Expression of miRNAs in Bull Spermatozoa Correlates with Fertility Rates

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Bull spermatozoa are rich in active miRNAs, and it has been shown that specific spermborne miRNAs can be linked to fertility. Thus, expression profiling of spermatozoa could be helpful for understanding male fertility and the ability of spermatozoa to initiate and sustain zygotic, embryonic and foetal development. Herein we hypothesized that bulls with moderate to high fertility can be identified by differences in amounts of certain miRNAs between their ejaculates. RNA samples from spermatozoa of eight brother pairs (one bull with high and one with moderate NRR in each pair) of the Holstein breed were prepared. miRNA was isolated, and the expression of 178 miRNAs was determined by RT-qPCR. Important findings were that highly expressed miRNAs, not linked to NRR status, were identified in the bull sperm samples, which indicate that these miRNAs have an important role in early embryogenesis. A large fraction of the targets genes were phosphoproteins and genes involved in the regulation of transcription. Seven miRNAs (mir-502-5p, mir-1249, mir-320a, mir-34c-3p, mir-19b-3p, mir-27a-5p and mir-148b-3p) were differentially expressed between bulls with moderate and high NRR with a strong tendency towards a higher expression of miRNAs in bulls with moderate fertility. Thus, bulls with a moderate NRR negatively regulate the expression of protein-coding genes, which leads to problems during the pregnancy.

Introduction

The ability of artificial insemination (AI) bulls to make cows pregnant, the field fertility, is affected by a number of factors, such as health, functional reproductive organs, semen quality, level of libido and proper nutrition (Parkinson 2004; Chapwanya et al. 2008; Menon et al. 2012). By existing quality examination methods, bulls with low fertility can be identified, but ranking those with moderate to high fertility is difficult. Only low to moderate correlation has been observed between the quality criteria currently used and the non-return rate (NRR). The quality criteria include sperm concentration, viability, motility and morphology, whereas NRR represents the proportion of served cows that are not subsequently re-bred within a specified period of time after insemination (Rodriguez-Martinez 2006).

Evidence of transcriptional activity has long been available for human-ejaculated spermatozoa, and also the presence of translational activity has been demonstrated (Pessot et al. 1989; Steger 1999; Dadoune et al. 2005; Gur and Breitbart 2006; Pan et al. 2007). The proteins resulting from these mRNAs are likely to affect the first steps of embryonic development (Miller et al.

2005; Milazzotto et al. 2012). Thus, expression profiling of spermatozoa has recently been used in attempts to identify novel biomarkers for the prediction of fertility (Govindaraju et al. 2012). In addition, expression profiling of spermatozoa could be helpful for understanding male fertility and the ability of spermatozoa to initiate and sustain zygotic, embryonic and foetal development (Ostermeier et al. 2002; Dadoune et al. 2005; Martins and Krawetz 2005; Miller et al. 2005; Carreau et al. 2007).

microRNAs (miRNAs) are known to have a large impact on the regulation of the transcriptome (Lau et al. 2001; Lee and Ambros 2001; Berezikov 2011; Van Wylsberghe et al. 2011), and it has been estimated that up to 30% of human genes may be regulated post-transcriptionally by miRNAs (Van Wylsberghe et al. 2011). As miRNAs interact directly with mRNAs, the global protein profile of a cell can rapidly change due to changes in miRNA expression (Grosshans and Filipowicz 2008). The functions of miRNA target genes include a broad range of important biological processes in development, differentiation and growth (Krutzfeldt et al. 2006; Tsai and Yu 2010; Van Wylsberghe et al. 2011). As miRNAs may be delivered to the oocyte during fertilization, their dysregulation in spermatozoa is likely to influence the early stages of embryonic development (Boerke et al. 2007; Jodar et al. 2013).

Robertson et al. (2008) found that bull spermatozoa are rich in active miRNAs and identified miRNAs with distinctly different expression levels in two bulls with pronounced differences in NRR, implicating that specific miRNAs can be linked to fertility. In another study, seven miRNAs were identified as differentially expressed in spermatozoa from high- and moderate-fertility bulls, which suggests that miRNAs play important roles in mammalian gametogenesis and early development (Govindaraju et al. 2012). Moreover, 68 miRNAs have been identified in human spermatozoa, of which 15 are only active during early embryonic development (Boerke et al. 2007). Therefore, it is obvious that information about miRNA activity in spermatozoa should be considered when developing diagnostic tools for male fertility (Boerke et al. 2007; Abdo et al. 2012).

Prediction of the fertilizing ability and the capability to maintain pregnancy is a scientifically challenging task, which is exceptionally important for AI companies and their customers. Herein we hypothesize that bulls with moderate to high fertility can be identified

by differences in amounts of certain miRNAs between their ejaculates. From the results, we expect to explain how bull spermatozoa transcripts contribute to maintaining the pregnancy during early embryonic development. miRNA profiling of spermatozoa is a promising new method for the prediction of NRR and is likely to increase our understanding of the biological processes involved in male fertility in cattle as well as in human.

The aim of this study was to identify spermborne miRNAs that are differentially expressed between brother pairs with high and moderate NRR and to create an expression signature based on a few marker miRNAs that can potentially be used for the prediction of field fertility in bulls.

For this purpose, RNA was isolated from spermatozoa of eight brother pairs (one bull with high and one with moderate NRR in each pair) of the Holstein breed. Using the isolated RNA samples, the expression of 178 bovine miRNAs was determined by RT-qPCR.

Materials and Methods

Material

Cryopreserved bull semen from semen sample straws from eight brother pairs of the Holstein breed was collected. Each pair consists of one bull with high and one with moderate NRR (VikingGenetics®, Skara, Sweden) (Table 1). NRR represents the proportion (%) of served cows that are not subsequently re-bred within a specified period of time after insemination, in this study 56 days. The calculations of NRR are based on >1000 inseminations for each bull. For brother pair

2, the number of inseminations was too few (<1000) to calculate NRR. Thus, this brother pair was omitted from analyses where NRR values were needed.

miRNA included

As bovine miRNA panels were not available, we used human miRNAs that were exact homologs to bovine miRNAs and included in the miRCURY LNA Pick-&-Mix microRNA panels (Exiqon, Vedbaek, Denmark). In total, 178 miRNAs with known validated homologs in bull were picked out aiming to explore the expression pattern of miRNAs in bull spermatozoa.

Sample preparation

BoviPure® density gradient (Nidacon, Sweden) was carried out for spermatozoa isolation according to the manufacturer's instructions. To avoid contamination of spermatozoa samples with somatic cells, a newly developed centrifuge tube (ProInsert™, Nidacon, Sweden) was used (Fourie et al. 2012). Four straws of cryopreserved semen were thawed in a 37°C water bath for 1 min and subsequently emptied in a Falcon tube using a pistollet. The thawed semen (approximately 24×10^6 motile spermatozoa in total) was loaded slowly onto the top layer of the BoviPure® gradient and centrifuged at $300 \times g$ for 25 min. After centrifugation, the spermatozoa pellets were transferred into an RNase-free 15-ml Falcon tube using a pellet retrieval pipette.

Total RNA isolation

A modified TRIzol reagent protocol was applied for total RNA isolation from the spermatozoa samples (Ambion®, Foster City, CA, USA). Briefly, 2 ml of TRIzol was added to each sample. The samples were homogenized in a 60°C water bath for 30 min and vortexed every 10 min. To remove extracellular materials, fat and protein from the samples, an additional isolation step was performed by centrifugation at $12\,000 \times g$ for 10 min at 4°C. The supernatant was collected in a new 15-ml RNase-free Falcon tube, and 400 µl of chloroform was added to each sample followed by 15 s of strong shaking by hand. The samples were subsequently incubated at room temperature for 2 min, and the aqueous phase was collected after centrifugation of samples at $12\,000 \times g$ for 15 min at 4°C. The precipitation of total RNA from the aqueous phase was performed by adding 1 ml of room-tempered isopropanol, followed by 10-min incubation at room temperature. After incubation, the samples were centrifuged at $12\,000 \times g$ for 10 min at 4°C, the supernatant was removed, and the remaining pellet was washed with 2 ml of 75% ethanol. After vortexing the samples, the RNA was re-pelleted by centrifugation at $7500 \times g$ for 5 min at 4°C. Following the exclusion of the supernatant, the pellet was air-dried for 5 min and thereafter resuspended in 50 µl of RNase-free water.

Table 1. Bulls included in the study

	Brother pair	Fertility	No inseminations	NRR (%)	dNRR	Name
P1HI	1	High	1216	106	1.19	Mälartorp
P1LO	1	Low	1217	89	0.84	Vagnabäck
P2HI	2	High	400	–	–	Kumlaby
P2LO	2	Low	25	–	–	Eto
P3HI	3	High	1392	99	1.10	Olle
P3LO	3	Low	3340	90	0.91	Odal
P4HI	4	High	2322	102	1.13	Sebbe
P4LO	4	Low	1382	90	0.88	Stuff
P5HI	5	High	1245	103	1.16	Karlslund
P5LO	5	Low	1203	89	0.86	Magi
P6HI	6	High	1380	99	1.18	Rachi
P6LO	6	Low	1400	84	0.85	Rabalder
P7HI	7	High	1039	107	1.29	Spänstad
P7LO	7	Low	1000	83	0.78	Amiral
P8HI	8	High	1018	107	1.13	
P8LO	8	Low	1008	95	0.89	

ID, Identity label used throughout the text. *Brother pair* indicates which of the eight brother pairs the bull belongs to. *Fertility* indicates which fertility group the bull belongs to; NRR, non-return rate (%) at 56 days from insemination; dNRR, ratio of NRR between the high- and low-fertility brother. The calculations of NRR is based on >1000 inseminations for each bull. Name, the bull's name. The NRR for brother pair 2 could not be calculated because too few inseminations were available (<1000).

The total RNA samples were treated with RNA Clean-Up and Concentration kit (Norgen Biotek Corp., Thorold, Ontario, Canada) according to the manufacturer's instructions. This was performed to remove traces of DNA and contaminating reagents remaining from the RNA isolation procedure, and also to concentrate the total RNA.

Purity verification

Total RNA purity of the samples was evaluated by SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, Mount Waverley, Australia) according to the manufacturer's protocol. RT-PCR was performed to decide whether the samples were contaminated with genomic DNA and/or somatic cell RNA. Two intron-spanning primers for the genes coding for protamine 1 (*PRM1*) and protein tyrosine phosphatase receptor type C (*PTPRC*) were used to assess the contamination. The resulting products were evaluated by loading on 1.5% agarose/TBE gel followed by electrophoresis at 70 V for 60 min and visualized by staining with GelRed (Biotium, Hayward, CA, USA). Contaminated RNA samples were discarded from further use.

Reverse transcription

The reverse transcription reactions (RT reactions) were performed using the miRCURY LNA Universal cDNA Synthesis Kit (Exiqon) according to the manufacturer's instructions. Briefly, for each reaction, 4 µl 5× reaction buffer, 9 µl nuclease-free water, 2 µl enzyme mix, 1 µl synthetic RNA spike-ins (later used for quality control) and 4 µl template total RNA were added to a 200-µl thin-walled PCR tube and mixed carefully. The RT reaction was performed on the Biometra TProfessional Basic Gradient 96 thermocycler (Biometra GmbH, Göttingen, Germany) using the following conditions: 42°C for 60 min for the RT reaction to take place and 95°C for 5 min to heat-inactivate the reverse transcriptase, followed by cooldown to 4°C.

Quantitative PCR

For each cDNA sample, 10 µl was mixed carefully with 40 µl ROX (Invitrogen) and 950 µl nuclease-free water to obtain a final 100× dilution. To determine the expression profile of 178 bovine miRNAs (Appendix, Table 1), a miRCURY LNA Pick-&-Mix microRNA panel (Exiqon) was used. Briefly, 2× PCR master mix and 100× diluted cDNA were combined in a 1 : 1 ratio. Next, 10 µl of the PCR master mix and cDNA mix was added to each well, and for automation, pipetting was done with an epMotion 5075 LH pipetting robot (Eppendorf, Hamburg, Germany). An interplate calibrator (IPC) was used to determine the technical variation between plates. Moreover, to verify that samples were free of contaminants, one sample was

also added to a well containing matching primers for the RNA spike-ins. Real-time amplification was performed on the QuantStudio 12K Flex (Life Technologies, Carlsbad, CA, USA), and the cycling conditions were as follows: initial activation step at 95°C for 10 min followed by 40 cycles of denaturation, annealing and amplification (95°C 10 s, 60°C 60 s).

Pre-processing and normalization

Raw data from the cycler were pre-processed using GENEx ver. 5.4.4 (MultiD Analyses AB, Gothenburg, Sweden). To adjust for possible technical variance between the plates/runs, the data were first calibrated using the output values for the IPC. The *C_q* cut-off was set to 38 and values higher than this considered as background signal, indicating lack of expression. miRNAs with undetectable expression for >75% of the samples were removed, leaving 95 miRNAs for further analysis. For these, all missing values were replaced with *C_q* = 39 to represent lack of expression. Normalization was done to the global mean expression (Mestdagh et al. 2009) of all miRNAs, excluding those with expression close to the detection threshold (*C_q* > 34).

Statistical analysis

Significant differential expression between moderate- and high-fertility bulls was identified using a two-tailed Student's *t*-test (*p* < 0.05). Correction for multiple testing was not applied, as the aim of the study was to identify candidates for further evaluation. Principal component analysis (PCA) was applied using GENEx on the whole set of miRNAs (*n* = 95), to evaluate the possibility of grouping bulls according to fertility using only expression data as input. Hierarchical clustering was applied on subsets of miRNAs using PERMUTMATRIX ver. 1.9.3, Bio-Soft Net. (Caraux et al., 2005).

Results and Discussion

Transcriptional miRNA activity in spermatozoa

Expression was measured for 178 miRNAs in bull spermatozoa. Expression levels above the detection threshold in a minimum of 25% of the samples (i.e. four of the 16 bulls) were found for 95 miRNAs. Of these, 62 miRNAs were expressed in at least half of the samples (Fig. 1). In terms of number of expressed miRNAs, the extent of transcriptional activity varied between bulls, from a minimum of 92 miRNAs (52%) in sample P4HI to 142 miRNAs (81%) in sample P1HI. The average numbers of expressed miRNAs in semen samples from high- and moderate-fertility bulls were 51.8 and 47.8, respectively, and the difference was not significant (*p* = 0.499).

The miRNAs that were most highly expressed on average across all samples did not show significant difference between moderate- and high-fertility bulls, except for miR-19b-3p, which was the tenth most highly

Fig. 1. Expression profiling of 178 miRNAs indicates extensive transcriptional activity in spermatozoa. The plot shows the number of miRNAs (y axis) detected as expressed in a certain number of samples, or higher (x axis). It was found that 62 miRNAs (35% if the total 176) were expressed in at least eight (50%) of the samples and that 95 miRNAs (52%) were expressed in at least four (25%) of the samples

expressed with weak significant differential expression ($p = 0.04$) (Table 2). The most highly expressed miRNA was miR-125b-5p, which was stably expressed at a high level in all 16 samples. This miRNA is a homolog of the first miRNA identified, lin-4 in *Caenorhabditis elegans*, and has been linked to various developmental stages in numerous studies (for a review, see Coppola et al. 2013). The observation that it is consistently highly expressed in bull sperm samples indicates an important role in early embryogenesis, while the absence of differential expression indicates that it is not linked to the differences in NRR among the bulls in our cohort. Interestingly, the miRNA genes of the two most highly expressed miRNAs, 125b-5p and 100-5p, are located on chromosome 15 in the bovine genome and within a distance of each other of only 54 kbp. This arrangement is preserved in human, where both genes are found within a 52-kbp segment on chromosome 11. In addition, miR-100-5p is located in a cluster (<10 kbp distance) with let-7a in both human and bovine, and this miRNA had the seventh highest expression in our data.

Differential expression between moderate- and high-fertility bulls

Seven miRNAs differed significantly in expression between moderate- and high-fertility bulls (Table 3).

Table 2. The five most highly expressed miRNAs

miRNA	Avg Cq	Expr. in	p
125b-5p	24.1	16	0.97
100-5p	26.4	15	0.55
20a-5p	27.0	16	0.55
15b-5p	27.4	15	0.53
93-5p	28.3	15	0.68

Avg Cq, raw Cq value (before global normalization); Expr. in, number of samples (of totally 16) in which the miRNA was expressed; p, p-value for differential expression in Student's t-test.

Table 3. microRNAs found to be significantly differentially expressed between samples from bulls with low and high NRRs

miRNA	p	FC	Expr. in	Chr.
502-5p	0.009	1.18	6	X
1249-3p	0.011	1.44	11	5
320a	0.023	1.35	13	8, 20
34c-3p	0.028	1.40	14	15
19b-3p	0.043	3.61	13	12
27a-5p	0.045	1.16	6	7
148b-3p	0.048	1.55	14	5

p, p-value in Student's t-test; FC, fold change; Expr. in, number of samples (of the total 14) that the miRNA is expressed in; Chr, chromosome on which the miRNA precursor gene is located.

All were lower expressed in the high-fertility group. For two of the miRNAs, 502-5p and 27a-5p, the fold change in average expression is due to the total absence of expression in the majority of high-fertility brothers. None of the differentially expressed miRNAs were co-located in the genome. Two of the miRNAs, 1249-3p and 148b-3p, were located on BTA5, but approximately 100 Mbp apart. One of the mature miRNAs, miR-320a, is encoded by two precursor genes located on different chromosomes. For two of the differentially expressed miRNAs, miR-320a and members of miR-34 family, it has been shown that these miRNAs are differentially expressed during implantation in the uterus and in spermatozoa (Xia et al. 2010; Tscherner et al. 2014). These facts indicate that an aberrant expression in spermatozoa could have implications at least during early stages of pregnancy.

Principal component analysis and clustering

Principal component analysis was performed using all 95 expressed miRNAs as input. The results indicate that five of the high-fertility bulls differ quite distinctly from the remaining bulls in terms of miRNA expression, as these tend towards the lower left corner (Fig. 2). On the

Fig. 2. Principal component analysis based on the 95 expressed miRNAs. Black boxes: High-fertility bulls. White boxes: Low-fertility bulls

other hand, the overlap between high- and moderate-fertility bulls for the remaining samples indicates that the general separation between the two groups is not very clear.

Hierarchical clustering was performed in PERMUTMATRIX ver. 1.9.3 using the 25 miRNAs with lowest p-values, that is the seven significant ones and the 18 with p-values closest to being significant. The expression values were first row-normalized using the *z*-score, followed by clustering based on Euclidean distance and average linkage. The clustering is consistent with the PCA results, as four high-fertility bulls (P2HI, P3HI, P4HI and P8HI) are again identified as clearly distinct from all other bulls (Fig. 3). The remaining four high-fertility bulls are intermixed within the larger cluster of moderate-fertility bulls. The distinct cluster of four high-fertility bulls is generally characterized in the heat map by under-expression of the vast majority of miRNAs. The remaining high-fertility bulls also show a tendency towards lower expression. This is particularly visible for bulls P7HI and P6HI in the cluster containing the significantly differentially expressed miRNAs 27a-5p and 502-5p.

Functional analysis of target genes for miRNAs expressed in spermatozoa

The miRDB prediction server (Wong and Wang 2015) was used to predict target genes for the 50 most highly expressed miRNAs. Using a minimum prediction score of 95, 438 target genes were predicted (on average 8.7 per miRNA), of which 44 (0.8 per miRNA) had a score of 100. The 438 targets were analysed for enriched functional annotation using DAVID ver. 6.7 (Huang et al. 2009). After the removal of duplicate genes in the target list (genes targeted by more than one miRNA), the remaining 298 genes were mapped to DAVID IDs and a functional chart generated, using default settings (Table 4).

The set of target genes was highly enriched in phosphoproteins, constituting almost 60% of the total ($p = 6 \times 10^{-12}$, Benjamini-Hochberg corrected), indicating that many of the genes are regulated by miRNAs. Further, a large proportion of the miRNA-regulated genes are also regulators, as terms such as DNA binding, transcription regulation, transcription, regulation of transcription, activator and repressor were among the most significantly enriched. Early stages of

Fig. 3. Two-way hierarchical clustering of the samples and the 25 miRNAs with lowest p-values. The suffix "SIGN" at the end of name labels indicates the seven significantly differentially expressed miRNAs ($p < 0.05$)

Table 4. Functional enrichment analysis of targets of the 50 highest expressed miRNAs using DAVID

Category	Term	Count	%	p (corr.)
SP_PIR_KEYWORDS	Phosphoprotein	176	59.1	6.20E-12
SP_PIR_KEYWORDS	Nucleus	108	36.2	2.90E-06
SP_PIR_KEYWORDS	Metal binding	81	27.2	1.30E-05
SP_PIR_KEYWORDS	DNA binding	57	19.1	4.70E-05
SP_PIR_KEYWORDS	Transcription regulation	59	19.8	7.20E-05
SP_PIR_KEYWORDS	Zinc finger	53	17.8	7.70E-05
SP_PIR_KEYWORDS	Transcription	60	20.1	7.80E-05
GOTERM_BP_FAT	Regulation of transcription	81	27.2	1.70E-04
SP_PIR_KEYWORDS	Activator	23	7.7	6.20E-04
GOTERM_BP_FAT	Regulation of transcription, DNA dependent	59	19.8	9.10E-04
GOTERM_BP_FAT	Regulation of RNA metabolic process	60	20.1	1.30E-03
SP_PIR_KEYWORDS	RNA-mediated gene silencing	6	2.0	1.50E-03
SP_PIR_KEYWORDS	Alternative splicing	148	49.7	1.90E-03
GOTERM_BP_FAT	Transcription	64	21.5	4.00E-03
SP_PIR_KEYWORDS	Zinc	55	18.5	6.50E-03
GOTERM_MF_FAT	DNA binding	67	22.5	9.20E-03
SP_PIR_KEYWORDS	Repressor	18	6.0	9.40E-03
SP_PIR_KEYWORDS	Protein transport	19	6.4	1.10E-02
GOTERM_MF_FAT	Chromatin binding	12	4.0	1.30E-02
SP_PIR_KEYWORDS	RNA binding	20	6.7	1.40E-02
GOTERM_MF_FAT	Transcription regulator activity	47	15.8	1.50E-02
GOTERM_MF_FAT	Metal ion binding	99	33.2	1.60E-02
GOTERM_MF_FAT	Transition metal ion binding	73	24.5	1.80E-02
GOTERM_MF_FAT	Ion binding	100	33.6	1.80E-02
GOTERM_MF_FAT	Cation binding	99	33.2	2.00E-02
GOTERM_BP_FAT	Negative regulation of gene expression	23	7.7	2.80E-02
GOTERM_BP_FAT	Gene silencing by miRNA	5	1.7	4.90E-02

differentiation and development are characterized by high activity of gene expression and cell division, which includes a high phosphorylation activity and regulation of transcription factors expressed early in embryo development (for reviews, see Becker 2012; Aye et al. 2013; Tonks 2013; Pera et al. 2014).

The relation between miRNA expression and differences in fertility between brothers was investigated using the *dNRR* measure (Table 1), defined as the ratio of NRR values. The correlation between *dNRR* and *C_q* was generally relatively low, reaching a maximum value of 0.59 ($p < 0.03$) for miR-502-5p (Fig. 4, left). For all six miRNAs with highest correlation to *dNRR*, average expression was lower in the high-fertility brothers (Fig. 4, right).

Conclusions

- There is an extensive expression of miRNA genes in the spermatozoa as 95 of 178 miRNAs included in the study were expressed in at least 25% of the samples.

Fig. 4. Scatter plots of *dNRR* (x axis) versus *C_q* (y axis) for miR-502-5p (left) and the six miRNAs that had highest correlation between the two measures (right). The six miRNAs included and their correlations were 502-5p (0.59), 378a-3p (0.52), 27a-5p (0.50), 202-5p (0.49), 210 (0.49) and 499a-3p (0.48). As trendlines show, all these miRNAs had a clear tendency of lower expression (i.e. higher *C_q*) in the high-fertility brothers, although with high variation between individuals

- Highly expressed miRNAs, not linked to NRR status, were identified in the bull sperm samples, which indicate that these miRNAs have an important role in early embryogenesis.
- For the miRNAs expressed in spermatozoa, a large fraction of the target genes were phosphoproteins and genes involved in the regulation of transcription. Seven miRNAs were differentially expressed between bulls with moderate and high NRR. There is a very strong tendency towards a higher expression of miRNAs in bulls with moderate fertility. In other words, bulls with a moderate NRR negatively regulate the expression of protein-coding genes, which leads to problems during the pregnancy.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Fagerlind performed the experiments, involved in manuscript writing and performed initial analysis of data. Klinga Levan together with Fagerlind designed the experiments and responsible for manuscript writing. Olsson performed bioinformatics analysis and involved in manuscript writing. Stålhammar contributed with bull spermatozoa and AI expertise and involved in manuscript writing.

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Appendix

Mature accession	Mature name	Mature accession	Mature name	Mature accession	Mature name	Mature accession	Mature name
MIMAT0004481	hsa-let-7a-3p	MIMAT0000757	hsa-miR-151a-3p	MIMAT0000688	hsa-miR-301a-3p	MIMAT0001627	hsa-miR-433-3p
MIMAT0000062	hsa-let-7a-5p	MIMAT0000438	hsa-miR-152-3p	MIMAT0000684	hsa-miR-302a-3p	MIMAT0001541	hsa-miR-449a
MIMAT0000063	hsa-let-7b-5p	MIMAT0000439	hsa-miR-153-3p	MIMAT0000717	hsa-miR-302c-3p	MIMAT0001545	hsa-miR-450a-5p
MIMAT0000064	hsa-let-7c-5p	MIMAT0000646	hsa-miR-155-5p	MIMAT0000716	hsa-miR-302c-5p	MIMAT00004762	hsa-miR-486-3p
MIMAT0000065	hsa-let-7d-5p	MIMAT0000417	hsa-miR-15b-5p	MIMAT0000718	hsa-miR-302d-3p	MIMAT00002178	hsa-miR-487a-3p
MIMAT0000067	hsa-let-7f-5p	MIMAT0000071	hsa-miR-17-3p	MIMAT00004685	hsa-miR-302d-5p	MIMAT00002804	hsa-miR-488-5p
MIMAT0000414	hsa-let-7 g-5p	MIMAT0000070	hsa-miR-17-5p	MIMAT0000088	hsa-miR-30a-3p	MIMAT00002816	hsa-miR-494-3p
MIMAT0000415	hsa-let-7i-5p	MIMAT0000270	hsa-miR-181a-3p	MIMAT0000693	hsa-miR-30e-3p	MIMAT00002817	hsa-miR-495-3p
MIMAT0000416	hsa-miR-1-3p	MIMAT0000256	hsa-miR-181a-5p	MIMAT0000510	hsa-miR-320a	MIMAT00002820	hsa-miR-497-5p
MIMAT0000098	hsa-miR-100-5p	MIMAT0000258	hsa-miR-181c-5p	MIMAT0005792	hsa-miR-320b	MIMAT00004772	hsa-miR-499a-3p
MIMAT0000099	hsa-miR-101-3p	MIMAT0000259	hsa-miR-182-5p	MIMAT0000755	hsa-miR-323a-3p	MIMAT00004773	hsa-miR-500a-5p
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