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MESP1 Mutations in Patients with Congenital Heart Defects

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Abstract

Identifying the genetic etiology of congenital heart disease (CHD) has been challenging despite being one of the most common congenital malformations in humans. We previously identified a microdeletion in a patient with a ventricular septal defect containing over 40 genes including *MESP1* (mesoderm posterior bHLH transcription factor 1). Because of the importance of *MESP1* as an early regulator of cardiac development in both in vivo and in vitro studies, we tested for *MESP1* mutations in 647 patients with congenital conotruncal and related heart defects. We identified six rare, non-synonymous variants not seen in ethnically matched controls and one likely race-specific non-synonymous variant. Functional analyses revealed that three of these variants altered activation of transcription by MESP1. Two of the deleterious variants are located within the conserved HLH domain and thus impair the protein-protein interaction of MESP1 and E47. The third deleterious variant was a loss of function frameshift mutation. Our results suggest that pathologic variants in MESP1 may contribute to the development of CHD and that additional protein partners and downstream targets could likewise contribute to the wide range of causes for CHD.

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Conflict of interest

The authors do not have any conflicts of interest to disclose.

Keywords

Mesoderm posterior bHLH transcription factor 1; Conotruncal heart defects; *MESP1*; Congenital heart disease

Introduction

Congenital heart defects (CHD) are the most common type of major birth defects with a complex and poorly understood etiology. Evidence strongly suggests a genetic component [Gelb and Chung, 2014] including chromosomal abnormalities, gene mutations and epigenetic insults [reviewed in Lalani and Belmont, 2014]. Chromosomal abnormalities including submicroscopic chromosomal aberrations (copy number variants, CNVs) contribute to disease risk for CHD in syndromic and seemingly non-syndromic cases [Lalani and Belmont, 2014]. Studies have found that sequence variants in genes disrupted by CNVs can likewise contribute to disease, including *TBX1* (MIM# 602054)[Yagi et al., 2003], *EMHT1* (MIM# 610253)[Kleefstra et al., 2006], *JAG1* (MIM# 601920), *NSD1* (MIM# 606681)[Cecconi et al., 2005], and *ELN* (MIM# 185500) [reviewed in Andersen et al., 2014]. To identify new disease-related genes, we have studied candidate genes disrupted by CNVs for potentially damaging variants in individuals with seemingly non-syndromic CHD. We previously reported a CNV spanning 4.3 Mbs on chromosome 15 in a case diagnosed with a posterior malalignment type ventricular septal defect VSD [Goldmuntz et al., 2011]. We hypothesized that mutations within genes contributing to cardiac development deleted by this CNV might contribute to CHD in humans.

Mesoderm posterior 1 (*MESP1*; MIM# 608689), a transcription factor of the basic helix-loop-helix (bHLH) family expressed in cardiac progenitors at different time points during their specification [Devine et al., 2014; Lescroart et al., 2014], was contained within this large CNV. MESP1 participates in the specification of the cardiac lineage and plays an essential role in early heart development in animal and *in vitro* studies [Bondue et al., 2008; David et al., 2008; Lindsley et al., 2008; Saga, 1998; Saga et al., 2000; Saga et al., 1999]. *MESP1*-null mice die in utero at 10.5 dpc due to anomalies in heart tube formation and heart looping, resulting in various degrees of cardiac bifida [Saga, 1998; Saga, et al., 1999]. Overexpression of MESP1 (mesoderm posterior protein 1) in two-cell *Xenopus laevis* embryos resulted in extra ectopic beating tissue in tadpoles [David, et al., 2008]. Similarly, overexpression of Mesp1 in mouse embryonic stem cells resulted in up- or down-regulation of specific gene sets and, subsequently, accelerated cardiovascular specification and premature appearance of beating cells. Among the up-regulated genes were core cardiovascular transcription factors such as *HAND2* (MIM# 602407), *GATA4* (MIM# 600576), *NKX2-5* (MIM# 600584), *TBX20* (MIM# 606061) and *MYOCD* (MIM# 606127) [Bondue et al., 2011; David, et al., 2008; He et al., 2011; Lindsley, et al., 2008]. Some of these genes, alone or in combination, are known to participate in human CHD [Garg et al., 2003; Granados-Riveron et al., 2012; Kirk et al., 2007; McElhinney et al., 2003; Posch et al., 2010; Reamon-Buettner and Borlak, 2010; Schott et al., 1998; Stallmeyer et al., 2010]. The potential upstream role of *MESP1* in regulating these critical cardiac transcription factors suggests that mutations in this gene might also play a role in human CHD. Based on our

CNV findings and the role of *MESP1* in cardiogenesis, we evaluated a cohort with the same CHD as the original case harboring the CNV, followed by cases with etiologically related conotruncal defects for potentially deleterious variants.

Material and Methods

Sample collection and DNA isolation

We studied a cohort of 280 cases with a primary diagnosis of ventricular septal defect (VSD) including conoventricular, malalignment and conoseptal hypoplasia type VSDs, and a cohort of 367 cases with conotruncal defects (predominantly tetralogy of Fallot, Table 1). Subjects with a recognizable genetic syndrome at the time of enrollment were excluded [Peyvandi et al., 2013]. Informed consent was obtained from all cases and parents following protocols approved by the Institutional Review Board for Human Research at The Children's Hospital of Philadelphia prior to collection of samples. DNA of ethnically matched control subjects was obtained from the Coriell Institute for Medical Research (Camden, NJ). DNA was extracted from whole blood or lymphoblastoid cell lines using standard methods (Gentra Puregene Blood kit by Qiagen, Valencia, CA).

Mutation analysis

The initial cohort of 280 cases was scanned for sequence variation in both coding exons of *MESP1* (NM_018670.3) by high-resolution melting curve analysis using a 96-well LightScanner™ (Idaho Technology Inc, Salt Lake City, UTAH). Analyzed amplicons spanned exonic sequences as well as exon/intron boundaries with a size range of 222–404 bp (see Supp. Table S1). Samples were amplified in a volume of 10 μl containing 1X LightScanner® Master Mix (Idaho Technolgy Inc.), 20 ng of DNA and 0.25 μM of each primer following the suggested PCR protocol. For some amplicons, we added a touchdown cycling step at the beginning starting 3°C above the annealing temperature and then decreasing 1°C per cycle for 3 cycles. Primer sequences were designed using PrimerSelect from the Lasergene Core Suite (DNASTAR, Madison, WI) and are listed together with annealing temperatures, and product size in Supp. Table S1. PCR products were then transferred to the LightScanner for melting analysis and the melting data analyzed for sequence variations using the LightScanner Software. Samples that showed variation in the melting curves were chosen for Sanger sequencing. PCR products were re-amplified in a volume of 20μl using AmpliTaq Gold Polymerase (Applied Biosystems, ThermoFisher Scientific, Pittsburgh, PA), with 20ng of DNA and a final concentration of 0.2mM dNTP, 2 mM $MgCl_2$, and 0.25 μM of each primer. PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA) before sequencing using BigDye™ Terminator version 3.1 on an ABI 3100 Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). Sequences were analyzed using Sequencher™ (Gene Codes, Ann Arbor, MI). Case, parental and control DNA sequences were compared to the reference sequences for *MESP1* (NM_018670.3) to identify sequence variations. Control samples were Sanger sequenced for exon 1 of *MESP1* and exon 2 was analyzed using high-resolution melting curve analysis as described above.

A second cohort of 367 cases with conotruncal defects underwent targeted whole exome sequencing by the NHLBI Resequencing & Genotyping Service at the Northwest Genomics Center at the University of Washington. The VCF files were analyzed using SNP & Variation Suite v8.1 (Golden Helix, Inc. Bozeman, MT, http://www.goldenhelix.com) for variants in *MESP1*. We performed Sanger sequencing to validate non-synonymous variations. Previously unreported variants have been submitted to dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Site directed mutagenesis

A clone containing the mRNA sequence of *MESP1* (NM_018670.3) was purchased from GenScript (Piscataway, NJ). The complete ORF sequence of *MESP1* was cloned into an expression vector pcDNA3.1/V5-His© Topo® (Invitrogen, ThermoFisher). Mutations in *MESP1* were introduced by site-directed mutagenesis using a QuikChange lightning site-directed mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA) and mutagenic primers (Supp. Table S2) that were designed using the QuikChange Primer Design Program (Agilent Technologies, Inc). A V5 tag was incorporated at the N-terminus for the constructs pcDNA3.1-*MESP1*, pcDNA3.1-*MESP1*-2745, pcDNA3.1-*MESP1*-2365, and pcDNA3.1-*MESP1*-2034 (Supp. Table S2) for protein analysis. All expression vectors were transformed into One Shot TOP 10 competent cells, grown in appropriate antibiotic media and isolated using a QIAprep Spin Miniprep Kit (Qiagen), and sequenced for verification. Expression of *MESP1* was verified by immunoblot analysis.

Dual luciferase reporter assay

Luciferase vector pGL4.23-*DKK1*-11: A triplicate of the E-box region *acCATATGgt* located approximately −11.6 kb upstream of *DKK1* (MIM# 605189)[David, et al., 2008] was inserted into the luciferase vector pGL4.23 [luc2/minP] (Promega, Madison, WI). A pCMV vector containing the sequence of *E47* (*TCF3*; MIM# 147141, X52078.1) was kindly provided by Dr. M. Atchinson, School of Veterinary Medicine, University of Pennsylvania. Human embryonic kidney (HEK) 293 cells were plated at a density of 60,000 cells per well on 24-well plates 24 hrs before transfection. Cells were co-transfected with 100 ng of empty pcDNA3.1 vector or pcDNA3.1 vector coding for either wildtype or mutant MESP1, 250 ng of pCMV-*E47*, 100 ng of either empty pGL4.23 or pGL4.23-*DKK1*-11 vector and 0.4 ng pGL4.75 (Renilla luciferase reporter vector) using FugeneHD (Promega) following the manufacturer's protocol. Transfection efficiency was ascertained using a co-transfected GFP expressing vector. Transfected cells were incubated for 48 to 60 hrs at 37°C with 5% CO_2 then washed and lysed using 1X passive lysis buffer for 15 min at RT provided by the Dual Luciferase Reporter Assay Kit (Promega). Dual luciferase assays (Promega) were performed according to the manufacture's protocol using a GloMax® 96 Microplate Luminometer w/ Dual Injectors (Promega). Firefly luciferase values were normalized relative to the Renilla luciferase values. At least three independent co-transfection experiments, all of which were done in triplicate, were performed to calculate average values and standard errors. A two-sample *t*-test was applied to assess statistical differences.

Mammalian Two-Hybrid assay

The ORFs of either the wildtype or mutant *MESP1* were cloned into the pCMV-BD plasmid (GAL4 insert) from the mammalian two-hybrid assay kit (Stratagene, La Jolla, CA). The *E47*–ORF was cloned into pCMV-AD (NF-κB insert). The control vectors supplied by the assay kit (pFR-Luc, pBD-NF-kB, pBD-p53, pAD-sv40T, pAD-TRAF) as well as the cloned vectors were amplified, isolated and sequence-verified as described above. The mammalian two-hybrid assay was performed similarly to the dual luciferase reporter assay described above. The total amount of DNA transfected per well was 500 ng, 100 ng of which was either wildtype pCMV-BD-*MESP1* or mutant pCMV-BD-*MESP1*, 200ng of pCMV-AD-*E47*, 200 ng pFR-Luc vectors and 0.5ng of pGL4.75 as the internal control. Cells were collected after 48h and luciferase activity measured using a GloMax® 96 Microplate Luminometer w/Dual Injectors (Promega). At least three independent co-transfection experiments, all of which were done in triplicate, were performed to calculate average values and standard errors. A two-sample *t*-test was applied to assess statistical differences.

Western Blot Assay

Human embryonic kidney (HEK) 293 cells were plated 24 hrs prior to transfection in 10 mm dishes or 6-well plates. Transfection of V5 or Gal4 tagged expression vectors was carried out using Fugene HD (Promega) according to the manufacturer's protocol. After 48 hrs incubation at 37°C with 5% CO_2, cells were washed with cold PBS and collected by scraping. Cells were then lysed in 100 μl 1 X cell lysis buffer (Cell Signaling Technology, Danvers, MA) collected, aliquotted and placed at −80°C. Single aliquots were then thawed on ice for the hours indicated. The supernatant was retained after 10 min centrifugation at 10,000 rpm at 4° C. 20μg of protein samples were separated by SDS-PAGE (NuPAGE 4–12% BT precast gels; Life Technologies, Carlsbad CA) and electrotransferred to Novex nitrocellulose (0.45μm; Life Technologies, ThermoFisher Scientific) or Immun-Blot PVDF membrane (BIO-RAD Laboratories, Inc., Hercules, CA). An Anti-V5 (Invitrogen, ThermoFisher Scientific) or Anti-GAL4 antibody (RK5C1, Santa Cruz Biotechnology, Inc, Dallas, Tx) was used for immunoblotting and visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, ThermoFisher Scientific).

Results

Identification of MESP1 variants in CHD

A previously reported subject harboring a large CNV encompassing *MESP1* was diagnosed with a VSD [Goldmuntz, et al., 2011]. Therefore, we studied a cohort of 280 subjects with VSDs for potentially pathogenic sequence variants in *MESP1*. The majority of the VSDs were classified as conoventricular and a smaller number were malalignment VSDs (Table 1). The cases were of mixed ethnicity, were not recognized to have a genetic syndrome at the time of enrollment, did not carry a 22q11.2 deletion and were reported to have an affected first-degree relative in 6% of cases [Peyvandi, et al., 2013]. To identify sequence variants in *MESP1* in the VSD cohort, we amplified short amplicons within exons and exon-intron boundaries and analyzed them for aberrant melting behavior using high resolution melting analysis. Due to the sequence variability in exon 1 (dbSNP, ExAC: exac.broadinstitute.org) this initial analysis resulted in a large number of variant curves.

Therefore, a high number of cases were selected for subsequent Sanger sequencing of exon 1, which identified previously reported synonymous as well as non-synonymous variants also present in our control groups (dbSNP, ExAC, [Lahm et al., 2013]). In addition we identified three non-synonymous variants and one frameshift mutation resulting in a premature stop codon in four unrelated cases (p.P27_D29dup, p.G70D, p.L147Pfs*9, p.K268N) that were not present in ethnically matched controls. We also identified one non-synonymous variant (p.D168G) present in 6 out of 97 ethnically matched controls (Table 2).

Thereafter, targeted exome sequence data for *MESP1* from an additional 367 cases with conotruncal defects who did not have a recognized genetic syndrome at the time of enrollment, did not carry a 22q11.2 deletion and were reported to have an affected first-degree relative in 10% of cases [Peyvandi, et al., 2013] were studied for deleterious mutations (Table 2). We identified two additional non-synonymous variants (p.E104K, p.L120P) that were not present in ethnically matched controls (Table 2). Six of the seven non-synonymous variants have allele frequencies less than 0.0009 in dbSNP and ExAC (Table 2). Testing of available parents for four trios revealed that one variant was *de novo* while three were inherited; the inheritance of the three remaining variants could not be determined (Table 2). None of the parental mutation carriers were reported to have congenital heart disease, but screening for subclinical cardiac anomalies was not done. Of these seven non-synonymous variants, five resulted in amino acid substitutions (Table 2), one was a duplication of three amino acids (c.79_87dup9; p.P27_D29dup), and one was a deletion of two base pairs that resulted in a frameshift and premature stop codon at position 155 (c.436_437delAG, p.L147Pfs*9). Two of the missense variants were located in the conserved bHLH domain and both were predicted to be probably damaging by Polyphen-2 (v2.2.2r398, see Supp. Table S3). Variant p.D168G was also predicted to be possibly damaging (Table 2). Since the frameshift mutation results in a stop codon in the penultimate exon, we hypothesized that it could lead to either nonsense-mediated decay or a truncated protein retaining the complete bHLH domain (Figure 1).

Assessment of variants on MESP1 transcriptional activity

Luciferase Reporter Assay—MESP1, a bHLH protein, is involved in the activation of several cellular processes via activation of transcription, including a cascade of cardiac-specific transcription factors [Bondue and Blanpain, 2010; Bondue, et al., 2008; Chan et al., 2013; David, et al., 2008; Lindsley, et al., 2008; Saga, et al., 2000; Wu, 2008]. Consequently, we studied whether the *MESP1* variants affected activation of downstream target genes.

Bondue et al. [2008] has shown that MESP1 binds to promoter regions containing putative bHLH binding sites (Ebox) of several of the cardiac transcription factors. However, it has also been described that MESP1 does not bind to Eboxes alone [Chan, et al., 2013; Takahashi et al., 2007] but forms heterodimers with E12 and E47 in order to activate transcription [Chan, et al., 2013; Lindsley, et al., 2008; Takahashi, et al., 2007]. E12 and E47 are isoforms of *TCF3* (MIM# 147141), a member of the ubiquitous E-protein family of bHLH transcription factors. We found that the *MESP1*/*E47* heterodimer activated transcription significantly more using a previously identified *MESP1* binding Ebox motif in

the DKK1 enhancer [David, et al., 2008] as compared to either one alone (Supp. Figure S1, Figure 2, Table 3). Using this assay, three of the seven MESP1 variants, namely p.E104K, p.L120P and p.L147Pfs*9, abolished activation of transcription ($p<0.005$, Figure 2).

Mammalian Two Hybrid Assay—The observation that both MESP1 and E47 are needed to activate transcription suggests that MESP1 needs to form an active heterodimer with E47 in order to activate transcription using the *DKK1*-11 Ebox construct. MESP1 mutants might abolish transcriptional activation by disrupting binding to E47. Alternatively, the mutations could disrupt DNA binding. To test if the protein-protein interaction between E47 and MESP1 was impaired by the three variants, p.E104K, p.L120P and p.L147Pfs*9, we performed a mammalian two-hybrid assay for all variants. In this assay, co-expression of MESP1 and E47 resulted in strong expression of luciferase while each alone was insufficient to drive expression, confirming interaction of the two proteins (Supp. Figure S2). Significantly reduced interaction was noted with the three MESP1 variants, p.E104K, p.L120P and p.L147Pfs*9 ($p<0.005$, Figure 3, Table 3). In addition, the variant, p.D168G, showed significantly reduced interaction in the mammalian two-hybrid assay. The two variants, p.E104K, and p.L120P, might disrupt protein-protein interaction and hence transcriptional activation by virtue of their location within the HLH domain. Alternatively, the mutant proteins might be unstable and thus unavailable to activate transcriptional activity. To test this, we incubated lysates on ice for 2 to 3 hrs and then performed immunoblotting. Indeed, we found that two variants, p.L120P and p.L147Pfs*9, affected MESP1 protein stability (Figure 4 A, B).

Discussion

Many studies demonstrate that the bHLH transcription factor, *MESP1*, participates in heart development [Bondue, et al., 2008; David, et al., 2008; Lindsley, et al., 2008; Saga, 1998; Saga, et al., 2000; Saga, et al., 1999]. We previously identified a large deletion that included *MESP1* in a patient with a VSD [Goldmuntz, et al., 2011]. We hypothesized that *MESP1* sequence variants might contribute to disease-risk for VSD and related conotruncal defects. As have others, we identified marked sequence variability in exon 1 (dbSNP, ExAC, [Lahm, et al., 2013]). Lahm et al. [2013] also identified a novel non-synonymous variant in exon 1 of *MESP1* in a patient population of 215 cases with a variety of congenital heart defects but it did not seem to have a deleterious effect. Our study of *MESP1* sequence variations in 647 patients with conotruncal lesions resulted in the identification of six very rare, non-synonymous and nonsense variants (MAF < 0.0009 ExAC/dbSNP) not seen in ethnically matched controls, and one non-synomymous variant that may be race-specific. Of these seven variants, three conferred functional changes, of which one was de novo, one inherited and the parental status unknown for the last. None of the first-degree relatives were reported to have CHD. Inherited variants from a seemingly normal parent may yet contribute to disease risk given that CHD are likely complex traits where several genetic variants contribute incrementally to disease-risk in any one person. In addition, seemingly normal parents can harbor subtle, clinically insignificant cardiac anomalies that are only detected by echocardiograms. Overall, these results suggest that approximately 1 in 200 cases with a VSD or tetralogy of Fallot carry a potential disease-related variant.

It is important and challenging to test whether sequence variants in a candidate gene change protein function and thus likely contribute to disease risk. We developed several assays to test whether the sequence variants in our cases conferred any functional change. We found that MESP1 alone would not activate transcription using several Ebox or Ebox containing regions. With the dimerization partner E47 [Lindsley, et al., 2008; Nakajima et al., 2006; Takahashi, et al., 2007], only the heterodimer MESP1/E47 activated transcription using a specific Ebox of the *DKK1* upstream region as a promoter [David, et al., 2008]. Using this assay three of the case-specific variants significantly affected *MESP1*-induced transcriptional activation. One of the variants (p.E104K) located in the bHLH region significantly affects the interaction of *MESP1* and *E47* as shown in the mammalian two-hybrid assay. It is difficult to say if the significantly lower activation of transcription with two other mutant MESP1 proteins (p.L120P, p.L147Pf*9) is due to protein instability or decreased dimerization, or a combination of both. Given that the premature stop codon of the variant p.L147Pfs*9 is in the penultimate exon, it is possible that the mRNA escapes nonsense-mediated decay and is translated into a truncated protein. Only one other variant (p.D168G) seemed to have a significant effect on dimerization of MESP1 and E47 but it did not affect the activation of transcription through the *DKK1* Ebox. Possible explanations for this discrepancy might be found in the difference in sensitivity of the two assays. Whereas the mammalian two-hybrid assay is more sensitive to protein interaction, the luciferase reporter assay is more sensitive to promoter occupancy and transcriptional activation. Alternatively the MESP1/E47 interaction may be stabilized by binding to Ebox motif DNA, which would not be the case for the mammalian two-hybrid assay, which utilizes the GAL4 DNA binding site. Three of the variants did not alter protein stability or transcriptional activation in this particular assay but may confer changes in different circumstances.

MESP1 is known to bind different Ebox-containing regulatory regions of known cardiac specific transcription factors including *NKX2.5*, *HAND2*, and *MYOCD* [Bondue, et al., 2008]. We evaluated several of these regions or single Eboxes contained within these regions for activation of transcription by MESP1 alone and the MESP1/E47 heterodimer but we did not see any activation (data not shown). Our findings suggest that the MESP1/E47 heterodimer likely prefers specific Ebox sequences, suggesting that MESP1 might use other dimerization partners to bind to other defined regulatory regions. Only a few dimerization partners of MESP1 are known so far, including Creb1 (MGI:88494) and E12 (MGI:98510) [Chan, et al., 2013; Shi et al., 2015]. However, recent studies suggest that additional cofactors influence MESP1 activity. For example embryonic stem cell-embryoid body differentiation differs depending upon the stage of differentiation of the cells and the signaling environment at the time point of induced MESP1 expression [Chan, et al., 2013]. Similarly, lineage-tracing experiments showed that the time point of MESP1 expression during development and the stage of specification of MESP1-expressing progenitor cells affects their cell fate. These findings suggest that different environmental cues during cardiac morphogenesis influence the ultimate fate of the progenitor cells [Devine, et al., 2014; Lescroart, et al., 2014]. Indeed, Lescroart et al. [2014] demonstrated differences in the molecular profile of the early and late expressing MESP1 progenitor cells in vivo. Further studies will show if co-expressed bHLH proteins, for example HAND1 protein, or other dimerization partners, interact or compete with MESP1 during early heart development

[Lescroart, et al., 2014; Vincentz et al., 2011]. Of interest, studies suggest that the Ebox sequence we used for our assays, acCATATGgt, is also a target for TWIST1 (MIM# 601622), a bHLH protein interacting with HAND2 and involved in heart development [Conway et al., 2010; Vincentz et al., 2008; Vincentz et al., 2013].

We also found the Ebox flanking bases to be critical for MESP1/E47 binding [Fisher et al., 1993; Gould and Bresnick, 1998; Kophengnavong et al., 2000]. Assays using the same *DKK1*-11 Ebox sequence but with different flanking bases failed to activate transcription (data not shown). Since we only tested E47 as a dimerization partner with only one specific Ebox sequence, it is possible that the mutant MESP1 proteins that did not show any significant change in this assay might have an effect on other MESP1 interactions.

Our results suggest that potentially deleterious variants in *MESP1* may contribute to the development of VSD and tetralogy of Fallot in humans. Additional studies testing a more extensive CHD population will help define the range of associated phenotypes. As with many genes implicated in CHD, the frequency of deleterious *MESP1* sequence variants is likely low in the CHD population [Zaidi et al., 2013]. However, these findings further clarify the cardiac transcription factor network and demonstrate that additional protein partners and down stream targets could likewise be disease-related. In addition, alternative methods might identify additional variants, such as indels, that were not detected by our screening method, and thus our results represent the minimum prevalence in this population. Given the significant sequence variability detected in exon 1 and variable reporting by different groups in public datasets, gene burden studies may be challenging to perform but clearly *MESP1* appears to be another important disease-related cardiac transcription factor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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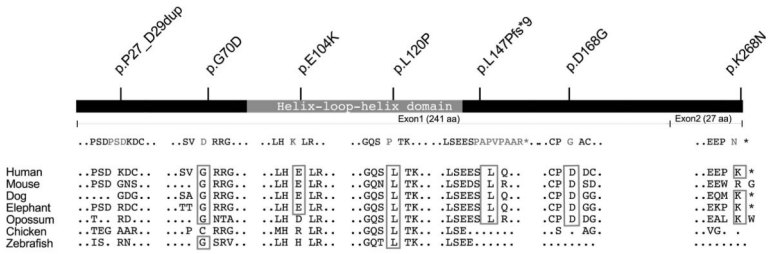

Figure 1.

Schematic showing the MESP1 protein indicating the conserved domains and exon locations. Location and description of the mutations studied in our cases are noted above. Comparative alignment of mutated regions in homologous proteins in various species indicating the affected amino acids in boxes.

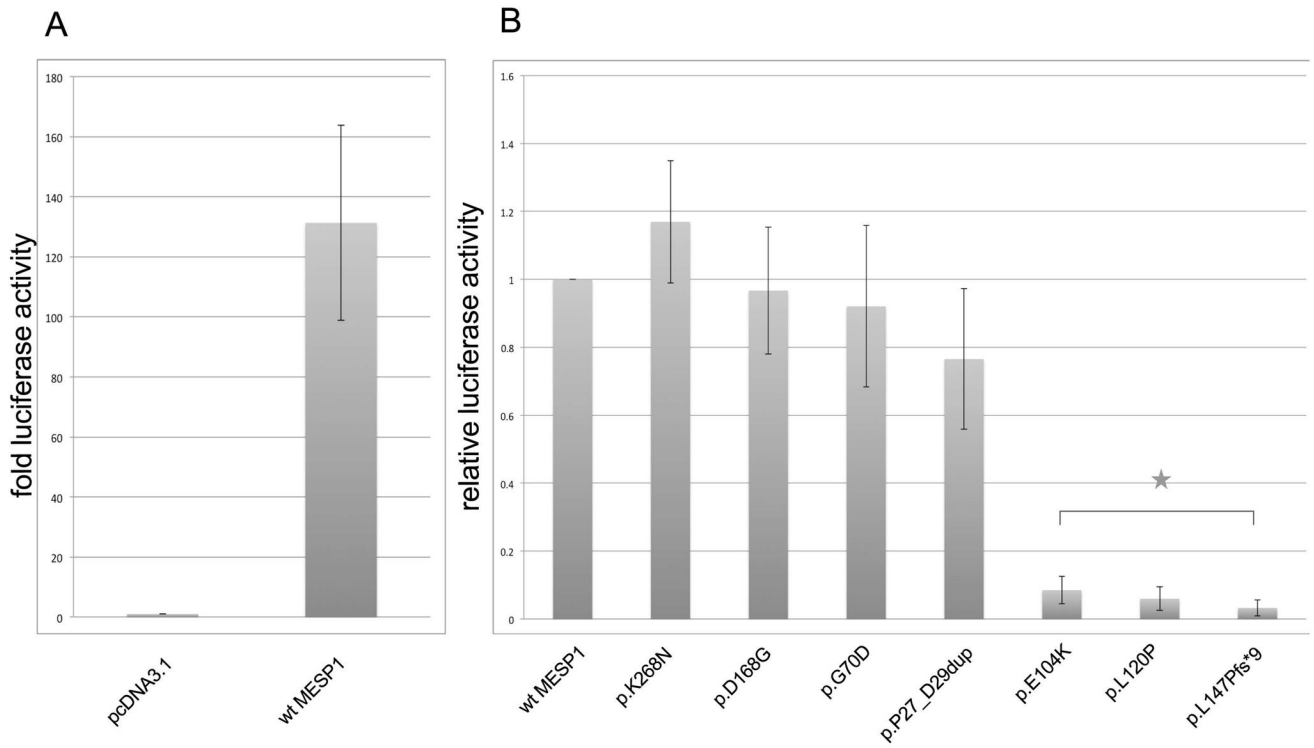

Figure 2.

Activation of transcription by the heterodimer MESP1/E47 using the *DKK1*-11 Ebox as enhancer. A: Activity of MESP1/E47 on *DKK1*-11 Ebox. Fold activation normalized to activation by empty expression vector with coexpression of E47. B: Relative luciferase activity of wildtype MESP1/E47 and mutant MESP1/E47 on *DKK1*-11 Ebox. * = p <0.005

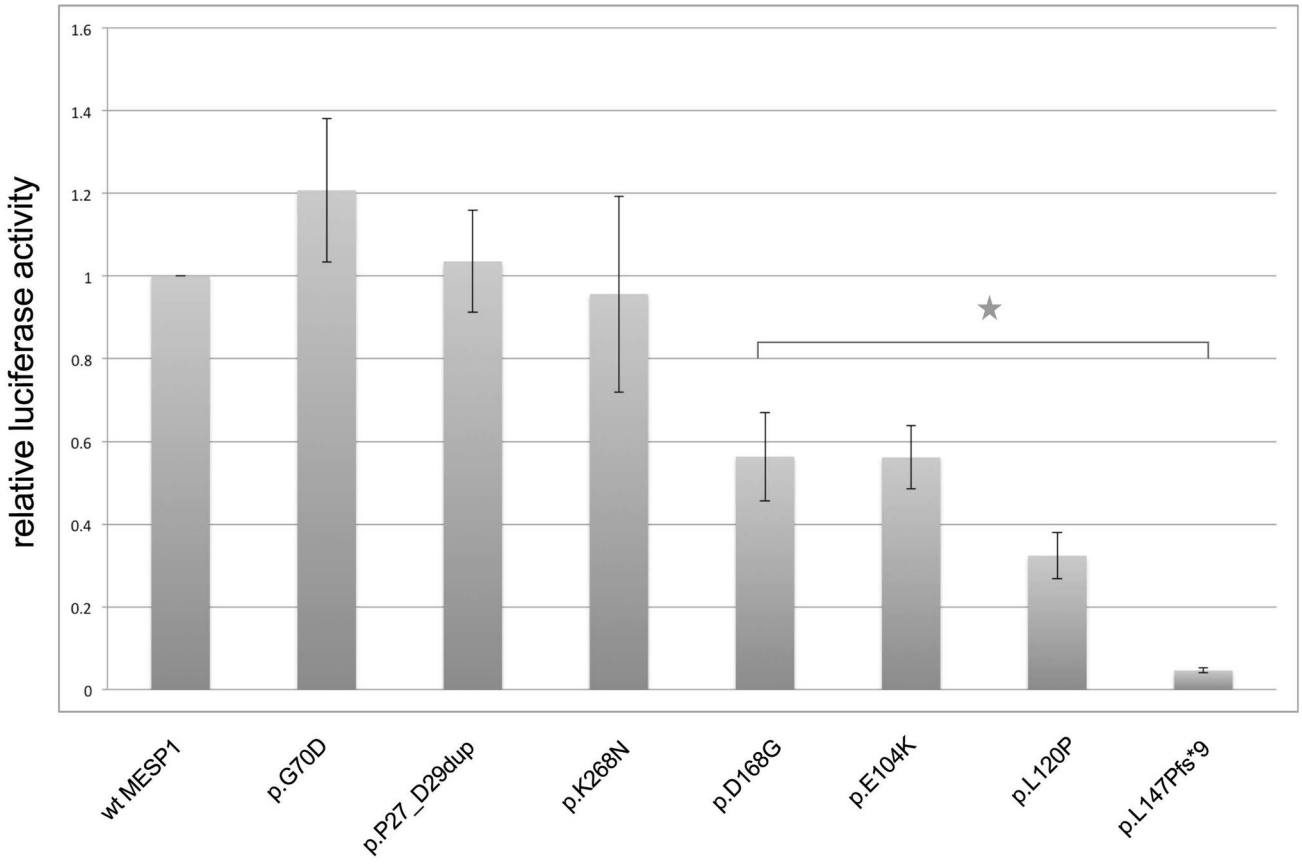

Figure 3.

Mammalian two-hybrid assays to evaluate effect of *MESP1* mutations on MESP1-E47 interactions. Relative luciferase activity was normalized to wildtype MESP1-E47 and shown as an average over three independent experiments. * $p < 0.005$

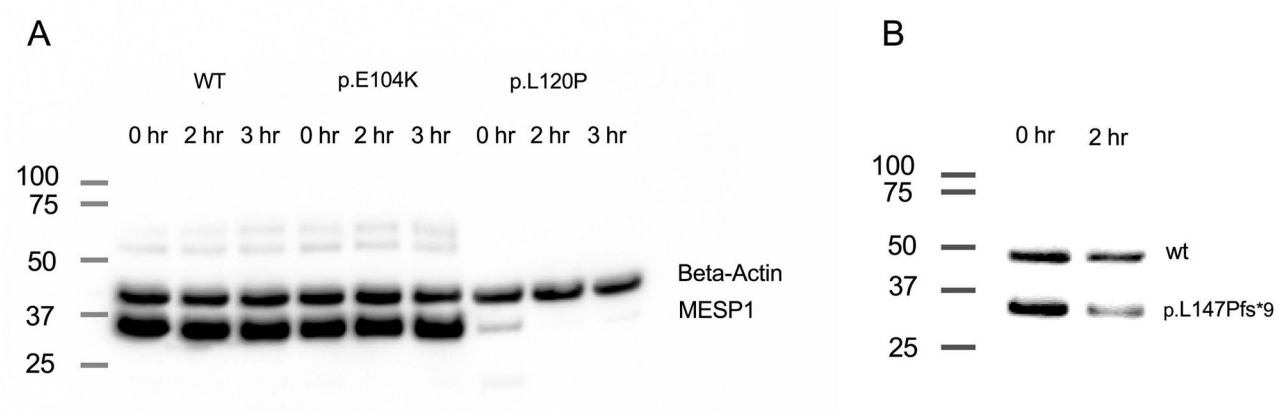

Figure 4.

Western blot analysis of wildtype and mutant MESP1 proteins. A. The V5-tagged MESP1 proteins, wildtype and mutant (p.E104K, p.L120P) each of which was transfected separately into HEK293 cells. Mutant p.L120P-MESP1 protein is not stable on ice. Beta-actin is labeled as an internal control B. Similar experiments with GAL4-tagged wildtype and mutant MESP1 protein, p.L147Pfs*9, which were co-transfected. After 2 hrs on ice the GAL4-tagged truncated protein shows degradation.

Table 1

Cardiac Diagnoses for Study Cohorts

Diagnosis	Numbers
Tetralogy of Fallot	318
Ventricular septal defect	292
Conoventricular	218
Posterior malalignment	51
Conal septal hypoplasia	19
Others[*]	4
Truncus arteriosus	21
Interrupted aortic arch type-B	13
Interrupted aortic arch type-A	3
Total	647

[*] Others included two unspecified VSD, one inlet VSD, and one VSD with virtually absent septum

Table 2

Mutations Identified in *MESP1*

Protein Q9BRJ9	Variant (based on cDNA) (NM_018670.3)	hg19	Region	Conserved Domain	dbSNP MAF (ExAC#)	Polyphen-2#	Sift#	CADD	Patient					Variant in Controls		
									Dx	Gender	Ethnicity	Father	Mother	CA	AA	Asian
p.P27_D29dup	c.79_87dup9	chr15:90294375_90294376insGTCGGAGGG	Exon1		rs752416885 0.00007477	N/A	N/A	N/A	cVSD	M	CA (no HISP)	Carrier	WT	0/95	0/87	
p.G70D	c.209G>A	chr15:90294254C>T	Exon1		rs770840637*	benign (0.447)	Tolerated	10.42	cVSD	M	AAm (Hisp)	N/A	WT	0/99	0/96	
p.E104K	c.310G>A	chr15:90294153C>T	Exon1	bHLH	rs771329728 0.0002231	probably damaging, 0.981	Tolerated	16.35	TOF	F	CA	WT	Carrier	0/99	0/97	
p.L120P	c.359T>C	chr15:90294104A>G	Exon1	bHLH	rs565846523 0.000817	probably damaging, 1.0	Damaging	15.83	TOF	F	CA	WT	WT	0/99	0/97	
p.L147Pfs*9	c.436_437delAG	chr15:90294027_90294028delTC	Exon1		rs777474430 2.106e-05	N/A	N/A	N/A	cVSD	F	AAm (no Hisp)	N/A	N/A	0/99	0/97	
p.D168G	c.503A>G	chr15:90293960T>C	Exon1		rs200810210 0.005209	possibly damaging (0.948)	Damaging	13.68	cVSD	M	AAm (no Hisp)	N/A	N/A	0/99	6/97	
p.K268N	c.804G>C	chr15:90293378C>G	Exon2		N/A	Benign (0.061)	Tolerated	13.9	mVSD/CoA/AA	M	AAm/CA/AS (Hisp)	WT	Carrier	0/198	0/98	0/96

N/A: Not available, Dx: diagnosis, CADD: Combined Annotation Dependent Depletion score (http://cadd.gs.washington.edu), cVSD: conoventricular VSD, mVSD: malalignment VSD, TOF: Tetralogy of Fallot, CoA: Coarctation of the Aorta, AA: Aortic atresia, AAm: African American, CA: Caucasian, WT: Wildtype; #ExAC:Exome Aggregation Consortium (exac.broadinstitue.org),

*not listed in ExAC, MAF given in dbSNP: 0.000; hg19: GrCh37.p13 (GCF_000001405.25),

#Values listed in Supp. Tables S3 and S4.

Table 3

Functional Consequences of Mutations Identified in *MESP1*

Q9BRJ9	Inheritance	Present in Controls	Relative Transcriptional Activation Compared to Wildtype MESP1 (Stdev)	
			Luciferase Reporter Assay	Mammalian Two-Hybrid
p.P27_D29dup	Paternal	N	0.77 (+/−0.21)	1.02 (+/−0.13)
p.G70D	N/A	N	0.92 (+/−0.24)	1.19 (+/−0.21)
p.E104K	Maternal	N	**0.08 (+/−0.04)***	**0.56 (+/−0.08)***
p.L120P	De Novo	N	**0.06 (+/−0.03)***	**0.32 (+/−0.06)***
p.L147Pfs*9	N/A	N	**0.03 (+/−0.02)***	**0.05 (+/−0.01)***
p.D168G	N/A	Y	0.97 (+/−0.19)	**0.53 (+/−0.11)***
p.K268N	Maternal	N	1.17 (+/−0.18)	0.90 (+/−0.26)

*Significant ($p<0.005$)