[1,3], Li-Jian Xie[1], Kaari L. Linask[2], Chen Zhang[1], Xiao-Qing Zhao[1,2], Yi Yang[1], Guo-Min Zhou[4], Ying-Jie Wu[2], Lucrecia Marquez-Rosado[7], Doff B. McElhinney[5,8], Elizabeth Goldmuntz[5], Chengyu Liu[6], Paul D. Lampe[7], Bishwanath Chatterjee[2,9], Cecilia W. Lo[2,9]

[1]Children's Hospital of Fudan University, [3]Institute of Biomedical Science of Fudan University, [4]Department of Anatomy and Histology, Shanghai Medical School of Fudan University, Shanghai, China, [2]Laboratory of Developmental Biology and [6]Transgenic Core Facility, NIBHL/NIH, Bethesda, Maryland 20892, [5]The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA, [7]Fred Hutchison Cancer Research Center, Seattle, WA, [8]Department of Cardiology, Children's Hospital, Boston, MA 02115, [9]Department of Developmental Biology, University of Pittsburgh School of Medicine, Pittshburgh, PA 15201, USA

Address for correspondence: Dr. Cecilia W. Lo, Department of Developmental Biology, University of Pittsburgh School of Medicine, Rangos Research Center, 530 45th Street, Pittsburgh, PA 15201, USA. E-mail: cel36@pitt.edum

ABSTRACT

Background: *GJA1* gene encodes a gap junction protein known as connexin 43 (Cx43). Cx43 is abundantly expressed in the ventricular myocardium and in cardiac neural crest cells. Cx43 is proposed to play an important role in human congenital heart disease, as *GJA1* knock-out mice die neonatally from outflow tract obstruction. In addition, patients with visceroatrial heterotaxia or hypoplastic left heart syndrome were reported to have point mutations in *GJA1* at residues that affect protein kinase phosphorylation and gating of the gap junction channel. However, as these clinical findings were not replicated in subsequent studies, the question remains about the contribution of *GJA1* mutations in human congenital heart disease (CHD). **Materials and Methods:** We analyzed the *GJA1* coding sequence in 300 patients with CHD from two clinical centers, focusing on outflow tract anomalies. This included 152 with Tetralogy of Fallot from over 200 patients exhibiting outflow tract anomalies, as well as other structural heart defects including atrioventricular septal defects and other valvar anomalies. Our sequencing analysis revealed only two silent nucleotide substitutions in 8 patients. To further assess the possible role of Cx43 in CHD, we also generated two knock-in mouse models with point mutations at serine residues subject to protein kinase C or casein kinase phosphorylation, sites that are known to regulate gating and trafficking of Cx43, respectively. **Results:** Both heterozygous and homozygous knock-in mice were long term viable and did not exhibit overt CHD. **Conclusion:** The combined clinical and knock-in mouse mutant studies indicate *GJA1* mutation is not likely a major contributor to CHD, especially those involving outflow tract anomalies.

Key words: Connexin43, congenital heart disease, gap junction, knock-in mouse model, outflow tract, phosphorylations

INTRODUCTION

The gene *GJA1* encodes a gap junction protein known as connexin 43 (Cx43). Cx43 is abundantly expressed in the ventricular myocardium, where it plays an important role in electrical conduction in the heart.[1,2] Cx43 is also proposed to play a role in congenital heart disease (CHD),

as *GJA1* knock-out mice die neonatally from outflow tract obstruction associated with conotruncal heart malformation. These malformations consist of pouches positioned at the base of the pulmonary outflow tract, a region known as the infundibulum. These pouches are comprised of elaborate intraventricular trabeculations forming lacunae and sinusoidal cavities that cause pulmonary outflow obstruction, with neonatal lethality resulting from failure to establish normal pulmonary circulation.[3] These mice also show various coronary artery anomalies[4] associated with dysregulation of coronary vasculogenesis.[5] Previous studies had shown that Cx43 plays an important role in modulating the migratory behavior of cardiac neural crest cells (CNC), and the resulting defects in the deployment of CNCs[6] underlies the conotruncal heart malformation.[7] Other studies in chick, *Xenopus* and mice suggest that the precise regulation of Cx43 function may be critical in other developmental processes as well.[8-13]

A possible role for *GJA1* in CHD has been suggested by the report of *GJA1* point mutations in a clinical study of patients with visceroatrial heterotaxia and hypoplastic heart syndrome. Six patients with visceroatrial heterotaxia[14] and eight patients with hypoplastic left heart syndrome (HLHS)[15] exhibited mutations involving serine and threonine residues that affect protein kinase phosphorylation and gating of the gap junction channel. However, as these findings were not replicated in subsequent clinical studies, the question remains about the contribution of *GJA1* mutations in human CHD.[16-20]

To further investigate the role of *GJA1* mutations in CHD in this study, we screened for *GJA1* mutations by direct sequencing the coding region of Deoxyribonucleic acid (DNA) obtained from 300 patients with CHD, over 200 of which have outflow tract anomalies. We also generated two mouse models to directly address the role of Cx43 phosphorylation in CHD with the targeted knock-in of Cx43 point mutations at serine residues subject to protein kinase C (PKC) or casein kinase 1 (CK1) phosphorylation. We chose to investigate these particular amino acid residues given previous studies showing that they can modulate gap junction conductance and/or trafficking of the Cx43 protein, respectively.[21-23] Both mouse models were found to be viable and fertile, and with no obvious decrease in viability or long term lifespan. The combined results from the mouse and human studies do not support a major role for *GJA1* mutations in human CHD, especially those involving the outflow tract.

MATERIALS AND METHODS

Patient recruitment

Patients were recruited with protocols approved by the Institutional Review Board (IRB) at The Children's Hospital of Philadelphia (CHOP), and the Fudan University Children's Hospital in China (FUCH). Cardiovascular diagnoses were confirmed by an attending pediatric cardiologist, who reviewed echocardiograms and/or echocardiogram reports, cardiac catheterization reports, and operative notes if applicable.

Deoxyribonucleic acid extraction, polymerase chain reaction amplification, and DNA sequencing

For FUCH patients, deoxyribonucleic acid (DNA) was obtained from peripheral blood in all subjects. DNA from patients at CHOP was prepared from blood or lymphoblastoid cell lines. Genomic DNA was extracted using standard techniques. The coding region of *GJA1* was polymerase chain reaction (PCR) amplified using primers designed to exclude the *GJA1* pseudogene in the human genome[24] [Figure 1a and Table 1]. At FUCH, each DNA sample was subjected to two rounds of nested PCR. In the first round, the whole coding region was amplified using F1 and R3 primers followed by amplification of three overlapping fragments using combination of all 6 pair of primers [Table 1]. At CHOP the whole coding region was amplified using two sets of overlapping primers [Table 1]. At both centres, 25-40 cycles of amplification was carried out by hot start DNA polymerase using annealing temperature of 58-60°C The resulting product was sequenced by Sanger sequencing using standard protocols.

Gja1 Knock-in mouse models

Targeting constructs with *Gja1,* point mutations were made with floxed neomycin selection cassette and either PGK-Neo (S368A) or PGK-Neo-Stop (S330A, S328Y, and S325A) and a thymidine kinase negative selection cassette [Figure 1b]. After electroporation, ES cells were screened with long range PCR using primers amplifying from the homology arms to the floxed neomycin cassette [Table 2], and homologous recombination was confirmed by Southern blotting [Figure 2]. PCR amplifications were carried out with Advantage 2 polymerase (clonetech) with initial annealing temp going down by 0.5°C per cycle starting from 64°C for 20 cycles followed by annealing at 52°C for next 18 cycles.
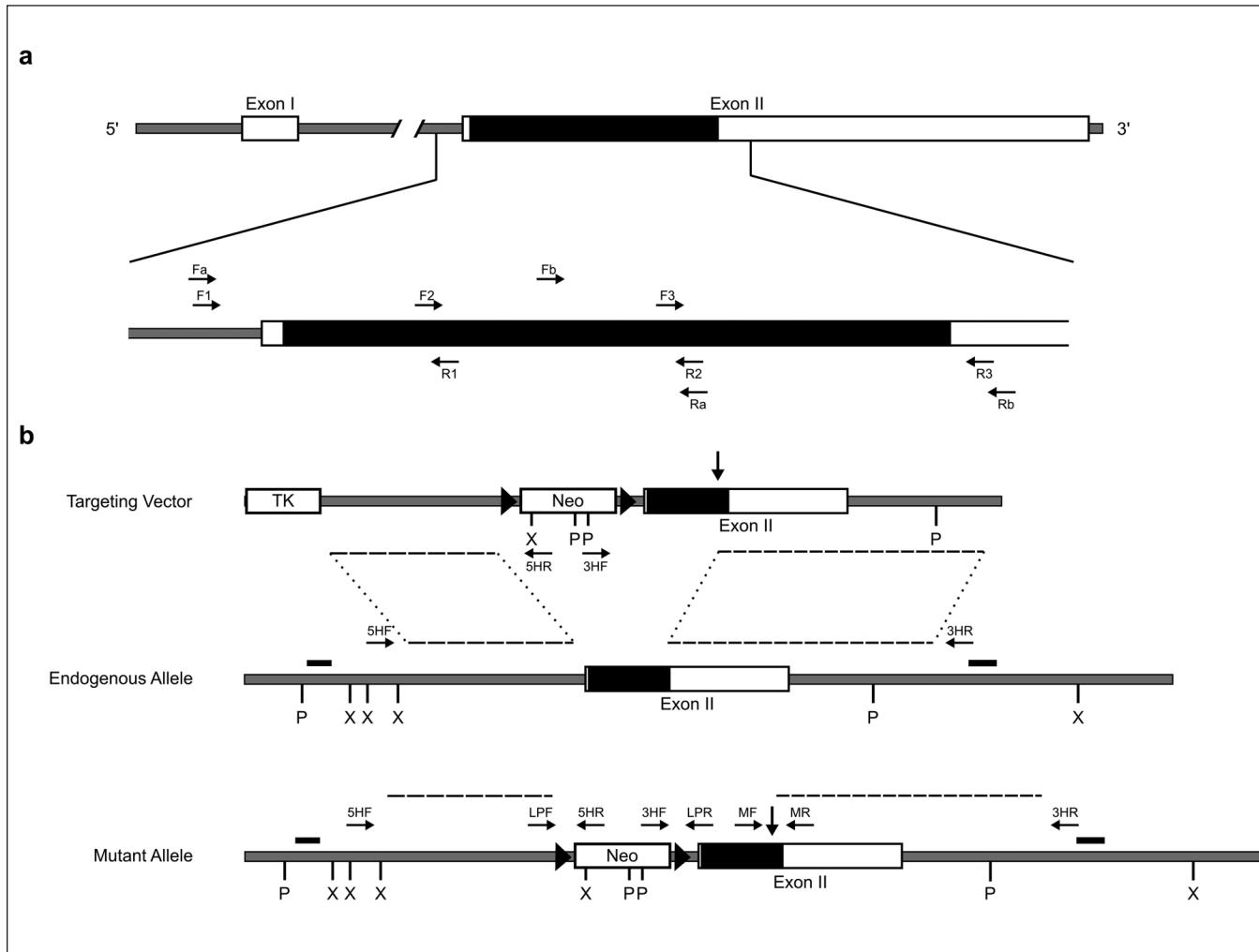
Figure 1: Genomic deoxyribonucleic acid (DNA) amplification and targeting construct design (a) *GJA1* genomic DNA amplification and sequencing. The location of primers used for polymerase chain reaction (PCR) amplification and sequencing of the coding region of *GJA1* (black region) are indicated. Primers were designed to avoid amplification of the pseudogene.[26] (b) *Gja1* knock-in construct design The knock-in vectors contained floxed PGK-Neo (S368A) or PGK-Neo-Stop (S330A/S328Y/S325A), a thymidine kinase cassette, and 5'/3' homology arms (dashed lines). Mutation in the mouse *Gja1* knock-in constructs (vertical arrow) consisted of S368A (AGC->GCT) or S330A:S328Y:S325A (TCC->GCA;TCC->TAC;AGC->GCA). Primers used for screening are described in Table 4.

Table 1: Primers for polymerase chain reaction amplification of patient deoxyribose nucleic Acid[†]

Primer sequence	Polymerase chain reaction product
F1:GAAATACGTGAAACCGTTGG R3:CCTGGTGCACTTTCTACAGC	1348bp
F1:GAAATACGTGAAACCGTTGG R1:TCTTGTTCAGTTTCTCTTCCTTTC	451bp
F2:TATTTGTGTCTGTACCCACACTCTTGT R2:CCTTAACCCGATCCTTAACGC	477bp
F3:GTGTCCCTGGCCTTGAATAT R3:CCTGGTGCACTTTCTACAGC	568bp
Fa:AGAATGTTAGAAATACGTGAAACC Ra: TTCCCTTAACCCGATCCTTA	862 bp
Fb: GCTGCGAACCTACATCATCAGTAT Rb: AAGGCTGTTGAGTACCAC	810 bp

[†]*F1-F3 and R1-R3: primers were used at FUCH; Fa-Fb and Ra-Rb: Primers were used at CHOP.*

Table 2: Primers for screening knock-in mice

Primer sequences		Polymerase chain reaction product
5HF:GCAAGATCCTGTGCTGAAAAACC	5HR:CTCGACTGTGCCTTCTAGTTGC	2.6kb
3HF:ACTGCCTTGGGAAAAGCGCCTC	3HR:AACCGCCTGTTCTGATGCTCTC	5.9kb
LPF:CTCACAGCTCTAATCCTCCTCCCTC	LPR:CTGACAGGCTCTACCTACCAAGTGC	WT: 461bp KI –Neo: 550bp KI +Neo: >2kb
MF:ACTCTCACCTATGTCTCCTCCT	MR:CCCTACTTTTGCCGCCTAGCTA	519bp

Table 3: Congenital heart defects in patient cohorts

Outflow tract defects	Valvar defects	Other defects
Tetralogy of fallot (152) ‡	Aortic atresia (3)	Coarctation of aorta (7)
Pulmonary atresia (6)	Tricuspid atresia (9)	Visceroatrial heterotaxy (11)
Truncus arteriosus (6)	Mitral atresia (3)	Single atrium (3)
Transposition of great arteries (31)	Atrio-ventricular septal defect (22)	Single ventricle (5)
Double-outlet right ventricle (29)		Interrupted aortic arch (1)
		Hypoplastic right heart syndrome (4)
		Total/Partial anomalous pulmonary venous drainage (4)
		Hypoplastic left heart syndrome (4)

‡ *Number in parenthesis equal to number of patients recruited in the study*
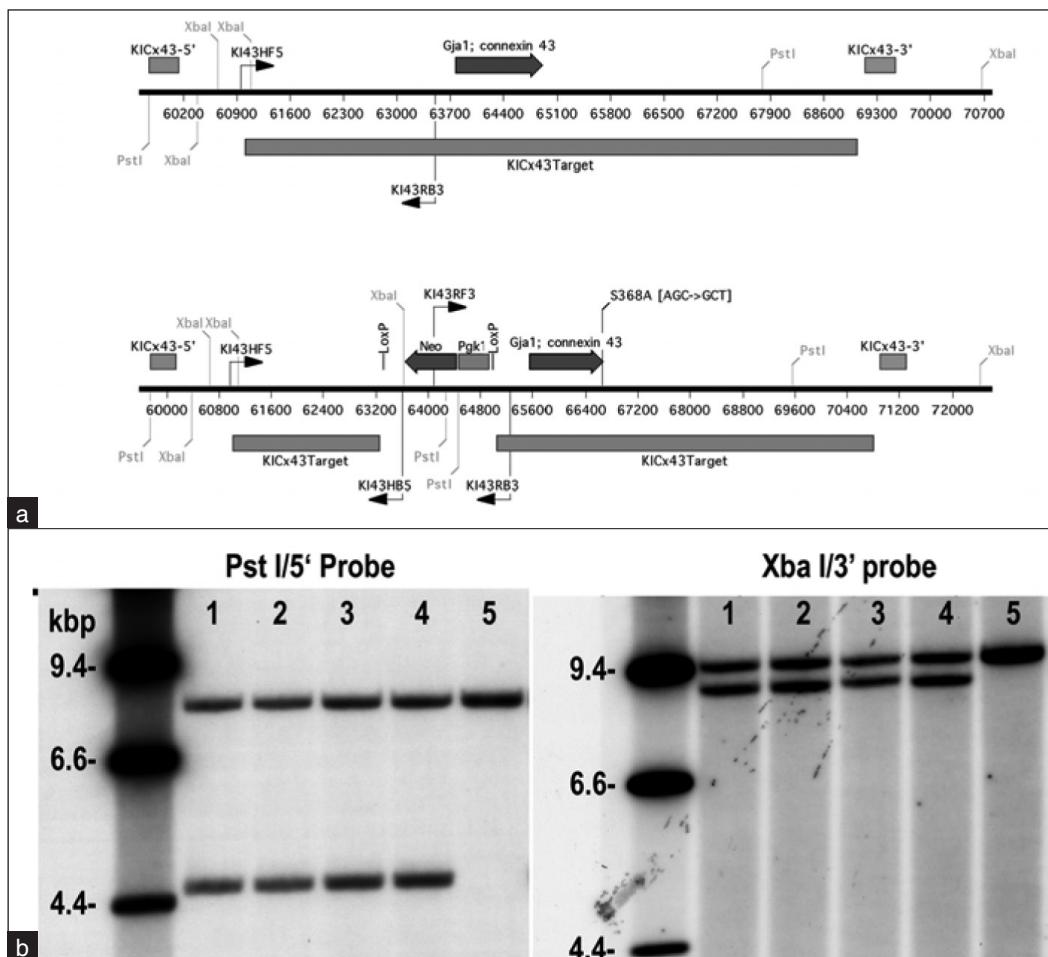
Figure 2: Southern blot analysis of Cx43 KI mice. (a) On the top shown is the representation of the genomic region of Cx43 gene with positions of diagnostic restriction site. Below shown is the representation of Cx43 KI construct and position of diagnostic restriction site. Also shown is the position of primers that was used for genotyping the mice. (b) Southern blot of genomic DNA digested with either PstI (left panel) or XbaI (right panel) and hybridized with e 5' and 3' probe respectively. The position of the probe was shown on map of panel A. Pst I digested genomic DNA hybridized to with 5' probe (left panel) should produce 8.0 Kb fragment for wildtype (lane 5) and 4.6 Kb fragment for KI mice (lane 1-4). Xba I digested genomic DNA hybridized to with 3' probe (right panel) should produce 9.5 Kb fragment for wildtype (lane 5) and 8.7 Kb fragment for KI mice (lane 1-4).

Immunoblotting and immunohistochemsitry

Western immunoblotting was performed as previously described and quantified using a Li-Cor Biosciences Odyssey infrared imaging system and associated software (inverted images are presented).[23] Cx43 was detected with our Cx43NT1 antibody[25] and the antibody against vinculin was obtained from Sigma Chemical Co. (V4505).

Immunohistochemistry was performed on formalin-fixed paraffin sections of hearts from wild-type, CK1 and S368A mice. Briefly, the de-paraffinized and hydrated histologic sections were subjected to heat-induced epitope retrieval for 20 minutes, blocked with 5% normal goat serum and 1% Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS) for 30 minutes, and then incubated with rabbit anti-Cx43 antibody (1:500) for 1

hour at room temperature.[26] Sections were washed in PBS and incubated with Alexa fluor 647 goat anti-rabbit secondary antibody (Invitrogen) for 30 minutes. Sections were washed three times and then were embedded in Prolong Gold Antifade reagent (Invitrogen). Fluorescence images were acquired using a TissueFax-System (TissueGnostics, Vienna, Austria) with far-red (Cy5, HyQ filter set: Excitation 590-650, emission 673-762) Zeiss PlanApo 20X/OD objective. After thresholding, the number of pixels in junctions (i.e., area) and intensity of the pixels were quantified using the public domain National Institutes of Health (NIH) Image program (developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

RESULTS

Sequencing for *GJA1* mutations in patients with congenital heart disease

We analyzed 300 patients with CHD recruited at FUCH and CHOP, including 224 patients with outflow tract anomalies, 152 of which had tetralogy of Fallot (TOF) that included the full range of pulmonary valve morphologies (stenosis, atresia, "absent") [Table 3]. Approximately 50% of the patients from both cohorts were less than 1 year of age [Table 4]. Double stranded DNA sequencing showed no amino acid altering mutations in these 300 patients. Two novel synonymous nucleotide substitutions were found in several TOF patients: G717A in one patient from FUCH, and C555T (1 patient) and G717A (6 patients) from the CHOP cohort. As *GJA1* mutations were suggested to arise *de novo* in somatic tissues in patients with CHD,[15] we also analyzed genomic DNA from surgically resected right ventricular outflow tract myocardium for 26 of the patients from FUCH; however, no difference in the DNA sequence was found.

Gja1 knockin mouse mutants show no overt congenital heart defects

Given limitation in the patient sample size, we made two *Gja1* knock-in constructs to assess whether specific *Gja1* mutations at important regulatory sites in Cx43 can cause CHD in mice. These included a construct [Figure 1b] containing S368A substitution at a PKC phosphorylation site in Cx43 that reduces channel conductance,[21] and a construct with S325A/S328Y/S330A (CK1) mutations at sites phosphorylated by casein kinase 1 known to regulate Cx43 assembly into a gap junction structure.[22,23] From previous work, we know that elimination of S368 or S325/328/330 would change the conductance properties of the resulting gap junction channels.[22,27-29] Mice from both knock-in constructs were heterozygous/homozygous viable [Figure 3; data not shown], with apparently normal lifespan (mice could be routinely aged > 12 months) and no obvious CHD. Surprisingly, immunostaining of mutant mice
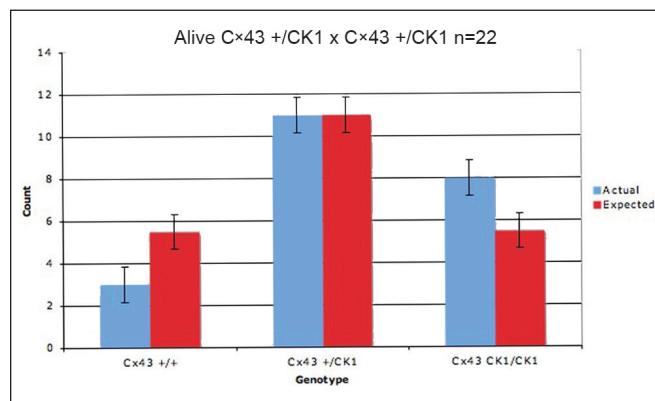

Figure 3: Genotype distribution of *Gja1* knock-in mice litters. Shown are the genotype distribution recovered in three litters of mice obtained from the mating of heterozygous *Gja1* knock-in mice with the S330A:S328Y:S325A (CK1) mutation. The genotype obtained (blue) is not significantly different from the expected distribution (red).

Table 4: Patients with complex heart defects from Children's Hospital of Philadelphia (CHOP) and Fudan University Children's Hospital in China (FUCH)

| Fudan University Children's Hospital in China				
Gender	**Age**			
	< 28D	**28D < 1Y**	**1Y < 3Y**	**>3Y**
Male	14 cases	51 cases	17 cases	33 cases
Female	6 cases	20 cases	18 cases	23 cases
% each age period	11.0%	39.0%	19.2%	30.8%
Children's Hospital of Philadelphia				
Gender	**Age**			
	< 28D	**28D < 1Y**	**1Y < 3Y**	**>3Y**	**Unknown**
Male	2 cases	18 cases	4 cases	8 cases	1 case
Female	1 case	7 cases	4 cases	9 cases	0 Case
% each age period	5.60%	46.3%	14.8%	31.5%	1.80 %
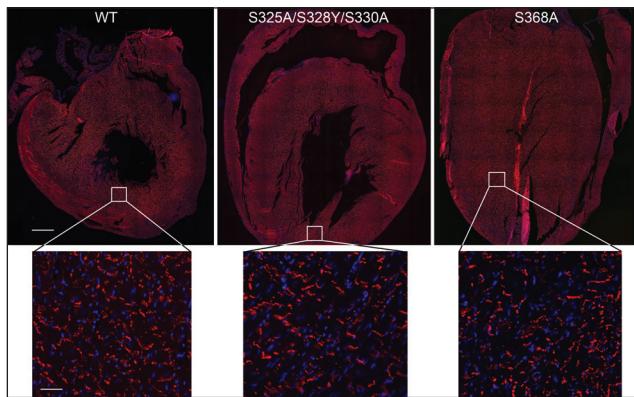
Figure 4: Cx43 localization in heart tissue from wildtype, S368A and S235A/S328Y/S330A mice. Paraffin-embedded sections of mouse hearts were stained with Cx43 primary and secondary as indicated in the Materials and Methods. 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei (bar = 500μm). The lower row of panels shows higher magnification views of the indicated portions from the panels above (bar = 60 μm)
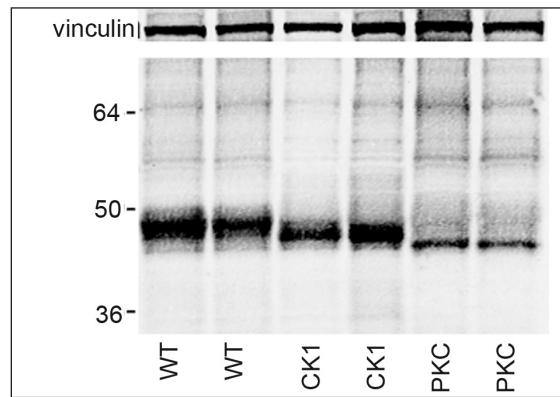

Figure 5: Characterization of total Cx43 in mouse hearts. Western blots of whole heart lysates, isolated from two WT (Wild type-Cx43), CK1 (S325A/S328Y/S330A) or two PKC (S368A) hearts, simultaneously probed for total Cx43. Membranes were also probed with anti-vinculin antibody, as an internal loading control. Notice that Cx43 protein levels are decreased in CK1 and PKC mice compared with WT. Positions of the molecular weight markers are shown on the left

Table 5: Reduction of Cx43 expression in Cx43 knock-in mice

		Cx43	Vinculin	Cx43/Vanculin
Lane 1	WT (1)	99.99	129.04	0.774876007
Lane 2	WT (2)	83	105.55	0.786357177
Lane 3	CK1 (1)	76.81	136.78	0.561558707
Lane 4	CK1 (2)	98.61	178.6	0.55212766
Lane 5	PKC (1)	41.67	144.88	0.287617338
Lane 6	PKC (2)	32.69	128.32	0.254753741

Where, WT = Wild type-Cx43; PKC = protein kinase C; CK1 = Casein kinase 1

showed no significant difference in Cx43 localization gap junctions; and, the density of Cx43 immunostaining did not appear to be significantly changed when compared to wildtype hearts [Figure 4]. The integrated densities of the area of junctional staining in arbitrary units were: Wild type-Cx43 (WT) = 18742±5191; CK1 = 17957±3851; PKC = 17352±1415.

When heart tissue was analyzed by Western immunoblotting, we observed the typical reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) migration of Cx43 due to its phosphorylation at sites such as S325/328/330 in the wild type mouse tissues,[23] that was much less prominent in heart tissue from the CK1 mice [Figure 5]. We had previously shown that phosphorylation at S368 does not affect SDS-PAGE migration.[30] The PKC1 mice, however, also showed a loss of these slower migrating species. Quantitation of the Western blot showed the Cx43 protein expression level (when normalized to vinculin) was reduced by more than 70% in the PKC mutant and nearly 30% in the CK1 mutant mouse hearts [Table 5]. Although, these mice could potentially show altered responses to acute signaling events, we did not detect any developmental defects or changes in their long-term viability or lifespan.

DISCUSSION

We found no amino acid altering mutations in *GJA1* gene in 300 patients with CHD, including 224 patients with outflow tract anomalies. However, two synonymous sequence variants were found - one is a novel Single Nucleotide Polymorphism [SNP (C555T)] and another is a known variant in National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism database [dbSNP (G717A, rs57946868)]. Given the neonatal lethality of the *Gja1* knockout mouse, it is possible that the disruption or perturbation of the Cx43 function may not be postnatal viable. We note that in both cohorts, half of the patients with complex CHD were less than 1 year of age.

Mutations in *GJA1* have been reported in patients with oculodentodigital dysplasia (ODDD),[31] a disorder causing craniofacial and limb dysmorphisms, neurodegeneration and deafness. Cardiac abnormalities were rare and consisted of conduction anomalies, with one ODDD individual exhibiting ventricular tachycardia and atrio-ventricular block associated with a Ile130Thr mutation, and another with atrioseptal defects associated with a Gly21Arg mutation. We noted a mutant mouse model with a N-ethyl-N-nitrosourea-induced point mutation in *Gja1*[G60S] exhibited phenotypes similar to ODDD.[32] Also

in these patients, there were no structural heart defects except for some cases of patent foramen ovale.

Surprisingly, our studies showed *Gja1* knock-in mice with point mutations at important regulatory serine residues *Gja1*^S368A or *Gja1*^S325A/S328Y/S330A were homozygous, viable and fertile. We chose to examine the S328Y mutation since early findings indicated that this site might be modified in a CHD patient,[14,15] although subsequent studies indicated this was not the case.[16-20] Findings from the previous *in vitro* studies would have predicted that these mice should show functional impairment for *Gja1* due to defects in Cx43 trafficking and or gating of the Cx43 gap junction channel. These unexpected findings show the importance of animal model studies to validate *in vitro* findings.[21,22] Together, our results indicate that *GJA1* mutations are not commonly associated with outflow tract anomalies. These findings do not preclude a role for *GJA1* mutations in a more complex or severe CHD condition associated with prenatal/neonatal lethality, a clinical population difficult to survey.

ACKNOWLEDGMENT

REFERENCES

1. Severs N. Cardiac muscle cell interaction: From microanatomy to the molecular make-up of the gap junction. Histol Histopathol 1995;10:481-501.
2. Severs N. Cardiovascular disease. Novartis Found Symp 1999;219:188-206; discussion 206-11.
3. Reaume A, de Sousa P, Kulkarni S, Langille B, Zhu D, Davies T, *et al.* Cardiac malformation in neonatal mice lacking connexin43. Science 1995;267:1831-4.
4. Li W, Waldo K, Linask K, Chen T, Wessels A, Parmacek M, *et al.* An essential role for connexin43 gap junctions in mouse coronary artery development. Development 2002;129:2031-42.
5. Walker D, Vacha S, Kirby M, Lo C. Connexin43 deficiency causes dysregulation of coronary vasculogenesis. Dev Biol 2005;284:479-98.
6. Lo C, Waldo K, Kirby M. Gap junction communication and the modulation of cardiac neural crest cells. Trends Cardiovasc Med 1999;9:63-9.
7. Hutson M, Kirby M. Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. Semin Cell Dev Biol 2007;18:101-10.
8. Ewart J, Cohen M, Meyer R, Huang G, Wessels A, Gourdie R, *et al.* Heart and neural tube defects in transgenic mice overexpressing the cx43 gap junction gene. Development 1997;124:1281-92.
9. Huang G, Wessels A, Smith B, Linask K, Ewart J, Lo C. Alteration in connexin 43 gap junction gene dosage impairs conotruncal heart development. Dev Biol 1998;198:32-44.
10. Levin M, Mercola M. Gap junctions are involved in the early generation of left-right asymmetry. Dev Biol 1998;203:90-105.
11. Levin M, Mercola M. Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of shh asymmetry in the node. Development 1999;126:4703-14.
12. Le M, Li Z, Cilley R, Donahue H, Chinoy M. Connexin 43 gene expression in mice with cardiopulmonary developmental defects. Front Biosci 2006;11:3014-25.
13. Rhee D, Zhao X, Francis R, Huang G, Mably J, Lo C. Connexin 43 regulates epicardial cell polarity and migration in coronary vascular development. Development 2009;136:3185-93.
14. Britz-Cunningham S, Shah M, Zuppan C, Fletcher W. Mutations of the connexin43 gap-junction gene in patients with heart malformations and defects of laterality. N Engl J Med 1995;332:1323-9.
15. Dasgupta C, Martinez A, Zuppan C, Shah M, Bailey L, Fletcher W. Identification of connexin43 (alpha1) gap junction gene mutations in patients with hypoplastic left heart syndrome by denaturing gradient gel electrophoresis (dgge). Mutat Res 2001;479:173-86.
16. Gebbia M, Towbin J, Casey B. Failure to detect connexin43 mutations in 38 cases of sporadic and familial heterotaxy. Circulation 1996;94:1909-12.
17. Debrus S, Tuffery S, Matsuoka R, Galal O, Sarda P, Sauer U, *et al.* Lack of evidence for connexin 43 gene mutations in human autosomal recessive lateralization defects. J Mol Cell Cardiol 1997;29:1423-31.
18. Splitt M, Burn J, Goodship J. Connexin43 mutations in sporadic and familial defects of laterality. N Engl J Med 1995;333:941.
19. Penman Splitt M, Tsai M, Burn J, Goodship J. Absence of mutations in the regulatory domain of the gap junction protein connexin 43 in patients with visceroatrial heterotaxy. Heart 1997;77:369-70.
20. De Luca A, Sarkozy A, Consoli F, De Zorzi A, Mingarelli R, Digilio M, *et al.* Exclusion of cx43 gene mutation as a major cause of criss-cross heart anomaly in man. Int J Cardiol 2010;144:300-2.
21. Lampe P, TenBroek E, Burt J, Kurata W, Johnson R, Lau A. Phosphorylation of connexin43 on serine368 by protein kinase c regulates gap junctional communication. J Cell Biol 2000;149:1503-12.
22. Cooper C, Lampe P. Casein kinase 1 regulates connexin-43 gap junction assembly. J Biol Chem 2002;277:44962-8.
23. Lampe P, Cooper C, King T, Burt J. Analysis of Cx43 phosphorylated at S325, S328, S330 in nomixic and ischemic heart. J Cell Sci 2006;119:3435-42.
24. Fishman G, Eddy R, Shows T, Rosenthal L, Leinwand L. The human connexin gene family of gap junction proteins: Distinct chromosomal locations but similar structures. Genomics 1991;10:250-6.
25. Sosinsky G, Solan J, Gaietta G, Ngan L, Lee G, Mackey M, *et al.* The C-terminus of connexin43 adopts different conformations in the Golgi and gap junction as detected with structure-specific antibodies. Biochem J 2007;408:375-85.
26. King T, Gurley K, Prunty J, Shin J, Kemp C, Lampe P. Deficiency in the gap junction protein connexin32 alters p27kip1 tumor suppression and mapk activation in a tissue-specific manner. Oncogene 2005;24:1718-26.
27. Moreno A, Fishman G, Spray D. Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels. Biophys J 1992;62:51-3.
28. Lampe P, TenBroek E, Burt J, Kurata W, Johnson R, Lau A. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. J Cell Biol 2000;149:1503-12.
29. Ek-Vitorin J, King T, Heyman N, Lampe P, Burt J. Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. Circ Res 2006;98:1498-505.
30. Solan J, Fry M, TenBroek E, Lampe P. Connexin43 phosphorylation at S368 is acute during S and G2/M and in response to protein kinase C activation. J Cell Sci 2003;116:2203-11.
31. Paznekas W, Boyadjiev S, Shapiro R, Daniels O, Wollnik B, Keegan C, *et al.* Connexin 43 (gja1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. Am J Hum Genet 2003;72:408-18.
32. Flenniken A, Osborne L, Anderson N, Ciliberti N, Fleming C, Gittens J, *et al.* A gja1 missense mutation in a mouse model of oculodentodigital dysplasia. Development 2005;132:4375-86.