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Mutation screening of *BMP4*, *BMP7*, *HOXA4* and *HOXB6* genes in Chinese patients with hypospadias

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Hypospadias, one of the most common congenital abnormalities of the male external genitalia with elusive etiology, are caused by a defect in the normal development of the urethra, foreskin and ventral aspect of the penis. Evidences indicate that *BMP4* and *BMP7*, two of those major factors in a signaling cascade involved in controlling the embryonic urethral development, play central roles in the normal development of the urethra, and that *HOXA4* and *HOXB6* play important roles in the development of skin in various tissues at the time course of the urethral development. We directly sequenced all these exons and exon–intron boundaries of the four genes in 90 unrelated Chinese patients with hypospadias. Thirteen different heterozygous nucleotide variations were identified for the first time in the four genes in 14 of 90 cases. Of the 13 variations, eight are missense: c.619C > G (p.H207D), c.668G > A (p.R223H), c.751C > T (p.H251Y) in *BMP4*; c.907C > T (p.R303C) in *BMP7*; c.385G > T (p.G129C), c.869C > G (p.S290C) in *HOXA4*; c.124C > A (p.P42T), c.367T > C (p.C123R) in *HOXB6*. None of these variations were found in 380 control chromosomes. Amino-acid sequence alignments showed most of these changed amino acids are conserved across various vertebrate species. In a word, these findings, together with the indicated roles of the four genes, imply that it should not be random events for so many nucleotide variations found in the present study. Further functional studies are required to make the associations clear between these variants and hypospadias.

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Introduction

Hypospadias (MIM# 146450) is one of the most common congenital anomalies occurring in 1 out of 300 to 1 out of 125 infant males in different ethnic populations.^{1,2} It can be defined as a defect in the normal development of the

urethra, foreskin and ventral aspect of the penis, and may be classified as simple (glandular or penile) and severe (scrotal or perineal) on the basis of the anatomical location of the urethral meatus. The cause of hypospadias is complex, and still elusive to date, especially in mild cases. Hypospadias is probably a complex disorder caused by both genetic and environmental influences, either alone or in a combination.^{2,3}

Because the formation of the male urethra is fully androgen-dependent, it seems that a more reasonable theory to explain hypospadias is an abnormality in the pathway of the androgen metabolism. Indeed, those defects in some molecules involved in the pathway, like

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SRY, SOX9, AR, SRD5A2 and WT1, might contribute to the abnormal male development including a subset of hypospadias.² The modes of inheritance of hypospadias might vary. For example, the inheritance of AR defects, is X-linked recessive, and SRD5A2 defects autosomal recessive.^{4,5} However, all these defects only account for no more than 30% of cases.⁶ Thus, the fact that the causes have not yet been identified for a large number of cases highlights the importance of seeking other possible genetic explanations for the congenital defect.

Knowledge about molecular mechanisms of the normal formation of the male urethra, including the glandular urethra might help to elucidate the etiology of hypospadias. To a great extent, the development of the male urethra is similar to those of palate and limbs. During the formation of these organs, tissue phenotypic transformations always occur. The classic mechanism of the phenotypic transformation is an epithelial-to-mesenchymal cell change or a mesenchymal-to-epithelial transformation. The transformation involves a signaling cascade composed of Hedgehog, TGF β signaling pathway as well as HOX molecules and so on. These molecules always act as the mediators of morphogenetic cell–cell interactions.^{3,7,8} This signaling cascade is conserved throughout organisms from *Drosophila* to humans and in many tissues, including skeleton, limb, lung, gut and genitalia. Among these signaling molecules of the cascade, SHH, HOXA13, BMP4, BMP7, FGF8 FGF10 and so on are major ones.^{7,9} Studies in mice have already shown that defects or loss of functions of these molecules could result in an altered genital phenotype including hypospadias, either alone or in a combination.^{7–11}

As mentioned above, the development of the penile shaft skin and the preputial skin is also fundamental stages in the formation of the male urethra. Any disturbance in these stages may also cause hypospadias.^{1,12} Although no direct evidence in the urogenital system, experimental evidences have suggested that HOXB6 (former Hox2.2) and HOXA4 play important roles in the development of skin of various tissues at week 10–17 of gestation, and that the time course of the development of the penile shaft skin and the preputial skin is just at week 10–17 of gestation.^{13,14} In addition, mice and chicken models also show that HOXB6 gene products are involved in controlling pattern formation in developing limbs.^{15,16}

Based on the above knowledge, we hypothesized that mutations in SHH, HOXA13, BMP4, BMP7, FGF8 and FGF10 genes might result in an altered genital phenotype including hypospadias. However, there have already been evidences excluding HOXA13 and some of the FGFs as candidate genes for isolated hypospadias.^{6,8} In the present study, we attempt to investigate whether there are some nucleotide alternations in BMP4 and BMP7, two downstream molecules of the above signaling cascade, and in HOXA4, HOXB6 in hypospadias.

Materials and methods

Patients and controls

Ninety sporadic Chinese patients enrolled in our experiments were identified in the Hypospadias Treating Center of the Plastic Surgery Hospital, PUMC. All patients included were 46,XY. Clinical phenotypes, family histories and complications of the 90 patients were shown in previous report. Some known candidate genes for hypospadias such as SRD5A2, WT1 and AR have already been studied in the 90 patients and some of these patients parents.⁴ A total of 190 Chinese normal males with no history of any abnormalities in urogenital system, limbs and palate, were recruited as a control group. The mean age of the control group was 27 years.⁴ Informed consent was obtained from all patients or their parents. Ethical approval for the study was obtained from the Ethical Committee of the National Center of Human Genome Research, Beijing.

Mutation detections and analysis

Polymerase chain reaction (PCR) amplification. Genomic DNAs were extracted from the peripheral leukocytes of the cases and controls using standard procedures. The sequences of *Bmp4*, *Bmp7*, *HoxA4* and *HoxB6* genes were obtained from <http://www.ncbi.nlm.nih.gov> or <http://www.genome.ucsc.edu>. PCR was employed to amplify all these exons and exon–intron boundaries of the four genes with primers designed using Premier 5.0 software. To facilitate their subsequent sequencing, some exons were divided to make PCR products less than 500–600 bp in length generally. PCR was performed in a 50 μ l volume and thermal cycling was performed in a GeneAmp PCR System 2700 (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) (detailed PCR conditions and sequences of primer sets are available upon request).

Sequencing and sequence analysis. PCR products were purified using a MultiScreen-PCR plate (Millipore Corporation, Bedford, USA). Sequencing was completed bidirectionally on an ABI 3730XL DNA analyzer (Applied Biosystems) using the amplification primers described above. When finding some nucleotide variations in some fragments of the cases, we also analyzed the corresponding fragments in controls using the same methods used in the cases. Sequence data were analyzed against respective reference sequence from NCBI or UCSC database (*BMP4*: NM_130851; *BMP7*: NM_001719; *HOXA4*: NM_002141; *HOXB6*: NM_156037) and compared to the normal controls using the Phred/Phrap/Consed software (University of Washington, Seattle, USA). All nucleotide changes were confirmed by repeating the PCR and sequencing reactions.

Multiple amino-acid sequence alignments of the four human genes with respective vertebrate orthologs were done with online ClustalW program (<http://www.ebi.ac.uk/clustalw/>).

Table 1 Genotype and phenotype of patients carrying variations^a

Gene	Patient no.	Nt. change ^b	Amino-acid change	Exon or intron	Phenotype			Others
					Hypospadias type	Micro-penis ^c	Bifid scrotal	
BMP4	30	c.619C>G	p.H207D	5	Penoscrotal	Yes	No	Not found
	51	c.668G>A	p.R223H	5	Penoscrotal	No	No	Not found
	26	c.751C>T	p.H251Y	5	Penile	Yes	No	Not found
BMP7	42	c.597G>A	p.Q199Q	2	Penile	No	No	Not found
	65 ^d	c.907C>T	p.R303C	4	Glandular	No	No	Not found
	95	c.1465T>A	3'UTR	7	Penoscrotal	Yes	No	Not found
	49	c.1567A>G	3'UTR	7	Penile	No	No	Not found
	40	c.612-33C>T		intron2	Penoscrotal	Yes	No	Not found
	67	c.1146+39C>T		intron6	Penoscrotal	No	No	Hernia
HOXA4	55	c.385G>T	p.G129C	1	Penoscrotal	Yes	No	Not found
	78	c.869C>G	p.S290C	2	Penile	No	Yes	Cryptorchidism
	73 ^e	c.869C>G	p.S290C	2	Penile	Yes	No	Not found
HOXB6	16 ^f	c.124C>A	p.P42T	1	Scrotal	Yes	Yes	Cryptorchidism
	61	c.367T>C	p.C123R	1	Penile	No	No	Not found

^aFor all the four genes in the table, numbering of the nucleotide position starts from A of the initiation codon (ATG).

^bAll alleles shown are heterozygous in the table.

^cMicropenis: length of penis is more than-2.5 SD from the average penile length in males of matched age.

^dThe patient also carries synonymous S159S mutation in *WT1* gene.⁴

^eThe patient also carries homozygous R227Q mutation in *SRD5A2* gene.⁴

^fThe patient also carries hemizygous A620V variation in *MID1* gene (unpublished data), heterozygous Q6X and G203S mutation in *SRD5A2* gene.⁴

Results

In the study, we found 13 different heterozygous nucleotide sequence variations, including three in BMP4, six in BMP7, two in HOXA4 and two in HOXB6, in all these exons and exon–intron boundaries of the four genes in 14 of the 90 patients. Of these variations, eight (c.619C>G, c.668G>A, c.751C>T; c.907C>T; c.385G>T, c.869C>G; c.124C>A, c.367T>C) are missense variations. The other five variations, c.597G>A, c.1465T>A (3'UTR), c.1567A>G (3'UTR), c.612-33C>T, c.1146+39C>T are silent because c.597G>A does not cause amino-acid change, whereas, c.1465T>A, c.1567A>G are located in the 3'UTR, and c.612-33C>T, c.1146+39C>T are in introns (Table 1, Figure 1).

None of these variations found in the study were found in 380 control chromosomes. Amino-acid sequence alignments of the four human genes with respective vertebrate orthologs available from NCBI show that p.H207, p.R223 and p.H251 in BMP4; p.Q199 and p.R303 in BMP7 are highly conserved across all their respective vertebrate orthologs available from NCBI database. p.G129 and p.S290 in HOXA4, p.P42 and p.C123 in HOXB6 are partly conserved across all their respective vertebrate orthologs available from NCBI database (Figure 2).

Discussion

Insights into possible important roles of some molecules of HOX and BMP families in urogenital and limb development provoked our investigation involvement of BMP4, BMP7, HOXA4 and HOXB6 genes in patients with hypospadias. To our knowledge, the genetic alterations of the four genes have not been previously described in humans.

In this study, we found a comparatively large amount of nucleotide variations (16%) in the four genes in our 90 patients. None of these variations were found in 380 control chromosomes. Eight missense variations appeared highly or partly conserved across all their respective vertebrate orthologs available from NCBI database. In addition, some animal models have already indicated that the four molecules are involved in the normal male urethral formation, or showed that the loss or defects of functions of these molecules could cause an altered genital phenotype including hypospadias elsewhere.^{1,7,11–16} Summing up these aspects, we believe it is possible that these variants, especially these eight missense ones, are associated with their phenotypes. However, those five silent variations are unlikely to have a major impact on their phenotypes. Of course, further functional studies are required to identify these possible associations in the future.

In addition, among the 14 patients, three also carry some variations located outside the *BMP4*, *BMP7*, *HOXA4* and *HOXB6* (Table 1 and 2). Patient 16 carries the hemizygous p.A620V in *MID1* (unpublished data), the heterozygous Q6X and G203S mutations in *SRD5A2*.⁴ Family studies found that, in the patient, the hemizygous p.A620V in *MID1* is *de novo*. In addition, family studies also found that his mother, the carrier of the heterozygous p.P42T in *HOXB6* and the heterozygous G203S in *SRD5A2*, and his father, the carrier of the heterozygous Q6X in *SRD5A2*, were all healthy.⁴ It is known that the G203S is pathogenic in patient 16.⁴ Because the defects in *MID1* is X-linked inheritance, we speculate p.A620V in *MID1* is also possibly pathogenic. If so, both G203S in *SRD5A2* and p.A620V in

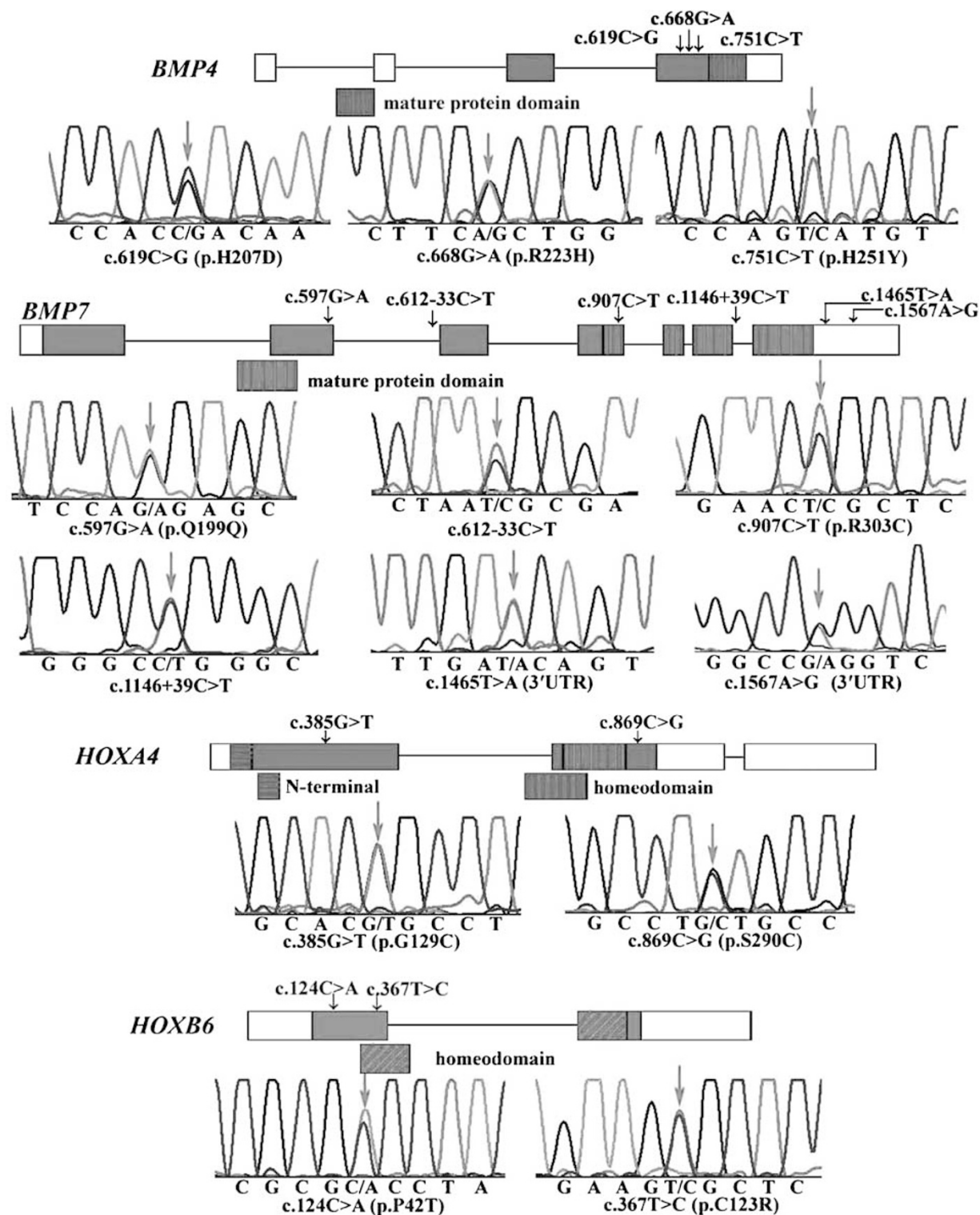


Figure 1 Linear diagrams showing exons (all boxes) and introns of *BMP4*, *BMP7*, *HOXA4* and *HOXB6* genes (accession number NM_130851, NM_001719, NM_002141, NM_156037, respectively), and the variation chromatograms of the four genes. All the coding regions are highlighted light green. Some important domains are shown by different boxes, which are explained below each of the linear diagrams. All the variations in the study are indicated by a black arrow above the linear diagrams and by a red arrow above the sequence chromatograms, respectively. Figures are drawn roughly to scale.

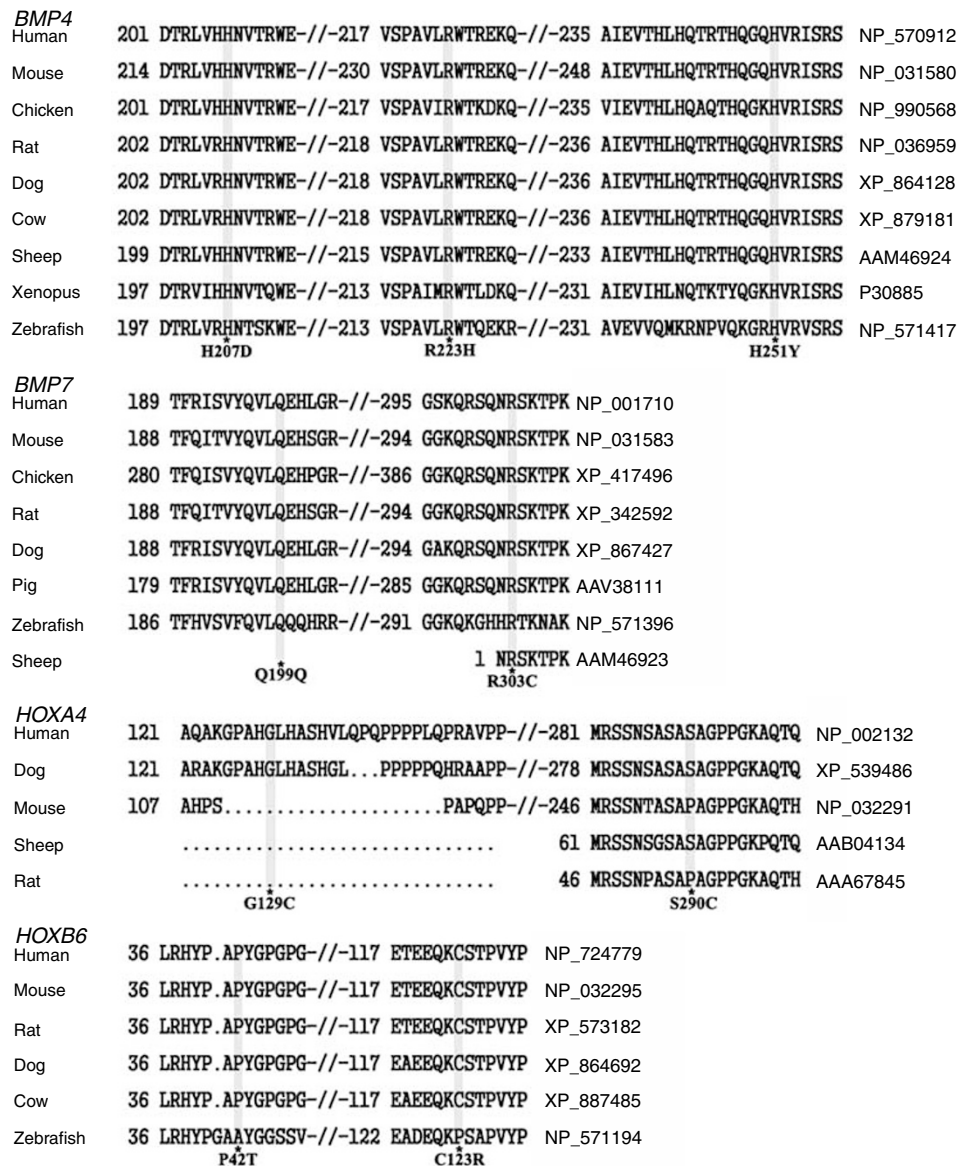


Figure 2 Amino-acid sequence alignments of BMP4, BMP7, HOXA4 and HOXB6 from respective vertebrate species. The changed residues are highlighted light gray and indicated by an asterisk (*). Amino-acid numbers are shown at the left of each of the selected regions. ‘-/-’ represents those residues omitted in every line, ‘...’ denotes that no corresponding residues were published in the NCBI protein databases.

Table 2 The detailed genetic information of the patient 16 and his parents

	HOXB6	SRD5A2	MID1
Index	p.P42T (Het)	p.Q6X (Het), p.G203S (Het)	A620V (Hem)
Mother	p.P42T (Het)	p.G203S (Het)	Not found
Father	Not found	p.Q6X (Het)	Not found

Het: heterozygous; Hem: hemizygous.

^aThe phenotype of the patient was the penile hypospadias (see Table 1), whereas his parents were healthy.

MID1 might contribute to his phenotype. And the severity of patient 16 is due to the combined contribution of mutations. But, owing to the unknown mode of inheritance of HOXB6 mutation in hypospadias and lack of functional data, we now cannot determine whether the p.P42T in HOXB6 also contributes to his phenotype. Patient 73 carries a known pathogenic homozygous mutation, R227Q in SRD5A2. Patient 65 carries a synonymous S159S mutation in WT1.⁴ Similarly, we also wonder if the p.S290C of HOXA4 and p.R303C of BMP7 in the present study have an effect on the phenotypes of patient

73 and 65, respectively. Likewise, these questions are also required for further functional identifications.

Also, based on the above idea of the combined effect, our samples in the present study are the same as those of our previous study, although some of the 90 patients have already got positive results in other known genes.

In conclusion, we have found 13 nucleotide sequence variations in 14 of 90 patients (16%) in the *BMP4*, *BMP7*, *HOXA4* and *HOXB6* genes. Among these nucleotide variations, eight are missense, and five are silent. Based their conservation data, the absences of all these variants in normal controls, and the roles of the four gene products in the formation of the male urethra indicated by other groups, we suggest that these variants in the present study are not random events in hypospadias, especially for the eight missense variations. However, further functional studies are required to make the associations clear between these variants and hypospadias. Our current work is a start in seeking the causes for hypospadias from development-related pathways. Future functional studies should include the assessment of the specific DNA-binding abilities for these *HOXA4* and *HOXB6* variants, and of the specific receptor-binding abilities for these *BMP4* and *BMP7* variants, or even the studies of all these variants by the transgenic animal models.

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