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Original Research Article

Sperm fertilizing ability *in vitro* influences bovine blastocyst miRNA content

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ABSTRACT

MicroRNAs (miRNAs) are small highly conserved non-coding RNA molecules that orchestrate a wide range of biological processes through post-transcriptional regulation of gene expression. During development, miRNAs play a key role in driving embryo patterning and morphogenesis in a specific and stage-dependent manner. Here, we investigated whether sperm from bulls with different fertilizing ability *in vitro* influence blastocyst quality and miRNA content. Results demonstrate that blastocysts obtained using sperm from high fertility sires (H group) display significantly greater cleavage and blastocyst development as well as greater transcript abundance in blastocysts for the developmental competence markers *CDX2*, *KRT8*, *NANOG*, *OCT4*, *PLAC8*, *PTGS2*, *SOX17*, and *SOX2*, compared to blastocysts generated using sperm from low fertility sires (L group). In parallel, high throughput deep sequencing and differential expression studies revealed that H blastocysts exhibit a greater miRNA content compared to L blastocysts, with hsa-miR-4755–5p and hsa-miR-548d-3p uniquely detected in the H group, and greater abundance of hsa-miR-1225–3p in the H group. Gene ontology (GO) and KEGG pathway analyses indicated that the 3 differentially expressed miRNAs identified are involved in the regulation of many biological mechanisms with a key role in aspects of early embryo development, including transcriptional regulation, cellular biosynthesis, nucleic acid metabolism, cellular differentiation, apoptosis, cytoskeleton remodeling, cell-to-cell interactions, and endocytosis. Overall, our results indicate that sperm fertilizing ability influences blastocyst developmental ability and miRNA content. In addition, we demonstrate an association between blastocyst quality and miRNA content, thus suggesting the possibility to score miRNA expression as biomarkers for improved routine embryo selection technologies to support assisted reproductive efforts.

1. Introduction

Reproductive efficiency drives profitability in all livestock farming systems and is particularly important in seasonal systems of production since the opportunity to become pregnant is limited to specific and restricted time periods [1]. In particular, in cattle, reduced fertility results in extended calving intervals, increased involuntary culling rates, decreased milk production, and delayed genetic progress, thus causing significant economic losses [2].

Based on this, over the years, reproductive biologists, nutritionists, and geneticists have focused their research on developing novel strategies to improve reproductive health and efficiency [3–5]. However, fertility is a multi-factorial trait influenced by complex interactions among genetic, environment and managerial factors, making it extremely difficult to determine the key molecular and/or physiological mechanisms involved in reproductive success [6,7].

To date, systems of *in vitro* embryo production are often based on the use of morphological criteria to predict both gamete and embryo quality, that, however, are subjective and controversial [8]. It is therefore necessary to identify novel specific and reliable biomarkers that allow for accurate and predictive assessment of oocytes, sperm, and embryos, thus improving overall pregnancy outcomes. Among the numerous possibilities, microRNAs (miRNAs) have emerged as one of the most promising candidates as a powerful screening tool.

Several studies have recently demonstrated that miRNAs play a key role in cell behavior by either directly or indirectly regulating mRNA

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transcript levels and related protein synthesis [9]. miRNAs are short non-coding RNAs of 19–24 nucleotides able to modulate gene expression by targeting their 3′-untranslated regions (3′UTRs) in an imperfect manner. Growing knowledge indicates that they are associated with a variety of biological functions, such as cell differentiation and proliferation, apoptosis, metabolism, aging, and development [10–13]. This makes them interesting candidates for the control of several critical pathways, including those essential for embryo development and differentiation. Studies in mice demonstrated that miRNAs derived both from oocytes and sperm decline in concentration from the zygotic to the 2-cell stage [14–18]. Subsequently, the newly synthetized miRNAs rapidly and steadily increase in a highly specific and stage-dependent manner from embryonic genome activation onwards [19–22]. Further studies support and expand these observations in cattle, demonstrating the key role played by miRNAs in the maternal-to-embryonic transition (MET) and, more in general, during early embryo development [23–29].

Here, we investigated whether *in vitro* sire fertility status influences developmental competence of blastocysts and their miRNA content. For this purpose, we fertilized *in vitro* matured oocytes using sperm with high (H group) or low (L group) fertilizing ability. We evaluate embryo morphology and analyze the transcription levels for the main embryo quality markers, namely CDX2, KRT8, NANOG, OCT4, PLAC8, PTGS2, SOX2, and SOX17 [30]. In parallel, we use high-throughput deep sequencing to characterize and compare miRNA expression profiles between H and L groups and to identify the differentially expressed miRNAs.

2. Materials and methods

All reagents were purchased from Sigma-Aldrich, Milan, Italy, unless otherwise indicated.

2.1. Ethical statement

Bovine ovaries were collected from an authorized local slaughterhouse. This study did not involve the use of living animals; therefore, ethical approval was not required.

Frozen semen straws from 8 Holstein bulls were randomly selected from an Artificial Insemination (AI) centre (INSEME, Zorlesco, Lodi, Italy).

2.2. Oocyte in vitro maturation

The details of methods for oocyte recovery and *in vitro* maturation (IVM) were previously described by Lonergan et al. [31]. Briefly, bovine ovaries obtained from a commercial abattoir were transported to the laboratory in phosphate-buffered saline (PBS) at 37 °C. Cumulus oocyte complexes (COCs) were isolated from surface visible antral follicles within 3–4 h of ovary collection, washed 3 times in modified PBS supplemented with 36 μg/L pyruvate, 50 μg/mL gentamycin, and 0.5 mg/mL BSA (Fraction V, cat. no. A-9647) and subsequently cultured in maturation medium consisting of Medium 199 (Thermo Fisher Scientific, Milan, Italy), 10 ng/mL epidermal growth factor, and 10% (v/v) fetal calf serum (FCS, Thermo Fisher Scientific, Milan, Italy) at 38.8 °C in 5% CO_2 for 24 h.

2.3. In vitro embryo production

In vitro embryo production (IVP) was carried out as previously described [31,32]. Briefly, a total of 150 *in vitro* matured COCs per replicate (n = 5) were washed twice in fertilization medium consisting of Tyrode's albumin lactate pyruvate (TALP) medium and 10 μg/mL heparin salt (Calbiochem, San Diego, CA, USA), transferred in groups of up to 50 into four-well dishes containing 250 μL/well of fertilization medium and fertilized with semen obtained from 8 different Holstein-Friesian bulls. Motile sperm were obtained by centrifugation of frozen-thawed sperm using a Percoll discontinuous density gradient (3 mL of 45% Percoll layered over 3 mL of 90% Percoll) for 10 min at 700×*g*, at room temperature. Viable sperm were collected at the bottom of the 90% Percoll fraction, washed in TALP and pelleted by centrifugation at 100×*g* for 10 min at room temperature. The supernatant was removed, sperm were counted using a haemocytometer (BRAND® counting chamber BLAUBRAND® Neubauer pattern) and diluted in an appropriate volume of TALP to a final concentration of 2×10^6 sperm/mL. 250 μL of sperm suspension were added to each fertilization well containing *in vitro* matured COCs to obtain a final concentration of 1×10^6 sperm/mL. Culture dishes were then incubated for 18–20 h in of 5% CO_2 at 38.8 °C in air with maximum humidity. Post-fertilization, 150 presumptive zygotes were denuded of cumulus cells by vortexing for 2 min in 2 mL PBS, washed twice in PBS, once in synthetic oviduct fluid medium (SOF) supplemented with 10% FCS, transferred into 25 μL drops of SOF medium under mineral oil in groups of 25, and cultured in 5% CO_2 and 5% O_2 at 38.8 °C. Drops were monitored daily, and blastocysts were collected after 7 days of culture (Day 7) for the subsequent analysis.

2.4. Blastocyst evaluation

2.4.1. Cleavage and blastocyst rates

At day 2 post-fertilization, cleavage rate was assessed, and all cleaved embryos were transferred into new droplets and cultured for a further 5 days. The rate of embryos developing to the blastocyst stage was assessed on Day 7 of culture. Data were generated from 5 replicates for each of the 8 bulls.

2.4.2. Gene expression analysis

Polyadenylated RNA [poly(A) + RNA] was extracted from single blastocysts (n = 10), using Dynabeads mRNA Direct microkit (Thermo Fisher Scientific, Milan, Italy) according to the manufacturer's instructions. Target genes were analyzed using predesigned primers and probe sets from TaqManGene Expression Assays (Table 1). *Histone H2A* and *PPIA* were used as reference genes [33,34]. Quantitative PCR was performed using CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Milan, Italy). Gene expression values were quantified with CFX Manager software (Bio-Rad Laboratories, Milan, Italy) and are reported with the highest expression set to 1 and the others relative to this value.

2.5. miRNA sequencing and bioinformatic data analysis

2.5.1. RNA extraction

Total RNA was extracted from pools of 30 blastocysts using the AllPrep RNA/DNA Micro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions and as previously described [35,36]. Briefly, 350 μL of Buffer RLT Plus supplemented with 1% beta-mercaptoethanol were added to each blastocyst and mixed by pipetting for 1 min. Lysates were homogenized using a syringe with 20-gauge needle and centrifuged at 16,000×*g* for 30 s at room

Table 1
List of primers used for quantitative PCR analysis.

Gene	Description	Cat.N.
CDX2	Caudal type homeobox 2	Bt03649157_m1
H2AFZ	H2A.Z variant histone 1	Bt03216348_g1
KRT8	Keratin 8	Bt03225175_g1
NANOG	Nanog homeobox	Bt03220540_m1
OCT4	POU class 5 homeobox 1	Bt03223846_g1
PLAC8	Placenta specific 8	Bt03220478_m1
PPIA	Peptidylprolyl isomerase A	Bt03224615_g1
PTGS2	Prostaglandin-endoperoxide synthase 2	Bt03214492_m1
SOX17	SRY-box transcription factor 17	Bt04291261_gH
SOX2	SRY-box transcription factor 2	Bt03278318_s1

temperature using the AllPrep DNA Mini spin column. Flowthroughs were recovered, 50 μL Proteinase K solution (20 mg/mL) were added and incubated for 1 min. Subsequently, 350 μL of 70% ethanol were added to the samples, transferred into an RNAeasy Mini spin column, and centrifuged at 16,000×*g* for 15 s at room temperature. Flowthroughs were discarded and columns were washed with Buffer RW1 (700 μl) and subsequently with Buffer RPE (500 μL). Tubes were then centrifugated at 16,000×*g* for 30 s at room temperature and flowthroughs were discarded. 500 μL 80% ethanol were added to the spin column and centrifuged 16,000×*g* for 2 min at room temperature. Lastly, RNA was eluted using 20 μL DNase/RNase-free water by centrifugation at 10,000×*g* for 1 min at room temperature. Samples were then concentrated into 5 μL using the RNA Clean and Concentrator kit (Zymo Research Corp, CA, USA).

2.5.2. Library preparation

Libraries were prepared from 5 μL of each of the RNA samples using the Truseq Small RNA kit (Illumina Inc., CA, USA) with some modifications. First, to minimize primer dimer formation, total RNA was mixed with half of the TruSeq Small RNA sample reagents, followed by 15 cycles of PCR to amplify the library. Then, 5 μL of each unique indexed library was pooled to create a 6-plex which was size selected by resolution on a Pippin gel cassette 3% Agarose Dye free (BluePippin, Sage Science, MA, USA). Library RNA fragments in the 140–160 bp size range (the length of miRNA inserts plus the 3′ and 5′ adaptors) were recovered in 40 μL of Pippin elution buffer and then purified by Qiagen MinElute PCR Purification kit (Qiagen, CA, USA). The indexed libraries were quantified in triplicate on an ABI9700 qPCR instrument using the KAPA Library Quantification Kit, according to the manufacturer's protocol (Kapa Biosystems, Woburn, MA, USA). Then, the pooled libraries were adjusted to a final concentration of 2 nM and 10 μL were used for sequencing on an Illumina HiSeq2000 using a 50 bp Single-Read sequencing protocol.

2.5.3. Bioinformatic analysis

The quality of raw reads was assessed using FastQC v0.11.2. Raw sequences were trimmed using Trimmomatic software to remove primer sequences. The quality control threshold was set as a minimum base quality of 15 over a 4 bases sliding window and only sequences longer than 15 nucleotides were retained. Trimmed miRNA sequences passing the threshold were annotated using miRDeep2 software. Known bovine and human miRNAs (mature and precursors) downloaded from the MirBase database were used as a reference in the discovery process. MiRNAs with a minimum of 1 read count in at least two of the three biological replicates were considered as detected. All known bovine and human miRNAs were used to quantify the frequency of miRNAs in each sample and to produce a list of miRNA IDs. The relative abundance of mapped reads was expressed as counts and the raw counts were analyzed using the edgeR package of R software.

2.5.4. Differential miRNA expression analyses

The differential miRNA expression analysis was perfomed using the R package 'EdgeR' (http://bioconductor.org/packages/release/bioc/html/edgeR.html) in order to identify differentially expressed miRNAs. MiRNAs with log2 fold change differences ≥1, *P-value* (*P*) ≤0.05 and False Discovery Rate (FDR) ≤ 0.1 were considered as differentially expressed. The interaction between differentially expressed miRNAs and their target mRNAs was predicted using miRWalk 2.0, a widely used web-based database to predict miRNA-target mRNA interactions. A total of 6 prediction programs were combined into a pipeline for the analysis as follows: miRanda-rel2010, PicTar2, PITA, RNA22v2, RNAhybrid2.1 and Targetscan 6.2. The lists of predicted target genes of individual miRNAs were imported to DAVID Bioinformatics systems, a freely available bioinformatic tool (http://david.abcc.ncifcrf.gov/).

2.5.5. miRNA target prediction and functional analysis

Gene ontology (GO) analysis was carried out using this tool to identify the most important enriched biological and cellular processes. The DAVID web-tool also facilitated the identification of the canonical signaling pathways significantly enriched with the predicted target genes of each miRNAs, which were then analyzed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. To better understand the functional significance of the results, KEGG pathway analysis was also performed using DIANA miRPath v2.0 with homologous human miRNA and gene union options. DIANA miRPath uses predicted miRNA targets (in CDS or 3′-UTR regions) provided by the DIANA-microT-CDS algorithm, or experimentally validated miRNA interaction derived from DIANA-TarBase v6.0. Furthermore, GO analysis and gene interaction analyses were performed using CluPedia plugin into Cytoscape V 3.2.1.

2.6. miRNA validation

Quantitative real-time PCR was used to validate differentially expressed miRNAs, as previously described [37]. Briefly, RNA was extracted from pools of 10 blastocysts obtained from the same bull sperm used for deep sequencing using the miRCURY RNA Isolation Kit (Exiqon, Denmark) and reverse transcribed with the miRCURY locked nucleic acid (LNA) Universal cDNA synthesis kit (Exiqon, Denmark). Real-time qPCR was performed in triplicate in a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Milan, Italy) using the SYBR Green master mix (Exiqon, Denmark) and specific LNA PCR primer sets for hsa-miR-4755–5p, hsa-miR-548d-3p, and bta-miR-1225–3p (Table 2). U6 snRNA, 5SrRNA, and RNU1A (Exiqon) were chosen as reference small RNAs for normalization, as previously described [38,39]. Melting curve analysis was run for each experiment to assess the specificity of the primer amplification. Expression values were quantified with CFX Manager software (Bio-Rad Laboratories, Milan, Italy) using the $2^{-\Delta\Delta Ct}$ method [40] and are reported with the highest expression set to 1 and the other relative to this.

2.7. Statistical analysis

Normality and homoscedasticity of the data was firstly checked before proceeding to perform statistical analysis. Thereafter, data were analyzed using one-way ANOVA (SPSS 19.1; IBM) followed by post-hoc Tukey's test. Data are presented as the Mean ± Standard Error of the Mean (SEM). Differences in $P \leq 0.05$ were considered significant.

3. Results

3.1. Blastocyst evaluation

Cleavage rate was significantly higher in embryos obtained after fertilization with semen from bulls A-E compared to those obtained from bulls F–H (Table 3, threshold = >70%). Nevertheless, Day 7 blastocyst evaluation revealed that the use of sperm from bulls A-C resulted in a higher blastocyst development compared to bulls D-H (Table 3). Based on this, bulls A, B and C were considered "high fertility" (H), while bulls F, G, and H were categorized as "low fertility" (L). Bulls D and E

Table 2
List of primer sets used for miRNA validation.

miRNA	GeneGlobe ID
hsa-miR-4755–5p	YP02103815
hsa-miR-548d-3p	YP00205608
bta-miR-1225–3p	YP02114803
U6 snRNA	YP02119464
5SrRNA	YP00203906
RNU1A	YP00203909

Table 3
Number of oocytes (N), cleavage and blastocyst rates following *in vitro* fertilization with semen from one of 8 Holstein bulls (A to E). [abc] Different superscripts indicate statistically significant differences (**$P \leq 0.05$**). * per total oocytes; ** per cleaved oocytes.

Bull ID	N	Cleavage rate	Blastocyst rate*	Blastocyst rate**
		% (mean ± SEM)		
A	295	75.9 ± 1.4^{a}	35.9 ± 3.7^{a}	47.3 ± 5.6^{a}
B	290	81.1 ± 2.5^{a}	33.2 ± 4.6^{a}	40.9 ± 4.8^{a}
C	318	74.8 ± 1.2^{a}	32.1 ± 3.7^{a}	42.9 ± 3.4^{a}
D	251	76.5 ± 1.5^{a}	22.7 ± 3.7^{b}	29.7 ± 4.8^{b}
E	481	78.8 ± 3.2^{a}	17.9 ± 4.9^{bc}	22.7 ± 6.3^{b}
F	283	68.6 ± 4.8^{b}	14.8 ± 3.2^{c}	21.6 ± 5.1^{b}
G	297	65.3 ± 3.6^{b}	13.5 ± 3.3^{c}	20.6 ± 4.6^{b}
H	379	62.8 ± 2.8^{b}	13.5 ± 2.8^{c}	21.4 ± 4.2^{b}

displayed discontinuous cleavage vs. blastocyst rate results and were not used for the subsequent analysis.

Gene expression studies indicated that *CDX2*, *KRT8*, *NANOG*, *OCT4*, *PLAC8*, *PTGS2*, *SOX2*, and *SOX17* genes were expressed in both experimental groups. Interestingly, embryos obtained after fertilization with sperm with high fertilizing ability (H group) displayed significantly higher transcription levels for all the genes analyzed, compared to blastocysts produced using L group semen (Fig. 1).

3.2. miRNA deep sequencing analysis

After filtering out low-quality reads, H group blastocysts displayed a mean of 24.6 million reads, 18.2 million (73.5 %) of which were mapped to the reference bovine genome, while L group blastocysts exhibited 12.8 million reads, 8.5 million (66.8 %) of which mapped (Table 4). In addition, a mean of 549,090 and 212,286 reads of H and L blastocysts, respectively, matched with known bovine and human miRNAs reported in miRBase release 22.1.

The most abundant length of all miRNAs mapped was 23 nucleotides (nt) in both experimental groups (Fig. 2).

Table 4
Sequencing data showing the number of filtered and mapped reads, % of mapped reads, number of known mapped miRNA reads and % of known mapped miRNA reads in blastocysts obtained after fertilization with sperm with high (H) or low (L) fertilizing ability.

Group	Bull ID	Filtered reads (n)	Mapped reads (n)	% Mapped reads	Known miRNA mapped reads (n)	% known miRNA mapped reads
H	A	38843847	29298950	75.4	913546	3.1
	B	16085421	11992592	74.6	352711	2.9
	C	18961284	13412562	70.7	381012	2.8
L	F	14440423	9506437	65.8	268079	2.8
	G	11468277	7850467	68.5	168995	2.2
	H	12365489	8243021	66.2	199785	2.4

3.3. miRNA expression analysis

A total of 692 and 377 known bovine and homologous human miRNA sequences were identified in the H and L groups, respectively. Specifically, 283 miRNAs were common to both groups, 409 were only identified in the H group and 94 were unique to the L group (Fig. 3).

Among the top 10 highly expressed miRNAs in the H and L groups, 8 miRNAs (bta-miR-10 b, bta-miR-423–5p, bta-miR-92a, bta-miR-191, bta-miR-378, bta-miR-148a, bta-miR-192, bta-miR-22) were common to both groups (Table 5). In particular, bta-miR-10 b represented the most abundantly expressed miRNA with 129,517 and 47,775 counted reads in the H and L group, respectively.

3.4. miRNA differential expression

miRNA expression analysis revealed that 3 miRNAs were differentially expressed between H and L groups (Table 6). More in details, two miRNAs, hsa-miR-4755–5p and hsa-miR-548d-3p, were exclusively expressed only in H group (FC = −10.5 and −10.1, respectively), while bta-miR-1225–3p was presented in both groups with a significantly

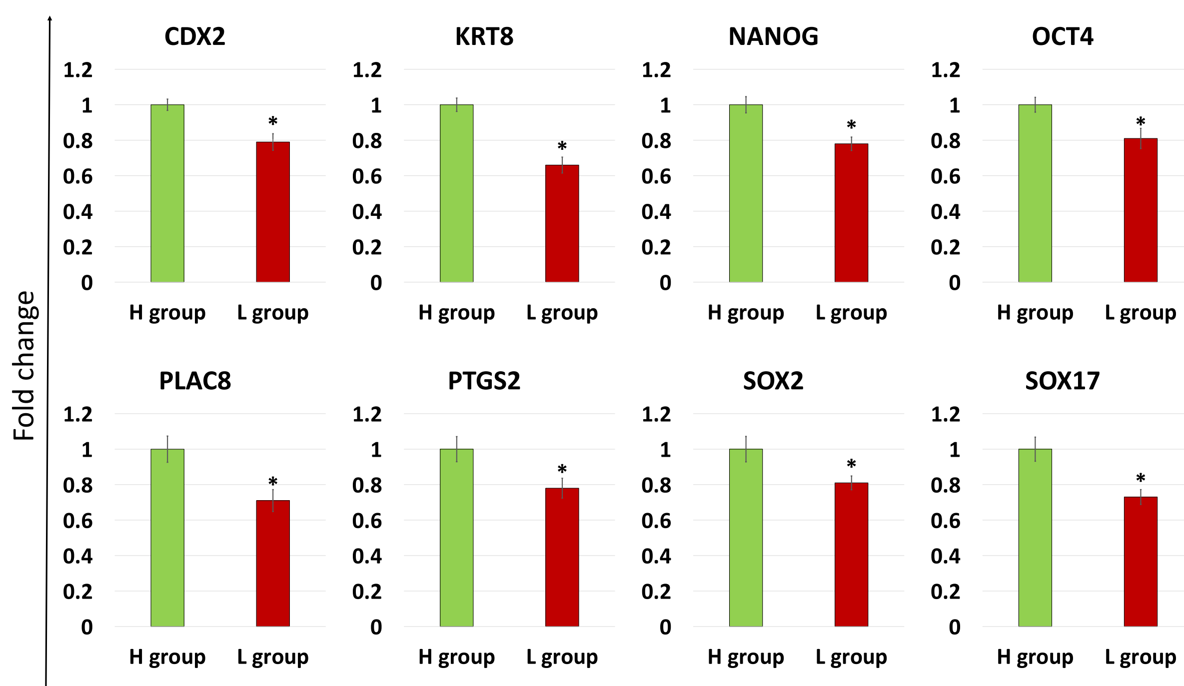

Fig. 1. Gene expression levels of *CDX2*, *KRT8*, *NANOG*, *OCT4*, *PLAC8*, *PTGS2*, *SOX2*, and *SOX17* genes in 10 single blastocysts obtained with sperm with high (H group, green bars) or low (L group, red bars) fertilizing ability. Data are expressed as the mean ± SEM. *asterisks indicate $P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

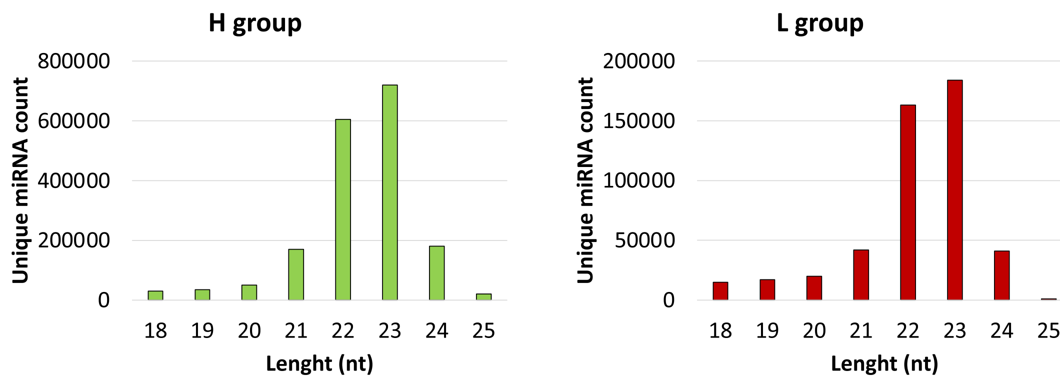

Fig. 2. Mapped reads reported as unique mature miRNAs and length distribution in blastocysts obtained after fertilization with sperm with high (H, green) or low (L, red) fertilizing ability. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

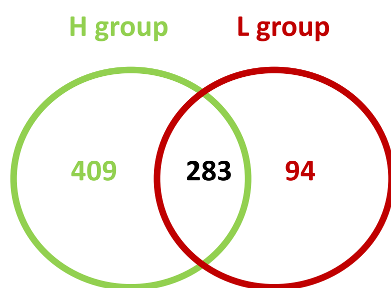

Fig. 3. Venn diagram showing the number of known miRNAs expressed in blastocysts obtained after fertilization with sperm with high (H) or low (L) fertilizing ability.

Table 5
Top 10 most abundant miRNAs in blastocysts obtained after fertilization with sperm with high (H) or low (L) fertilizing ability. miRNAs are reported as miRNA ID number and average read counts.

H		L	
miRNA ID	Avarage read counts	miRNA ID	Avarage read counts
bta-miR-10b	129517	bta-miR-10b	47775
bta-miR-423–5p	36460	bta-miR-423–5p	21813
bta-miR-92a	51670	bta-miR-92a	25310
bta-miR-191	29438	bta-miR-191	11804
bta-miR-378	47152	bta-miR-378	16875
bta-miR-148a	23568	bta-mR-148a	7253
bta-miR-192	18989	bta-miR-22	5686
bta-miR-22	18735	bta-miR-192	5600
bta-miR-30d	11657	bta-miR-16b	4844
bta-miR-30e	11029	bta-miR-92b	4680

Table 6
Differentially expressed miRNAs in blastocysts obtained after fertilization with sperm with high (H group) or low (L group) fertilizing ability. Fold change (FC) = Log2 Fold Change, *P-value* (*P*), False Discovery Rate (FDR).

miRNA	FC	*P*	FDR	Group
hsa-miR-4755–5p	−10.5	<0.0001	0.005	H
hsa-miR-548d-3p	−10.1	0.0002	0.037	H
bta-miR-1225–3p	−6.0	0.0002	0.037	H and L

lower level in the L group compared to the H one (FC = −6.0).

3.5. Gene ontology and pathway enriched by differentially expressed miRNAs

A total of 4382 genes were predicted as targets of the differentially expressed miRNAs. GO analysis revealed that biological processes associated with transcription regulation, cellular biosynthetic processes, including nucleic acid metabolism and cell and embryonic morphogenesis, were over-represented (Table 7).

Moreover, KEGG pathway analysis identified 56 enriched canonical signaling pathways (Table 8) associated with cellular processes: cell adhesion (Regulation of actin cytoskeleton, Tight Junction and cell adhesion molecules), cell proliferation (MAPK signaling pathway), and cell survival and growth (TGF-beta, apoptosis and neurotrophin pathways); cell communication (Endocytosis and calcium pathways); cell metabolism (Insulin and phosphatidylinositol pathways).

Functional analysis carried out on hsa-miR-4755–5p and hsa-miR-548d-3p simultaneously (Table 9) and on hsa-miR-1225–5p alone (Table 10) using DIANA mirPath revealed other important pathways reported in the tables below.

3.6. Deep sequencing data validation

RT-qPCR analysis confirmed the quality of deep sequencing data. Specifically, the expression levels of hsa-miR-4755–5p, hsa-miR-548d-3p, and bta-miR-1225–3p detected in H and L groups were consistent with those obtained with miRNA differential expression analysis. Specifically, hsa-miR-4755–5p and hsa-miR-548d-3p were undetectable in blastocysts from the L group and bta-miR-1225–3p was expressed at significantly lower levels in the L group compared to the H group (Fig. 4).

4. Discussion

In the present study, we demonstrate that blastocysts obtained after fertilization with sperm with high or low *in vitro* fertility, as assessed by differential cleavage and blastocyst rates following IVF, exhibit differential transcript abundance of several developmental competence markers, and differential miRNA content. This is in agreement with previous studies demonstrating critical influence of sire on oocyte developmental potential [41,42]. Consistent with this observation, gene expression studies indicate that blastocysts derived from high fertility sires display higher transcript levels for all the embryo quality markers analyzed, namely *CDX2*, *KRT8*, *NANOG*, *OCT4*, *PLAC8*, *PTGS2*, *SOX2*, and *SOX17*. Interestingly, such genes are known to play a fundamental role in a variety of important pathways for the developmental potential

Table 7
Results of Gene Ontology (GO) analysis. Gene count indicates how many times the genes in the pathway were observed in the data. Gene Ontology (GO) terms, *P-value* (*P*), False Discovery Rate (FDR).

GO Term	Gene count	*P*	FDR
GO:0006350~transcription	623	$9.41e^{+13}$	$1.79e^{+06}$
GO:0045449~regulation of transcription	750	$1.02e^{+13}$	$1.94e^{+06}$
GO:0006357~regulation of transcription from RNA polymerase II promoter	251	$1.37e^{+14}$	$2.61e^{+07}$
GO:0010557~positive regulation of macromolecule biosynthetic process	227	$1.02e^{+14}$	$1.93e^{+07}$
GO:0009891~positive regulation of biosynthetic process	238	$1.43e^{+14}$	$2.72e^{+07}$
GO:0031328~positive regulation of cellular biosynthetic process	235	$1.58e^{+14}$	$3.00e^{+07}$
GO:0010604~positive regulation of macromolecule metabolic process	282	$2.42e^{+13}$	$4.61e^{+07}$
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	217	$2.49e^{+14}$	$4.74e^{+08}$
GO:0051173~positive regulation of nitrogen compound metabolic process	222	$3.73e^{+14}$	$7.10e^{+07}$
GO:0010628~positive regulation of gene expression	203	$7.40e^{+14}$	$1.41e^{+08}$
GO:0045941~positive regulation of transcription	198	$8.10e^{+13}$	$1.54e^{+09}$
GO:0031175~neuron projection development	105	$2.91e^{+14}$	$5.54e^{+08}$
GO:0045893~positive regulation of transcription, DNA-dependent	171	$2.92e^{+14}$	$5.56e^{+08}$
GO:0051254~positive regulation of RNA metabolic process	172	$3.22e^{+14}$	$6.13e^{+08}$
GO:0007167~enzyme linked receptor protein signaling pathway	131	$3.64e^{+13}$	$6.94e^{+08}$
GO:0048666~neuron development	130	$3.92e^{+14}$	$7.47e^{+08}$
GO:0051252~regulation of RNA metabolic process	526	$6.40e^{+14}$	$1.22e^{+09}$
GO:0007242~intracellular signaling cascade	380	$1.42e^{+14}$	$2.71e^{+10}$
GO:0006355~regulation of transcription, DNA-dependent	512	$2.42e^{+14}$	$4.61e^{+09}$
GO:0030030~cell projection organization	136	$2.53e^{+14}$	$4.82e^{+10}$
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	92	$3.74e^{+14}$	$7.13e^{+09}$
GO:0006468~protein amino acid phosphorylation	220	$3.92e^{+14}$	$7.46e^{+09}$
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	136	$4.60e^{+14}$	$8.76e^{+09}$
GO:0048812~neuron projection morphogenesis	87	$1.41e^{+14}$	$2.68e^{+11}$
GO:0030182~neuron differentiation	153	$2.01e^{+14}$	$3.84e^{+11}$
GO:0006793~phosphorus metabolic process	297	$4.62e^{+13}$	$8.81e^{+09}$
GO:0006796~phosphate metabolic process	297	$4.62e^{+13}$	$8.81e^{+09}$
GO:0048858~cell projection morphogenesis	95	$5.92e^{+14}$	$1.13e^{+12}$
GO:0048667~cell morphogenesis involved in neuron differentiation	84	$6.02e^{+14}$	$1.15e^{+12}$
GO:0007409~axonogenesis	79	$6.24e^{+14}$	$1.19e^{+12}$
GO:0007267~cell-cell signaling	196	$6.79e^{+14}$	$1.29e^{+11}$
GO:0032990~cell part morphogenesis	98	$7.45e^{+14}$	$1.42e^{+12}$
GO:0000902~cell morphogenesis	126	$1.76e^{+14}$	$3.35e^{+11}$
GO:0016192~vesicle-mediated transport	186	$3.71e^{+13}$	$7.07e^{+10}$
GO:0048598~embryonic morphogenesis	110	$5.75e^{+13}$	0.001
GO:0032989~cellular component morphogenesis	135	$8.21e^{+14}$	0.002
GO:0000904~cell morphogenesis involved in differentiation	91	$8.62e^{+14}$	0.002
GO:0033674~positive regulation of kinase activity	86	$1.93e^{+14}$	0.004
GO:0045184~establishment of protein localization	233	$2.87e^{+14}$	0.005
GO:0045860~positive regulation of protein kinase activity	83	$3.00e^{+14}$	0.006
GO:0015031~protein transport	231	$3.03e^{+14}$	0.006
GO:0016310~phosphorylation	238	$9.43e^{+14}$	0.018
GO:0008104~protein localization	259	$1.06e^{+14}$	0.020
GO:0051347~positive regulation of transferase activity	86	$1.09e^{+14}$	0.021
GO:0019220~regulation of phosphate metabolic process	153	$1.80e^{+14}$	0.034
GO:0051174~regulation of phosphorus metabolic process	153	$1.80e^{+14}$	0.034
GO:0010629~negative regulation of gene expression	158	$1.90e^{+14}$	0.036
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	92	$2.58e^{+14}$	0.049
GO:0016481~negative regulation of transcription	145	$2.81e^{+14}$	0.053
GO:0048729~tissue morphogenesis	67	$2.96e^{+14}$	0.056
GO:0044057~regulation of system process	103	$4.59e^{+14}$	0.087
GO:0046578~regulation of Ras protein signal transduction	75	$4.77e^{+14}$	0.091
GO:0046907~intracellular transport	196	$5.25e^{+14}$	0.100

of pre-implantation embryos, in several species, including cattle [30,43], suggesting that the use of high fertilizing sperm may lead to the generation of high-quality blastocysts, while, in contrast, low fertilizing sperm may cause the formation of low-quality embryos. In agreement with this, low *in vitro* fertility bulls have been shown to cause polyspermy and to trigger overexpression of genes correlated with apoptosis and cellular damages [44].

Interesting information derive in parallel from the molecular analysis with deep sequencing data indicating that all samples belonging to both H and L groups were successfully sequenced with the libraries prepared. In particular, distribution analysis length reveals that the identified miRNAs exhibit a similar pattern distribution, and the majority of reads are 23nt in length, which is consistent with the typical size of mature cleaved products [45]. In addition, bioinformatic studies demonstrate that H group blastocysts display a higher miRNA content compared to those of L group. Although we have no clear explanation and further experiments are needed to better clarify how sperm may affect the molecular profile of produced embryos, we hypothesize that several miRNAs are not expressed in blastocysts belonging to L group with respect to the blastocysts obtained from the L group. In agreement with this, cluster analysis indicates that only 283 miRNAs are common to both groups, while 409 are mainly detected in H group and 94 are solely expressed in L group. Nevertheless, 8 out of 10 most abundantly expressed miRNAs are common to both groups, thus suggesting their fundamental key role in early embryonic development. It is interesting to note that these data are in line with previous observations demonstrating the involvement of these same miRNAs in bovine early embryogenesis, using three different and distinct procedures [36].

An in-depth evaluation of the molecular data obtained led to the identification of 3 differentially expressed miRNAs. Specifically, hsa-miR-4755–5p and hsa-miR-548d-3p were not detectable in L group blastocysts, while bta-miR-1225–3p was significantly reduced in the L group. Interestingly, while the latter has been annotated in the bovine, the former have not yet been annotated in cattle [46,47], making any functional interpretation of the finding challenging. It is tempting to speculate that hsa-miR-4755–5p and hsa-miR-548d-3p might exert a meaningful role in early embryonic development in cattle as well.

When gene prediction tools are applied to explain a possible role of the 3 differentially expressed miRNAs identified, important biological processes, including transcriptional regulation, cellular biosynthesis, nucleic acid metabolism, cellular differentiation, and embryonic morphogenesis, are over-represented. Furthermore, KEEG analyses indicate the correlation with signaling pathways that play a key role in early embryo development, such as regulation of actin cytoskeleton, tight Junction and CAMs, MAPK, TGF β, apoptosis, neurotrophin, endocytosis, calcium, GnRH, insulin, and phosphatidylinositol. In particular, the actin cytoskeleton pathway is reported to be essential in

Table 8
Results of Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis. Gene count indicates how many times the genes in the pathway were observed; abbreviations: *P-value* (*P*).

KEGG pathway	Gene count	*P*
hsa04310:Wnt signaling pathway	69	1.57e^{+07}
hsa04010:MAPK signaling pathway	92	2.11e^{+11}
hsa04020:Calcium signaling pathway	65	4.11e^{+11}
hsa04012:ErbB signaling pathway	37	1.08e^{+12}
hsa04920:Adipocytokine signaling pathway	30	1.96e^{+12}
hsa04370:VEGF signaling pathway	30	0.002
hsa04720:Long-term potentiation	27	0.004
hsa04514:Cell adhesion molecules (CAMs)	45	0.005
hsa04340:Hedgehog signaling pathway	23	0.005
hsa04960:Aldosterone-regulated sodium reabsorption	17	0.017
hsa04350:TGF-beta signaling pathway	30	0.019
hsa04540:Gap junction	30	0.026
hsa04730:Long-term depression	24	0.035
hsa04630:Jak-STAT signaling pathway	47	0.035
hsa05210:Colorectal cancer	45	4.57e^{+05}
hsa05200:Pathways in cancer	113	2.02e^{+10}
hsa05214:Glioma	32	5.08e^{+09}
hsa05213:Endometrial cancer	28	5.70e^{+09}
hsa05215:Prostate cancer	39	3.04e^{+11}
hsa05223:Non-small cell lung cancer	27	4.74e^{+10}
hsa05220:Chronic myeloid leukemia	34	4.86e^{+11}
hsa05217:Basal cell carcinoma	26	2.12e^{+11}
hsa05212:Pancreatic cancer	31	3.44e^{+11}
hsa04912:GnRH signaling pathway	39	3.49e^{+11}
hsa04270:Vascular smooth muscle contraction	43	4.05e^{+12}
hsa04150:mTOR signaling pathway	24	6.10e^{+11}
hsa05218:Melanoma	30	6.39e^{+11}
hsa05211:Renal cell carcinoma	27	0.006
hsa05014:Amyotrophic lateral sclerosis (ALS)	21	0.011
hsa05221:Acute myeloid leukemia	22	0.017
hsa05222:Small cell lung cancer	29	0.021
hsa05216:Thyroid cancer	13	0.022
hsa04520:Adherens junction	39	3.98e^{+08}
hsa04144:Endocytosis	65	1.87e^{+11}
hsa04510:Focal adhesion	68	5.33e^{+11}
hsa04210:Apoptosis	35	5.96e^{+11}
hsa04914:Progesterone-mediated oocyte maturation	33	0.002
hsa04810:Regulation of actin cytoskeleton	69	0.002
hsa04530:Tight junction	46	0.004
hsa04114:Oocyte meiosis	38	0.008
hsa04910:Insulin signaling pathway	58	5.11e^{+08}
hsa04916:Melanogenesis	43	1.42e^{+11}
hsa04722:Neurotrophin signaling pathway	50	2.85e^{+11}
hsa04360:Axon guidance	50	9.44e^{+10}
hsa04660:T cell receptor signaling pathway	41	7.37e^{+11}
hsa04062:Chemokine signaling pathway	62	0.002
hsa04666:Fc gamma R-mediated phagocytosis	36	0.002
hsa04664:Fc epsilon RI signaling pathway	30	0.004
hsa04670:Leukocyte transendothelial migration	41	0.005
hsa04662:B cell receptor signaling pathway	26	0.028
hsa00310:Lysine degradation	20	0.003
hsa04070:Phosphatidylinositol signaling system	27	0.013
hsa05414:Dilated cardiomyopathy	32	0.013
hsa04930:Type II diabetes mellitus	19	0.014
hsa04130:SNARE interactions in vesicular transport	16	0.018
hsa04120:Ubiquitin mediated proteolysis	42	0.039

actin remodeling that takes place during oocyte maturation as well as early embryogenesis [48]. Similarly, tight junction and CAM signaling pathways are known to control cell interactions which are fundamental during blastocyst formation and its subsequent development [49,50].

In conclusion, in the present study, we show that bovine sperm fertilizing ability *in vitro* impacts on cleavage and blastocyst rates as well as gene transcription of the main developmental competence markers and affects blastocyst miRNA content. The results confirm and extend previous findings that demonstrate the role of miRNA during embryogenesis [51] further suggesting a paternal influence that extends its effects through early embryogenesis and may impact on the further development. These results suggest the possibility to score miRNA expression as biomarkers for improved routine embryo selection technologies to support assisted reproductive efforts.

Table 9
Results of KEGG pathways from DIANA miRPath v2.0 for the target genes of hsa-miR-4755–5p and hsa-miR-548d-3p. Gene count indicates how many times the genes in the pathway were observed; abbreviations: *P-value* (*P*).

KEGG pathway	*P*	Target genes	miRNA ID
ErbB signaling pathway (hsa04012)	0.0001389508	*CAMK2D, ERBB2, SHC1, BTC*	hsa-miR-4755–5p hsa-miR-548d-3p
Transcriptional misregulation in cancer (hsa05202)	0.001395331	*HMGA2, CCND2, NCOR1, DDX5, PPARG, MEIS1*	hsa-miR-4755–5p
African trypanosomiasis (hsa05143)	0.002354351	*SELE, APOL1*	hsa-miR-4755–5p
Ubiquitin mediated proteolysis (hsa04120)	0.005949636	*RFWD2, UBA6, PARK2, BIRC2*	hsa-miR-4755–5p
Apoptosis (hsa04210)	0.009663038	*CSF2RB, IRAK1, BIRBC2*	hsa-miR-4755–5p
Huntington's disease (hsa05016)	0.01833565	*CREB5, AP2A2, PPARG, CNAQ, PPARGC1A, SDHD*	hsa-miR-4755–5p
Ubiquinone and other terpenoid-quinone biosynthesis (hsa00130)	0.04608754	*NQO1*	hsa-miR-4755–5p

Table 10
KEGG pathways from DIANA miRPath v2.0 for the target genes of hsa-miR-1225–5p.

KEGG pathway	*P*	Target genes	miRNA ID
Protein processing in endoplasmic reticulum (hsa04141)	0.003416487	*SSR1, DERL1, ERO1LB*	hsa-miR-1225–3p
Proteasome (hsa03050)	0.007827826	*PSME4, IFNG*	hsa-miR-1225–3p
Amphetamine addiction (hsa05031)	0.009343598	*CAMK4, GRIA4*	hsa-miR-1225–3p
Biosynthesis of unsaturated fatty acids (hsa01040)	0.0284081	*ELOVL2*	hsa-miR-1225–3p
Type I diabetes mellitus (hsa04940)	0.0284081	*IFNG*	hsa-miR-1225–3p
Protein digestion and absorption (hsa04974)	0.0284081	*ATP1A4, DPP4*	hsa-miR-1225–3p
Lysine degradation (hsa00310)	0.03504665	*KMY2C*	hsa-miR-1225–3p
Graft-versus-host disease (hsa05332)	0.04021567	*IFNG*	hsa-miR-1225–3p

Declarations of competing interest

None.

Data availability

All data generated or analyzed during this study will be available on request.

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CRediT authorship contribution statement

Rolando Pasquariello: Writing – original draft, Visualization, Validation, Methodology, Investigation. **Georgia Pennarossa:** Writing – original draft, Methodology, Investigation. **Sharon Arcuri:** Visualization, Validation, Methodology. **Beatriz Fernandez-Fuertes:** Writing – review & editing, Visualization, Methodology. **Patrick Lonergan:**

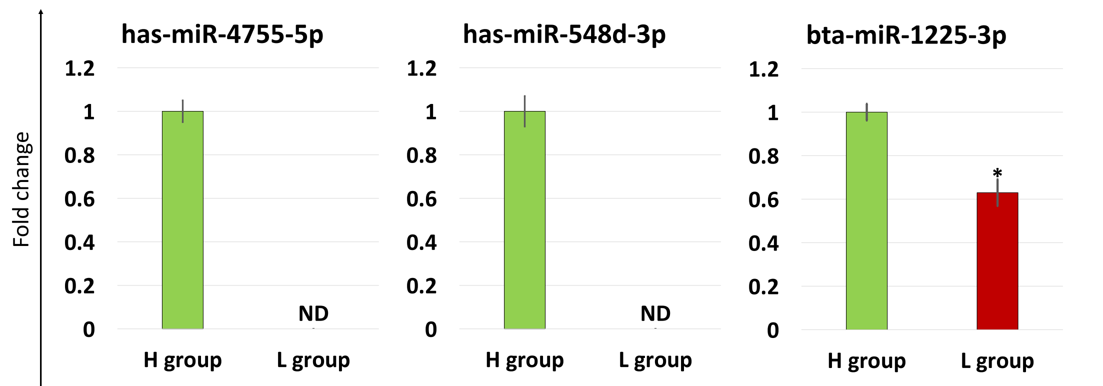

Fig. 4. Expression levels of hsa-miR-4755–5p, hsa-miR-548d-3p, and bta-miR-1225–3p in blastocysts obtained after fertilization with sperm with high (H group, green bars) or low (L group, red bars) fertilizing ability. Data are expressed as the mean ± SEM. * asterisk indicates $P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Writing – review & editing, Conceptualization. **Tiziana A.L. Brevini:** Writing – review & editing, Methodology, Conceptualization. **Fulvio Gandolfi:** Writing – review & editing, Conceptualization.

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