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Research Project Proposal

Sperm transcriptome profiling for assessment of boar semen freezability

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Abstract

In this project transcriptome analysis on high-throughput RNA-sequencing (RNA-Seq) data of boar spermatozoa will be utilized to identify novel genetic markers associated with semen freezability. Three boars each with poor and good semen freezability will be subjected to transcriptome studies on sperm RNA-seq data, and in combination with bioinformatics screening, will select candidate genes associated with low or high sperm cryo-tolerance. A validation scheme will be used to enhance the analysis of the d**ifferentially expressed (DE) genes,** putative markers for single nucleotide polymorphisms (SNPs) and the protein expression levels of the respective mRNA transcripts on a large animal population. Identification of sperm-derived genetic markers, associated with sperm freezability, will improve the cryopreservation technology of boar semen.

***Key words**: spermatozoa, RNA-seq, d**ifferentially expressed genes**, SNPs, bioinformatics studies*

1. Relevance of the project

The cryopreservation process causes morphological and biochemical changes in spermatozoa, which compromise their fertilizing ability [6,14-15]. Moreover, cryo-induced changes in spermatozoa significantly differ among individuals, suggesting that sperm attributes, such as motility, viability, capacitation and acrosome reaction, might have differential response to the cryopreservation process [13]. An objective identification of sperm freezability markers for boars with high cryo-tolerance and fertilizing capacity, and the application of these markers in the breeding programmes will have the potential to improve the cryopreservation technology of boar semen, which will be of great economic importance for the commercial pig industry. Selection of boars with poor or good semen freezability ejaculates depends on routine semen analysis, which sometimes do not always accurately predict the fertility of frozen-thawed spermatozoa [6,15]. Molecular markers linked to genes controlling the freezability of boar semen have been reported, and there is evidence suggesting that there is a genetic basis for the significant male-to-male differences in sperm cryo-tolerance [7,13]. Consistent with these findings, it has been suggested that sperm freezability markers might be useful in the selection of boars for freezing. A complex population of RNAs has been detected in mature spermatozoa, and the functions of the majority of the sperm-derived RNAs still have not been elucidated as yet [9-10]. It is suggested that transcriptome analysis of RNA-sequencing (RNA-seq) data on spermatozoa of the domestic swine (*Sus scrofa*) will provide valuable information about key factors that are responsible for variations in sperm cryo-tolerance. Even though several transcripts present in spermatozoa have been identified [2-4], unequivocal evidence of their role in sperm biological function is rare. Moreover, the levels of expression of several mRNA transcripts have been associated with sperm functions, such as motility, mitochondrial function, capacitation and chromatin condensation, and the sperm-derived RNAs are implicated in fertilization and embryo development [1-4,9]. In the literature there is a lack of studies regarding the combination of transcriptomic and proteomic data on spermatozoa to provide more insights in the mechanisms

implicated in sperm cryo-tolerance among boars. This research project employs the utilization of new biotechnological genetic tools and the application of key experimental design strategies to provide a comprehensive investigation of the sperm-derived genes associated with the freezability of boar semen. To the best of our knowledge, this is the first research project that will provide a thorough analysis of transcript profiling of spermatozoa from the Polish Large White (PLW) boars by high-throughput RNA-seq, using the next generation sequencing (NGS) technology, which seems to be a more sensitive approach to accurately explain the differences in semen freezability. Therefore, it is hypothesized that transcriptome profiling of boar spermatozoa, in combination with functional genomics techniques, would advance our understanding of the biological significance of the specific-sperm gene transcripts that are implicated in poor- and good-freezability ejaculates.

Semen freezability and RNA isolation

Ejaculates were collected from 40 PLW boars and frozen using a standard cryopreservation protocol [6]. The frozen samples were stored in liquid nitrogen (-196^{o}C) for a week-period, prior to post-thaw semen analysis. Boars with poor and good semen freezability ejaculates were identified using a plethora of sperm phenotype parameters, such as motility characteristics analyzed by the computer–assisted semen analysis (CASA) system (total and progressive motility, TMOT and PMOT, respectively), mitochondrial membrane potential, MMP (JC-1/PI assay) [14], plasma membrane integrity, PMI (SYBR-14/PI assay) [8], and DNA fragmentation [6]. A thorough analysis of the sperm phenotypic parameters following cryopreservation demonstrated that 19 and 21 boars showed poor and good semen freezability, respectively. Three boars from each freezability group will be subjected to transcriptome studies on sperm RNA-seq data. The sperm phenotype parameters of the 3 boars with poor semen freezability were significantly lower ($P < 0.05$) compared with those with good semen freezability (Table 1).

In this project the first major task will be to isolate highly-purified RNA from spermatozoa of 3 boars each with poor and good semen freezability, respectively (Table 1). Following RNA isolation from the raw or frozen-thawed semen, the high-throughput RNA-seq technology will be employed, using the NextSeq 500 Illumina platform on the highly-purified RNA isolate. It is noteworthy that an effective RNA-extraction protocol is required for the isolation of high-quality RNA for successful RNA-seq data analysis [5,12]. Preliminary studies showed that a slight modification of a previously described RNA extraction protocol [12], comprising the conventional TRIzol protocol and Lysis Buffer (PureLink RNA mini kit, Ambion, USA), proteinase K and β-mercaptoethanol, allowed the removal of highly abundant ribosomal RNA (rRNA) from spermatozoa, regardless of their source.

Table 1. Phenotype parameters of boar spermatozoa following cryopreservation.

Sperm parameters (%)	Semen freezability	
	Poor *(n=24)*	Good *(n=27)*
TMOT	24.3 ± 1.9	44.9 ± 1.8*
PMOT	18.6 ± 2.1	33.5 ± 2.5*
MMF	34.9 ± 3.9	55.7 ± 1.6*
PMI	39.0 ± 1.2	53.9 ± 1.9*
DNA fragmentation	10.9 ± 1.4	5.1 ± 0.3*

means ± SEM. *P < 0.05 denotes significance difference.

RNA-seq and transcriptome studies

Isolation of high-quality RNA from fresh and frozen-thawed semen is a crucial prerequisite to assess the sperm gene expression by RNA-seq followed by bioinformatics analysis [5,12]. RNA-seq of spermatozoa has allowed the identification, profiling, and quantification of the complete transcript profile of the sperm cell [3,10]. Accumulating evidence has shown that RNA-seq technology is been widely used for a number of molecular genetic analyses, such as global gene expression, detection of alternative splicing, and identification of single nucleotide polymorphisms [10-11]. In this regard, analysis of boar sperm transcriptome on RNA-seq data will include library construction, template preparation and sequencing of the libraries. It is noteworthy that the error probabilities derived from the NGS-based RNA-seq data will be standardized and the statistical distribution analysis will be performed using several software packages. An important aspect of this research study will be the re-assembly or mapping of the boar sperm genome to the *Sus scrofa* reference genome to elucidate which transcripts that will be highly expressed in the poor- and good-freezability

ejaculates. It should be emphasized that transcriptome profiling of *Sus scrofa* spermatozoa will allow to develop a catalogue of mRNAs transcripts, determine the transcriptional structure of genes and quantify the varying expression levels of each identified transcript. Furthermore, a battery of bioinformatics analytical tools, including transcriptome assembling**,** differentially expressed (DE) genes analysis**,** and detection of single nucleotide polymorphisms (SNPs), will be utilized to thoroughly assess the sperm transcript sequencing. Using bioinformatics screening, it is envisaged that several candidate DE genes (both up-regulated and down-regulated, and with no regulation according to the RNA-seq data) and putative SNP markers potentially associated with sperm freezability will be selected. An efficient validation scheme will be achieved through the implementation of the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) for analysis of the candidate DE genes, and KASP™ assay (LGC Genomics, Middlesex, UK) to detect the putative SNP markers on a large animal population (n=40). Furthermore, the RNA-seq data analysis will be integrated with the Western blotting and immunofluorescent staining to elucidate the functional significance of the differentially expressed proteins, which are markers for sperm cryo-tolerance, such as motility, mitochondrial function, plasma membrane integrity, acrosome and structural DNA integrity 91,6-7,13-15].

2. Concluding remarks

The research project presents a novel approach based on the use of the RNA-seq technology as an efficient tool for bioinformatics screening of sperm-related genes linked to the freezability of boar semen. It is envisaged that transcriptome studies on sperm-RNA will offer new perspectives in the technology of semen cryopreservation and will allow the discovery of novel genetic markers, particularly for reproductive traits that are difficult to select by routine semen analyses. It is suggested that such an innovative approach, based on the utilization of NGS-based RNA-seq data tools, in conjunction with advanced bioinformatics screening, will aid in the improvement of the cryopreservation technology of boar semen.

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