Identification of common genetic variants

influencing spontaneous dizygotic twinning

and female fertility

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Abstract

Spontaneous dizygotic (DZ) twinning occurs in 1-4% of women, with familial clustering and unknown ~~patho~~physiological pathways and genetic origin. DZ twinning may index increased fertility and has distinct health implications for mother and child. We performed ~~the first~~ a GWA study in 1,980 mothers of spontaneous DZ twins and 12,953 controls. Findings were replicated in a large Icelandic cohort and tested for association across a broad range of fertility traits in women. Two SNPs were identified (rs11031006 near *FSHB*, $p = 1.54 \times 10^{-9}$, and rs17293443 in *SMAD3*, $p = 1.57 \times 10^{-8}$) and replicated ($p = 3 \times 10^{-3}$ and $p = 1.44 \times 10^{-4}$, respectively). Based on ~90,000 births in Iceland, the relative risk of a mother delivering twins increased by 18% for each copy of ~~the~~ allele rs11031006-G, and 9% for ~~the~~ rs17293443-C. A higher polygenic risk score (PRS) for DZ twinning, calculated based on the results of the DZ twinning GWAS, was significantly associated with DZ twinning in Iceland ($p = 0.001$). A higher PRS was also associated with having children ($p = 0.01$), greater lifetime parity ($p = 0.03$) and earlier age at first child ($p = 0.02$). ~~The~~ Allele rs11031006-G was associated with higher serum FSH levels, earlier age at menarche, earlier age at first child, higher lifetime parity, lower PCOS risk, and earlier age at menopause. Conversely, ~~the~~ rs17293443-C was associated with later age at last child. We identified ~~the first~~ robust genetic risk variants for DZ twinning: one near *FSHB*, and a second within *SMAD3*, ~~—~~the product of which plays an important role in gonadal responsiveness to FSH. These loci contribute to crucial aspects of reproductive capacity and health.

Introduction

DZ twinning (MIM: 276400) is defined as the concomitant conception and development of two independent zygotes during one pregnancy. Mothers of spontaneous (conceived without assisted reproductive technology) DZ twins have a predisposition to multiple ovulation events due to interference with single dominant follicle selection, a biological mechanism fundamental for the human species.[1] DZ twinning is common, with large regional differences from six per 1000 births in Asia to 40 per 1000 births in Africa,[2] whereas monozygotic twinning occurs around the world at a constant rate of around three to four per 1000 births.[3; 4] DZ twinning rates also vary substantially over time. For example, in the US, the observed incidence of twin births increased by a factor of 1.9 between 1971 and 2009.[5] While a considerable part of the increase is attributable to fertility treatments, with an estimate of 36% of all twins born in the USA in 2011 resulting from assisted reproduction, the majority of twins are still conceived spontaneously. In addition to fertility treatments, increases in maternal age contribute to increases in twinning incidence.[3; 6]

Twinning is associated with increased risks to mother and offspring, including higher risks of stillbirth, neonatal death and premature birth. Compared to singleton children, twins use more hospital resources, especially during the first year of life,[7] with hospital costs in the first five years of life being as much as 3·3-fold higher.[8]

Mothers of DZ twins differ from other women in that they are taller, have an increased BMI, are more often overweight and more often smoke before the twin pregnancy.[9] Family history, increased parity and gravidity all increase the risk of spontaneous DZ twinning.[1; 3] Remarkably, twinning rates do not reflect average nutritional status of a population, as established from longitudinal studies in countries that experienced periods of starvation, such as the Dutch hunger winter. Above a specific, yet undetermined, threshold, nutrition seems to be of minimal importance for reproduction in general and also for twinning.[10] These

observations all point to spontaneous twinning being a heritable trait and suggest the potential for polygenic inheritance. In a landmark study of twinning based on data from genealogic records from Utah,[11] White and Wyshak established that the genotype of the mother, but not that of the father, affects the frequency of DZ twinning.

The underlying physiological mechanism for DZ twinning is the release and fertilization of two or more oocytes. Ovarian folliculogenesis and determination of ovulation quota are controlled both by circulating concentrations of follicle-stimulating hormone (FSH) and by intra-ovarian factors including the two oocyte growth factors, *GDF9* (MIM: 601918) and *BMP15* (MIM: 300247), as well as their cognate receptors.[12] In the common marmoset monkey (subfamily Callitrichinae), DZ twins comprise the predominant litter size, and singletons are rarely, if ever, observed. On DNA sequencing, specific nonsynonymous substitutions were identified in *GDF9*, *BMP15*, *BMP4* (MIM: 112262), and *WFIKKN1* (MIM: 608021) as having a role in Callitrichine twinning.[13] These genes are among a larger set of 63 candidate genes with a potential involvement in regulation of ovulation number and/or control of growth and body size.[14]

Efforts to characterize the genes that contribute to DZ twinning in humans have not been successful. Candidate gene and genome-wide linkage studies failed to uncover common variants associated with DZ twinning,[15-19] although one study reported rare variants in *GDF9* to be associated with DZ twinning.[18]

DZ twinning has been suggested as a measure of human fertility both at the individual and at the population level.[20] Spontaneous DZ twinning may be considered as a marker of high fertility, as it reflects the frequency of double ovulation, the probability of coitus within the appropriate time frame with fertilization of both ova, and maintenance of a multiple pregnancy.

The aim of this study was to perform ~~the first~~ a genome-wide association study (GWAS) in mothers of spontaneous DZ twins to identify relevant genomic regions and test their significance across a broad range of female fertility and reproductive traits including age at menarche, age at natural menopause, age at first and last child, and lifetime parity. Three twin registries, from the Netherlands, Australia and Minnesota (USA) had detailed information on spontaneous twinning in mothers of DZ twins, as well as genotype data. Replication of top hits for twinning was possible in the Icelandic population and for other measures of reproductive ageing in several large-scale population meta-analyses[21-24].

Material and Methods

~~Details of methods and associated references can be found in the supplemental data.~~

Descriptions of Participating Studies

Netherlands Twin Register (NTR)

The NTR sample consisted of 806 cases and 4,535 controls from the Netherlands Twin Register (2,776 participants) and the Netherlands Study of Depression and anxiety (NESDA; 2,565 participants). NTR participants were ascertained by the presence of liveborn twins or triplets in the family and consist of multiples, their parents, siblings and spouses. Twins were born in all strata of society and NTR represents a general sample from the Dutch population[25-28]. NESDA is a longitudinal study focusing on the course and consequences of depression and anxiety disorders. Subjects for NESDA were recruited from the general population, mental health organizations and general practices. The sample includes subjects selected for depression and anxiety, as well as healthy controls[29]. Zygosity of twins was confirmed by DNA genotyping. Data on mode of pregnancy were available from several data collection waves including surveys sent out to mothers of twins, a survey to parents upon registration of young twins, and telephone interview as part of a project on DZ twinning[9]. The comparison of

the survey data with the hospital records showed that mothers can accurately report on the mode of conception of their twins[30]. Participants were excluded if they reported the use of assisted reproductive technology at one or more occasions. In case no reports on mode of pregnancy were available, data were excluded unless the twins were born prior to 1985.

QIMR Berghofer Medical Research Institute (QIMR)

The sample used in this analysis consisted of 606 cases and 6,656 controls. The individuals were drawn from families containing (any type of) twins recruited for prior studies, either from around Australia from the Australian Twin Registry (ATR) (generally twins born before 1971) or from south-eastern Queeensland (generally twins born after 1980). Study recruitment was predominantly population based (any family where the twins were willing to participate) with no screening performed on reproductive phenotypes apart from selecting families with twins. Studies for the older cohort typically were focused on personality traits but not selected for them. Zygosity of twins was reported at time of recruitment; during phenotyping studies; and tested by genotyping (on SNP arrays or Sequenom assays). New phenotyping excluded mothers ('cases') from Queensland cohort, who used Assisted Reproductive Technology (ART–typically IVF or hormone treatment) to become pregnant. Screening questions asked of the mother during clinical sessions for phenotyping, were the primary basis for excluding ART cases. A smaller subset of mothers was contacted specifically to establish this information where it was not otherwise available. For the older (ATR) cohort, at the time of the twins' birth, IVF was not yet in clinical use and other ART was rare.

Minnesota Center for Twin and Family Research (MCTFR)

All subjects in this sample were independently ascertained through vital records of the State of Minnesota in an effort to construct a population-based twin registry[31; 32]. The sample for the current study consisted of 568 mothers of DZ twins and 1,862 controls who were the parents of MZ twins from 1,062 families, including 800 complete parental pairs, 203 mothers

and 59 fathers. Most of the twins were born in the 70s or early 80s, when even though fertility treatment was available in the US, it was expensive and few had access to it. Genotyping was population-based and independent of phenotypes other than twinning. About 92% of the registry, and 100% of both case and control samples, are of primarily European ancestry.

Iceland (deCODE)

Mothers of twins or other multiples ("twins") were selected from among those taking part in deCODE's genetic studies based on a nationwide genealogical database. To increase the proportion of these mother of twins who were mothers of dizygotic twins, twins who had been genotyped and shown to be monozygotic were not used to identify mothers. Controls were individuals participating in deCODE's genetic research from which both mothers of twins and the mothers' first-degree relatives had been removed. For the prediction of twinning using polygenic risk scores, mothers having opposite sex or verified dizygotic twins were compared with mothers who did not have twins.

Study Design, Genome-Wide Association Study and Replication

We established the Twinning GWAS Consortium (TGC) to characterize the genetic basis of DZ twinning in humans and performed ~~the first~~ a genome-wide association study (GWAS) for DZ twinning utilizing data from 1,980 mothers of DZ twins (MODZT) and 12,953 controls from ~~The Netherlands, US and Australian~~ European ancestry cohorts. Sample sizes and study characteristics are described in table ~~S~~1. All cases underwent screening to exclude mothers who received assisted reproductive techniques. Controls were screened to exclude pedigrees containing DZ twins. In the replication stage, significant findings from the meta-analysis were tested in large Icelandic cohort of 3,597 mothers of twins and 297,348 controls. All participants provided written informed consent, including consent for genotyping and

analysis, and were recruited according to the protocols approved by the institution review board of each institution.

Fertility Traits Measures

Fertility measures (having children, number of children, age at first and last child and average birth interval) were defined in females born before 1970 based on the Icelandic genealogy. Data for age at menarche, age at menopause and polycystic ovary syndrome were derived from previously published GWAS consortia[21-24]. Sample sizes and study characteristics are described in table 2.

Genotyping, Quality Control and Imputation

Each participating cohort performed participant-level genotyping of single nucleotide polymorphisms (SNPs), that included standard quality-control measures for genotyping and imputation (Table S~~2~~1 and Supplemental Methods). All imputations were performed using the 1000 Genomes Project March 2012 release as the reference panel.

~~**Statistical analysis**~~

~~GWA analysis was performed in each cohort using logistic regression under an additive genetic model with adjustment for principal components of genetic ancestry and specific covariates for each study. GWA results for each SNP (odds ratio [OR] and 95% confidence interval [CI]) were combined across cohorts using fixed-effect meta-analysis with inverse variance weighting. The most significant SNP at each genome-wide significant ($p<5\times10^{-8}$) locus was tested in the replication stage and replicating SNPs were examined for association with FSH levels and other fertility traits.~~

Association Tests

Each genome-wide association analysis from the three cohorts was conducted using logistic regression under an additive genetic model with adjustment for principal components of genetic ancestry. Because the GWAS data include family members we added the –family option in the analysis, which takes the familial structure of the data into account using a sandwich estimator[33]. Imputed SNPs were analyzed using PLINK software[34] and genotype imputation uncertainty was accounted for by using allelic dosage in PLINK.

Meta-analysis was performed using the fixed-effects inverse variance method based on the regression β estimates and standard errors from each study implemented in METAL[35]. The presence of heterogeneity between cohorts for the effect sizes of risk alleles was investigated using the Cochran's *Q*-test as implemented in METAL. To determine whether the genome-wide significant signal at each locus with low LD in the same chromosomal region (defined as $r^2 < 0.05$ in a 750-kb region) could be accounted for by a single SNP, we carried out conditional analysis. Each cohort performed a genome-wide analysis for MODZT using logistic regression adjusting for the top signal at each of the three associated regions to determine whether potential second signals remained significant even after adjusting for these variants. Results from each individual study were meta-analyzed to determine whether these potential second signals were truly independent (that is, if $p < 5 \times 10^{-8}$). In Iceland, being a mother of twins was tested for association with the top alleles using logistic regression and including age, age-squared and county of birth as covariates as described previously[36]. Associations between FSH level and genotype was assessed using linear regression as described previously[36]. To study the association of fertility measures and SNP genotype, we used logistic regression (having children), Poisson log-linear regression (number of children), or linear regression (age at first child, age at last child, and average birth interval). In these analyses, birth cohort (as a factor for each five year interval), county of birth and six principal components were included as covariates. Relatedness was controlled for by using genomic

control in all Icelandic association analyses[37]. The combined p value of the meta-analysis and the replication study was calculated using Fisher's combined probability test[38].

FSH Serum Level Measure

Serum levels of follicle-stimulating hormone (FSH) were measured in 2,411 men (1,275 genotyped persons; 1,136 close relatives of genotyped individuals) and 15,586 women (9,738 genotyped persons; 5,848 close relatives of genotyped individuals) referred to three clinics in Iceland. FSH testing was undertaken primarily to investigate possible gonad impairment. Hormone levels were measured by electrochemiluminescence immunoassay, using reagents and analytical instruments from Roche Diagnostics GmbH, according to the manufacturer's instructions.

***In silico* Functional Annotation**

We used a number of publicly available bioinformatics tools and datasets to identify putative functional effects of the top associated SNPs at each locus, including: Combined Annotation Dependent Depletion (CADD)[39], HaploReg2 v4[40] and Variant effect predictor (VEP)[41].

Gene Based Test

Results of the MODZT meta-analysis were used to perform a gene-based test of association for the 63 candidate genes from Harris et *al.* using the *Knowledge-based mining system for Genome-wide Genetic studies* (KGG) software Version 3.5[42; 43]. This approach uses an extended Simes test that integrates prior functional information and the meta-analysis association results when combining the SNP p values within a gene in order to obtain an overall association p value for each entire gene. As we tested for genetic association for 63 genes, the significance level was set at 7.93×10^{-4} (Bonferroni correction; 0.05/63).

Polygenic Risk Scores (PRS)

Polygenic risk scores were calculated based on the results of the MODZT meta-analysis. Only markers having info > 0.9 in all groups and MAF > 0.01 were included. To obtain effect sizes taking LD into account, the LDpred method developed by Vilhjálmsson and colleagues was used[44]. As suggested by Vilhjálmsson et al, we calculated multiple sets of LD-modified effect sizes based on a grid of values for the fraction of causal markers (α = 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1). The resulting scores were then tested in a validation data set of Icelandic mothers of dizygotic twins. The score producing the most significant result in the validation data set were subsequently used to test for association with five fertility-related traits ("has children", "number of children", "age at first child", "age at last child" and "average birth interval").

Results

Genome-wide association results, replication and gene based analyses

The overall GWAS meta-analysis genomic control statistic (λ) was 1.01, indicating no appreciable inflation due to population structure. The quantile-quantile (Q-Q) plot of genome-wide p values showed a strong deviation from the null hypothesis of no association (Figure S1). The results are represented in the Manhattan plot (Figure 1). Three chromosomal regions contained genome-wide significant SNPs ($p < 5 \times 10^{-8}$). Twenty-two SNPs on chromosome 11p13 showed genome-wide significant associations. Of these, the strongest signal (rs11031006, $p = 1.54 \times 10^{-9}$ and OR 1.41, 95% CI 1.29-1.53) lies in the region 5' of the transcription start site of *FSHB* (encoding the FSH, beta polypeptide, [MIM: 136530]). A second locus was represented by five SNPs on *1q42.13* covering an intergenic region flanked by *PGBD5* (MIM: 616791) and *COG2* (~~[~~MIM: 606974~~]~~), ($p = 1.23 \times 10^{-8}$, OR 1.43, 95% CI

1.31-1.55 for strongest signal, rs12064669). A third locus on *15q22.33*, mapped to the first intron of *SMAD3* (SMAD family member 3, [MIM: 603109]) (rs17293443, $p = 1.57 \times 10^{-8}$ and OR 1.27, 95% CI 1.19-1.35). No significant heterogeneity in SNP effects was observed across cohorts for the top SNPs ($p > 0.1$, Cochran's *Q* test) ~~;~~ Tables ~~1~~ 3 and S2~~3~~). The regional association plots for these loci are shown in figure S2. After conditioning on the top SNPs at each locus, no secondary signals were observed (all $p > 0.05$; Tables S~~4~~3, S~~5~~4 and S~~6~~5). We sought validation of these three top signals in an independent replication study from Iceland (deCODE) totaling 3,597 mothers of twins and 297,348 controls. The *FSHB* (rs11031006, $p = 3 \times 10^{-3}$, OR 1.14, 95% CI 1.06-1.22) and *SMAD3* (rs17293443, $p = 1.44 \times 10^{-4}$, OR 1.15, 95% CI 1.07-1.23) loci replicated, but rs12064669 (p=0.88) was not confirmed (Table ~~1~~3 and Figure S~~3~~2). We also investigated whether any of the proposed 63 candidate genes[14] was associated with human DZ twinning. In a gene-based test, five genes demonstrated a nominally significant association ($p < 0.05$; *BMPR1A* [MIM: 601299], *BMPR1B* [MIM: 603248], *IGF1* [MIM: 147440], *FSHB* and *FSHR* [MIM: 136435]). After correction for multiple testing, only *FSHB* remained significant (Table S~~7~~6).

Functional *in silico* annotation of associated variants

We explored plausible functional effects of our associated variants using Combined Annotation Dependent Depletion (CADD).[39] The *FSHB* SNP rs11031006 had a high Phred scaled C-score (22.4), indicating that it is among the top 1% of SNPs in the human genome most likely to have a functional effect. The Phred score for the *SMAD3* SNP rs17293443 was only 2.71 indicating that it is among the bottom 50% of SNPs in the human genome likely to have a functional effect (Table S~~10~~7). Examination of individual constituents of the CADD scores showed particularly high conservation-based scores for rs11031006 (Figure S~~6~~3). To further investigate possible functional effects we examined data from the ENCODE project.[45]

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The *FSHB* SNP rs11031006 alters the sequence of 11 protein-binding motifs including that of the Estrogen receptor alpha, indicating a possible effect on hormonal feedback inhibition (Table S~~11~~8). Variant Effect Predictor (VEP)[41] identified rs17293443 as a regulatory region variant within a promoter flanking region (ENSR00000410126) (Figure S~~7~~4). SNP rs17293443 was contained in a *DNase I* hypersensitive site suggesting open chromatin, although it did not alter any of the transcription factor binding sites present in the promoter flanking region. Together, these data indicate that rs11031006 and rs17293443 may have direct functional roles.

SNPs associated with FSH serum level

We analyzed serum FSH measurements from 17,997 genotyped Icelanders and their close relatives. SNP rs11031006 was significantly associated with higher serum FSH levels ($p = 2.3 \times 10^{-10}$), with each G allele conferring an increase in FSH level of about 0.11 SD units (Figure S~~5~~4). Notably, the allele (rs11031006-G) conferring the strongest association with FSH levels in the FSH GWAS is the same allele that conferred the greatest chance of having DZ twins in our MODZT GWAS. No association was seen between the *SMAD3* signal and serum FSH levels ($p = 0.30$).

Relative risk of twin birth and polygenic risk score prediction of DZ twinning and fertility measures

We estimated that the relative risk of a twin birth, based on approximately 90,000 births in Iceland between 1950 and 1991, was increased by 18% for each maternal rs11031006-G allele and by 9% for each rs17293443-C allele ~~(Table S8)~~. A higher polygenic risk score (PRS) for DZ twinning, calculated based on the results of the DZ twinning GWAS, was significantly associated with DZ twinning in our independent Icelandic cohort ($p = 0.001$) (Table S7). A higher PRS was also associated with a higher likelihood of having children ($p =$

0.01), higher lifetime number of children (p = 0.03), and an earlier age at first child (p = 0.02) (Table S~~9~~8). A re-calculated PRS, excluding the 1 Mb regions surrounding the two replicated variants, remained associated with DZ twinning (p = 0.02) and with the likelihood of having children (p = 0.03). These results reflect the polygenic contribution to the susceptibility to DZ twinning and its association with greater reproductive ability.

SNPs associated with female reproduction traits

Table ~~2~~4 reports on the two loci robustly implicated in DZ twinning and other reproductive traits in women. Consistent with its effects on higher circulating FSH levels, the rs11031006-G allele is also associated with earlier age at menarche,[22; 46] earlier age at first child and higher total lifetime number of children, lower risk of polycystic ovary syndrome (PCOS),[23] and earlier age at natural menopause.[24; 47] Also, the DZ twinning SNP rs11031006 is correlated with a variant (rs10835638, *FSHB*-211G>T) located in the promoter of *FSHB* (r^2 = 0.62) that is associated with timing of breast development in girls.[48] In contrast, the rs17293443-C allele in *SMAD3* was associated only with a later age at last child (Figure S~~6~~5).

~~**Functional *in silico* annotation of associated variants**~~

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Discussion

Here we report the ~~first~~ compelling evidence that sequence variation at the *FSHB* and *SMAD3* loci increases the odds of DZ twinning. A number of studies in mothers of DZ twins, but not all,[49] have found higher FSH levels responsible for multiple follicle growth.[50] The associations of *FSHB* rs11031006-G with earlier ages at breast development, menarche, menopause, first child, and higher lifetime parity indicates that this locus plays an important role in multiple reproductive aspects. Female carriers of rs11031006-G likely may have a more advanced depletion of the ovarian follicular pool and hence would have an increased risk of premature ovarian failure (POF, [MIM: 612964]). Indeed advanced ovarian aging is a recognized feature of familial DZ twinning, with reported lower levels of anti-Mullerian Hormone (AMH) a marker of lower ovarian primordial follicular reserve.[51] The rs17293443-C allele in *SMAD3* also increases chances of DZ twinning, but this effect appears independent of circulating FSH levels.

Of the 63 suggested candidate genes for twinning, only *FSHB* was associated in gene-based tests after correcting for multiple testing. Emerging data in human and non-human primates suggest that mechanisms underlying multiple ovulation may differ among species, explaining why in some species twinning is accompanied by unique evolutionary adaptations enabling offspring's survival, such as the marmoset diminutive fetal size in a simplex uterus,

and subsequent alloparenting.[14] Conversely, in humans, multiple gestations remain an independent risk factor for preterm birth, pregnancy loss, and fetal growth restriction. Thus loci common to species and strains with higher rates of DZ twinning will not necessarily be shared, as the rate, complications, and future reproductive fitness of those twin gestations differ.

The third genome-wide hit from the discovery was not replicated in Iceland. SNP rs120644669 is located 149kb from the angiotensinogen (*AGT*, [MIM: 106150]), which influences ovulatory capacity in mice,[52] and 89kb from component of oligomeric golgi complex 2 (*COG2*) affecting protein glycosilation, which regulates the biological activity of the pituitary gonadotrophins.[53]

Genetic variants near *FSHB* (rs11031005 and rs11031002, which are highly correlated with rs11031006) are reportedly associated not only with higher serum FSH, but also lower LH levels.[54] This agrees with the known response of the gonadotrophic cell to a high frequency hypothalamic pulsatile GnRH signal that reciprocally controls secretion of both hormones.[55] Furthermore, under normal conditions suppression of FSH in the early follicular phase and higher LH levels in late phase typically favor mono-ovulation in the human.[56] In a study aiming to identify genetic predictors for IVF success or IVF-controlled ovarian stimulation (COS), rs611246 located in *FSHB* ($r^2 = 0.3$ with rs11031006), was reported significantly associated with measured early follicular phase FSH values and also with the probability of clinical pregnancy, suggesting that these genetic variants are potential predictor candidates that could be considered in clinical ovarian reserve and function assessment in assisted reproduction.[57]

A recent linkage study in cattle reported only one strong signal ($p < 1 \times 10^{-28}$) for ovulation rate in a region spanning *SMAD3*, *SMAD6* (MIM: 602931) and *IQCH* (MIM: 612523).[58] *SMAD3* encodes one of a family of proteins that function as signal transducers and

transcriptional modulators that mediate multiple signaling pathways. Observations in mice have established an essential role for Smad3 in mediating TGFbeta and activin signals in the ovarian granulosa cell and also in the pituitary[59] to maintain a favorable environment for oocyte maturation.[60] Smad3 is strongly expressed in the human ovary, where it promotes granulosa cell proliferation and steroidogenesis possibly by upregulating gonadotrophin receptor signaling pathways.[61] Thus, sequence variation in *SMAD3* may increase chance of DZ twinning by increasing responsiveness to FSH. Understanding the role of *SMAD3* will offer novel opportunities to optimize responsiveness and minimize risk among assisted reproduction technology (ART) recipients for example through adjustment of hormonal stimulation and thus contributes to prevention of life-threatening ovarian hyper stimulation syndrome in hyper responding female carriers of the rs17293443-C allele and conversely prevention of a poor response in ~~patients~~ individuals with allelic variants that lead to a poor response.

The potential applications of this work in reproductive medicine are multifold. Firstly, it reveals a well-defined set of loci for DZ twinning in the general population. Secondly, twinning is associated with common perinatal morbidities such as preterm birth, discordant twin growth, latter prenatal asymmetric intrauterine growth restriction, and placental abruption. Multiple gestations are also related to a higher prevalence of maternal morbidities such as preeclampsia, postpartum hemorrhage, and ensuing complications. By understanding the genetic basis of DZ twinning, we concomitantly identify loci conferring susceptibility (or conversely resistance) to these prevalent perinatal comorbidities. The involvement of *SMAD3* is of relevance in the light of a possible phenotype in male DZ twins namely the repeatedly reported more frequent occurrence of testicular seminoma, a gonadal tumor in which the mitogenic cyclin D2 is overexpressed.[62; 63] In animal experimental studies knockout of *SMAD3* significantly attenuates cyclin D2 and tumor proliferation.[64] This indicates to the

likely clinical potential of *SMAD3* stretching beyond that of fertility physiology and treatment.

This study ~~The sequence variants found to influence spontaneous DZ twinning and their relationship with other fertility-related measures,~~ provides important insights into ovarian functioning and the control of natural multiple follicle growth and reproductive aging. This has important implications for fertility, including improved outcome prediction and novel avenues of fertility treatment. Other strengths include the rigorous phenotype inclusion of mothers of DZ twins with a documented history of spontaneous DZ twinning. It is worth mentioning that analyses without excluding mothers who conceived their twins with hormone induction of multiple ovulation or other ART, did not yield to any genome-wide significant results (results not shown). We thus recommend twins registries collect data on mode of pregnancy of twins. There are also some limitations. The current effort focused on unraveling the genetic basis of DZ twinning in European-ancestry populations only. However, the highest incidence of DZ twins was reported in the Nigerian population and some other countries from Africa. Next steps in unraveling the genetic cause of DZ twinning need to include mothers of DZ twins originating from these regions, and if possible also from regions where DZ twinning is a rare trait, such as Japan[2].

In summary, we identified *FSHB* and the ~~novel~~ *SMAD3* locus as maternal susceptibility loci for DZ twinning. These loci are also significantly and specifically associated with several other aspects of reproductive capacity and health.

Supplemental Data

Supplemental data include ~~seven~~ six figures and ~~11~~eight tables

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Web Resources

1000GenomesProject, ftp://ftp-trace.ncbi.nih.gov/1000genomes/ ftp/release/20110521/

CADD, http://cadd.gs.washington.edu/

HaploReg, http://www.broadinstitute.org/mammals/haploreg/haploreg.php

Variant Effect Predictor (VEP), http://www.ensembl.org/info/docs/tools/vep/index.html

Knowledge-based mining system for Genome-wide Genetic studies (KGG), http://grass.cgs.hku.hk/limx/kgg/

ENCODE, https://genome.ucsc.edu/ENCODE/

OMIM, http://www.omim.org/

LDpred, https://bitbucket.org/bjarni_vilhjalmsson/ldpred

MACH, http://www.sph.umich.edu/csg/abecasis/MACH/

Minimac, http://genome.sph.umich.edu/wiki/Minimac

Beagle, https://faculty.washington.edu/browning/beagle/b3.html

PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/

LocusZoom, http://csg.sph.umich.edu/locuszoom/

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Figures titles and legends

Figure 1. Manhattan Plot for the genome-wide association meta-analysis of mothers of dizygotic twins. SNPs are plotted on the *x*-axis according to their position on each chromosome against the significance of the association on the *y*-axis (shown as –log10 p

values). The dotted red line denotes $p=5\times10^{-8}$ statistical significance. The arrows point to the chromosomal region that reached genome-wide significance level**.**

Figure 2. Forest plots depicting risk allele odds ratio (OR) estimates at (A) rs11031006 (near FSHB), (B) rs17293443 (SMAD3) and (C) rs12064669 from each study, the meta-analysis, deCODE (replication) and the overall combined results. Black squares indicate the OR and horizontal lines represent the 95% CIs. The combined results are indicated by the black diamond.

Tables titles and legends

Table 1. Number of cases and controls included in the meta-analysis and replication; mean maternal age at delivery

Table 2. Sample characteristics for fertility measures traits

Table ~~1~~3. Genome-wide significant loci in the meta-analysis of mothers of spontaneous DZ twins (n = 1,980) and replication (n = 3,597) versus screened controls

Table ~~2~~4. Association results for the implicated DZ twinning SNPs in fertility-related measures

Table 1. Number of cases and controls included in the meta-analysis and replication; mean maternal age at delivery

Cohort	No. of subjects		Mean maternal age at delivery (SD)
	Cases	Controls	
Netherlands	806	4,535	29.7 (4.1)
Australia	606	6,556	29.8 (4.8)
Minnesota	568	1,862	28.3 (4.5)
Total discovery	1,980	12,953	29.3 (4.5)
Replication			
Iceland	3,597 (1,356 genotyped; 2,241 *in silico*)	297,348 (76,342 genotyped; 221,006 *in silico*)	30.3 (5.8)

Table 2. Sample characteristics for measure of fertility

Traits	Sample size	Mean (SD)
Age at menarche	259,247	-[1]
Age at menopause	69,360	-[1]
FSH levels	17,997	-
Has children	41,946	0.94[2]
Number of children	41,946	2.9 (1.6)
Age at first child	41,946	23.0 (4.7)
Age at last child	41,946	32.1 (5.5)
Average birth interval	41,946	4.7 (2.5)
Polycystic ovary syndrome	5,184 cases / 82,759 controls	-[1]

[1] this is a large meta-analysis of summary statistics and sample mean is not available
[2] fraction with children;

Table ~~1~~3. Genome-wide significant loci in the meta-analysis of mothers of spontaneous DZ twins (n=1,980) and replication (n=3,597) versus screened controls

SNP	Locus	Position[a]	Gene	Annotation	Risk allele	Meta-analysis			Replication			Combined
						RAF	OR (95%CI)	p	RAF	OR (95% CI)	p	p
rs11031006	*11p14.1*	30226528	*FSHB*	5' upstream	G	0.85	1.41 (1.29-1.53)	1.54×10^{-9}	0.85	1.14 (1.06-1.22)	3×10^{-3}	1.25×10^{-10}
rs17293443	*15q22.33*	67437863	*SMAD3*	Intron	C	0.24	1.27 (1.19-1.35)	1.57×10^{-8}	0.21	1.15 (1.07-1.23)	1.44×10^{-4}	6.29×10^{-11}
rs12064669	*1q42.13*	230688643		Intergenic	C	0.10	1.43 (1.31-1.55)	1.23×10^{-8}	0.07	1.01 (0.89-1.13)	0.88	2.09×10^{-7}

[a]SNP position according to NCBI Human Genome Build 37; RAF, risk allele frequency; OR, odd ratio; 95% CI, 95% confidence interval

Table 24. Association results for the implicated DZ twinning SNPs in fertility-related measures

	rs11031006-G allele (near *FSHB*)			rs17293443-C allele (in *SMAD3*)		
DZ twinning and fertility-related measure	**Effect**	**OR (95% CI) / Beta (95% CI)**	**p value**	**Effect**	**OR (95% CI) / Beta (95% CI)**	**p value**
DZ twinning[a]	Increase	1.41 (1.29, 1.53)	1.54×10^{-9}	Increase	1.27 (1.19, 1.35)	1.57×10^{-8}
FSH levels (SD units)[b]	Increase	0.11 (0.078, 0.15)	2.3×10^{-10}	–	0.016 (-0.014, 0.045)	0.30
Age at menarche (years)[c,d]	Earlier	-0.04 (-0.012, 0.011)	8.5×10^{-10}	Earlier	-0.001 (-0.127, 0.010)	0.84
Age at menopause (years)[e]	Earlier	-0.2165 (-0.065, 0.052)	8.5×10^{-14}	–	0.009 (-0.048, 0.049)	–0.71
Has children (yes/no)[f]	–	1.07 (0.98, 1.17)	0.12	–	0.96 (0.89, 1.04)	0.34
Number of children[f]	Increase	0.014 (0.00091, 0.27)[h]	0.03	–	0.0048 (-0.0062, 0.016)[h]	0.39
Age at first child (years)[f]	Earlier	-0.20 (-0.31, -0.086)	5.3×10^{-4}	–	0.032 (-0.065, 0.13)	0.51
Age at last child (years)[f]	Earlier	-0.097 (-0.21, 0.015)	0.08	Later	0.14 (0.043, 0.24)	4.7×10^{-3}
Average birth interval (years)[f]	–	0.015 (-0.037,	0.57	–	0.0051 (-0.040,	0.82

		0.067)			0.050)	
PCOS[g]	Decrease	0.90 (0.84, 0.95)	~~1~~2.~~3~~9×10^{-49}	–	1.00 (0.96,1.05)	0.78

[a]MODZT GWAS; [b]deCODE sample of 17,997 individuals with FSH levels; [c,d]~~Lunetta~~ [d]Day *et al*~~.~~ . and *Perry et al.* (ref 21 and 22~~21) and Perry *et al.* (ref 22~~); [e]Day *et al*. (ref ~~24~~24) ~~and Stolk *et al.* (ref 25)~~; [f]deCODE sample of 41,946 women; [g]Polycystic ovary syndrome, ~~Hayes~~ Day *et al*. (ref 2~~2~~3); [h] factor of increase from log-linear model.~~.~~