Single Nucleotide Polymorphisms in Candidate Genes Associated with Fertilizing Ability of Sperm and Subsequent Embryonic Development in Cattle[1]

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ABSTRACT

Fertilization and development of the preimplantation embryo is under genetic control. The present goal was to test 434 single nucleotide polymorphisms (SNPs) for association with genetic variation in fertilization and early embryonic development. The approach was to produce embryos from 93 bulls using in vitro procedures (n = 3–6 replicates per bull) and relate cleavage rate (CR) and development of cleaved embryos to the blastocyst stage (BDRC) to the genotype for each SNP. Bulls were selected to have either high or low estimates for predicted transmitted ability for daughter pregnancy rate (DPR), an estimate of female fertility. The repeatability was 0.84 for CR and 0.55 for BDRC. Semen extender affected CR, with lower results for milk extender than yolk extender. There was no significant correlation between DPR and either CR or BDRC. A total of 100 SNPs had a minor allele frequency sufficiently high (>5%) to allow association analysis. There were nine genes with SNPs associated with CR (*AVP, DEPP, EPAS1, HSD17B6, NT5E, SERPINE2, SLC18A2, TBC1D24*, and a noncharacterized gene) and 12 genes with SNPs associated with BDRC (*C1QB, FAM5C, HSPA1A, IRF9, MON1B, PARM1, PCCB, PMM2, SLC18A2, TBC1D24, TTLL3*, and *WBP1*). Results demonstrate that in vitro fertilization and blastocyst development are under genetic control and point out the potential importance of some previously unknown genes in these processes. Selection of cattle based on the genotype at one or more of these 19 loci may prove useful in conjunction with other genetic markers for improving genetic ability for fertility.

embryo, fertility, fertilization, genetics, preimplantation development, SNP

INTRODUCTION

Fertilization and development of the preimplantation embryo is under genetic control. In cattle, several single nucleotide polymorphisms (SNPs) have been associated with these traits. For example, SNPs in *FGF2* [1], *STAT3* [2], *STAT5A* [3–5], *GHR* [4], *SPP* [4], *DNAJC27* [6], and *PGR* [7] have been associated with in vitro fertilization. Furthermore, SNPs in *STAT5A* [3, 4, 6], *STAT3* [3], *FGF2* [1, 8], *PGR* [7], *GHR* [4], *PRLR* [4], *SERPINA14* [4], *SPP* [4], and *DNAJC15* [6] have been associated with in vitro development of embryos to the blastocyst stage.

The existence of genes controlling fertilization and embryonic development is indicative that some of the historical decline in fertility in dairy cattle [9, 10] may be the result of increased frequency of alleles that inhibit development. There are SNPs in cattle for which the beneficial allele for milk production is the less desirable allele for reproduction [11, 12]. Therefore, selection for production traits may have reduced the frequency of alleles that benefit fertility. Identification of genes controlling embryonic development could lead to new methods for genetic selection to improve fertility in dairy cattle, as well as increase understanding about genes with function critical for the processes of fertilization and early development.

In a previous study, SNPs in 434 genes related to reproductive processes were evaluated for their relationship to fertility and reproduction traits in dairy cattle [12]; a total of 40 genes significantly related to fertility were identified. The goal of the current study was to test whether any of the 434 SNPs previously studied are associated with genetic variation in fertilization and early embryonic development. The approach was to produce embryos from 93 bulls using in vitro procedures and relate cleavage rate and development to the blastocyst stage with the genotype at each SNP.

MATERIALS AND METHODS

Selection of Bulls

Straws of semen were obtained from 93 Holstein bulls born between 1973 and 2008. These bulls, which represent a subset of bulls from a previous study [12], were chosen based on predicted transmitting ability (PTA) for daughter pregnancy rate (DPR). PTA is the average genetic value for a trait that an animal transmits to its offspring, and DPR is an estimate of the percentage of a bull's daughters eligible to become pregnant in a 21-day period that actually become pregnant. Bulls in the high-DPR group (n = 47) had PTA for DPR that ranged from 2.0 to 5.3 (average = 3.0), and bulls in the low-DPR group (n = 46) had PTA for DPR that ranged from −4.6 to −2.0 (average = −3.1). Reliability of estimates of PTA for DPR ranged from 0.64 to 0.99 (average = 0.82). Semen samples were obtained from the USDA National Animal Germplasm Program (NAGP; 23 bulls; Fort Collins, CO), Alta Genetics (16 bulls; Watertown, WI), Taurus-Service Inc. (16 bulls; Mehoopany, PA), Select Sires Inc. (13 bulls; Plain City, OH), ABS Global Inc. (11 bulls; DeForest, WI), Genex Cooperative Inc. (five bulls; Shawano, WI), Semex (five bulls; Madison, WI), and Accelerated Genetics (four bulls; Baraboo, WI). Semen extender type was classified based on appearance: white and milky was classified as milk, yellow was classified as egg yolk, and extenders of other colors were classified as unknown.

SNP Genotyping

Bulls were genotyped for 434 SNPs located in genes involved in reproductive processes as described elsewhere [12]. Genes were chosen based on one or more of the following criteria: well known to be involved in reproductive processes, physically close to SNPs related to reproductive traits in other studies, containing SNPs previously linked to fertility, and

differentially regulated between physiological conditions in a variety of reproductive tissues. SNPs were also chosen based on the likelihood that a change in protein function would result (nonsense mutations, frameshifts, and nonsynonomous mutations that were predicted to cause a large change in protein function). Only SNPs that had a call rate >70% in the larger study and had a minor allele frequency (MAF) >0.05 among the 93 bulls in the current study were analyzed. A list of the SNPs and associated MAFs is presented in Supplemental Table S1 (available online at www.biolreprod.org).

Evaluation of Fertilizing Ability and Competence of Embryos to Develop to the Blastocyst Stage

Each bull was evaluated for ability of sperm to fertilize matured oocytes, as determined by percentage of oocytes exposed to sperm that underwent cleavage (cleavage rate [CR]) and on the ability of cleaved oocytes (i.e., newly formed embryos) to become a blastocyst at Day 7 after insemination (blastocyst development rate of cleaved embryos [BDRC]). For each bull, traits were evaluated in a total of three to six separate in vitro fertilization (IVF) and embryo culture procedures.

Techniques for fertilization of matured oocytes and culture of the resultant embryos to the blastocyst stage were as follows. Bovine ovaries were collected from a local abattoir and transported to the laboratory within 5 h after slaughter. Follicles 2–10 mm in diameter were lacerated, and cumulus-oocyte complexes (COCs) were collected in oocyte-collection medium (Medium-199 with Hanks salts [Sigma-Aldrich, St. Louis, MO] supplemented with 350 mg/L sodium bicarbonate [Sigma-Aldrich], 2.38 g/L HEPES [Sigma-Aldrich], 2% [vol/vol] bovine steer serum [Pel-Freez, Rogers, AR] containing 2 U/ml heparin [Sigma-Aldrich], 100 U/ml penicillin-G [Caisson Labs, North Logan, UT], 100 mg/ml streptomycin [Caisson Labs], and 1 mM Glutamax [Life Technologies, Grand Island, NY]). The COCs were incubated in groups of 10 in 50 µl of maturation medium (Medium-199 with Earle salts [Life Technologies] supplemented with 10% [vol/vol] bovine steer serum, 0.22 g/µl sodium pyruvate [Sigma-Aldrich], 2 µg/ml estradiol 17-β [Sigma-Aldrich], 25 µg/ml bovine follicle-stimulating hormone [Folltropin-V; AgTech Inc., Manhattan, KS], 22 µg/ml sodium bicarbonate, 50 µg/ml gentamicin sulfate [Life Technologies], and 1 mM Glutamax) overlaid with mineral oil for 22 h at 38.5°C in an atmosphere of 5% (vol/vol) CO_2 in humidified air.

On the day of fertilization (designated d 0), matured COCs were washed in HEPES-Tyrodes albumin lactate pyruvate (HEPES-TALP) [13] supplemented with 3 mg/ml bovine serum albumin (Sigma-Aldrich), 22 µg/ml sodium pyruvate, and 15 µg/ml gentamicin; divided into groups of about 100; and transferred to a fertilization medium consisting of 1.7 ml of SOF-IVF fertilization medium [14] and 80 µl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 mM epinephrine [all three from Sigma-Aldrich] in 0.9% [w/v] NaCl). Fertilization was initiated by addition of 120 µl Percoll-purified sperm suspended in HEPES-TALP from a single bull to achieve a final concentration of 1.1×10^6 sperm per milliliter. The COCs were incubated with sperm for 12 h at 38.5°C in an atmosphere of 5% (vol/vol) CO_2 in humidified air. Cumulus cells were then removed by vortexing COCs for 4 min in 1429 U/ml hyaluronidase (Sigma-Aldrich) in HEPES-TALP. Then, putative zygotes (i.e., oocytes exposed to sperm) were cultured in groups of ~30 in 50-µl drops of SOF-BE1 [15] overlaid with mineral oil. Embryos were incubated at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO_2 and 5% (vol/vol) O_2, with the balance N_2, until d 7. The CR was determined on d 3, and the BDRC was determined on d 7.

In total, 308 IVF procedures were performed. For each procedure, two to six bulls were evaluated, with the number depending on the number of oocytes obtained. Each day, bulls were randomly chosen from the low- and high-DPR groups so that at least one low- and one high-DPR bull were used on each fertilization day. After all bulls were tested once, the experiment was repeated an additional two times for all bulls, with bulls again being selected at random within a DPR group for each series. A few bulls were used in a total of four (n = 27), five (n = 3), or six (n = 1) IVF procedures because earlier results were considered unreliable due to results that deviated widely from expectations. Unreliable procedures were most often ascribed to a defective medium used for the procedure.

Statistical Analysis

MAF was determined using the FREQ procedure of SAS (version 9.2; SAS Institute, Inc., Cary, NC). Repeatability of CR and BDRC was determined by calculating the ratio of the between-animal variance to the sum of the between-animal and the residual variances. The association between SNP genetic variants and each trait was evaluated using the GLM procedure of SAS. The full model included effects of extender, semen company, birth year, DPR class (high or low), and SNP. All nonsignificant variables were removed, and the

final model for CR included

$$Y_i = \text{extender}_j + \text{company}_l + \beta\text{SNP}_k + \epsilon_i, \qquad (1)$$

and the final model for BDRC included

$$Y_i = \beta\text{SNP}_k + \epsilon_i, \qquad (2)$$

where Y_i is the trait of interest (CR or BDRC, averaged for all runs for an individual bull) for the ith bull ($i = 1, 2, \ldots, 93$), extender_j is the effect of the jth type of extender ($j = $ milk, egg yolk, unknown) used on the ith bull, company_l is the effect of the lth semen company, β is the linear regression coefficient for the kth SNP, SNP_k is the number of copies ($k = 0, 1,$ or 2) of the major allele, and ϵ_i is the random residual effect.

Allele substitution effects were computed using two mixed-model analyses that included random animal effects [16] and either fixed or continuous SNP terms. In the first, genotype was considered a continuous variable to determine the allele substitution effect (the linear effect of the number of copies of the major allele). In the second, genotype was considered a class variable, and orthogonal contrasts were used to estimate dominant effects ((AA + aa)/2 vs. Aa).

Correlations were calculated between average values of CR and BDRC with each other as well as with the SD of these traits and with DPR using the PROC CORR procedure of SAS.

Averages are reported as least-squares means ± SEM as derived from PROC GLM, except for results for individual bulls (Figs. 1 and 2), in which case data represent arithmetic means ± SEM.

RESULTS

Minor Allele Frequencies

Of the 434 SNPs, 405 previously passed the call rate requirements in a previous study [12]. Of those 405 SNPs, 100 had MAF >0.05 among the bulls tested in the current study (Supplemental Table S1) and were used for further analyses.

Characteristics of In Vitro Fertilization and Development

The average CR ± SEM for each bull is plotted in Figure 1. The CR did not differ between bulls of high (73.0% ± 2.1%) and low (73.7% ± 2.0%) DPR (P = 0.76). The repeatability of CR was 0.84. Semen extender affected CR (P = 0.004). Semen that was extended in milk extender had the lowest CR (63.9% ± 2.7%), followed by semen in egg extender (73.8% ± 2.3%), and semen in unknown extender (82.3% ± 4.0%). Furthermore, semen company of the bull had an effect on CR (P = 0.01), with differences between companies varying from a low of 64.1% ± 3.2% to a high of 85.4% ± 8.1%.

The average BDRC ± SEM for each bull is plotted in Figure 2. The BDRC did not differ between bulls of high (40.8% ± 1.5%) and low (41.7% ± 1.4%) DPR (P = 0.58) . The repeatability of BDRC was 0.55. Neither extender (P = 0.45) nor semen company (P = 0.67) affected BDRC.

Correlations between in vitro development measurements, DPR, and within-bull SD of in vitro development measurements are shown in Table 1. DPR was not correlated with any in vitro measurements that were assessed in the current study. CR was positively correlated with BDRC (correlation, 0.23; P = 0.02). CR was negatively correlated with the SD of CR (correlation, −0.59; P < 0.0001); therefore, bulls with greater CR had less variability in percent cleavage than bulls with lower CR. There was no correlation between BDRC and SD of BDRC (correlation, −0.02).

SNP Effects

Each of the 100 SNPs with MAF > 0.05 were analyzed for effects on in vitro embryo development. Two types of analyses were performed: a regression analysis to determine the linear allele substitution effect of each SNP (0, 1, or 2 copies of the major allele) and orthogonal contrasts (heterozygote vs.
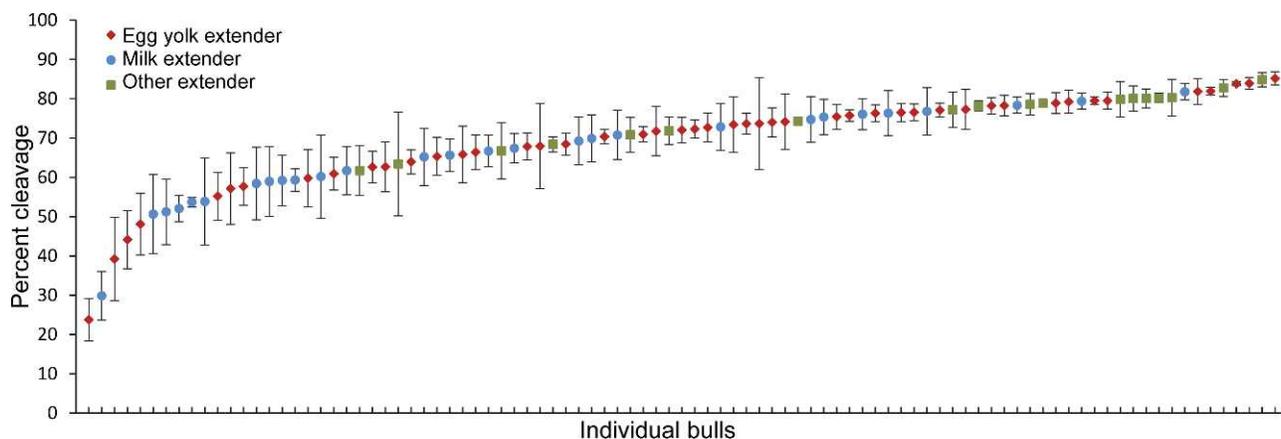
FIG. 1. Variation among bulls used for in vitro fertilization in the percentage of oocytes exposed to sperm that underwent cleavage. Each point on the graph represents the average percentage of cleavage ± SEM of an individual bull. The red diamonds represent bulls whose semen was in egg yolk extender, blue circles represents bulls with semen in milk extender, and green squares represent bulls with semen in an unknown extender.

average of the two homozygotes) to determine the dominance effect.

Cleavage rate. Results for SNPs significantly associated with CR are shown in Table 2. Allele substitution effects were significantly different from 0 for 5 genes (*C28H10orf10* [predicted to encode for DEPP], *HSD17B6, NT5E, SERPINE2*, and *TBC1D24*). The largest magnitude for SNP effects was for *NT5E*, where the difference in CR between homogygotes was 21.2%. For this gene, only two bulls were homozygous for the deleterious allele. The other large SNP effects were seen for *SERPINE2*, where homozygotes differed by 12.8%, and *SLC18A2*, where homozygotes differed by 10.1%.

Two SNPs with significant substitution effects, *NT5E* and *SERPINE2*, also exhibited dominance. In both cases, the heterozygote had a phenotype similar to that of the desirable homozygote. Four other genes exhibited dominance. In two cases, for *C7H19orf60* and *SLC18A2*, the heterozygote had a phenotype similar to that of the desirable homozygote. For the other two cases, *AVP* and *EPAS1*, the heterozygote was lower than either homozygote.

Development of cleaved embryos to the blastocyst stage. Results are presented in Table 3. Allele substitution effects were significant for nine genes (*FAM5C, HSPA1A, IRF9, MON1B, PCCB, PMM2, SLC18A2, TTLL3*, and *WBP1*). Of these, *SLC18A2* was also significant for CR, but the

desirable allele was different. There were large SNP effects for *MON1B* and *WBP1*, but the number of observations for the deleterious homozygote was only 1–2. The greatest SNP effect for the remaining seven genes was for *IRF9*, in which the difference between homozygotes was 6.3%, and *HSPA1A*, in which the difference was 6.0%.

There were no dominant effects for genes in which allele substitution effects were significant, but there were significant dominance effects for an additional three genes (*C1QB, PARM1*, and *TBC1D24*). For *C1QB*, the heterozygote had a phenotype similar to that of the desirable homozygote, but the fact that there was only one bull that was homozygous for the deleterious allele adds uncertainty to the conclusion regarding dominance. For *PARM1* and *TBC1D24*, the heterozygote had higher BDRC than either homozygote.

Relationship Between SNP Effects and SNPs Previously Associated with In Vivo Measurements of Fertility

The SNPs studied here were also examined by Cochran et al. [12] for association with three measures of fertility in vivo: DPR, heifer conception rate (HCR) [17], and cow conception rate (CCR) [17]. We evaluated whether any of the 20 SNP that were associated with CR or BDRC in the current study were the same as SNPs related to DPR, HCR, or CCR. Four

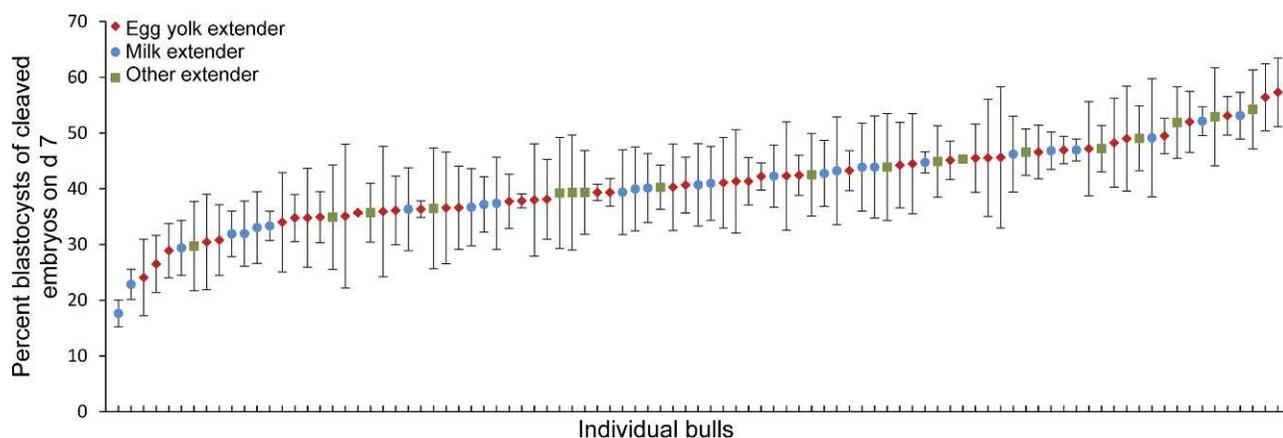FIG. 2. Variation among bulls used for in vitro fertilization in the percentage of cleaved embryos that became blastocysts. Each point on the graph represents the average percentage of cleavage ± SEM of an individual bull. The red diamonds represent bulls whose semen was in egg yolk extender, blue circles represents bulls with semen in milk extender, and green squares represent bulls with semen in an unknown extender.

TABLE 1. Correlation of in vitro measurements of fertilization and embryonic development with DPR.[a,b]

	DPR	CR	SD of CR	SD of BDRC
CR	−0.06 (0.54)		−0.59 (<0.0001)	
BDRC	−0.12 (0.25)	0.23 (0.02)		−0.02 (0.85)

[a] The P value of each correlation is in parentheses.
[b] Abbreviations are as follows: BDRC, blastocyst development rate from cleaved embryos; CR, percent cleavage; DPR, daughter pregnancy rate; SD, standard deviation.

individual SNPs that were associated with CR (*C7H19orf60*, *SERPINE2*, *SLC18A2*, and *TBC1D24*) and eight SNPs that were associated with BDRC (*C1QB*, *MON1B*, *PARM1*, *PCCB*, *PMM2*, *SLC18A2*, *TBC1D24*, and *WBP1*) had earlier been shown to be related to DPR, HCR, or CCR. The majority of those associations were in the opposite direction for CR or BDRC as compared to effects on DPR, HCR, or CCR. In only four cases was the desirable allele the same in vitro and in vivo: *C7H19orf60* for CR, and *C1QB*, *PARM1*, and *SLC18A2* for BDRC. Note that the desirable allele for *SLC18A2* was different for CR than it was for BDRC.

Five SNPs examined in the current study had previously been associated with fertility in dairy or beef cattle. These were SNPs in *FGF2* [18], *HSPA1A* [19], *PAPPA2* [20], *PGR* [21], and *STAT5A* [18]. One of these, *PAPPA2*, was not analyzed due to low MAF. Of the other four, none were significantly related to CR, and only one (*HSPA1A*) was significantly associated with BDRC. However, the favorable genotype for

BDRC (−/−) was the opposite as the favorable genotype (C/C) reported earlier for calving rate and Julian calving date [19] (Table 3).

Relationship Between SNP Effects and SNPs Previously Associated with In Vitro Fertilization and Development

Two SNPs examined (*PGR* and *STAT5A*) had been associated with CR in earlier studies [5, 7]. There were no significant associations of either SNP with CR in the current study, although the favorable allele for PGR in the earlier study [7] (G) was associated with numerically greater CR in the current study. The CR for *PGR* was 74.22% ± 4.03%, 74.66% ± 2.05%, and 71.18% ± 2.16% for G/G, G/C, and C/C, respectively. However, the favorable allele for *STAT5A* in a previous study was C for CR [5], and the CR for *STAT5A* was 74.60% ± 2.99%, 73.23% ± 2.08%, and 71.91% ± 2.66% for G/G, G/C, and C/C, respectively (least-squares means ± SEM). Three SNPs examined here had previously been related to blastocyst development in vitro, including *FGF2* [1], *PGR* [7], and *STAT5A* [3]. None of these three SNPs were significantly related to BDRC in the current study. The least-squares means for BDRC for each of these genes were as follows: *FGF2*, 42.46% ± 2.23%, 39.92% ± 1.29%, and 40.69% ± 1.07% for A/A, A/G, and G/G, respectively; *PGR*, 40.67% ± 2.52%, 40.82% ± 1.10%, and 40.75% ± 1.26% for G/G, G/C, and C/C, respectively; and *STAT5A*, 40.73% ± 1.85%, 39.90% ± 1.10%, and 41.85% ± 1.35% for G/G, G/C, and C/C, respectively (least-squares means ± SEM).

TABLE 2. SNPs associated with percentage of oocytes that cleaved.[a]

SNP	Gene	Least-squares means ± SEM[b]			Linear		Dominance P value
		0	1	2	Effect	P value	
rs43114141	*AVP*	76.92 ± 2.82 (n = 16)	70.22 ± 2.20 (n = 31)	74.56 ± 2.16 (n = 34)	−0.82	0.6075	0.0350
rs134150850	*C28H10orf10*	69.51 ± 2.77 (n = 16)	73.11 ± 1.93 (n = 45)	77.70 ± 2.41 (n = 31)	4.18	0.0105	0.8229
rs109332658	*C7H19orf60*	66.17 ± 3.60 (n = 9)	76.79 ± 2.29 (n = 32)	72.86 ± 1.97 (n = 50)	1.07	0.5530	0.0086
rs43676052	*EPAS1*	80.24 ± 5.03 (n = 5)	70.78 ± 2.28 (n = 34)	74.50 ± 1.87 (n = 53)	0.64	0.7512	0.0316
rs109769865	*HSD17B6*	78.50 ± 5.94 (n = 4)	77.64 ± 2.59 (n = 26)	71.51 ± 1.85 (n = 61)	−5.06	0.0285	0.4571
rs42508588	*NT5E*	53.25 ± 7.50 (n = 2)	72.51 ± 2.34 (n = 32)	74.47 ± 1.83 (n = 58)	4.44	0.0485	0.0464
rs43321188	*SERPINE2*	62.33 ± 3.68 (n = 9)	74.20 ± 2.24 (n = 31)	75.08 ± 1.83 (n = 52)	4.30	0.0126	0.0381
rs110365063	*SLC18A2*	63.32 ± 5.38 (n = 4)	76.46 ± 2.59 (n = 24)	73.40 ± 1.82 (n = 63)	0.75	0.7217	0.0251
rs110660625	*TBC1D24*	68.76 ± 3.29 (n = 14)	71.41 ± 2.12 (n = 39)	76.12 ± 2.19 (n = 35)	3.88	0.0229	0.6724

[a] SNP represented as the rs number given by the National Center for Biotechnology Information data base SNP.
[b] Number of copies of the major allele.

TABLE 3. SNPs associated with percentage of cleaved embryos that became a blastocyst.[a]

SNP	Gene	Least-squares means (SEM)[b]			Linear		Dominance P value
		0	1	2	Effect	P value	
rs135390325	*C1QB*	17.64 ± 7.07 (n = 1)	40.82 ± 1.52 (n = 22)	40.86 ± 0.75 (n = 68)	11.61	0.2140	0.0038
rs135071345	*FAM5C*	36.61 ± 5.15 (n = 2)	38.24 ± 1.52 (n = 23)	41.56 ± 0.89 (n = 67)	3.07	0.0445	0.7807
HSP70C895D	*HSPA1A*	45.27 ± 2.43 (n = 9)	41.31 ± 1.14 (n = 41)	39.29 ± 1.11 (n = 43)	−2.62	0.0259	0.5823
rs110953315	*IRF9*	46.01 ± 4.22 (n = 3)	42.53 ± 1.53 (n = 23)	39.71 ± 0.91 (n = 65)	−2.95	0.0428	0.8996
rs41859871	*MON1B*	26.51 ± 7.29 (n = 1)	39.05 ± 1.55 (n = 22)	41.50 ± 0.87 (n = 70)	3.34	0.0451	0.2091
rs111027720	*PARM1*	39.61 ± 1.64 (n = 28)	42.22 ± 1.07 (n = 44)	38.33 ± 1.35 (n = 19)	−0.95	0.3799	0.0339
rs109813896	*PCCB*	36.78 ± 2.27 (n = 10)	39.88 ± 1.08 (n = 44)	42.21 ± 1.21 (n = 35)	2.59	0.0281	0.8210
rs109629628	*PMM2*	37.77 ± 1.98 (n = 14)	40.35 ± 1.15 (n = 41)	42.33 ± 1.22 (n = 37)	2.21	0.0459	0.8533
rs110365063	*SLC18A2*	43.97 ± 3.69 (n = 4)	43.12 ± 1.51 (n = 24)	39.64 ± 0.93 (n = 63)	−2.87	0.0388	0.5892
rs110660625	*TBC1D24*	36.96 ± 1.79 (n = 14)	43.47 ± 1.07 (n = 39)	39.45 ± 1.13 (n = 35)	0.13	0.9041	0.0008
rs135236119	*TTLL3*	38.95 ± 2.91 (n = 6)	37.85 ± 1.30 (n = 30)	42.03 ± 0.97 (n = 54)	2.77	0.0255	0.1922
rs134282928	*WBP1*	50.59 ± 4.91 (n = 2)	43.75 ± 2.01 (n = 12)	39.43 ± 0.92 (n = 57)	−4.87	0.0057	0.6956

[a] SNP represented as the rs number given by the National Center for Biotechnology Information data base SNP.
[b] Number of copies of the major allele.

DISCUSSION

There are a few reports, with smaller numbers of bulls than examined here, that the success of in vitro fertilization and embryonic development varies with sire [22–24]. In the current study, there was a large range among bulls in average CRs (23.8%–85.2%) and BDRCs (17.6%–57.3%). Much of this variation is probably due to environmental differences among bulls in conditions for spermatogenesis, semen collection and processing, and semen storage. Present results indicate there is also a genetic component to this variation as well. Using a candidate gene approach, 19 genes were identified that were significantly associated with CR (a measure of fertilization) and BDRC. Many of these genes are likely to encode for proteins that play an important role in the process whereby sperm fertilize an oocyte and the resultant embryo develops to the blastocyst stage. Moreover, it is possible that changes in allele frequency for some of these genes contributed to changes in fertility of dairy cattle.

Identification of an association between a SNP in a candidate gene and a phenotype does not necessarily mean the SNP is causative. Associations could also be due to proximity of the genotyped SNP to the actual causative SNP. Nonetheless, many of the genes that were associated with CR and BDRC have functions consistent with a role in these processes. Genes affecting CR could do so by participating in sperm formation and maturation in the male reproductive tract, sperm survival after ejaculation and cryopreservation, or sperm function during fertilization. Nine genes were significantly associated with CR. *HSD17B6* encodes for an enzyme with oxidoreductase and epimerase activity toward androgens and estrogens [25, 26] and likely regulates steroid action in the male reproductive tract. Another gene, *SLC18A2,* is a neurotransmitter transporter for monoamines [27], and mono-aminergic nerves may be involved in regulation of blood flow in the testis [28]. Other genes associated with CR have functions suggesting they play a role in survival of sperm in vivo after environmental insults or in vitro following semen processing and cryopreservation. For example, *AVP* is antiapoptotic [29, 30], and *EPAS1* encodes for a transcription factor induced by hypoxia [31]. Furthermore, *SERPINE2* can prevent capacitation [32] and may act to prevent premature capacitation. Another gene associated with CR, *NT5E,* encodes for an enzyme that converts adenosine monophosphate to adenosine [33], which could conceivably affect energy availability or signal transduction. Also, TBC1D24 interacts with ARF6, a member of the RAS superfamily that is involved in vesicular trafficking and remodeling of membrane lipids [34]. Perhaps *TBC1D24* is involved in the membrane reorganization occurring during capacitation and the acrosome reaction [35]. The gene might also play a role in recovery of sperm from freezing, since membrane architecture is disrupted by that process [35]. It is unclear what roles *C28H10orf10* or *C7H19orf60* might play in CR. The protein encoded by *C28H10orf10* is orthologous to DEPP, which is expressed in several tissues and is involved in activation of the Elk1 transcription factor [36]. The protein encoded by *C7H19orf60* is not known.

The 12 genes associated with development to the blastocyst stage are involved in a wide variety of functions that could be important for the preimplantation embryo. At least four of the genes may be involved in signaling systems that control embryonic development. One, *IRF9,* is a transcription factor involved in the IFNA signaling pathway [37]. The bovine blastocyst produces the type I interferon, IFNT2 [38], and perhaps IFNT signaling in the embryo is important for

regulation of embryonic development. The *FAM5C* gene encodes for a secreted protein that promotes differentiation of osteoblasts [39]. As mentioned previously, *SLC18A2* is a neurotransmitter transporter for monoamines [27], and mono-amines can participate in regulation of preimplantation development [40]. Another gene associated with blastocyst development, *WBP1*, interacts with the kinase gene *YAP* to positively or negatively regulate cell growth, differentiation, and migration [41].

Based on upregulation of gene expression in the trophec-toderm [42], outer cells of the preimplantation embryo are very active in endocytosis, lipid biosynthesis, and transmembrane transport. Three genes associated with development to the blastocyst stage are involved in these processes. The gene *TBC1D24* interacts with *ARF6* to regulate vesicular trafficking and remodeling of membrane lipids [34], *MON1B* regulates autophagy and processing of ingested cells [43, 44], and *TTLL3* is a tubulin glycine ligase that participates in cilia assembly [45].

Three other genes related to embryonic development are involved in protection from cellular stress. *HSPA1A* protects cells from heat shock, in large part by blocking apoptosis [46], and is upregulated in preimplantation bovine embryos in response to heat shock [14, 47, 48]. Addition of an antibody against HSPA1A to culture medium reduced the proportion of bovine embryos developing to the blastocyst stage [49]. *PARM1* is antiapoptotic [50] and *MON1B* is required for autophagy [43].

An SNP in *PMM2* was also associated with blastocyst development. It may be involved in cell-cell interactions in the embryo, because it encodes for an enzyme that isomerizes mannose 6-phosphate into mannose 1-phosphate [51], which in turn is used to make fucosylated glycans [52]. It is likely that *PCCB* is involved in embryo energy metabolism, because it encodes for an enzyme that converts proponyl CoA to methylmalonyl CoA during gluconeogenesis [53]. The possible role of the complement gene *C1QB* in embryonic development is less clear. There was only one homozygote of the minor allele, and the conclusion regarding association with BDRC is tentative.

When testing for dominance, three types were identified: 1) dominance where inheritance of one copy of the dominant allele resulted in the same phenotype as having two copies of the dominant allele (*C1QB, C7H19orf60, NT5E, SERPINE2*, and *SLC18A2*); 2) heterozygote advantage (*PARM1* and *TBC1D24*), where the heterozygote was better than either homozygote; and 3) heterozygote disadvantage (*AVP* and *EPAS1*). Heterozygote advantage could theoretically result if the optimal level of gene expression is intermediate between that for the two homozygotes, if the desirable allele is different in different cell types, or if heterozygosity increases ability of proteins to interact with other molecules, for example binding of MHC class I to peptide antigens [54]. It is unknown why heterozygote disadvantage could occur.

There was no association between CR or BDRC and in vivo fertility, as determined by a lack of difference between bulls of high and low DPR as well as nonsignificant correlations of CR or BDRC with DPR. Moreover, there were only three SNPs (*C1QB, PARM1*, and *SLC18A2*) in which the allele associated with improved BDRC was also associated with higher fertility in vivo [12]. In one case, *HSPA1A*, the favorable allele for BDRC, was the unfavorable allele for fertility in beef cattle [19]. It was not expected that CR would be related to DPR, because CR is determined by the bull, whereas DPR is a measure of female fertility. In contrast, it was hypothesized that BDRC would be related to DPR, because competence of the

embryo to develop to the blastocyst stage is one of the determinants of whether a female reproduces successfully. The lack of a relationship could be due to inaccuracies in estimates of in vitro development (repeatability for BDRC was only 0.55), as well as the fact that early embryonic development is only one of many physiological events that determine in vivo fertility. In addition, some genes that affect embryonic development in vitro may do so by controlling processes that would not be important for development in vivo (for example, genes that help embryos survive stressful culture conditions). One implication of the lack of relationship between CR or BDRC with DPR and other estimates of fertility in vivo is that it is unlikely that bull differences in daughter fertility can be identified by screening procedures based on in vitro fertilization.

Three SNPs examined in the current study had previously been associated with in vitro fertilization and development. These were *PGR* [7] and *STAT5A* [5] for CR, and *FGF2* [1, 8], *PGR* [7], and *STAT5A* [3, 5] for BDRC. None of these relationships were significant in the current study, although the trend was as expected for *PGR* and CR. Some of the lack of repeatability could be due to different measurements of development in vitro as well as the fact that only the sire was genotyped in the current study versus genotyping of the dam [1, 7, 8], embryo [5], or sire and dam [3] in the other studies. However, inability to repeat SNP effects on biological traits is a common phenomenon [55], and confidence in the genetic relationship between SNPs and phenotype will be increased with independent replication.

Semen extender had an effect on the fertilizing ability of the semen, with slightly superior results for semen in egg yolk-based extenders as compared to semen in milk extenders. This result is in contrast to what is seen in the goat, where semen extended in milk resulted in better in vitro fertilization rates compared to semen extended in egg yolk [56], and what is seen in the human, where there was no difference in fertilization rates between semen extended in milk or egg yolk [57]. More research is required to determine whether the interaction between extender and species is real and, if so, the mechanism responsible. The enhanced fertilizing ability of semen extended in yolk extender in the current study may be due to in vitro conditions, because there was no consistent difference between milk- and egg yolk-extended semen for cows subjected to artificial insemination [58, 59].

In conclusion, the current study showed variation in in vitro fertilization and blastocyst development among different bulls. Moreover, this variation was due, at least in part, to genetic variation. SNPs were identified in 19 genes that were associated with in vitro fertilization and embryo development. Selection of cattle based on the genotype at one or more of these 19 loci may prove useful in conjunction with other genetic markers for improving genetic ability for fertility. In addition, these results point out the potential importance of some previously unknown genes for control of the processes leading to fertilization and embryonic development.

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