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Genome-wide Association Study Identifies Candidate Genes for Male Fertility Traits in Humans

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Despite the fact that hundreds of genes are known to affect fertility in animal models, relatively little is known about genes that influence natural fertility in humans. To broadly survey genes contributing to variation in male fertility, we conducted a genome-wide association study (GWAS) of two fertility traits (family size and birth rate) in 269 married men who are members of a founder population of European descent that proscribes contraception and has large family sizes. Associations between ~250,000 autosomal SNPs and the fertility traits were examined. A total of 41 SNPs with $p \leq 1 \times 10^{-4}$ for either trait were taken forward to a validation study of 123 ethnically diverse men from Chicago who had previously undergone semen analyses. Nine (22%) of the SNPs associated with reduced fertility in the GWAS were also associated with one or more of the ten measures of reduced sperm quantity and/or function, yielding 27 associations with p values < 0.05 and seven with p values < 0.01 in the validation study. On the basis of 5,000 permutations of our data, the probabilities of observing this many or more small p values were 0.0014 and 5.6×10^{-4}, respectively. Among the nine associated loci, outstanding candidates for male fertility genes include *USP8*, an essential deubiquitinating enzyme that has a role in acrosome assembly; *UBD* and *EPSTI1*, which have potential roles in innate immunity; and *LRRC32*, which encodes a latent transforming growth factor β (TGF-β) receptor on regulatory T cells. We suggest that mutations in these genes that are more severe may account for some of the unexplained infertility (or subfertility) in the general population.

Introduction

Infertility is a common reproductive disorder, affecting 10%–15% of couples in Western countries, with nearly equal contributions from male and female partners.[1] This high prevalence is not surprising, considering the precise regulation of diverse processes required for successful reproduction. For example, defects in reproductive tract development, gametogenesis, sex determination, sexual behavior, and endocrine and immunologic dysfunction can limit fertility in both sexes.[2] Almost a quarter of infertility cases are unexplained, mostly due to our poor understanding of basic molecular mechanisms underlying male and female fertility.[2] Mutagenesis screens in animal models have identified hundreds to thousands of genes that influence reproductive success,[2–5] and it has been suggested that up to 50% of infertility in humans can be attributed to genetic abnormalities.[6] Moreover, evidence for genes that also influence normal variation in fertility is provided by quantitative-trait loci (QTL) mapping studies in mice, flies, pigs, and cattle; these studies have identified genetic loci that affect reproductive characteristics in those species.[7–15] However, in most QTL mapping studies, the associated regions were too broad to allow for the discovery of specific genes that affect reproductive traits.

Additionally, studies of fertility traits in humans are further complicated by the many nongenetic factors, such as socioeconomic status, education level, cultural beliefs, and religious dictates, that influence human reproductive behavior. Accordingly, the concept of "cultural inheritance" has been put forth to explain correlations in family sizes between parents and offspring in some studies.[16–21] As a result, nearly all previous genetic studies of human reproduction have been candidate-gene studies in men or women with infertility (or subfertility) and fertile controls (e.g., references [22–27]), which led to the identification of mutations in only a handful of genes essential for reproduction.[28] However, such studies have not been able to identify novel genes or pathways that contribute to variation in natural human fertility.

To overcome the limitations inherent in genetic studies of human fertility, we focused our studies on the Hutterites, a founder population of European descent.[29,30] The Hutterite communal lifestyle and strict adherence to religious doctrine ensure that variation in nongenetic factors that affect reproductive practices is minimized between individuals, providing an ideal population in which to study the genetics of normal human fertility. In particular, Hutterites traditionally proscribe contraception and uniformly desire large families. As a result, median sibship size was > 10 in the 1960s,[31] and the mean interbirth interval was < 2 years during the same period.[32] With relatively few (~2%) childless couples, the Hutterites are among the most fertile human populations.[32,33] As a first step in assessing genetic contributions to human fertility, we defined heritable measures of fertility in the Hutterites and confirmed the presence of significant genetic components of natural variation in fertility,[34] leading us to propose that reproductive traits should be amenable to genetic mapping studies in this population.

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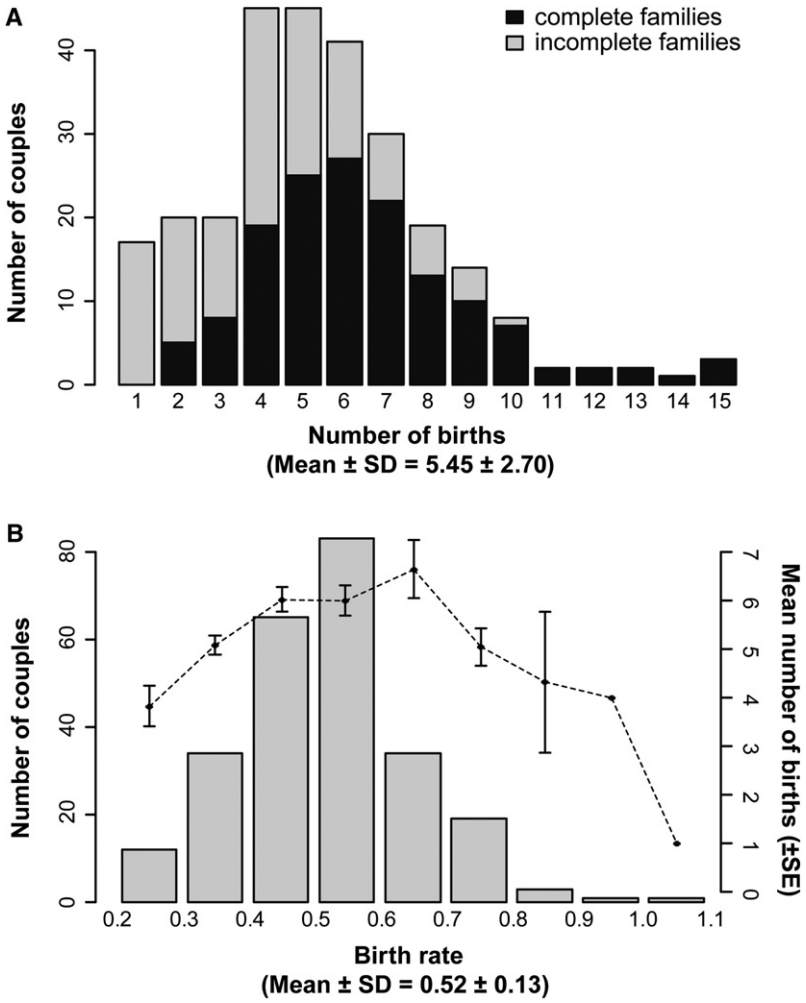

Figure 1. Distribution of Reproductive Phenotypes in 269 Hutterite Men

(A) Family sizes. Black bars represent completed families (either the wife is > 45 years old, or the couple has not had a child in > 6 years; n = 146), light gray bars represent incomplete families (n = 123). Due to the high number of couples who have not completed their families, the number of years from marriage to last birth was used as a covariate in the analysis of family size, in order to adjust for the length of the reproductive period.

(B) Birth rates, defined as number of births per year of marriage. Mean number of births for the couples in each interval are shown in diamonds (right x axis); SE is shown as a vertical bar if there are more than three couples for each interval.

Here, we present the results of a genome-wide association study (GWAS) for two reproductive phenotypes in Hutterite men: family size and birth rate. To validate the most significant associations in the Hutterites and to assess the functional or clinical relevance of associated loci, we genotyped the most highly associated SNPs in DNA from 123 ethnically diverse men from Chicago, who had previously undergone semen analyses. We report the discovery of nine loci that influence both overall fertility in Hutterite men and sperm parameters, which are indicators of testicular function, reproductive development, and fertilizing potential in ethnically diverse men. We propose that mutations in these genes that are more severe may account for some cases of unexplained infertility in the population at large.

Subjects and Methods

The Hutterite Sample and Measures of Fertility

The Hutterites are a young founder population that originated in South Tyrol in the 16th century and migrated to the United States in the 1870s. Today, their more than 40,000 descendants live on communal farms in the northern United States and western Canada.[29,30] The subjects of this study are 269 married Hutterite men living in South Dakota who are a subset of the couples previously described[34] and have a DNA sample available for genotyping. All of these subjects can be traced back to 64 ancestors and are related to each other in a 13-generation pedigree consisting of 3,657 individuals.

The details of data compilation and measures of fertility are reported in detail elsewhere.[34] In brief, we obtained birth, death, and marriage dates from records compiled by the Hutterite ministers, and we obtained reproductive histories during in-person interviews with married or widowed women that elicited information on births, miscarriages, fertility treatment, birth-control use, ages at menarche and last menses, medication use, and other potentially confounding conditions.[35–37] All of our studies of the Hutterites were population-based, and participation within each colony was high (>95%); therefore, there are no known ascertainment biases that could affect the interpretation of our results.

We included in this study Hutterite men with at least one child and considered two quantitative measures of fertility: family size and birth rate (Figure 1). Family size refers to the number of births (counting multiple births as one); birth rate was calculated for each couple with two or more children (n = 534), as (number of births − 1) / (sum of the interbirth intervals). For both traits, we fit a linear regression model, using the following covariates: wife's birth year (which is highly correlated with husband's birth year; Pearson r = 0.98) to adjust for cohort effects,[33] wife's age at marriage to adjust for maternal age effects (mean ± SD = 23.0 ± 2.8 years), and years from marriage to last birth to adjust for the length of the reproductive period and the incompleteness of some families (mean ± SD = 10.4 ± 5.4 years). Residuals from the regression model for both family size and birth rate were normally distributed and used to estimate variance components and in tests of association. Childless couples were excluded from the analyses because the cause of infertility in most cases was unknown and could therefore add noise to the data and potentially mask the effects of male-specific fertility genes. Both traits are highly heritable in Hutterite men (broad heritability of family size [H^2] = 0.72, SE = 0.20; broad heritability of birth rate [H^2] = 0.65, SE = 0.20), consistent with our previous report.[34] The two traits are highly, but not perfectly, correlated (Pearson r = 0.72). Therefore, we conducted

a GWAS of both traits to maximize our chances of finding associations in this relatively small sample. These studies were approved by the institutional review board at the University of Chicago.

Genotyping and Statistical Analyses in the Hutterites

Of the 553 Hutterite husbands whose wives were interviewed, 269 with DNA available were genotyped with one of three Affymetrix SNP arrays: GeneChip 500K Mapping Array (n = 158), Genome-Wide Human SNP Array 5.0 (n = 14), or Genome-Wide Human SNP Array 6.0 (n = 97). Because the number of SNPs on each array differed (and therefore not all men had genotypes at every SNP), we only included SNPs that were common to all three genotyping arrays (corresponding to at least 240 genotyped men or ~90% of the total sample size). From the combined set of 369,487 autosomal SNPs present on all three genotyping platforms, 94,471 were removed prior to analyses because they either were monomorphic in the sample (n = 31,246) or had minor allele frequencies $\leq$ 5% (n = 63,137). An additional 26,894 were excluded because of low call rates (<95%; n = 6,433), high Mendelian error rates ($\geq$5; n = 14,496), or deviations from Hardy-Weinberg expectations at $p < 0.001$ after correcting for the Hutterite inbreeding and population structure (n = 5,965).[38] The remaining 248,210 SNPs were included in the GWAS.

Using the genotypes at the same markers for ~1,400 Hutterites available in our lab (including most of the wives and children of the men included in this study), we performed Mendelian error checks using PedCheck[39] and confirmed the accuracy of the pedigree relationships using PREST.[40] On the basis of these quality checks and the fact that the Hutterites are a strictly monogamous community, we were able to confirm paternity in all families.

Associations with male fertility traits were tested using a regression-based test, designed for large, complex pedigrees,[41] as previously described.[42,43] When reviewing the GWAS results, we grouped together SNPs that were near each other and had the same "closest" gene, and we referred to the associated region as an independent locus. We then picked one SNP per locus that showed the most significant evidence of association for the subsequent validation studies.

In addition, we had exome-sequence data for 25 Hutterites who were not first degree relatives of each other, and who had the largest number of descendants in the (Affymetrix) genotyped portion of the pedigree.[44] To assess the effect of coding variation at each validated locus (see next section), we imputed genotypes to other members of the pedigree using the method described in Uricchio et al.[45] Association tests were repeated for the imputed SNPs as described above. One SNP, which showed a nominal association with family size (rs3739474, $p < 1 \times 10^{-3}$), was genotyped with a TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) in all Hutterite individuals for the validation of the imputation accuracy and maximization of the number of genotypes available for association testing.

Validation Studies in Outbred Men from Chicago

To validate associations detected in the GWAS and to assess the functional or clinical effects of associated SNPs, we obtained DNA from semen samples from 123 men who had undergone semen analyses at the University of Illinois at Chicago's (UIC) Andrology Laboratory. Because these were anonymized samples, only age, ethnicity, and the results of semen analysis were available for each subject. The composition of this sample was 58.5% (n = 72) Hispanic, 26.0% (n = 32) African American, 8.9% (n = 11) Middle Eastern, 4.1% (n = 5) individuals of European ancestry, and 2.4% (n = 3) Asian. Although we did not have information on the reason for the referral, we assume that most of the subjects were referred for infertility evaluation. DNA was extracted from semen samples at the University of Chicago after analyses were completed at the UIC. These studies were approved by the institutional review boards at the University of Chicago and the UIC.

Semen samples were obtained after 3–4 days of sexual abstinence, allowed to liquefy at room temperature, and analyzed within 1 hr of sample collection by the same technologist with a computer-assisted sperm analyzer, which measures four parameters of sperm count (sperm concentration, total volume, total sperm count, and total motile sperm count) and six parameters of sperm motility (% motility, % progressive motility, average velocity, mean amplitude of lateral head displacement [ALH], linearity, and beat frequency; Table S1 available online). Pairwise correlations between sperm parameters are shown in Table S2. We note that there was considerable variation between men for each of the parameters assessing sperm count and sperm motility.

Genotyping and Statistical Analyses in Men from Chicago

One SNP with the strongest association at each of the 41 loci, at $p < 10^{-4}$ in either GWAS, was selected for validation studies in the men from Chicago. Genotyping in this cohort was performed with TaqMan allelic discrimination assays (Applied Biosystems) or the iPLEX MassARRAY platform (Sequenom, San Diego, CA, USA), in accordance with the manufacturers' instructions. Two targeted SNPs, rs12049958 and rs10088000, failed the assay design and were replaced by rs1514673 and rs13265504, respectively, which are in perferct linkage disequilibrium ($r^2 = 1$) with the two targeted SNPs in the HapMap CEU (Utah residents with ancestry from northern and western Europe) population.

To minimize deviation from a normal distribution, we subjected each sperm parameter to a square or square-root transformation, as appropriate. Ethnicity of the patient was included as a covariate for all traits, except for volume and beat frequency (for which ethnicity was not a significant predictor). Age was a significant predictor only of volume and was included as an additional covariate for this trait. We tested associations between each validation SNP and semen parameters through linear regression (or Student's t test with Welch correction for unequal variances, where appropriate), requiring that the model being tested (additive, recessive, or dominant) and the direction of the effect for a given allele (increased or decreased fertility) were consistent with the initial association observed in the Hutterites. For the assessment of significance of the associations, 5,000 permutations were performed in which the genotypes for 41 SNPs were considered together and randomly permuted between individuals. The resulting permutations retained correlations between both phenotypes and genotypes in our data. From these permutations, p values were calculated as the proportion of test statistics rejected, where the test statistic considered the model and direction of effect observed in the Hutterites. These empirical p values account for multiple testing (ten sperm parameters × 41 SNPs). All statistical analyses were conducted with R statistical software.[46]

Results

GWAS in the Hutterites

No associations reached genome-wide levels of significance (approximately $p \leq 10^{-7}$) in either GWAS (Figure S1). Therefore, we used a liberal threshold of $\leq 10^{-4}$ to identify SNPs for validation studies in the men from Chicago. We expect, on average, approximately 25 p values to be smaller than 10^{-4} by chance alone. We observed 61 SNPs at this level of significance in the GWAS of family size and 25 in the GWAS of birth rate (Table S3).

The 61 SNPs with p values $\leq 10^{-4}$ in the GWAS of family size were located at 28 independent loci. Forty-three of these SNPs are located within the coding regions or in close vicinity of (±100 kb) 23 different genes at 22 loci; the remaining 18 SNPs (9 loci) were located > 100 kb (range = 104–646 kb) from the closest known gene (Table S3A). The 25 SNPs with p values $\leq 10^{-4}$ in the GWAS of birth rate defined 15 loci. Twenty-three of these SNPs are within or close to (±100kb) 18 different genes at 15 loci; the remaining two SNPs were > 100 kb (102 and 572 kb) from the closest known gene (Table S3B). Despite the fact that the phenotypes of family size and birth rate were highly correlated ($r = 0.72$), the GWAS p values were less so (Spearman's $\rho = 0.30$), and only four SNPs in two regions had p values $\leq 10^{-4}$ in both GWAS.

In summary, the 82 SNPs with $p \leq 10^{-4}$ in either GWAS represent 41 independent loci for family size (n = 26), birth rate (n = 13), or both (n = 2). We then selected the most significant SNP at each locus for validation studies and to assess the potential functional or clinical significance of these associations.

Association with Semen Analysis Parameters in the UIC Cohort

Alleles or genotypes for nine of the 41 SNPs that were associated with reduced family size (n = 5) or reduced birth rate (n = 4) in the Hutterites were also associated with reduced measures of sperm count and/or motility in the men from Chicago, at $p < 0.05$ (after correcting for multiple testing by permutation) (Table 1; complete results for all 41 loci and ten traits are shown in Table S5). Six of these SNPs were associated with multiple parameters, reflecting the high correlation between many of these measures (Table S2). Mean ALH showed the largest number of associations, with five SNPs having $p < 0.05$, while % progressive motility had no significant associations with any of the SNPs (Table 1). Overall, we observed 27 associations with p values < 0.05 and seven associations with p values < 0.01 (on the basis of 5,000 permutations that maintain the correlation structure between the ten parameters and 41 genotypes). The fractions of permutations yielding as large or larger numbers of associations with a $p < 0.05$ or $p < 0.01$ were 0.0014 and 5.6×10^{-4}, respectively. That is, on average, only seven of 5,000 permutations yielded 27 or more p values < 0.05, and three yielded seven p values < 0.01. Box plots in Figure 2 show the most significant associations at each of these nine SNPs in the Chicago sample and at that same SNP with birth rate or family size in the Hutterite sample.

Because the men from Chicago were ethnically diverse, it is possible that the associations we observed were due to population substructure if, for example, both phenotypes and allele frequencies differed between the groups in the same direction as that observed in the Hutterite GWAS. To address this possibility, we normalized each phenotype within each ethnic group to obtain distributions centered around zero and with standard deviations of one and then repeated the association tests as described above. We observed only small changes in p values (data not shown), which did not affect the overall interpretation of our results. In addition, when we restricted the analyses to Hispanic individuals only (the only population with a sample size large enough to perform analyses), we observed that the direction of effect remained the same for all traits at all loci, with 18 of the 27 associations remaining significant at $p < 0.05$, despite a halving of the sample size (data not shown). Therefore, we ruled out population structure as a potential confounder in the association studies of the men from Chicago.

Lastly, there is no gametic disequilibrium between the nine SNPs associated with sperm parameters (pairwise disequilibrium, as measured by r^2, < 0.05), and therefore, we expect these SNPs to have (statistically) independent effects on fertility traits. In support of this expectation is the observation that the mean phenotypic values decrease with increasing number of risk genotypes carried by Hutterite and Chicago men (Figure 3, Figure S2).

Exome Sequence Results

Of the 11 genes (in nine regions) associated with male-fertility traits, five harbored exonic SNPs in 25 Hutterite exomes. The 11 SNPs in these five genes were imputed, on average, to 144 married Hutterite men (range = 72–226) (Table S6). Only one synonymous SNP in *PSAT1* (MIM 610936), rs3739474, showed nominal association with family size in Hutterite men ($p = 5.0 \times 10^{-4}$; n = 200). To assess imputation quality and to obtain the maximum sample size, we genotyped this SNP in the Hutterites using a TaqMan assay. There was a very high concordance (99.5%) between imputed genotypes and those obtained by TaqMan genotyping in 934 Hutterites. The number of men with genotypes at rs3739474 increased by 95 after TaqMan genotyping, so we repeated the test of association with this marker. The strength of the association did not change substantially ($p = 4.6 \times 10^{-4}$; Table S6). Importantly, this exonic SNP was less associated with family size than the SNPs near this gene on the Affymetrix arrays that showed the original associations (smallest $p = 1.0 \times 10^{-5}$). Therefore, we concluded that the observed associations in these nine regions were not driven by previously untyped coding variation in the genes located near the associated SNPs.

Table 1. Annotation of Validation SNPs and Association Statistics with Reproductive Phenotypes in Hutterites and with Sperm Parameters in Chicago Men

						Association in the Hutterites			Association with Semen Analysis Parameters in the Chicago Men (Empirical p Values from 5,000 Permutations)											
SNP	Chr	Position	Closest Gene(s)[a]	Relationship to Closest Gene	Dist. (kb)	Assoc. Trait	p Value	Model	Allele: Genotypes (2/1/0 Copies)	Freq.	Conc.	Vol.	Total Sperm Count	Total Motile Sperm Count	% Motil.	% Prog. Motil.	Avg. Veloc.	Mean ALH	Linear.	Beat Freq.
rs10966811	9	25,223,484	*TUSC1*	dwnst.	445.4	FS	5.57×10^{-06}	recessive	T: 23/49/47	0.40										0.012
rs7867029	9	80,210,238	*PSAT1*	dwnst.	75.4	FS	1.04×10^{-05}	dominant	G: 9/38/65	0.25	0.042				0.004		0.045	0.032		
rs12870438	13	42,378,205	*EPSTI1*	intron	–	FS	2.07×10^{-05}	recessive	A[b]: 5/32/85	0.17	0.005		0.024	0.023			0.001	0.001		
rs7174015	15	48,504,360	*USP8*	intron	–	FS	3.57×10^{-05}	recessive	T: 39/52/24	0.57		0.016	0.001	0.006			0.040	0.010		
rs10129954	14	72,220,454	*DPF3*	intron	–	FS	3.86×10^{-05}	recessive	T: 50/60/12	0.66				0.034					0.016	0.044
rs680730	11	116,980,443	*DSCAML1*	intron	–	BR	2.45×10^{-05}	additive	T[b]: 12/58/50	0.34									0.005	
rs11236909	11	76,116,716	*TSKU, LRRC32*	upst., upst.	55.2, 58.0	BR	6.11×10^{-05}	additive	C[b]: 8/43/70	0.24							0.018	0.015	0.035	
rs10488786	11	100,245,404	*ARHGAP42*	intron	–	BR	8.70×10^{-05}	additive	T: 0/11/106	0.05										0.021
rs724078[c]	6	29,597,027	*MAS1L, UBD*	upst., dwnst.	33.7, 34.4	BR	9.95×10^{-05}	recessive	T: 26/59/34	0.47			0.023	0.018	0.041			0.027		

Chr, chromosome; Dist., distance; Freq., frequency; Conc., sperm concentration; Vol., total volume; % Motil., % motility; % Prog. motil., % progressive motility; Avg. Veloc., average velocity; ALH, amplitude of lateral head displacement; Linear., linearity; Beat Freq., beat frequency; dwnst., downstream; upst., upstream; FS, family size; BR, birth rate.

Only SNPs for which there was at least one significant association (p value ≤ 0.05, after 5,000 permutations) with the sperm parameters in the Chicago men are shown; complete results for all 41 SNPs are presented in Table S5. For each SNP, physical location and the closest gene are shown. Distances are reported from the 5′-end of the gene if the SNP is located upstream and the 3′-end of the gene if the SNP is located downstream with respect to the gene. In the Hutterite men, the trait that showed initial association in the GWAS (family size or birth rate) and the association model for the minor allele in this population (Table S3) are shown. In the Chicago men, genotype counts and the frequencies of the Hutterite minor alleles are reported first, followed by empirical p values after 5,000 permutations for all ten sperm parameters tested (model and direction of effect are matched with the Hutterite associations). All sperm parameters were adjusted for race, and in addition, total volume was adjusted for age (see Subjects and Methods). The reported alleles are associated with lower fertility measures, unless otherwise noted.

[a]Gene names (commonly used alternative gene symbols are shown in parentheses): *TUSC1*, tumor suppressor candidate 1; *PSAT1*, phosphoserine aminotransferase 1; *EPSTI1*, epithelial stromal interaction 1; *USP8* (*UBPy*), ubiquitin-specific peptidase 8; *DPF3*, D4, zinc and double plant homeodomain fingers, family 3; *DSCAML1*, Down syndrome cell-adhesion-molecule-like 1; *TSKU*, Tsukushi small leucine-rich proteoglycan homolog (*Xenopus laevis*); *LRRC32* (*GARP*), leucine-rich repeat-containing 32; *ARHGAP42*, Rho GTPase-activating protein 42; *MAS1L*, MAS1 oncogene-like; *UBD* (*FAT10*), ubiquitin D.

[b]Reported allele is associated with increased fertility.

[c]SNP is a predicted *cis* eQTL for *DDR1* in lymphoblastoid cell lines.

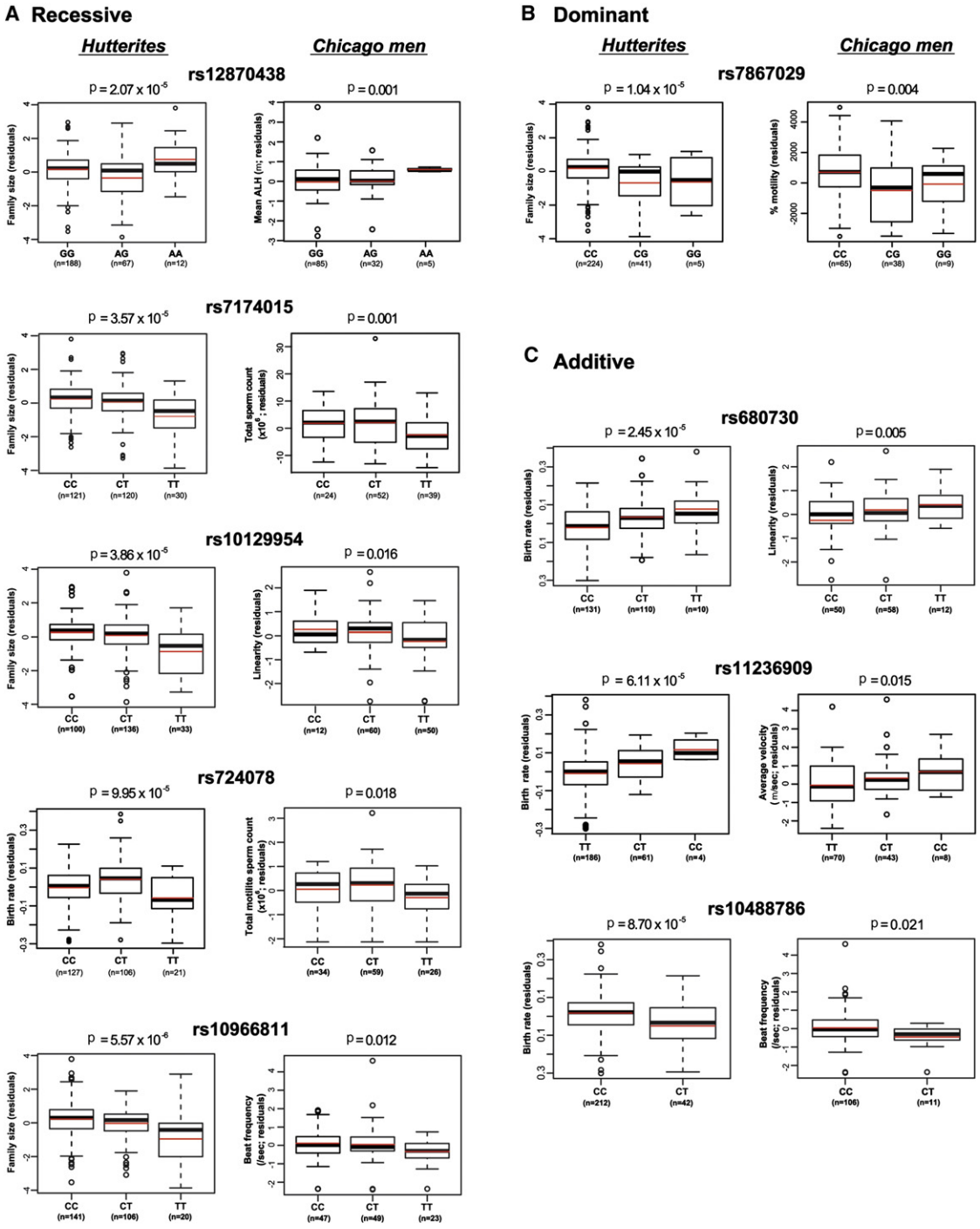

Figure 2. Effects of the Associated SNPs on Family Size and Birth Rates in the Hutterite Men; Sperm Count and Motility Parameters in the Chicago Men

Most significantly associated parameters are shown for each SNP. SNPs are grouped according to whether the minor allele in the Hutterite men shows an (A) recessive, (B) dominant, or (C) additive effect. The boxes in the boxplots show the first and third quartiles; the whiskers extend to the minimum and maximum values excluding outliers. Black horizontal lines show the medians and red horizontal lines show the means for each genotype group. Sample sizes are shown under each genotype on the x axes. p values correspond to the single-locus SNP-specific GWAS p values in the Hutterite men and empirical p values from 5,000 permutations in the Chicago men.

Discussion

Human fertility is a complex phenotype influenced by both environmental and genetic factors. The contribution of the latter is supported by numerous studies on model organisms,[5] as well as by the many genetic disorders that also affect human fertility (Online Mendelian Inheritance in Man [OMIM]). However, genetic studies of natural fertility in human populations have been challenging because family sizes are often deliberately limited due to

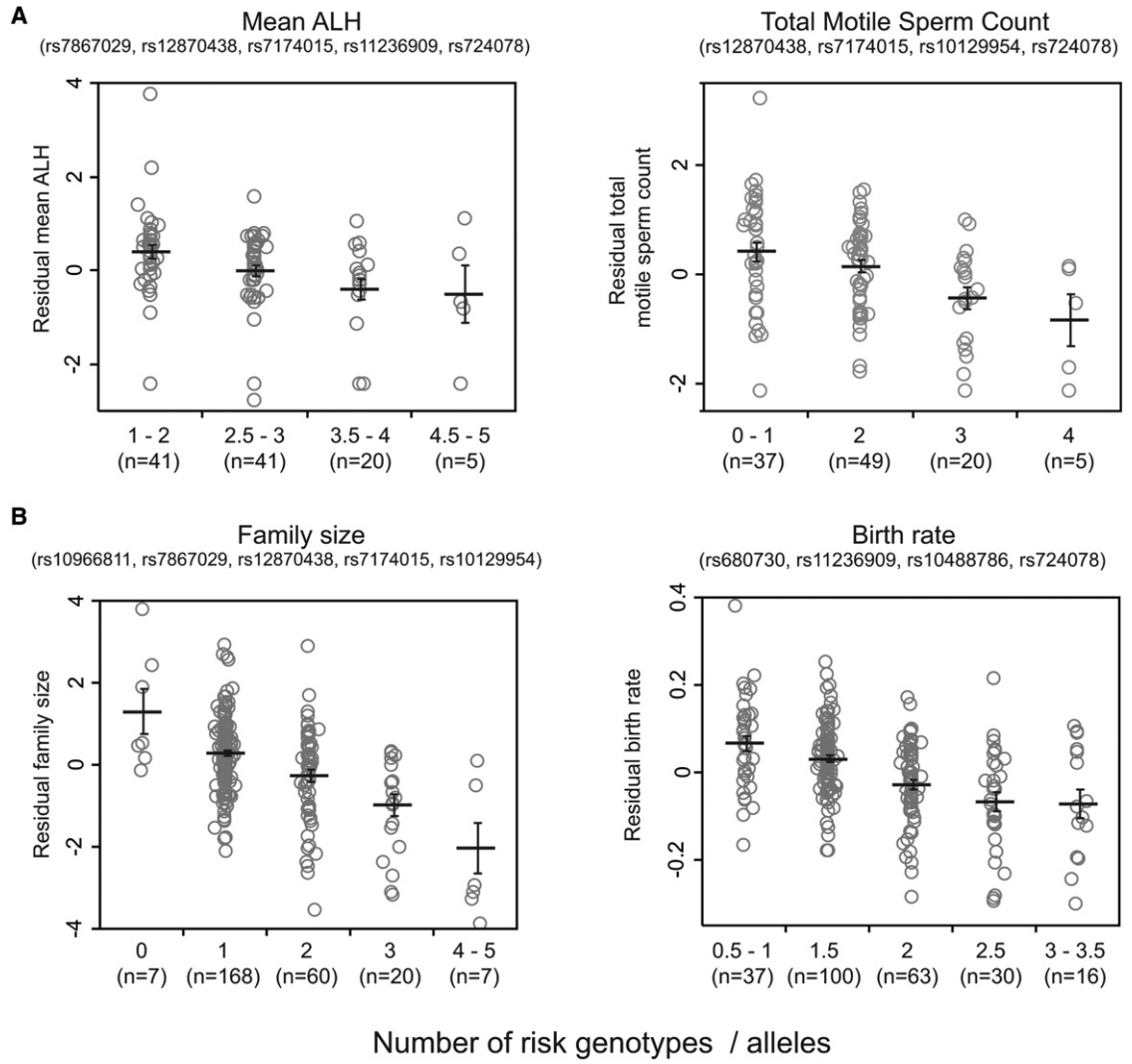

Figure 3. Combined Effects of SNPs Associated with Fertility Phenotypes

The associated SNPs for each phenotype were assessed (Table 1), and the total number of risk genotypes or alleles an individual carries were counted as presence (=1) or absence (=0) of that genotype at each locus, if the SNP had a recessive or dominant effect. For the SNPs with additive effects, presence of each risk allele was counted as 0.5 (i.e., 0 for homozygotes for the nonrisk allele, 0.5 for heterozygotes, and 1 for homozygotes for the risk allele). The counts for each trait were summed up, and the total number of risk genotypes or alleles an individual carries is shown on the x axes in each panel; sample sizes are shown in parentheses. When there were fewer than five individuals for a given count, we combined those individuals with the individuals in the adjacent group. The y axes show the distribution of each phenotype, with black bars representing the means and SE.

(A) Mean ALH and total motile sperm count in Chicago men; all eight traits with two or more associated SNPs are shown in Figure S2.

(B) Family size and birth rate in Hutterite men.

economic, sociocultural, or other nongenetic reasons. The Hutterites are an excellent population in which to study the genetics of fertility in humans because their family sizes and birth rates are likely to approximate the true human reproductive potential.[29,30,32] Yet, because the Hutterites are a relatively young population and our sampling strategy was population-based, there were only 269 married men (and only slightly more married women) with proven fertility among the Hutterites for our genetic studies. Thus, our sample size was relatively small for GWAS.

To address this limitation, we utilized a two-stage strategy. We first conducted GWAS for family size and birth rate in the Hutterites and identified candidate SNPs for validation studies. We used a relatively liberal threshold of $p < 10^{-4}$ in the first stage and carried forward one SNP from each of 41 independent regions associated with either family size or birth rate (or both) in the Hutterites. Our validation studies of these 41 SNPs were conducted in ethnically diverse men from the Chicago area. To facilitate the interpretation of our results and provide an additional level of stringency, we tested associations in the second stage using the same model and direction of effect that we observed in the Hutterites. Using this approach, we identified SNPs in nine regions that showed associations with both reduced fertility in the Hutterites (five with

family size, four with birth rate) and reduced sperm parameters in the ethnically diverse men from Chicago.

Semen analysis, including analysis of the 10 parameters considered in this study, is routinely performed in the assessment of male fertility. For example, low sperm counts (i.e., concentration), one of the leading causes of male infertility,[47] can indicate testicular dysfunction or abnormal endocrine profiles,[48] whereas low semen volume may result from congenital absence of seminal vesicles and/or vas deferens or obstruction in ejaculatory ducts.[48] Low sperm motility, on the other hand, may be due to abnormal spermatogenesis, sperm maturation, or sperm transport, and abnormal motility kinetics (i.e., velocity, linearity, ALH, and beat frequency) may affect cervical-mucus-penetration ability and fertilization potential of the sperm.[49,50] Although to our knowledge there are no studies exploring how these parameters affect reproductive outcome in men with proven fertility, we reason that variants associated with normal development of the male reproductive system and/or sperm function could help us to identify genes whose functions are essential for normal fertility and provide mechanistic insights into the associations observed in the Hutterites.

Overall, we observed 27 associations with p values < 0.05 and seven with p values < 0.01. The likelihood of observing this many or more p values < 0.05 or < 0.01 in these data was 0.0014 and 5.6×10^{-4}, respectively, reflecting a significant enrichment of small p values and indicating that many of the observed associations are unlikely to be type 1 errors. The validation of associations initially observed in the Hutterites in this ethnically diverse sample further indicates the generalizability of our results, which appear to be robust to racial or ethnic backgrounds or to differences in allele frequencies, background genes, or environment.

Six of the sperm parameter traits showed associations with multiple SNPs, and the average phenotypic values of each of those traits decreased with increasing numbers of risk genotypes or alleles (Figure 3A). Similar trends were observed for family size and birth rate in the Hutterites, when considering the five or four SNPs associated with each trait, respectively (Figure 3B). These combined results suggest that the genetic architecture of these phenotypes is likely to be polygenic, with contributions from multiple independent loci. Moreover, using exome-sequence data, we were able to exclude the possibility that coding variation in any of these genes present in the Hutterites is driving the observed associations with the fertility phenotypes. Rather, we suggest that the effects of variation at these nine loci are regulatory in nature.

To investigate this possibility further, we used publicly available gene expression data in lymphoblastoid cell lines from HapMap CEU and YRI (Yoruba in Ibadan, Nigeria) populations (SNP and CNV Annotation Database). Three of the nine SNPs were predicted expression QTLs (eQTLs) for five genes in HapMap CEU, and four SNPs were predicted eQTLs for 19 genes in HapMap YRI populations (p value $\leq 1 \times 10^{-4}$). Only one SNP, rs724078, that was associated with four sperm parameters is predicted to be a *cis* eQTL, regulating expression of *DDR1* (MIM 600408, $p = 1.0 \times 10^{-4}$; Table S7) and located approximately 1.36 Mb away. Interestingly, an alternative transcript of *DDR1* is expressed exclusively in postmeiotic germ cells in rat testes.[51] All the other predicted eQTLs regulate genes located on chromosomes other than those that harbor the associated SNP (i.e., in *trans*); therefore, interpretation of these potential associations is less straightforward. Lastly, these are eQTLs in lymphoblastoid cell lines, which may not be representative of the regulatory landscape in relevant tissues; however, they provide a promising starting point for understanding possible mechanisms for how these variations could be affecting male fertility.

In addition to *DDR1*, there are also several outstanding candidates for fertility genes in the nine associated regions. The association between an intronic SNP in ubiquitin-specific peptidase 8 (*USP8* [MIM 603158], also referred to as *USPy*), rs7174015, and five sperm parameters (total volume, total sperm count, total motile sperm count, average velocity, and mean ALH) is particularly noteworthy. *USP8* is an essential gene for survival in mice[52] and encodes a crucial enzyme for deubiquitinating proteins and sorting endosomal cargo in neuronal and spermatogenic cells, where it is highly expressed. Ubiquitination pathways have a critical role in cellular homeostasis through regulation of protein synthesis and activity at transcriptional, epigenetic, and posttranslational modification levels.[53] It affects a wide range of physiological functions in the cell, including cell-cycle regulation (including spermatogenesis), apoptosis, DNA repair, and embryogenesis.[54,55] Moreover, Berruti et al. recently showed that *USP8* has an important role in assembling acrosomes in differentiating sperm cells and in shaping the sperm head through direct interaction with other sorting complexes, labeled vesicles, and microtubules.[56] Overall, *USP8* is a compelling candidate for a male fertility gene. The remaining candidates reflect the importance of immune processes in male fertility. For example, rs724078, which is associated with four traits (total sperm count, total motile sperm count, % motility, and mean ALH), is located approximately 34 kb downstream of a gene in the ubiquitin-like modifier family, ubiquitin D (*UBD* [MIM 606050]; also referred to as *FAT10*), and has been implicated in the regulation of the cell cycle, as well as cytokine response through the activation of the NF-κB pathway.[57] A regulatory polymorphism, located immediately downstream of this gene and resulting in upregulation of *UBD* in the intestinal mucosa, is associated with celiac disease (MIM 212750), an immune-mediated disorder of the small intestine.[58] Another intriguing association was observed between an intronic SNP (rs12870438) in the epithelial-stromal interaction 1 gene (*EPSTI1* [MIM 607441]) and five sperm parameters (sperm concentration, total sperm count, total motile sperm count, average velocity, and mean ALH). Initially identified for its expression in breast

tumors, *EPSTI1* is an interferon response gene whose expression is associated with systemic lupus erythematosus, and transcript levels in the peripheral blood correlate with lymphocyte counts.[59,60] *EPSTI1* is expressed at high levels in the testes; however, its function in this tissue is not known.[61] Lastly, we observed an association between rs11236909, located approximately 58 kb upstream of the leucine-rich repeat-containing 32 gene (*LRRC32* [MIM 137207]; also referred to as *GARP*), and three sperm-motility parameters (average velocity, mean ALH, and linearity). The protein product of this gene is found on the regulatory T cells and functions as a receptor for latent transforming growth factor β (TGF-β) molecules.[62,63]

The associations with three genes (*UBD*, *EPSTI1*, and *LRRC32*) that have roles in immune regulation are particularly exciting. Testicular tissue has unique immunologic features due to the need for tight regulation of immune responses in this tissue for the survival of germ cells. For example, testicular inflammation due to infection or injury can lead to disruption of spermatogenesis and androgen production, resulting in impaired fertility.[64,65] Interestingly, certain cytokines are present at high levels in the testes even in the absence of an inflammatory response; they are thought to contribute to the development and normal functioning of testes, act as growth and differentiation factors of testicular cells, and regulate steroidogenesis.[65,66] For example, tumor necrosis factor alpha (TNF-α) inhibits steroid synthesis in the Leydig cells[66] and regulates spermatogenesis by inhibiting germ cell apoptosis.[67] Another large family of cytokines, interferons, has a role in the antiviral defense system and is found in the Leydig and Sertoli cells, macrophages, and peritubular cells in the testes.[68] Similar to TNF-α, interferons also inhibit testosterone production in the Leydig cells.[69] Intriguingly, both TNF-α and interferon gamma (IFN-γ) are shown to upregulate *UBD* expression in liver and colon cancer cells, suggesting a function of *UBD* in proinflammatory immune response.[70] It has also been observed that increased leukocyte counts and increased concentrations of certain cytokines (especially TNF-α and IFN-γ) in the seminal fluid are associated with decreased sperm parameters (including count, motility, and normal morphology) and compromise fertilization rates,[71–73] although these findings were not consistently replicated (for a review, see Diemer et al.[74]). Nevertheless, a relationship between immune regulation and reproductive function in the testicular tissue is evident. The associations we report here between sperm count and motility parameters and the three SNPs located in or near genes whose products have roles in immune responses are consistent with those observations.

The two-stage strategy we utilized in this study allowed us to identify associations meeting the following two criteria: the genetic model at each associated SNP (i.e., additive, recessive, or dominant) is the same in both Hutterite and Chicago men, and associations showed the same direction of effect (i.e., increased or decreased fertility associated with the same allele) in both groups of men (Figure 2). Therefore, we may have missed other true associations because they did not fulfill both requirements. Among the possible reasons for this are differences in allele frequencies between the Hutterite and Chicago men and the relatively small sample sizes, both of which would affect power. In addition, variation in the haplotype structure around these SNPs in the Hutterites and in the ethnically diverse men from Chicago could affect the fit of the specified genetic model and the accuracy of tagging the same causal variants in both samples. Furthermore, some of the associated SNPs in the Hutterite men may influence fertility through mechanisms other than those affecting sperm quantity or motility. Such genes might have effects on other aspects of sperm biology that were not considered in this study (e.g., sperm morphology or penetration capacity) or on factors that regulate different aspects of reproduction, such as hormonal profiles or sexual behavior. In particular, the only two SNPs that were associated with both family size and birth rate in the Hutterites at $p < 10^{-4}$ (rs12339229 and rs10893363) were not associated with any of the sperm parameters (Table S5). We might also have missed some true associations with some SNPs in our initial GWAS because their p values were greater than 1×10^{-4}. For example, we previously reported an association in Hutterite men between birth rate and polymorphism in a candidate gene (*CFTR*).[75] Carriers of the Val allele at the Met470Val variant had increased birth rates ($p = 0.0029$) and larger families ($p = 0.0002$) compared to men with the Met/Met genotype. Even though the fertility effect attributable to this polymorphism was robust and was supported by signatures of positive selection at this locus, we would have missed this association in the current study. Thus, it is likely that other true associations are present in our data that may be revealed in the future through enlargement of the samples sizes or through assays of additional clinical phenotypes. Lastly, we acknowledge that reproductive outcome is a highly complex phenotype that is determined by both partners, and our study design allows us to capture only the male-specific component of this phenotype. However, the phenotypes included in this study were previously shown to have significant heritabilities in Hutterites,[34] which provided the rationale for conducting the studies presented here. Similar studies considering females only and/or simultaneous analysis of the genotypes of husband and wife will probably reveal additional genes and pathways that are important for human fertility.

Our understanding of the genetic causes of male infertility is still quite incomplete. Currently, genetic testing for the diagnosis infertility in men is limited to cytogenetic studies, detection of Y chromosome deletions, and *CFTR*-mutation analysis.[6] In the absence of positive findings, semen analysis reveals severe motility defects and/or oligozoospermia in more than half of infertile men. However, only a handful of genes have been associated with either

measure, and these cases are relatively rare (reviewed in Hwang, et al.[6]). In this study, we identified at least nine genes that harbor common variation that influences reproductive phenotypes in Hutterite men and in ethnically diverse men from Chicago. Further study is required to determine whether mutations in these genes that are more severe result in male infertility and whether sequencing studies of these genes will reveal mutations that could serve as useful clinical markers for men with unexplained infertility.

Supplemental Data

Supplemental data include two figures and seven tables and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

SNP and CNV Annotation Database (SCAN), http://scan.bsd.uchicago.edu/

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