Familial Left Ventricular Non-Compaction Is Associated With a Rare p.V407I Variant in Bone Morphogenetic Protein 10

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Background: Left ventricular non-compaction (LVNC) is a heritable cardiomyopathy characterized by hypertrabeculation, inter-trabecular recesses and thin compact myocardium, but the genetic basis and mechanisms remain unclear. This study identified novel LVNC-associated mutations in NOTCH-dependent genes and investigated their mutational effects.

Methods and Results: High-resolution melting screening was performed in 230 individuals with LVNC, followed by whole exome and Sanger sequencing of available family members. Dimerization of bone morphogenetic protein 10 (BMP10) and its binding to BMP receptors (BMPRs) were evaluated. Cellular differentiation, proliferation and tolerance to mechanical stretch were assessed in H9C2 cardiomyoblasts, expressing wild-type (WT) or mutant BMP10 delivered by adenoviral vectors. Rare variants, p.W143*-NRG1 and p.V407I-BMP10, were identified in 2 unrelated probands and their affected family members. Although dimerization of mutant V407I-BMP10 was preserved like WT-BMP10, V407I-BMP10 pulled BMPR1a and BMPR2 receptors more weakly compared with WT-BMP10. On comparative gene expression and siRNA analysis, expressed BMPR1a and BMPR2 receptors were responsive to BMP10 treatment in H9C2 cardiomyoblasts. Expression of V407I-BMP10 resulted in a significantly lower rate of proliferation in H9C2 cells compared with WT-BMP10. Cyclic stretch resulted in destruction and death of V407I-BMP10 cells.

Conclusions: The W143*-NRG1 and V407I-BMP10 variants are associated with LVNC. Impaired BMPR-binding ability, perturbed proliferation and differentiation processes and intolerance to stretch in V407I-BMP10 mutant cardiomyoblasts may underlie myocardial non-compaction.

Key Words: Bone morphogenetic protein 10 (BMP10); Cardiomyocyte differentiation; Left ventricular non-compaction; Proliferation; Stretch

Left ventricular non-compaction (LVNC) is a heritable cardiomyopathy characterized by myocardial hypertrabeculation, inter-trabecular recesses, and thin compacted LV wall. LVNC occurs in 0.05% or 1/2,000 of adults. Clinically, LVNC is heterogeneous, categorized by ventricular size, thickness, function, atrial size, rhythm, outcomes and whether there is associated congenital heart disease (CHD). Progressive cardiac dysfunction, arrhythmias, or thromboembolism are common manifestations. Evidence of clinical LVNC coinciding with other types of cardiomyopathies (dilated [DCM], hypertrophic, restrictive or arrhythmogenic) and CHD suggests multiple mechanisms that may interact, culminating in overlapping phenotypes. Genetically, autosomal dominant and X-linked recessive inheritance are reported, with familial pattern present in approximately 40% of LVNC cases. Despite this, only a few LVNC-associated mutations have been reported in LBD3/ZASP, DTNA, TAZ/G4.5, LMNA, MYH7, ACTC, TPM1 and TNNT2, indicating that genetic etiologies and underlying mutation-induced mechanisms remain largely unexplored.

Imbalanced myocardial development regulated by NOTCH signaling is widely accepted as associated with LVNC. Bone morphogenetic protein 10 (BMP10), a cardiac-specific ligand belonging to the transforming growth factor-β superfamily, is expressed by endothelial and myoblast cells

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briefly and only in embryonic myocardium at E9.0–E13.5 and its expression (pre-pro-mRNA→pro-mRNA→pro-BMP10) is regulated by NOTCH signaling in a spatiotemporal and dosage-dependent manner. Secreted into the cytoplasm, pro-BMP10 protein dimerizes and forms a mature ligand-homodimer, which is capable of binding to BMP receptors (BMPRs). In cardiomyocytes, BMPR consists of activin A receptors (ACVR) and activin receptor-like kinases (ALKs). Receptors BMPR1a (ALK3), BMPR1b (ALK6) and BMPR2 are specific to BMP10. Binding BMP10 to its BMPRs regulates the downstream SMAD1/5/8 (canonical) and MAPK (non-canonical) pathways, subsequently activating cardiogenic factors (NFKX2-5, MEF2c, TBX20) and inhibiting CDKN1c/p57-kip2. Ablation of Bmp10 in mice resulted in hypoplastic ventricular walls and embryonic death at E10.5. In contrast, BMP10 overexpression caused LVNC and ventricular septal defect in vivo, highlighting its importance in the development of LVNC.

We performed screening of NOTCH-dependent genes in 230 individuals with LVNC and their family members, and identified 2 very rare heterozygous variants: missense c.1219G>A (p.V407I) in Tbx20 and c.230A>G (p.H77D) in Nkx2-5. Interestingly, expression of the V407I-BMP10 variant is associated with LVNC, and that further in vivo studies are needed to comprehensively understand its roles in LVNC pathogenesis.

Methods

Human Subjects and Clinical Diagnostic Criteria
After obtaining informed consent, patients and healthy control individuals were recruited in this study as described in Supplementary Materials. Clinical evaluation of probands and their first-degree relatives and collection of family history were performed. Blood was collected from 230 patients and their relatives (Latino, n=43; Caucasian, n=52; Asian, n=100; African, n=35; female, n=118; male, n=112; families, n=73) and 272 ethnicity-matched controls. Genomic DNA was extracted and biobanked in the Pediatric Cardiomyopathy Specimen Repository (PCSR).

High-Resolution Melting (HRM) Analysis, Whole Exome Sequencing (WES) and Sanger Sequencing
To effectively select LVNC candidate genes, we first selected genes using an algorithm based on association with NOTCH signaling followed by Protein Interaction Network Analysis2 (PIN2A) at http://cbg.garvan.unsw.edu.au/pina/. Then, 50 genes underwent HRM using AB17500 ifast (Applied Biosystems). We performed WES in a proband carrying the V407I-BMP10 variant. All non-synonymous or nonsense variants with minor allele frequency (MAF) <0.01 were prioritized using in silico algorithms. Then, the exons containing the deleterious variant were amplified in an exon-by-exon manner using the 3730xl Analyzer (Applied Biosystems). Experimental details of HRM, WES and bioinformatics analysis are described in Supplementary Materials.

Cell Culture and Animals
H9C2, HEK293F and C2C12 cells were obtained from the American Type Culture Collection. HEK293F cells were used for purification of BMP10 protein and immunoprecipitation (IP), while H9C2 and C2C12 were used for siRNA knockdown, adenoviral infection, cellular proliferation and stretch studies. All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin G and 100 μg/mL streptomycin. All animal work was conducted according the protocols approved by Institutional Animal Care and Use Committee (IACUC), Cincinnati Children’s Hospital Medical Center (CCHMC).

Site-Directed Mutagenesis, Protein Production and Purification
The human BMP10 cDNA (1.5 kb) was cloned into a pcDNA3.1/Myc-His expression vector (Invitrogen) and the V407I-BMP10 variant was introduced using site-directed mutagenesis. Then, WT or mutant BMP10 expressed in HEK293F cells was extracted using the FreestyleTM 293 system (Invitrogen), as described in Supplementary Materials.

IP and Immunoblotting
HEK293F cells were transfected/co-transfected with WT or mutant BMP10 (pcDNA3.1/Myc-His) and/or BMPR (pcDNA-1) constructs. Forty-eight hours after transfection, cells were collected and cell lysates were processed with IP and Co-IP and immunoblotted with the anti-FLAG-M2, anti-Myc-9E10, anti-HA-3F10 (Boehringer Mannheim), or anti-phosphosine antibodies (Zymed) as described in Supplementary Materials. Data were analyzed using results from 3 pull-down experiments.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) and siRNA Knockdown
We first performed qPCR to assess expression of BMP10, BMPR and GAPDH (control) in H9C2 cells in triplicates at least twice (Prism7500, Applied Biosystems). Then, BMPR siRNA knockdown in H9C2 cells followed by BMP10 treatment was used to test whether H9C2 cardiomyoblasts can serve as an appropriate in vitro model of BMP10 and BMPR interactions. Experimental details and analysis are described in Supplementary Materials.

Adenoviral Construction, Cellular Differentiation and Cell Proliferation Assay
Adenoviral vectors containing mature WT- or V407I-BMP10 were constructed, and infection of H9C2 cells was performed. The rate of cellular proliferation of the H9C2 cardiomyoblasts was assessed in an MTT assay using the SynergyH1 reader (BioTek).

Immunohistochemistry and Cell Death Analysis
H9C2 cells grown in 10% FBS (proliferation condition) or 2% heat inactivated horse serum (HIHS, differentiation condition) on chamber slides (Lab-Tek) were infected with WT-BMP10 or V407I-BMP10 vectors. Immunohistochemistry using anti-HA (Sigma), anti-BMPR1a and anti-BMPR2 (Santa Cruz), anti-SMAD1/5/8 (Cell Signaling), anti-a-actinin2 (Invitrogen) antibodies and 0.5 μg/mL diaminido-2-phenylindole (DAPI; Sigma) was performed.
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(82.2%) had isolated LVNC (Supplementary Table 1). LVNC accompanied by cardiomyopathy, CHD or other systemic diseases accounted for 17.8%. Due to the large size of the patient cohort, we first performed HRM analysis on 50 NOTCH-associated candidate genes (Supplementary Table 2). HRM identified 2 very rare heterozygous variants in BMP10 and NRG1. A missense variant c.1219G>A (p.V407I) in BMP10 (rs148561995, MAF=0.00007783) was identified in a proband with isolated LVNC and her mother with LVNC/DCM from a Latino family (Figure 1A). The second variant, c.661G>A (p.W143*) in NRG1 (rs913880282, MAF=0.00002) was identified in a proband and his mother with isolated LVNC, while the proband’s unaffected brother was negative (Figure 1B). The p.W143* variant resulted in premature termination of NRG1 type II (NG_012005.2), resulting in ablation of glial growth factor-2 (GGF2), the precursor pro-NRG1 expressed in many tissues, including the heart.12 Notably, a GGF2-knockout mouse model has demonstrated abnormalities in cardiac and neuron development, supporting that p.W143* is an LVNC-associated variant.13

Therefore, we selected the V407I-BMP10 variant for days 0, 1, 3 and 5 as described in Supplementary Materials.

Cyclic Stretch
H9C2 cells were infected with WT-BMP10 or V407I-BMP10 vectors (multiplicity of infection or MOI=5). After 48h, H9C2 cells were plated on 6-well BioFlex plates in DMEM containing 10% FBS. When cells reached 80% confluency, they underwent cyclic 10% mechanical stretch for 1h/day for 3 consecutive days using FlexCell5000 system (FlexCell International) as previously described.11 On days 1 and 3, the cells were fixed, immunostained and visualized using a Zeiss710 confocal microscope.

Statistical Analysis
Student t-test, 2-way ANOVA or repeated-measures ANOVA over time were used to analyze data, and P<0.05 was considered significant.

Results
Patient Cohort and Mutational Analysis
On analysis of the 230 individuals with LVNC, the majority (82.2%) had isolated LVNC (Supplementary Table 1). LVNC accompanied by cardiomyopathy, CHD or other systemic diseases accounted for 17.8%. Due to the large size of the patient cohort, we first performed HRM analysis on 50 NOTCH-associated candidate genes (Supplementary Table 2). HRM identified 2 very rare heterozygous variants in BMP10 and NRG1. A missense variant c.1219G>A (p.V407I) in BMP10 (rs148561995, MAF=0.00007783) was identified in a proband with isolated LVNC and her mother with LVNC/DCM from a Latino family (Figure 1A). The second variant, c.661G>A (p.W143*) in NRG1 (rs913880282, MAF=0.00002) was identified in a proband and his mother with isolated LVNC, while the proband’s unaffected brother was negative (Figure 1B). The p.W143* variant resulted in premature termination of NRG1 type II (NG_012005.2), resulting in ablation of glial growth factor-2 (GGF2), the precursor pro-NRG1 expressed in many tissues, including the heart.12 Notably, a GGF2-knockout mouse model has demonstrated abnormalities in cardiac and neuron development, supporting that p.W143* is an LVNC-associated variant.13

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### Table. Genes Re-Sequenced on Sanger Sequencing

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<th>Gene</th>
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<th>Father</th>
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*Found in the proband and her affected mother. AA, amino acid; dbSNP, The Single Nucleotide Polymorphism Database; No, negative for a variant; YES, positive for a variant.

**Figure 2.** Dimerization and receptor-binding of wild-type (WT) and mutant bone morphogenetic protein 10 (BMP10). (A) Western blotting of WT-rhBMP10 and V407I-rhBMP10. First lower arrow, predicted 12.2-kDa mature rhBMP10 monomer. Upper arrows, dimers and polymers of rhBMP10. C, control; M, molecular weight marker; V407I, mutant rhBMP10. (B) Immunoprecipitation (IP) and co-IP of BMP10 and BMP receptors (BMPRs). (Upper panel) Combinations of WT- or V407I-BMP10 fused with GFP (green fluorescent protein) and receptors, BMPR1a or BMPR2, fused with FLAG epitope. (+) Expression of a construct. (Lower panel) Western blotting, IB, immunoblotting. Arrow and asterisk, increase in intensity of the band, representing the amount of BMP10 pulled down by BMPR1 and BMPR2-FLAG, respectively. (C) Relative ratio of signal strength of WT or V407I BMP10 pulled by BMPR1a or BMPR2. Average ratios of BMP10-GFP to BMPR1-FLAG or BMPR2-FLAG from 3 pull-down experiments, quantified using ImageJ. *P<0.05.
Myocardial Non-Compaction and BMP10 Mutation

ATP5SL and NDUFB11 encode the components of mitochondrial complex 1. Interestingly, the same variant c.361G>A (p.E121K) in NDUFB11 identified in the present patients was previously reported in a premature male infant carrying hemizygous E121K-NDUFB11 on the X chromosome, who died soon after birth due to mitochondrial complex I deficiency (MCID). Most patients with MCID do not survive beyond early childhood, whereas the present patients carrying the heterozygous E121K-NDUFB11 had no evidence of metabolic disorder.

NRP1 is reported to play roles in angiogenesis, axon guidance, and cell survival, and CTNNA2 may function as a cadherin receptor and cytoskeleton linker in the nervous system. Therefore, we excluded ATP5SL, NDUFB11, CTNNA2, and NRP1 from further studies. Based on the very low MAF in the non-Puerto Rican Latino population and potential deleterious effects on BMP10 function in silico (Supplementary Table 4), we hypothesized that V407I-BMP10 is associated with LVNC, and finally selected this variant for further investigation on protein and cellular levels.

Preserved Dimerization and Polymerization of Mutant V407I-BMP10

The V407I variant (Figure 1C, red arrow) is located at the C-terminus of BMP10, which is responsible for binding to BMPR2. The BMP10-monomer has a structure of “a wrist with 2 fingers”. This means that the N-terminus forms the first finger-shaped strand, the rod-domain forms the α-helix (wrist) and the C-terminus forms the second finger-tip strand. The BMP10 monomers of 12.2 kDa form a dimer, stabilized by a disulphide bond, and only dimerized further genetic studies. This variant has been reported as a rare single-nucleotide polymorphism (SNP) in Latino (MAF=0.0003), Asian (MAF=0.00006) and non-Finnish European ethnicity (MAF=0.00007; https://gnomad.broadinstitute.org/variant/2-69092819-C-T), while no SNP have been reported in other races. Interestingly, high MAF (0.03) was reported in the Puerto Rican population only (http://grch37.ensembl.org), although no reports on LVNC frequency are available in this region. In this study, we found no V407I-BMP10 in the father or in 272 Latino non-Puerto Rican unrelated control individuals (546 alleles). Thus, to discover other potential LVNC-associated variants, we performed WES using the proband’s genomic DNA. A total of 8.6 Gb of raw sequence data were generated, of which 98.64% was mapped onto the human reference genome with a 93X median read depth. Of the coding variants, 11,378 synonymous, 10,468 non-synonymous, 94 stop-gain, and 11 stop-loss variants were identified. After variant prioritization by SIFT, Polyphen2, LRT, MutationTaster, FATHMM, PROVEAN, and MetaSVM, 54 deleterious variants in 49 genes were retained (Supplementary Table 3). Then, based on 2 criteria, that is, whether the gene is expressed in the heart and whether there is functional evidence of cardiovascular disease according to DAVID (https://david.ncifcrf.gov/) and VarElect (https://ve.genecards.org/), we selected 13 variants (Table) for resequencing, and identified 5 deleterious variants in ATP5SL, BMP10, CTNNA, NDUFB11, and NRPL, which were co-segregated in the proband and her mother and negative in her father. Then, we compared tissue expression, biological consequences, structural and functional features of those 5 variants using online sources (NCBI, ExAC, OMIM, GAD, UNIPROT). ATP5SL and NDUFB11 encode the components of mitochondrial complex 1. Interestingly, the same variant c.361G>A (p.E121K) in NDUFB11 identified in the present patients was previously reported in a premature male infant carrying hemizygous E121K-NDUFB11 on the X chromosome, who died soon after birth due to mitochondrial complex I deficiency (MCID). Most patients with MCID do not survive beyond early childhood, whereas the present patients carrying the heterozygous E121K-NDUFB11 had no evidence of metabolic disorder. NRP1 is reported to play roles in angiogenesis, axon guidance, and cell survival, and CTNNA2 may function as a cadherin receptor and cytoskeleton linker in the nervous system. Therefore, we excluded ATP5SL, NDUFB11, CTNNA2, and NRPL from further studies. Based on the very low MAF in the non-Puerto Rican Latino population and potential deleterious effects on BMP10 function in silico (Supplementary Table 4), we hypothesized that V407I-BMP10 is associated with LVNC, and finally selected this variant for further investigation on protein and cellular levels.

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mature BMP10 bind the BMPR. Therefore, we first purified recombinant human WT-BMP10 and V407I-BMP10 proteins, blotted and detected using His-Tag to determine whether V407I alters the dimerization of BMP10. As shown in Figure 2A, His blotting patterns were identical in the WT-BMP10 and V407I-BMP10 lanes, with the lowest molecular weight bands of approximately 18 kDa representing monomers, the higher bands of approximately 37 kDa representing dimers, and the approximately 75 kDa bands representing polymers, suggestive of the ability of V407I-BMP10 to form dimers similar to WT-BMP10.

Pull-Down of BMPR1a and BMPR2 by V407I-BMP10 Is Decreased

Given that dimerization was preserved in V407I-BMP10, binding of mutant BMP10 to BMPRs was expected. We further hypothesized that mutation may have effects on BMP10 binding to BMPR2 specifically. To test if the pulling of receptors by V407I-BMP10 is altered, we tagged WT-BMP10 or V407I-BMP10 with GFP (green fluorescent protein), and BMPR1a or BMPR2 with FLAG epitope. Equal amounts of each construct were transfected into HEK293F cells and IP and Co-IP were performed. As shown in Figure 2B, pulling of BMPR1a-FLAG by FLAG was identical in WT- and V407I-BMP10 co-transfected cells. Bands of WT-BMP10-GFP pulled by FLAG (Figure 2B arrowhead), however, had higher intensity compared with the other 3 lanes, suggesting that WT-BMP10 has stronger binding to BMPR1a compared with V407I-BMP10. Furthermore, the intensity of bands corresponding to the pulled BMPR2-FLAG by FLAG was significantly higher in V407I-BMP10 cells (Figure 2B asterisk) than in WT-BMP10 cells. This suggests the possibility of a higher concentration of ligand-free BMPR2 in mutant cells available for FLAG pulling. Thus, we quantified the ratio of band intensity corresponding to BMP10-GFP to the intensity of BMPR1a or BMPR2 from 3 independent pull-down experiments (Figure 2C), and noted a lower ratio in V407I-BMP10 compared with WT-BMP10. We therefore assumed that V407I-BMP10 had impaired receptor-binding ability, and further investigated the effects using cellular models.

**H9C2 Cells Express BMP10 and BMPRs Similar to Mouse Embryonic Hearts**

BMP10 expression regulates the balanced proliferation and differentiation processes in myoblasts during the particular embryonic period when myocardium develops under mechanical and shear stretch, and BMP10-BMPR interactions specifically reduce proliferation of cardiomyoblasts in an autocrine and paracrine manner. To corroborate, we first determined the **BMP10** and **BMPRs** co-expression pattern in mouse embryonic hearts from E6.5 to E16.5 using qPCR. We found that expression of **BMP10** was signifi-
Myocardial Non-Compaction and BMP10 Mutation

V407I-BMP10 Form Aggregates in H9C2 Cells

To visualize BMP10, BMPR1a and BMPR2 expression, we infected H9C2 cells with adenoviral vectors expressing WT-BMP10 or V407I-BMP10, and then carried out immunohistochemical analysis followed by confocal microscopy. We found that WT-BMP10, V407I-BMP10, BMPR1a and BMPR2 were all detectable after 24h of infection at baseline (data not shown). To determine the long-term effects of V407I-BMP10 on cellular processes, we continued culturing the infected cells for 5 days in 10% FBS media, and evaluated the co-expression of BMP10 and α-actinin2 used to assess the cytoskeletal assembly, as described previously. On day 0, no BMP10 was detected in any cells, while α-actinin2 was seen mainly in the periphery of H9C2 myoblasts in all groups (Figure 4a–c). On day 1, diffuse cytoplasmic expression of BMP10 (green) and α-actinin2 (red) was detectable in WT-BMP10 (Figure 4e) and V407I-BMP10 (Figure 4f) cells. On day 3, control cells (Figure 4g) retained a myoblast-like appearance with no BMP10 expression. The WT-BMP10 cells (Figure 4h) were elongated, and diffuse cytoplasmic and nuclear expression of BMP10 was noted. Expression of α-actinin2 was seen in the cellular periphery and throughout the cytoplasm, with a tendency toward a striation-like arrangement. The V407I-BMP10 cells (Figure 4i) in contrast, retained a round myoblast-like shape with V407I-BMP10 seen as dotted spots, suggesting the possibility of mutant BMP10 aggregates formed in the cytoplasm (Figure 4i arrows, Lower right panel). Scattered α-actinin2 expression was seen in the cytoplasm compared with WT-BMP10 cells. On day 5, control cells (Figure 4j) continued being seen as myoblast-like cells, similar to days 0–3. Diffuse cytoplasmic α-actinin2 overlapped with BMP10 in WT cells (Figure 4k). In contrast, V407I-BMP10 cells contained vast vacuoles in the cytoplasm and nuclei. Mutant BMP10 was mainly localized in nuclear and perinuclear areas, whereas

![Figure 5](http://example.com/image5.png)

**Figure 5.** Rates of proliferation in H9C2 cells, expressing wild-type (WT)- or V407I-bone morphogenetic protein 10 (BMP10). Proliferating condition, 10% fetal bovine serum (FBS) and dotted lines. Differentiating condition, 2% heat inactivated horse serum (HIHS) and solid lines. *P<0.05. The data are standardized according to the control condition on day 1. Cytoskeleton assembly, as described previously. On day 0, no BMP10 was detected in any cells, while α-actinin2 was seen mainly in the periphery of H9C2 myoblasts in all groups (Figure 4a–c). On day 1, diffuse cytoplasmic expression of BMP10 (green) and α-actinin2 (red) was detectable in WT-BMP10 (Figure 4e) and V407I-BMP10 (Figure 4f) cells. On day 3, control cells (Figure 4g) retained a myoblast-like appearance with no BMP10 expression. The WT-BMP10 cells (Figure 4h) were elongated, and diffuse cytoplasmic and nuclear expression of BMP10 was noted. Expression of α-actinin2 was seen in the cellular periphery and throughout the cytoplasm, with a tendency toward a striation-like arrangement. The V407I-BMP10 cells (Figure 4i) in contrast, retained a round myoblast-like shape with V407I-BMP10 seen as dotted spots, suggesting the possibility of mutant BMP10 aggregates formed in the cytoplasm (Figure 4i arrows, Lower right panel). Scattered α-actinin2 expression was seen in the cytoplasm compared with WT-BMP10 cells. On day 5, control cells (Figure 4j) continued being seen as myoblast-like cells, similar to days 0–3. Diffuse cytoplasmic α-actinin2 overlapped with BMP10 in WT cells (Figure 4k). In contrast, V407I-BMP10 cells contained vast vacuoles in the cytoplasm and nuclei. Mutant BMP10 was mainly localized in nuclear and perinuclear areas, whereas
Given that BMP10 induced proliferation in cardiomyoblasts,17 we then compared the rate of proliferation in WT-BMP10 and V407I-BMP10 myoblasts grown in 10% FBS (proliferating condition) and 2% HIHS (differentiating condition). Cells were cultured for 5 days and the rates of MTT absorption measured on days 1, 3 and 5 were equated to that of the control cells on day 1 (Figure 5). On day 1, the rate of MTT absorption was similar in all cell groups. On day 3, the rate of MTT absorption was equally increased in uninfected control, WT or mutant cells in proliferating conditions (Figure 5, dashed lines), indicating an increase in proliferation. In differentiating conditions (Figure 5, solid lines), only WT-BMP10 cells preserved a high proliferation rate similar to that in proliferating conditions, suggesting that WT-BMP10 may transiently promote cellular proliferation during cardiomyoblast differentiation. In contrast, mutant cells had a significantly lower proliferation rate compared with control and WT-BMP10 cells (P<0.05), suggesting that the V407I-BMP10 mutation may inhibit proliferation of differentiating cardiomyoblasts. On day 5, a-actinin2 was seen mainly in the cellular periphery (Figure 4I). Taken together, the experiments demonstrated that V407I causes alterations in BMP10 and a-actinin2 expression and localization.

**Effects of BMP10 in Differentiating H9C2 Cells**

To examine whether V407I affects cellular differentiation and proliferation processes, we carried out differentiation of H9C2 cells with HIHS media for 5 days. On day 1, a-actinin2 expression was seen in the periphery and cytoplasm of WT-BMP10 and V407I-BMP10 cells (Supplementary Figure 3). In WT cells, BMP10 was diffusely expressed (Supplementary Figure 3b), while V407I-BMP10 formed aggregates in mutant cells (Supplementary Figure 3c). On day 3, WT- and V407I-BMP10 cells (Supplementary Figure 3c,f) appeared elongated, diffusely expressing BMP10 and a-actinin2 throughout the cytoplasm, suggesting that they are undergoing successful differentiation. On day 5, WT-BMP10 and V407I-BMP10 cells continued being elongated and expressing diffuse cytoplasmic a-actinin2. Taken together, this indicates that V407I-BMP10 did not affect differentiation of H9C2 cells (days 3–5), despite the V407I-BMP10 aggregates seen on day 1.

**V407I-BMP10 Decreases Proliferation of Differentiating H9C2 Cells**

Given that BMP10 induced proliferation in cardiomyoblasts,17 we then compared the rate of proliferation in WT-BMP10 and V407I-BMP10 myoblasts grown in 10% FBS (proliferating condition) and 2% HIHS (differentiating condition). Cells were cultured for 5 days and the rates of MTT absorption measured on days 1, 3 and 5 were equated to that of the control cells on day 1 (Figure 5). On day 1, the rate of MTT absorption was similar in all cell groups. On day 3, the rate of MTT absorption was equally increased in uninfected control, WT or mutant cells in proliferating conditions (Figure 5, dashed lines), indicating an increase in proliferation. In differentiating conditions (Figure 5, solid lines), only WT-BMP10 cells preserved a high proliferation rate similar to that in proliferating conditions, suggesting that WT-BMP10 may transiently promote cellular proliferation during cardiomyoblast differentiation. In contrast, mutant cells had a significantly lower proliferation rate compared with control and WT-BMP10 cells (P<0.05), suggesting that the V407I-BMP10 mutation may inhibit proliferation of differentiating cardiomyoblasts. On day 5, a-actinin2 was seen mainly in the cellular periphery (Figure 4I). Taken together, the experiments demonstrated that V407I causes alterations in BMP10 and a-actinin2 expression and localization.

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no change in proliferation rates is seen proliferating cells, while proliferation significantly decreased in all cell groups in the differentiating condition, with the lowest seen in mutant cells. Taken together, we demonstrated no effects of WT-BMP10 or V407I-BMP10 on proliferation of H9C2 cells at baseline. In differentiating conditions, WT-BMP10 transiently promoted the proliferation up to day 3, while V407I-BMP10 steadily inhibited the proliferation of differentiating cells.

**V407I-BMP10 Cells Are Intolerant to Cyclic Stretch**

Maturation of the multi-layered compacted myocardium occurs under mechanical and shear stretch created by cyclic contractions and blood flow.18 Thus, we tested whether V407I-BMP10 alters cellular tolerance to mechanical cyclic stretch by applying biaxial 10% cyclic stretch to H9C2 cells, followed by immunohistochemistry using anti-α-actinin2 to observe the cytoskeletal assembly19 and anti-SMAD1/5/8 to assess stretch-associated responses.20 On day 1, uninfected control cells were round and resisted cyclic stretch well, diffusely expressing α-actinin2 (Figure 6a). No SMAD1/5/8 expression was detected in uninfected cells. In contrast, WT-BMP10 and V407I-BMP10 cells (Figure 6b,c) were small, elongated and separated from each other. We assumed that this is due to adenosival infection. Expression of Smad1/5/8 was detected in both WT- and V407I-BMP10 cells. On day 3, control cells continued resisting cyclic stretch and formed cell clusters with enlarged and elongated cells, expressing cytoplasmic α-actinin2 (green, Figure 6d). Smad1/5/8 was expressed as well, suggestive of stretch-associated activation of the BMP10-downstream pathway. WT-BMP10 cells (Figure 6e) were also elongated but separated from each other and expressed α-actinin2, suggesting the cellular ability to resist the mechanical stretch. In contrast, mutant cells (Figure 6f) were distorted, damaged and most of them underwent cell death. To evaluate cell death, we counted the number of DAPI-stained nuclei per field and assessed nuclear morphology (fragmentation and chromatin condensation) as described previously,21 and found a significant reduction in the number of DAPI-stained nuclei and an increase in the percentage of damaged nuclei in mutants from day 0 (9%) to day 1 (52%) and 3 (70%) in response to stretch (Figure 6, Right lower panel) compared with the control cells (4%, 4% to 7%, Right upper panel) or WT cells (6%, 8% to 11%, Right middle panels), respectively. These data suggest the detrimental effects of V407I-BMP10 on cellular tolerance to mechanical stretch.

**Discussion**

The genetic basis of the LVNC phenotype is extremely heterogeneous, and the abnormal process of maturation and compaction in the last stage of embryonic endomyocardial morphogenesis is a widely accepted hypothesis for the development of LVNC.29 BMP10 is one of the critical NOTCH-associated molecules involved in embryonic cardiomyoblast proliferation and differentiation.30 An increase in BMP10 expression enhances cardiomyocyte proliferation in embryonic myocardium, resulting in hypertrabeculation and non-compaction of ventricular walls via direct upregulation of Tbx20 and Hey2 and downregulation of p57kip2.31 In the postnatal myocardium, persistent overexpression of BMP10 has been shown to prevent cardiomyocyte hypertrophic growth.32 In contrast, BMP10 deficiency in embryonic heart results in the development of a hypoplastic ventricular wall and embryonic lethality of Bmp10-KO mice at E10.5.33 A similar phenotype was seen in Bmpr1a-deficient mice, suggesting the importance of BMP10 and BMPR1a in BMP10 signaling.34

The aim of this study was to identify novel NOTCH-associated gene mutations in patients with LVNC and clarify mutation-induced mechanisms(s) using genetic, molecular and in vitro studies. We screened 50 NOTCH-associated genes in 230 patients with LVNC and identified p.W143*-NRGI and p.V407I-BMP10 variants. Based on results of WES of a proband and direct sequencing in family members, we propose that the V407I-BMP10 variant is potentially associated with the LVNC phenotype in this family. Given that only mature BMP10 dimer retains an ability to bind BMPR,35 we tested whether the mutant V407I-BMP10 dimerizes, and, if so, whether it binds to BMPRs. We showed that V407I-BMP10 may form dimers to interact with BMPR, but that the binding abilities of mutant BMP10 to BMPR1a and BMPR2 were decreased compared with that of WT-BMP10. Thus, we further investigated the effects of V407I-BMP10 on proliferation and differentiation in the cellular model.

First, we examined if rat ventricular H9C2 cardiomyoblasts can serve as an appropriate model for BMP10 studies. For this purpose, we performed comparative gene expression analysis of BMP10 and BMPR1a in mouse embryonic heart, adult LV myocardium, H9C2 cells and C2C12 myoblasts followed by siRNA knockdown of BMPR1a in H9C2 and C2C12 cells. We found that BMPR1a and BMPR2 responded selectively to BMP10 in H9C2 cells, but not in C2C12 myoblasts derived from skeletal muscle. Thus, we used H9C2 cells and found several important mutation-induced effects. We showed that V407I-BMP10 is expressed as dotted spots, unlike the diffuse expression of WT-BMP10, suggesting that mutant protein may form aggregates in the cytosol. We also found that mutant cells contained vast cytosolic and nuclei vacuoles, suggesting that V407I may affect endosomal trafficking of BMP10. BMP-induced SMAD phosphorylation in early endosomes and spatial BMP/SMAD segregation due to inhibition of dynamin-dependent endocytosis have been shown to affect differentiation in C2C12 cells.36 This suggests that an advanced investigation of how the V407I-BMP10 affects endocytic pathways may clarify the mechanisms of the vacuole accumulation in the present mutant cells.

Regarding cellular proliferation, we showed that overexpression of WT-BMP10 transiently preserved a high rate of proliferation in differentiating H9C2 cells, consistent with previously observed enhanced cardiomyocyte proliferation in embryonic myocardium in response to increased expression of BMP10.22 In contrast, differentiating mutant cells had a persistently suppressed rate of proliferation, suggestive of inhibitory effects of mutant V407I-BMP10 on the proliferation process. During differentiation, myofilament organization is regulated by α-actinin2, which links the membrane-associated proteins with actin, initiating the assembly of primitive Z-bodies.37 In the present study, immunohistochemistry demonstrated diffuse cytoplasmic overlapping of WT-BMP10 with α-actinin2 in H9C2 myoblasts. Conversely, we observed less overlap between V407I-BMP10 and α-actinin2 due to the fact that α-actinin2 remained at the cellular periphery, suggestive of disturbed cytoskeletal differentiation in mutant cells.

Finally, development of the compacted myocardium occurs in the mid-gestational period of cardiac develop-
ment, under constant mechanical and shear stretch created by cyclic contractions and blood flow. Therefore, we also tested the impact of mechanical cyclic stretch in H9C2 single cell cultures and observed detrimental effects of the mutant V407I-BMP10 on cellular tolerance to cyclic stretch. We have therefore demonstrated that the V407I variant in BMP10 disturbed protein (reduced receptor binding), expression/trafficking (forming dotted spots) and cellular function (vacuoles in the cytoplasm and nuclei, altered proliferation/differentiation and intolerance to mechanical stretch).

Conclusions

The V407I-BMP10 variant is most likely associated with LVNC and may contribute to detrimental alterations in cardiac myocytes, maturing under contractile and shear stretch. These effects are potentially associated with decreased binding of mutant V407I-BMP10 to both BMPR1a and BMPR2 receptors.

Study Limitations

The present genetic studies identified the V407I-BMP10 variant in a Latino family with a history of LVNC. The molecular studies showed promising data on disturbed BMP10 protein functions and associated cellular processes in vitro, but we were unable to fully analyze genotype–phenotype correlations in the extended family, because the maternal relatives were not available for genetic screening. In addition, not all protein interactions and physiological consequences were studied, and definitive pathogenetic mechanisms are still lacking. We emphasize that the creation of a mutant animal model of the V407I-BMP10 variant will greatly contribute to the understanding of the pathogenesis and pathophysiology of LVNC.

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Disclosures

The authors declare no conflicts of interest.

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Supplementary Files