J. Dairy Sci. 92:2238–2247
doi:10.3168/jds.2008-1767

Single gene and gene interaction effects on fertilization and embryonic survival rates in cattle

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ABSTRACT

Decrease in fertility and conception rates is a major cause of economic loss and cow culling in dairy herds. Conception rate is the product of fertilization rate and embryonic survival rate. Identification of genetic factors that cause the death of embryos is the first step in eliminating this problem from the population and thereby increasing reproductive efficiency. A candidate pathway approach was used to identify candidate genes affecting fertilization and embryo survival rates using an in vitro fertilization experimental system. A total of 7,413 in vitro fertilizations were performed using oocytes from 504 ovaries and semen samples from 10 different bulls. Fertilization rate was calculated as the number of cleaved embryos 48 h postfertilization out of the total number of oocytes exposed to sperm. Survival rate of embryos was calculated as the number of blastocysts on d 7 of development out of the number of total embryos cultured. All ovaries were genotyped for 8 genes in the POU1F1 signaling pathway. Single-gene analysis revealed significant associations of *GHR*, *PRLR*, *STAT5A*, and *UTMP* with survival rate and of *POU1F1*, *GHR*, *STAT5A*, and *OPN* with fertilization rate. To further characterize the contribution of the entire integrated POU1F1 pathway to fertilization and early embryonic survival, a model selection procedure was applied. Comparisons among the different models showed that interactions between adjacent genes in the pathway revealed a significant contribution to the variation in fertility traits compared with other models that analyzed only bull information or only genes without interactions. Moreover, some genes that were not significant in the single-gene analysis showed significant effects in the interaction analysis. Thus, we propose that single genes as well as an entire pathway can be used in selection programs to improve reproduction performance in dairy cattle.
Key words: survival rate, fertilization rate, candidate pathway, candidate gene

INTRODUCTION

The concentrated focus on selection for production traits in Holstein and other breeds in the past may have led to genotypes in dairy cattle that are suboptimal for reproductive competence (Royal et al., 2000; Lucy, 2001). Recently, however, reproductive performance has been included in the selection indices of many breeding organizations (Miglior et al., 2005). There appear to be about 50 QTL affecting milk production traits (Bagnato et al., 2008; Lipkin et al., 2008). Restructuring of the dairy cattle genome over the past 30 yr caused by intense selection for production traits may have resulted in a hitchhiking effect on a large number of loci affecting fertilization rate and embryonic survival. The decrease in dairy cattle fertility is a worldwide problem and a major cause of economic loss and cow culling in the global dairy herd. Many reasons account for this reduced reproductive efficiency, but the most important component seems to be a reduction in embryonic survival and fertilization rates (Santos et al., 2004). There appears to be an important genetic basis for this decline (Veerkamp and Beerda, 2007), therefore genetic approaches may help alleviate this problem. As such, there is an urgent need to identify the genetic factors responsible for the decline in embryo survival rate. Identifying these factors would enable reduction in the frequency of the deleterious alleles at these loci by marker- or gene-assisted selection, preventing further decline or even improving reproductive status of the global dairy herd.

In previous studies, the effectiveness of the candidate pathway approach in choosing candidate genes affecting milk production traits has been demonstrated (Khatib et al., 2008a; Wang et al., 2008). In the candidate pathway-based approach, candidate genes affecting

Received September 30, 2008.
Accepted December 20, 2008.
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quantitative traits can be identified by tracking their biological action through effector pathways. It is readily recognized that phenotypic variation of quantitative traits results from combined effects of many genes. Therefore, there is an increased interest in the use of multigenic pathway-based approach in which gene interactions can be successfully identified (Horikawa et al., 2008). Also, recently an in vitro fertilization (**IVF**) experimental system in cattle has been developed that enables the association of SNP in candidate genes with fertilization rate and embryonic survival (Khatib et al., 2008a,b). Using this system, 2 genes, fibroblast growth factor 2 (*FGF2*) and signal transducer and activator of transcription 5 (*STAT5A*), were found to be significantly associated with variation in fertilization and embryonic survival rates (Khatib et al., 2008a,b). The *FGF2* gene was chosen from the interferon-tau (IFNT) pathway and *STAT5A* was chosen from the POU1F1 signal transduction pathway using the candidate pathway approach. The IFNT pathway plays a key role in regulating the expression of genes involved in initiation of pregnancy in ruminants, in embryo implantation, and in protection of the conceptus against maternal rejection (Martal et al., 1997). In previous studies, it has been shown that members of the POU1F1 pathway—osteopontin, uterine milk protein, STAT5A, POU1F1—are associated with milk production and health traits (Leonard et al., 2005; Khatib et al., 2007a, b, 2008a; Huang et al., 2008). The objectives of this study were 1) to investigate additional genes in the POU1F1 pathway for individual associations, 2) to analyze the effects of gene-gene interactions on fertilization and embryonic survival rates, and 3) to investigate the relationship between milk production and fertility at the gene level.

MATERIALS AND METHODS

Gene Selection and Genotyping

The genes *POU1F1*, *GH*, *PRL*, *GHR*, *PRLR*, *STAT5A*, *OPN*, and *UTMP* (Figure 1) were chosen for association tests with fertility traits because they are members of the POU1F1 pathway. Table 1 shows SNP information and references for these genes. Genotyping of genes was performed as described in the literature (Table 1) except for *GHR*, for which we used primers, GHR-F ctttggaatacttgggctagcagtgaca"a"tat and GHR-R gtctctctgtggacacaaca, that amplify a 230-bp genomic fragment. The original T nucleotide at position −4 of the SNP was mutated to an A nucleotide in the forward primer to create an *Ssp*I recognition site. Restriction enzyme digestions were carried out according to the manufacturer's instructions.

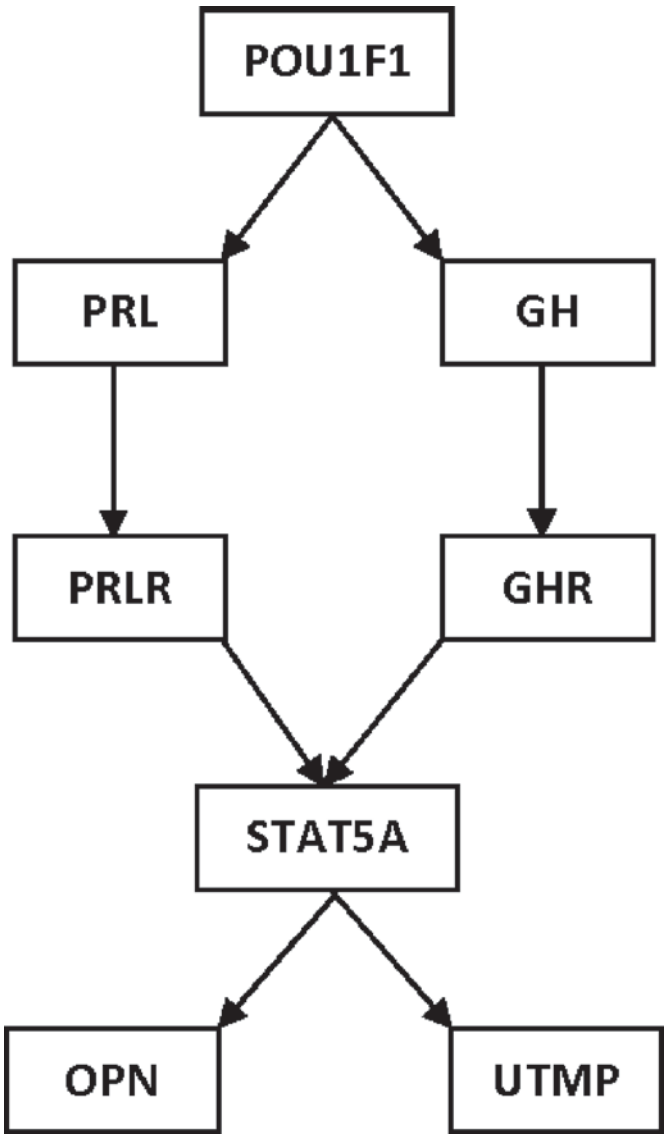

Figure 1. Genes of the POU1F1 pathway investigated for association with fertility traits. Each arrow represents a biological interaction and direction of arrowhead indicates the direction of the signaling cascade. Genes are as described in Table 1.

Fertility Data Collection

Ovaries from mature cows were collected from a local abattoir and immediately used in the IVF experiments as described in Khatib et al. (2008a,b). Briefly, oocytes were aspirated from antral follicles (>2–6 mm) and immediately incubated in maturation medium. On average, about 15 oocytes were aspirated from each ovary. On d 2, all oocytes were fertilized with frozen-thawed Percoll-separated semen that had been adjusted to a final concentration of 1 million sperm/mL. Fertilization rate was calculated as the number of cleaved embryos at 48 h postfertilization out of the total number of oo-

Table 1. Gene name, SNP location, and references used to genotype genes

Gene	Chromosome	SNP (location)	Reference
POU class 1 homeobox 1 (*POU1F1*)	1	A/C (exon 3)	Huang et al. (2008)
Growth hormone (*GH*)	19	A/B[1] (intron 3)	Zhou et al. (2005)
Growth hormone receptor (*GHR*)	20	A/T (exon 8)	Blott et al. (2003)
Prolactin (*PRL*)	23	A/G (exon 4)	Brym et al. (2005)
Prolactin receptor (*PRLR*)	20	A/G (exon 3)	Viitala et al. (2006)
Signal transducer and activator 5A (*STAT5A*)	19	C/G (exon 8)	Khatib et al. (2008)
Osteopontin (*OPN*)	6	C/T (intron 4)	Leonard et al. (2005)
Uterine milk protein (*UTMP*)	21	A/G (exon 4)	Khatib et al. (2007)

[1]*GH* alleles were designated as A and B for consistency with the reports in the literature.

cytes exposed to sperm. Survival rate of embryos was calculated as the number of blastocysts on d 7 of development out of the number of total embryos cultured. Viability was determined as a function of the embryo's ability to attain the morphological stage of blastocyst on d 7 of development. Embryos that failed to show cellular compaction (morula stage) on d 5 or d 6 were considered nonviable. Therefore, only embryos exhibiting adequate compaction followed by the formation of a blastocele on d 7 were considered viable. Ovaries from which fewer than 4 oocytes were harvested were discarded and not further analyzed. A total of 7,413 fertilizations were performed using oocytes from a total of 504 ovaries (collected from 504 cows) and semen from 10 different bulls.

Statistical Analysis

Association of Individual Genes with Fertilization and Survival Rates. Associations of individual genes in the POU1F1 pathway (Figure 1) with fertilization and survival rates were analyzed using the following logistic regression model:

$$\log\left[p\,/\left(1-p\right)\right]_i = \beta_0 + \beta_{1j}\text{Bull}_j + \beta_{2k}\text{Genotype}_k \quad [1]$$

where $\log[p/(1-p)]_i$ ($i = 1, 2, \ldots, n$) is the natural logarithm of odds of survival rate or fertilization rate, β_0 is a general constant, β_{1j} is the fixed effect associated with the jth bull (Bull_j); and β_{2k} is the ovary genotype effect associated with the kth genotype (Genotype_k) of the gene analyzed. This model was fitted by maximum likelihood approach. Association between the gene and survival/fertilization rate was tested using a likelihood ratio test (**LRT**) by comparing to a reduced model without the genotype effect.

Model Selection for Genes in the Pathway With or Without Interaction. Our goal is to select the models that best explain survival and fertilization rates by polymorphisms in genes in the pathway. A stepwise model selection procedure based on Akaike's information criterion (**AIC**) was employed. To select for the interactions that can best fit the data, interaction between each pair of genes was parameterized as follows: 1) Code each gene by 3 variables, "additive" (AA = 0, AB = 1, BB = 2); "Adom" (AA = 1, AB = 1, BB = 0) and "Bdom" (AA = 0, AB = 1, BB = 1); these 3 variables are redundant, and so are the interactions, but they won't enter the model simultaneously; 2) The interaction variables were obtained by the cross products between parameters of 2 genes; and 3) These interaction variables were subjected to an AIC-based stepwise selection. There are indeed many other types of parameterizations, but this type of partitioning effect gives the best interpretability of the selected model. The stepwise selection procedure started with a simple model. At each step, the selection may happen in both directions, either adding one variable not in the model or removing one variable already in the model, depending on which change produces the smallest AIC. This step is repeated until no variable can be removed or added to arrive at an optimal model. To keep hierarchy, all single genes were forced to be in the model when selecting for interactions. The final tuning of the selected candidate model involved removing insignificant interactions and main effects that did not have interaction terms in the model, thus retaining the hierarchy. Likelihood ratio tests were then applied to successively compare selected models with or without interactions. The null model (model 2) that has only bull effect was

$$\log\left(\frac{p}{1-p}\right)_i = \beta_0 + \beta_{1j}\text{Bull}_j, \quad [2]$$

where the notations are as defined for model 1.

To test whether individual interaction between adjacent genes in the pathway was significant, a reduced additive model

$$\log\left(\frac{p}{1-p}\right)_i = \beta_0 + \beta_{1j}\text{Bull}_j + \beta_{2mp}\text{Genotype}_{mp} + \beta_{3nq}\text{Genotype}_{nq} \quad [3]$$

Table 2. Association tests (*P*-values) between individual genes and embryo survival rate, genotypes of ovaries, number of embryos, and observed survival rates

Gene	*P*-value	Genotype	Ovaries, n	Embryos, n	Survival rate
POU1F1		CC	279	3,442	0.36
	0.286	AC	51	622	0.38
		AA	1	14	0.50
GH		AA	289	3,287	0.34
	0.223	AB	69	908	0.34
		BB	3	21	0.52
GHR		TT	256	3,131	0.37
	3.80E-06***	AT	125	1,426	0.29
		AA	17	153	0.28
PRL		GG	231	2,772	0.35
	0.175	AG	97	1,173	0.33
		AA	12	132	0.41
PRLR		AA	99	1,216	0.34
	0.0314	AG	117	1,519	0.33
		GG	91	1,068	0.38
STAT5A		GG	87	902	0.31
	1.37E-07***	GC	232	2,762	0.33
		CC	85	1,113	0.40
UTMP		GG	140	1,735	0.30
	0.000394***	GA	167	1,924	0.36
		AA	112	1,266	0.36
OPN		TT	142	1,734	0.33
	0.228	TC	204	2,503	0.35
		CC	48	457	0.34

***Adjusted *P*-value <0.01.

was compared with a full interactive model

$$\log\left(\frac{p}{1-p}\right)_i = \beta_0 + \beta_{1j}\text{Bull}_j + \beta_{2mp}\text{Genotype}_{mp} + \beta_{3nq}\text{Genotype}_{nq} + \beta_{4mnpq}\text{Genotype}_{mp} : \text{Genotype}_{nq} \quad [4]$$

In the full model (model 4), there are variables representing bull information (β_{1j} for jth bull), genotype information (β_{2mp} and β_{3nq} are the genotypic effects of the pth genotype of the mth gene and of the qth genotype of the nth gene, respectively), and interaction between genes (β_{4mnpq} is the effect for interaction between the pth genotype of the mth gene and the qth genotype of the nth gene). In contrast, there was no interaction term in the reduced model (model 3). Likelihood ratio testing was performed to test for significance of the interaction term. All significances were declared after Šidák adjustment (adjusted *P*-value $= 1 - (1 - p)^{1/n}$; n = number of tests) where applicable. All statistical analysis was implemented in R, version 2.7.2 (www.r-project.org) using functions from packages "stats" and "MASS."

RESULTS

Association of Individual Genes with Fertilization Rate and Embryonic Survival

The *GHR*, *PRLR*, *STAT5A*, and *UTMP* genes showed significant associations with survival rate (Table 2). For *GHR*, the survival rate of embryos produced from TT ovaries was 9% higher than that of embryos produced from AA ovaries. For *STAT5A*, CC ovaries showed 9 and 8% higher survival rates than that of GG and GC ovaries, respectively. The *PRLR* and *UTMP* genes showed 4 and 6% survival rate differences between their genotypes (Table 2). Table 3 shows the association of individual genes with fertilization rate. Ovaries carrying the TT genotype of *OPN* showed a 70% fertilization rate versus a 62% rate for ovaries carrying the CC genotype. The CC genotype of *STAT5A*, which was also associated with high survival rate, showed significant association with fertilization rate versus the GC and GG genotypes. Similarly, although less statistically significant, *GHR* ($P = 0.0647$) and *POU1F1* ($P = 0.0516$) also showed associations with fertilization rate.

Effect of the Pathway Genes and Their Interactions on Fertility Traits

To quantify the contribution of the POU1F1 pathway genes to fertilization rate and early embryonic survival rate, a model with only bull information was compared with a more complex model which had selected genes in the pathway, additively and without any interaction term. Adding gene information additively significantly improved the model fit for both fertilization rate and early embryonic survival rate (Table 4). Furthermore, when selected interactions between genes were included in the models, the model fits improved significantly for

Table 3. Association tests (*P*-values) between individual genes and fertilization rate, genotypes of ovaries, number of fertilizations, and observed fertilization rate

Gene	*P*-value	Genotype	Ovaries, n	Fertilizations, n	Fertilization rate
POU1F1		CC	279	4,821	0.71
	0.0516	AC	51	918	0.68
		AA	1	19	0.74
GH		AA	289	4,880	0.67
	0.621	AB	69	1,308	0.69
		BB	3	30	0.70
GHR		TT	256	4,473	0.70
	0.0647	AT	125	2,154	0.66
		AA	17	223	0.69
PRL		GG	231	3,956	0.70
	0.956	AG	97	1,668	0.70
		AA	12	185	0.71
PRLR		AA	99	1,747	0.70
	0.551	AG	117	2,138	0.71
		GG	91	1,530	0.70
STAT5A		GG	87	1,360	0.66
	0.00371**	GC	232	4,028	0.69
		CC	85	1,574	0.71
UTMP		GG	140	2,580	0.67
	0.546	GA	167	2,784	0.69
		AA	112	1,830	0.69
OPN		TT	142	2,481	0.70
	0.00529*	TC	204	3,601	0.70
		CC	48	739	0.62

*Adjusted *P*-value <0.10; **adjusted *P*-value <0.05.

both fertilization rate ($P = 2.11E{-}05$) and early embryonic survival rate ($P = 2.21E{-}08$; Table 4). Here, the overall model fits including potentially significant genes and interactions selected by a stepwise procedure were compared instead of looking at individual genes or interactions present in the model, enabling us to understand the effects of genes or interactions in general and within the network of the pathway.

Identification of Favorable Genotype Combinations

To test whether the 2-way interaction between each of the 8 direct interactions in the POU1F1 pathway was significantly associated with fertilization and survival rate, LRT was used to assess the significance of the interaction term in the model with 2 genes. Table 5 shows that 5 of 8 gene-gene interactions were significantly associated with survival rate, while only 2 interactions were associated with fertilization rate. However, after Šidák correction of *P*-values, only 4 interactions (*POU1F1*/*PRL*; *GHR*/*STAT5A*; *PRLR*/*STAT5A*; and *STAT5A*/*UTMP*) were still significant for survival rate. It is worth noting that neither *POU1F1* nor *PRL* was statistically significant for survival rate when analyzed individually. For fertilization rate, only one interaction (*STAT5A*/*UTMP*) was still significant after Šidák correction of *P*-values. For significant interactions, the observed survival/fertilization rate and the 95% confidence intervals for each genotype combination of the 2-way interactions were calculated (Table 6). The difference between the highest and the lowest observed survival rate for genotype combinations ranged from a 5% difference for CC/AG and AC/AG of *POU1F1*/*PRL* to a 20% difference for AT/GC and AT/CC of *GHR*/*STAT5A*. Likewise, the difference between CC/GG and GG/AA of *STAT5A*/*UTMP* was 11% for fertilization rate (Table 6).

DISCUSSION

In previous studies, we demonstrated the efficiency of the candidate pathway approach in choosing candidate genes (e.g., *STAT5A* and *FGF2*) affecting milk production and fertility traits (Khatib et al., 2008a,b; Wang et al., 2008). Both *STAT5A* and *FGF2* are members of the IFNT and POU1F1 pathways. In this study, we extended our investigation to include additional genes in the POU1F1 pathway (Figure 1). Recently, we reported the association between *STAT5A* and fertility traits using records of 1,551 IVF embryos produced from 3 bulls and 160 ovaries (Khatib et al., 2008a). To validate these results, in the current study we used 504 ovaries and semen samples from 10 different bulls that produced about 4,780 embryos.

Single Gene Effects

Single gene association analysis revealed significant associations of *STAT5A*, *UTMP*, and *OPN* and, to a lesser extent *POU1F1*, with fertilization rate, and of

Table 4. Model selection with or without interactions of genes, model degrees of freedom (df), deviance, the selection criterion (AIC; Akaike's information criterion), and significance of model comparisons

Selected model[1]	Deviance	df	AIC	*P*-value[2]
Survival rate				
Only bull	801.86	9	1412.8	NA
Genes without interactions[3]	745.13	19	1376.1	1.50E−08
Genes with interactions[4]	687.16	30	1340.1	2.21E−08
Fertilization rate				
Only bull	734.60	9	1517.6	NA
Genes without interaction[5]	702.28	13	1493.3	1.64E−06
Genes with interactions[6]	647.00	32	1476.0	2.11E−05

[1]The models are presented as variables selected by the stepwise selection procedure (see Materials and Methods). "BULL" represents bull effects; gene names represent effects of genes. An interaction is presented in the format of "Gene.Mode:Gene.Mode" where "Gene" is the name of the gene and "Mode" is one of the component variable of the gene (see Materials and Methods). For example, PRL.add:PRLR.Gdom represents the interaction between the additive component of the PRL gene and the G-allele dominant component of PRLR.

[2]The *P*-values are from likelihood ratio tests comparing two models successively (with genes versus no gene and with interactions versus no interactions).

[3]BULL + GHR + PRLR + STAT5A + UTMP + OPN.

[4]BULL + GH + GHR + PRL + PRLR + STAT5A + UTMP + OPN + PRL.add:PRLR.Gdom + PRL.Adom:PRLR.Gdom + PRLR.add:STAT5A.add + GHR.add:STAT5A.Cdom + STAT5A.Cdom:UTMP.Adom + STAT5A.add:UTMP.Adom + STAT5A.Cdom:OPN.add.

[5]BULL + POU1F1 + STAT5A.

[6]BULL + POU1F1 + GHR + PRL + PRLR + STAT5A + UTMP + OPN + OU1F1.add:PRL.add + PRL.Gdom:PRLR.Adom + PRLR.add:STAT5A.add + PRLR.Gdom:STAT5A.Gdom + PRLR.Adom:STAT5A.Cdom + GHR.add:STAT5A.Gdom + STAT5A.Cdom:UTMP.Adom + STAT5A.Gdom:UTMP.add + STAT5A.Gdom:OPN.Cdom.

GHR, *PRLR*, and *STAT5A* with survival rate. The causative mutations responsible for these associations have not been identified; therefore we assume that the observed effects are due to linkage disequilibrium between the typed polymorphisms and causative mutations affecting fertilization and early embryonic survival. Given that these effects were discovered at early stages of embryonic development, we questioned whether genes associated with fertilization are expressed in eggs and sperm and whether genes associated with survival are expressed in the blastocyst or in prior stages of development. Indeed, several reports have demonstrated the involvement of these genes in the fertilization process and in early embryonic development. Nakasato et al. (2006) have shown that *STAT5A* is expressed in oocytes at the metaphase II stage (before fertilization) and in the 2-cell, 4-cell, morula, and blastocyst stages. These results are consistent with effects of *STAT5A* on fertilization and embryonic development found in our study. The OPN protein contains the arginine-glycine-aspartic acid peptide sequence which is known for its ability to block fertilization (Campbell et al., 2000). Recently, it was reported that fertilization in cows was blocked as a result of OPN-antibody binding in both spermatozoa and oocytes (Gonçalves et al., 2007, 2009). The authors concluded that OPN has a potential role in sperm-egg binding and in early embryonic development. For *UTMP*, which was found to be associated with fertilization rate in this study, there are no reports on its expression in the developing embryo. However, in a previous study, we reported the predominant expression of this gene in the bovine reproductive tissues

Table 5. Likelihood ratio tests (LRT) between the models with and without interaction, degrees of freedom (DF), and significance of interactions (*P*-values) for survival and fertilization rates

	Survival rate			Fertilization rate		
Gene pair	LRT	DF	*P*-value	LRT	DF	*P*-value
POU1F1:GH	3.16	1	0.0754	1.67	1	0.197
POU1F1:PRL	11.6	2	0.00297**	2.31	2	0.316
GH:GHR	0.250	3	0.969	4.82	3	0.186
PRL:PRLR	11.5	4	0.0212	2.68	4	0.612
GHR:STAT5A	24.1	4	7.60E-05***	5.24	4	0.264
PRLR:STAT5A	18.1	4	0.00118**	8.74	4	0.0680
STAT5A:UTMP	36.7	4	2.07E−07***	29.8	4	5.42E−06***
STAT5A:OPN	4.56	4	0.336	11.7	4	0.0195

**Adjusted *P*-value <0.05; ***adjusted *P*-value <0.01.

endometrium and ovary which suggests an important role for *UTMP* in reproductive success (Khatib et al., 2007a). Although not statistically significant, *GHR* ($P = 0.0647$) and *POU1F1* ($P = 0.0516$) showed association with fertilization rate. These results are consistent with recent reports on the expression of these genes in oocytes and on their importance in embryonic development. Izadyar et al. (2000) showed that *GHR* is expressed in bovine oocytes and embryos in different developmental stages. Joudrey et al. (2003) reported the expression of the bovine *GH* and *POU1F1* in both immature and mature oocytes and in the early embryo from fertilization to the blastocyst stage. Also, *PRLR* was reported to be highly expressed in metaphase II-stage oocytes and in lower expression levels between the 4-cell and blastocyst stages (Nakasato et al., 2004).

Given that *GH* and *PRL* play important roles in cell differentiation and proliferation, that *POU1F1* is a transcription factor that binds and activates promoters of *GH* and *PRL*, and that all these genes were found be associated with fertilization and early embryo survival in our study, these results suggest an important role of the integrated pathway in regulating embryo development.

Interaction Effects in the POU1F1 Pathway

To further understand the contribution of the entire integrated POU1F1 pathway to the variation in fer-

Table 6. Observed survival/fertilization rates, numbers of ovaries and embryos, and confidence intervals for genotype combinations

Genes/trait	Genotype combination	Ovaries, n	Embryos, n	Observed rate	95% CI
Survival rate					
POU1F1:PRL	CC:GG	175	2,147	0.36	[0.3414, 0.3819]
	CC:AG	72	910	0.32	[0.2934, 0.3538]
	CC:AA	11	101	0.33	[0.2376, 0.4158]
	AC:GG	33	389	0.36	[0.3136, 0.4087]
	AC:AG	15	185	0.37	[0.2973, 0.4378]
	AC:AA	1	31	0.68	[0.5161, 0.8387]
GHR:STAT5A	TT:GG	56	596	0.34	[0.3020, 0.3775]
	TT:GC	139	1,693	0.36	[0.3420, 0.3881]
	TT:CC	53	745	0.40	[0.3611, 0.4309]
	AT:GG	27	264	0.27	[0.2159, 0.3220]
	AT:GC	71	840	0.25	[0.2178, 0.2762]
	AT:CC	24	284	0.45	[0.3979, 0.5106]
	AA:GG	2	16	0.25	[0.0625, 0.5000]
	AA:GC	11	103	0.26	[0.1845, 0.3495]
	AA:CC	3	22	0.27	[0.0909, 0.4545]
PRLR:STAT5A	AA:GG	22	225	0.25	[0.1956, 0.3111]
	AA:GC	57	705	0.32	[0.2879, 0.3560]
	AA:CC	20	286	0.45	[0.3986, 0.5140]
	AG:GG	27	256	0.23	[0.1836, 0.2891]
	AG:GC	71	963	0.35	[0.3146, 0.3749]
	AG:CC	19	300	0.35	[0.2933, 0.4000]
	GG:GG	26	267	0.38	[0.3258, 0.4419]
	GG:GC	46	539	0.37	[0.3321, 0.4137]
	GG:CC	19	262	0.40	[0.3397, 0.4580]
STAT5A:UTMP	CC:AA	17	208	0.35	[0.2885, 0.4183]
	CC:GA	40	536	0.38	[0.3396, 0.4216]
	CC:GG	26	356	0.44	[0.3876, 0.4888]
	GC:AA	63	731	0.37	[0.3379, 0.4090]
	GC:GA	89	1,010	0.37	[0.3356, 0.3950]
	GC:GG	79	1,010	0.25	[0.2228, 0.2762]
	GG:AA	28	288	0.34	[0.2847, 0.3958]
	GG:GA	30	286	0.29	[0.2343, 0.3392]
	GG:GG	29	328	0.30	[0.2561, 0.3567]
Fertilization rate					
STAT5A:UTMP	CC:AA	17	305	0.68	[0.6295, 0.7344]
	CC:GA	40	759	0.71	[0.6732, 0.7378]
	CC:GG	26	483	0.74	[0.6977, 0.7764]
	GC:AA	63	1,020	0.72	[0.6892, 0.7441]
	GC:GA	89	1,430	0.71	[0.6825, 0.7301]
	GC:GG	79	1,555	0.65	[0.6257, 0.6733]
	GG:AA	28	441	0.65	[0.6077, 0.6961]
	GG:GA	30	454	0.63	[0.5859, 0.6740]
	GG:GG	29	465	0.71	[0.6645, 0.7462]

tilization and embryonic survival, a model comparison procedure was applied. First, a simple model (model 2) with only bull information was fitted, representing the situation in which bull information and fertilization and survival rate alone are available. Second, this simplest model (model 2) was compared with a more complex model (model 3), which has all 8 genes in the pathway acting additively. The individual genes additively in model 3 clearly improved the fit for both survival and fertilization rate. Finally, a more complicated model (model 4), which contained all 8 individual genes and all interactions between adjacent genes in the pathway, revealed a significant improvement in explaining the variation in both fertilization and survival rates. Thus, genes of this pathway, individually and in interactions with other genes, clearly influence embryo fertilization/survival rates.

One of the drawbacks of the conventional methods of gene mapping is that they focus on the average genetic effects of the genotypes of a QTL or individual genes, not taking into account the possibility that these effects can be influenced by other loci (Carlborg and Haley, 2004). Indeed, recent studies in human, mouse, plants, and *Drosophila* indicate that epistatic interaction contributes to the regulation of quantitative traits. There are different reports in which disease occurs not through any single gene, but only through a combination (interaction) of 2 or more genes. For example, the combination of the KIR3DS1 allele of the killer immunoglobulin-like receptor gene and the HLA-BW4–80ile allele is associated with delayed progression of AIDS in individuals infected with HIV-1 (Martin et al., 2002). The authors found that in the absence of KIR3DS1 alleles, the HLA-BW4-ile80 allele did not show any significant effects on any of the AIDS traits. In our study, single gene analysis showed no significant associations between *POU1F1* and *PRL* and survival rate. In contrast, the 2-way interaction analysis showed that some genotypic combinations of these genes were found to be associated with significant effects on early embryonic survival (Table 6). Likewise, the genotypic combination of AT/CC of STAT5A/GHR was associated with 45% survival rate versus 25% for the combination of AT/GC (Table 6). Taken together, these results suggest that genes in the pathway are important genetically for fertilization or early embryonic survival and that genotypic combinations should not be ignored in marker-assisted selection programs.

Our results suggest that both polymorphisms in genes and gene interactions are important in determining the phenotypic variation in fertility traits. It should be noted that these results do not rule out the possibility of involvement of many other genes in controlling embryonic survival and fertilization, which are both complex traits. Instead, the results of this study testify to the power of the IVF experimental design and to the efficiency of including gene information and their interactions in the statistical analysis. Additionally, these results emphasize the effectiveness of the candidate pathway approach in choosing candidate genes. Genes are chosen based on their biological functions in the metabolic pathway. When one gene of a pathway affects our target traits, other genes of the same pathway are likely to do so as well. Recent studies show the power of epistatic models in identification of novel QTL, in identification of QTL without individual effects, and in detection of QTL with small effects which may be responsible for a large portion of trait variation (Kroymann and Mitchell-Olds, 2005). Indeed, our results show that gene interactions of *POU1F1* and *PRL* were found to be associated with embryonic survival rate, whereas single-gene analysis of these genes did not show significant associations.

Milk Production and Fertility in Dairy Cattle

It is widely accepted that there is a strong association between high milk production and low fertility in dairy herds. Washburn et al. (2002) analyzed the relationship of conception rate and milk production over more than a 20-yr time period (1976–1999) in dairy herds in the southeastern United States. It was clear that conception rates decreased from about 55% to about 35% during this time period as milk production dramatically increased. López-Gatius (2003) reported that each 1,000-kg increase in milk yield per cow was associated with a decrease of 3.2 to 6% in pregnancy rate, 4.4 to 7.6% in the number of cycling cows, and with a 4.6 to 8% increase in the incidence of inactive ovaries. In a recent review of the literature, Dobson et al. (2008) concluded that over the past 30 to 50 yr, intensive selection to milk yield traits has led to a reduction in first-service pregnancy rate from 70 to 40%.

The genes investigated in this study were chosen for association tests with fertility traits because they are members of the POU1F1 signaling pathway and because of previous studies relating some members of this pathway with fertilization success and embryonic survival. Moreover, all investigated genes have been reported to be associated with milk production traits in dairy cattle. Therefore, for this fertility study, we chose the same polymorphisms reported in milk production association studies to test whether or not they are also associated with fertility traits.

The antagonistic relationship between high milk production and fertility that has been observed for many years in dairy cattle (López-Gatius, 2003; Dobson et al., 2008) was detected for some of the genes investi-

gated in this study. In the Viitala et al. (2006) study, the AA and AG genotypes of *PRLR* were reported to be associated with significantly higher protein, fat, and milk yields versus the GG genotype. In our study, the AA and AG genotypes of *PRLR* were associated with a lower survival rate versus the GG genotype. In a previous study (Khatib et al., 2007a), we reported the association of the G allele of the *UTMP* gene with longer productive life in 2 different Holstein dairy cattle populations. In this study, the GG genotype of *UTMP* was associated with a significantly lower embryo survival rate. For *GHR*, the AA genotype of *GHR* has been reported to be associated with higher milk yield (Blott et al., 2003), while in this study, AA was associated with a lower survival rate. The G allele of *STAT5A* was associated with lower milk fat and protein percentages (Khatib et al., 2008a) and with lower fertilization and survival rates in this study. Given that milk fat and protein percentages are negatively correlated with milk yield traits, the interpretations of *STAT5A* regarding the antagonistic relationship between milk production and fertility are consistent with those of *GHR*, *PRLR*, and *UTMP*. However, for *OPN*, where the C allele has been reported to be associated with significant increases in fat and protein percentages (Leonard et al., 2005), the CC genotype in this study was associated with a decrease in fertilization rate. This result for *OPN* is inconsistent with the other 4 genes investigated in this study. The OPN protein is a glycophosphoprotein cytokine that has multiple functions including cell migration and adhesion, tissue remodeling and wound healing, promoting differentiation of precursor cells to become osteoclasts and enhancing the activity of osteoblasts, acting in inflammation by attracting macrophages and T cells and accelerating early acute immune responses, and functioning in cell survival and proliferation (reviewed in Johnston et al., 2008). Because of these multiple functions, it is unlikely that *OPN* has been under selection pressure similar to other milk production genes.

CONCLUSIONS

In summary, in this study we demonstrated the effectiveness of the candidate pathway strategy and the usefulness of the experimental IVF population developed in our laboratory in identifying candidate genes affecting fertility traits. To test the effect of the integrated POU1F1 pathway on the variation of the investigated traits, interaction effects were estimated using a model comparison procedure. Some genes that were not significant using single gene analysis (e.g., *POU1F1* and *PRL* for survival rate) showed significant effects in the interaction analysis. Moreover, the analysis that contained all 8 individual genes and all interactions between adjacent genes in the pathway revealed a significant improvement in explaining the variation in both early embryonic survival and fertilization rates. Nonetheless, there is a need for a field test to confirm and validate the in vitro results obtained in this study. Validation of the field test will provide the platform for an extensive future research on the genomic basis of reduced embryonic survival and on improving reproductive efficiency in dairy cattle using modern molecular genetic techniques of gene-assisted and pathway-assisted selection.

ACKNOWLEDGMENTS

This study was supported by USDA Hatch grant No. WIS-04895 from the University of Wisconsin-Madison.

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