A Deletion Mutation in GDF9 in Sisters with Spontaneous DZ Twins

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A loss of function mutation in growth differentiation factor 9 (GDF9) in sheep causes increased ovulation rate and infertility in a dosage-sensitive manner. Spontaneous dizygotic (DZ) twinning in the human is under genetic control and women with a history of DZ twinning have an increased incidence of multiple follicle growth and multiple ovulation. We sequenced the GDF9 coding region in DNA samples from 20 women with DZ twins and identified a four-base pair deletion in GDF9 in two sisters with twins from one family. We screened a further 429 families and did not find the loss of function mutation in any other families. We genotyped eight single nucleotide polymorphisms across the GDF9 locus in 379 families with two sisters who have both given birth to spontaneous DZ twins (1527 individuals) and 226 triad families with mothers of twins and their parents (723 individuals). Using case control analysis and the transmission disequilibrium test we found no evidence for association between common variants in GDF9 and twinning in the families. We conclude that rare mutations in GDF9 may influence twinning, but twinning frequency is not associated with common variation in GDF9.

Women with a history of dizygotic (DZ) twinning have an increased incidence of multiple follicle growth and multiple ovulation (Gilfillan et al., 1996; Martin et al., 1991). Current models suggest multiple ovulation is controlled by both endocrine and paracrine factors (Baird, 1983; Baird & Campbell, 1998; Baker & Spears, 1999; Campbell et al., 1995; Macklon & Fauser, 2000). The concentrations of follicle-stimulating hormone (FSH) above some threshold at the critical phase of the menstrual cycle contribute to selection of the dominant follicle (Baird, 1983; Macklon & Fauser, 2000) and paracrine signaling within the ovary influences FSH action in the follicle to regulate granulosa cell differentiation, follicle development and twinning rate (Erickson & Shimasaki, 2001; Matzuk et al., 2002). Concentrations of FSH are higher in mothers of DZ twins (MODZT) in some (Lambalk et al., 1998; Martin et al., 1984), but not all (Gilfillan et al., 1996) studies. No obvious differences were seen in concentrations of inhibin A or B in MODZT with or without double ovulations (Gilfillan et al., 2003), suggesting changes in endocrine signaling to the ovary may not be the primary mechanism responsible for DZ twinning.

Two closely related members of the TGFβ family of growth factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), are expressed exclusively by the oocyte in several species (Laitinen et al., 1998; McGrath et al., 1995; Otsuka et al., 2000). Both GDF9 and BMP15 have six conserved cysteine residues characteristic of the TGFβ superfamily, but lack the additional cysteine residue that strengthens dimerisation in other members of the bone morphogenetic proteins (Vitt et al., 2001). GDF9 is autosomal (human chromosome 5q31.1) and BMP15 is located on the X chromosome. Deletion of GDF9 leads to infertility in female mice caused by a block in folliculogenesis during early follicle development (McGrath et al., 1995). Loss of function mutations in BMP15 in independent lines of sheep cause increased ovulation rate and infertility in a dosage-sensitive manner (Galloway et al., 2000; Hanrahan et al., 2004). Recently a mutation in GDF9 was identified in the prolific Cambridge and Belclare sheep breeds in Ireland (Hanrahan et al., 2004) that increased ovulation rate and caused infertility in a dosage-sensitive manner identical to BMP15 mutations. An activating mutation in a third gene from the BMP signaling pathway, the receptor BMPR1B, also increases ovulation rate and twinning in sheep (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001). The results of these genetic studies in sheep demonstrate that both GDF9 and BMP15 signaling pathways are critical for follicle selection and control of ovulation rate. The role of these pathways in control of DZ twinning in the human is not known.

DZ twinning in the human clusters in families and appears to be under genetic control (Bulmer, 1970; Lewis et al., 1996; Meulemans et al., 1996). The frequency of DZ twinning varies at least fivefold between populations with the highest incidence in African populations and the lowest in Asian populations (Bortolus et al., 1999; Bulmer, 1970). Taken together, the risk to first-degree female relatives is in excess of two (Bulmer, 1970; Lewis et al., 1996; Meulemans et al., 1996), comparable with breast cancer (Claus et al., 1991). Family studies suggest that DZ twinning in the human is not X-linked (Meulemans et al., 1996). We therefore analyzed GDF9 in mothers of DZ twins and transmission of single nucleotide polymorphisms (SNPs) across the GDF9 locus in families with DZ twins to determine whether variation in GDF9 is associated with DZ twinning.

Experimental Subjects

Study subjects were 1527 individuals from 379 families in which two sisters had both given birth to spontaneous DZ twins and 723 individuals from 226 case families with 309 mothers of DZ twins. Some mothers of index cases were themselves mothers of DZ twins. Mothers of DZ twins (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 (Boomsma et al., 2002; Meulemans et al., 1996). Mothers were explicitly asked about fertility treatments and all such cases were excluded. For control samples in case-control studies we also genotyped the markers in parents from 512 families (1024 individuals) recruited from the Australian population as part of a genetic study on endometriosis (Treloar et al., 2002). Study protocols were reviewed and approved by the QIMR Human Research Ethics Committee. Participation was voluntary and each patient gave written informed consent.

Materials and Methods

Genomic DNA was extracted (Miller et al., 1988) from peripheral venous blood samples. Zygosity of the twins was determined by 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). The probability of dizygosity given concordance of all markers in the panel was < 10^{-4}.

The coding region of GDF9 was sequenced from one mother of twins in 20 pedigrees with the highest density of DZ twins. PCR primers were designed to amplify the complete coding region in three fragments including more than 20 bp of flanking sequence. PCR reactions were performed in 20μl volumes containing 15 ng of DNA, 1 × PCR buffer, 15 μM each primer, 2 mM of $MgCl_2$, 200 nM of dNTPs and 1 U Amplitaq Gold (Applied Biosystems, Foster City, CA, USA). PCR products were purified by Microcon-PCR Centrifugal Filter devices (Millipore, Billerica, MA, USA). Dye terminator reaction premix (Applied Biosystems, Foster City, CA, USA) was used for cycle sequencing and purified products sequenced on an ABI377 automated sequencer. Sequence traces were aligned using Vector NT (InforMax Inc., Frederick, MD, USA) and base changes were confirmed by sequencing of the reverse strand.

Denaturing High Performance Liquid Chromatography (DHPLC) Analysis

The PCR products for exon 1 were amplified from 15 ng of genomic DNA using AmpliTaq Gold (PE Applied Biosystems) in a final volume of 20 μL. Amplicons were then denatured at 95°C for five minutes and cooled to 60°C over 30 minutes (1°C/min) prior to DHPLC. PCR products were injected onto a Varian Helix System (Varian, Walnut Creek, CA). Samples were 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, 0.1mMEDTA and 25% acetonitrile) with a flow rate of 0.45 mL/min. The buffer B gradient was 45% to 50% (0–0.5 minutes), 50% to 68% (0.5–6 minutes), 68% (6–7 minutes) and 68% to 45% (7–8 minutes). DHPLC was carried out at the nondenaturing temperature, 50°C. Analysis was performed using the Star Workstation version 5 (Varian, Walnut Creek, CA).

SNP Genotyping

Assays were designed to type eight SNPs (Table 1, Figure 1) across the GDF9 locus using a Sequenom MassARRAY genotyping system (Sequenom, San Diego, CA). DNA oligonucleotides were purchased

Table 1

Single Nucleotide Polymorphisms at the GDF9 Locus Genotyped in Families of Mothers of DZ Twins

SNP ID	dbSNP #	Location	Change (transcribed strand)	Frequency minor allele
1	rs30177	5′ region	C>G	0.261
2	rs30179	5′ region	T>C	0.423
3	rs30180	5′ region	T>C	0.435
4	rs39830	5′ region	T>C	0.422
5	rs254285	Intron–1	C>G	0.092
6	rs254286	Exon–2	T>C	0.425
7	rs10491279	Exon–2	G>A	0.171
8	rs809140	Genomic	G>T	0.094

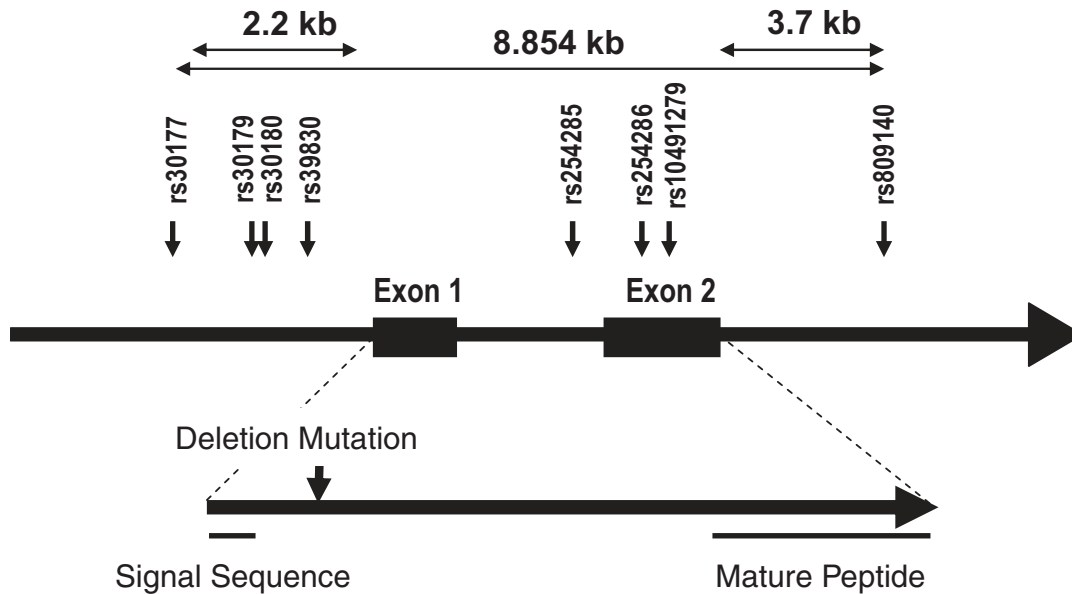Note: Locations of SNPs in the GDF9 gene are shown in Figure 1.

Figure 1

Schematic representation for the genomic structure of GDF9 gene located on human chromosome 5q31.1, showing the position of a deletion mutation identified in one family of mothers of twins and locations of eight SNPs genotyped in our twinning families.

from GeneWorks (Australia). PCR primers have a 10–base tag (5'–ACGTTGGATG–3') added so that they will not interfere in mass spectra. The final concentration of PCR primers varied from 50 nM to 200 nM for different SNPs. PCR reactions were performed in 5μl volumes, which contained 15ng DNA, PCR reaction buffer, 200 μM dNTPs, 2.5 mM MgCl2 and 0.1U of HotStar Taq Polymerase (Qiagen, Valencia, CA). The PCR conditions were: 15 minutes at 95°C, 45 cycles of 20 sec at 95°C, 30 sec at 56°C and 1 minute at 72°C, followed by extension at 72°C for 3 minutes. To remove excess dNTPs from the PCR products, shrimp alkaline phosphatase (Sequenom, San Diego, CA) was added and the reactions were incubated at 37°C for 20 minutes (followed by 5 minutes at 85°C for enzyme inactivation). Homogeneous Mass Extension (h–ME) reactions were performed in 9 μl volumes, which contained 300 nM extension primers, termination mix and 0.576 U ThermoSequenase (Sequenom, San Diego CA). The h–ME conditions were: 94°C for 2 minutes, followed by 94°C for 5 seconds, 52°C for 5 seconds, and 72°C for 5 seconds for 55 cycles. All reactions were carried out in GeneAmp 9700 thermocycler (Applied Biosystems).

SpectroCLEAN resin (Sequenom, San Diego, CA) was used to desalt h–ME products before reaction products were dispensed onto 384-format SpectroCHIPs (Sequenom, San Diego, CA) by Robodesign (Carlsbad, CA, USA). A Biflex matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometer (Bruker, Billerica, MA) was used to determine h–ME reaction product masses. Both spectra and genetic information were automatically interpreted from the MassARRAY genotype analyzer.

Data Analysis

The program Sib-pair (http://orpheus.qimr.edu.au/Programs/sib-pair.html) was used to calculate allele and genotype frequencies. SNPs were genotyped within panels of families collected for linkage analysis. We have therefore tested for association taking account of the family nature of the data using a 'measured genotypes' approach (Boerwinkle & Sing, 1987). We used case control comparisons to test genotype association by contingency χ^2 testing with p values simulated allowing for the family nature of the

Table 2

Genotypic Association between Dizygotic Twinning and Single Nucleotide Polymorphisms at the GDF9 Locus Genotype

SNP	N genotyped	Genotypic c2	p-value*
rs30177	3092	6.3	.060
rs30179	3069	3.1	.260
rs30180	3114	1.6	.556
rs39830	3115	2.5	.318
rs254285	3125	0.7	.769
rs254286	3158	4.9	.142
rs10491279	3140	0.7	.741
rs809140	3079	0.8	.909

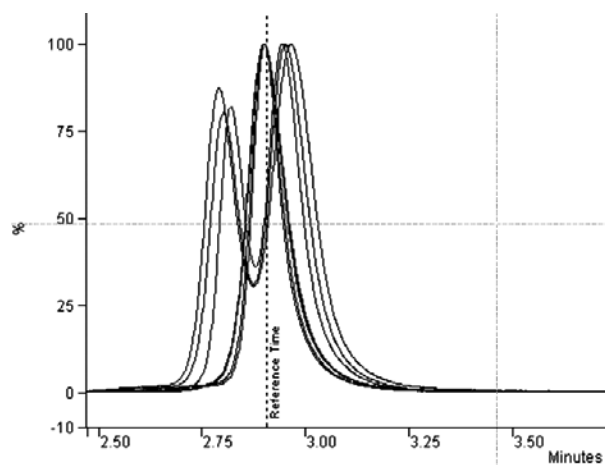Note: *Empiric p-values simulated allowing for the family nature of the data.

Figure 2

Elution curves from DHPLC analysis of individual PCR products from GDF9 spanning exon 1 and the region containing a four-base pair-deletion mutation.
Five samples show the normal allele with a single peak centered on the dotted line marked reference time. Three samples are heterozygous for the deletion and show a characteristic double peak flanking the peak for the normal allele.

data (gene dropping). Two-parent, one-locus transmission/disequilibrium tests (TDT) to examine allele transmission to MODZT were performed using Sib-pair. Haplotypes were imputed using SNPHap (Chiano & Clayton, 1998) and haplotype transmission was analyzed using Sib-pair.

Results

Complete sequence data were obtained for 20 mothers of twins from families with a high twin density. Forward and reverse sequencing for one individual from a Dutch twinning pedigree suggested the presence of an insertion/deletion variant in the first exon. The two alleles for this individual were cloned and several clones were sequenced. A four-base pair deletion was confirmed on one allele. The deletion begins 207 base pair (bp) from the start of exon 1 (third position in codon 69) and alters the reading frame for the protein introducing a stop codon at codon position 88 of exon 1 (Figure 1).

The four-base pair deletion could be readily detected by DHPLC (Figure 2). The original case was from an extended family including a sister, aunts and a cousin with spontaneous DZ twins (Figure 3). Samples were available from the parents, one aunt and a cousin with twins. DHPLC analysis of these samples (Figure 2) confirmed the presence of the heterozygous deletion in the proband and showed the deletion was also carried by her sister and her father. The deletion was passed by the father to both daughters. The mother and other related family members with DZ twins did not carry the deletion mutation. We screened DNA

samples available from all MODZT from 429 twinning families using DHPLC. No other carriers of the deletion mutation were observed.

Complete DNA sequences for GDF9 were aligned from the remaining mothers of twins. No variants were identified that were common to all mothers of twins from these twinning families. DNA sequencing demonstrated that three SNPs from public databases (rs254285, rs254286, rs10491279) were polymorphic in our families. Two SNPs in exon 2 (rs254286, rs10491279) do not change the amino acide composition of the protein.

Further SNPs were identified from public databases and eight SNPs across the GDF9 locus were typed in our families (Figure 1) using the Sequenom MassARRAY genomics platform. The assays were designed in three multiplex sets. Minor allele frequencies for the eight SNPs ranged from 0.092 to 0.435 (Table 1). Genotype data for all SNPs were in Hardy–Weinberg equilibrium. There were no differences in allele frequencies between samples from ANZ and Netherlands families. Association between GDF9 and twinning was tested by comparing genotype and allele frequencies between mothers of twins and controls. We tested genotypic association where the effects of each genotype (penetrance ratios) are estimated (Table 2) and allelic association which estimates a multiplicative effect on the penetrance for different alleles. There was no evidence for association between DZ twinning and individual GDF9 SNPs. We also tested association using a TDT analysis in the twinning families and found no association between DZ twinning and GDF9 (data not shown).

There was strong linkage disequilibrium across the GDF9 locus. One haplotype accounted for 57% of GDF9 chromosomes in our families and four common haplotypes accounted for more than 95% of chromosomes (Table 3). The haplotype allele frequencies for mothers of twins were similar and not significantly

Table 3

Frequency of GDF9 Haplotypes (rs30177, rs30179, rs30180, rs39830, rs254285, rs254286, rs10491279, rs809140)

Haplotype	Controls	Mothers of twins*	Overall proportion
CTTTCTGC	0.568	0.578	0.571
CCCCCCAC	0.156	0.156	0.158
GCCCCCGC	0.147	0.139	0.146
GCCCGCGT	0.082	0.082	0.080
GTTTCTGC	0.012	0.010	0.010
CCCCCCGC	0.010	0.011	0.010
CTCTCTGC	0.011	0.002	0.007
GCCCCCAC	0.007	0.005	0.006
Other*	0.010	0.017	0.012

Note: *There were no significant differences between controls and mothers of twins.
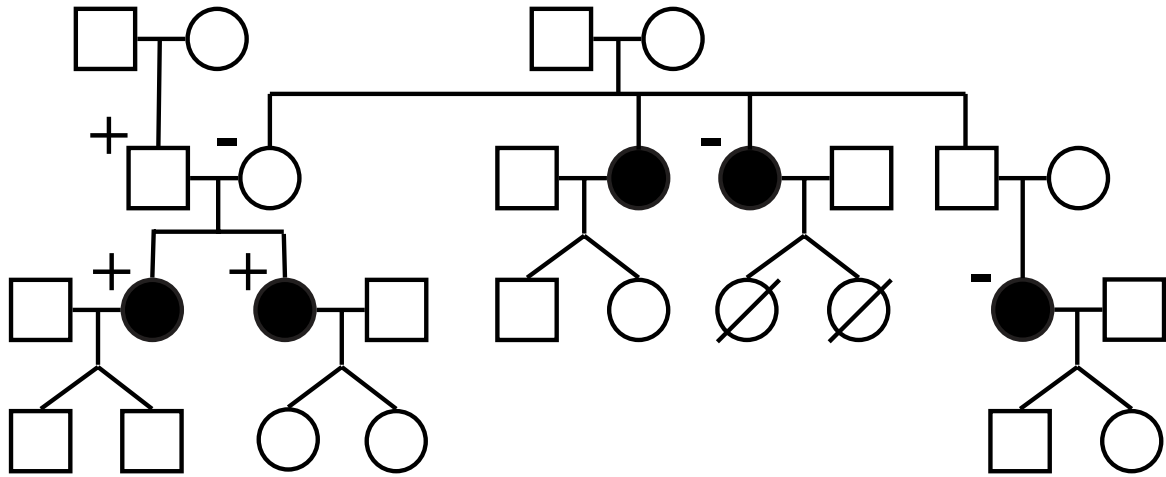
Figure 3

Family with deletion mutation in GDF9.

Mothers of DZ twins are shown with black circles. Individuals with a + symbol are heterozygous for the deletion, while those with a – symbol do not carry the deletion. DNA samples were not available for individuals without symbols.

different from the allele frequencies for controls (Table 3). The frequencies of transmitted and nontransmitted haplotypes to MODZT in both ANZ and Netherlands families where parents were informative were similar and did not differ significantly (Table 4).

Discussion

GDF9 is an oocyte-derived growth factor essential for follicle growth. The GDF9 protein stimulates primordial and primary follicle growth through roles in granulosa and theca cell function at multiple stages of follicle development (Vitt et al., 2000). We sequenced GDF9 in women with DZ twins from 20 of the families with the highest density of twins. We identified a four-base pair deletion mutation on one copy of the GDF9 gene in three individuals from a family with five sets of DZ twins. The mutation was located in the prepro region of GDF9. It alters the reading frame and introduces a stop codon 18 amino acids downstream of the deletion before the sequence coding for the mature protein. Two sisters with spontaneous DZ twins and their father were heterozygous for the deletion mutation, which arose on the most common

haplotype (Table 3). The mother did not carry the mutation despite the presence of three other sets of DZ twins in the family on the mother's side.

GDF9 and BMP15 are closely related and expressed specifically in oocytes in several species (Laitinen et al., 1998; McGrath et al., 1995; Otsuka et al., 2000). Heterozygous loss of function mutations in both BMP15 and GDF9 increase the frequency of twins in sheep (Galloway et al., 2000; Hanrahan et al., 2004). In the sheep, homozygous carriers of the loss of function mutation in GDF9 are infertile (Hanrahan et al., 2004). Species differences in the roles of GDF9 and BMP15 have been reported. Sheep carrying homozygous knockout mutations in BMP15 are infertile (Galloway et al., 2000), similar to GDF9 knockout mutations in the mouse (Dong et al., 1996). However, BMP15 knockout mice are subfertile rather than infertile (Yan et al., 2001). The data might suggest BMP15 plays a more critical role in the sheep, whereas GDF9 is more essential in the mouse. However, it appears that heterozygous loss of function mutations in both BMP15 and GDF9 in the sheep result in very similar increases in ovulation rate

Table 4

Common Haplotypes Transmitted to Affected Sisters with DZ Twins from Australia and New Zealand (ANZ) and the Netherlands

Haplotypes	Proportion transmitted	ANZ families		Netherlands families	
		Trans	p-value	Trans	p-value
CGTTTCTGC	0.493 (0.441–0.544)	127/267	.463	49/90	.461
CGCCCCCAC	0.534 (0.463–0.603)	85/150	.120	18/43	.360
GACCCCCGC	0.518 (0.442–0.593)	70/129	.379	15/35	.500
GACCCGCGT	0.531 (0.439–0.622)	35/73	.815	24/38	.143

and twinning (Galloway et al., 2000; Hanrahan et al., 2004). Based on evidence from the sheep, the mutation identified in this family is likely to contribute to the incidence of twins in the affected sisters. However, it cannot be the cause of the other sets of twins in the family and we did not find this deletion mutation in mothers of DZ twins from any of our other families.

Genetic factors contributing to DZ twinning are probably complex (Bulmer, 1970; Meulemans et al., 1996). It is likely that contributions arise from variation of small to moderate effects in many genes and variation contributing to twinning at more than one gene could be segregating within a family. The presence of a deletion mutation in GDF9 in sisters with twins that is not present in mothers of twins from elsewhere in the family is compatible with complex inheritance models expected for traits such as twinning. An alternative explanation is the deletion mutation is not associated with twinning in the sisters and a gene (or genes) inherited from the mother predisposes to twins. Variants inherited from the mother could act together with the deletion in GDF9 (Hanrahan et al., 2004).

We compared the sequence for the coding region of GDF9 in mothers of twins to sequences from public databases and control individuals. We detected three previously reported SNPs, but did not detect any variants specific to mothers of twins. We did not sequence the promoter region or the large intron in GDF9. Analysis of eight SNPs across the GDF9 locus including SNPs in the promoter and 3'UTR showed strong linkage disequilibrium with four haplotypes accounting for 95% of chromosomes. Tests of association between SNPs in GDF9 and DZ twinning phenotype were not significant. There was no evidence that common variation within the human GDF9 locus contributes to variation in DZ twinning.

The loss of function mutation in GDF9 may increase twinning while common variation does not alter gene expression or protein function sufficiently to influence the twinning phenotype. The dose of intact BMP15 and GDF9 alleles influences the destiny of the oocyte during folliculogenesis and in the peri ovulatory period (Yan et al., 2001). In the sheep, variants in BMP15 and GDF9 known to increase twinning are all loss-of-function mutations (Galloway et al., 2000; Hanrahan et al., 2004), suggesting that changes in concentration or complex interactions of these growth factors are necessary to increase twinning. In vitro studies suggest GDF9 and BMP15 form homo- and heterodimers and that the point mutation in BMP15 in Inverdale sheep interferes with processing of heterodimers (Liao et al., 2003). We have demonstrated that loss of function mutations in GDF9 exist in the human. We did not find this deletion in other families. However, we have not conducted a systematic screen for mutations in GDF9 in all our families and a low frequency of other mutations altering GDF9 expression or protein function could exist in some twinning families.

In conclusion, we identified a heterozygous loss of function mutation in GDF9 in two sisters with DZ twins. The mutation may contribute to an increased frequency of twins, but was not present in other families with DZ twins. Rare mutations in GDF9 may influence twinning, but there was no evidence that common variation at the human GDF9 locus contributes to the frequency of dizygotic twins.

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