A Novel PITX2c Gain-of-Function Mutation, p.Met207Val, in Patients With Familial Atrial Fibrillation

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Genome-wide studies have associated several genetic variants upstream of *PITX2* on chromosome 4q25 with atrial fibrillation (AF), suggesting a potential role of *PITX2* in AF. Our study aimed at identifying rare coding variants in *PITX2* predisposing to AF. The Polymerase chain reaction sequencing of PITX2c was performed in 60 unrelated patients with idiopathic AF. The p.Met207Val variant was identified in 1 of 60 French patients with early onset AF and in none of 389 French referents. This variant, located in the inhibitory domain 1 distal to the homeodomain, was evaluated by the software MutationTaster as a disease-causing mutation with a probability of 0.999. Reporter gene assays demonstrated that p.Met207Val caused a 3.1-fold increase in transactivation activity of PITX2c in HeLa cells in comparison with its wild-type counterpart. When the variant was coexpressed with wild-type PITX2c in the HL-1 immortalized mouse atrial cell line, this *gain-of-function* caused an increase in the mRNA level of *KCNH2* (2.6-fold), *SCN1B* (1.9-fold), *GJA5* (3.1-fold), *GJA1* (2.1-fold), and *KCNQ1* in the homozygous form (1.8-fold). These genes encode for the IKr channel α subunit, the β-1 Na^+ channel subunit, connexin 40, connexin 43 and the IKs channel α subunit, respectively. These conditions may contribute to the propensity to AF found in patients carrying the p.Met207Val variant. In conclusion, the present report is the first to associate a *gain-of-function* mutation of PITX2c with increased vulnerability to AF, therefore, restoration of normal PITX2c function may be a potential therapeutic target in AF patients. **(Am J Cardiol 2019;123:787−793)**

The physiopathology of atrial fibrillation (AF) is poorly understood and the therapeutic measures available are not sufficient. The SNP most significantly associated with AF is located in a noncoding region of chromosome 4q25, approximately 150,000 base pairs upstream of the gene coding for the paired-like homeodomain transcription factor 2 (*PITX2,* MIM 180500).[1] PITX2c is the major protein isoform expressed in the hearts of mice[2] and humans[3,4] and is involved in generating and maintaining morphologic and electrical cardiac asymmetry.[3,5,6] PITX2c deficiency causes changes in atrial electrical properties that favor AF.[3,6−8] These observations suggest a link between the PITX2c isoform and AF. Our objective was therefore to search for genetic variants in PITX2 in our cohort of patients with AF.

Methods

A brief description of the methods follows, a complete account of methods is provided in the Supplemental Material available online. The experimental protocols were approved by the local institutional ethics committee on human subject research of the Louis Pradel Hospital (Protocol reference number: CAL 2012-030). A sample of 60 unrelated patients with idiopathic AF was recruited at the Cardiologic Hospital of Lyon. Other recognized cardiovascular diseases, hypertension, and metabolic or pulmonary diseases had been excluded. Genomic DNA from all participants was extracted from whole blood samples. The entire coding sequence of *PITX2* was sequenced using Sanger sequencing. Sequencing of genes previously associated with AF was also performed: *KCNQ1, KCNH2, KCNA5, SCN5A, GJA1, GJA5,* and *GJC1*. To evaluate the transactivation activity of PITX2c proteins, Henrietta Lacks' cervical tumor cells (HeLa) were cotransfected with a PITX2c plasmid and a SLC13A3 luciferase reporter plasmid including PITX2 binding site A.[9] The transactivation activity of wild-type (WT) and variant PITX2c was evaluated by luminometry. We evaluated the ability of WT and mutated PITX2c proteins to interact with the DNA bicoid binding site by an electrophoretic mobility shift assay. Nondenaturating polyacrylamide gel electrophoresis was

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See page 792 for disclosure information.

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performed on a mixture of double-stranded Cy3-labeled DNA (at 0.5 μM) and 40 μg of whole-cell extracts of HeLa cells transfected with a PITX2c plasmid. The amount of PITX2c protein expressed by the cells was monitored by a western blot assay. The effect of the variant on mRNA transcription of ion channel genes was measured 24 hours after transfection with WT or variant PITX2c plasmids of immortalized mouse atrial cells (HL-1, obtained from Dr. Claycomb).[10] Total RNA (0.5 μg) was used to prepare cDNA and quantitative real-time polymerase chain reaction measurement was performed using appropriate primers. Quantitative analyses of Western blot and mRNA data were performed using Prism 6 software (GraphPad, California). Statistical analyses of quantitative experimental data were performed using the unpaired Student *t* test. A 2 sided p value <0.05 was considered statistically significant. The following web resources have been used: Exome Variant Server, NHLBI GO Exome Sequencing Project, Seattle, Washington (URL: http://evs.gs.washington.edu/EVS/) (October 2018 accessed). Exome Aggregation Consortium (ExAC), Cambridge, Massachusetts (URL: http://exac.broadinstitute.org) (October 2018 accessed). Online Mendelian Inheritance in Man, (URL: http://www.omim.org/) (October 2018 accessed). Single Nucleotide Polymorphism database, the National Center for Biotechnical Information (http://www.ncbi.nlm.nih.gov/SNP/). The Online Mendelian Inheritance in Man accession number for the PITX2 sequence reported in this work is MIM 180500.

Results

The clinical characteristics of the study samples are provided in Table 1. Mutation screening of PITX2 resulted in the identification of a nonsynonymous variant c.619A>G (p.Met207Val, rs138163892) (Figure 1). The amino acid position of the variant is depicted in Figure 1. The alignment of a segment of the amino acid sequences of PITX2c across several species (Supplemental Table S4) shows that

Table 1
Clinical characteristics of the study sample

Variable	Cases n = 60	Referents n = 389
Male	41 (69%)	324 (83%)
Age at onset/inclusion*, (years)	47 ± 13	57 ± 12
Height, (cm)	175 ± 9	171 ± 8
Weight, (kg)	86 ± 22	77 ± 13
BMI, (kg/m^2)	28 ± 7	26 ± 4
Blood pressure, (mm Hg)		
Systolic	129 ± 12	-
Diastolic	79 ± 13	-
AF type		
Paroxysmal	64.4%	NA
Persistent	15.2%	NA
Permanent	18.6%	NA
First degree relative with AF	34	-

All numbers are reported as mean ± standard deviation unless otherwise noted. -, not available; NA = not applicable.

* For cases we report age at first diagnosis of AF, for referents we report age at inclusion.

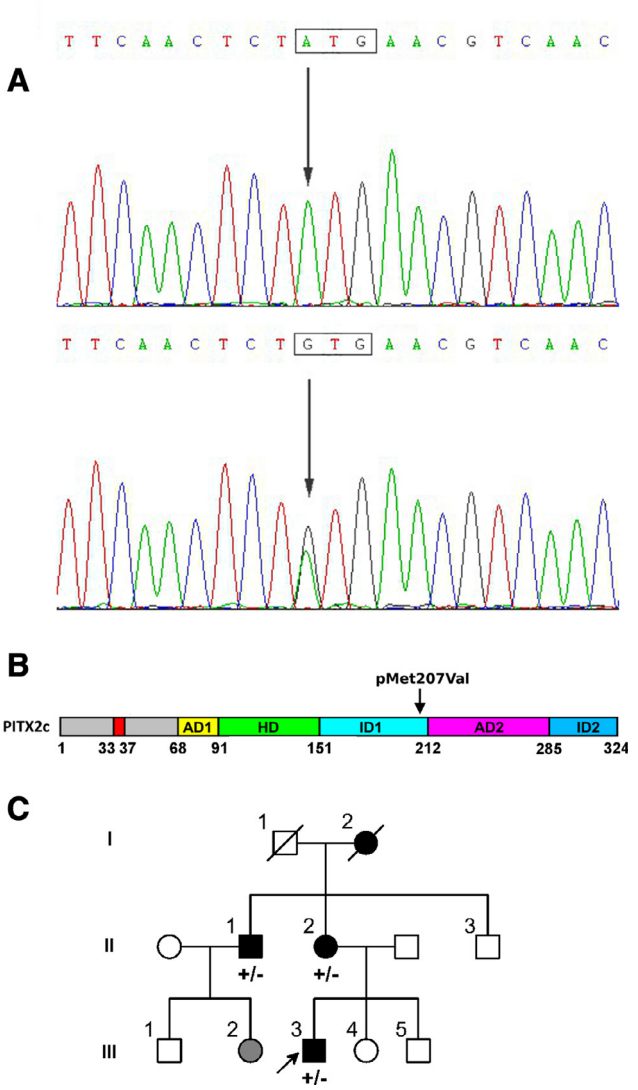

Figure 1. Detection of the *PITX2c* variant, p.Met207Val, in patients with familial AF. (*A*) The sequence electropherogram of the patient carrying the heterozygous c.619A>G variant. (*B*) Schematic representation of *PITX2c* and location of the detected variant. Numbers are in amino-acid residues. p.Met207Val is located in the C-terminal domain. AD1: common sequence, HD: homeodomain, ID1: transcriptional inhibitory domain 1, AD2: second common sequence, ID2: transcriptional inhibitory domain 2. The 5-AA sequence important for transcriptional activity of the N-terminal of PITX2c (33 to 37) is LAMAS. (*C*) Pedigree showing familial aggregation of p.Met207Val. The mother of the proband and her brother were diagnosed with AF (as did their mother) and both carried p.Met207Val. Arrow = the proband (III-3); ± = heterozygous for the mutation; squares = male gender; circles = female gender; black-filled symbols = documented AF; gray-filled symbol = arrhythmia symptoms but no documented AF; open symbols = no AF; crossed symbols, not alive at inclusion.

the methionine residue at position 207 is highly conserved. The p.Met207Val variant was found in a patient and in none of the 389 unrelated referents. In addition, we investigated p.Met207Val cosegregation with atrial fibrillation in our pedigree and found that p.Met207Val cosegregated with the AF phenotype (Figure 1). According to the Exome Variant Server database, p.Met207Val was found in 3

patients of a general population of 8,597 European Americans and in no individual of a general population of 4,406 African Americans. Of note, atrial fibrillation is not an exclusion criterion of these cohorts. ExAC reports 37 heterozygous carriers out of a total European population of 121,304 patients (allele frequency $3.05*10^{-4}$). A search within the Single Nucleotide Polymorphism database or within 1,000 Genomes yielded no occurrence of the p. Met207Val variant. No variant was detected in *KCNQ1, KCNH2, KCNA5, SCN5A, GJA1, GJA5,* and *GJC1* in our propositus. Few variants were found in *KCNH2*, *GJA1*, and *GJA5* in other patients of our AF sample. Using gene reporter assays, WT-PITX2c showed a level of activation about 13-fold higher than the empty expression vector (Figure 2). The mutated PITX2c showed significantly increased activation of the reporter in comparison with WT PITX2c (~3.1-fold). However, the transfection with the variant construct in HeLa cells resulted in the production of ~1.7-fold more PITX2c protein than with the WT construct (Figure 2). Taking this effect into account, we estimated that the normalized activity of the variant protein was ~1.8-fold higher than that of the WT. The transactivation effects did not result from a change in intracellular trafficking of the variant protein (Figure 2). In the electrophoretic mobility shift assay shown in Figure 3, WT and mutated PITX2c proteins were shifted similarly in the presence of a DNA oligomer probe containing the canonical PITX2 binding element TAATCC (upper panel). The intensities of these bands were parallel to those in Western blots of PITX2c proteins (lower panel), indicating a similar affinity to the DNA probe. In the HL-1 cardiac atrial cell line, we assessed the transcriptional effect of the variant on several genes that encode key proteins involved in AF (*GJA5, GJA1, SCN1B, KCNQ1, KCNH2,* and *SCN5A*) using qRT-PCR (Figure 4). This was done under 3 conditions: in cells transfected with either a WT plasmid or the variant *PITX2c* plasmid (200 ng/well) or with both plasmids in combination (100 ng/well of each), to imitate the heterozygous situation. The mRNA levels with the p. Met207Val alone or in combination with the WT were normalized to those obtained with the WT alone. There were no significant differences in the amount of PITX2c expressed in HL-1 cells under these 3 conditions, using quantitative Western blot (Figure 4). As a negative control, nontransfected cells did not yield detectable amounts of PITX2c and the mRNA levels of the target genes explored were not changed by the empty vector (Figure 5). The relative changes in mRNA levels caused by the variant are depicted in Figure 4 and recapitulated in Table 2. These effects indicate that p.Met207Val had a larger potency than the WT in activating the transcription of connexin and ion channel genes tested, as seen with the reporter gene assays in HeLa cells (Figure 2). Further, apart from *KCNQ1*, similar changes were observed when the variant was cotransfected with the WT, which corresponds to its heterozygous presentation in the carriers.

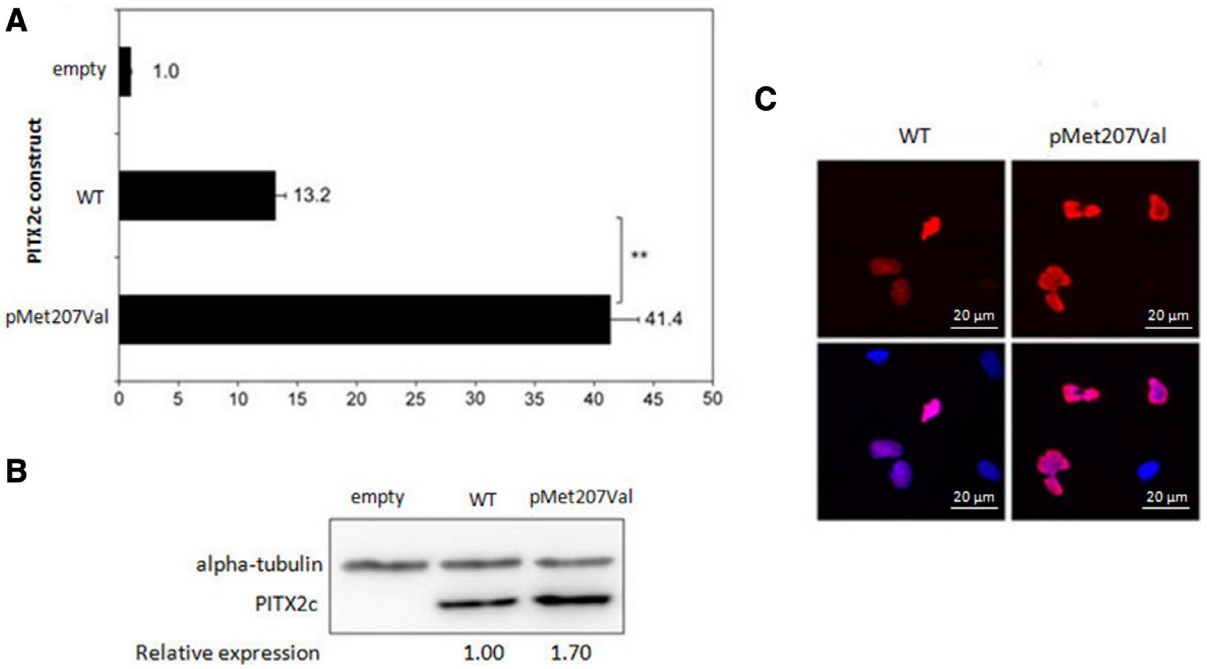

Figure 2. Reporter gene assay of *PITX2c* variants in HeLa cells. Efficiency of activation of the SLC13A3-reporter plasmid by PITX2c proteins in HeLa cells. (*A*) Average values for 3 measurements in each of 3 separate transfections of each plasmid (n = 9) are shown. HeLa cells were transfected with either the empty vector, a vector encoding the WT PITX2c, or a vector encoding the p.Met207Val PITX2c variant. These cells were co-transfected with the SLC13A3 reporter containing the PITX2 binding site (black bars). Asterisks (**) denote that activation was significantly stronger with p.Met207Val versus WT ($p < 0.001$) as calculated from comparisons with Student's *t* test. (*B*) Quantitative immunoblot of PITX2c protein expression in lysates of HeLa cells transfected with WT or p.Met207Val plasmids or with the empty vector (empty). Densities were normalized to the expression of co-transfected alpha-tubulin and expressed with reference to the value for WT PITX2c. (*C*) Immunofluorescence detection of recombinant PITX2c in HeLa cells. Upper panels show the localization of Xpress-PITX2c labeling by mouse Anti-Xpress Ab (1:500) detected by goat Anti-Mouse-IgG coupled to Cyanine3 (1:500) in cells transfected with the WT cDNA (left) or the p.Met207Val variant cDNA (right). Lower panels show the same images merged with the corresponding images of nuclei labeled with DAPI.

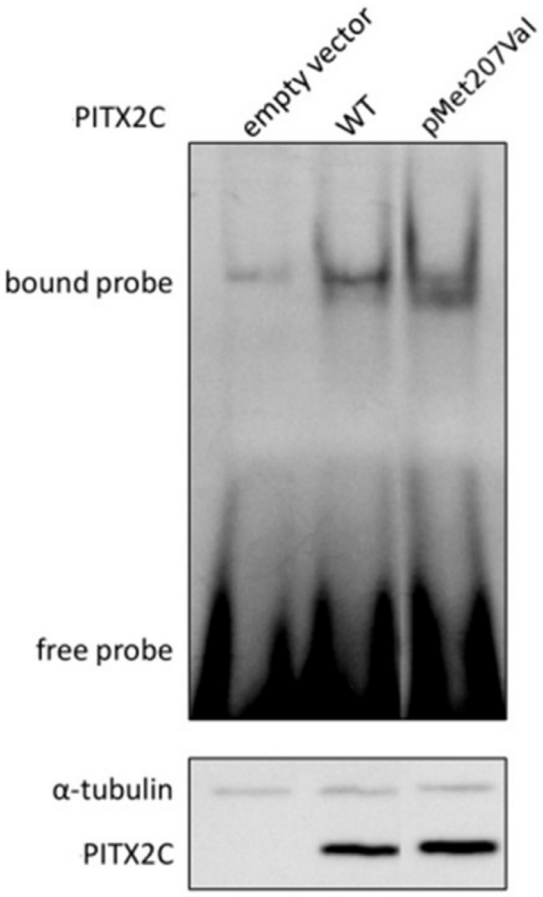

Figure 3. Electrophoretic mobility shift assay in HeLa cells. Electrophoretic mobility shift assay of whole-cell extracts from HeLa cells transfected with pcDNA4: Xpress-PITX2c plasmids (upper panel). Empty expression vector is shown as a negative control displaying a single low-mobility shifted background band due to unknown endogenous protein binding to the Cy3-labeled DNA oligomer probe (containing the canonical PITX2 binding element TAATCC). Complexes of probe bound to recombinant PITX2c constructs are shifted to the same location in the respective lanes, with unbound probe migrating to the bottom of the gel. In the lower panel, Western analysis shows the expression levels of the Xpress-PITX2c constructs in HeLa cells, in relation to the alpha-tubulin internal lane control.

Discussion

In the present study, we report a variant in *PITX2*, p.Met207Val, in association with familial atrial fibrillation. In HeLa cells, the variant p.Met207Val induced a gain of function as revealed by the increase in the transactivation activity of PITX2c (Figure 2). In the HL-1 cardiac cell line, p.Met207Val resulted in increased mRNA levels of *KCNH2* when coexpressed with WT PITX2c (Figure 4). Mutations in *KCNH2* associated with familial AF have been shown to cause *gain-of-function* effects on the I_{Kr} current.[11,12] Such a *gain-of-function* may trigger AF through shortening of the atrial action potential duration and, hence, favour re-entry mechanisms. The variant p.Met207Val also increased the level of *SCN1B* mRNA 1 compared with WT, whereas *SCN5A* mRNA levels were not significantly affected. An increase in the expression of the $Na_v\beta1$ regulatory subunit may nevertheless cause an increase in the amount of fast Na^+ current by facilitating surface expression of the $Na_v1.5$ α-subunit together with its $\beta1$ regulatory subunit.[13] Such a *gain-of-function* effect was found associated with AF.[14] Additionally, overexpression of *SCN1B* in nerve cells increased the density of $K_v4.2$ channels[15] and SCN1Bb, a splice variant of the SCN1B gene, when coexpressed with $K_v4.3$ in TsA201 cells, increased the outward current.[16] In the human atrium, the transient outward current I_{to} is carried by heterotetramers of $K_v4.2$ and $K_v4.3$ subunits,[17] and increasing their expression might contribute to shortening the action potential duration.[17] The variant p.Met207Val increased *GJA5* and *GJA1* mRNA levels. In human atrial tissue from AF patients, increased levels of Cx40 and Cx43[18] have been found and an increase in internal resistivity was correlated with the ratio Cx40/(Cx40 + Cx43).[19] Changes in the expression of Cx40 have been shown to either increase or decrease atrial conduction velocity,[20] which may actually cause AF either way according to a recent study associating both shortened and prolonged PR-intervals to AF.[21] Our data are supported by the findings of Y. Huang et al[22] showing >800 differentially expressed genes, including increased transduction of KCNH2 (9-fold) and KCNQ1 (3.3-fold), when PITX2c was overexpressed in HEK293 cells. Analysis of their data using gene ontology (GO) database biologic processes shows 2 GO terms related to cardiac function among the most enriched terms ("regulation of heart rate by cardiac conduction" and "cardiac muscle cell action potential involved in contraction"). Moreover, in another study, PITX2 CHIPseq analysis revealed peaks near several genes related to FA (KCNQ1, Cav1, and Zfhx3) suggesting a direct regulation by PITX2.[23] Our reporter gene assays showed a *gain-of-function* of p.Met207Val in comparison to the wild type construct (Figure 2). A number of previous studies have shown a link between AF and decreased expression or activity of PITX2c in the left atrium in humans and transgenic mice,[7,8] or with loss-of-function *PITX2* mutations.[24,25] However, *PITX2* expression has been found to be increased in the left atrium[26] and in right atrial myocytes,[27] from AF patients. Thus both PITX2 gain and loss of function are associated with AF. Besides, in >50 mutations of PITX2 related to the Axenfeld-Rieger syndrome most are *loss-of-function* mutations, but 6 of them are *gain-of-function* mutations.[28–30] In conclusion, we identified a rare variant (p.Met207Val) in *PITX2* that was associated with familial AF. The variant increased transcriptional activity of PITX2c and increased the transcription of *GJA1, GJA5, KCNH2,* and *SCN1B* in an atrial cardiac cell line. As for several other genes, *gain-of-function* as well as *loss-of-function* of PITX2c might favor AF, pointing out to the rupture of the balance of PITX2c activity as a unifying concept. Thus, restoration of a balanced PITX2c activity might be a new goal for AF prevention and therapy.

Our study is limited by the low frequency of p.Met207Val in publicly available databases (minor allele frequency = 0.03% in European samples in ExAC, See Web Resources, and in the Exome Variant Server, See Web Resources), in combination with the limited size of our case sample. This warrants replication in a larger study of

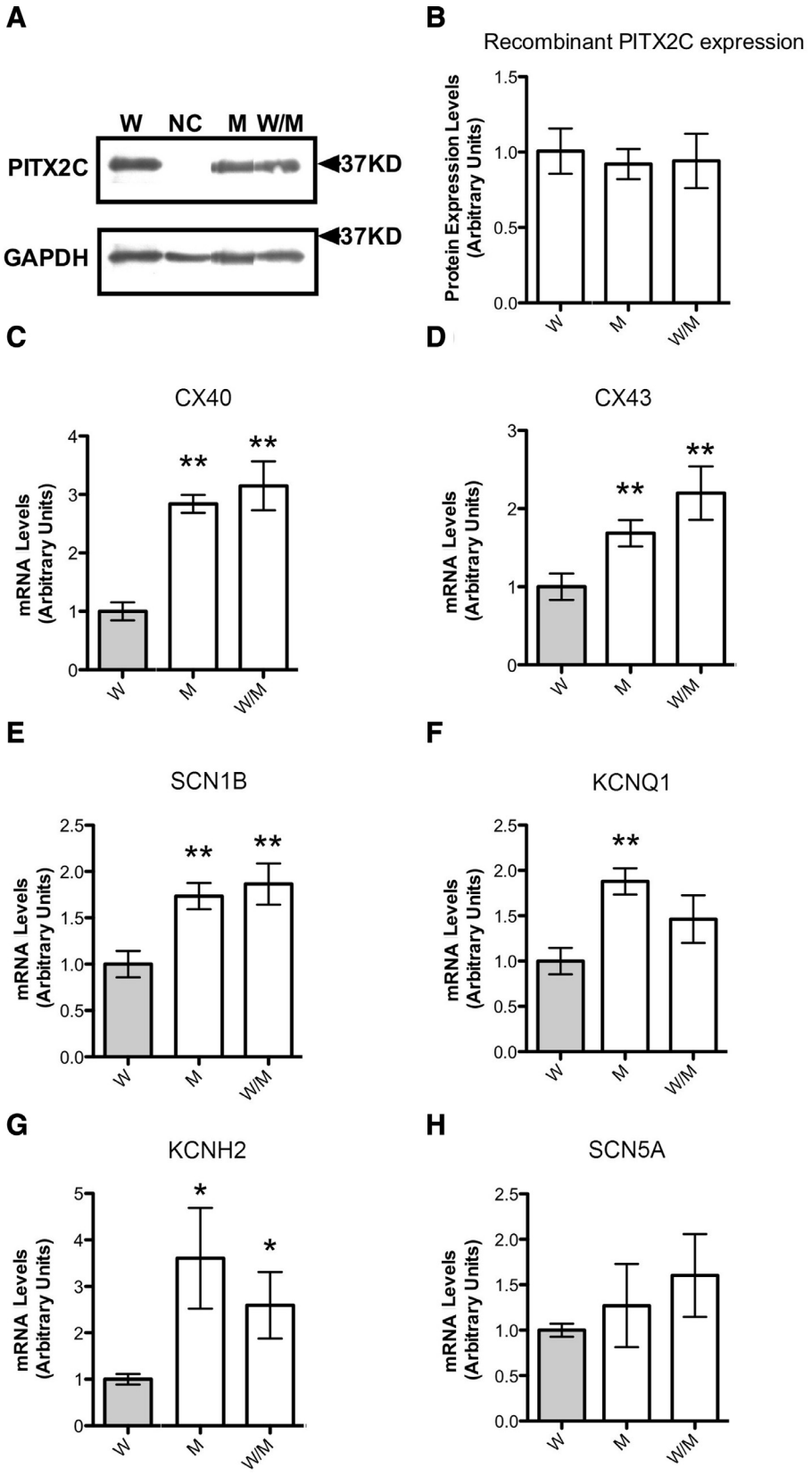

Figure 4. Effects of the PITX2c variant on mRNA transcription in cardiac HL-1 cells. Effect of recombinant PITX2c overexpression on the mRNA levels of *Cx40, Cx43, SCN1B, KCNQ1, KCNH2,* and *SCN5A*. (*A*) Western blot analysis of V5-tagged PITX2c recombinant proteins expressed in HL-1 cells. WT, WT; NC, non-transfected control; M, p.Met207Val; WT/M, WT/p.Met207Val. GAPDH served as the internal loading control. Each plasmid transfection was done 3 times. (*B*) Semi-quantitative illustration of recombinant PITX2c protein expression normalized to GAPDH corresponding to WT. qRT-PCR analysis of Cx40 (*C*), Cx43 (*D*), SCN1B (*E*), KCNQ1 (*F*), KCNH2, (*G*) and SCN5A (*H*) mRNAs with PITX2c recombinant plasmid transfected in HL-1 cells. Mean ± SEM are shown. Asterisks illustrate the degree of significance (*$p < 0.05$; and **$p < 0.01$) of the differences from WT as evaluated with Student's *t* test. Comparisons of WT/M versus M revealed no significant differences ($p > 0.05$).

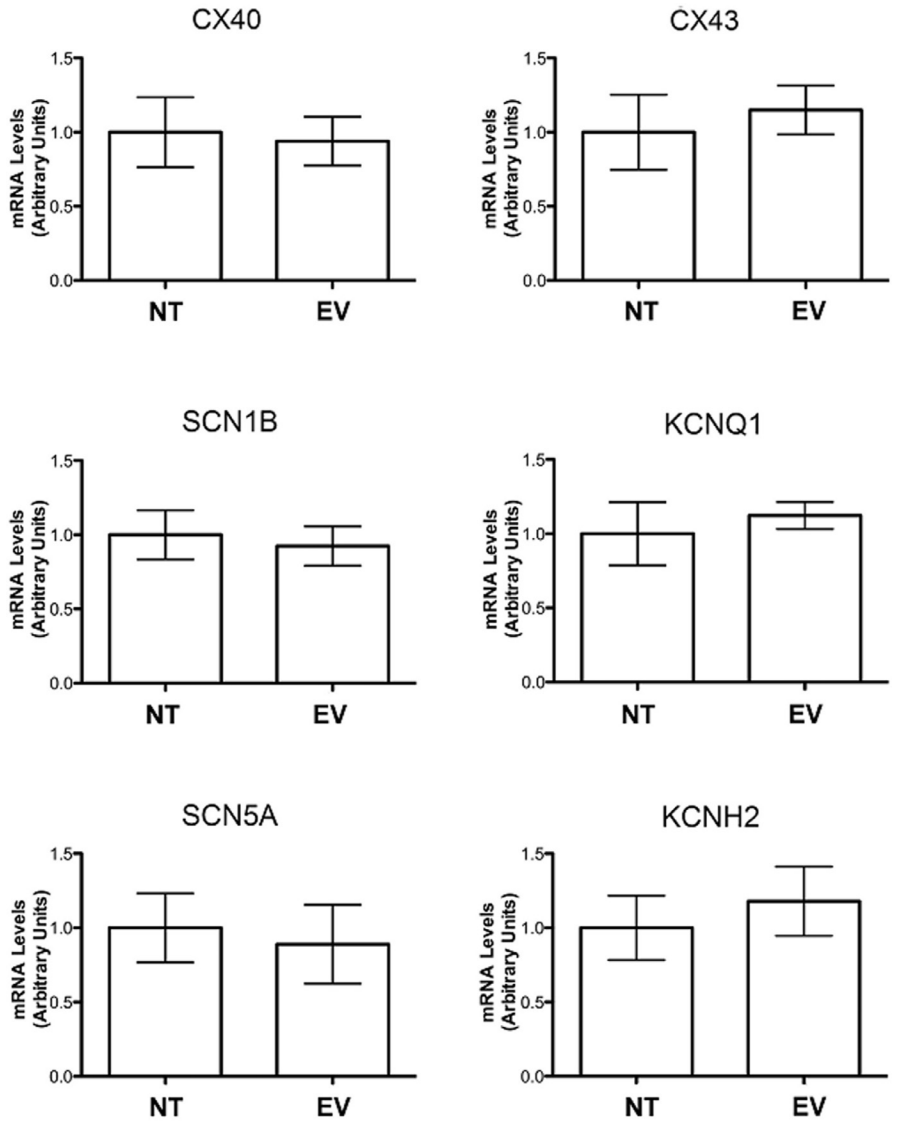

Figure 5. Comparison of nontransfected and mock-transfected HL-1 cells. Levels of connexins and ion channels mRNA in nontransfected (NT) HL-1 cells and in HL-1 cells transfected with the V5-Tag-pcDNA3.1/Zeo vector that did not contain any PITX2 sequence (EV). Note that the empty vector did not cause any change in any of the mRNAs explored, as compared with NT. Each plasmid transfection was done 3 times.

Table 2
PITX2c variant effects on the mRNA levels corresponding to key proteins involved in AF

	GJA5 (Cx40)	GJA1 (Cx43)	SCN1B	KCNQ1	KCNH2	SCN5A
p.Met207Val	2.8-fold **	1.7-fold **	1.7-fold **	1.8-fold **	3.6-fold *	NS
p.Met207Val +WT	3.1-fold **	2.1-fold **	1.9-fold **	NS	2.6-fold *	NS

The levels of mRNA were measured when transfected alone (p.Met207Val *PITX2c)* or mixed with the WT (p.Met207Val *PITX2c*+WT *PITX2c)* and normalized to the mRNA levels observed with WT *PITX2c* as plotted in Figure 4 panels C-H. NS = no significant changes. *p <0.05; **p <0.01.

patients with early onset AF, to confirm the association observed for p.Met207Val. Our discussion of the possible proarrhythmic consequences is made under the assumption that the transcriptional changes would cause corresponding changes in the functional activity of the proteins, which we did not investigate. Further studies should also address the direct and indirect pathways from mutated PITX2c through mRNAs transcription down to expression of end point proteins.

Disclosures

The investigators have no conflicts of interest to disclose.

Acknowledgment

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.amjcard.2018.11.047.

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