Differential expression of heat shock protein genes and their splice variants in bovine preimplantation embryos

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ABSTRACT

Heat shock proteins (HSP) are among the first proteins produced during embryonic development and are crucial to cell function; their activities include folding, unfolding, transport, and localization of proteins and differentiation and regulation of the embryonic cell cycle. Although HSP have been extensively studied in humans and mice, limited information exists on the roles of these genes in bovine embryos. As such, the objectives of this study were to profile the expression of HSP and their splice variants in bovine embryos (degenerates vs. blastocysts) and to carry out association analysis with fertility traits. Quantitative real time PCR analysis revealed differences in expression that ranged from 1.5- to 7.6-fold in degenerate embryos compared with blastocysts. Interestingly, all members of the HSP40 family were found to be upregulated in degenerate embryos. Analysis of current bovine Ensembl data showed that 13 HSP genes have 1 transcript each, 2 genes have 2 transcripts each, and 2 have 3 transcripts each. Some splice variants show differential expression between degenerate embryos and blastocysts, whereas others were not expressed at all in embryos, which implies different functions of these transcripts in embryonic development. The most significant differentially expressed genes were further investigated for association with fertility and development traits. Single nucleotide polymorphisms in *DNAJC15* and *DNAJC27* were found to be associated with blastocyst rate and fertilization rate, respectively. Collectively, the roles of HSP in fertilization and early development of cattle embryos are important.

Key words: heat shock protein, bovine, embryo, alternative splicing

INTRODUCTION

Heat shock proteins (HSP) are among the most highly conserved proteins in nature and have been

found in all organisms studied from bacteria to humans (Becker and Craig, 1994). The structure and roles of HSP as molecular chaperones in folding, transport, and assembly of proteins, as well as in protecting the cell under different stress conditions, have been extensively studied and reported in the scientific literature (Becker and Craig, 1994; Nollen and Morimoto, 2002; Qiu et al., 2006). The fact that HSP are essential in the folding, stability, and cellular localization of newly synthesized proteins implies key roles of these proteins in apoptosis, cell differentiation, and regulation of the embryo cell cycle (Luft and Dix, 1999; Lanneau et al., 2007). Strong evidence that has accumulated on the expression of HSP during spermatogenesis, oogenesis, and embryogenesis suggests they have important functions in fertilization and during the preimplantation period (Neuer et al., 1999). Mouse embryos cultured with monoclonal antibodies to HSP have been found to display a significantly decreased blastocyst rate (Neuer et al., 1998). Al-Katanani and Hansen (2002) reported that the addition of antibodies for the induced form of HSP70 decreased the blastocyst rate of cattle embryos, suggesting that HSP70 is involved in proper embryonic development. Matwee and colleagues (2001) showed that fertilization—measured as the number of spermatozoa tightly bound to a zona pellucida—and embryo development in cattle were significantly affected by the presence of different concentrations of anti-HSP70 antibodies.

Although strong evidence exists in the literature on the roles of HSP in early embryonic development, most of the studies have focused on the mouse and only a few HSP genes have been studied in cattle embryos. Given that HSP are among the first proteins produced during embryonic development (Neuer et al., 1999), determining expression patterns of these genes in developed versus arrested embryos could lead to the identification of specific genes involved in reproductive success. This is of particular interest in cattle because of decreasing fertility over the past few decades (Dobson et al., 2007; Leroy et al., 2008). Furthermore, recent studies have shown that low fertilization rates and embryonic loss seem to be the main factors in dairy cattle infertil-

Received December 28, 2010.

Accepted April 21, 2011.

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ity (Santos et al., 2004; Morris and Diskin, 2008). To characterize genetic factors involved in embryonic loss, and hence infertility, an in-vitro fertilization (IVF) experimental system in cattle has been used to associate candidate genes and pathways with fertilization rate and embryonic survival at both the genomic and gene expression levels (Khatib et al., 2008a,b, 2009; Huang et al., 2010a,b; Huang and Khatib, 2010).

The present study hypothesizes that the high structural and functional conservation of HSP during evolution suggests crucial roles in fertilization, embryo development, and thus, fertility in cattle. Also, some HSP are considered housekeeping genes that are essential for many cell functions. Hence, the objectives of this study were to evaluate the expression levels of 17 HSP genes in biological replicate bovine embryo pools that differ in their morphology and developmental statuses and to look for associations of polymorphisms in significant genes with fertility traits. In addition, recent studies have shown that more than 90% of the human multi-exon genes encode multiple transcripts (Wang et al., 2008) and that alternative splicing is considered to be a key factor in the evolution of phenotypic complexity (Blencowe, 2006). However, limited information exists on the pattern of alternative splicing in cattle or its roles in embryonic development. Recently, using high-throughput sequencing of transcriptomes (RNA-seq), thousands of alternative splicing events were detected and tested for their association with the status of embryonic development (Huang and Khatib, 2010). Thus, another objective of this study was to compare the expression levels of splice variants of HSP in degenerate embryos and healthy blastocysts.

MATERIALS AND METHODS

In Vitro Maturation, Fertilization, and Embryo Culture

In this study, fertilization and embryo production were performed in 2 different experiments. The first experiment is a comparison of expression profiles of 17 HSP between 2 populations of embryos differing in their morphology, and the second experiment is a genomic association analysis of genes—found to be differentially-expressed in the first experiment—with fertilization and blastocyst rates.

Experiment I: Expression Analysis of HSP Genes in Cattle Embryos

Ovaries were obtained from a local abattoir and processed for IVF using standard protocol (Khatib et al., 2008b; Huang et al., 2010b). Briefly, oocytes were aspirated from 2 to 6-mm follicles and washed in Tyrode's

albumin lactate pyruvate (TALP)-Hepes medium, after which they underwent maturation in supplemented M199 media (Khatib et al., 2008b). Incubation was completed at 5% CO₂ in air at 39°C and high humidity for 22 to 24 h, at which time oocytes were then washed in TALP-Hepes medium and moved into fresh IVF-TALP medium (BioWhittaker Inc., Walkersburg, MD). Semen samples from 2 different bulls were used for fertilization; sperm underwent Percoll separation (45–90% gradient) and were adjusted to a final concentration of 1×10^6 /mL (Parrish et al., 1995). Fertilization was marked as d 0 of development and completed by combining sperm, heparin, and penicillamine, hypotaurine, and epinephrine with the oocytes in 44-μL drops of media (Khatib et al., 2008b). Incubation was then continued for 22 to 24 h, after which the putative zygotes were washed in TALP-Hepes medium, denuded of remaining cumulus cell complexes, and placed into supplemented synthetic oviductal fluid (BioWhittaker Inc.) and returned to incubation (Khatib et al., 2008b).

Morphological Assessment

Putative zygotes were cultured over an 8-d period, during which assessments of their developmental progress were made. By d 5 of development, a bovine embryo should attain approximately 16 to 32 cells and show signs of cellular compaction deeming it as morula. Embryos failing to show these characteristics were assumed to have been arrested in development and were excluded from analysis. Morulas were then continued in culture until d 8. By this time, an embryo should show evidence of a fluid-filled cavity (blastocoele), which gives rise to the differentiation of the inner cell mass and the trophectoderm, qualifying it as a blastocyst. For expression analysis, 2 populations of embryos were collected. The first consisted of embryos that attained a compacted morula status by d 5 but failed to form a blastocoele by d 8, referred as degenerate embryos. The second consisted of embryos that developed into blastocysts by d 8. Embryos from each morphological group were collected in pools of 20 and preserved in RNAlater (Ambion Inc., Austin, TX). Two bulls were used with 2 sets of biological replicate pools (total of 4 biological replicates) from each to prevent maternal crossover.

Quantitative Real-Time PCR

A previous study in our laboratory using microarray expression analysis has revealed that many genes are differentially expressed between blastocysts and degenerate embryos (Huang et al., 2010b). Seventeen HSP genes that showed 1.5-fold or higher differences in expression between the embryo groups described in the

Table 1. Primers used for total expression and alternative splicing analysis of heat shock protein (HSP) genes using quantitative real-time PCR (qRT-PCR) and RT-PCR

Item	Family ¹	Forward primer 5'–3'	Reverse primer 5'–3'	Amplicon size (bp)
Gene				
<i>GAPDH</i>		TGCCCAGAATATCATCCC	AGGTCAGATCCACAACAG	134
<i>DNAJC5</i>	HSP40	CTACGACAAGTACGGCTCAC	GGCAGCAGCAACAGTAGC	149
<i>DNAJC15</i>	HSP40	AGGTCGCTACGCATTTCAG	GACTTGCTTCTCGCCTACTC	137
<i>DNAJC19</i>	HSP40	GGACTGACCATTGCTGCTG	CAAACCCACCTCTGTAATAGC	140
<i>DNAJC24</i>	HSP40	GAAATATGGGACCAGTAGATGC	TGTAACCTTCTTCTGCTTCATCC	134
<i>DNAJC27</i>	HSP40	AACAAGCGGACACCATTTCG	TGAAGCAGCAGCAAGC	124
<i>HSPA5</i>	HSP70	CAACCAACTGTTACCATCAAGG	AAAGGTGACTTCAATCTGTGG	133
<i>HSPA9</i>	HSP70	GACCAACTGCCTGCTGATG	GATGCCGCCTGCCTTATG	113
<i>HSPA8</i>	HSC70	CGCAGAAGCCTACCTTGG	GTTGAGACCAGCAATAGTTCC	115
<i>HSPA14</i>	HSP70	AACCTTAGCACAGTACCTAGC	TGTCAGCACCGTTCATCAG	101
<i>HSBP1</i>	HSBP	CATGTCCGACCAGATCATTG	TTCAGTGTCCAGCTCTTCC	115
<i>HSPE1</i>	HSP10	GCAAGCAACGGTGGTAGC	ACTTTGGTGCCTCCATATTCTG	117
<i>DNAJB1</i>	HSP40	GAGGAGAAGTTCAAGGATCG	TTAGTACCGCCGCTGCTC	134
<i>DNAJB12</i>	HSP40	GCAAACTAGCCCTCAAATTCC	CCTTGTCATCACCGAACTGG	141
<i>DNAJC14</i>	HSP40	GTGAATGAGTTTCTGTCCAAGC	GTATCTGGCACTCTTAGGTTCC	114
<i>HSPA2</i>	HSP70	ACGCTGTGGAGTCTATACC	TTCCGCCATCTGGTTCCG	146
<i>HSPA4</i>	HSP70	TCCTGCCTTAGAAGAGAAACC	CCCAGTGTTGTGTCAAAATGC	132
<i>HSPH1</i>	HSP100	ATGTTGAGTTGCCTATTGAAGC	CCTCCACCGCATTCTTAGC	141
Transcript				
DNAJC5–667		AGGAGACGGAGTTTCTATG	CACGTTTCACACCTCAAC	126
DNAJC5–725		GGCCCTGTTTCATCTTCTG	GGCACAGACCCCTCTCAT	181
DNAJC5–1049		GGGTTCAACTAAATCCAGGA	ACGCCATCTCTGTGACTA	79
DNAJC19–475		GGATCTCCTTATATAGCAGCCAAA	AGCCTTCCCTCCCAGTGA	93
DNAJC19–536		ATGCTCATCGGCGAATTATG	AGCTGGAACGCATAAGAGAA	161
DNAJC24–628		CTCATTTTAATGGAAGATG	GTATCACAAAGAAATCAGT	176
DNAJC24–2172		CATCCAGATAAACAGAGT	GGTCCCATATTTCTTAGAT	161
DNAJC24–1893		CAAAAGAAAGTATCTCATTC	TAACTTCTTCTGCTTCATC	176
DNAJB12–1220		ACCGACTGTCAGAGACTATG	CGGCCTCCAATTCCATTT	127

¹HSC = heat shock constitutive protein; HSBP = heat shock factor binding protein.

study of Huang et al. (2010b) were chosen for validation of differential expression and further investigation in new sets of blastocysts and degenerate embryo pools using quantitative real-time PCR (qRT-PCR) analysis. Primers (Table 1) were designed to amplify fragments spanning more than one exon to exclude the possibility of genomic DNA contamination in the qRT-PCR reactions using the Beacon Designer software (Premier Biosoft International, Palo Alto, CA). Total RNA was extracted from pools of embryos using RNAqueous-Micro (Ambion Inc.) and quality controlled using a RNA 6000 Pico Chip (Agilent Technologies Inc., Santa Clara, CA). Messenger RNA was amplified using MessageAmp II (Ambion Inc.), followed by cDNA synthesis using iScript (Bio-Rad Laboratories Inc., Hercules, CA) according to manufacturers' instructions. Reactions of qRT-PCR were run on a DNA Engine Opticon 2 Detection System (MJ Research Inc., Watertown, MA) using iQ SYBR Green Supermix kit (Bio-Rad Laboratories Inc.). Each sample was run in quadruplicate and all 4 expression results were averaged. Analysis of gene expression levels was conducted using the $2^{-\Delta\Delta C_t}$ (where C_t is the cycle threshold) method (Livak and Schmittgen, 2001). The selection of the housekeeping gene

GAPDH as an endogenous control was as described in Huang et al. (2010b).

Experiment II: Association Analysis of Differentially Expressed HSP with Fertilization and Blastocyst Rates

To further investigate the involvement in fertility of genes (*DNAJC15*, *DNAJC19*, *DNAJC24*, and *DNAJC27*) that showed the highest-fold change in expression in degenerate embryos versus blastocysts, an association analysis between SNP in these genes and 2 main fertility traits, fertilization rate and blastocyst rate, was performed. Using the IVF experimental system, several genes were found to be significantly associated with variation in fertilization and embryonic survival rates (Khatib et al., 2008a,b; 2009).

Phenotypic Data. To generate phenotypic data for association analysis, a total of 6,893 IVF were performed using oocytes from 399 ovaries (obtained from 399 Holstein cows) and semen samples from 12 Holstein bulls. For 92 ovaries, oocytes were fertilized by 2 different bulls each. Fertilization rate was calculated as the number of cleaved embryos 48 h postfertilization out

of the total number of oocytes exposed to sperm. Blastocyst rate was calculated as the number of embryos that reached blastocyst stage out of the total embryos cultured by d 8.

Genotyping. The DNA was extracted from ovaries ($n = 399$) using standard phenol/chloroform protocols (Sambrook and Russell, 2001). The DNA concentrations were measured using a spectrophotometer (Ultra-spec 2100; Amersham Biosciences, Uppsala, Sweden). For polymorphism identification, 3 DNA pools were constructed from 20 different ovary/cow samples to contain 25 ng of DNA from each sample. The DNA pools were amplified with different sets of primers designed from the coding and 5' and 3' untranslated regions (UTR) of the 4 candidate genes (Supplementary Table 1). Amplification, sequencing of PCR products, and SNP identification were as described in Khatib et al. (2008a,b). Single nucleotide polymorphisms identified in *DNAJC15*, *DNAJC19*, *DNAJC24*, and *DNAJC27* were genotyped for the 399 ovary/cow samples at GeneSeek Inc. (Lincoln, NE).

Statistical Analysis

For expression analysis, normalized gene expression values (ΔCt) were analyzed using a general linear model including the fixed effects of the bull, the type of embryo (blastocyst or degenerate embryo), and the random effect of the pool. Association between the normalized gene expression and the type of embryo was tested using a likelihood ratio test by comparing this model to a reduced model without the embryo effect. The mean and the range of the fold change for each gene were calculated as $2^{-\Delta\Delta\text{Ct}}$, using the estimated $\Delta\Delta\text{Ct}$ value $\pm$ standard error.

For genomic association analysis between SNP and fertilization and blastocyst rates, the following mixed linear model was used:

$$y_{ijk} = \mu + o_i + b_j + \text{SNP}_{ijk} + e_{ijk},$$

where y_{ijk} represents, in turn, the fertilization or survival rate of oocytes k from ovary i fertilized with semen from bull j ; μ represents a general mean for the trait considered; o_i represents the random effect of the individual ovary from which oocytes were harvested; b_j represents the random effect of the sire used in the fertilization; SNP_{ijk} represents the fixed effect of the genotypic class for the SNP considered; and e_{ijk} represents the residuals, assumed normal, independent, and identically distributed with mean 0 and variance $\text{I}\sigma_e^2$. Ovaries and bulls were assumed uncorrelated with variance structures $\text{I}\sigma_o^2$ and $\text{I}\sigma_b^2$, respectively. The associa-

tion between the SNP and fertilization or blastocyst rates was tested using a likelihood ratio test by comparing the full model to a reduced model without the SNP effect. All analyses were performed using the lme4 package of R language/environment (R Development Core Team, 2009).

RESULTS

Expression Profiling of HSP Genes in Embryos

Expression differences between degenerate embryos and blastocysts were estimated for 17 HSP genes in 4 sets of biological replicates using qRT-PCR (Figure 1). A range of 1.5- to 7.6-fold difference in expression was observed between embryo groups. Interestingly, all HSP40 gene family members were found to be upregulated in degenerate embryos compared with blastocysts (Figure 1). For example, *DNAJC15* ($P < 0.0001$), *DNAJC19* ($P < 0.0001$), *DNAJC24* ($P = 0.002$), and *DNAJC27* ($P = 0.0098$) showed an average of 7.6-, 4.8-, 3.3-, 4.33-fold differences in expression, respectively. In contrast, only 2 members of the HSP70 family (*HSPA14* and *HSPA9*) showed upregulation in degenerate embryos, whereas 4 HSP70 members (*HSPA2*, *HSPA4*, *HSPA5*, and *HSPA8*) showed higher expression levels in blastocysts than in degenerate embryos (Figure 1).

Differential Expression of Alternatively Spliced Transcripts

Splice variants of HSP genes were identified using the genebuild procedure of Ensembl (<http://uswest.ensembl.org>). In this procedure, annotation of transcripts is based on mRNA and protein sequences deposited in public scientific databases. An Ensembl search revealed 1 transcript per gene for 13 genes, 2 transcripts each for *DNAJB12* and *DNAJC19*, and 3 transcripts each for *DNAJC5* and *DNAJC24*. Expression levels of splice variants of *DNAJC5*, *DNAJC19*, *DNAJC24*, and *DNAJB12* (Figure 2) were estimated in 4 sets of biological replicates of blastocysts and degenerate embryos using qRT-PCR. Transcripts DNAJC5-1049 and DNAJC5-667 (Figure 2) showed 2.53- and 1.67-fold higher expression, respectively, in degenerate embryos compared with blastocysts, whereas the DNAJC5-725 transcript was not detected in any of the embryo groups. For *DNAJC19*, only transcript DNAJC19-536 was detected in embryos. For *DNAJC24*, the DNAJC24-2172 transcript showed a 3.22-fold higher expression in degenerate embryos compared with blastocysts, whereas transcript DNAJC24-628 was lowly expressed in all embryo samples. The DNAJC24-1893 transcript could not be detected in embryos. The sequences of

Figure 1. Fold change differences in expression of heat shock protein genes in bovine embryos using quantitative real-time PCR (qRT-PCR). Data are shown as mean $\pm$ maximum- and minimum-fold changes. Upregulation in degenerate and blastocyst embryos is represented by bars above and below, respectively, the x-axis. The qRT-PCR was performed in 4 sets of biological replicates of blastocysts and degenerate embryos.

the 2 *DNAJB12* transcripts were overlapping so that only transcript DNAB12–1220 could be amplified using transcript-specific primers. The DNAB12–1220 transcript showed a 1.95-fold higher expression in degenerate embryos versus blastocysts.

Association of HSP Polymorphisms with Fertilization and Blastocyst Rates

Single nucleotide polymorphisms in genes *DNAJC15*, *DNAJC19*, *DNAJC24*, and *DNAJC27* were tested for association with fertilization and blastocyst rates. These genes showed the highest-fold differences in expression between embryo groups (Figure 1) and the highest statistical significance in the general linear model analysis ($P < 0.01$). Using the pooled DNA sequencing approach, 5 SNP were identified in the 3' UTR of *DNAJC15*, 1 SNP in exon 5 of *DNAJC19*, 1 SNP and one 4-bp deletion in the 3' UTR of *DNAJC24*, and 3 SNP in the 3' UTR of *DNAJC27*. All SNP in each of the *DNAJC15* and *DNAJC27* genes showed high linkage disequilibrium with each other (Figure 3). Polymorphisms in *DNAJC19* and *DNAJC24* did not show significant associations with fertility traits. Table 2 shows fertilization and blastocyst rates for the genotypic classes of the *DNAJC15* and *DNAJC27* SNP. For *DNAJC27* SNP36016, oocytes collected from genotype GG ovaries showed a 69.3% fertilization rate versus 62.2% for oocytes collected from CC ovaries ($P = 0.034$). For *DNAJC15* SNP85146, the blastocyst rate of embryos produced from GG dams was 40.1 vs. 31.0 and 28.1% for embryos produced from AA and AG dams, respectively (Table 2).

DISCUSSION

The HSP are highly conserved across most organisms; therefore, it is conceivable to hypothesize that they play a vital role in survival and development. The objective of this study was to investigate the roles of HSP genes in early embryonic development and fertility in cattle at both genomic and gene expression levels. A total of 17 candidate genes were chosen based on differential expression results observed in a previous microarray analysis of bovine embryos. To test whether or not these genes have roles in early development of cattle embryos, their expression levels were quantified and compared between blastocysts and degenerate embryos. All 17 genes showed expression differences that ranged between 1.5- and 7.6-fold between the embryo groups.

All HSP40 family genes were found to be upregulated in degenerate embryos versus blastocysts. Although the specific functions of HSP40 have not been reported in cattle, studies from other species have shown that these genes play important roles in protecting cells under stress conditions (Lanneau et al., 2007). It is well established that ATP hydrolysis is necessary for protein-folding activity of HSP70 and that HSP40 stimulate ATPase activity and stabilize the interaction of HSP70 with their substrates (Qiu et al., 2006). Gotoh and colleagues (2004) reported that HSP40 combine with HSP70 to act as chaperones to protect cells from apoptosis. Degenerate embryos in the IVF system are growth-arrested embryos and seem to undergo partial apoptosis. Research has shown that HSP are upregulated to prevent apoptosis triggered by different stimuli

DNAJC5 ***DNAJC19*** ***DNAJC24*** ***DNAJB12***

Figure 2. Splice variants of *DNAJC5*, *DNAJC19*, *DNAJC24*, and *DNAJB12* genes. Positions of start and stop codons are indicated by vertical arrows and positions of primers used in the quantitative real-time PCR (qRT-PCR) are indicated by horizontal arrows. Black boxes represent coding sequences and white boxes represent untranslated regions. Due to overlap for *DNAJB12*, only transcript DNAB12-1220 could be amplified using transcript-specific primers. F = forward; R = reverse.

Table 2. Blastocyst and fertilization rates for genotypic classes of *DNAJC15* and *DNAJC27* genes

Gene	Genotype (number of ovaries)	Blastocyst rate ($\pm$ SE)	P-value
<i>DNAJC15</i> SNP			
SNP 85146(G > A)	AA (202)	0.310 $\pm$ 0.025	0.014
	AG (206)	0.281 $\pm$ 0.025	
	GG (53)	0.401 $\pm$ 0.040	
SNP 85161(G > A)	AA (202)	0.309 $\pm$ 0.023	0.022
	AG (206)	0.283 $\pm$ 0.024	
	GG (61)	0.390 $\pm$ 0.037	
SNP 85216(A > G)	GG (203)	0.312 $\pm$ 0.022	0.059
	AG (201)	0.282 $\pm$ 0.023	
	AA (63)	0.373 $\pm$ 0.036	
SNP 85292(C > A)	AA (204)	0.310 $\pm$ 0.023	0.012
	AC (201)	0.279 $\pm$ 0.024	
	CC (62)	0.395 $\pm$ 0.037	
SNP 85300(G > A)	AA (208)	0.312 $\pm$ 0.023	0.066
	AG (201)	0.280 $\pm$ 0.023	
	GG (63)	0.369 $\pm$ 0.037	
<i>DNAJC27</i> SNP			
SNP 35728(G > A)	AA (126)	0.621 $\pm$ 0.029	0.042
	AG (257)	0.654 $\pm$ 0.025	
	GG (103)	0.691 $\pm$ 0.030	
SNP 36016(C > G)	CC (129)	0.622 $\pm$ 0.029	0.034
	CG (251)	0.653 $\pm$ 0.026	
	GG (103)	0.693 $\pm$ 0.030	
SNP 38867(A > G)	AA (133)	0.626 $\pm$ 0.028	0.049
	AG (246)	0.650 $\pm$ 0.026	
	GG (104)	0.693 $\pm$ 0.030	

by interacting with key factors of the apoptotic signaling pathways (Lanneau et al., 2007). Therefore, it is feasible that upregulation of HSP40 genes observed in degenerate embryos is a response to stress on these embryos so they can maintain protein homeostasis.

The qRT-PCR analysis revealed moderate-fold change expression of *HSPE1*, *HSPH1*, *HSB1*, and 6 HSP70 genes. The HSP70 genes *HSPA2*, *HSPA4*, *HSPA5*, and *HSPA8* showed higher expression in blastocysts than in degenerate embryos, whereas expression levels of *HSPA9* and *HSPA14* were higher in degenerate embryos. Although the specific functions of most HSP70 have not been reported in cattle, studies in humans and mice have indicated important roles for these genes in early embryogenesis. It has been reported that *Hspa2* is essential for normal spermatogenesis and for growth and survival of cancer cells and that *Hspa8* knockout mice are not viable because of the housekeeping functions of this gene (Daugaard et al., 2007). Human *HSPA5* is involved in the folding and transport of proteins into the endoplasmic reticulum and *Hspa5* knockout mice embryos die at d 3.5, and therefore it, too, is considered a housekeeping gene (Daugaard et al., 2007). Furthermore, Matwee et al. (2001) have reported a decreased blastocyst rate and an increased apoptosis of embryos cultured in the presence of anti-HSP70 antibodies. Thus, upregulation and downregulation of

HSP in bovine embryos observed in the present study imply that these genes play vital roles in early embryo development.

Given that alternative splicing is a major source of phenotypic complexity in mammals (Wang et al., 2008), the Ensembl annotation of the bovine genome was searched and it was found that only 4 out of 17 HSP candidate genes have annotated splice variants. In contrast, for the 17 human and mouse counterparts, 15 and 13 genes, respectively, have 2 or more splice variants. The low number of alternative transcripts identified in cattle is presumably due to the incomplete annotation of the bovine genome. Indeed, in a previous study, thousands of alternative splicing events were characterized in blastocysts and degenerative embryos, and some of those events were found to be associated with the developmental status of embryos (Huang and Khatib, 2010).

To explore the effects of the different splice variants of HSP on embryo development, expression levels of the identified variants were estimated and compared between blastocysts and degenerate embryos. The results clearly show that different splice variants display different expression levels, which imply different functions in embryo development. For example, although the long variant of *DNAJC24* was highly expressed in degenerate embryos compared with blastocysts, the short

Figure 3. A heat map of linkage disequilibrium (r^2) between SNP in *DNAJC15* and *DNAJC27*. Single nucleotide polymorphisms 85146, 85161, 85216, 85292, and 85300 are located in *DNAJC15*, whereas SNP 35728, 36016, and 38867 are located in *DNAJC27*. Color version available in the online PDF.

variant of this gene was found to be lowly expressed in both embryo groups. Also, for *DNAJC5*, one transcript could not be detected using qRT-PCR. These results are consistent with human studies in which only some of the splice variants of genes were found to be associated with a disease state (Wang and Cooper, 2007). Collectively, these results testify to the importance of alternative splicing as a key regulator of phenotypic variation in mammals.

To better understand the involvement of the HSP genes in fertility traits, 4 genes that showed the most significant expression differences between embryo groups were tested for SNP association. Recently, an IVF experimental system in cattle has been developed in our laboratory (see Methods) aimed at identifying genetic factors affecting fertilization and embryo development. The effectiveness of this system in identifying genes and pathways associated with developmental and fertility traits has been demonstrated in several studies (Khatib et al., 2008a,b, 2009; Driver et al., 2009; Wang et al., 2009; Huang et al., 2010a). In this study, analysis of *DNAJC15* and *DNAJC27* SNP revealed significant associations with blastocyst and fertilization rates, respectively. These results are of particular significance because these 2 genes showed a remarkable difference in expression between blastocysts and degenerate em-

bryos, providing strong evidence for the involvement of these genes in embryo production and development in cattle. These results also provide further evidence for the roles of HSP in the fertilization process and blastocyst rates observed in mice and cattle in previous studies. Neuer et al. (1998) found that the presence of anti-HSP60 monoclonal antibodies had significant effects on blastocyst rates of mouse embryos. Matwee et al. (2001) reported that the addition of anti-HSP70 monoclonal antibodies to the culture medium decreased the mean number of spermatozoa bound to zona pellucida by about 50% compared to that of controls. Also, the authors reported that anti-HSP70 antibodies significantly decreased the number of bovine embryos that reached the blastocyst stage (Matwee et al., 2001). A recent study by Rosenkrans et al. (2010) reported an association between SNP in the promoter region of HSP70 and calving percentages in Brahman cows. Although a small sample size was used in the association analysis, the results point to an important role of HSP70 in cow fertility.

CONCLUSIONS

The identification of specific HSP genes contributing to embryo survival can be a unique opportunity to improve protection of IVF embryos from different toxic conditions and to enhance pregnancy rates in cattle (Hansen, 2007). The HSP genes investigated in this study were found to be differentially expressed between blastocysts and degenerate embryos and SNP in 2 genes were associated with fertility traits. Blastocysts and degenerate embryos have distinct morphological and developmental features. As such, the present study provides a set of candidate genetic markers for preimplantation embryo development. The question whether the expression differences between blastocysts and degenerate embryos observed in this study have significant effects on development necessitates further research. However, it has been reported that relatively small changes (decrease or increase) in expression levels of HSP can result in growth abnormalities and cell death (Nollen and Morimoto, 2002). Also, the assessment of the expression of alternative transcript isoforms in the embryos examined in this study testifies to the importance of inclusion of this approach in studying gene expression.

ACKNOWLEDGMENTS

This study was supported by USDA Hatch grant WIS-142-PRJ17PH from the University of Wisconsin-Madison. Bao Zhang was supported by the China Scholarship Council (Beijing).

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