

Variation in bone morphogenetic protein 15 is not associated with spontaneous human dizygotic twinning

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BACKGROUND: Spontaneous dizygotic (DZ) twinning in humans is under genetic control. In sheep, heterozygous loss of function mutations in bone morphogenetic protein 15 (*BMP15*) increase ovulation and hence twinning rates. **METHODS:** To investigate the role of *BMP15* in human twinning, we typed 14 common variants, 4 rare novel variants initially detected by sequencing 279 mothers of DZ twins (MODZT) and 17 variants previously associated with premature ovarian failure (POF) in 933 DZ twinning families. We also typed five additional POF associated *GDF9* variants. **RESULTS:** There was some evidence for association between DZ twinning and a common intronic *BMP15* variant (rs3897937), but this was not significant after correction for multiple testing. Three of the four novel variants (p.Pro174Ser, p.Ala311Thr and p.Arg392Thr) occurred in 1–5 MODZT but were not detected in 1512 controls. We also detected three POF associated mutations in both *BMP15* and *GDF9* at low frequencies in MODZT and controls. **CONCLUSIONS:** We conclude that neither rare nor common *BMP15* variants play a significant role in the variation in human DZ twinning.

Keywords: dizygotic twinning; *BMP15*; variation; genetic association; primary ovarian failure

Introduction

The transforming growth factor signalling pathway within the ovary is critical for the regulation of ovarian function, ovulation rate and fertility (Moore *et al.*, 2004; Shimasaki *et al.*, 2004). Two genes in this pathway are growth differentiation factor 9 (*GDF9*) on chromosome 5 and bone morphogenetic protein 15 (*BMP15*) on the X chromosome. Loss of function of *Gdf9* in female mice blocks folliculogenesis during early follicle development and leads to infertility (McGrath *et al.*, 1995). In sheep, the effects of mutations in both *GDF9* and *BMP15* are sensitive to copy number. Heterozygous mutations in both genes increase the frequency of twins and higher order multiples, whereas homozygous loss of function mutations results in ovarian dysgenesis and infertility (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004).

This pathway is also essential for human fertility (Di Pasquale *et al.*, 2004). Two sisters with hypergonadotrophic ovarian failure due to ovarian dysgenesis were found to carry a non-conservative amino acid substitution in the pro region of *BMP15* (p.Tyr235Cys) which acts in a dominant negative fashion by altering *BMP15* processing (Di Pasquale *et al.*,

2004). Rare variants in both *BMP15* and *GDF9* contribute to premature ovarian failure (POF) (Di Pasquale *et al.*, 2004, 2006; Dixit *et al.*, 2005, 2006; Laissue *et al.*, 2006). Additionally, we found that some mothers of spontaneous dizygotic (DZ) twins (MODZT) carry rare deletions and missense mutations in the coding region of *GDF9* that are significantly associated with twinning (Montgomery *et al.*, 2004; Palmer *et al.*, 2006).

BMP15 is expressed in oocytes in several mammalian species (Laitinen *et al.*, 1998; Galloway *et al.*, 2000; Otsuka *et al.*, 2000). The protein has six conserved cysteine residues characteristic of the TGF β superfamily, but lacks the additional cysteine residue that strengthens dimerization in other members of the bone morphogenetic protein family (Vitt *et al.*, 2001). *BMP15* stimulates granulosa cell proliferation (Otsuka *et al.*, 2000), inhibits follicle-stimulating hormone receptor (FSHR) expression (Otsuka *et al.*, 2001) and stimulates KIT ligand expression (Otsuka and Shimasaki, 2002).

Five different heterozygous mutations in the coding region of *BMP15* in different lines of sheep increase ovulation rate and litter size (McNatty *et al.*, 2004). It is unknown if variants

in *BMP15* contribute to variation in human twinning. The human *BMP15* sequence has a number of variants that alter the predicted protein sequence, including rs41308602 (c.308A>G) located in the second position of codon 103 of the preproprotein that changes the amino acid from an asparagine to a serine. We hypothesized that variation in *BMP15* may contribute to the variation in human twinning and genotyped common variants identified from the literature and public databases in families of MODZT and controls. We also searched for rare variants by conducting a mutation screen of the coding region of the *BMP15* gene in a subset of MODZT and typed these novel variants in additional families to determine whether these are associated with DZ twinning. Further, we typed rare *BMP15* and *GDF9* variants previously reported in patients with POF to determine whether these POF associated variants might contribute to the risk for DZ twinning.

Materials and Methods

Experimental subjects

Study subjects were Caucasians recruited from 933 families with a history of DZ twinning (770 families from Australia and New Zealand and 163 families from the Netherlands) with 3450 individuals available for genotyping, including 1693 MODZT. We recruited 406 families with two or more sisters who had given birth to spontaneous DZ twins (Duffy *et al.*, 2000) and also 527 families with a single case where at least one-third degree female relative had spontaneous DZ twins. Samples were also obtained from parents of MODZT where available and from additional sibs. MODZT and their families were identified through records from our genetic epidemiology studies using twins and their families in Australia (Lewis *et al.*, 1996), through organizations for mothers of twins in Australia and New Zealand, and through appeals in the media in both countries. In the Netherlands, ascertainment was population-based through community records as part of a systematic recruitment to the Netherlands Twin Register (Meulemans *et al.*, 1996; Boomsma *et al.*, 2002). Mothers were explicitly asked about fertility treatments and all such cases were excluded.

Genetic investigation was extended to a population-based control group of 1512 Caucasian females and males (unselected for twinning history), selected at random from the electoral roll in Australia. Study protocols were reviewed and approved by the Human Research Ethics Committee of the Queensland Institute of Medical Research and the Ethics Committee of the Vrije Universiteit Hospital. Participation was voluntary and each participant gave written informed consent.

Genomic DNA was extracted (Miller *et al.*, 1988) from peripheral venous blood samples. Zygosity of the mothers' twin offspring was determined from differences in sex, eye colour or hair colour and, in equivocal cases, by typing nine independent microsatellite markers (AmpFLSTR[®] Profiler Plus[™], Applied Biosystems, Foster City, CA, USA). The probability of dizygosity given concordance of all markers in the panel was $<10^{-4}$.

Denaturing high performance liquid chromatography (DHPLC) analysis

PCR fragments covering the entire coding sequence and intron-exon junctions of the *BMP15* gene were analysed in 279 MODZT, where one sister was drawn from each of 279 affected sister pair families. PCR reactions were performed in 20 μ l volumes containing 15 ng of DNA, 1 \times PCR buffer, 16 pmol of each primer, 1.5 mM MgCl₂, 200 μ M dNTPs and 1 U Amplitaq Gold (Applied Biosystems). Prior

to DHPLC, amplicons were denatured at 95°C for 5 min and cooled to 60°C, dropping by 5°C increments with 4 min at each temperature. PCR products were then injected into a Varian Helix System (Varian, Walnut Creek, CA, USA) and eluted within a linear acetonitrile gradient consisting of buffer A [0.1 M triethylammonium acetate (TEAA) and 0.1 mM EDTA] and buffer B (0.1 M TEAA, 0.1 mM EDTA and 25% acetonitrile) with a flow rate of 0.45 ml/min. The buffer B gradient was 45–50% (0–0.5 min), 50–68% (0.5–6 min), 68% (6–7 min) and 68–45% (7–8 min). DHPLC was carried out, on a fragment specific basis, at the optimal temperature as determined by the Stanford Genome Technology Centre DHPLC melt program (<http://insertion.stanford.edu/melt.html>). Analyses were performed using the Star Workstation version 5 (Varian). The appearance of additional peaks was interpreted as indicative of a mismatch in the PCR fragment. For these samples, new PCR products amplifying the entire exon of interest were generated and purified by Microcon-PCR Centrifugal Filter devices (Millipore, Billerica, MA, USA). BigDye[®] Terminator v3.1 terminator premix (Applied Biosystems) was used for cycle sequencing with purified PCR products analysed with a capillary based genetic analyser.

SNP genotyping

All common, novel and POF associated SNPs were typed in the 3450 individuals from 933 twinning families and 1512 controls using the Sequenom[™] iPLEX[™] protocol. Genotyping assays were designed using MassARRAY Assay Design software (Sequenom Inc., San Diego, CA, USA). The 2.5 μ l PCR reactions were performed in standard 384-well plates using 10 ng genomic DNA, 0.5 U *Taq* polymerase (HotStarTaq, Qiagen, Valencia, CA, USA), 500 μ mol of each dNTP and 100 nmol of each PCR primer. PCR thermal cycling in an ABI-9700 instrument was 15 min at 94°C, followed by 45 cycles of 20 s at 94°C, 30 s at 56°C, 60 s at 72°C. The completed PCR reactions were then incubated with 0.15 U shrimp alkaline phosphatase for 30 min at 37°C followed by inactivation for 5 min at 85°C. After adjusting the concentrations of extension primers to equilibrate signal-to-noise ratios, the post-PCR primer extension reaction of the iPLEX assay was performed in a final 5 μ l extension reaction containing 0.1 μ l of termination mix, 0.02 μ l of DNA polymerase (Sequenom Inc.) and 600–1200 nM extension primers. A two-step 200 short-cycles program was used for the iPLEX extension reaction: initial denaturation was 30 s at 94°C followed by five cycles of 5 s at 52°C and 5 s at 80°C. An additional 40 annealing and extension cycles were then looped back to 5 s at 94°C, 5 s at 52°C and 5 s at 80°C. A final extension was carried out at 72°C for 3 min and then the sample was cooled to 20°C. The iPLEX reaction products were desalted by diluting samples with 15 μ l of water and 3 μ l of resin to optimize mass spectrometric analysis. Products were spotted on a SpectroChip (Sequenom Inc.), and processed and analysed in a Compact Mass Spectrometer using MassARRAY Workstation (version 3.3) software (Sequenom Inc.).

Statistical analysis

The program Sib-pair (<http://www.qimr.edu.au/davidD/sib-pair.html>) was used to calculate preliminary allele and genotype frequencies. Since variants were genotyped in families, the case-control comparisons of allele frequencies to test association allowing for the family nature of the data were carried out using the program MENDEL 7.0 (Lange *et al.*, 2001). To make the comparison as clear as possible, a case was defined as an MODZT, whereas a control was a member (male or female) of our population-based sample; other relatives of cases were treated as having unknown phenotypes. We fitted allelic association models (i.e. assuming multiplicative effects on penetrance in females), and so could utilize male

controls (remembering that *BMP15* is X-linked). For association analysis of rare variants discovered via sequencing (where only cases were sequenced), we carried out ascertainment correction (conditioning the family likelihood on that of the sequenced proband). Reconstruction of haplotypes consisting of four common SNPs surrounding and within exon 1, and the analysis of haplotype frequencies in MODZT and controls were performed using MENDEL 7.0.

To predict the functional significance of missense mutations, we constructed a multiple sequence alignment for mammalian *BMP15* protein sequences for human (NP_005439.1), macaque (XP_001083980), chimpanzee (XP_529247), sheep (Q9MZE2), cow (NP_001026922), pig (NP_001005155), mouse (NP_033887) and rat (NP_067702) sequences and compared the amino acid substitutions using the Align-GVGD program (Mathe *et al.*, 2006) available at <http://agvgd.iarc.fr>. The program uses an extension of the Grantham difference and compares the amino acid substitutions taking into account composition, polarity and volume of amino acid substitutions within the context of a multiple sequence alignment for the protein. Amino acid changes are classified as to their likelihood of interfering with protein function on a scale from class C65 (most likely) to C0 (least likely).

Results

Common SNPs

We typed 14 common SNPs located across the *BMP15* locus identified from public databases (all SNPs with rs designations) and the literature (c.-673C>T, Moron *et al.*, 2006; Fig. 1). Two SNPs (rs6614369 and rs6614608) were monomorphic in our samples and were omitted from all analyses. Genotype data for the remaining 12 common SNPs were in Hardy–Weinberg equilibrium, and overall minor allele frequencies ranged from 0.001 to 0.327 (Table I). The analysis of allelic association for individual SNPs identified some evidence of association between the DZ twinning phenotype and the intronic SNP rs3897937 (IVS1+905G>A) with a significantly higher frequency of the A allele in MODZT than in controls (0.325 versus 0.294, $P = 0.010$, Table II). A SNP in the promoter region (rs3810682; c.-9C>G) also showed some evidence of

association. However, the differences in allele frequencies between MODZT and controls were small and the effects were not significant after correcting for multiple testing of all SNPs.

We estimated the frequencies of haplotypes including four common SNPs located around and within exon 1 of *BMP15*, including rs3810682 and rs3897937, following recent evidence that a haplotype comprising these SNPs is associated with the production of increased numbers of follicles (≥ 12) during ovarian stimulation (Moron *et al.*, 2006). In our samples only four haplotypes had frequencies $>5\%$ in both MODZT and controls (Table III). One common haplotype accounted for 67% of *BMP15* chromosomes among our MODZT, with the two most frequent haplotypes accounting for $>82\%$ of chromosomes. There were no differences in the frequencies of haplotypes between MODZT and controls (Table III).

Novel variants in MODZT

A search for rare variants in the *BMP15* gene in 279 MODZT identified four rare missense alterations resulting in putative changes in the amino acid sequence. Two variants (c.520C>T, p.Pro174Ser and c.581T>C, p.Phe194Ser) were located in the pro-region of *BMP15* and two variants (c.931G>A, p.Ala311Thr and c.1175G>C, p.Arg392Thr) in the mature protein region. The p.Arg392Thr variant was predicted as ‘most likely’ to interfere with protein function (Align-GVGD class C65), p.Phe194Ser and p.Ala311Thr as slightly less likely to affect protein function (class C55, one class below C65), whereas p.Pro174Ser is predicted to have no effect (class C0).

These variants were then typed in all samples (Table IV). The p.Phe194Ser variant had a higher frequency in controls than in MODZT (0.003 versus 0.001, $P = 0.054$). The other three variants were seen in twinning families but not controls; the p.Pro174Ser variant in additional members of the family in which it was first found, the p.Ala311Thr variant in members of the original and one additional family and the p.Arg392Thr

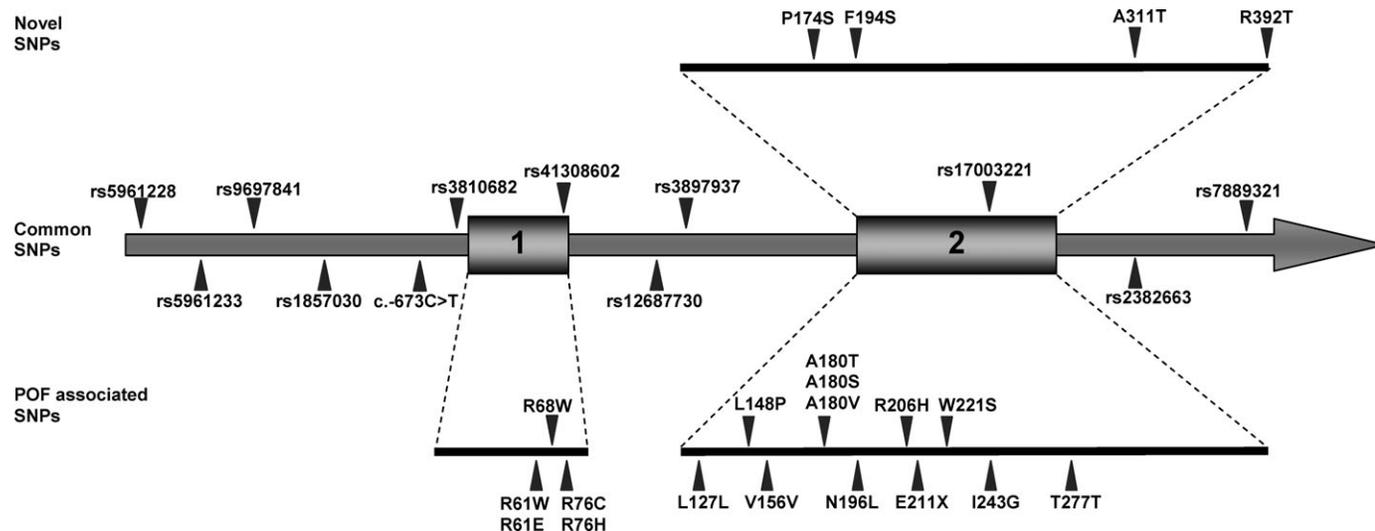


Figure 1: Schematic representation of the genomic structure of the *BMP15* gene showing the locations of the SNPs genotyped in our DZ twinning families, including 12 common SNPs, 4 rare variants identified in mutation screening of MODZT and 17 POF associated variants. Common SNPs that were not polymorphic in our samples are not shown, and only the exons are drawn to scale.

Table I. Common SNPs at the *BMP15* locus genotyped in 933 twinning families and 1512 controls.

SNP	Position*	Location	Amino acid change	Change (transcribed strand)	Minor allele frequency
rs5961228	50656568	5' genomic	—	C>T	0.135
rs5961233	50663804	5' genomic	—	C>A	0.101
rs9697841	50664677	5' genomic	—	T>G	0.061
rs1857030	50667633	5' genomic	—	T>A	0.061
c.-673C>T	50671179	5' genomic	—	C>T	0.240
rs3810682	50670515	5' UTR	—	C>G	0.232
rs41308602	50670830	Exon 1	Asn103Ser	c.308A>G	0.086
rs12687730	50671338	Intron 1	—	T>C	0.001
rs3897937	50671756	Intron 1	—	T>C	0.294
rs17003221	50676020	Exon 2	Ser248Ser	c.852C>T	0.005
rs2382663	50679039	3' genomic	—	G>A	0.327
rs7889321	50692668	3' genomic	—	A>G	0.038

*Genomic position according to NCBI Build 36.1.

Table II. Allelic association between dizygotic twinning and common SNPs at the *BMP15* locus.

SNP	N genotyped	Minor allele frequency		Allelic χ^2	P-value ¹
		MODZT N = 1693	Controls N = 1512		
rs5961228	4648	0.137	0.141	2.032	0.154
rs5961233	4633	0.102	0.103	0.002	0.952
rs9697841	4628	0.065	0.059	1.250	0.289
rs1857030	4637	0.064	0.057	1.161	0.281
c.-673C>T	4680	0.250	0.230	2.390	0.122
rs3810682	4534	0.255	0.232	3.503	0.061
rs41308602	4596	0.088	0.086	0.414	0.519
rs12687730	4613	0.0001	0.002	2.628	0.105
rs3897937	4581	0.325	0.294	6.705	0.010
rs17003221	4649	0.004	0.005	1.971	0.160
rs2382663	4563	0.319	0.328	0.614	0.433
rs7889321	4648	0.038	0.038	0.421	0.516

¹Asymptotic P-values were estimated allowing for the family nature of the data using the measured genotype approach implemented in MENDEL 7.0 (Lange *et al.*, 2001).

Table III. Frequencies of observed haplotypes and odds ratios for MODZT (cases) and controls for four common *BMP15* SNPs.

Haplotype c.-673C>T–rs3810682–rs41308602–rs3897937	Frequency MODZT	Frequency controls	Odds ratio
C C A A	0.668	0.692	1.000
T G A G*	0.155	0.136	0.846
T G G G*	0.077	0.092	1.153
C C A G	0.063	0.058	0.888
Rare haplotypes	0.034	0.021	0.704

Haplotypes with frequencies ≤ 0.01 in both MODZT and controls were collapsed and analysed as a single group of 'rare haplotypes'.

*A haplotype associated with the production of high numbers of follicles during assisted reproduction by Moron *et al.* (2006), TGG, is composed of markers c.-673C>T–rs3810682–rs3897937, incorporating either allele of rs41308602.

variant only in the sample in which it was first detected. Corrected allele frequencies taking ascertainment bias into account were not significantly different between MODZT and controls (Table IV).

POF associated variants in MODZT

Seventeen variants in the coding regions of *BMP15* and six in *GDF9* that have been previously reported in POF patients were also typed in our 933 twinning families and 1512 controls (Table V). Three POF associated *BMP15* variants were detected in our samples, at approximately similar minor allele frequencies for both MODZT and controls (p.Arg68Trp, 0.0003 versus 0.002, $P = 0.303$; p.Ala180Thr, 0.014 versus 0.016,

$P = 0.253$; p. Leu263_Arg264insLeu, 0.005 versus 0.005, $P = 0.406$).

Three POF associated *GDF9* variants were each detected in either a single control (c.-8C>T and p.Lys67Glu) or MODZT (p.Ser70Ser; Table V). Previously, the p.Pro103Ser variant had been found in this cohort at a higher frequency among our MODZT than among controls (Palmer *et al.*, 2006; Table V).

Discussion

BMP15 is a strong candidate gene likely to contribute to the variation in human twinning. The gene is specifically expressed in the oocytes of developing follicles (Laitinen *et al.*, 1998;

Table IV. *BMP15* variants identified by DHPLC analysis of 279 MODZT.

<i>BMP15</i> variant	Protein variant	MODZT		Controls		<i>P</i> -value
		Carriers	Allele freq	Carriers	Allele freq	
c.520C>T	Pro174Ser	2	0.0005	—	—	0.112
c.581T>C	Phe194Ser	5	0.0012	11	0.0036	0.054
c.931G>A	Ala311Thr	3	0.0008	—	—	0.066
c.1175G>C	Arg392Thr	1	0.0002	—	—	0.295
Any of the above	—	11	0.0032	11	0.0036	0.168

Numbers of carriers and minor allele frequencies were determined by Sequenom MALDI-TOF SNP analysis of 933 DZ twinning families with 1693 MODZT and 1512 controls. The *P*-values were calculated for the likelihood ratio test (MENDEL binomial link measured genotype model) testing for effect of the variant on the likelihood of having twins under a multiplicative model.

Table V. Summary of mutations identified in women with premature ovarian failure (POF) in the *GDF9* and *BMP15* genes.

Gene and sequence change	Amino Acid Change	Initial study population	No. POF mutations	References	No. of Carriers	
					MODZT	Controls
<i>GDF9</i>						
c.-8C>T	—	Indian	2/195	Dixit <i>et al.</i> (2005)	—	1
c.199A>C	Lys67Glu	Indian	5/195	Dixit <i>et al.</i> (2005)	—	1
c.205C>T	Ser70Ser	Indian	1/195	Dixit <i>et al.</i> (2005)	1	—
c.307C>T	Pro103Ser	Caucasian	1/61	Kovanci <i>et al.</i> (2007)	30*	13*
c.646G>A	Val216Met	Indian	2/195	Dixit <i>et al.</i> (2005)	—	—
c.1353C>T	Cys461Cys	Indian	3/195	Dixit <i>et al.</i> (2005)	—	—
<i>BMP15</i>						
c.181C>T	Arg61Trp	Indian	2/202	Dixit <i>et al.</i> (2006)	—	—
c.182G>A	Arg61Glu	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.202C>T	Arg68Trp	Italian	1/166	Di Pasquale <i>et al.</i> (2006)	2	3
c.226C>T	Arg76Cys	Indian	5/202	Dixit <i>et al.</i> (2006)	—	—
c.227G>A	Arg76His	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.381A>G	Leu127Leu	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.443T>C	Leu148Pro	Mixed origins	1/203	Laissue <i>et al.</i> (2006)	—	—
		African-American	2/14	Di Pasquale <i>et al.</i> (2006)	—	—
c.468G>A	Val156Val	Mixed origins	1/203	Laissue <i>et al.</i> (2006)	—	—
c.538G>A	Ala180Thr	Italian	5/166	Di Pasquale <i>et al.</i> (2006)	46	32
		Indian	3/202	Dixit <i>et al.</i> (2006)	—	—
		Mixed origins	2/203	Laissue <i>et al.</i> (2006)	—	—
c.538G>T(+c.539C>T)	Ala180Phe/Ser+ Val	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.588T>A	Asn196Lys	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.617G>A	Arg206His	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.631C>T	Glu211X	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.661T>C	Trp221Arg	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.727A>G	Ile243Gly	Indian	1/202	Dixit <i>et al.</i> (2006)	—	—
c.788_789insTCT	Leu263_Arg264insLeu	Indian	9/202	Dixit <i>et al.</i> (2006)	16	12
c.831T>C	Thr277Thr	Mixed origins	1/203	Laissue <i>et al.</i> (2006)	—	—

SNPs were screened in 933 DZ twinning families (including 1693 MODZT) and 1512 controls.

*Found previously in this cohort, Palmer *et al.* (2006).

Galloway *et al.*, 2000; Otsuka *et al.*, 2000) and several loss of function mutations in sheep increase ovulation rates and litter sizes in heterozygous carriers (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004). To test for association between *BMP15* variants and human twinning, we typed a total of 35 common, rare and novel *BMP15* SNPs in our DZ twinning families. The frequency of the C allele of one intronic SNP (rs3897937) was higher in MODZT than in controls, although this was not significant after accounting for multiple testing. The analysis of the SNPs across the *BMP15* locus showed strong linkage disequilibrium, with two haplotypes accounting for 82% of chromosomes in MODZT. There were no significant associations between haplotype frequencies and the DZ twinning phenotype.

We previously identified rare mutations in the *GDF9* gene including both insertion/deletion and missense mutations with higher frequencies in MODZT compared with controls (Montgomery *et al.*, 2004; Palmer *et al.*, 2006). We carried out a similar screen of *BMP15* in 279 probands from our most twin dense families. We found no insertion/deletion mutations, but did identify four rare missense *BMP15* mutations in our MODZT. One of these (p.Phe194Ser) was more frequent in controls than in MODZT, and hence can probably be considered a rare polymorphism. The other three variants were seen only in twinning families. However, the variant most likely to affect protein function, p.Arg392Thr, was carried by one MODZT but not by her sister, also an MODZT. Conversely, p.Ala311Thr and p.Pro174Ser were detected in both MODZT

and their ‘unaffected’ sisters. It is possible that these variants are twinning associated alleles with reduced penetrance; but although both are predicted to interfere to some degree with protein function, whether or not they actually have an effect is unknown. Additionally, the carrier status of all MODZT for the p.Ala311Thr variant within one of the two families in which it was detected cannot be determined due to the incomplete sampling of this very large extended pedigree. Taken together, our results suggest that the contribution of rare variants in *BMP15* to the variability in human twinning would be small.

There are species differences in the actions of BMP15. Loss of function mutations in humans and sheep has dramatic consequences on follicle development. A mutation at codon 235 in the preproregion of human BMP15 associated with hypergonadotrophic ovarian failure in two sisters with streak ovaries (Di Pasquale *et al.*, 2004) acts in a dominant negative fashion, abolishing the effects of wild-type BMP15 in stimulating the growth of granulosa cells (Di Pasquale *et al.*, 2004). In contrast, the targeted deletion of the second exon of the *Bmp15* gene in mice has limited effects on folliculogenesis (Yan *et al.*, 2001). Female *Bmp15* knockout mice are subfertile due to defects in the ovulation process and the ability of oocytes to develop into normal embryos (Liao *et al.*, 2004). The differences between these species may reflect differences in the relative importance of BMP15 and GDF9 in regulating events of folliculogenesis (Liao *et al.*, 2004) or may reflect the nature of the specific mutations. Studies with a recombinant human BMP15 p.Ile31Asp substitution (Liao *et al.*, 2003), which mimics the p.Val31Asp variant in the Inverdale strain of sheep (Galloway *et al.*, 2000), show that the variant form of the protein interferes with proteolytic processing of both wild-type BMP15 and also GDF9. The p.Tyr235Cys mutation in human BMP15 associated with hypergonadotrophic ovarian failure (Di Pasquale *et al.*, 2004) also interferes with the processing and secretion of bioactive proteins.

Our data suggest variation in human *BMP15* plays at best a very minor role in human DZ twinning. This contrasts with evidence for sheep where at least five different *BMP15* mutations are associated with high ovulation rates and litter sizes in different flocks, where they have attained high frequencies due to artificial selection or genetic drift (McNatty *et al.*, 2004). These variants have been identified in prolific breeds of sheep, and to date there has been no systematic screen for variants in either *BMP15* or *GDF9* in the wider sheep population to estimate the frequency of rare variants or the relative importance of variation in these two growth factors influencing ovulation rate. We have earlier shown that rare variants in human *GDF9*, including both insertion/deletion and missense mutations, account for ~2.4% of the attributable risk for twins (Palmer *et al.*, 2006). Taken together, our results suggest *GDF9* plays a more important role than *BMP15* in the regulation of twinning in humans (Montgomery *et al.*, 2004; Palmer *et al.*, 2006). Associations between DZ twinning and variants in other genes including *FMR* (Vianna-Morgante, 1999; Marozzi *et al.*, 2000) and *SERPINA1* (Clark and Martin, 1982; Boomsma *et al.*, 1992) have been reported, but none have been replicated in large samples.

Four of the common *BMP15* variants analysed here have recently been associated with high response to treatment with recombinant follicle-stimulating hormone (FSH) during assisted reproduction (Moron *et al.*, 2006). Minor alleles for variants c.-673C>T plus rs3810682 (the two markers are in almost complete linkage disequilibrium) and rs3897937, as well as the resulting ‘TGG’ haplotype, were significantly over-represented among 35 high FSH responders (producing ≥12 follicles), and particularly in the subset of 11 high responders who developed ovarian hyperstimulation syndrome. Although the numbers of women involved were small (11.4% and 3.6% of the women tested, respectively), this suggests a role for *BMP15* variants in the control of follicle numbers in response to high exogenous FSH concentrations, possibly through a functional variant linked to the TGG haplotype (Moron *et al.*, 2006). Raised FSH concentrations during the follicular phase have been documented in MODZT (Nylander, 1974; Martin *et al.*, 1984; Lambalk *et al.*, 1998), and it may be that with this haplotypic background *BMP15* also influences follicle number in response to high levels of endogenous FSH. However, we found no evidence that the frequency of the TGG haplotype was increased in MODZT.

Conversely, a number of recent studies identified variants in both *BMP15* and *GDF9* associated with POF (Dixit *et al.*, 2005, 2006; Di Pasquale *et al.*, 2006; Laissue *et al.*, 2006). MODZT reach menopause significantly earlier than mothers of monozygotic twins, with the difference resulting from some MODZT reaching menopause before age 40 (Martin *et al.*, 1997). This small increase in the frequency of POF in MODZT could be explained by mutations in *GDF9* and/or *BMP15* influencing both aspects of ovarian function. To test this hypothesis, 23 *GDF9* and *BMP15* variants previously associated with POF were typed in our 933 twinning families. We have earlier shown that the *GDF9* variant p.Pro103Ser, also reported in one woman with POF (Kovanci *et al.*, 2007), is significantly associated with twinning (Palmer *et al.*, 2006). We detected three additional POF associated variants in each gene in our MODZT and/or controls—*BMP15* variants p.Arg68Trp (Di Pasquale *et al.*, 2006), p.Ala180Thr (Di Pasquale *et al.*, 2006; Dixit *et al.*, 2006; Laissue *et al.*, 2006) and p.Leu263_Arg264insLeu (Dixit *et al.*, 2006) and *GDF9* variants c.-8C>T, p.Lys67Glu and p.Ser70Ser (Dixit *et al.*, 2005). In previous reports, the p.Leu263_Arg264insLeu was associated with POF in one study (Dixit *et al.*, 2006), but not in two others (Di Pasquale *et al.*, 2006; Laissue *et al.*, 2006). This and the other variants we detected may be rare polymorphisms rather than mutations, and further studies are now required to confirm whether these variants are in fact associated with POF.

The association of the *GDF9* p.Pro103Ser variant with both twinning (Palmer *et al.*, 2006) and POF (Kovanci *et al.*, 2007) might, however, support the hypothesis that earlier menopause in mothers of twins and the twinning phenotype are related to mutations in *GDF9* and that other mutations/genes causing POF may also be candidates for twinning. We found no globally significant association between twinning and any *BMP15* variant or haplotype, but the possibility that multiple variants might affect either POF or twinning is intriguing. Further

studies will be required to determine if such differences reflect the rare frequencies of these variants in each condition or whether variants can influence POF and twinning independently.

In conclusion, we found no evidence that either rare or common variants in *BMP15* play any significant role in the variation in human DZ twinning. We cannot entirely rule out weak effects or epistatic interactions with other variants, but very large studies will be required to examine these effects. We identified rare variants in *BMP15* in MODZT, but the frequencies of these were very low. There is evidence for an association between early menopause and twinning and although most POF variants were not found in MODZT, the GDF9 p.Pro103Ser variant associated with twinning (Palmer *et al.*, 2006) has been reported in one POF patient (Kovanci *et al.*, 2007). Further studies should examine the relationships between twinning and POF.

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