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Identification of plasma miRNA biomarkers for pregnancy detection in dairy cattle

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ABSTRACT   A pregnancy diagnosis is an important standard for control of livestock's reproduction in paricular dairy cattle. High reproductive performance in dairy animals is a essential condition to realize of high life-time production. Pregnancy diagnosis is crucial to shortening the calving interval by enabling the farmer to identify open animals so as to treat or re-breed them at the earliest opportunity. MicroRNAs are short RNA molecules which are critically involved in regulating gene expression during both health and disease. This study is sought to establish the feasible of circulating miRNAs as biomarkers of early pregnancy in cattle. We applied Illumina small-RNA sequencing to profile miRNAs in plasma samples collected from 12 non-pregnant cows ("open" cows: samples were collected before insemination (non-pregnant state) and after pregnancy check at the indicated time points) on weeks 0, 4, 8, 12 and 16. Using small RNA sequencing we identified a total of 115 miRNAs that were differentially expressed weeks 16 relative to non-pregnancy ("open" cows). Weeks 8, 12 and 16 of pregnancy commonly showed a distinct increase in circulating levels of miR-221 and miR-320a. Through genome-wide analyses we have successfully profiled plasma miRNA populations associated with pregnancy in cattle. Their application in the field of reproductive biology has opened up opportunities for research communities to look for pregnancy biomarker molecules in dairy cattle.

Keywords: dairy cattle, miRNA, pregnancy diagnosis

INTRODUCTION

Mammals should reproduce to be able to lactate. Estrus detection, artificial insemination (AI) and pregnancy diagonosis are routinely performed. Good reproductive management in dairy farms is reliant on early and precise diagnosis of pregnancy. Currently, several pregnancy diagnosis tools including the rectal palpation, ultrasonography, milk progesterone test, and preganncy-associated glycoproteins have been widely utilized. Any direct or indirect method for pregnancy diagnosis must accurately distinguish between pregnant and non-pregnant animals. Pregnancy diagnosis can identify open cows, help expect calving dates, and help producers make culling decisions. Early identification of non-pregnant dairy cattle post AI can improve reproductive performance and pregnancy rate by decreasing the interval between AI services and increasing AI service rate. Identification of animals in a herd that fails to conceive within 3 weeks after insemination would reduce economic losses. Finally it extends the

calving interval and have contributed to the decline in profitability. Thus, new technologies to identify pregnant dairy cattle early after artificial insemination (AI) may play a key role in economically viable reproductive management decision to improve reproductive performance and profitability to commercial dairy farms.

Currently, several pregnancy diagnosis tools use molecules including pregnancy-associated glycoproteins (PAGs), protein B, DG29 and preliplantation factor (PIF). Research to develop commercial indirect mehods for pregnancy diagnosis continues because these methods are non-invasive and the tests can be marketed to and performed by dairy farmers or herd employees. We aimed at finding micro RNA (miRNAs) biomarkers by hypothesis-free, small RNA next generation sequencing (NGS). miRNAs are small non-coding RNAs molecule (16-27 nt long) that act as post-transcriptional gene regulators and play important roles in regulation of gene expression. The research for easily accessible biomarkers of several diseases and physiological condition has recently focused on circulating microRNAs (miRNA). The differentially expressed miRNAs could serve as feasible biomarkers for not only early diagnosis of pregnancy but also for various livestock health and disease (Hale et al., 2014). As miRNAs are mainly secreted into small extracellular vesicles and miRNAs have been found in numerous biofluids ranging from serum and amniotic fluid to urine and milk (Reid et al., 2011; Pohler et al., 2015). They can therefore be taken as a liquid biopsy and represent ideal molecules for the use of non-invasive biomarker of disease. (Reid et al., 2011; Buschmann et al., 2016).

MicroRNAs are expeled from cells of most tissue types in plasma membrane bound extracellular vesicles (EV), in particular exosomes. The packaging of miRNA in EVs or exosomes is important in terms of a detection standpoint as ribonuclease (RNA-ases) are unable to penetrate and breakdown the miRNA allowing them to be extracted from blood and serum (Reid et al., 2011). Exosomes and EVs play a crucial role in intercellular communication, including promotion of sperm maturation, regulation of immune function, release of miRNA for a wide array of regulatory functions, as well as other roles currently under reserach (Raposo and Stoorvogel, 2013). Plasma and whole blood have found an appropriate resource of EV-derived miRNA profiles, thus offering a feasible blood-borne biomarker candidate for several disease and physi-

ological condition (Häusler et al., 2010; Reid et al., 2011).

Human based disease resaerch has found significant differences in profusion of miRNAs for many cancers (Lawrie et al., 2008; Häusler et al., 2010), heart disease (Tijsen et al., 2010) and sepsis (Wang et al., 2010). Furthermore, earlier studies in humans have shown that circulating miRNA profiles related to pregnancy become more pronounced as pregnancy progresses. Circulating miRNAs in maternal serum have been regarded as feasible biomarkers of pregnancy condition due to their significant impact on gene expression and regulation (Chim et al., 2008). A study by Gilad et al. (2008) identified miRNAs that are increased in profusion in pregnant humans but not in non-pregnant females. This finding led to the sharp progress of identifying miRNAs that were unique to pregnancy and across various species, although none have been thoroughly explained.

This thesis is aimed to focus on physiological role of miRNAs during pregnancy, alsso emphasizing their feasible for being biomarkers for pregnancy detection. The object of the present study was to determine the expression pattern of circulatory miRNAs in plasma of pregnant and non-pregnant dairy cows.

MATERIALS AND METHODS

Animals selection and sampling

The selected animals and the experimental protocol were approved by institutional animal ethical committee of the National Institute of Animal Science (NIAS). A total of 30 dairy cow were used according to their health condition, parity ($\geq$ 2) and with a BCS of approximately 3.5. After pregnancy diagnosis, non-pregnant cows were excluded from the analysis. For the present investigation, the blood samples were collected from individual animal (n = 12) on different weeks of pregnancy (0, 4, 8, 12 and 16 weeks). Day 0 represents the control (collection of blood before artificial insemination: AI). Following AI, blood was collected from the cows till the 16 weeks of pregnancy. Briefly, about 10 mL whole blood was collected in EDTA tubes, and then the samples were incubated at room temperature for 1 h. The collected blood sample was centrifuged at 3,000 rpm for 15 min at 4℃ to obtain plasma. After this step, the circulating cell-free nucleic acid was in the supernatant (plasma) and then the obtained plasma was transferred into a fresh 2.0 mL tube and the plasma samples were stored at -80℃ until further processing.

Confirmation of pregnancy

Pregnancy was diagnosed by palpation per rectum of the uterine contents between days 50 and 60 after AI or ultrasonic examination to determine pregnancy status. Ultrasonography was carried out using a B-Mode ultrasound scanner (MyLab™OneVET, esaote) equipped with a 5.0-MHz linear array probe. Pregnancy diagnosis was confirmed by observation of embryocoele and allantoic fluid (Abdullah et al., 2014). The ovaries were also scanned for the presence of corpus luteum.

Total RNA isolation

Before RNA isolation, the exosomes in plasma were firstly isolated by using Total Exosomes Isolation kit (Invitrogen, Carlsbad, CA). Exosomal total RNA, including mRNA and miRNA, was isolated by using miRNeasy Mini Kit (Qiagen) following manufacturer's protocos. The concentration of RNA was measured by using the Qubit microRNA assay kit (Invitrogen, Carlsbad, CA). RNA integrity of all RNA eluates was assessed. And high quality (RNA integrity number ≥ 7) samples from isolated total RNA were selected. The total RNA with lowest quality was not used for further study. The RNA samples were stored at -80℃ until further processing.

Illumina sequencing and data analysis

Total RNA (5 μL) from each sample was used to construct miRNA library by using the NEXTflex Small RNA Sequencing Kit (Illumina, San Diego, CA) according to the manufacturer's instruction. Small RNA libraries were then pooled together in equal volumes for gel purification. The pooled library was sequenced by using the HiSeq 2500 system (Illumina) as 50 bp single reads. Read quality (adaptor removal, and size selection) was assessed using FastQC v0.11.5 (http://www.bioinformatics.babraham. ac.uk/projects/fastqc/) and cutadapt (Martin, 2011). The sequences with read length larger than 18 nucleotides (nt) were aligned against bovine miRNA database (miR-Base, release version 21) with the default parameters to identify known miRNAs using Bowtie2 (Langmead and Salzberg, 2012). Each library was processed separately. The expression level of miRNAs in each library was estimated by sRNAbench, which normalized reads count number of each miRNA reads per million (RPM) by the following foumula: RPM = (miRNA reads number/total mapped reads per library) × 1,000,000. The differentially expressed (DE) miRNAs were investigated by using bioinformatics tool edgeR v3.10.2 (Robinson et al., 2010). The DE miRNAs were determined by Log2 fold change (FC) > 1 OR < -1 and false discovery rate (FDR) < 0.05 based on counting reads by using HTSeq (Anders et al., 2015).

Identification of novel miRNA prediction

Prediction of novel miRNAs was performed by using the miRDeep2 software. Briefly, quality-trimmed reads from all samples were combined into a single reads file followed by the preprocessing, mapping, and novel miRNA prediction steps through mapper.pl and miRDeep2.pl scripts. Next, the FASTA files of predicted novel miRNA and quantifier.pl script were used to determine the readcounts/expression values for each novel miRNA from each sample. Combined count matrix for novel miRNAs was generated by using the custom scripts. Differential expression between control and pregnant groups was calculated by using the DESeq2 package (version 1.12.4).

RESULTS

We sequenced a total of 60 blood samples from non-pregnancy and 16 weeks of pregnancy. On average, the high throughput Illumina sequencing resulted in 22.4 million raw reads per sample from the exosomes of all stages (Table 1). 71.5% reads were uniquely mapped to annotate miRNAs in bovine genome.

Table 1. Sequencing and miRNA profiling statistics of normal and days of pregnancy samples

Sample	Total reads	Mapped reads (%)	Precursor miRNA reads	Mature miRNA reads	Known precursor with ≥ 5× coverage	No. known miRNA	No. novel miRNA
Normal	24773796.0 ± 3962727.1	73.2 ± 5.6	305494.7 ± 81383.0	7713350.2 ± 2613944.7	361.7 ± 45.0	334.8 ± 27.4	491.8 ± 138.4
4 weeks	19421596.7 ± 3587596.6	74.3 ± 4.7	179172.5 ± 146647.2	8038306.5 ± 3180088.2	385.5 ± 57.1	307.3 ± 37.2	367.3 ± 178.1
8 weeks	24122975.9 ± 4669481.7	70.7 ± 12.5	239792.4 ± 154469.7	6806040.9 ± 3069368.0	383.9 ± 53.6	314.4 ± 43.0	410.0 ± 179.6
12 weeks	22139499.4 ± 3132885.2	68.8 ± 5.8	155846.3 ± 56288.9	7785174.4 ± 1930464.7	374.7 ± 28.2	306.1 ± 19.7	320.7 ± 72.9
16 weeks	21845616.1 ± 2814855.6	70.5 ± 6.8	140514.6 ± 54922.5	7386644.1 ± 1905019.6	343.1 ± 39.2	307.0 ± 35.0	316.7 ± 83.6

The average data of all five analyzed samples for each animal is displayed.

Venn diagrams were used to demonstrate the overlap between pregnant groups (Fig. 1). Pregnant 8, 12 and 16 weeks groups had 2 common miRNAs (bta-miR-221 and bta-miR-320a).

To determine the miRNA expression patterns in blood samples, miRNA microarray analysis was conducted. By using small RNA sequencing we identified a total of 115 miRNAs that were differentially expressed on pregnancy compared to non-pregnancy. A total of 115 miRNAs were identified to be significantly differentially expressed between the groups; 91 miRNAs were upregulated and 26 miRNAs were downregulated (Table 2). And 91 upregulated and 26 downregulated miRNAs are listed (Table 3).

Gene ontology (GO) and pathway enrichment analysis were used to explore the functions of differentially expressed genes in bta-miR-221 (Fig. 2A). The target genes were enriched in a total of 167 GO terms, which included 12 molecular function (GO:MF), 132 biological process (GO:BP), and 23 cellular component (GO:CC) terms, in addition to reactomes (REAC), and WiKiPathways (WP). The target genes of differentially expressed bta-miR-320a were significantly enriched in a total of 144 GO terms, which included 23 molecular function (GO:MF), 98 biological process (GO:BP), and 23 cellular component (GO:CC) terms, in addition to reactomes (REAC), and WiKiPathways (WP).

Fig. 1. Venn diagrma showing the overlap of the number and percentage of miRNAs detected in plasma samples.

DISCUSSION

This study aimed to characterize the earliest changes in miRNA expression for the purpose of define a marker for early pregnancy detection. We determined the expression pattern of circulatory miRNAs in plasma of pregnant and non-pregnant dairy cattle. Varing expression patterns of circulating miRNAs in the regulation of pregnancy has been determined in bovines (Cai et al., 2017). Many researchers are now investigating miRNAs as biomarkers for pregnancy diagnosis in the cow. There is increasing evidence that pregnancy specific miRNAs exist and may be feasible markers for pregnancy diagnosis. In 2015, exosomal miRNAs were reported to be differentially expressed in pregnant versus non-pregnant dairy cattle and dairy cattle undergoing early embryonic mortality (Pohler et al., 2015). A recent study by Fiandanese et al. (2016) identified a feasible miRNA, bta-miR 140, as an early biomarker for pregnancy diagnosis in high producing dairy cows. At day 19, bta-miR 140 was up regulated in all pregnant dairy cattle, and at day 13 onwards, it was upregulated in pregnant, non-lactating dairy cattle (Fiandanese et al., 2016). Similarly, Ioannidis and Donadeu (2016) proved different stages of the estrous cycle (day 16: bta-miR-26a, bta-miR-29c, bta-miR-138, bta-miR-204. Day 24: bta-miR-1249, day 16 & 24: hsa-miR-4532) that were differentially expressed in pregnant heifers and miR-26a was differentially upregulated on Day 16 pregnant relative to non-pregnant heifers. The expression pattern of miR-496 and miR-125a has significantly varied during formation of bovine conceptus. This clearly suggests the role of these miRNAs in maternal-to-zygotic transcription translation (Tesfaye et al., 2009). Likewise, various miRNAs including miR-27a and miR-92b are differentially expressed during the formation of the placenta (Su et al., 2010). In the present study, we report the results of circulating bta-miR-221 and bta-miR-320a were notably expressed at over 8 weeks of pregnancy. The expres-
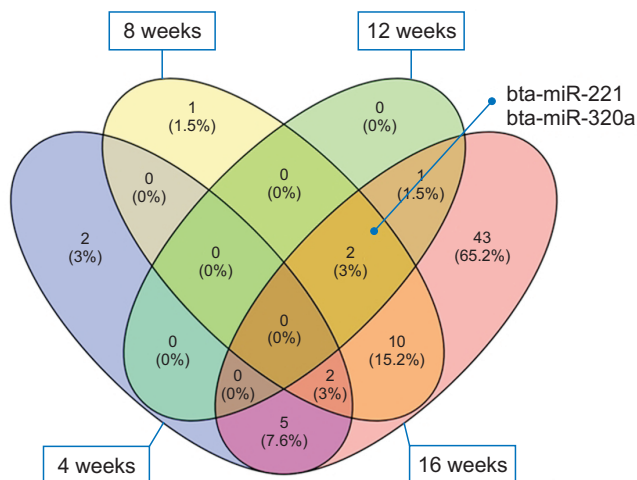
Table 2. Differential miRNA profile expression between normal and weeks of prengnancy groups (p-value < 0.05)

Differentially expressed miRNAs	Up	Down
4 weeks	9	8
8 weeks	16	8
12 weeks	3	2
16 weeks	63	8

Table 3. List of significantly differentially expressed plasma circulatory miRNAs in pregnant cows

Week	Gene_id	a.value	*p*.value	q.value
Up-regulation				
4 weeks	bta-miR-455-5p	1.07186493	0.04259614	0.98472459
	bta-miR-193b	9.60083749	0.00309688	0.50066183
	bta-miR-133a	9.90982635	0.03399751	0.98472459
	bta-miR-296-5p	11.135414	0.01974089	0.98472459
	bta-miR-1307	11.6237296	0.02810407	0.98472459
	bta-miR-342	15.4400631	0.03737714	0.98472459
	bta-miR-150	16.0778023	0.00042426	0.20576638
	bta-miR-339a	16.2270717	0.00568494	0.6892985
	bta-miR-339b	16.5113771	0.00992402	0.80219133
8 weeks	bta-miR-2349	5.52978713	0.03643007	0.80182568
	bta-miR-2299-3p	10.9842492	0.03782667	0.80182568
	bta-miR-877	11.5808517	0.02669041	0.80182568
	bta-miR-4286	12.1609216	0.0491537	0.80182568
	bta-miR-378	12.3958376	0.01719906	0.78481392
	bta-miR-143	13.0927918	0.0201574	0.78481392
	bta-miR-2284z	13.62152	0.04436674	0.80182568
	bta-miR-2284aa	14.0033362	0.03916645	0.80182568
	bta-miR-451	14.588654	0.04087686	0.80182568
	bta-miR-328	14.599698	0.02207844	0.78481392
	bta-miR-375	15.1485213	0.00697631	0.6188333
	bta-miR-339a	16.2840769	0.00289751	0.57394257
	bta-miR-150	16.3453427	0.01200376	0.78481392
	bta-miR-150	16.5251687	0.00168736	0.57394257
	bta-miR-221	17.0721993	0.00767149	0.6188333
	bta-miR-320a	18.4741794	0.0035575	0.57394257
12 weeks	bta-miR-221	16.7979849	0.00410378	1
	bta-miR-26a	14.9168773	0.03069057	1
	bta-miR-320a	18.2924635	0.03597042	1
16 weeks	bta-miR-2481	1.14963851	0.03041101	0.28213644
	bta-miR-133b	2.86869902	0.0030251	0.06646495
	bta-miR-2346	5.76241916	0.0423637	0.32562789
	bta-miR-127	5.97836278	0.04310756	0.32562789
	bta-miR-1584-3p	7.20287377	0.04827621	0.34076361
	bta-miR-6119-5p	7.20686333	0.04194146	0.32562789
	bta-miR-146a	7.34224067	0.02060563	0.24081834
	bta-miR-23b-5p	7.50414778	0.010668	0.16368713
	bta-miR-200b	7.55021804	0.00922451	0.15405228
	bta-miR-1296	8.48194252	0.02129413	0.24081834
	bta-miR-133a	8.68207222	0.00108619	0.04102443
	bta-miR-211	8.93880225	0.04927539	0.34076361
	bta-miR-2284w	9.14777305	0.02051713	0.24081834
	bta-miR-744	9.22386786	0.00669801	0.12180462
	bta-miR-769	9.79008083	0.00627157	0.12144622
	bta-miR-193b	9.7992275	0.00941256	0.15405228
	bta-miR-2284ab	10.7000489	0.03586982	0.29850986
	bta-miR-32	10.7206389	0.03160388	0.28213644
	bta-miR-2299-3p	10.7875179	0.00237651	0.0648259
	bta-miR-296-5p	10.8219527	0.00012621	0.00834593
	bta-miR-29b	11.114811	0.00184855	0.06050908

Table 3. Continued

Week	Gene_id	a.value	*p*.value	q.value
16 weeks	bta−miR−574	11.1383229	0.03148469	0.28213644
	bta−miR−877	11.2242015	0.00229709	0.0648259
	bta−miR−1307	11.3036664	0.00015298	0.00834593
	bta−miR−345−3p	11.3975095	0.03964558	0.3191144
	bta−miR−2285f	11.4813384	0.00310742	0.06646495
	bta−miR−98	11.7713234	0.03485827	0.29850986
	bta−miR−4286	11.7812685	0.00084086	0.03440499
	bta−miR−16a	12.0324417	0.00028575	0.01403053
	bta−miR−126−3p	12.034786	0.00739763	0.12972271
	bta−miR−425−3p	12.1519812	0.01700118	0.22677729
	bta−miR−885	12.1930632	0.02627789	0.27452004
	bta−miR−326	12.3306069	0.0488033	0.34076361
	bta−miR−185	12.3441055	0.03792451	0.31034894
	bta−miR−378	12.4227355	0.04301751	0.32562789
	bta−miR−2285k	12.7770755	0.03126496	0.28213644
	bta−miR−16b	13.0378558	0.00146122	0.05124697
	bta−miR−652	13.1575626	0.02967302	0.28213644
	bta−miR−26b	13.2076922	0.01328624	0.18851546
	bta−miR−2284y	13.3654816	0.02049432	0.24081834
	bta−miR−143	13.3976177	0.00116375	0.00399116
	bta−miR−2284z	13.4231816	0.01864053	0.24081834
	bta−miR−2284aa	13.8486383	0.02880073	0.28213644
	bta−miR−874	13.9459277	0.00197643	0.00399116
	bta−miR−27a−3p	13.9867105	0.00388976	0.07957799
	bta−miR−10b	14.2690092	0.00977644	0.15484621
	bta−miR−215	14.3511691	0.02158046	0.24081834
	bta−miR−328	14.4005258	0.01343797	0.18851546
	bta−miR−197	14.4655493	0.02483939	0.27102538
	bta−miR−1306	14.5382078	0.02137329	0.24081834
	bta−miR−29a	14.9644205	0.00311343	0.06646495
	bta−miR−375	15.0306074	0.0027077	0.06646495
	bta−miR−26a	15.1268421	0.00202395	0.06210982
	bta−miR−451	15.1997736	0.00037921	0.01692671
	bta−miR−142−5p	15.3504092	0.00015229	0.00834593
	bta−let−7g	15.6507868	0.01342206	0.18851546
	bta−miR−339a	15.9390783	0.00090517	0.00142078
	bta−miR−30e−5p	15.9534442	0.0342503	0.29850986
	bta−miR−150	16.1951035	0.00254583	0.06578964
	bta−let−7b	16.2247572	0.04634703	0.33964761
	bta−miR−339b	16.230831	0.00197238	0.00399116
	bta−miR−221	16.7451505	0.00136249	0.00142078
	bta−miR−320a	18.1878667	0.00135029	0.00399116
Down−regulation				
4 weeks	bta−miR−2454−5p	−4.28939694	0.00150232	0.36431335
	bta−miR−2415−5p	−4.28939694	0.0077144	0.74829656
	bta−miR−7861	−4.28939694	0.02419468	0.98472459
	bta−miR−196a	−4.28939694	0.02515021	0.98472459
	bta−miR−4449	−4.28939694	0.03433801	0.98472459
	bta−miR−338	−4.28939694	0.03612927	0.98472459
	bta−miR−1277	−4.28939694	0.03627568	0.98472459
	bta−miR−17−3p	−4.28939694	0.04710053	0.98472459

Table 3. Continued

| Week	Gene_id	a.value	*p*.value	q.value
8 weeks	bta-miR-2313-5p	−4.48412073	0.00678968	0.6188333
	bta-miR-4449	−4.48412073	0.01430248	0.78481392
	bta-miR-2284h-3p	−4.48412073	0.01739699	0.78481392
	bta-miR-2378	−4.48412073	0.01801676	0.78481392
	bta-miR-154b	−4.48412073	0.02270123	0.78481392
	bta-miR-381	−4.48412073	0.03092379	0.80182568
	bta-miR-187	−4.48412073	0.03258187	0.80182568
	bta-miR-99a-3p	−4.48412073	0.04675656	0.80182568
12 weeks	bta-miR-346	−4.17303705	0.03826548	1
	bta-miR-187	−4.17303705	0.0468653	1
16 weeks	bta-miR-196a	−4.24490661	0.00643096	0.12144622
	bta-miR-2377	−4.24490661	0.01708912	0.22677729
	bta-miR-2378	−4.24490661	0.02605244	0.27452004
	bta-miR-2446	−4.24490661	0.02746247	0.2809182
	bta-miR-346	−4.24490661	0.0304451	0.28213644
	bta-miR-4449	−4.24490661	0.03566852	0.29850986
	bta-miR-188	−4.24490661	0.04480545	0.33332536
	bta-miR-592	−4.24490661	0.04783157	0.34076361
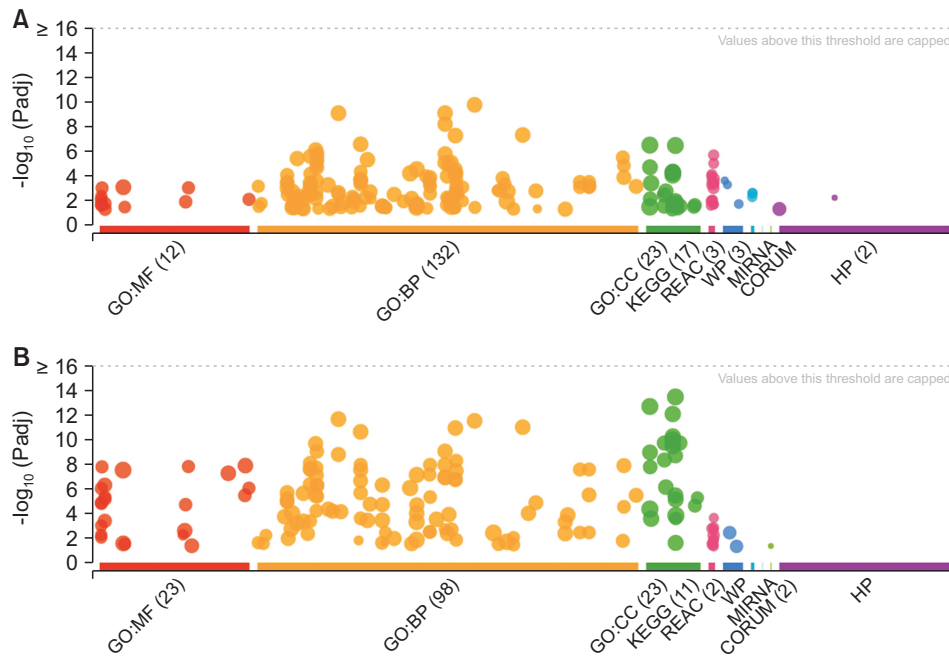
Fig. 2. Manhattan plot illustrating the differentially expressed gene-enriched GO terms (MF, molecular function; BP, biological process; and CC, cellular component) and KEGG pathways across reactome pathways (REAC), WiKi-Pathways (WP), transcription factor (TF), microRNA target base (MIRNA), and human phenotype ontology (HP) term categories. (A) bta-miR-221 enriched in GO terms and pathways. (B) bta-miR-320a enriched in GO terms and pathways.

sion of miR-221 during pregnancy indicates the interface between maternal and fetal. Also miR-320 may promote uterine migration of endometrial stromal cells during gestation (Bidarimath et al., 2014).

Increading proofes indicated the presence of human placenta-specific miRNAs in maternal circulation. The expression of placenta-specific human chromosome 19 miRNA cluster (C19MC); hsa-miR-515-3p, hsa-miR-517a, hsa-miR-517c, hsa-miR-518b, and hsa-miR-526b increased in maternal circulation during the third trimester of pregnancy and decreased after parturition (Kotlabova et al., 2011). The villous of trophoblast cells shed the C19MC miRNA cluster encapsulated in exosomes and could be the main source of placenta-specific miRNAs

in maternal circulation (Luo et al., 2009; Donker et al., 2012). Furthermore, according to Morales-Prieto et al. (2013) the human chromosome 14 miRNA cluster (C14MC) is also announced to be related to pregnancy. Other miRNAs involving miR-141, miR-149, miR-299-5p, and miR-135, which are offered to be aplenty expressed in placenta were also augmented in plasma of pregnant women and their concentration declines after parturition (Chim et al., 2008). Earlier study expressed that 25 miRNAs were differentially expressed between exosomes of maternal serum derived from non-pregnant and day 30 and 90 pregnant ewes (Cleys et al., 2014).

Even though purification is essential to accuarate optimal miRNA for pregnancy detection, results mean that miRNAs have feasible as an early pregnancy diagnosis tool. In addition, miRNA may offer information to represent embryonic viability. A study indicate dairy cattle that go through embryo mortality compared to dairy cattle that have a successful pregnancy have a significantly rose abundance of unique miRNAs at days 17 and 24 of conception (Reese et al., 2016). Future studies are needed to evaluate the repeatability of these result and to decide unique miRNA most appropriate for embryo viability survey.

Two new and generally available technologies for reproductive management include hormonal protocols such as Ovsynch (Pursley et al., 1995, 1997) and Presynch/Ovsynch (Moreira et al., 2001; Navanukraw et al., 2004) that synchronize ovulation and allow for TAI, and use of transrectal ultrasonography for early detection of non-pregnant cows. Dairy farms must schedule and administer artificial inseminations, hormone injections, and pregnancy tests for a multitude of animals on a daily or weekly basis. Detection of non-pregnant dariy cattle early after breeding can only improve reproductive performance when together with a operating schedule to quickly present non-pregnant dairy cattle for a subsequent AI service. Accurate detection of non-pregnancy is administered to synchronize estrus or ovulation to reduce the interval to the subsequent AI service.

CONCLUSION

In conclusion, comprehensive profiling of miRNAs in plasma of pregnant and non-pregnant dairy cattle found

specific miRNA expression pattern. Much studies and development efforts are being made toward advancement of a pregnancy diagnosis for dairy cattle. Our study provides an experimental basis to reveal the feasible role of miRNAs as biomarkers in pregnancy diagnosis. This specific pregnancy differentially expressed miRNAs marker can be used as the retrospective detection of early pregnancy biomarkers. Pregnancy-associated microRNA profiling at 8 weeks in bovine was described for the first time and can be used for comparative studies. These miRNAs may have similar function in mammalian species and can be feasible molecular markers for evolution. Coupling a non-pregnancy diagnosis with a management strategies to quickly reinitiate. AI service may improve reproductive performance by decreasing the interval between AI services and the effectiveness of hormonal ovulation and estrus control protocols initiated at certain physiologic stages post AI breeding. Future experiments are needed in this area to truly understand early identification of pregnancy diagnosis through miRNA biomarkers.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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