

Research Article

MicroRNAs in amniotic fluid and maternal blood plasma associated with sex determination and early gonad differentiation in cattle[†]

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[†]**Grant Support.** Supported by Science Foundation Ireland (13/IA/1983) and grant RTI2018–093548-BI00 from the Spanish Ministry of Science and Innovation. IG-R was supported by a predoctoral fellowship from the Spanish Ministry of Science and Innovation (BES-2016-077794), and BP was supported by a Marie Skłodowska-Curie ITN European Joint Doctorate in Biology and Technology of Reproductive Health (REP-BIOTECH 675526).

Conference Presentation: Presented in part at the 46th Annual Conference of the International Embryo Technology Society (IETS) held in New York City, USA, 16–19 January, 2020.

Received 8 February 2021; Revised 22 March 2021; Accepted 12 April 2021

Abstract

We hypothesized that sexually dimorphic differences exist in the expression of miRNAs in amniotic fluid (AF) and maternal blood plasma (MP) in association with the process of sex determination and gonad differentiation in cattle. Amniotic fluid and MP were collected from six pregnant heifers (three carrying a single male and three a single female embryo) following slaughter on Day 39 postinsemination, coinciding with the peak of *SRY* expression. Samples (six AF and six MP) were profiled using an miRNA Serum/Plasma Focus PCR Panel. Differentially expressed (DE) miRNAs were identified in AF ($n = 5$) and associated MP ($n = 56$) of male vs. female embryos ($P < 0.05$). Functional analysis showed that inflammatory and immune response were among the 13 biological processes enriched by miRNAs DE in MP in the male group ($FDR < 0.05$), suggesting that these sex-dependent DE miRNAs may be implicated in modulating the receptivity of the dam to a male embryo. Further, we compared the downstream targets of the sex-dependent DE miRNAs detected in MP with genes previously identified as DE in male vs. female genital ridges. The analyses revealed potential targets that might be important during this developmental stage such as *SHROOM2*, *DDX3Y*, *SOX9*, *SRY*, *PPP1CB*, *JARID2*, *USP9X*, *KDM6A*, and *EIF2S3*. Results from this study highlight novel aspects of sex determination and embryo–maternal communication in cattle such as the potential role of miRNAs in gonad development as well as in the modulation of the receptivity of the dam to a male embryo.

Summary sentence

Sex-dependent differentially expressed miRNAs identified in amniotic fluid and maternal plasma on Day 39 of pregnancy are associated with gonad development as well as the modulation of the receptivity of the dam to a male embryo.

Key words: circulating miRNAs, amnion, pregnancy, cattle.

Introduction

Gonad differentiation is a critical step in the sexual development of vertebrates [1]. In eutherian mammals, sex determination is the process through which a bipotential gonad (the somatic precursor of which is known as the genital ridge) develops into a testis or an ovary depending on the sex chromosome content of the embryo, specifically by the presence of the *SRY/Sry* gene (sex-determining region of the Y-chromosome; recently reviewed by Capel [2]; Zhao and Yao [3]). An intricate cascade of gene expression and interactions between signaling pathways takes place in the genital ridges [4–7], the complexity of which suggests that additional layers of gene regulation must act downstream to mediate this process [8, 9].

MicroRNAs (miRNAs) are short non-coding RNAs that post-transcriptionally regulate gene expression [10] and are involved in diverse functional roles including development, differentiation, apoptosis, and immunity [11, 12]. MicroRNAs are present in practically all body fluids, including blood, saliva, urine, amniotic fluid (AF), and milk [13–15]. Interest in using miRNAs as potential biomarkers of disease and other physiological states has increased considerably in various research fields, associated with the fact that circulating miRNAs can be assayed in a non-invasive, relatively easy, manner and are potentially predictive, specific, sensitive, and robust [16, 17]. For example, placenta-specific miRNAs are detectable in the maternal circulation during pregnancy in women [18, 19], mares [20], and cows [21–23]. Furthermore, we have recently reported that heifers with divergent responses to ovarian superstimulation exhibit differential plasma extracellular vesicle miRNA profiles [24].

The crucial germ line decision to commit to either a male or a female fate has been partially explained by genetic and *ex vivo* studies in mice that have implicated a complex network of regulatory genes, numerous factors, and pathways [4, 25]. However, there is a paucity of information relating to large mammalian species such as cattle, despite the similarity in bovine sex determining gene expression patterns and gonadal histology to that of humans [5, 7]. The study of gonadal development in the bovine model may, therefore, provide insights into human sex determination and gonadogenesis that could be pivotal for understanding the etiology of human developmental diseases affecting the gonads.

Sexual dimorphism in terms of embryo metabolism has been demonstrated very early in development by the differential uptake of amino acids between male and female bovine embryos *in vitro* [26]. In addition, alteration of glucose availability in culture medium induces a skew in the sex ratio of bovine blastocysts [27, 28]. Furthermore, secreted miRNAs have been detected in embryo culture media *in vitro* as early as the blastocyst stage [29], in a sexually dimorphic manner, and induce a transcriptomic response when applied to endometrial epithelial cells *in vitro* [30]. In contrast to culture *in vitro*, *in vivo*, the embryo/fetus is surrounded by the amniotic fluid (AF), which is formed at the very early stages of pregnancy and is present throughout the remainder of gestation [31]. The composition of AF is a dynamic indicator of embryonic/fetal metabolism and most of the proteins or peptides identified in AF have activities associated with cellular growth and proliferation (reviewed by Tong [32]). However, to the best of our knowledge, its role during gonad development has not been studied in cattle.

The genital ridges first appear in the bovine embryo at Day 32, *SRY* expression begins at Day 37, peaking at Day 39, and the

testis cords are distinguishable by Day 42 [5]. We hypothesized that divergent miRNA profiles would be detectable in AF surrounding male and female embryos and in the maternal blood plasma (MP) of pregnant heifers around this critical time in development. The objectives of this study were (i) to characterize the miRNA profile in AF and MP of heifers carrying a single male or female embryo on Day 39 (corresponding to the peak of *SRY* expression in cattle); (ii) to characterize sex-dependent differential expression (DE) of miRNAs in both fluids and to identify potential target genes of DE miRNAs, with a focus on immune-related target genes in the MP; and (iii) to compare these putative target genes with our previous data on the transcriptome landscape of male and female bovine embryo gonads collected on Days 35, 39, and 43 (to capture all of the early molecular events related with sex determination in cattle) [7]. To our knowledge, this is the first study investigating AF and MP miRNA profiles during sex determination and early gonad differentiation in cattle and provides novel data that further our understanding of the regulatory networks underpinning these critical processes. In addition, our study will be of interest to researchers studying the mechanisms underlying fetomaternal immunity and pregnancy outcome associated with fetal sex. Finally, applied benefits potentially include new opportunities to identify biomarkers for early prediction of embryo/fetal sex in commercial practice to aid in management decisions at farm level.

Material and methods

All experimental procedures involving animals were sanctioned by the Animal Research Ethics Committee of University College Dublin and were licensed by the Health Products Regulatory Authority, Ireland, in accordance with Statutory Instrument No. 543 of 2012 under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes.

Experimental animals and procedures

The experimental procedures are illustrated in Figure 1. This study builds on our previous work describing gene expression profiles of bovine genital ridges during sex determination and early differentiation of the gonads in cattle [7]. In that study, Charolais- and Limousin-cross heifers (26–34 months old and 606.5 ± 10.7 kg; mean \pm SEM) were synchronized using an 8-day intravaginal P4 device (PRID, 1.55 g P4; Ceva Santé Animale, Libourne, France). On the day of PRID insertion, each heifer received a 2 ml intramuscular injection of an analogue of gonadotrophin-releasing hormone (GnRH; Ovarelin, Ceva Santé Animale, equivalent to 100 μ g gonadorelin). One the day before PRID removal, all heifers received a 5 ml intramuscular injection of an analogue of prostaglandin F2 alpha (PGF2 α ; Enzaprost, Ceva Santé Animale, equivalent to 25 mg dinoprost) to induce luteolysis. Only those heifers observed in standing estrus (Day 0) were artificially inseminated with frozen-thawed semen from the same bull 12 and 24 h after estrus onset.

All inseminated animals were ultrasound scanned using a portable ultrasound machine (Easi-Scan; BCF Technology Ltd, Bellshill, Scotland, UK) fitted with a 4.5–8.5-MHz linear array transducer on Day 28 to assess pregnancy status. Pregnant heifers were slaughtered in a commercial abattoir on Day 39, corresponding to the peak of *SRY* expression in cattle. The reproductive tracts were

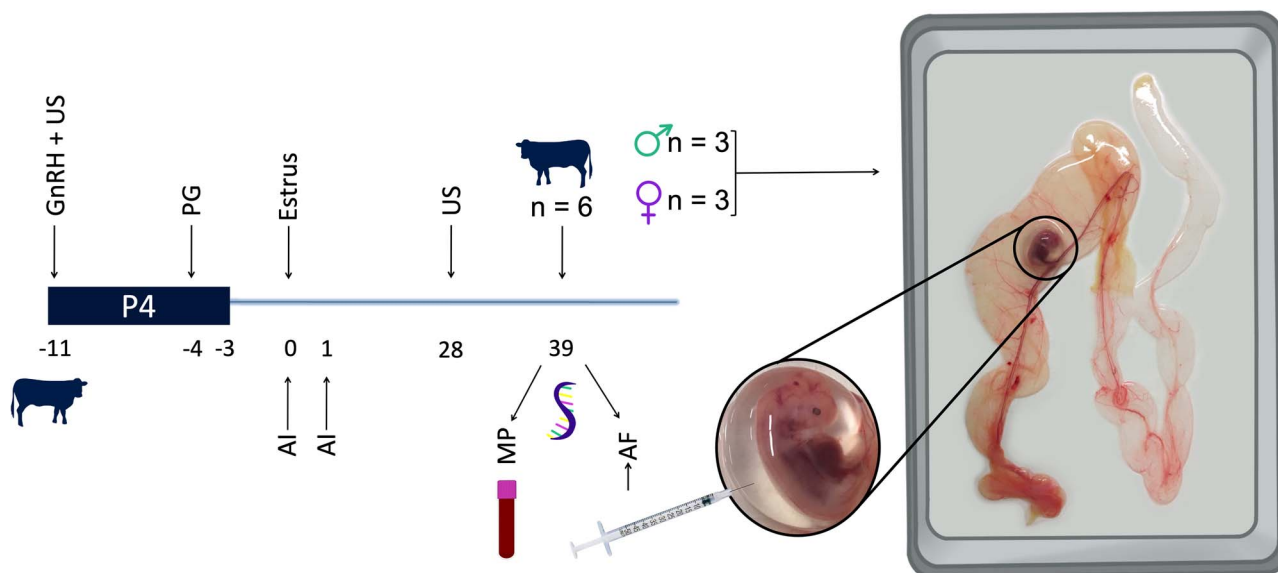


Figure 1. Experimental design. Amniotic fluid (AF) and maternal blood plasma (MP) were collected from each of six pregnant heifers (three carrying a single male embryo and three a single female embryo) on Day 39 (corresponding to the peak of *SRY* expression in cattle). Embryos and extraembryonic membranes were removed from the uterine horns and placed on a dissection tray prior to amniotic sac isolation and AF aspiration. US = ultrasound scanning; P4 = progesterone releasing device; GnRH = gonadotrophin-releasing hormone; PG = prostaglandin F_{2α}; AI = artificial insemination. Day 0 was considered the day of the estrus onset.

recovered, brought back to the laboratory within 2 h, and gently dissected in order to completely isolate both uterine horns. The uterine horns were opened and the embryo and extraembryonic membranes were removed (Figure 1). For the current study, we collected AF and MP from six heifers (three carrying a single male and three a single female embryo), which were among those processed for genital ridge gene expression analysis in our previous study [7]. Only clear AF samples (i.e., not contaminated with blood) were used.

Amniotic fluid and maternal plasma blood sample collection

Maternal plasma was collected into non-heparinized tubes (EDTA BD Vacutainer, Vaud, Switzerland) on the day of slaughter, maintained on ice, and spun immediately after collection (at 4°C) for 10 min at 1900 g plus a second spin at 16 000 g for 10 min before freezing at -20°C in plastic pour off tubes. For AF collection, a 30 G needle connected to a 1 ml syringe was used to pierce the amnion and aspirate the fluid (Figure 1). The AF was placed into RNase/DNase-free tubes (Thermo Fisher Scientific, Waltham, USA), centrifuged at 16 000 g for 10 min at 4°C, and the supernatant placed into new RNase/DNase-free tubes, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

Sex determination

Genomic DNA was extracted from tail tips and the sex of the embryos was determined by PCR with primers for the bovine amelogenin gene located on the sex chromosomes as described by Planells et al. [7].

RNA isolation from plasma and amniotic fluid

A total of six AF and six MP samples (from three heifers carrying a single male and three a single female embryo) were used. RNA was isolated from both biological fluids using the miRNeasy

Serum/Plasma Kit (Qiagen, Manchester, UK) with some minor modifications. Frozen aliquots of biofluids were thawed gently on ice and spun briefly at 5000g for 5 min to remove cryoprecipitates; 200 µl of the fluid was combined with 1000 µl of Qiazol, vortexed, and incubated at room temperature for 5 min. After 5 min, 1.0 µl of MS2 carrier RNA (0.625 ng/µl) and 1.0 µl of spike-in controls (UniSp 2,4, and 5 prepared as per manufacturer's instructions) were added to each sample. All remaining steps were performed exactly as per the manufacturer's protocol and the miRNA was eluted in a final volume of 30 µl.

cDNA synthesis and qPCR

cDNA was prepared using the miRCURY LNA RT Kit (Qiagen) as per the manufacturer's instructions. Total reaction volume was 20 µl, using 4 µl of RNA per reaction, and 1 µl of cel-miR-39-3p (0.002 fmol/µl) and 1 µl of UniSp6 included as positive controls. MicroRNA expression analysis was performed using the miRCURY LNA miRNA Serum/Plasma Focus PCR Panels (Qiagen). The panel consists of two 96-well plates and contains 179 pre-validated assays for miRNAs commonly present in human serum and plasma, as well as 7 potential reference genes in addition to assays for the spike in controls and cel-miR-39-3p. qPCR was performed using the ABI7500 FAST system as per the manufacturer's instructions. Briefly, 20 µl of cDNA was combined with 2000 µl of miRCURY LNA SYBR Green Master Mix (Qiagen) and 10 µl was aliquoted per well across the two plates. The plates were sealed, spun briefly, and run consecutively on the same instrument.

Data analysis

Data were analyzed using the Qiagen Data Analysis Center, setting the female plasma samples as control, as the panel we used specifically targets relevant serum/plasma miRNAs. A cutoff of 35 threshold cycle value (Ct) was established, and an miRNA was considered as expressed when it was present in at least 75% of

the samples. Normalization was performed using the “Normfinder” Qiagen’s normalization tool, which evaluates the adequate reference miRNAs for a given set, and also considers the grouping of the samples to perform the normalization to compensate any possible biases. Principal component analysis (PCA) was carried out to determine how the samples were grouped.

Differential expression analysis of miRNAs was performed for both MP and AF, comparing the samples from heifers carrying a single male embryo with those carrying a single female embryo for each biological fluid. miRNA family information was retrieved from the miRbase database [33]. Then, the corresponding putative target genes were extracted. Finally, these putative target genes were compared with our previous data on the transcriptome of male and female bovine embryo gonads collected on Days 35, 39, and 43 to capture all of the early molecular events related to sex determination in cattle [7]. PCA and volcano plots were generated using R [34]. Potential miRNA-gene interactions were determined by matching differentially expressed miRNAs with TarBase v.8 [35]. In order to reduce the noise in our analysis, we kept only those interactions occurring with genes that are expressed in the blood (information obtained from Tissues 2.0 database [36]).

Gene Ontology (GO), InterPro domains, and pathway enrichment analyses were carried out for those genes targeted by at least five upregulated miRNAs in each case, to improve the accuracy of our analysis. DAVID software v6.8 was used for this analysis [37, 38], considering a term as significantly enriched when their adjusted *P*-value (Benjamini correction) was ≤ 0.05 . Enrichment of target genes in sex determination was performed using Fisher’s exact test. In-house python scripts were used to check 1000 iterations over all annotated genes, selecting 14 046 random genes each iteration and calculating the mean percentage of these genes overlapping genes that are involved in sex determination [7].

Results

Global miRNA profile in maternal blood plasma and amniotic fluid

Of the 179 miRNAs investigated, 168 and 163 miRNAs were detected in MP and AF, respectively (Figure 2A and Supplemental Table S1); 161 miRNAs were commonly detected in both fluids. Nonetheless, PCA showed a clear separation of their expression patterns in both fluids (Figure 2B). Only one (miR-485-3p) and four miRNAs (let-7b-3p, miR-195-5p, miR-497-5p, miR-29b-3p) were detected exclusively in AF and MP, respectively, (Supplemental Table S1).

Sex-specific differentially expressed miRNAs in amniotic fluid

Expression analysis of the detected miRNAs in AF revealed that two miRNAs (miR-324-5p [Log Fold Change, LogFC, 1.78; $P < 0.01$] and miR-150-5p [LogFC 1.81; $P < 0.05$]) were upregulated in heifers carrying male embryos (Figure 3A). In contrast, three miRNAs (miR-362-3p, miR-151a-5p, and miR-320d [Log FC 1.45, 1.41, and 1.28, respectively; $P < 0.05$]) were upregulated in heifers carrying female embryos (Figure 3A).

Sex-specific differentially expressed miRNAs in maternal blood plasma

The evaluation of miRNA expression in MP of pregnant heifers on Day 39 revealed that 53 miRNAs were upregulated in heifers

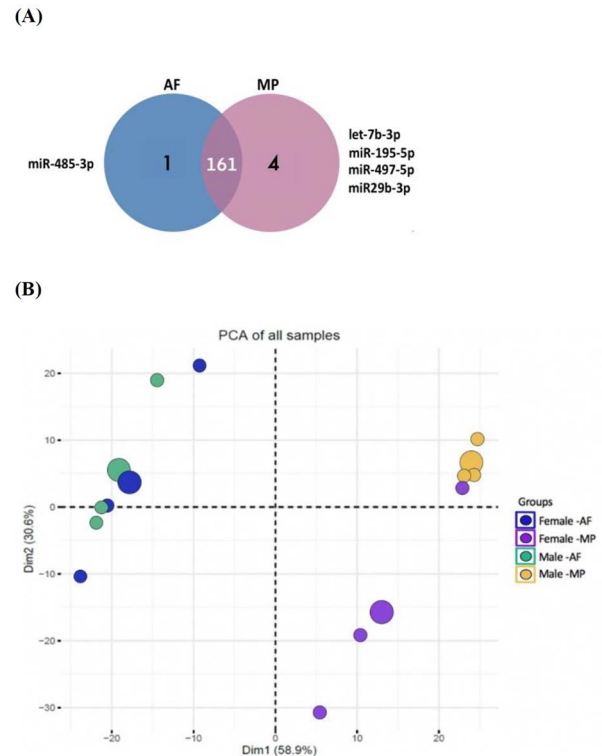


Figure 2. (A) Venn diagram showing the number of miRNAs detected in amniotic fluid (AF) and maternal blood plasma (MP). (B) Principal component analysis (PCA) illustrating miRNA expression in AF (represented in blue and green) and MP (purple and yellow) on Day 39 of pregnancy (corresponding to the peak of *SRY* expression in cattle) in heifers carrying a single male embryo (green and yellow) or a single female embryo (blue and purple). The largest circle within the same color group represents the mean expression of that particular group.

carrying a single male embryo (Table 1). Among the upregulated miRNAs, miR-877-5p, miR-2110, and miR-1260a showed the highest FC (LogFC ≥ 4 ; $P < 0.02$; Table 1). On the other hand, only three miRNAs (miR-148b-3p [LogFC 2.91; $P \leq 0.01$], miR-93-5p [LogFC 2.02; $P < 0.02$], and miR-106b-5p [LogFC 1.96; $P < 0.04$]) were upregulated in MP of heifers carrying a single female embryo (Figure 3B).

Sex-specific DE miRNAs in MP of heifers carrying a single male embryo were subjected to miRNA family analysis (Table 2). An miRNA family is a group of miRNAs that share a similar ancestry or show evolutionary conservation in their sequences, therefore potentially sharing biological functions [39].

Main targets of differentially expressed miRNAs in amniotic fluid and in silico-based functional analysis

To understand the functions of these sex-specific DE miRNAs in AF, we first identified their putative target genes. Accordingly, 528 and 1437 genes were predicted to be targeted by the upregulated miRNAs in AF of male and female embryos, respectively (Supplemental Table S2). Among the 528 genes, Prickle Planar Cell Polarity Protein 4 (*PRICKLE4*), GRB10 Interacting GYF Protein 2 (*GIGYF2*), SET Domain Containing 5 (*SETD5*), TGFB Induced Factor Homeobox 1 (*TGIF1*), and Trinucleotide Repeat Containing Adaptor 6A (*TNRC6A*) were among the predicted genes targeted by both upregulated miRNAs in the AF of male embryos

Table 1. Upregulated miRNAs on Day 39 of pregnancy (corresponding to the peak of *SRY* expression in cattle) in maternal blood plasma of heifers carrying a single male embryo compared with plasma from heifers carrying a single female embryo.

miRNA Name	Fold regulation	P-value	miRNA name	Fold regulation	P-value
miR-877-5p	4.50	0.0088	miR-136-5p	2.67	0.0340
miR-2110	4.36	0.0176	miR-205-5p	2.67	0.0297
miR-1260a	4.00	0.0121	miR-335-3p	2.66	0.0342
miR-485-3p	3.94	0.0304	miR-378a-3p	2.65	0.0088
miR-146a-5p	3.94	0.0304	miR-320a	2.62	0.0349
miR-590-5p	3.94	0.0304	miR-16-2-3p	2.60	0.0337
miR-339-3p	3.94	0.0304	miR-24-3p	2.57	0.0100
miR-154-5p	3.94	0.0304	miR-874-3p	2.50	0.0100
miR-20b-5p	3.94	0.0304	let-7b-3p	2.48	0.0256
miR-208a-3p	3.94	0.0304	miR-151a-5p	2.44	0.0391
miR-629-5p	3.94	0.0304	miR-376c-3p	2.40	0.0077
miR-501-3p	3.94	0.0304	miR-375	2.34	0.0189
miR-766-3p	3.94	0.0304	miR-425-3p	2.33	0.0177
miR-584-5p	3.94	0.0304	miR-130a-3p	2.29	0.0027
miR-483-5p	3.94	0.0304	let-7a-5p	2.28	0.0453
miR-32-5p	3.83	0.0304	miR-22-3p	2.13	0.0267
miR-454-3p	3.61	0.0317	miR-29a-3p	1.94	0.0164
miR-127-3p	3.53	0.0304	miR-223-5p	1.94	0.0416
miR-363-3p	3.53	0.0163	miR-28-3p	1.93	0.0091
miR-382-5p	3.41	0.0169	miR-29c-3p	1.87	0.0288
miR-144-5p	3.25	0.0306	miR-193a-5p	1.80	0.0139
miR-320d	3.09	0.0104	miR-23a-3p	1.77	0.0013
miR-320c	3.05	0.0139	miR-361-5p	1.66	0.0289
miR-93-3p	2.97	0.0452	miR-326	1.64	0.0073
miR-320b	2.84	0.0165	miR-27a-3p	1.52	0.0211
miR-502-3p	2.80	0.0062	miR-23b-3p	1.47	0.0101
miR-22-5p	2.76	0.0102			

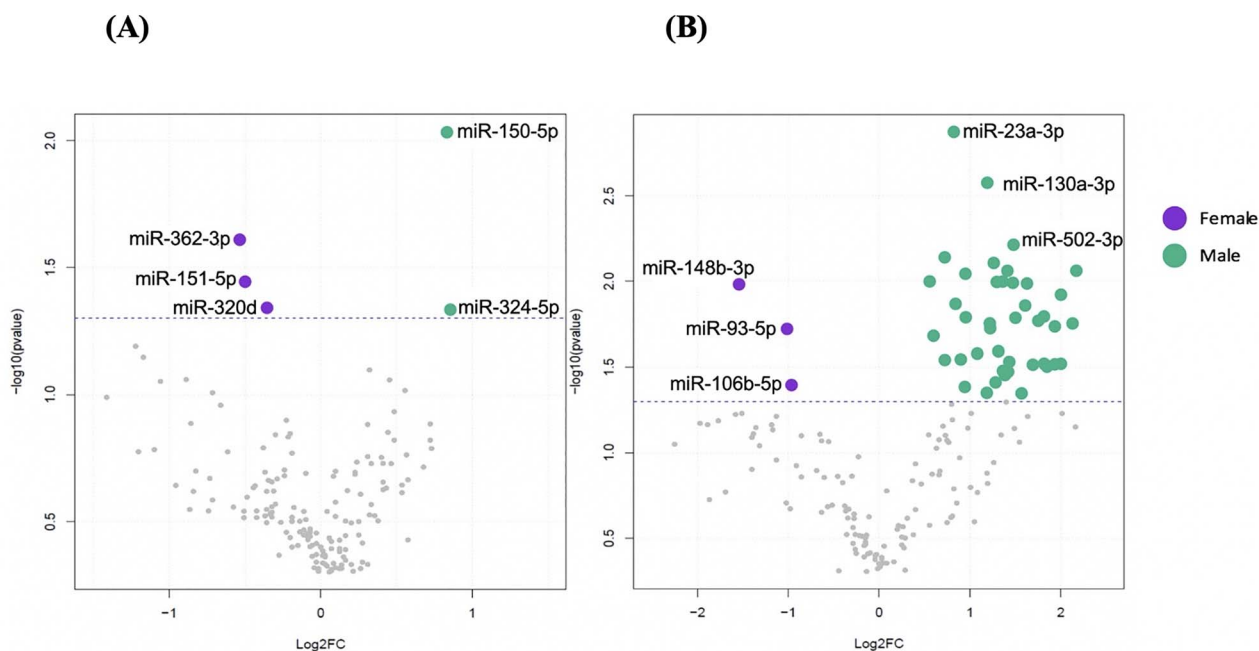


Figure 3. Volcano plots diagram showing the Log2 fold change (FC) and the P-value distribution of all the miRNAs detected in amniotic fluid (A) and maternal blood plasma (B) of heifers on Day 39 of pregnancy (corresponding to the peak of *SRY* expression in cattle). miRNAs upregulated in those heifers carrying a single male or a single female embryo are represented in green and purple, respectively.

Table 2. Differentially expressed miRNA families in maternal plasma of heifers carrying a single male embryo on Day 39 of pregnancy (corresponding to the peak of *SRY* expression in cattle).

Accession	Family name	miRNAs	Fold change	P-value
MIPF0000001	mir-17	miR-20b-5p	3.94	0.030445
		miR-93-3p	2.97	0.045204
MIPF0000002	let-7	let-7a-5p	2.28	0.045341
		let-7b-3p	2.48	0.025589
MIPF0000009	mir-29	miR-29a-3p	1.94	0.016421
		miR-29c-3p	1.87	0.028822
MIPF0000018	mir-154	miR-154-5p	3.94	0.030445
		miR-382-5p	3.41	0.016897
MIPF0000027	mir-23	miR-23a-3p	1.77	0.001347
		miR-23b-3p	1.47	0.010119
MIPF0000053	mir-22	miR-22-3p	2.13	0.026681
		miR-22-5p	2.76	0.010227
MIPF0000057	mir-28	miR-151a-5p	2.44	0.039066
		miR-28-3p	1.93	0.009095
MIPF0000139	mir-500	miR-502-3p	2.8	0.006159
		miR-501-3p	3.94	0.030445
MIPF0000163	mir-320	miR-320b	2.84	0.016533
		miR-320a	2.62	0.034911
		miR-320d	3.09	0.010407
		miR-320c	3.05	0.013924

(miR-324-5p and miR-150-5p). In addition, AT-Rich Interaction Domain 1 (*ARID1A*), Cyclin Dependent Kinase 6 (*CDK6*), G3BP Stress Granule Assembly Factor 1 (*G3BP1*), HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase Family Member 1 (*HERC1*), and Heterogeneous Nuclear Ribonucleoprotein A2/B1 (*HNRNPA2B1*) were among the predicted genes targeted by the three upregulated miRNAs in the AF of female embryos (miR-362-3p, miR-151a-5p, and miR-320d).

As only five sex-specific DE miRNAs were identified in the AF, GO analysis of the target genes was not performed.

Main targets of differentially expressed miRNAs in maternal blood plasma and in silico-based functional analysis

Totals of 14 046 and 3951 genes were identified as potential targets of the upregulated miRNAs in MP of heifers carrying male and female embryos, respectively (Supplemental Table S3).

Due to the high number of target genes identified in MP of heifers carrying a male embryo, we extracted those genes targeted by at least 5 DE miRNAs (3435 genes) and selected them for subsequent analysis, to improve the reliability of our findings. GO analysis did not reveal any significant enrichment among the target genes of miRNAs upregulated in MP of heifers carrying a female embryo. Interestingly, analysis of enrichment among the putative target genes of miRNAs upregulated in MP of heifers carrying a male embryo revealed that “inflammatory response” and “immune response” were among the 13 highly enriched GO biological processes ($P < 0.01$; Figure 4A; Supplemental Table S4). In addition, 23 InterPro domains were enriched by this set of miRNAs including “Immunoglobulin subtype,” “Immunoglobulin-like fold,” and “Immunoglobulin subtype 2” ($P < 0.01$; Figure 4A; Supplemental Table S5). Finally, pathway analysis of this set of miRNAs revealed 36 enriched pathways including “Rap1 signaling pathway,” “TGF-beta signaling pathway,” “cytokine-cytokine receptor interaction,”

“T-cell receptor signaling pathway,” “TNF signaling pathway,” and “chemokine signaling pathway” ($P < 0.01$; Supplemental Table S6).

Comparison between putative target genes of differentially expressed miRNAs in amniotic fluid and differentially expressed genes in genital ridges of bovine embryos

To understand the potential role of miRNAs in sex determination and early gonad differentiation in cattle, we compared the list of putative target genes of the sex-specific DE miRNAs identified in AF with the transcriptome of male and female bovine embryo gonads collected on Days 35, 39, and 43 from our previous study [7]. Such an approach could highlight a sex-dependent local communication between the embryo and the mother and/or a novel transport pathway for miRNAs, as the embryo is surrounded by this fluid.

Among the putative targets of the two miRNAs upregulated in the AF surrounding male embryos (miR-324-5p and miR-150-5p), 32 genes were upregulated in genital ridges of male ($n = 24$) or female ($n = 8$) embryos (Supplemental Table S7A). Of note, only three of these genes were DE in the genital ridges on Day 39 (GRB10 Interacting GYF Protein 1 [*GIGYF1*], Leukocyte Receptor Cluster Member 8 [*LENG8*], and Trio Rho Guanine Nucleotide Exchange Factor [*TRIO*]). However, most (22/32) were upregulated in one or other sex on Day 43. Only one of the two miRNA targeted each of the 32 genes.

Comparison of the putative target genes for the three upregulated miRNAs in AF of female embryos (miR-362-3p, miR-151a-5p, and miR-320d) with the list of sex-dependent DE genes in the genital ridges revealed 87 genes in common (Supplemental Table S7B). Of note, 16 (5 upregulated in male embryos; 11 upregulated in female embryos), 8 (7 upregulated in male embryos; 1 upregulated in female embryos), and 63 (48 upregulated in male embryos; 15 upregulated in female embryos) genes were upregulated in genital ridges of bovine embryos on Days 35, 39, and 43, respectively (Supplemental Table S7B). While the majority of these target genes were upregulated in

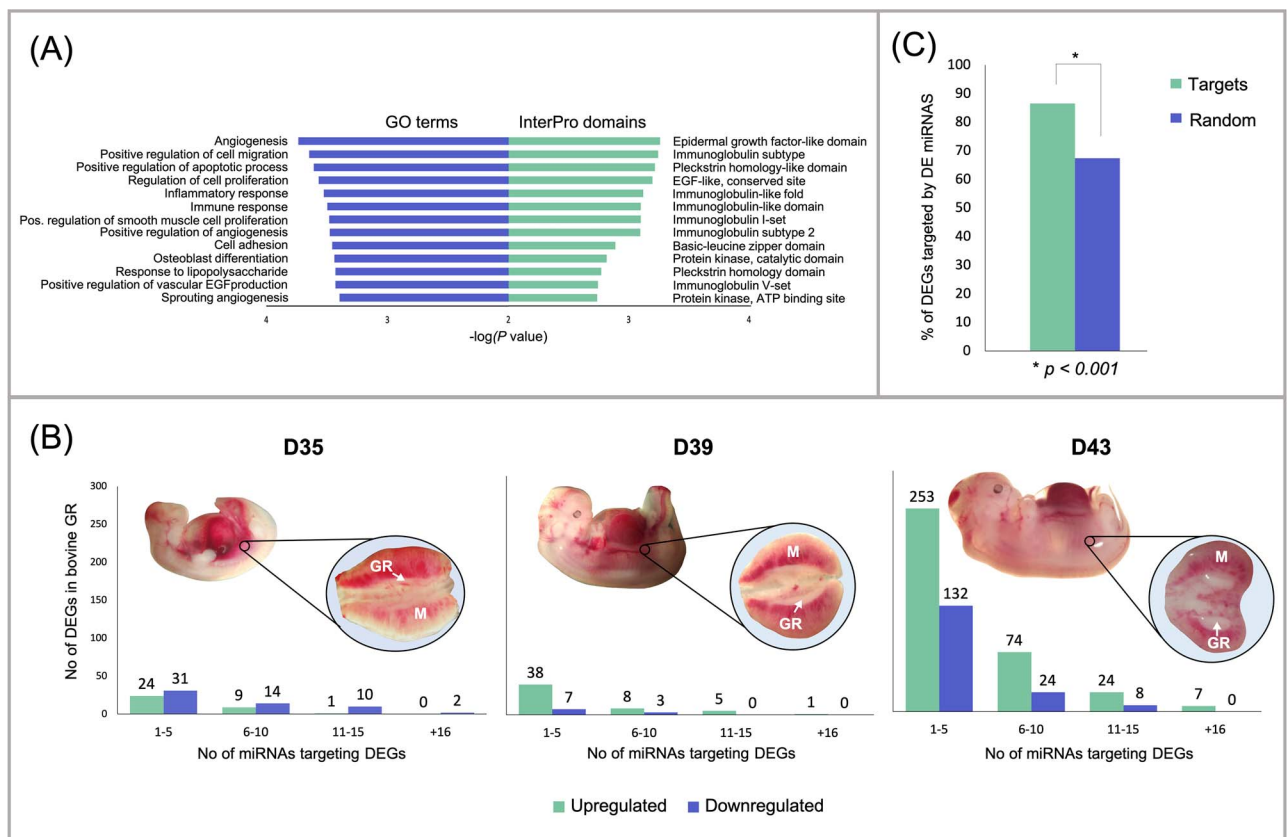


Figure 4. (A) Gene ontology (GO) and InterPro domains analysis for miRNAs enriched in maternal blood plasma of heifers carrying a single male embryo (FDR < 0.05). (B) Number of miRNAs upregulated in maternal blood plasma of heifers carrying single male embryos targeting sex-dependent differentially expressed genes (DEGs) in bovine genital ridges (GR) on Days (D) 35, 39, and 43. M = Mesonephros. Images modified from [7]. (C) Percentage of sex-dependent DEGs in bovine genital ridges targeted by differentially expressed miRNAs in maternal blood plasma of heifers carrying single male embryos, compared to 1000 randomizations of the same gene list size. Enrichment was calculated by Fisher's exact test.

embryos of either sex at only one time-point, Eukaryotic Translation Initiation Factor 2 Subunit Gamma (*EIF2S3*), Jumonji and AT-Rich Interaction Domain Containing 2 (*JARID2*), and IQ Motif Containing GTPase Activating Protein 2 (*IQGAP2*) were upregulated at more than one time-point (Supplemental Table S7B). Of these 87 genes, only *HNRNPA2B1* was targeted by the 3 upregulated miRNAs in AF of female embryos (Supplemental Table S7B).

Finally, only 7 genes were putative target genes for miRNAs upregulated in AF of both male and female embryos and DE in genital ridges of bovine embryos, including Exportin 1 (*XPO1*), *GIGYF1*, *LENG8*, *TRIO*, Zinc Finger Protein 703 (*ZNF703*), Cadherin 1 (*CDH1*), and *IQGAP1* (Table 3).

Of note, purple- and green-colored cells indicate that a particular gene was upregulated in genital ridges of female or male embryos on the corresponding day of pregnancy (D35, D39, and D43) [7].

Comparison between putative target genes of differentially expressed miRNAs in maternal blood plasma and differentially expressed genes in genital ridges of bovine embryos

In order to determine if a sex-dependent systemic embryo-maternal communication also occurs, we compared those genes differentially expressed between male and female gonads on Days 35, 39, and 43 with the potential target genes of the sex-specific miRNAs identified as differentially expressed in MP on Day 39.

The analysis revealed 651 upregulated genes in genital ridges of male and/or female embryos as potential targets for the miRNAs upregulated in MP of heifers carrying a single male embryo (Supplemental Table S8A). Of these 651 genes, 76 were potential targets for 10 or more miRNAs upregulated in MP in the male group. Of note, 91 (34 upregulated in male embryos; 57 upregulated in female embryos), 62 (52 upregulated in male embryos; 10 upregulated in female embryos), and 522 (358 upregulated in male embryos; 164 upregulated in female embryos) of these genes were also upregulated in genital ridges of embryos on Days 35, 39, and 43, respectively (Figure 4B; Supplemental Table S8A). This constitutes a significant enrichment of the identified target genes in sex-dependent differentially expressed genes (Fisher's exact test, $P < 0.001$), compared to what would be expected for our gene list size (Figure 4C). While the majority of these target genes were upregulated in embryos of either sex at only one time-point, *JARID2*, Ubiquitin Specific Peptidase 9 X-linked (*USP9X*), DEAD-Box Helicase 3 Y-Linked (*DDX3Y*), Protein Tyrosine Phosphatase Non-Receptor Type 14 (*PTPN14*), Lactate Dehydrogenase A (*LDHA*), Lysine Demethylase 6A (*KDM6A*), *SRY*, Protein Phosphatase 1 Catalytic Subunit Beta (*PPP1CB*), Shroom Family Member 2 (*SHROOM2*), Neurofilament Medium (*NEFM*), *IQGAP2*, Thymocyte Selection Associated High Mobility Group Box (*TOX*), and *SRY*-Box Transcription Factor 9 (*SOX9*) were among the upregulated at more than one time-point (Table 4A).

Table 3. Putative target genes among the sex-dependent differentially expressed genes in bovine genital ridges on Days (D) 35, 39, and 43, for miRNAs upregulated in the amniotic fluid of both male and female bovine embryos on Day 39 of pregnancy (corresponding to the peak of *SRY* expression in cattle).

Gene Symbol	D35	D39	D43	n° of miRNAs	miRNAs targeting this gene
<i>XPO1</i>	■			2	miR-324-5p, miR-362-3p
<i>GIGYF1</i>		■		2	miR-324-5p, miR-362-3p
<i>LENG8</i>		■		2	miR-324-5p, miR-362-3p
<i>TRIO</i>		■		2	miR-324-5p, miR-320d
<i>ZNF703</i>			■	3	miR-324-5p, miR-362-3p, hsa-miR-320d
<i>CDH1</i>			■	2	miR-324-5p, miR-320d
<i>IQGAP1</i>			■	2	miR-150-5p, miR-151a-5p

Table 4. Potential target genes among the sex-dependent differentially expressed genes in bovine genital ridges on Days (D) 35, 39, and 43 upregulated at more than one time-point, for miRNAs upregulated in maternal blood plasma of male embryos (A) or female embryos (B).

(A)					(B)				
Gene Symbol	D35	D39	D43	n° of miRNAs targeting this gene	Gene Symbol	D35	D39	D43	n° of miRNAs targeting this gene
<i>JARID2</i>	■		■	15	<i>USP9X</i>	■		■	2
<i>USP9X</i>	■			14	<i>JARID2</i>	■			1
<i>DDX3Y</i>	■	■	■	12	<i>DNAJC21</i>			■	1
<i>PTPN14</i>		■	■	10	<i>PPP1CB</i>	■			1
<i>LDHA</i>	■			10	<i>DDX3Y</i>	■	■	■	1
<i>KDM6A</i>	■	■	■	8	<i>IQGAP2</i>	■		■	1
<i>EIF2S3</i>	■	■		6	<i>PTPN14</i>		■	■	1
<i>IQGAP2</i>	■		■	6					
<i>PPP1CB</i>	■		■	6					
<i>CCT8</i>		■	■	4					
<i>LRRN1</i>		■	■	2					
<i>SHROOM2</i>	■	■	■	2					
<i>SOX9</i>	■	■	■	2					
<i>TOX</i>	■	■	■	2					
<i>DNAJC21</i>	■		■	2					
<i>NEFM</i>	■		■	2					
<i>UTY</i>	■	■	■	2					
<i>SRY</i>	■	■	■	1					
<i>FAM71F1</i>	■		■	1					
<i>FREM1</i>	■	■		1					

For full list of potential target genes and miRNAs, see Supplemental Table S8

Of note, purple- and green-colored cells mean that a particular gene was upregulated in genital ridges of female or male embryos on the corresponding day of pregnancy (D35, D39, and D43) [7]. For full list of potential target genes and miRNAs, see Supplemental Table S8.

We found 222 upregulated genes in genital ridges of male and/or female embryos as potential targets for the miRNAs upregulated in MP of heifers carrying a single female embryo (Supplemental Table S8B). Only 24 of these genes, including High Mobility Group Box 3 (*HMGB3*), Low-Density Lipoprotein Receptor

(*LDLR*), Ankyrin Repeat Domain 50 (*ANKRD50*), and DEAD-Box Helicase 6 (*DDX6*), were potential targets for the three miRNAs upregulated in MP in the female group (Supplemental Table S8B). In addition, 32 (9 upregulated in male embryos; 23 upregulated in female embryos), 21 (only upregulated in genital ridges of male embryos), and 177 (120 upregulated in male embryos; 57 upregulated in female embryos) genes were upregulated in genital ridges of embryos on Days 35, 39, and 43, respectively (Supplemental Table S8B). Only *USP9X*, *JARID2*, DnaJ Heat Shock Protein Family (Hsp40) Member C21 (*DNAJC21*), *PPP1CB*, *DDX3Y*, *IQGAP2*, and *PTPN14* were upregulated at more than one time-point (Table 4B).

Finally, 219 genes were common putative target genes for miRNAs upregulated in MP of heifers carrying a single male or female embryo and DE in genital ridges of bovine embryos. Only three genes, Mannosidase Alpha Class 1C Member 1 (*MAN1C1*), Doublesex and Mab-3-Related Transcription Factor 2 (*DMRT2*), and Opioid Receptor Kappa 1 (*OPRK1*), were specific putative target genes for miRNAs upregulated in MP of heifers carrying a single female embryo and DE in genital ridges of bovine embryos.

Discussion

In contrast to what occurs in mice, the similarity in bovine sex-determining gene expression patterns and gonadal histology compared to humans [5, 7] highlights the utility of the bovine model for the study of this important developmental stage and the mechanisms underlying fetomaternal immunity. Moreover, the importance of the sex of the calf in the dairy and beef industry [40] prompts, not only a better understanding of the processes of sex determination and early gonad differentiation, but also the search for new tools for embryo/fetal sexing that ensure more efficient livestock management programs. The current approach deepens our understanding of the molecular aspects that regulate bovine sex determination and embryo–maternal communication through the study of the miRNA profiles in AF and MP of heifers carrying a single male or female embryo on Day 39 (corresponding to the peak of *SRY* expression in cattle). The main findings were that: (i) even though most of the evaluated miRNAs were detected in both AF and MP, PCA showed clear separation of their expression in both fluids (Figure 2B); (ii) sex-dependent DE miRNAs were identified in both biological fluids but, surprisingly, the number of those sex-specific miRNAs was higher in MP ($n = 56$) compared to AF ($n = 5$); (iii) GO analysis showed that although no enrichment was detected for DE miRNAs in AF (in either sex) or in MP in heifers carrying a female embryo, 13 biological processes were enriched by DE miRNAs in MP of heifers carrying a male embryo. Inflammatory response and immune response were among them, suggesting that these sex-dependent DE miRNAs may be implicated in modulating the receptivity of the dam to a male embryo; (iv) finally, the comparison of downstream targets of the sex-dependent DE miRNAs detected in MP with those DE genes previously identified in male vs. female genital ridges on Days 35, 39, and 43 from our previous study [7] revealed potential targets that might be important during this developmental stage such as *SHROOM2*, *DDX3Y*, *SOX9*, *SRY*, *PPP1CB*, *JARID2*, *USP9X*, *KDM6A*, and *EIF2S3*.

Role of AF miRNAs in embryo/fetal development

In cattle, AF first accumulates after closure of the amnion on Day 22 [41]. Apart from the role in nutrition, metabolism, and homeostasis,

AF provides a protective space around the developing embryo/fetus that allows its movement and growth. The current approach identified five DE miRNAs in AF of heifers carrying a single male embryo compared with those carrying a single female embryo (miR-324-5p and miR-150-5p upregulated, and miR-362-3p, miR-151a-5p, and miR-320d downregulated). Hui et al. [42] reported that AF cell-free fetal RNA reflects real-time developmental fetal physiology. Given the fact that AF can be obtained by amniocentesis in many species, the assessment of its mRNA and miRNA composition could represent an interesting tool to study key stages of fetal development. Secretion of miRNAs into the extracellular environment represents a potential signaling mechanism involved in the regulation of embryo development; indeed, miRNAs have been detected in culture media in vitro as early as the blastocyst stage [29]. Furthermore, secretion of miRNAs at the early blastocyst stage is sexually dimorphic and these miRNAs induce a transcriptomic response when applied to primary bovine endometrial epithelial cells in culture [30]. In agreement with these studies, our results showing sex-dependent DE miRNAs in AF of pregnant heifers suggest that AF (as a vehicle) and miRNAs (as regulators) may play important roles in embryo/fetal development.

Embryo–maternal communication through miRNAs during sex determination

It is known that miRNAs can be secreted from a cell through extracellular vesicles, via apoptotic bodies, or by being bound to Argonaute proteins [43]. In contrast to an endocrine effect, the paracrine mode of cell-to-cell signaling for extracellular miRNAs appears to be more likely [43]. In the current study, although most of the evaluated miRNAs were detected in both AF and MP, PCA showed a clear separation of their expression in both fluids (Figure 2B). While male and female samples clustered separately in MP, this distinction was not apparent in AF. Although sex-dependent DE miRNAs were identified in both biological fluids, somewhat surprisingly, the number of sex-specific miRNAs was higher in MP ($n = 56$) compared to AF ($n = 5$). This bias is unlikely due to the miRNA panel used in this study that includes miRNAs previously detected in serum/plasma as most of the evaluated miRNAs (161 of 179) were detected in both fluids. However, an endocrine route is a possibility as an accumulation of orally administered bovine miRNAs in the murine placenta and embryo has been demonstrated recently [44]. Previously, the same group reported distinct miRNA tissue distribution patterns and accumulation that could be regulated by the abundance of complementary mRNAs [45]. On the other hand, Sheller-Miller et al. [46] demonstrated that exosomes can traffic from the AF into the placenta and systemically spread through maternal circulation. Thus, circulating miRNAs detected in the MP in the current study could participate in an endocrine mode of cell-to-cell signaling controlling this developmental process. This mechanism warrants further research.

To shed some light on the potential role of DE miRNAs detected in AF and MP during the process of sex determination and early gonad differentiation, we compared those genes differentially expressed between male and female gonads on Days 35, 39, and 43 identified in our previous study [7] with the predicted target genes of the sex-specific miRNAs identified as differentially expressed in AF and MP in the current study. Interestingly, the proportion of target genes for miRNAs upregulated in MP and AF that were upregulated in the genital ridges was higher at advanced developmental stages (see Supplemental Tables S7 and S8). Overall, ~80% (521/651) and ~76% (82/108) of the putative target genes for miRNAs

upregulated in MP and AF, respectively, were upregulated in genital ridges on Day 43. This may indicate that miRNAs upregulated in MP and AF in this study (Day 39) may have a role in early gonad differentiation, which occurs around Day 43 in cattle [5].

Common sex-specific DE miRNAs found in AF and MP during sex determination

Only two sex-specific DE miRNAs in AF miR-151a-5p and miR-320d were also found as sex-specific DE miRNAs in MP. Moreover, the expression level of those miRNAs differed between the two biological fluids, while they were upregulated in female AF, miR-151a-5p and miR-320d were downregulated in female MP. Interestingly, four miR-320 family members (miR-320a, miR-320b, miR-320c, and miR-320d) were also downregulated in MP of heifers carrying a single female embryo. In mice, miR-320 is one of the most downregulated miRNA in ovarian granulosa cells after TGF- β 1 treatment [47] and regulates steroid production during follicular development [48]. TGF- β signaling plays important roles in ovarian follicular development in mammals [49] and was one of the most enriched pathways for the set of miRNAs upregulated in MP of heifers carrying a male embryo in the present study. In addition to the miR-320 family, the miR-22 family also exhibited sexual dimorphism in the present study, in agreement with Gross, Kropp, and Khatib [30]. In that study, Day 7 bovine blastocysts showed sex-dependent secretion of miR-122, miR-22, and miR-320a in culture media, and the addition of those miRNAs to primary bovine endometrial epithelial cell culture altered the expression of progesterone receptor transcript.

Downstream analysis of common sex-specific DE miRNAs found in AF and MP during sex determination

Results revealed that Sp1 transcription factor (*SP1*), transcriptional repressor GATA binding 1 (*TRPS1*), phosphatase and tensin homolog (*PTEN*), and tet methylcytosine dioxygenase 3 (*TET3*) are potential target genes for several sex-specific DE miRNAs found in both AF and MP (> 15 sex-specific DE miRNAs targeting each of these genes); however, they were not DE in genital ridges of male or female embryos [7]. *SP1* is a regulator of transcription and has been implicated in the expression of numerous genes involved in cell metabolism, cell growth, differentiation, angiogenesis, and apoptosis regulation [50]. Wang et al. [51] reported the major function of *Trps1* as a sex-specific regulator of bone mineral density of the femur and tibia in mice. *PTEN* is a dual protein and lipid phosphatase that interferes with the insulin-signaling pathway via its lipid phosphatase activity. Samaan et al. [52] showed that muscle *PTEN* is regulated in a sex-specific manner. Sex differences in the expression of Tet enzymes (*Tet1*, *Tet2*, and *Tet3*), involved in DNA demethylation, were seen in several brain regions during the first week of life in mice suggesting that they may play important roles in sexual differentiation of the brain [53]. Thus, some of the sex-specific DE miRNAs found in AF and MP, although not directly involved in the sex determination process, may control other biological, metabolic, and/or developmental processes, clearly biased by the sex of the embryo, such as skeletal and brain development.

Sex-dependent DE miRNAs in MP during sex determination

Our approach revealed 56 sex-dependent DE miRNAs in MP of pregnant heifers. Recently, Murri et al. [54] reported a strong positive association of miR-877-5p (the most upregulated DE miRNA observed in MP in our study) with serum androgens and negative

associations with estradiol and sex hormone-binding globulin concentrations. In addition to miR-877-5p, and consistent with our findings, expression pattern of circulating miRNAs let-7a-5p, miR-27a-3p, miR-326 exhibited sexual dimorphism in adult humans [54]. A recent comprehensive review summarized the role of several of the DE miRNAs observed in the current study in mammalian reproduction [55]. Of note, the let-7 family, which was upregulated in the MP in the male group in our study, is overexpressed in primordial germ cells (PGC) for male germ line commitment, indicating that let-7 members may contribute to differentiation of male PGCs [56]. Here, miR-22 family members miR-22-5p and miR-22-3p were also upregulated in MP of heifers carrying a male embryo, while miR-148b-3p was upregulated in MP of heifers pregnant with a female embryo. Estrogen receptor 1 (*ESR1*) is a potential target for miR-22-3p and miR-148b-3p. miR-22 suppresses *ESR1*-mediated estrogen signaling in ovine fetal testes, which is necessary for male gonad establishment [57]. Garverick et al. [58] demonstrated that the fetal cattle ovary (on Day 45 but not on Day 35 of gestation) expresses the enzyme (P450 aromatase) necessary for the final synthetic step of converting androgens to estradiol-17 and the potential to respond to the estradiol-17 via estrogen receptors. In agreement with this, we did not observe differential expression of *ESR1* between male and female genital ridges in our previous study, where samples were collected up to Day 43 [7]. Pathway analysis for miRNAs enriched in MP of heifers carrying a single male embryo in the current study revealed estrogen signaling pathway among the most enriched pathways (Supplemental Table S6). These findings suggest that estrogen signaling could be important in fetal ovarian development and further support our hypothesis that DE miRNAs in MP could regulate these signaling pathways in further developmental stages (early gonad differentiation).

Comparison between downstream targets of the sex-dependent DE miRNAs detected in MP and DE genes in genital ridges of bovine embryos

In order to detect those circulating miRNAs potentially involved in the process of sex determination and early gonad differentiation, we explored the downstream targets of the sex-dependent DE miRNAs detected in MP and compared them with those DE genes previously identified in male vs. female genital ridges [7]. The analyses revealed targets that may be important during this developmental stage such as *SHROOM2*, *DDX3Y*, *SOX9*, *SRY*, *PPP1CB*, *JARID2*, *USP9X*, *KDM6A*, and *EIF2S3* (Table 4 and Supplemental Table S8). Of note, *DDX3Y*, *SHROOM2*, *SOX9*, and *SRY* were upregulated in male genital ridges at all developmental stages studied (Days 35, 39, and 43). Ramathal et al. [59] proposed that *DDX3Y* is a testis-specific gene that acts as an early spermatogenesis regulator. In cattle, *DDX3Y* is one of the Y genes from X-degenerate (X-d) regions, which do not recombine, and hence diverge over time, allowing for the possibility of sex-specific selection [60]. Therefore, Y genes from X-d regions may be involved in sexual dimorphism [61]. Here, we identified 12 miRNAs upregulated in the MP of heifers carrying a single male embryo that can regulate the expression of *DDX3Y*. Interestingly, miR-485-3p, one of the most upregulated circulating miRNAs detected in the male group, has *SOX9* as a potential target. Although we have recently reported differences in terms of *SOX9* expression during bovine sex determination with respect to the classic mouse model [7], it is well known that this transcription factor plays an important role in orchestrating testis morphogenesis in many species [4, 5, 25, 62–65]. Recently, increased levels of circulating miR-485-3p have been reported in polycystic

ovary syndrome patients [66], but its role during the sex determination process has yet to be determined. In line with this, miR-146a-5p, one of the most upregulated miRNAs in MP of heifers carrying a single male embryo, targets *SRY*, the key gene in the sex determination process. This miRNA has a sex-specific role in renal and cardiac pathology by modulating the effect of gonadal hormones [67]. Our study also highlighted that *PPP1CB* may have important functions during this developmental stage in cattle as this gene is a potential target for six miRNAs upregulated in the MP of heifers carrying a single male embryo. Furthermore, we previously reported that *PPP1CB* was upregulated in female genital ridges on Day 35 and upregulated in male genital ridges on Day 43. Targeted disruption of the *Ppp1cc* gene in mice results in male infertility due to impaired spermatogenesis while females appear normal, suggesting that *PPP1CA* and *PPP1CB* can substitute for the absence of *PPP1CC* isoforms in all tissues except testis [68]. In cattle, its role in sex determination remains unknown.

Of note, *JARID2*, *USP9X*, *KDM6A*, and *EIF2S3* were upregulated in female genital ridges at several developmental stages from Day 35 to Day 43. We detected 15, 14, 8, and 6 upregulated miRNAs in MP of heifers carrying a male embryo as potential regulators of these genes, respectively, suggesting that these miRNAs may act by suppressing their expression in male genital ridges during sex determination. Intron retention of *JARID2* (a Jumonji family gene) is implicated in reptile temperature-dependent sex determination and sex reversal [69]. In mammals, a closely related Jumonji family member (*Kdm3a*) is a direct regulator of *SRY*, and *Kdm3a* dysfunction causes male-to-female sex reversal in mice [70]. In the present study, five upregulated miRNAs in MP of heifers carrying a male embryo and one circulating miRNA upregulated in the female group were also detected as potential regulators of *KDM3A*. Interestingly, *KDM6A* (also known *UTX*) and *EIF2S3X* are X-linked genes that escape X inactivation in somatic tissues in humans and mouse [71, 72]. Genes that escape X inactivation represent exceptional genes with higher expression in females vs. males, in agreement with our results, suggesting that they may be important for female-specific functions [73]. *USP9X* is also an X-linked gene with a sex- and stage-dependent expression during murine gonadal development, implicating a transient and restricted expression pattern of *Usp9x* from 10.5 to 13.5 days postcoitum in the germ cells of XY gonads [74]. Although the importance of some of these genes in sex determination in cattle is yet to be determined, here, we have identified circulating miRNAs that may act as regulators of their expression and, therefore, could be key players during the process. In addition, these circulating miRNAs could be used as potential markers for early prediction of embryo/fetal sex in commercial practice. Due to the importance of calf gender in the dairy and beef industry, the knowledge generated here could potentially have a significant impact in cattle production systems and deserves further investigation.

Sex-dependent DE miRNAs as potential modulators of the dam's receptivity to a male embryo

Finally, to obtain a better insight into the underlying biology of DE miRNAs, GO analysis was carried out. Interestingly, analysis of enrichment among the putative target genes of miRNAs upregulated in MP of heifers carrying a male embryo revealed that “inflammatory response” and “immune response” were among the 13 highly enriched GO biological processes. In addition, pathway analysis of this set of miRNAs revealed 36 enriched pathways including “Rap1 signaling pathway,” “TGF-beta signaling pathway,”

“cytokine–cytokine receptor interaction,” “T-cell receptor signaling pathway,” “TNF signaling pathway,” and “chemokine signaling pathway”. Pregnancy is characterized by substantial changes in maternal immune parameters. In agreement with our results, several studies in women have demonstrated differences in the maternal immune response between pregnancies with male or female fetuses [75–77]. Specifically, and consistent with the pathways enriched in our study, male fetal sex was associated with higher levels of pro-inflammatory cytokines and angiogenic factors and female fetal sex was associated with higher levels of regulatory cytokines in the maternal plasma [75]. Multiple miRNAs act together to regulate these biological processes and pathways. Interestingly, a recent study has shown that specific miRNAs can regulate macrophage tolerization [78]. Taken all together, sex-dependent DE miRNAs detected in the current study may be implicated in modulating the receptivity of the dam to a male embryo. Further studies evaluating the effects of these miRNAs on their relevant mRNA targets are required to confirm their involvement in the aforementioned biological processes and pathways. Nonetheless, results of the current study will be of interest to researchers studying the mechanisms underlying fetomaternal immunity and their effects on pregnancy outcome based on fetal sex given the fact that (i) women carrying male fetuses have higher rates of preterm births, higher birth weights, and greater fetal mortality [79, 80] as occurs in cattle [81]; (ii) studies examining associations between fetal sex and maternal immune parameters are limited, especially during early-pregnancy; and (iii) the similarity in bovine sex determining gene expression patterns and gonadal histology to that of humans [5, 7].

In conclusion, results highlight novel molecular aspects of sex determination in cattle and demonstrate for the first time differences in miRNA expression in AF between male and female bovine embryos, suggesting that AF may play an important role in sex determination and early gonad differentiation through miRNA trafficking. While some miRNA families such as miR-22, miR-320, and let-7 may have an important role in the sex determination process in cattle, some other DE miRNAs detected in AF and MP, although not directly involved in the sex determination process, may control other biological, metabolic, and/or developmental processes, clearly biased by the sex of the embryo. In silico-based functional analysis of the putative target genes of miRNAs upregulated in MP of heifers carrying a male embryo also suggests a distinct immune response of the dam depending on the fetal sex. Finally, circulating miRNAs in MP may offer new opportunities to investigate biomarkers for early prediction of embryo/fetal sex in commercial practice.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Author Contributions

JMS and PL conceived the study. JMS, BP, and JAB were involved in sample collection and RNA and miRNA extractions. IGR and AGA analyzed the data. JMS wrote the first draft of the manuscript. All authors commented on the draft manuscript.

Acknowledgments

The authors thank the farm staff at UCD Lyons Research Farm for their collaboration and acknowledge the excellent assistance of Michael McDonald, Mary Wade, Dr Sandra Bagés-Arnal, and Dr Beatriz Fernández-Fuertes during

sample collection and processing. Also, the authors thank Ceva Santé Animale for their collaboration.

Conflict of interest: The authors declare no conflicts of interest.

Data availability

All raw and processed data will be publicly available at ArrayExpress. Accession number E-MTAB-10130.

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