



# Variation in *BMPR1B*, *TGFRB1* and *BMPR2* and Control of Dizygotic Twinning

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Genes in the TGF9 signaling pathway play important roles in the regulation of ovarian follicle growth and ovulation rate. Mutations in three genes in this pathway, growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*) and the bone morphogenetic protein receptor B 1 (*BMPRB1*), influence dizygotic (DZ) twinning rates in sheep. To date, only variants in *GDF9* and *BMP15*, but not their receptors transforming growth factor  $\beta$  receptor 1 (*TGFRB1*), bone morphogenetic protein receptor 2 (*BMPR2*) and *BMPR1B*, have been investigated with respect to their roles in human DZ twinning. We screened for rare and novel variants in *TGFRB1*, *BMPR2* and *BMPR1B* in mothers of dizygotic twins (MODZT) from twin-dense families, and assessed association between genotyped and imputed variants and DZ twinning in another large sample of MODZT. Three novel variants were found: a deep intronic variant in *BMPR2*, and one intronic and one non-synonymous exonic variant in *BMPRB1* which would result in the replacement of glutamine by glutamic acid at amino acid position 294 (p.Gln294Glu). None of these variants were predicted to have major impacts on gene function. However, the p.Gln294Glu variant changes the same amino acid as a sheep *BMPR1B* functional variant and may have functional consequences. Six *BMPR1B* variants were marginally associated with DZ twinning in the larger case-control sample, but these were no longer significant once multiple testing was taken into account. Our results suggest that variation in the TGF9 signaling pathway type II receptors has limited effects on DZ twinning rates in humans.

■ **Keywords:** *TGFRB1*, *BMPR2*, *BMPR1B*, dizygotic twinning, high resolution melt

Genes in the TGF9 signaling pathway play important roles in the regulation of ovarian follicle growth and ovulation rate (Juengel & McNatty, 2005; Moore et al., 2003). Heterozygous mutations in two genes from this pathway, bone morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (*GDF9*) increase ovulation rate and twinning frequency in sheep, with multiple independent mutations being reported in both genes (Galloway et al., 2000; Hanrahan et al., 2004). The pathway is also critical for ovarian function in women. A dominant-negative mutation in Italian sisters caused ovarian dysgenesis (Di Pasquale et al., 2004) and rare variants in both *BMP15* and *GDF9* have been reported in patients with premature ovarian failure (Dixit et al., 2005; Dixit et al., 2006; Laissue et al., 2006). We previously investigated whether genetic variants in *GDF9* or *BMP15* contribute to variation

between women in the frequency of dizygotic (DZ) twinning. We genotyped common variants in both genes in mothers of DZ twins (MODZT) and found no evidence for association between common variants in *GDF9* or *BMP15* and DZ twinning (Montgomery et al., 2004; Zhao et al., 2008). We also screened the coding regions of both genes and identified novel insertion/deletion mutations in *GDF9* that segregated with the DZ twinning phenotype in families (Montgomery et al., 2004; Palmer et al., 2006). Rare coding variants in *GDF9* were present at higher fre-

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quencies in mothers of DZ twins (Palmer et al., 2006), but no such association was seen between DZ twinning and rare variants in *BMP15* (Zhao et al., 2008).

GDF9 and BMP15 proteins signal through type II and type I serine/threonine kinase receptors. GDF9 initiates signaling by binding to bone morphogenetic protein receptor 2 (*BMPR2*), a type II serine-threonine kinase receptor (Vitt et al., 2002). *BMPR2* then phosphorylates the glycine and serine rich domain of the type I serine-threonine kinase receptor, transforming growth factor  $\beta$  receptor 1 (*TGFBR1*), also known as activin-like kinase receptor 5 (*ALK5*) (Pangas & Matzuk, 2008). The activated *TGFBR1* phosphorylates either receptor-activated SMADs 2 or 3 and goes on to form a complex with SMAD 4. The complex translocates to the nucleus and binds to promoter sequences of target genes at SMAD-binding DNA elements to regulate gene transcription (Derynck & Zhang, 2003). *BMP15* operates in a similar manner through *BMPR2* and the type I receptor bone morphogenetic protein receptor type 1B (*BMPR1B*), also known as activin-like kinase 6 (*ALK6*), and receptor-SMADs 1, 5 or 8 (Moore et al., 2003).

A mutation (p.Glu249Arg) in the highly conserved intracellular kinase signaling domain of *BMPR1B* increases ovulation rate and litter size in a dosage sensitive manner (Mulsant et al., 2001; Wilson et al., 2001). It is possible that variants in *BMPR1B*, *BMPR2* and *TGFBR1*, the type I and II receptors for *GDF9* and *BMP15*, may influence DZ twinning rates in women. The aim of the present study was to screen for both rare and common variants in the three receptors and to test for association between these variants and DZ twinning in a large sample of MODZT.

## Material and Methods

### SCREENING FOR NOVEL VARIANTS IN *BMPR1B*, *BMPR2* AND *TGFBR1*

#### Subjects

The coding regions of *BMPR1B*, *BMPR2* and *TGFBR1* were sequenced in DNA samples from one MODZT from 20 pedigrees drawn from Australian and Dutch families previously recruited for studies on the genetics of DZ twinning (Duffy et al., 2001; Painter et al., 2010; Palmer et al., 2006). MODZT in these families were explicitly asked about fertility treatments and all such cases were excluded. Zygosity was unequivocally determined for all cases from differences in sex, eye colour or hair colour and 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  $<10^{-4}$  (Nyholt, 2006). Study protocols were reviewed and approved by the Human Research Ethics Committee of the Queensland Institute of Medical Research (QIMR) and the Ethics Committee of the Vrije Universiteit Hospital. Participation was voluntary and each participant gave written informed consent.

To maximise the chance of discovering variants associated with DZ twinning, two strategies were used to select samples. Pedigrees were first examined to identify families with the highest numbers of MODZT. Ten families were chosen where at least three sisters and other relatives were all MODZT. The mean number of MODZT per family was 3.9, and one MODZT from each family was sequenced for all three genes. Additional families were selected based on information from a recent linkage study (Painter et al., 2010). For each gene, 10 families with linkage peaks over the gene regions were selected. One MODZT from each family was then sequenced for either *BMPR1B*, *BMPR2* or *TGFBR1*. As *BMPR1B* is a known twinning gene, we included a further 80 samples from MODZT-dense families and screened these using High Resolution Melt (HRM) analysis.

#### PCR Amplification and Sequencing

Genomic DNA was extracted (Miller et al., 1988) from peripheral venous blood samples. For each gene PCR primers were designed to amplify the 3'UTR, non-coding and coding exons, including at least 50 bp of intronic sequence either side of each exon to cover intron-exon boundaries, using the Primer 3.0 program (Rozen & Skaletsky, 1999). Each amplicon was then amplified in a 15  $\mu$ l reaction containing 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 1 U AmpliTaq Gold (all Applied Biosystems), 200  $\mu$ M each dNTP (Promega, Madison, WI, USA), 1  $\mu$ M each of forward and reverse primers and 50 ng of DNA. PCR cycling conditions, incorporating a 'touchdown' protocol where the annealing temperature was reduced by .5°C per cycle for the first 20 cycles, were as follows: initial denaturation at 95°C for 5 mins for one cycle; touchdown annealing at 95°C for 30 secs, 60°C (-.5 per cycle) for 30 secs and 72°C for 30 secs for 20 cycles; further annealing at 95 for 30 secs, 50°C for 30 secs and 72°C for 30 secs for 15 cycles; final extension at 72°C for 10 minutes. Products were verified by electrophoresis through 2% agarose gels, cleaned using Exonuclease I and Shrimp Alkaline Phosphatase (both Fermentas, Burlington, Ontario, Canada) and sequenced using BigDye 3.0 terminator chemistry (Applied Biosystems). All details, including protocols and primer sequences, are available from the authors upon request.

#### High Resolution Melt (HRM) Assay

Non-coding and coding exons, exon/intron boundaries and the 3'UTR of *BMPR1B* were screened for variants using HRM on a Rotor-Gene 6000 Realtime Rotary Analyser (Corbett Research, QIAGEN, Hilden, Germany). HRM is a technique with 96-100% sensitivity to detect single-base changes and 1-3 base insertions, deletions or duplications in DNA sequence, with a low risk of false positives (Krenkova et al., 2009; Purcu et al., 2010; Reed & Wittwer, 2004; Wittwer, 2009). Results are visualized as melt curves, the appearance of which depends on the

overall DNA sequence, GC content, length and heterozygosity of the amplicon (White & Potts, 2006).

HRM reactions were performed in 16 µl containing 1 µl DNA (at a concentration of 25 ng/µl), 7.5 µl Sensimix (containing heat-activated DNA polymerase, 6 mM MgCl<sub>2</sub>, and dNTPs; Quantace, Finchley, UK), .5 µl of forward and reverse primers at 10 µM, .8 µl Evagreen fluorescent dye (Biotium, Hayward, CA, USA) and 5.7 µl H<sub>2</sub>O. Positive controls were included in each HRM run, where available, for two known heterozygous variant samples, two known homozygous variant samples, two known homozygous wildtype samples and two negative controls with no DNA. The HRM assay included an initial denaturation step of 10 minutes at 95°C, followed by 35 cycles with 30 seconds denaturation at 95°C, 30 seconds annealing at 57°C, and 30 seconds extension at 72°C. The melting profile for each amplicon was calculated using the OligoCalc program, with a range ± 10°C of the 'nearest neighbour' melting temperature (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). Melting curves were analyzed using the Rotor-Gene 6000 analysis software v 1.7 (Corbett Research).

#### Estimation of Allele Frequencies for Novel Variants

Allele frequencies for the novel variants in the *BMPR1B* gene were estimated in 100 control samples from mothers of monozygous twins (MOMZT) via HRM.

The novel variant in *BMPR2* intron 2 could not be detected by HRM possibly due to its position close to the

rs6742403 SNP (9 bases away) or because individuals heterozygous for both variants carry the same DNA bases (AG). Allele frequencies were instead estimated by restriction fragment length polymorphism in the 100 MOMZT controls as this variant introduced a restriction site for the BsmBI enzyme. The region was amplified by PCR and 5 µl of PCR product was digested using 10 U of BsmBI (New England BioLabs, MA, USA) in 25 µl with 1X restriction enzyme buffer, and 10 µg BSA. Reactions were incubated at 55°C for 2 hours and bands resolved by electrophoresis through 2% agarose gels. Bands were visualized on a UV transilluminator.

#### CANDIDATE GENE ASSOCIATION STUDIES

To widen the search for variants in *BMPR1B*, *BMPR2* and *TGFBR1* associated with DZ twinning, we analyzed genome-wide association (GWA) data from a different set of 727 MODZT (as cases) and 935 mothers and fathers of MZ twins (as controls). These samples were part of a larger collection of samples genotyped for GWA studies by QIMR; data were available for > 2.4 million directly genotyped and imputed SNPs genome wide (Medland et al., 2009). To focus on the genes screened in this study, association analyses were then performed including SNPs 20Kb up- and downstream of *BMPR1B*, *BMPR2* and *TGFBR1* using the PLINK program (Purcell et al., 2007). For all analyses *P* values were corrected for multiple testing using the Bonferroni procedure. The number of independent SNPs in each gene region was determined using the

**TABLE 1**

Variants Identified During the Resequencing of *TGFBR1*, *BMPR2* and *BMPR1B* in Twenty Mothers of Dizygotic Twins

SNP name	Location	Nucleotide variant	Amino acid change	HapMap MAF*	MAF in 20 MODZT	MAF in 100 MODZT	MAF in 100 MOMZT
<b>TGFBR1</b>							
rs334354	Intron 7	c.1255+24 G>A		.217	.175		
rs67687202	Intron 8	c.1386+90_1386+94del (del TCTTT)			.125		
<b>BMPR2</b>							
Novel	Intron 2	c.247+239G>A			.025		0
rs6742403	Intron 2	c.247+248A>G			.100		
rs71425943	Intron 3	c.418+212A>C			.250		
rs10714063	Intron 3	c.419-43delT			.175		
rs7575056	Intron 4	c.529+64G>C		.138	.175		
rs55722784	Exon 5	c.600A>C	p.L200L	.025	.050		
rs77506341	Intron 5	c.621+103C>G		.060	.050		
rs41271467	Intron 6	c.852+88T>C		.040	.025		
rs10559648	Intron 7	c.968-120_968-118del3 (del CTT)			.075		
rs17199235	Intron 11	c.1587-116A>G		.125	.050		
rs1061157	Exon 12	c.2810G>A	p.A937A	.117	.075		
rs6435155	Intron 12	c.2866+228C>T			.125		
<b>BMPR1B</b>							
rs17500892	Intron 1	c.-30 G>A		.125		.135	
Novel	Intron 1	c.-5 G>A				.005	
rs71979769	Intron 4	c.141-76(AC/in-del)			.150		0
rs35973133	Exon 9	c.86 G>A	p.R224H			.005	0
rs56083112	Exon 9	c.120 C>T	p.T235T			.005	
Novel	Exon 9	c.160 C>G	p.Q249E			.005	0
rs1365691	Intron 12	c.1383+32A>T		.448	.275		
rs1434536	3'UTR	c.141C>T		.488	.400	.490	
rs1836261	3'UTR	c.196A>G		.488	.400	.490	

Note: \* MAF = minor allele frequency.

'clump' option in PLINK, grouping all SNPs with pairwise  $r^2$  values  $> .5$  (indicating a moderate to high degree of linkage disequilibrium between them).  $P$  values were adjusted according to the number of independent 'clumps'.

## Results

### SEARCHING FOR RARE AND NOVEL VARIANTS IN 20 MOTHERS OF DIZYGOTIC TWINS

#### TGFRB1

Eight coding exons (exons 2-9) plus the intron/exon boundaries of *TGFRB1* were successfully amplified and sequenced for 20 MODZT. Two previously reported intronic variants, rs334354 and rs67687202, were detected (Table 1).

#### BMPR2

Twelve coding exons (exons 2-13) plus the intron/exon boundaries of *BMPR2* were successfully amplified and sequenced. Twelve variants were detected, two of which were exonic (Table 1). One was a previously reported synonymous A to C base change in exon 5 (rs55722784) resulting in no change to the amino acid at position 200 (p.Leu200Leu). This SNP was present in heterozygous form in two MODZT. The other variant was a synonymous G to A base change (rs1061157) in exon 12 (p.Arg937Arg). Of the 10 intronic variants nine had been previously reported, while one was novel and does not appear in genomic databases. This was a heterozygous G to A change located 239 bases into intron 2, and was present in one MODZT. As this was novel we screened this variant in 100 MOMZT but it was not found among our controls.

#### BMPR1B

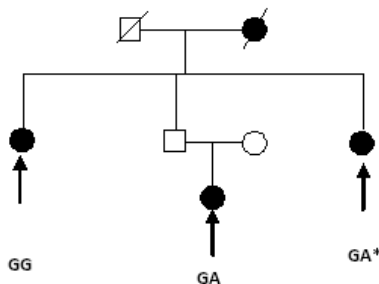
Two non-coding exons (exons 2-3), 10 coding exons (exons 4-13), the 3'UTR, and the intron/exon boundaries of *BMPR1B* were amplified and sequenced in 20 MODZT, and screened by HRM in a further 80 MODZT. A total of nine variants were detected amongst 100 MODZT. Seven of the variants have been previously reported, including

three intronic variants and two in the 3'UTR (Table 1). The remaining two SNPs were coding SNPs in exon 9, detected in two MODZT each. One variant (rs35973133) was a non-synonymous G to A change that would change the amino acid at position 224 from arginine to histidine. The other (rs56083112) was a synonymous C to T base change leaving the amino acid threonine at position 235 unchanged.

Two novel variants were discovered. One was a G to A base change located in intron 1, 5 bases from the beginning of exon 2 (non-coding), found in one MODZT in heterozygous form. The second novel variant was in the coding region of exon 9, a non-synonymous C to G change resulting in an amino acid change from glutamine to glutamic acid at position 249 (p.Gln249Glu). This novel variant was also detected in one MODZT in heterozygous form. Additional MODZT in the two families were then screened via sequencing for the presence of these variants, but neither was present in all other available MODZT: the intron 1 variant was present in only one of two additional MODZT screened (Figure 1), while the exon 9 variant was present only in the individual in which it was originally detected (Figure 1).

The possible effect of both novel variants was examined by *in silico* analyses. The intron 1 variant was predicted to have no effect on splicing using the Exonic Splicing Enhancer finder ([http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese\\_finder.cgi](http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese_finder.cgi)). The exon 9 p.Gln249Glu substitution was predicted to be possibly damaging by the Polyphen program (<http://genetics.bwh.harvard.edu/pph/>) with a PSIC score of 1.665, while it was predicted to be tolerated by the Sorting Intolerant from Tolerant (SIFT) program (<http://www.blocks.fhcrc.org/sift/SIFT.html>), with a score of .42. Both the PANTHER PSEC classification system ([https://panther.appliedbiosystems.com/methods/csnp\\_ScoreForm.jsp](https://panther.appliedbiosystems.com/methods/csnp_ScoreForm.jsp)) and Molecular Modeling & Bioinformatics Group (MMB) (<http://mmb2.pcb.ub.es:9080/PMut/>) predicted the p.Gln249Glu substitution to be neutral.

A. Pedigree of the MODZT family with a novel variant in intron 1



B. Pedigree of the MODZT family with a novel variant in exon 9 (Gln249Glu)

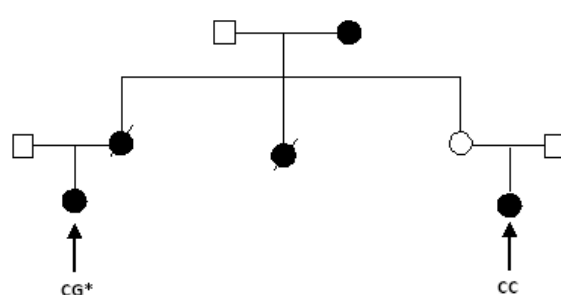
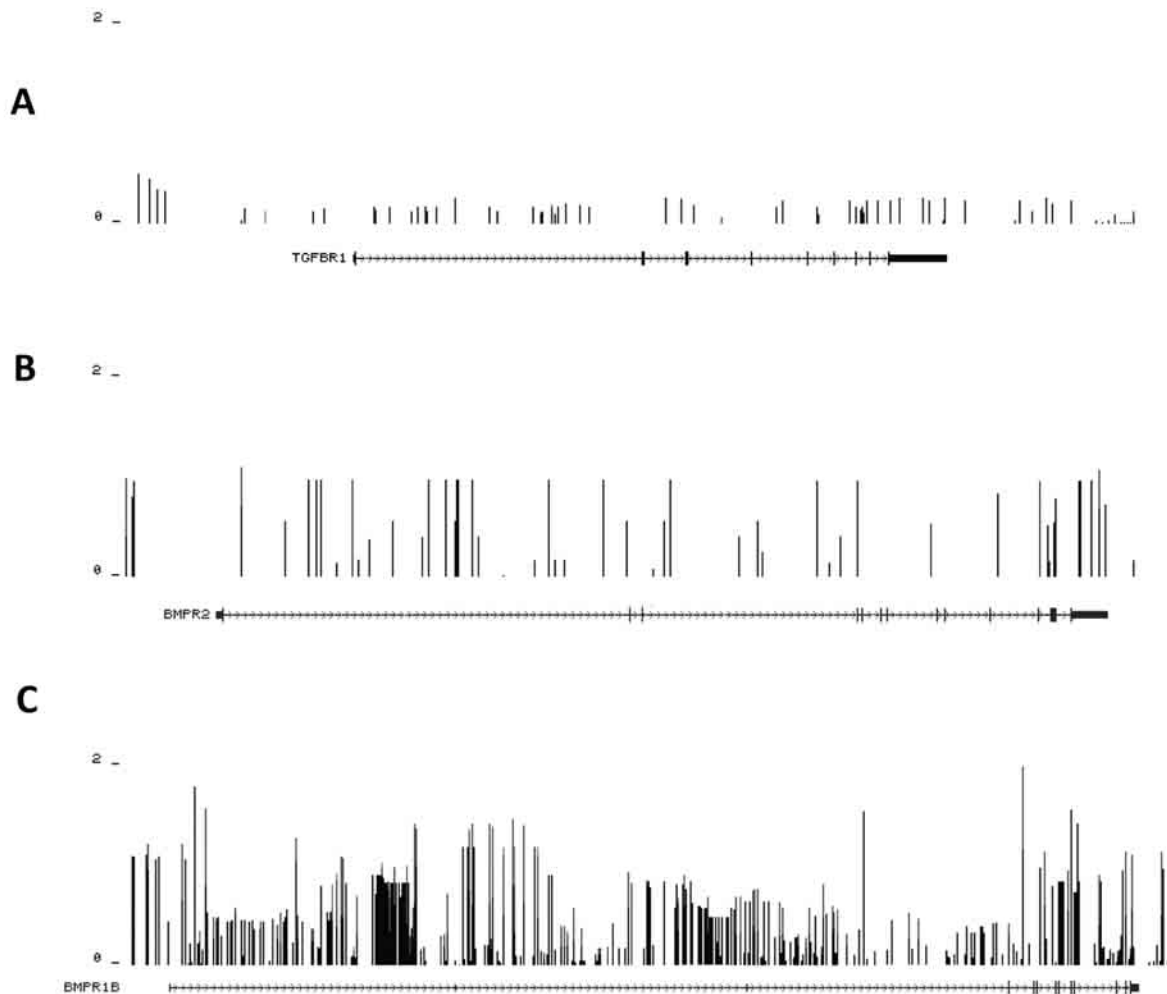


FIGURE 1

Pedigrees of mothers of dizygotic twins (MODZT) carrying novel variants in the *BMPR1B* gene.

Note: A solid circle indicates a MODZT (twins themselves are not shown in these simplified pedigrees). The family members in whom the variants were originally detected are indicated by \*.

**FIGURE 2**

Case-control association analysis results for the imputed genotype datasets for *TGFBR1* (A), *BMPR2* (B), and *BMPR1B* (C) comparing 727 mothers of DZ twins (cases) with 930 mothers and fathers of MZ twins (controls).

Note: Uncorrected  $P$  values are indicated on the Y axis as  $-\log_{10} P$  values.

## GENE-BASED ASSOCIATION ANALYSES IN A LARGE COHORT OF MOTHERS OF DIZYGOTIC TWINS

### TGFBR1

All association analyses were performed on a separate sample set from that studied above that included 727 MODZT as cases and 930 mothers and fathers of MZ twins as controls. The *TGFBR1* region contained genotypes for a total of 73 SNPs which were grouped by PLINK into nine independent clumps defined by pairwise  $r^2$  values  $> .5$ . No association between any *TGFBR1* SNP and DZ twinning was detected (Figure 2).

### BMPR2

The *BMPR2* region contained 56 SNPs in the imputed dataset, grouped into 12 independent clumps. As for *TGFBR1*, no association was detected between any of the *BMPR2* SNPs and DZ twinning (Figure 2).

### BMPR1B

The *BMPR1B* region contained a total of 465 SNPs in the imputed dataset, grouped into 39 independent clumps. Association analyses detected six SNPs with significant unadjusted  $P$  values (Table 2, Figure 2), separated into three clumps: rs3796433 and rs3796442 (clump  $P = .016$ ), rs17022120 and rs17022139 (clump  $P = .016$ ), and rs17023081 and rs17022924 (clump  $P = .028$ ). Following correction for multiple testing (corrected significance threshold  $P < .001$ ) none of these six SNPs remained significant (Table 2).

## Discussion

We screened the type I and type II receptors in the ovarian TGF $\beta$  signaling pathway *BMPR1B*, *TGFBR1* and *BMPR2* for association with DZ twinning. We first sequenced the coding exons and intron/exon boundaries for all three genes in 20 MODZT, and screened a further 80 MODZT



for *BMPR1B* using HRM, to identify novel variants not yet reported in SNP databases. We detected three such variants, a deep intronic variant in *BMPR2* and two novel variants in *BMPR1B*, one of which was located 5 bases from the start of exon 2 and the other, a non-synonymous change, in exon 9. We next screened for association with common variants making use of genome-wide association data available for a separate sample of 727 MODZT and 935 mothers and fathers of MZ twins. Analysing SNPs located within the regions 20 Kb up- and downstream of each gene suggested no association with *TGFRB1* or *BMPR2*, while six *BMPR1B* SNPs had significantly different allele frequencies between cases and controls before correction for multiple testing.

Sequencing 20 individuals chosen at random has little power to detect rare variants (frequencies less than .5%) and a comprehensive screen for rare variants would require mutation detection or sequencing of a much larger sample. However, to increase the chance of detecting physiologically relevant variants, we selected DNA samples from our most MODZT-dense families and also from families that showed evidence for segregation over the relevant genes. This strategy was successful in an earlier screening of *GDF9* where sequencing twenty individuals from our most MODZT-dense families detected a four base pair deletion in *GDF9* in two sisters with DZ twins (Montgomery et al., 2004). Subsequent mutational screening in a larger set of samples found further variants including insertion/deletion and mis-sense variants associated with DZ twinning (Palmer et al., 2006).

*TGFRB1* is a type-one serine-threonine kinase receptor which is activated through the phosphorylation of its glycine-serine rich domain (by *BMPR2*) and binds to either receptor-SMADS 2 or 3. Both *BMPR2* and *TGFRB1* are directly involved in signal transduction for *GDF9* and play a crucial role in ovarian follicle development. During our screen of *TGFRB1* and *BMPR2* we found only one novel variant, located in a deep intronic region of *BMPR2*, 239 bases from exon 2, and unlikely to have an effect on the function of this gene. Mutations in *TGFRB1* mainly cause abnormalities of cardiovascular, craniofacial, neurocognitive and skeletal development (Loeys et al., 2005), while

*BMPR2* mutations cause sporadic primary pulmonary hypertension (Lane et al., 2000; Thomson et al., 2000). Taken together with our results, it appears that these genes do not have major roles in human DZ twinning.

A larger set of samples including 100 MODZT was screened for variants in the *BMPR1B* gene using high resolution melt (HRM), a technique with 98–100% sensitivity and specificity in mutation discovery (van der Stoep et al., 2009). *BMPR1B* is a latent receptor for BMP15, regulating follicular growth and ovulation rate (Moore et al., 2003). Of the three genes screened in this study *BMPR1B* is potentially the most interesting due to the large effect seen in sheep, where mutations have been shown to increase the frequency of multiple ovulation by up to 95% (McNatty et al., 2004).

We found two novel variants in human *BMPR1B*, one intronic and one exonic. The intronic variant is located five bases from exon 2, a non-coding exon. While the effect of this variant on gene expression is unknown, it has no predicted effect on splicing and did not segregate with the twinning phenotype in the family. It is interesting to note that the exon 9 p.Gln249Glu substitution is located at the same amino acid position as the p.Gln249Arg substitution responsible for the hyperproliferacy seen in a number of sheep breeds (Davis et al., 2006; Davis et al., 2002; Wilson et al., 2001). The exon 9 p.Gln249Glu substitution we observed has no consistent predicted effect on protein structure. However, we would not expect *BMPR1B* variants with a large effect on ovulation rate in humans because of effects on viability, and the p.Gln249Glu substitution could influence DZ twinning. Our results show the substitution did not segregate with twinning in the family, although not all MODZT were available for testing. There could be sporadic cases within the family, but both non-carriers were less than 26 years of age when their twins were born. Additionally, all currently known *BMPR1B* mutations in humans cause bone-related developmental disorders, type A2 brachydactyly (Lehmann et al., 2006; Lehmann et al., 2003) and acromesomelic chondrodysplasia with genital anomalies (Demirhan et al., 2005). On current evidence the p.Gln249Glu substitution is not likely

**TABLE 2**Association Results for Imputed and Genotyped *BMPR1B* Variants for 727 MODZT and 930 Mothers and Fathers of MZ Twins

SNP*	Location (NCBI36/hg18)	Nucleotide variant (Major>Minor allele)	MAF cases (n = 727)	MAF controls (n = 930)	Unadjusted P value
rs3796433 <sup>1</sup>	96250478	C>A	.123	.092	.015
rs17022120 <sup>2</sup>	95909023	A>G	.032	.017	.016
rs17023081 <sup>3</sup>	96270327	G>A	.024	.013	.028
rs17022924 <sup>3</sup>	96184678	A>G	.024	.013	.028
rs3796442 <sup>1</sup>	96273050	A>C	.101	.075	.037
rs17022139 <sup>2</sup>	95913339	T>C	.029	.017	.032

Note: Only SNPs with significant (unadjusted) P values are shown. No association remained significant after correction for multiple testing.

\*SNP clumps are indicated by the numbers 1–3.

to be associated with twinning, but could be tested in future association studies.

There are several limitations to our study. While HRM is a sensitive method routinely used to detect variants in DNA sequences, it is possible that some variants may have been missed. This is due to physical limitations of the HRM method, where, depending on the base changes involved, variants may not be detected if they occur within 10 bases of another SNP, or are close to the end of an amplicon (Reed & Wittwer, 2004). Each experiment is also typically optimized for the variant under study, and this is difficult when the method is used for variant detection. Additionally, as we are searching for rare variation (< .5% frequencies) contributing to DZ twinning, a thorough investigation of these genes should include a much larger number of MODZT. We found suggestive evidence of association with common variation in *BMP1B* in an independent group of MODZT and controls, although these signals were no longer significant once multiple testing was taken into account. Bonferroni correction is a highly conservative correction method, and with 465 SNPs grouped into 39 clumps our sample would have only ~12% power to detect an association to an allele with 10% frequency at the corrected level of significance ( $P < .001$ ). However, as this is an important candidate gene both common variants and the novel variants found in this study should be tested for association with DZ twinning in the future.

To date, the only gene confirmed to play a role in human DZ twinning, and therefore the multiple ovulation that underlies this trait, is *GDF9*. We found no evidence for the involvement of *TGFBR1* and *BMP2* in DZ twinning. The situation is less clear for *BMP1B*. Rare variants, particularly in *BMP1B*, may still contribute to human DZ twinning, but a much larger sample of MODZT would need to be screened to detect these. Our results suggest that mutations in the TGF $\beta$  signaling pathway type II receptors have limited contributions to variation in DZ twinning rates in humans.

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