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MicroRNA expression in specific segments of the pig periovulatory internal genital tract is differentially regulated by semen or by seminal plasma

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ABSTRACT

microRNAs play pivotal roles during mammalian reproduction, including the cross-talk between gametes, embryos and the maternal genital tract. Mating induces changes in the expression of mRNA transcripts in the female, but whether miRNAs are involved remains to be elucidated. In the current study, we mapped 181 miRNAs in the porcine peri-ovulatory female reproductive tract: Cervix (Cvx), distal and proximal uterus (Dist-Ut, Prox-Ut), Utero-tubal-junction (UTJ), isthmus (Isth), ampulla (Amp), and infundibulum (Inf) when exposed to semen (natural mating (NM) or artificial insemination (AI-P1)) or to infusions of sperm-free seminal plasma (SP): the first 10 mL of the sperm rich fraction (SP-P1) or the entire ejaculate (SP-E). Among the most interesting findings, NM decreased mir-671, implicated in uterine development and pregnancy loss prior to embryo implantation, in Cvx, Dist-UT, Prox-UT, Isth, and Inf, while it increased in Amp. NM and SP-E induced the downregulation of miR-let7A-1 (Dist-UT, Prox-UT), a regulator of immunity during pregnancy. miR-34C-1, a regulator of endometrial receptivity gene expression, was increased in Dist-UT, UTJ and Amp (NM), in Prox-UT (AI-P1), and in Amp (SP-P1). miR-296, a modulator of the inflammatory response and apoptosis, was upregulated in the UTJ (all treatments). NM elicited the highest miRNA activity in the sperm reservoir (UTJ), suggesting that key-regulators such as miR-34c or miR-296 may modulate the metabolic processes linked to the adequate preparation for gamete encounter in the oviduct. Our results suggest that SP should be maintained in AI to warrant miRNA regulation within the female genital tract for reproductive success.

1. Introduction

Mating alters gene expression in different tissues of the internal female genital tract, in several species (Alvarez-Rodriguez et al., 2020b). Moreover, the presence of spermatozoa and/or seminal plasma (SP) during the pre/*peri* ovulatory porcine estrus period is also recognized to differentially impact on endometrial gene expression (Rodriguez-Martinez et al., 2021), particularly on immune-related genes (Álvarez-Rodríguez et al., 2020). The genes displaying changes include those ruling the synthesis of proteins such as the cortisol receptor, prostaglandins, and other complex molecules as the RNA binding molecules, with active roles in the maternal immune response to spermatozoa (and SP) in the reproductive tract (Gardela et al., 2020; Ruiz-Conca et al., 2020). During natural mating (NM) in pigs, the ejaculate is expelled into the female genital tract in well-defined fractions with specific compositions: sperm-peak (P1), sperm-rich, and post-sperm-rich fractions (Rodríguez-Martínez et al., 2009), eliciting inflammatory uterine responses (Rodriguez-Martinez et al., 2011). The immune response by the female against semen is paradoxical, comprising primary rejection while issuing immune tolerance to guarantee pregnancy success. Mating implies, therefore, a crucial decision by the female immune system, which is to maintain protection against pathogens and up to 80% of surplus

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foreign spermatozoa while at the same time allowing the survival of potentially fertile spermatozoa in the sperm reservoir (Rodriguez-Martinez et al., 1990), as well as triggering an influx of antigen-presenting cells responsible for presenting paternal antigens to elicit activation of lymphocytes in the adaptive immune compartment, exerting a state of immune tolerance in the latter stages of embryo development (Robertson et al., 2003).

Several families of non-coding RNAs (ncRNAs): microRNAs (miRNAs), short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), and transfer RNA (tRNA) have been identified across species (Chen et al., 2017; Hua et al., 2021). The most described family (miRNAs) is a class of ncsRNA, roughly 21–22 nucleotides in length, which are master gene regulators (Chen et al., 2017; Hua et al., 2021). miRNAs are intracellularly located but they can also be secreted -mainly via extracellular vesicles- to the extracellular environment, establishing a well-described cross-talk between cells and their extracellular environment (Ng et al., 2013; Valadi et al., 2007; Vilella et al., 2015). miRNAs control gene expression post-transcriptionally, by hybridizing to target mRNAs and thereby regulating their translation or stability (Filipowicz et al., 2008; Hausser et al., 2013). Moreover, miRNAs influence protein production and translation (Vasudevan et al., 2007), and one single miRNA could target multiple mRNAs (Pritchard et al., 2012). miRNAs are widely recognized as key regulators of many reproductive processes and pathways, including embryo development and implantation, placentation, and sperm capacitation, among others (Liang et al., 2017; Nothnick, 2012; Robertson et al., 2017; Salas-Huetos et al., 2019). Moreover, miRNAs are modulators of several immunological pathways during pregnancy (Baltimore et al., 2008) and help maintain the maternal immune environment required for pregnancy success in several species (Robertson et al., 2017; Schjenken et al., 2016).

In the porcine species, the miRNA expression profile has been explored in the context of pregnancy failure and embryo loss by studying the differences in miRNA expression in the implantation site of healthy and arrested embryos during different periods of pregnancy. miRNA expression profile in the pig endometrium has been characterized on days 15 (implantation period), 26 (placentation period) and 50 (mid-gestation period), three critical stages for embryo/fetal loss in the species. The miRNAs differentially expressed across these days were involved in several pathways, such as focal adhesion, cell proliferation and tissue remolding, such as miR-181a and miR-181c which play relevant roles in the regulation of genes and pathways known as involved in embryo implantation and placentation through immune signaling (Su et al., 2014).

On the male side, differentially expressed miRNAs were identified between fresh and capacitated boar spermatozoa. From a total of 204, 12 miRNAs were found to be involved in sperm capacitation-related PI3K-Akt, MAPK, cAMP-PKA and Ca^{2+} signaling pathways (Li et al., 2018a). Moreover, different miRNAs have been identified within the different ejaculated fractions using high-output small RNA sequencing (Martinez et al., 2022). Four miRNAs (mir-1285, mir-92a, mir-34c, mir-30), were differentially expressed among spermatozoa sourced from ejaculate fractions. These miRNAs target genes with key roles in fertility, sperm survival, immune tolerance, or cell cycle regulation, among others. On the other hand, different fractions of the SP, have been shown to elicit changes in the miRNAs profile secreted by explants of the female genital tract (uterus, utero-tubal junction and isthmus) in vitro. Dysregulated miRNAs, miR-34b, miR-205, miR-4776-3p and miR-574-5p, found in the explants were involved in functions and pathways related to immune responses (Barranco et al., 2020).

Against this background, it is reasonable to wonder whether the different fractions of the ejaculate may exert differences in the miRNA profile of the female genital tract after natural mating (NM) or artificial insemination (AI) and whether it may impact on latter stages of pregnancy. To address the hypothesis that spermatozoa, and/or SP components modulate miRNA expression sperm survival during colonization of the sperm reservoir (utero-tubal junction, UTJ) and sperm transit to the site of fertilization, we examined the effect of semen (entire ejaculate-mating or AI-P1 fraction) or sperm-free SP deposition (from the entire ejaculate or the P1 fraction) on the expression of miRNA transcripts across segments of the preovulatory sow internal genital tract (cervix to infundibulum).

2. Materials and methods

2.1. Ethics approval

Animal handling and experiments were carried out in accordance with the European Community Directive 2010/63/EU, 22/09/2010, and current Swedish legislation (SJVFS 2017:40). The study was accepted by the Regional Committee for Ethical Approval of Animal Experiments (Linköpings Djurförsöksetiska nämnd, Linköping, Sweden). Permits number 75–12 (10/02/2012), ID1400 (02/02/2018), and Dnr 03416–2020 (26/03/2020).

2.2. Animal management

At the Translational Medicine Centre (TMC/CBR-3) of Linköping University, weaned sows (parity 1–3, $n = 20$) and young matured boars (9–11 months of age, $n = 5$) of the Swedish Landrace breed (*Sus scrofa* domestica) were housed in individual pens under temperature and light (12 h:12 h light/dark cycle) control. Commercial feed was used to feed the animals, and water was available at all times. While pro-estrus and estrus behavior signals were observed, experienced staff applied back pressure to sows twice daily. Sows were also subjected to snout-to-snout contact with nearby boars.

2.3. Experimental design

Sows were randomly assigned to one of the five experimental groups when they displayed an estrus reflex, on the first day of behavioral estrus. Sows were at the onset of estrus and randomly allowed to be mounted by an individual boar or cervically inseminated/infused with the sperm-peak ejaculate fraction (containing 25% of the total spermatozoa of the ejaculate) or SP-harvested after double centrifugation at 1500 $\times g$ for 10 min and microscopically checked for absence of spermatozoa, either from the sperm-peak fraction or from the whole ejaculate.

In brief, females were naturally mated with a single male (NM, $n = 4$), or cervically infused with pooled sperm rich-fraction of the ejaculate (AI-P1, $n = 4$), with pools of sperm-free SP of the entire ejaculate (SP-E group, $n = 4$), sperm-free SP harvested from pools of sperm-peak fractions (SP-P1 group, $n = 4$), as shown in Fig. 1. The control group (Control, $n = 4$) received cervical infusions of BTS-extender. Sows were given general anesthesia for the tissue collection process after receiving each treatment for 24 h.

2.4. Tissue collection

All sows were subjected to mid-ventral laparotomies to collect mucosal tissue samples 24 h after the procedures (pre/peri-ovulation period), as previously described (Alvarez-Rodriguez et al., 2019). Briefly, sows were sedated by the intramuscular administration of a mixture of 5 mg dexmedetomidine (Dexdomitor, Orion Pharma Animal Health, Sollentuna, Sweden) and 100 mg tiletamine hydrochloride/zolazepam hydrochloride (Zoletil vet, Virbac A/S, Kolding, Denmark) followed by induced intravenous anesthesia with sodium thiopental

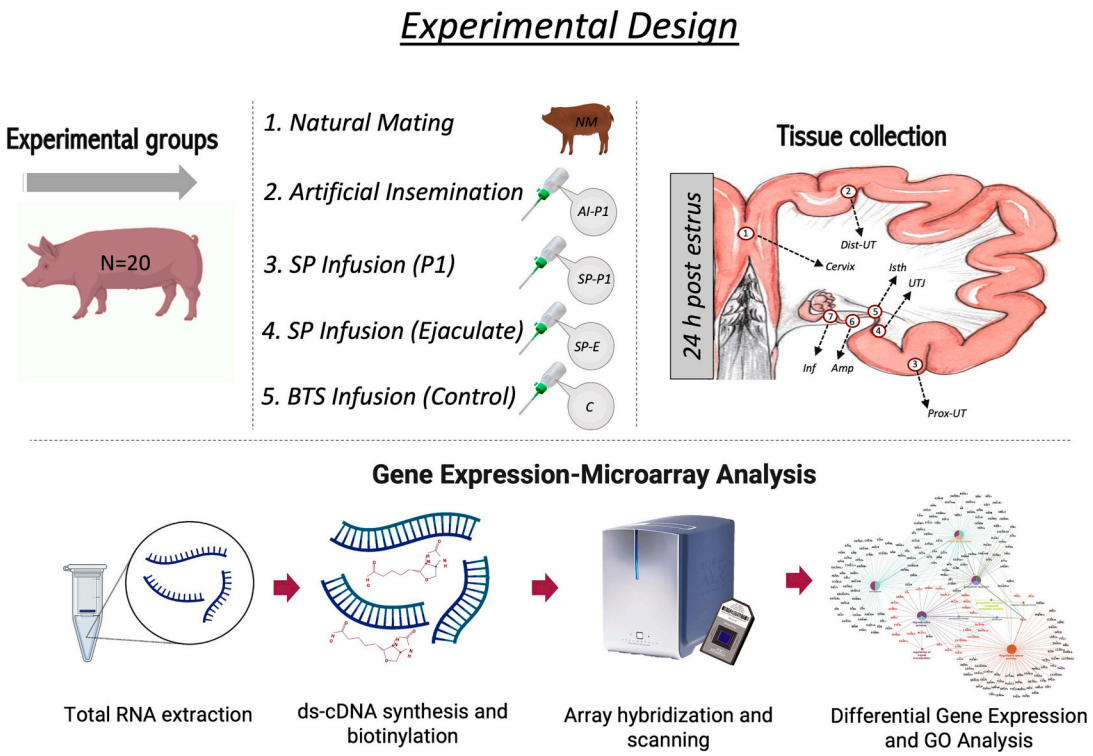

Fig. 1. Schematic representation of the experimental design. Sows were subjected to natural mating (NM); insemination with the first 10 mL of the sperm rich fraction (AI-P1); infusion with seminal plasma from the first 10 mL of the sperm rich fraction (SP-P1); infusion with seminal plasma from the whole ejaculate (SP-E); or infusion of BTS extender (Control). 24 h after treatments, tissue samples were collected from different segments of the reproductive internal genital tract: Cervix (Cvx), distal uterus (Dist-Ut), proximal uterus (Prox-Ut), utero-tubal junction (UTJ), and the oviductal segments isthmus (Isth), ampulla (Amp), and infundibulum (Inf). BTS: Beltsville Thawing Solution (Pursel and Johnson, 1975).

(Abbot Scandinavia AB, Solna, Sweden, 7 mg/kg BW) and maintained with isoflurane (3.5%–5%, Baxter Medical AB, Kista, Sweden) administered via a tracheal cuffed tube by a close-circuit PVC-ventilator (Servo ventilator 900 D, Siemens-Elema AB, Solna, Sweden). Peripheral blood plasma was analyzed (ELISA) for progesterone (P4) and 17ß-estradiol (E2) contents, confirming the sows were in pre/peri-ovulatory estrus (P4 = 0.77 ± 0.35 pg/mL; E2 ranging 294.2–376.50 ± 27.76 pg/mL, $p > 0.05$ among sows/groups). The ovarian follicles were visually counted. The mean was 22.30 ± 7.29 (mean ± standard deviation) follicles per sow, without significant differences between sow groups or sides. Samples of the mucosa, including the lining epithelium and the subjacent connective tissue, were collected under a surgical microscope (Wild M651, Heerbrugg, Switzerland) from specific segments: endocervix (Cvx), right distal uterine horn (Dist-UT), right proximal uterine horn (Prox-UT), right utero-tubal junction (UTJ; as a reference tissue for its role as sperm reservoir in the pig (Rodriguez-Martinez et al., 1990; Rodríguez-Martínez et al., 2005), right isthmus (Isth), right ampulla (Amp), and right infundibulum (Inf), from all the specimens. Mucosal samples were directly plunged into liquid nitrogen and stored in cryovials at −80 °C in RNAlater (Ambion, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) until analyzed.

2.5. Transcriptome analysis

Total RNA from reproductive samples was extracted following a TRIzol (Invitrogen, Carlsbad, CA, USA) modified protocol (Álvarez-Rodríguez et al., 2020). RNA concentration, integrity evaluation, cDNA synthesis, and microarray analyses (GeneChip® Porcine Gene 1.0 ST Array, Affymetrix Inc., Santa Clara, CA, USA) were performed according to methods previously described (Álvarez-Rodríguez et al., 2020). Only samples with RIN (RNA integrity number) values larger than 9 were employed for microarray hybridization. The GeneChip®Whole Transcript Plus reagent kit (Affymetrix, Santa Clara, CA, USA) was used to synthesize cDNA (250 ng/reaction). An initial incubation of the hybridization cocktail at 99 °C for 5 min was done after a fall to 45 °C before loading the array chip (GeneChip® Porcine Gene 1.0 ST Array, Affymetrix Inc., 3420 Central Expressway, Santa Clara, CA, USA). The cocktail hybridization solution (130 μL) was then put into each array chip and incubated for 16 h at 45 °C under 60 rotations/min. The hybridized cartridge array was unloaded after incubation and washed and stained with the GeneChip® Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA) before being scanned with the Affymetrix GeneChip® Scanner GCS3000 (Affymetrix, Santa Clara, CA, USA).

2.6. Bioinformatics and enrichment analyses

Transcriptomic results were processed following a previously described protocol (Alvarez-Rodriguez et al., 2020a). Briefly, the array chip data were processed using robust multi-array average (RMA) normalization, computing average intensity values by background adjustment, quantile normalization among arrays, and finally, log2 transformation for extracting the expression values of each transcript in the probe set. The normalized RNA expression data of the 181 detected transcripts were analyzed using the Transcription Analysis Console (TAC, Affymetrix). Differentially expressed transcripts were calculated using a linear model and the empirical Bayes' approach implemented in the package limma, included in the TAC console. A principal component analysis-based *p*-value correction was used, establishing a fold change (FC) >1 or < −1. From this set of differentially expressed miRNAs, 8 miRNAs were selected for further analysis based on their biological roles in reproductive and other physiological processes. GO terms and pathways were analyzed by PANTHER (Mi et al., 2013) based on the KEGG database (Kanehisa and Goto, 2000).

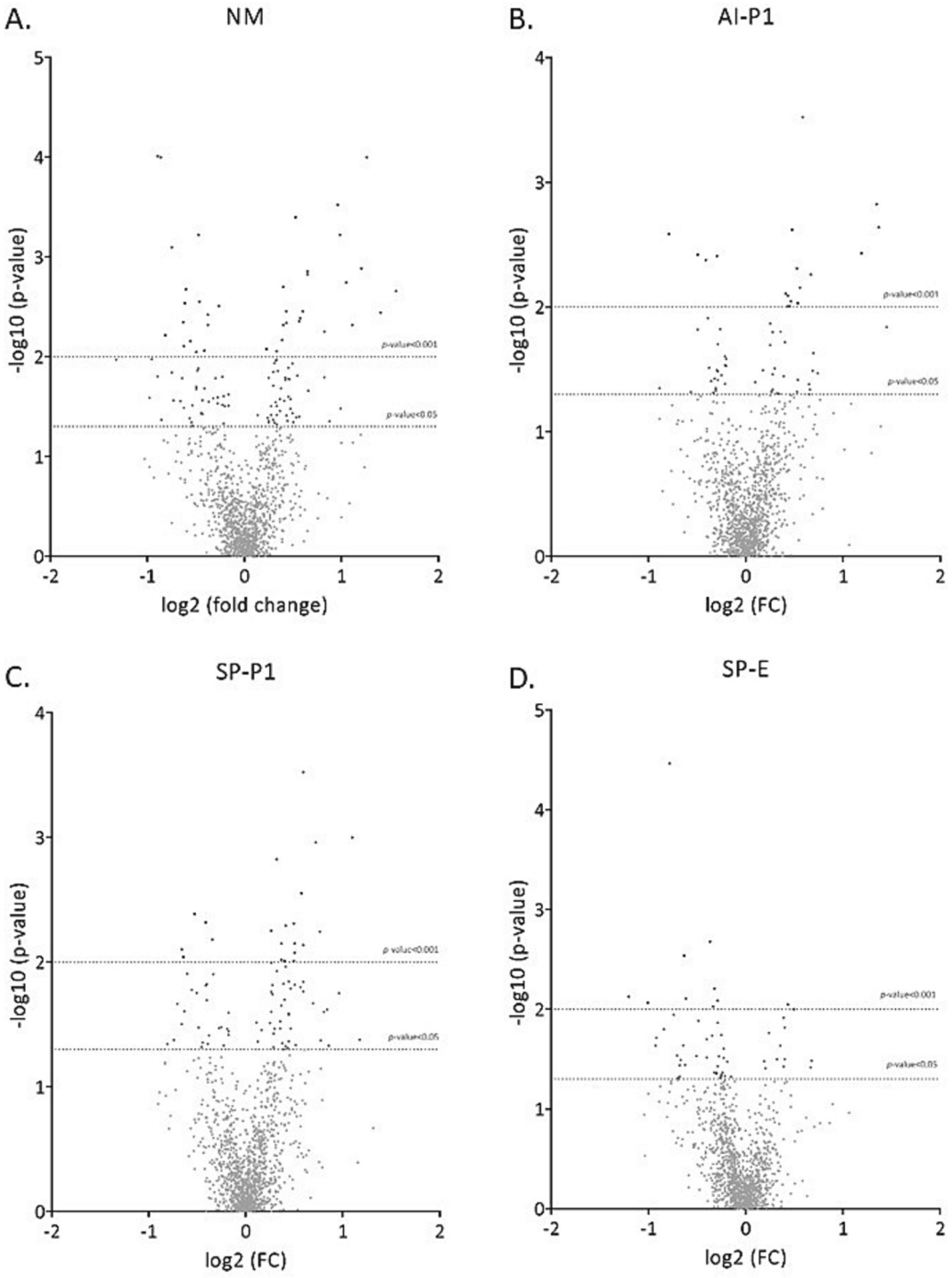

Fig. 2. Volcano plot depicting a summary of the differential miRNA transcripts cargo in the reproductive internal genital tract segments (cervix, distal uterus, proximal uterus, utero-tubal junction, isthmus, ampulla, and infundibulum), 24 h after natural mating (NM; A), insemination with the first 10 mL of the sperm rich fraction (AI-P1; B), infusion with seminal plasma from the first 10 mL of the sperm rich fraction (SP-P1; C), and infusion with seminal plasma from the whole ejaculate (SP-E; D), compared to unmated, Sham, infusion of BTS extender (Control). The x-axis shows the log2 fold-changes in expression and the y-axis the statistical significance (−log10 *p*-value). This figure depicts $p < 0.05$ and $p < 0.01$ relative to control.

3. Results

3.1. Mucosal miRNAs were differentially expressed among segments of the female reproductive tract in response to mating, artificial insemination, or infusion of different fractions of seminal plasma

A total of 181 miRNAs were detected among endometrial and oviductal mucosal tissue segments within groups. From these, miRNAs up-regulated and down-regulated, depending on the treatment tested, are depicted in the volcano plot (Fig. 2, Supplementary Table 1). The principal component analysis explained 29.9%, 28.3%, 31%, 30.7%, 26.8%, 26.8% and 24% of the variation in the two components, in Cvx, DistUt, ProxUt, UTJ, Isth, Amp and Inf, respectively (Supplementary Fig. 1). The hierarchical clustering analysis through a heap map representation (Supplementary Fig. 2) showed a heterogeneous grouping pattern in some of the tissues, with the endometrial tissues being the ones showing a more discrete grouping pattern.

3.2. Commonly dysregulated miRNAs among the different tissues and treatments

Results on the co-detection of altered miRNA transcripts across tissues after comparing the different treatments with the Control group are depicted in Fig. 3. Only one transcript, miR296, is commonly altered (up-regulated) in UTJ by all treatments (Fig. 3D).

Results on the co-detection of altered miRNA transcripts across treatments after comparing the different tissue segments with the UTJ (used as a reference Control), and 24 h after mucosal samples (including the lining epithelium and the subjacent connective tissue) are depicted in Fig. 4. Natural mating showed 1 dysregulated miRNA in all tissues (miR-671).

3.3. A specific set of miRNAs was studied further based on their biological role within the reproductive tract

Differentially expressed miRNAs across reproductive tissues in response to mating, artificial insemination, or infusion of different fractions of seminal plasma were selected based on their biological function, and a specific set of miRNAs with key roles in reproductive processes was further investigated (Fig. 5, Supplementary Table 2). Target genes for the selected set of miRNAs are shown in Table 1. miR-671 was significantly decreased in Cvx, Dist-UT, Prox-UT, Isth, and Inf, while it was up-regulated in Amp in response to natural mating. It was also downregulated in UTJ, Amp and Inf in response to artificial insemination (AI-P1), and only in UTJ when SP from the first 10 mL of the sperm rich fraction was infused (SP-P1). miR-let7A-1 was found downregulated in Dist-UT and Prox-UT when sows were exposed to mating and SP from the entire ejaculate, while up-regulated in Amp in the AI-P1 treatment. miR-34C-1 increases in Dist-UT, UTJ and Amp in response to mating, in Prox-UT in response to artificial insemination (AI-P1), and in Amp after SP infusion (SP-P1). miR-101 is overexpressed in Cvx (SP-E), and Inf (NM and AI-P1), and repressed in Amp (NM). miR-628 was up-regulated in UTJ (NM and SP-P1), Inf (NM and AI-P1), and downregulated in Amp (NM). miR-191, was up-regulated in UTJ (NM and SP-P1), and down-regulated in Dist-UT (SP-E). miR-27B was down-regulated in Dist-UT (SP-P1 and SP-E), Amp (NM), up-regulated in Prox-UT (NM), UTJ (AI-P1), Isth (SP-P1), Amp (AI-P1) and Inf (NM). miR-296 was upregulated by all treatments in UTJ.

3.4. Main biological functions of selected miRNAs target genes

From the broader set of miRNAs reported in this study, we selected 8 miRNAs based on the biological roles of their target genes. These miRNAs hold the potential to regulate the expression of genes with crucial roles in reproductive processes, encompassing inflammatory responses, uterine angiogenesis, vascular remodeling, and various other functions. The top biological terms and pathways associated with these target genes are visually represented in Fig. 6 and Supplementary Fig. 3, respectively.

4. Discussion

miRNAs play pivotal roles in a plethora of different biological and molecular processes, including many aspects of mammalian reproduction. During the pre/peri-ovulatory period in the porcine species, miRNA expression and function across the female genital tract may depend on the presence or absence of semen, as well as either the type of semen deposition (NM vs. AI), or even the fraction of the ejaculate deposited. To test our working hypothesis, we hereby studied the miRNAs differential abundance in the different segments of the female genital tract in response to different treatments: exposure to semen (by NM or AI), or exposure to sperm-free seminal plasma (from the entire ejaculate or from the rich fraction). A total of 181 miRNAs were detected across the entire genital tract in response to the different treatments.

Semen exposure by mating triggered the downregulation of miR671 in all tissues studied (from Cvx to Inf, despite in Amp where it was up-regulated). However, semen exposure by AI only motivated changes in miR-671 expression in the UTJ and the oviduct (Amp and Inf), all down-regulated. miR-671 has been identified as a relevant regulator of the inflammatory response observed in humans with inflammatory skin disorders (Nunes et al., 2018). It has also been associated with suppression of NF-κB activity and inhibition of interleukin-8 and CXCL3 messenger RNA expression in colonic epithelial HCT116 cells during Crohn's disease (Chuang et al., 2014). A recent study in porcine monocyte-derived dendritic cells (Mo-DC) demonstrated that miR-671 was upregulated in Mo-DC stimulated with *Bifidobacterium lactis* BB12 while showing a downregulation on IL-10 profile (Bravo-Parra et al., 2023). Interestingly, the downregulation of miR-671 observed in the present study agrees with previous findings from our group where we addressed an increase of IL-10 in the pre/peri-ovulatory UTJ (Gardela et al., 2022). It is well known that the act of mating induces a local inflammatory response that is tightly regulated and requires a switch in gene expression, leading to a downregulation of inflammatory mediators (Robertson et al., 1997). It is reasonable to assume that AI does not trigger such a strong inflammatory response as the one observed after mating since several infection-triggers as pathogens or sperm debris are eliminated from the equation, thus potentially explaining the smaller dysregulation of miR-671 along the genital tract in this group. One interesting note is the upregulation of Fibroblast Growth Factor Receptor Two (*FGFR2)* gene expression observed in uterine and oviductal tissues after mating in a previous study from our laboratory (Gardela et al., 2022). *FGFR2* is a key target of miR-671 and its repression is implicated in alterations of uterine development and pregnancy loss prior to embryo implantation in mice (Filant and Spencer, 2014).

Another important regulator of key reproductive processes (miR-34c) was found to be overexpressed in Dist-UT, UTJ and Inf after mating. Only in Dist-UT after AI and in Inf after SP-P1 deposition. It has been proposed as an important regulator of the expression of some marker genes of endometrial receptivity (Liu et al., 2020). Achievement of endometrial receptivity, indispensable for embryo implantation (Galán et al., 2000) involves dynamic processes of morphological and physiological changes (Cheng et al., 2009; Heng et al., 2010). Previous studies have shown that miRNAs can induce endometrial cell (EC) apoptosis in mice and goat (Ma et al., 2022; Nie et al., 2017; Yuan et al., 2019). Interestingly, miR-34c was found to inhibit EC proliferation and induce EC apoptosis in dairy goats by downregulating the antiapoptotic gene *BCL2* (Li et al., 2018c; Liu et al., 2020). In addition, EC's treated in vitro with miR-34c mimic or inhibitor showed changes in vascular endothelial growth factor (VEGF), forkhead box M1 (FOXM1), prostaglandin-endoperoxide synthase 2 (PTGS2), and osteoporin (OPN) protein levels, strongly involved in the maternal immune tolerance and uterine receptivity (Liu et al., 2020). The levels of VEGF, FOXM1, and OPN were

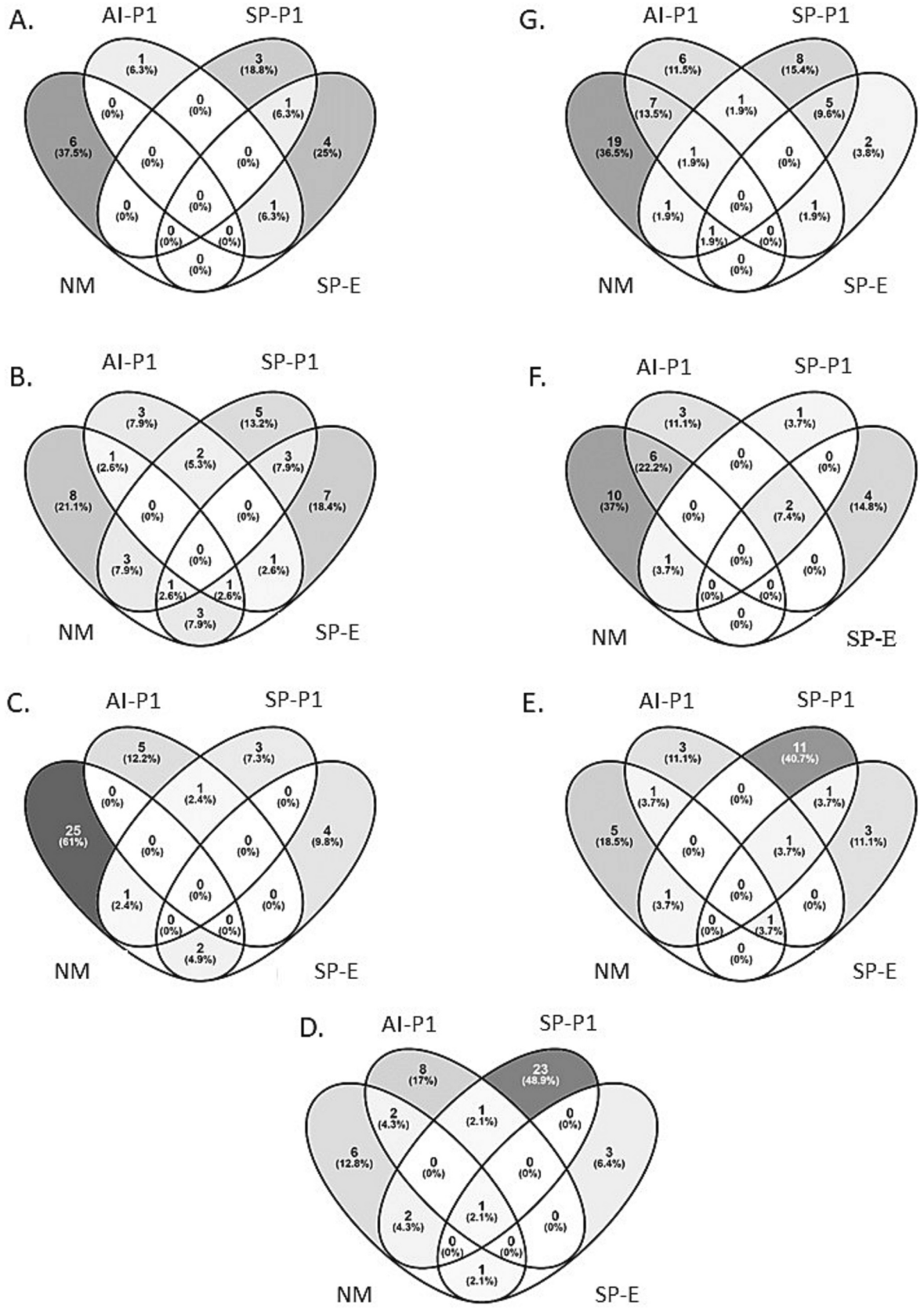

Fig. 3. Venn diagram plot depicting a summary of the differential miRNA transcripts in the reproductive internal genital tract segments (cervix (Cvx; A), distal uterus (Dist-UT; B), proximal uterus (Prox-UT; C), utero-tubal junction (UTJ; D), isthmus (Isth; E), ampulla (Amp; F), and infundibulum (Inf; G)), 24 h after natural mating, insemination with the first 10 mL of the sperm rich fraction (AI-P1), infusion with seminal plasma from the first 10 mL of the sperm rich fraction (SP-P1), and infusion with seminal plasma from the whole ejaculate (SP-E), compared to unmated, Sham, infusion of BTS extender (Control).

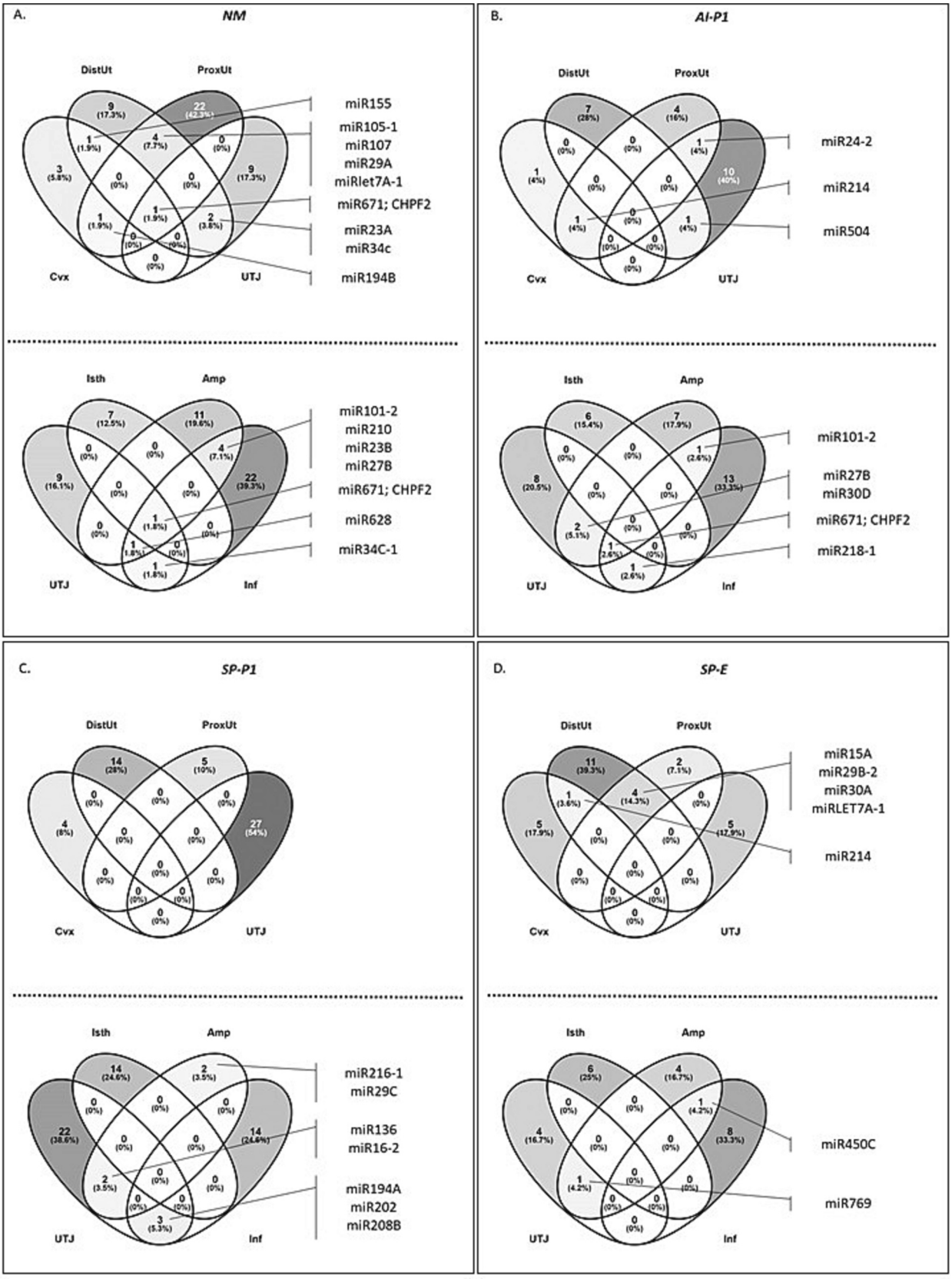

Fig. 4. Venn diagram plot depicting a summary of the differential miRNA transcripts found when comparing the different reproductive internal genital tract segments (cervix (Cvx), distal uterus (DistUt), proximal uterus (ProxUt), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp), and infundibulum (Inf)), 24 h after Natural Mating (NM; A), insemination with the first 10 mL of the sperm rich fraction (AI-P1; B), infusion with seminal plasma from the first 10 mL of the sperm rich fraction (SP-P1; C), or infusion with seminal plasma from the whole ejaculate (SP-E; D) compared to unmated, Sham, infusion of BTS extender (Control).

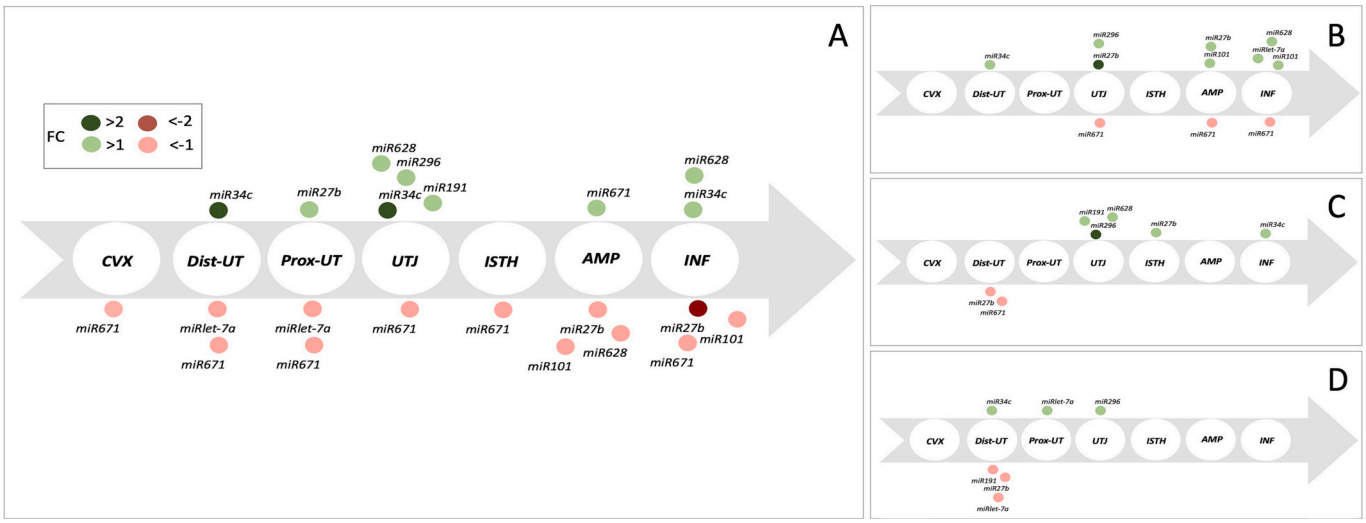

Fig. 5. Schematic representation of selected miRNAs up or down-regulated among the different reproductive internal genital tract segments (cervix (Cvx), distal uterus (Dist-UT), proximal uterus (Prox-UT), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp), and infundibulum (Inf)), 24 h after Natural Mating (NM; A), insemination with the first 10 mL of the sperm rich fraction (AI-P1; B), infusion with seminal plasma from the first 10 mL of the sperm rich fraction (SP-P1; C), or infusion with seminal plasma from the whole ejaculate (SP-E; D), compared to unmated, Sham, infusion of BTS extender (Control). FC: Fold Change.

significantly upregulated by miR-34c mimic, whereas the inhibitor only decreased the OPN protein levels. In addition, the miR-34c mimic decreased the PTGS2 protein levels, whereas the miR-34c inhibitor increased these levels. In the present study, mating seemed to potentiate the strongest signaling of this miRNA across the genital tract suggesting a better preparation of the endometrial environment than that achieved by AI. We know that although AI procedure is effective, i.e. yielding fertility outcomes similar to those achieved by NM (Roca et al., 2016). But, could it be even more efficient and bust economic benefit if we mimicked natural conditions? In this regard, AI bypasses the requirement for seminal fluid as a transport medium for spermatozoa, which is absent or highly extended in buffer solutions (as BTS) and is not required for fertilization. However, an increasing amount of evidence suggests that seminal fluid has a substantial effect on the female reproductive tract and potentially changes the immune function of T-cells, B cells, NK cells and macrophages in several species to allow paternal antigen recognition and embryo tolerance (Bromfield, 2014).

In line with our hypothesis, the results showed miR-let-7a-1 was downregulated in Dist-UT and Prox-UT after mating and SP-E deposition indicating a clear influence of SP on the regulation of this miRNA. Let-7 family has been associated with the immunology of pregnancy. It is down-regulated during preimplantation embryo development in mice (Ma et al., 2010) and in dormant blastocysts at 3 h post oestradiol activation (Liu et al., 2012). Garcia-Lopez et al., highlighted a continuous decrease in the expression of primary and mature miR-let-7a (García-López and del Mazo, 2012) during preimplantation embryo development, indicating that a low level of miR-let-7a is needed during implantation. As the down-regulation of the maternal immune system is required prior to embryo implantation to avoid embryo rejection (Martinez et al., 2020), the downregulation of miR-let-7a 24 h after NM or SP deposition showed in the present study suggests that SP components might have a key role in paternal antigen recognition and sperm survival. miR-let-7a also suppresses angiogenesis via down-regulation of *TGFBR3* expression (Wang et al., 2019), a novel target for miR-let-7a (Yan et al., 2016), leading our results to suggest a positive influence of SP in the process of angiogenesis during the early inflammatory process. This is not surprising, since several studies have demonstrated that TGFβ is the active compound in SP responsible for the increased expression of endometrial inflammatory mediators and ultimately post-insemination inflammation (Ibrahim et al., 2019; Sharkey et al., 2012).

The present study analyzed a particular timeframe, the pre/peri-ovulatory period when spermatozoa have already colonized the female sperm reservoir in the UTJ (Rodríguez-Martínez et al., 2005). Notably, pro-angiogenic miRNA (miR-296) was overexpressed only in UTJ in all treatments tested. This implies that SP itself has the potential to alter the pattern of expression of this miRNA in the sperm reservoir. Fluid production and composition in the UTJ and the oviduct undergo dynamic changes throughout the oestrous cycle associated with increased vascular permeability (Saint-Dizier et al., 2019) and contribute to the establishment of the specific environment necessary for gamete transport, fertilization and embryo development. The target genes of miR-296 are involved in the modulation of angiogenesis, inflammatory response, hypertension, cellular proliferation and apoptosis (Li et al., 2018b; Peng et al., 2018). Upregulation of miR-296 has been linked to the induction of morphologic characteristics associated with angiogenesis of human endothelial cells (Würdinger et al., 2008) by increased expression levels of VEGF and VEGFR2 which are crucial in angiogenesis (Lungu and Mehedinti, 2023). These results suggest that components of SP have the potential to facilitate oviductal environment preparation for gamete transport via miRNA regulation.

5. Conclusions

Here, the major changes in miRNA expression were elicited by NM when compared to AI or SP infusion. This fact suggests that the synergic effect of both sperm and SP from the entire ejaculate (highly extended when performing AI) is crucial for miRNA regulation within the female genital tract, and whether these miRNAs have the potential to impact the offspring's outcomes needs further evaluation. In addition, The UTJ-sperm reservoir exhibited a notable increase in miRNA activity, particularly after mating. This observation raises the possibility that key-regulator miRNAs, including miR-34c or miR-296, could not only relate to sperm survival (sperm metabolism, apoptosis blockade) but also modulate metabolic and cell adhesion processes, preparatory for gamete encounter in the upper oviduct. However, further confirmation of this hypothesis is required.

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Fig. 6. List of most significant Gene Ontology (GO) biological processes (Bp) associated with selected target genes of miRNAs up or down-regulated among the different reproductive internal genital tract segments (cervix, distal uterus, proximal uterus, utero-tubal junction, isthmus, ampulla, and infundibulum, 24 h after Natural Mating, insemination with the first 10 mL of the sperm rich fraction, infusion with seminal plasma from the first 10 mL of the sperm rich fraction, or infusion with seminal plasma from the whole ejaculate, compared to unmated, Sham (infusion of BTS extender) controls. The functional enrichment analysis was performed using g:Profiler (version e109_eg56_p17_1d3191d) with g:SCS multiple testing correction method applying a significance threshold of 0.01 (Raudvere et al., 2019).

Table 1
List of target genes for the selected differentially expressed miRNAs.

miRNA	N	Predicted Targets Name
miRlet7A	377	*ARID4B; RICTOR; PLAG1; TUT4; CCDC126; PUM2; PPM1A; CASP8AP2; DCUN1D4; HMGXB4; SOX9; EEA1; PTCH1; NR3C1; TAOK1; ITGA6; ANK3; WAC; DCAF7; USP25; NEUROG2; LRP6; DOCK1; IER5; NR2F2; PNISR; HECTD2; FRS2; MAST4; NFYB; SMARCA5; PPP4R2; CEMIP2; NUDT4; PPM1L; TRIM63; QKI; PIAS1; DGKH; YY1; NBEA; MON2; CNOT6L; TVP23C; APOOL; ICK; NLGN1; KIAA1217; PIK3R4; PFKFB3; PSD3; RAB11FIP2; NCKAP1; EMP2; BTAF1; PCDH8; CCNA2; COLCA2; F3; CEP76; BDNF; ACTR3; APP; BST1; INO80D; DOP1A; TVP23B; AKAP9; AKTIP; ARHGAP44; LYSMD3; GTF3C3; FRMD4B; PTPRE; TMEM132B; ATAD5; RAB10; SLAIN2; NKTR; FAM3C; HOOK3; ITM2B; FOXO1; ZNF800; POLR2K; DCLRE1B; RBM12B; MARCH6; OPA1; HEATR5B; ZFYVE16; BTBD3; GDAP2; MMS22L; CAMTA1; ARHGAP20; WNT5B; TSHZ3; AAK1; PDE10A; FUT9; NR5A2; ACVR2B; TCF3; AMMECR1; KDM6A; FBXO34; PIK3C2A; ZMYM4; RABGGTB; NASP; GPR37; EGFL6; CDK19; SH3GL3; SDC2; RC3H1; KIAA0408; RPRD1A; DAAM1; SNAPC1; EDIL3; EPHB4; B3GNT2; CLIP1; ARIH1; TOB1; DLL4; PTBP3; TLE1; MNX1; DCAF6; KLF3; LATS1; TAF4B; LEMD3; DACT1; NUFIP2; IRX3; GLS; PPP4R3B; PRH1-TAS2R14; WASF3; ARAP2; RALGDS; C11orf54; ABI1; OLFML2B; RIMKLB; GNAQ; PDLIM5; SCAMP1; SFPQ; CRISPLD1; MARK1; SEPT9; ZNF367; GABRG1; HES1; SPOCK3; NDC1; COL4A1; PSD2; ITCH; ZNF506; FOXN2; HMGCR; LRP1B; PPP3CA; MINDY3; DLX2; TRPM7; FNDC3B; PAXBP1; REV3L; CPEB3; ZNF827; SLC4A5; UBE3C; UNC119B; CORO1C; DKK3; LIPG; SRSF; CTU2; RAD21; KLF4; FZD6; TLE4; SLC18A2; CARF; BRD1; CIAO2A; SORBS1; SEPHS1; GRHL3; CNOT6; SNCAIP; USP12; NAA16; BMPER; MEX3B; DCLK3; SCAI; PANK3; RNF24; SGTB; MED6; PLCL2; C1QTNF7; ZMYM6; ERF; PUM1; UBTF; ACTL6A; PI15; TEAD1; KLHL2; GRM5; RHOBTB3; TBC1D2B; CEPT1; CACNA1C; MBLAC2; TET2; SLC35A3; HOXA9; C1QBP; BCL6; HECTD1; ZNF292; BRF2; DERL1; VMA21; BTF3L4; NAA25; U2SURP; TMTC2; AHR; IGFBP1; GTF2A1; BHLHE40; ULBP1; ANO4; TCERG1L; DOCK11; CREBZF; SRSF1; SUN1; CFTR; PRPF8; HNRNPD; ARFGEF2; IKZF2; EBF3; CD38; LILRA1; DSG2; DCC; GSK3B; MED14; SLC6A17; HIP1; CTNNB1; MLLT10; B3GLCT; ZFYVE21; NRP2; NRP1; SPRED1; GIGYF2; SLC34A2; NPY; EIF4ENIF1; CCNE2; NGDN; AP1S3; ZMIZ1; AKAP12; E2F8; PAN3; KIN, SDHAF3, CRY1; LIN9; UBE2B; ANKRD44; GATA3; PPP1R3F; CNNM4; VEZF1; FREM2; FYTTD1; DPM1; ZBTB39; FGF7; OXR1; SP8; CLASP2; SGO1; AKAP6; CDH20; SRSF11; PM1D; ST5; ATG2B; ZBTB14; CHD3; ZMYND8; SEC22C; F2RL1;HNRNPA1; SPEN; DUSP6; UTRN; IREB2; FGFR2; TSLP; KIF3A; COL11A1; FAM117B; NADK2; SCARF1; FSTL5; PAK2; MALT1; KDM2B; GOLIM4; TTF2; SRBD1; OTUD4; SEMA3C; BAG2; ZNF236; CLDN12; RTN1; SATB1; CLDN16; TGFB3; RAB6D; NFAT5; ICE1; DLL1; CTR9; JAG1; BMPR2; FBLN5; SCX; ZDHHC20; USP32; AASDH; ZFY; SPOPL; E2F5; ZNF569; SLC6A4; VAPA; FAM76B; SYNGR3; GNB4; GLCCI1; LRRC32; PCSK2; RNF216; CNTN1; RPS16; DACH1; SYF2; KDM7A; CCNY; SPRY2; RHOT1; ROCK1; NAA20; LIN54; FNBP4; FSHB*
miR27B	306	*AFF4; GXYLT1; ARFGEF1; GCC2; DCUN1D4; PLK2; TNPO1; TRPV3; GAB1; GRIA4; BEX3; MBTD1; PDS5B; AKIRIN1; RPS6KA5; RGPD6; PHLPP2; TAB3; SOS1; FBXW7; CDS1; RGPD5; SEMA6A; NRK; USP42; ABHD17C; GPAM; ARHGEF26; RGPD4; KCNK2; EYA1; RGPD8, ZBTB34; KIAA1109; ST6GALNAC3; EYA4; PLEKHJ1; SBF2; PRR3; ONECUT2; TMBIM6; FOXA3; TG2; ADAMTSL3; RUNX1; ACVR1C; TMEM170B; TMCC1; EHF; GRIN2D; USP46; C2CD2; WNK3; MMD; HOXA5; SLC7A11; RNF139; GATC; DNAJC27; KDM7A; PNKD; KCTD8; SLITRK1; PDIA5; ABCA1; EDRF1; KLF3; SMAD9; TFAP2B; HIPK2; UNKL; PLEKHH1; PLCL2; ROR1; SEMA7A; CKAP4; VAV2; CDR2; FBXO10; LIFR; ABHD6; ZFHX3; ADORA2B; GOLM1; EPS8; CCNK; UGCG; IPMK; HIVEP3; PEG10; SZRD1; SLC35F1; ZHX1; GNS; MARK1; ZNF800; RREB1; PDE7B; C20orf194; CREBRF; DNAJC13; TSC22D2; GSPT1; B4GALT3; CCNG1; NR2F6; ITSN2; CSRP2; NECAP1; ID4; RO60; RALGAPA2; MAP1B; GRM5; ST3GAL6; ASPH; BRPF3; SSH1; STYK1; KDM3A; COG7; EDEM3; SLC25A25; SNRNP27; DOT1L; KIAA1211L; BICC1; INSM2; ZCCHC24; GALNT7; B3GNT7; APPBP2; PPARG; UBE2V1; RMND5A; SHE; NIPAL4; ARF3; CTH; KCNA4; ENDOU; PCNX1; TNRC18; INO80D; PTGER3; KITLG; USP25; ZSCAN26; NPAS3; SLC39A11; GRIA3; PCDH9; AGFG1; VSIG10; MIER3; C1orf52; ADAMTS10; SLC6A1; HMGCR; PALM2; GAREM1; HBEGF; REPS1; EPB41L4A; SFRP1; CREB1; TPR; ELFN2; FAM184A; ATAD2B; STAB2; MTURN; PAQR9; DIPK1A; MKNK2; FBXO34; PARD6B; ARHGAP32; STAG1; NABP1; RPS6KB1; TRIM23; RCAN2; ACTA2; HOXA10; MATN3; TEAD1; RAPH1; NPEPPS; LPAR6; CLCN3; PANK1; RNF141; TLK2; YWHAQ; LPCAT1; MAP2K4; ING5; AK4; NEMP2; CACNG2; NR5A2; CEP135; DTNA; BRWD3; SNAP25; MYT1; LPIN1; E2F7; PPP4R2; PDE10A; PLPP; HAPLN1; CLK2; TNRC6B; SOX11; CA10; SCAF11; SRGAP2; MARC1; MED12L; LONRF1; DYNC2LI1; MTMR4; KMT2C; GOLGA1; CIPC; FAM133B; IKZF2; NGFR; RGS8; SNX10; PIK3CA; PSEN1; OAF; GALNT3; PLCH1; NDUFS4; SORL1; MOCS3; HIC1; GPD2; PKIA; SUCO; SOGA1; SMOC2; ARL4C; TXLNG; EGFR; FRS3; TMEM189-UBE2V1; NXT2; STAC; SLC22A23; LCOR; NPTN; RELN; KLHL29; COLEC10; PTGDR; ANK1; PAX9; ZMYM4; SETD5; CCM2; COMMD3-BMI1; ATP2B1; USF3; OTULIN; FZD4; HSD17B12; HOXC6; CAPN15; NEURL4; TRIM50; GATA6; RARA; FRMD6; ZNF329; SIX1; AQP11; PSPC1; NEK6; PDPK1; COLGALT2; SLC9B1; PLXND1; PATZ1; PLPPR1; MED14; RSBN1L; ZNF268; RSPO3 ;NRBF2; LITAF; TRAPPC8; RPGRIP1L; ZDHHC17; NCALD; PF4V1; EPB41; KPNB1; TMEM9B; MKLN1; TCIM; CDH11*
miR34-C	103	*HCN3; FAM76A;MDM4; DLL1; FKBP1B; SYT1; E2F5; PPP1R11; RAP1GDS1; FAM167A; SDK2; SATB2; SCN2B; MYCN; NECTIN1; CELF3; MGAT4A; LGR4; NAV3; NAV1; MET; FLOT2; XYLT1; AHCYL2; TGIF2; PACS1; PKP4; CACNA1E; MLLT3; FUT9; RRAS; PITPNC1; MPP2M; VAMP2; ABR; SLC25A27; FOXP1; CAMTA1; SRPRA; MEX3C; JAKMIP1; ELMOD1; TOB2; FUT8; LEF1; SHANK3; NPNT; KIAA1217; GPR22; DAAM1; ASIC2, GALNT7; NUMBL; TBL1XR1; BMP3; GABRA3; TNRC18; UBP1; PPARGC1B; CUEDC1; ZMYM4; ARID4B; FAM117B; ATMIN; CYREN; HNF4A; SFT2D1; CDK6; NRN1; EML5i; SAR1A; TMEM255A; FOXN2; TASOR; TPPP; FGD6; PDE7B; ADO; ANK3; UNC13C; LMAN1; CTNND2; POGZ; KDM5D; SNAI1; AKIP1; SLC4A7; CBX3; TAF4B; FGF23; RPS6KL1; SERPINF2; STRN3; DNM1L; UCN2; PPP2R3A; AMER1; FOXJ2; PPFIA1; ADIPOR2; GPR158; SMIM15; CREB3L2*
miR101-2	1	*SARD3*
miR191	5	*NEURL4; TAF5; CREBBP; CASTOR2; TMOD2*
miR296	9	*ZNF99; ZNF117; AZI2; ZFP90; ITM2A; ZNF138; ABLIM3; C1QTNF7; CADPS*
miR628	32	*RBM41; SINHCAF; MAMDC2; PAIP1; EPHA7; BEND4; ATRX; LSM12; SLC40A1; ABCA13; ZNF197; SPAG1; DDX5; RICTOR; MED23; SEPT7; PPP3CB; SGCD; HSPBAP1; PTTG1IP; VAMP2; DGKI; ALKAL1; RCN1; DERA; ZNF606; ZFP82; KLB; AIDA; ITCH; ALG9; LIN54*
miR671	45	*USP46; SATB2; AAK1; CAMKK2; C16orf72; TBL2; ST8SIA5; CA7; VSNL1; KRT38; C12orf77; CFL2; C10orf25; EDN1; SSBP1; ACTR2; GID4; CBS; KRT9; KIT; KCNMA1; SLC30A6; SGK2; RAG1; MAP3K19; MARK1; FGFR2; ZNF805; JADE1; GALNT10; LIN9; FOXP2; SPTBN1; FADS1; SPOCK1; DVL3; ADCY1; MAML2; LOC102724951; ACBD6; LOC102723360; LOC102724219; SPPL3; LOC102724843; LGALS3BP*

* Only target genes with a score >90 (miRbase) were selected.

** Genes highlighted in red were selected based on the importance of their biological role.

) with g:SCS multiple testing correction method applying a significance threshold of 0.01 (Raudvere et al., 2019).

Author contributions

All authors provided contributions to study conception and design, acquisition of data or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published.

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Manuel Álvarez-Rodríguez: Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Conceptualization, Data curation. **Cristina A. Martinez-Serrano:** Software, Writing – original draft, Investigation. **Jaume Gardela:** Formal analysis, Investigation, Methodology, Software, Writing – review & editing. **Helena Nieto:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Eduardo de Mercado:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Heriberto Rodríguez-Martínez:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

The data presented in the study are deposited in the Harvard Dataverse public repository, accession number DVN/FJM7F4_2023 (link: https://doi.org/10.7910/DVN/FJM7F4).

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