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# Transcripts of enriched germ cells responding to heat shock as potential markers for porcine semen quality

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**Abstract**

A cDNA microarray-assisted experiment was conducted to survey genes that respond early to heat shock in enriched immature porcine germ cells; the 5′-UTR flanking the highest upregulated gene, heat shock 105/110 kDa protein 1 (*Hsph1* or *Hsp105*), in response to heat shock was also investigated. We established a porcine testis cDNA microarray with 9944 transcripts from two libraries constructed from the testes of mature boars, with or without heat shock. After a mild heat shock treatment (39 °C for 1 h and recovered at 34 °C for 2 h), 380 transcripts demonstrated significant gene expression in enriched immature germ cells; 326 were upregulated and 54 were downregulated. Ten transcripts of interest exhibiting significance analysis of microarrays (SAM) scores higher than the median were subjected to quantitative real-time PCR; three (*Hsp105*, *Hspa4l* and *Thap4*) were upregulated >1.5-fold. The sequence of the 5′-UTR of *Hsp105*, the highest upregulated transcript, was cloned and analyzed. A single nucleotide polymorphism (SNP) was found at position −762 (C or T) upstream of the translational start site (ATG codon). Only two genotypes (CC or TC) were found in the mature boars that were studied ($n = 31$). A heterozygous genotype (TC) at this SNP site revealed an elevated percentage of morphologically normal sperm during hot and cold seasons; this SNP may be a useful marker for semen quality in boars. Furthermore, the cell-model established from enriched primitive germ cells has potential for the study of reproduction in mature animals.
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## 1. Introduction

Elevated environmental temperatures can markedly reduce male fertility [1,2]; high ambient temperatures

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and humidity have negative effects upon sperm morphology in boars [3,4]. Many heat shock proteins (HSPs), e.g. Hsp70 [5], Hsp90 [6] and Hsp110 [7], exhibit protective roles in germ cells confronted with heat stress. In male germ cells, the expression of HSPs is activated by heat shock factor (HSF) in a lower temperature threshold [8]. This is especially prominent among germ cells, resulting in initiation of the protective process in advance. In our previous study on the *Hsp70.2* promoter, we reported a significant correlation between single nucleotide polymorphisms (SNPs) and semen quality in boars [9]. However, the exact molecular mechanism associated with heat-tolerance in reproduction remains obscure.

To date, 1675 Quantitative Trait Loci (QTL), representing 281 different traits, have been discovered in pigs (PigQTLdb, http://www.animalgenome.org/QTLdb/pig.html) [10]. No QTL is related to male reproductive traits. Because the decline in fertility caused by heat shock impacts AI, it would be beneficial to clarify mechanisms behind heat-tolerance and discover essential biomarkers. The ratio of normal to abnormal sperm cells declines over the summer. Nevertheless, spermatogonia are more tolerant to heat than other types of germ cells involved in sperm development [1,11]. Therefore, it would be of interest to identify these differences at a molecular level. Early responding genes in the advanced stages of spermatogenesis may play important roles in this protective machinery.

In order to investigate the transcripts that may contribute to the heat-tolerance of germ cells, a cDNA microarray-assisted approach was utilized, using an *in vitro*-enriched primitive germ cell model. The relative expressions of some highly regulated transcripts were confirmed by real-time PCR. The sequence of the 5′-untranslated region (5′-UTR) of the highest upregulated transcript was cloned and analyzed for single nucleotide polymorphisms (SNP). The genotypes in the reported polymorphism site of mature boars were determined and correlated to semen quality during both hot and cold seasons.

## 2. Materials and methods

### 2.1. Animals

Testes from four purebred Duroc pigs (84 $\pm$ 15 d) were used for germ cell isolation and real-time PCR analysis. Three of the four animals (77 $\pm$ 11 d) were randomly selected for microarray analysis. Immature piglets, 2–3-mo-old, were selected to maximize the yield obtained during primitive germ cell isolation [12,13]. To collect the testes, the animals were castrated following the guidelines published by the *National Science Council*, Republic of China. All assays, e.g. heat shock, microarray hybridization, and real-time PCR, were conducted individually with cells or total RNA isolated from each animal.

For analysis of SNP genotyping, the semen from mature purebred Duroc boars (1–2-y-old) was collected, during both summer and winter, from a local AI center in northern Taiwan. Semen characteristics, including sperm motility and the percentage of morphologically normal sperm, were determined with phase-contrast microscopy, as described [9]. The percentage of morphologically normal sperm was defined as the percentage of sperm without any plasma droplets and acrosome defects. Semen from 31 animals, with the above-mentioned characteristics (>50% in both hot and cold seasons), were used for the correlation analysis of semen quality and boar genotype.

### 2.2. Cell isolation

Approximately 3 g of testis tissue was minced and processed by enzymatic digestion in a final volume of 30 mL, as described previously, with minor modifications [14,15]. Enzymatically dispersed cells, with viability greater than 90%, were suspended in 30 mL of PBS with 0.5% BSA (concentration from $5 \times 10^6$ to $1 \times 10^7$ cells/mL), and separated using the STAPUT method [14,15] in a cold room (4 °C). After 2–3 h of sedimentation, cells were collected in fractions of 30 mL at a flow rate of 10 mL/min. Fractions of similar cell size were pooled and cultured overnight on tissue culture plates in DMEM/F12 media, 10% fetal bovine serum (FBS) at 34 °C to eliminate contaminated somatic cells. Non-adherent cells were collected and defined as enriched primitive germ cells for further study. The isolated cells, seeded at a density of approximately $1 \times 10^6$ cells/mL, were cultured at 34 °C for another day before proceeding to heat shock treatment. The purity of isolated cells was determined by negative immunostaining to vimentin, a cytoplasmic protein expressed in somatic cells, but not germ cells [16].

### 2.3. Immunohistochemical and immunocytochemical characterization

Testis sections were incubated with mouse anti-vimentin monoclonal antibody (diluted to 1:200, MAB3400, Chemicon International Inc., Temecula,

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