Original Article
Discovery and functional investigation of BMP4 as a new causative gene for human congenital heart disease

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Abstract: Objective: Aggregating evidence highlights the strong genetic basis underpinning congenital heart disease (CHD). Here BMP4 was chosen as a prime candidate gene causative of human CHD predominantly because BMP4 was amply expressed in the embryonic hearts and knockout of Bmp4 in mice led to embryonic demise mainly from multiple cardiovascular developmental malformations. The aim of this retrospective investigation was to discover a novel BMP4 mutation underlying human CHD and explore its functional impact. Methods: A sequencing examination of BMP4 was implemented in 212 index patients suffering from CHD and 236 unrelated non-CHD individuals as well as the family members available from the proband carrying a discovered BMP4 mutation. The impacts of the discovered CHD-causing mutation on the expression of NKX2-5 and TBX20 induced by BMP4 were measured by employing a dual-luciferase analysis system. Results: A new heterozygous BMP4 mutation, NM_001202.6:c.318T>G;p.(Tyr106*), was found in a female proband affected with familial CHD. Genetic research of the mutation carrier’s relatives unveiled that the truncating mutation was in co-segregation with CHD in the pedigree. The nonsense mutation was absent from 236 unrelated non-CHD control persons. Quantitative biologic measurement revealed that Tyr106*-mutant BMP4 failed to induce the expression of NKX2-5 and TBX20, two genes whose expression is lost in CHD. Conclusion: The current findings indicate BMP4 as a new gene predisposing to human CHD, allowing for improved prenatal genetic counseling along with personalized treatment of CHD patients.

Keywords: Congenital heart disease, molecular genetics, signal transduction, BMP4, reporter gene assay

Introduction

Congenital heart disease (CHD) is the most frequent type of human birth malformation globally, occurring in approximately 0.8%-1.2% of all live births and roughly 10% of miscarriages globally, accounting for nearly 33% of all congenital deformations [1-3]. Notably, if minor cardiac developmental aberrations are encompassed, such as patent foramen ovale and aortic bicuspid valve, the prevalence of CHD rises to around 5% of all live births [4-6]. As an array of cardiovascular developmental deformations, CHD is clinically categorized into > 30 distinct isoforms, encompassing double-outlet right ventricle (DORV) and ventricular septal defect (VSD) [2, 7-14]. Though certain mild/minor forms of CHD may resolve spontaneously [2], severe/complex forms of CHD usually lead to worse quality of life [15-17], reduced exercise performance [18-21], neurodevelopmental delay and structural brain anomaly [22-26], ischemic/thromboembolic stroke [27, 28], acute renal injury/chronic kidney disease [29-32], hepatic fibrosis [33, 34], pulmonary dysplasia/pulmonary arterial hypertension [35-37], bacte-
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During vertebrate embryogenesis, cardiac organogenesis undergoes an exceedingly complex biologic process that predominantly relies on precise spatiotemporal interactions between different multipotent heart progenitors [66]. A finely-coordinated sophisticated signaling network, which principally consists of transforming growth factor-β (TGFβ), bone morphogenetic protein (BMP), Wnt family member (WNT), nodal growth differentiation factor (NODAL), and fibroblast growth factor (FGF), induces expression of a key cluster of cardiac transcription factors encompassing NKX2.5, TBX20/5/1, and GATA5/6/4 that work in a mutually-reinforcing cascade to drive cellular lineage restriction and proliferation, differentiation, and migration of different progenitor cell populations to the proper chambers for specific types of cardiac cells [66]. Both environmental pathogenic factors and inheritable defects can disturb the heart-development process, giving rise to a diverse array of CHD [1, 3, 66-71]. It is believed that non-inherited risk factors account for about 30% of CHD, although the molecular mechanisms of CHD caused by detrimental environmental exposure are largely elusive [71]. Well-recognized non-genetic factors that enhance vulnerability to CHD include maternal disease, maternal ingestion of medication, maternal consumption of toxic chemical, and maternal exposure of gaseous pollutants, atmospheric particulate substances, and heavy metals during early pregnancy [71]. However, increasing evidence demonstrates that genetically compromised components are responsible for most CHD [1, 3, 66, 67]. In addition to chromosomal aneuploidies and copy number variations, a growing body of deleterious mutations in > 100 genes, including NKX2.5 and TBX20, have been found to contribute to CHD [1, 3, 66, 67, 72-96]. Nevertheless, known genetic causes of CHD can explain < 40% of CHD patients, and in most (> 60%) cases, the genetic determinants for CHD remain uncertain [66, 96].

Recent investigations have established the essential role of BMP4 as an important member of the TGFβ superfamily of polypeptide signaling molecules in regulating the cardiovascular morphogenetic process, particularly at the early stages of cardiac development [97-101]. In mice, knockout of Bmp4 results in embryonic lethality, mainly because of abnormal cardiac development [98]; while conditional deletion of Bmp4 in cardiomyocytes leads to DORV, VSD, and atioventricular canal defect [99]. Moreover, murine embryos with compound heterozygous deletion of Bmp4 and Bmp2 also manifest VSD [100, 101]. Furthermore, in humans, BMP4 was found to be highly expressed in the heart with a similar expression level in the healthy and CHD-affected hearts (with no BMP4 mutation), and sustained mRNA and protein expressions of BMP4 were observed in CHD patients [97]. These findings highlight the crucial role of BMP4 in normal cardiac organogenesis. This suggests that genetically defective BMP4 predisposes to CHD in humans.

Materials and methods

Recruitment of research participants

The current retrospective case-control investigation was accomplished in line with the Declaration of Helsinki. The ethical review committee of Tongji Hospital in Shanghai approved the protocols involved in human research (with an approved protocol code of LL(H)-09-07 and an ethical approval date of July 27, 2009). After ethical approval, the research subjects or their parents signed an informed consent form, at
the time of enrollment before the commencement of the current study. For the present human research, 212 probands suffering from CHD and 236 unrelated non-CHD volunteers were recruited from the Chinese Han-ethnicity population. The pedigree members available from the CHD-affected probands were also enlisted. Each research subject underwent a comprehensive clinical evaluation at study entry, including a systemic review of personal and familial histories along with medical records, meticulous physical examination, echocardiographic/electrocardiographic images, and routine laboratory tests. For all research subjects, CHD was diagnosed based on the echocardiographic images and/or operative reports. All the patients had an echocardiogram-documented CHD, of whom most had medical records indicating surgical or catheter-based treatment for CHD. The affected individuals’ CHD was categorized based on the international nomenclature [102]. The inclusion and exclusion criteria for the CHD patient group and non-CHD control group are described elsewhere [103]. The patients with syndromic CHD were excluded from the current study just because the genetic causes for most syndromic CHD were known and no evidence indicated the association of a BMP4 defect with syndromic CHD. CHD was diagnosed based on the echocardiographic images and/or operative reports. All the patients had an echocardiogram-documented CHD, of whom most had medical records indicating surgical or catheter-based treatment for CHD. The affected individuals’ CHD was categorized based on the international nomenclature [102]. The inclusion and exclusion criteria for the CHD patient group and non-CHD control group are described elsewhere [103]. The patients with syndromic CHD were excluded from the current study just because the genetic causes for most syndromic CHD were known and no evidence indicated the association of a BMP4 defect with syndromic CHD. Patients with known causes (including chromosomal aneuploidy and a copy number variation) associated with CHD were also excluded. Clinical data and demographic information together with 1-3 mL of venous blood were acquired from every research participant.

Sequencing assay of BMP4

Isolation of genomic DNA from the research participants’ circulating leukocytes was completed by utilizing a genomic DNA purification kit (Thermo Fisher, USA) according to the manual. The oligonucleotide primers applied to the amplification of the coding exons along with the splicing donors/acceptors of human BMP4 (GenBank accession number: NC_000014.9) are given in Table 1. Polymerase chain reaction (PCR)-amplification of BMP4 from a research participant’s genomic DNA was fulfilled on a thermal cycler apparatus (Bio-Rad, USA) with a Taq DNA polymerase kit (New England Biolabs, USA) as well as the BMP4-specific primers mentioned above. The amplicons were fragmented through 1.6% agarose gel electrophoresis and purified utilizing a gel extraction kit (Invitrogen, USA) following the manufacturer’s procedures. Direct PCR-DNA sequencing assay of the purified amplicons was performed as described elsewhere [103-105]. Additionally, for a detected human BMP4 mutation, the gnomAD database along with the SNP database was accessed to confirm its novelty as described previously [103].

Production of gene-expressing constructs

As described previously [106], cDNA was produced by reverse transcription of the mRNA isolated from the excised myocardial issue, which originated from a case experiencing surgical therapy for Fallot’s tetralogy. A 1368-bp fragment including the entire coding region of wild-type human BMP4 (Nucleotide accession number: NM_001202.6) was PCR-amplified from human heart cDNA using the AccuPrime™ Taq DNA Polymerase Kit (Invitrogen, USA) and a human BMP4-specific pair of primers of 5’-GTCGAATTCAACGCACTGCTGCAGCTTC-3’ (forward) and 5’-GTCTCTAGAGTGTATATCTGTC-TATCCTC-3’ (reverse). The amplified fragment containing whole human BMP4 cDNA and the pcDNA™3.1(+) vector (Invitrogen, USA) were doubly cut by EcoRI and XbaI, gel-purified, and recombined by T4 DNA ligase to construct the wild-type human BMP4-pcDNA™3.1(+) vector. Using the wild-type human BMP4-pcDNA™3.1(+) vector as a template, the

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**Table 1. Oligonucleotide primers for amplifying the coding sequences along with flanking introns of the human BMP4 gene**

<table>
<thead>
<tr>
<th>Coding exon</th>
<th>Forward (from 5’ to 3’)</th>
<th>Reverse (from 5’ to 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAGTTTGGGACAGGATCAC</td>
<td>GGCTCGAGATAGCTGACG</td>
<td>546</td>
</tr>
<tr>
<td>2</td>
<td>GGTTGAGACTTTCCACGACT</td>
<td>TAAAGGAGGTCCGACGGAAG</td>
<td>670</td>
</tr>
<tr>
<td>3</td>
<td>TGCTTTTCACTGGGCTCCT</td>
<td>CTGGACACTGGGGCTTGTG</td>
<td>606</td>
</tr>
<tr>
<td>4-a</td>
<td>GCTTTTGGTAGCATGCCACT</td>
<td>CCCCCTGAGTGACGAGC</td>
<td>602</td>
</tr>
<tr>
<td>4-b</td>
<td>GGCTAGCCATTGAGGTGACT</td>
<td>ATAAAGGTGCTAAGGAAGC</td>
<td>658</td>
</tr>
</tbody>
</table>
BMP4 mutation in congenital heart disease

Tyr106*-mutant human BMP4-pcDNA™3.1(+) vector was yielded employing a site-targeted mutagenesis kit (Thermo Scientific, USA) along with a complimentary pair of oligonucleotide primers (forward: 5’-CACTGGTCTTGAGTAGCCT-GAGGCGCCGCCC-3’; backward: 5’-GGCCGGGCGC-GCTAGGTCTTGACAGCGGT-3’). The NKX2-5-luc and TBX20-luc constructs were generated as previously described [106]. All constructed vectors were verified by DNA sequencing analysis.

Cellular transfection with gene-expressing vectors and dual-luciferase analysis

HeLa cells (a cervical cancer cell line derived from Henrietta Lacks) were routinely maintained as described elsewhere [106]. Cells were counted using a hemocytometer (Invitrogen, USA) and seeded in a 24-well plate (Greiner Bio-One, USA), cultivated for 36 h, then transient cellular transfection with gene-expressing vectors was conducted using the Lipofectamine® LTX & PLUS™ Reagent (Invitrogen, USA). Specifically, cells were transfected with 15 ng of pGL4.75, 1.5 μg of NKX2-5-luc or TBX20-luc, and 0.6 μg of each gene-expressing vector (empty pcDNA™3.1(+), wild-type human BMP4-pcDNA™3.1(+), or Tyr106*-mutant human BMP10-pcDNA™3.1(+), alone or together). Here, the renilla luciferase-expressing plasmid of pGL4.75 (Promega, USA) was employed as an internal control to balance transfection efficiency. The empty pcDNA™3.1(+) plasmid was utilized as an external negative control. HeLa cells transfected with gene-expressing plasmids were harvested 36 h after cellular transfection and lysed in a lysis buffer (Promega, USA). Cell lysates were applied to the measurement of firefly/renilla luciferase activities, respectively, as described in detail elsewhere [106]. For each gene-expressing vector, a cellular transfection experiment was accomplished in three independent replicates.

Statistics

Quantitative variables (age and promoter activity) are given as means ± standard deviations (X ± SD) throughout. Qualitative values (sex, race and positive family history of CHD) are expressed as frequency numbers (n) and percentages (%). An independently measured Student’s t-test was applied to compare continuous variables between two groups. To compare continuous variables among over three groups, a one-way analysis of variance followed by the Tukey-Kramer post-hoc test was used. Categorical variables were compared between two groups using Fisher’s exact test or Pearson’s chi-square (χ²) test when indicated. Statistical assay was done with SPSS v17 (SPSS, USA). In all cases, a 2-sided P < 0.05 denoted a statistical difference.

Results

Basic features of the CHD-affected proband cohort

In the current human investigation, 212 index patients with miscellaneous forms of CHD (including 95 female index patients and 117 male index patients, with an average age of 5.8 years) was evaluated clinically in comparison with 236 unrelated non-CHD volunteers without family history of CHD (including 106 female volunteers and 130 male volunteers, with an average age of 5.7 years). All the study individuals were enlisted from the Chinese Han race population. The included index patients had echocardiographic/surgical documentation suggesting the presence of CHD, while the included volunteers showed normal echocardiograms, with no proof suggesting cardiovascular structural deformities. Of the 212 CHD-affected probands, 61 probands reported a positive familial history of CHD, whereas none of the 236 volunteers employed as control subjects had it. No research subjects had known non-heritable factors susceptible to CHD, including maternal obesity, diabetes mellitus, hypothyroidism, phenylketonuria, pre-eclampsia, primary hypertension, nutritional deficiency, epilepsy, connective tissue disease, acute febrile illness, along with exposure to therapeutic medications, toxicants, and ionizing radiation during gestation, and the vast majority of CHD-affected probands experienced catheter-based interventional/surgical treatment for CHD. The basic demographic and phenotypic features of the 212 probands with a wide spectrum of CHD are summarized in Table 2.

Identification of a CHD-causative mutation in BMP4

By DNA sequencing examination of human BMP4 in 212 probands with a diverse array
Table 2. Basic characteristics of the research cohort comprising 212 index patients affected with a wide spectrum of congenital heart disease

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number or mean ± SD</th>
<th>Percentage or range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female index patients</td>
<td>95</td>
<td>44.8</td>
</tr>
<tr>
<td>Male index patients</td>
<td>117</td>
<td>55.2</td>
</tr>
<tr>
<td>Age at initial recruitment (years)</td>
<td>5.8 ± 3.27</td>
<td>0.6-11.3</td>
</tr>
<tr>
<td>Having a family history of CHD</td>
<td>61</td>
<td>28.8</td>
</tr>
<tr>
<td>Distribution of distinct forms of CHD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VSD</td>
<td>53</td>
<td>25.0</td>
</tr>
<tr>
<td>ASD</td>
<td>48</td>
<td>22.6</td>
</tr>
<tr>
<td>PDA</td>
<td>16</td>
<td>7.5</td>
</tr>
<tr>
<td>TOF</td>
<td>12</td>
<td>5.7</td>
</tr>
<tr>
<td>DORV</td>
<td>10</td>
<td>4.7</td>
</tr>
<tr>
<td>TGA</td>
<td>4</td>
<td>1.9</td>
</tr>
<tr>
<td>HLV</td>
<td>4</td>
<td>1.9</td>
</tr>
<tr>
<td>TAPVC</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>PS</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>CoA</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>PTA</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>AS</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>VSD + PDA</td>
<td>18</td>
<td>8.5</td>
</tr>
<tr>
<td>DORV + VSD</td>
<td>16</td>
<td>7.5</td>
</tr>
<tr>
<td>VSD + ASD</td>
<td>9</td>
<td>4.2</td>
</tr>
<tr>
<td>ASD + PDA</td>
<td>5</td>
<td>2.4</td>
</tr>
<tr>
<td>TOF + ASD</td>
<td>5</td>
<td>2.4</td>
</tr>
<tr>
<td>TGA + VSD</td>
<td>4</td>
<td>1.9</td>
</tr>
<tr>
<td>PTA + VSD</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Dysrhythmias</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVB</td>
<td>15</td>
<td>7.1</td>
</tr>
<tr>
<td>AF</td>
<td>11</td>
<td>5.2</td>
</tr>
<tr>
<td>Medical management</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catheter-based treatment for CHD</td>
<td>117</td>
<td>55.2</td>
</tr>
<tr>
<td>Surgical procedures for CHD</td>
<td>72</td>
<td>34.0</td>
</tr>
<tr>
<td>Follow-up observation</td>
<td>23</td>
<td>10.8</td>
</tr>
</tbody>
</table>

AF: atrial fibrillation; AS: aortic stenosis; ASD: atrial septal defect; AVB: atrioventricular block; CHD: congenital heart disease; CoA: coarctation of the aorta; DORV: double-outlet right ventricle; HLV: hypoplastic left ventricle; PDA: patent ductus arteriosus; PS: pulmonary stenosis; PTA: persistent truncus arteriosus; TAPVC: total anomalous pulmonary venous connection; TGA: transposition of the great arteries; TOF: tetralogy of Fallot; VSD: ventricular septal defect.

The DNA sequencing chromatograms displaying the heterozygous c.318T>G mutation in BMP4 in contrast to its wild type are illustrated in Figure 1A. The structural motifs of the wild-type and Tyr106*-mutant human BMP4 proteins are shown in Figure 1B. The pedigree of the proband carrying the discovered human BMP4 mutation is shown in Figure 1C. In Family C002, there were 21 family members available, including 10 male and 11 female members, with ages varying from 1 to 78 years. All the six affected members suffered DORV and VSD and underwent surgery for CHD. No recognized environmental factors vulnerable to CHD were unmasked in each pedigree member. The clinical characteristic profile as well as BMP4 mutation status of the living relatives with CHD from Family C002 are summed in Table 3.

As presented in Figure 2, in grown HeLa cells expressing multiple constructs, encompassing empty pcDNA™3.1(+) as an external negative control (+), wild-type human BMP4-
Figure 1. A new BMP4 mutation causing familial congenital cardiovascular malformations. A. Sequence chromatogram traces revealing the heterozygous BMP4 mutation in the CHD-affected proband (Mutant) along with corresponding homozygous control in an unaffected family member of the proband (Wild type). A vertical arrow points to the nucleotide position where the heterozygous BMP4 mutation (c.318T>G) occurs. B. Schematic diagrams delineating the structural domains of human BMP4 proteins. The Tyr106*-mutant BMP4 protein (Mutant) was predicted to lose 303 amino acids at the carboxyl terminus (COOH). TGFβ: transforming growth factor-beta; NH2: amino
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terminus. C. Pedigree manifesting autosomal-dominant inheritance of ventricular septal defect and double-outlet right ventricle. An oblique arrow points to the index patient. A family member’s genotype is marked with “+” or “-,” of which “+” signifies a member harboring the heterozygous BMP4 mutation, while “-” signifies a member with no BMP4 mutation.

| Table 3. Clinical characteristic profile and BMP4 mutation status of the living relatives from Family C002 suffering congenital heart defects |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Individual (Family C002)       | Sex             | Age (years)     | Cardiovascular structural deformities | BMP4 mutation (Tyr106*) |
| II-3                           | Male            | 54              | DORV, VSD                   | +/-              |
| II-8                           | Female          | 50              | DORV, VSD                   | +/-              |
| III-3                          | Male            | 27              | DORV, VSD                   | +/-              |
| III-7                          | Male            | 26              | DORV, VSD                   | +/-              |
| IV2                            | Female          | 2               | DORV, VSD                   | +/-              |

VSD: ventricular septal defect; DORV: double-outlet right ventricle; +/-: heterozygote for the human BMP4 mutation.

pcDNA™3.1(+) (BMP4), and Tyr106*-mutant human BMP4-pcDNA™3.1(+) (Tyr106*), alone or together, BMP4 and Tyr106* induced the transcriptional activity of the NNX2-5 promoter by ~19-fold and ~2-fold, respectively (BMP4 versus Tyr106*: t = 12.7416; P = 0.0002). When BMP4 and Tyr106* were co-expressed, the induced transactivation on the NNX2-5 promoter was ~10-fold (BMP4 versus Tyr106* + BMP4: t = 5.9808; P = 0.0039). Equivalent statistical results were obtained when multiple comparisons were carried out (F = 94.983, P = 6.514 × 10^-8). Specifically, for (-) versus BMP4, t = 17.1833; P < 0.0001; for (-) versus Tyr106*, t = 0.3167; P = 0.9978; for (-) versus BMP4 + (-), t = 8.9933; P < 0.0001; for (-) versus Tyr106* + BMP4, t = 8.6367; P < 0.0001; for BMP4 versus Tyr106*, t = 16.8667; P < 0.0001; for BMP4 versus Tyr106* + BMP4, t = 8.5467; P < 0.0001; for Tyr106* versus BMP4 + (.), t = 8.1900; P = 0.0001; for BMP4 versus Tyr106* + BMP4, t = 5.1867; P = 0.0002; for BMP4 versus Tyr106* + BMP4, t = 4.7833; P = 0.0011; for BMP4 versus Tyr106*, t = 12.0867; P < 0.0001; for BMP4 versus BMP4 + (-), t = 6.3100; P = 0.0001; for BMP4 versus Tyr106* + BMP4, t = 7.4809; P = 0.0017). Parallel statistical results were achieved when multiple comparisons were made (F = 76.560, P = 1.847 × 10^-7). Specifically, for (-) versus BMP4, t = 12.1267; P < 0.0001; for (-) versus Tyr106*, t = 0.0400; P = 1.0000; for (-) versus BMP4 + (-), t = 5.8167; P = 0.0002; for (-) versus Tyr106* + BMP4, t = 4.7833; P = 0.0011; for BMP4 versus Tyr106*, t = 12.0867; P < 0.0001; for BMP4 versus BMP4 + (-), t = 6.3100; P = 0.0001; for BMP4 versus Tyr106* + BMP4, t = 7.3433; P < 0.0001; for Tyr106* versus BMP4, t = 5.7767; P = 0.0002; for Tyr106* versus Tyr106* + BMP4, t = 4.7433; P = 0.0012; for BMP4 + (-) versus Tyr106* + BMP4, t = 1.0333; P = 0.7116.

Discussion

In humans, the BMP4 gene is mapped at chromosome 14q22.2, which codes for a growth factor comprising 408 amino acids, a secreted ligand with multiple functions belonging to the TGFβ superfamily. It consists of over 30 ligands recognized so far [97]. The TGFβ superfamily is classified into several subcategories, including BMPs, TGFβs, growth and differentiation factors (GDFs), as well as activins/inhibins. TGFβ superfamily signaling occurs through a heterodimeric complex comprising two types of receptors (types I and II) [97]. BMPs were initially demonstrated to account for the development of bone as well as cartilage. Further research has substantiated their pivotal roles
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in embryonic development with a diverse array of effects, encompassing growth and differentiation along with apoptosis of multiple cell types, such as chondroblasts, osteoblasts, epithelial cells, and neuronal cells [97]. In the heart, BMP signaling first activates the endocardium by the establishment of a proper environment, followed by the promotion of epithelial-mesenchymal transition as well as invasion of the mesenchymal cells into the endocardial cushion with the aid of TGFβ as well as Notch signaling [97]. The BMP ligands bind receptors (type II), resulting in activation of receptors (type I), which phosphorylates the SMAD signal transducers, playing a crucial role in inducing transcription of target genes [97]. Additionally, non-SMAD signaling pathways are also involved in governing BMP signaling [97, 107]. Although all BMP proteins possess a similar protein structure, each BMP protein has a unique tissue expression spectrum along with a distinct physiologic function [108, 109]. To date, six BMP members have been validated to be amply expressed in the embryonic heart, encompassing BMP4, BMP6, BMP2, BMP5, BMP7, and BMP10, of which BMP4 was shown to be highly expressed in the mesoderm as well as the outflow tract myocardium [110]. It exerts multiple functions during cardiac organogenesis, particularly during the early period of cardiac organogenesis [97, 103, 111]. Specifically, BMP4 functions to induce the formation of endocardial cushion and outflow tract cushion mainly by increasing epithelial-mesenchymal transition of endocardial cells at the outflow tract and inducing the invasion of car-

Figure 2. No induction of NKX2.5 expression by Tyr106*-mutant BMP4. In maintained HeLa cells, dual-luciferase measurement of the expression of the NKX2.5 promoter-driven firefly luciferase (NKX2.5-luc) in the presence of wild-type human BMP4-pcDNA3.1(+)-vector (BMP4) or Tyr106*-mutant human BMP10-pcDNA3.1(+)-vector (Tyr106*), separately or in combination, showed that Tyr106* failed to induce the expression of NKX2.5. For every expression vector, functional experiments were performed three times in triplicate. Here, “a” means P < 0.001, and “b” means P < 0.005, in comparison to BMP4 (600 ng).

Figure 3. Inability of Tyr106*-mutant BMP4 to induce TBX20 expression. Dual-reporter analysis of the expression of the TBX20 promoter-driven firefly luciferase (TBX20-luc) in grown HeLa cells in the presence of wild-type human BMP4-pcDNA3.1(+)-vector (BMP4) or Tyr106*-mutant human BMP10-pcDNA3.1(+)-vector (Tyr106*), singly or in both, revealed that Tyr106* failed to induce the expression of TBX20. For each expression vector, three independent biological measurements were conducted in triplicate. Here, “c” signifies P < 0.001, and “d” indicates P < 0.005, when compared with BMP4 (600 ng).
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diac neural crest into the outflow tract and aortopulmonary septum cushions during embryogenesis [97, 99]. BMP4 was shown to play an essential role in regulating cardiovascular morphogenesis by interaction with transcription factors [97]. Furthermore, recent investigations have shown that BMP4 can induce the expression of multiple important downstream genes, encompassing NKX2-5 and TBX20 [97, 112-114], which encodes transcription factors crucial for normal cardiovascular development, and disease-causing mutations in both NKX2-5 and TBX20 have been involved in the occurrence of CHD [85, 115-117]. In the current study, the discovered Tyr106* mutation was anticipated to yield a truncated BMP4 protein lacking pivotal structural motifs. Results from functional experiments showed that Tyr106*-mutant BMP4 failed to induce the expression of NKX2-5 as well as TBX20. Collectively, these findings suggest that BMP4 haploinsufficiency is a molecular determinant of CHD in humans.

In experimental mice, functionally defective BMP4 results in CHD. Deletion of Bmp4 caused embryonic lethality, mainly due to cardiovascular developmental abnormalities [98]; while conditional ablation of Bmp4 in cardiomyocytes caused early embryonic death, along with DORV, VSD, and atrioventricular canal defect [99]. Moreover, loss of Bmp4 from the anterior heart field in mice led to a remarkably reduced number of cells in the developing endocardial cushions within the outflow tract, abnormal cushion remodeling, persistent truncus arteriosus, VSD, and semilunar valve deformity [118]. Additionally, murine embryos with the compound heterozygous knockout of both Bmp4 and Bmp2 also manifested VSD [100, 101]. Conditional deletion of both Bmp4 and Bmp7 in the murine second heart field led to defective epithelial to mesenchymal transition, decreased cardiac neural crest ingress, and persistent truncus arteriosus [119]. Conditional inactivation of Bmp4 from TBX1-expressing cells in mouse embryos resulted in a spectrum of malformations resembling the cardiovascular anomalies of patients with DiGeorge syndrome, mainly affecting the remodeling of outflow tract and pharyngeal arch arteries [120]. Taken together, these experimental animal studies underscore the essential role of BMP4 in cardiovascular development, so that BMP4 insufficiency may be a molecular basis of CHD.

In humans, BMP4 was validated to be amply expressed in the heart, with a similar expression level in normal and CHD-affected hearts (with no BMP4 mutation). Sustained mRNA and protein expressions of BMP4 were observed in CHD patients [97]. Furthermore, a common BMP4 intronic SNP (rs762642) was reported to confer an enhanced vulnerability to sporadic CHD in a Chinese population [119], though the functional effect of the rs762642 polymorphism was not explored [121]. These results support that genetically compromised BMP4 contributes to CHD.

Limitations of the current study are as follows. First, we could not rule out that other genetic defects might also contribute to CHD in the patients harboring a BMP4 mutation. Whole-genome or whole-exome sequencing analysis would be needed to assess this. Second, more cellular functional experiments should be performed, especially for the further cell experiments performed in cardiomyocytes. Third, establishment of a mouse model with the BMP4 mutation knocked in would help to validate the causative effects of the BMP4 mutation. Finally, BMP4 should be screened in populations of different ethnicities to evaluate the mutational spectrum and prevalence of BMP4.

Conclusion

This research indicates BMP4 as a causative gene responsible for human CHD and unveils a new molecular mechanism underpinning human CHD. This has hypothetical clinical implications for prenatal diagnosis and personalized prophylaxis of CHD in a subgroup of patients.

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Disclosure of conflict of interest

None.
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References


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[57] Moisa SM, Burlacu A, Brinza C, Țărcă E, Butnariu LI and Trandafir LM. An up-to-date narrative review on congenital heart disease percutaneous treatment in children using con-
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BMP4 mutation in congenital heart disease

tar: findings from the sidra cardiac registry. Genes (Basel) 2022; 13: 1369.
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