Arch. Anim. Breed., 60, 199–204, 2017
https://doi.org/10.5194/aab-60-199-2017

Open Access
Archives Animal Breeding

Identification and expression analysis of *miR-144-5p* and *miR-130b-5p* in dairy cattle

Zhixiong Li[1,2], Hongliang Wang[3], Ling Chen[1], Mengxing Zhai[1], Si Chen[1], Na Li[1], and Xiaolin Liu[1]

[1]College of Animal Science and Technology, Northwest A&F University, Shaanxi Key Laboratory of Molecular Biology for Agriculture, Yangling, Shaanxi 712100, P.R. China
[2]College of Life Science and Technology, Southwest University for Nationalities, Chengdu 610041, P.R. China
[3]State Key Laboratory for Molecular Biology of Special Economic Animals, Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun 130112, P.R. China

Correspondence to: Xiaolin Liu (liuxiaolin@nwsuaf.edu.cn)

Received: 9 October 2016 – Revised: 10 May 2017 – Accepted: 13 June 2017 – Published: 19 July 2017

Abstract. MicroRNAs (miRNAs) can coordinate the main pathways involved in innate and adaptive immune responses by regulating gene expression. To explore the resistance to mastitis in cows, *miR-144-5p* and *miR-130b-5p* were identified in bovine mammary gland tissue and 14 potential target genes belonging to the chemokine signaling pathway, the arginine and proline metabolism pathway and the mRNA surveillance pathway were predicted. Subsequently, we estimated the relative expression of *miR-144-5p* and *miR-130b-5p* in cow mammary tissues by using stem-loop quantitative real-time polymerase chain reaction. The results showed that the relative expression of *miR-144-5p* and *miR-130b-5p* in the mastitis-infected mammary tissues ($n = 5$) was significantly downregulated 0.14-fold ($p < 0.01$) and upregulated 3.34-fold ($p < 0.01$), respectively, compared to healthy tissues ($n = 5$). Our findings reveal that *miR-144-5p* and *miR-130b-5p* may have important roles in resistance to mastitis in dairy cattle.

1 Introduction

Bovine mastitis, defined as "an inflammation of the mammary gland", is a prevalent and complex infectious disease affected by genetics and pathogens that can result in significant dairy cattle losses (Nash et al., 2003). Mastitis can be caused by many bacteria, including *Staphylococcus aureus* and *Escherichia coli*. The primary defense against pathogens relies on the appropriate expression of antigen-presenting molecules triggering the release of effector molecules in the innate immune system. The immune system, as the central host determinant for dictating the outcome of intramammary infection, can defend against in-breaking pathogens as the first line once the pathogens penetrate the physical barrier (Bannerman et al., 2009).

Recent studies have shown that microRNAs (miRNAs) play important roles in regulating and modulating innate and adaptive immune responses (Zhou et al., 2012; Gu et al., 2012). Mature miRNAs are a class of small non-coding RNA molecules that are ∼ 22 nucleotides (nt) long processed from ∼ 70 nt long precursor miRNAs (pre-miRNAs) that form hairpin secondary structures and are evolutionarily conserved (Bartel et al., 2004; Cullen et al., 2004; Kim et al., 2005). miRNAs are post-transcriptional regulators that inhibit the translation or induce the degradation of protein-coding protein mRNAs that contain complementary sequences to miRNAs (Berezikov, 2011; Bartel et al., 2009).

miR-144 plays a crucial role in hemoglobin synthesis during primitive erythropoiesis and is associated with anemia severity in sickle-cell diseases (Fu et al., 2009; Sangokoya et al., 2010). *miR-130b* inhibits cell proliferation and invasion in pancreatic cancer through targeting *STAT3* and targets *DICER1* for aggression in endometrial cancer (Zhao et al., 2013; Li et al., 2013). *miR-130b* is associated with poor prognosis in colorectal cancer and is a prognostic marker (Colangelo et al., 2013). Our previous research showed two differentially expressed miRNAs matched to bta-mir-144 and bta-mir-130b that were detected in the peripheral blood of healthy and mastitis-infected dairy cattle (Li et al., 2014a).

Table 1. The primer sequences of the stem-loop qPCR experiments.

miRNA name	Primer	Primer sequence	Product size (bp)
miR-144-5p	Loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTACAGT	59
	FW primer	CCGGGATATCATCATATACTGTAAG	
	RW primer	GTGCAGGGTCCGAGGT	
miR-130b-5p	Loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTAGTGC	59
	FW primer	CGGACTCTTTCCCTGTTGCACTACT	
	RW primer	GTGCAGGGTCCGAGGT	
18S-snRNA	FW primer	GTGGTGTTGAGGAAAGCAGACA	79
	RW primer	TGATCACACGTTCCACCTCATC	

Note: the underlined letters are the sequences from *miR-144-5p* and *miR-130b-5p*.

However, our knowledge of the differential expression of the two miRNAs in cattle mastitis resistance remains largely limited. The two miRNAs may have important roles in the development of the immune system against pathological changes in mammary tissue in dairy cattle.

The aims of this study were (1) to investigate whether *miR-144-5p* and *miR-130b-5p* are present in bovine tissues, (2) to predict the target genes *miR-144-5p* and *miR-130b-5p* and (3) to analyze whether *miR-144-5p* and *miR-130b-5p* are differentially expressed in healthy and mastitis-infected mammary tissues.

2 Materials and methods

2.1 Animal samples

Samples were collected from five healthy and five mastitis-infected Chinese Holstein cows of first lactation from a commercial bovine slaughter farm. The selection of mastitis-infected cows was carried out as previously described (Li et al., 2014b). A part of the 10 mammary tissue samples was collected and stored in liquid nitrogen for RNA isolation; others were used for the identification of the pathogen. All 10 mammary tissue samples were used for analysis of the expression profile of *miR-144-5p* and *miR-130b-5p*. The liver, heart, lung, kidney and spleen from five healthy samples were used to analyze the expression pattern. This study was approved by the Northwest A&F University Animal Care and Use Committee.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol Reagent (Invitrogen, USA) following the manufacturer protocol. RNA purity was verified by measuring the absorbance at 260 and 280 nm with an ND-1000 spectrophotometer (NanoDrop Technologies, USA). First-strand cDNA synthesis was performed in a 20 µL volume using a PrimeScript RT reagent kit (Takara, Japan) following the manufacturer protocol with a specific stem-loop primer. The primers for the reverse transcription polymerase chain reaction (RT-PCR) are shown in Table 1.

To identify *miR-144-5p* and *miR-130b-5p* expressed in the mammary tissue of dairy cattle, primers were designed according to the sequences previously detected (Li et al., 2014). PCR was performed in a total volume of 25 µL containing 50 ng of cDNA, 2.5 µL 10 × PCR buffer, 2.1 mM $MgCl_2$, 0.1 mM dNTPs, 0.25 mM of each primer, 0.2 µL Easy Taq DNA polymerase and ddH_2O run for 32 cycles at 95° for 40 s, 60° for 30 s and 72° for 30 s, followed by incubation at 72° for 10 min. PCR products were ligated into the T-Vector pMD19 (Takara, Japan) after gel extraction and then transformed into competent *E. coli* DH5α. Finally, 10 randomly selected positive clones were sequenced. The experiment was repeated twice to confirm the result.

2.3 Sequence analysis

Sequence alignment was performed to verify *miR-144-5p* and *miR-130b-5p* using DNAman (version 6.0) software. To obtain the potential target genes of *miR-144-5p* and *miR-130b-5p*, the prediction of the target gene was performed with MIREAP software. The predicted target genes were classified by KEGG functional annotations; the identified pathways were actively regulated by *miR-144-5p* and *miR-130b-5p* in healthy and mastitis-infected dairy cattle.

2.4 Quantitative analysis of miRNAs

Stem-loop quantitative real-time polymerase chain reaction (stem-loop qPCR) was used to analyze miRNAs according to Chen et al. (2005). The stem-loop qPCR was performed in the Bio-Rad CFX96 Real-Time PCR Detection System using the SYBR Green PCR kit (Takara, Japan) according to the manufacturer instructions. 18S rRNA was used as the reference gene in the stem-loop qPCR detection of bovine miRNAs, and all reactions were run in triplicate. The relative expression level of *miR-144-5p* and *miR-130b-5p* was calculated according to the method of Livak and

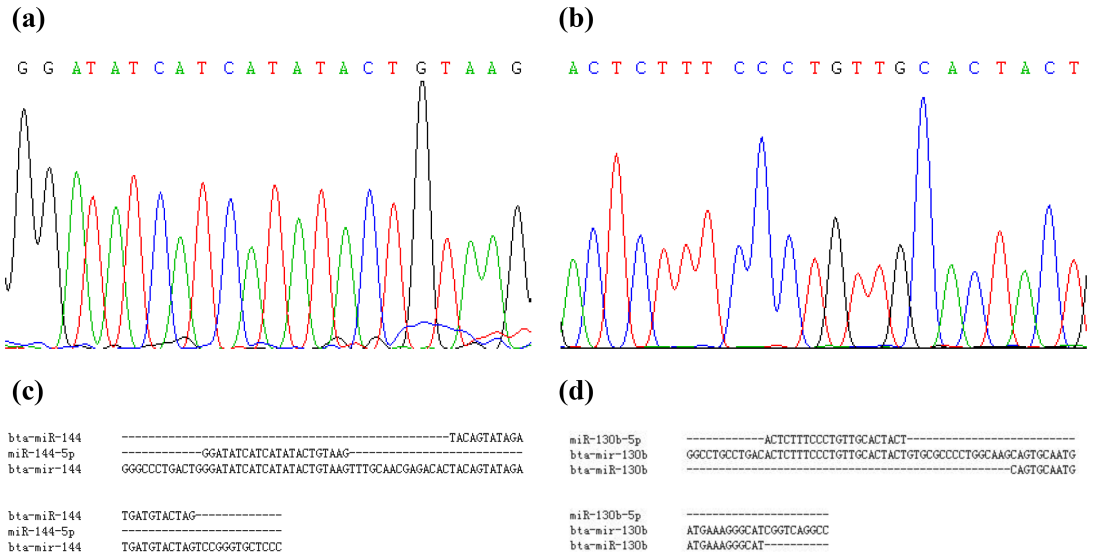

Figure 1. **(a)** Sequencing chromatograms for *miR-144-5p*. **(b)** Sequencing chromatograms for *miR-130b-5p*. **(c)** The bta-mir-144 sequence comparison with *bta-miR-144* and *miR-144-5p*. **(d)** The bta-mir-130b sequence comparison with *bta-miR-130b* and *miR-130b-5p*.

Schmittgen (2001). The primers for the qPCR are shown in Table 1.

2.5 Statistical analysis

The value of the relative quantity was presented as fold change. The means of two groups were compared by a Student's paired-samples t test. The analysis was performed with SPSS software (version 20.0); $p < 0.05$ was regarded as significant.

3 Results

3.1 Identification of *miR-144-5p* and *miR-130b-5p*

RT-PCR and sequencing were used to identify *miR-144-5p* and *miR-130b-5p* with specific primers (Fig. 1a and b). The miRNAs of *bta-miR-144* and *bta-miR-130b* from miRBase (http://www.mirbase.org/) are matched to the 3′ of bta-mir-144 and bta-mir-130b. The cloned miRNAs are totally matched to the 5′ of bta-mir-144 and bta-mir-130b (Fig. 1c and d), so we called them *miR-144-5p* and *miR-130b-5p*.

3.2 Target gene prediction of *miR-144-5p* and *miR-130b-5p*

Twenty potential target genes of *miR-144-5p* and *miR-130b-5p* were predicted using MIREAP software (Table 2). Ten target genes of *miR-144-5p* and *miR-130b-5p* belong to the chemokine signaling pathway, which plays an important role in inflammatory responses and cancer (Coussens and Werb, 2002; Charo and Ransohoff, 2006; Baggiolini and Loetscher, 2000). Six target genes of *miR-144-5p* and *miR-130b-5p* belong to the arginine and proline metabolism pathway, which is closely involved in conceptus metabolism, growth and development. Four target genes of *miR-130b-5p* belong to the mRNA surveillance pathway, which ensures the viability and quality of mRNA.

Table 2. Target genes of *miR-144-5p* and *miR-130b-5p*.

miRNA name	Gene name	KEGG pathway name
miR-144-5p	CXCL2 CRK GNB5	Chemokine signaling pathway
	NOS2 ARG1	Arginine and proline metabolism
miR-130b-5p	AMD1 SAT2 ARG1 GLS2	Arginine and proline metabolism
	PPP2R2B PPP2CB SMG1 SAP18	mRNA surveillance pathway
	CHUK CXCL2 CXCL6 GNAT2 CCL11 NRAS BRAF	Chemokine signaling pathway

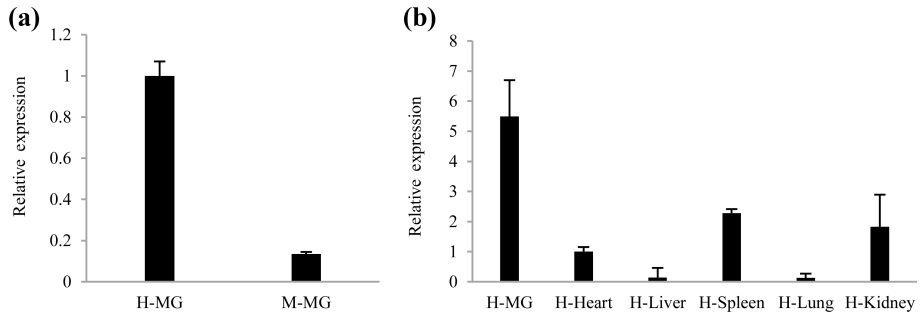

Figure 2. Relative expression of *miR-144-5p*. **(a)** Relative expression of *miR-144-5p* in healthy and mastitis-infected mammary gland tissues using qPCR. H-MG denotes healthy mammary gland and M-MG denotes mastitis-infected mammary gland. **(b)** Relative expression of *miR-144-5p* in a variety of healthy tissues using qPCR. H denotes the healthy cow group. The vertical bar represents the standard error.

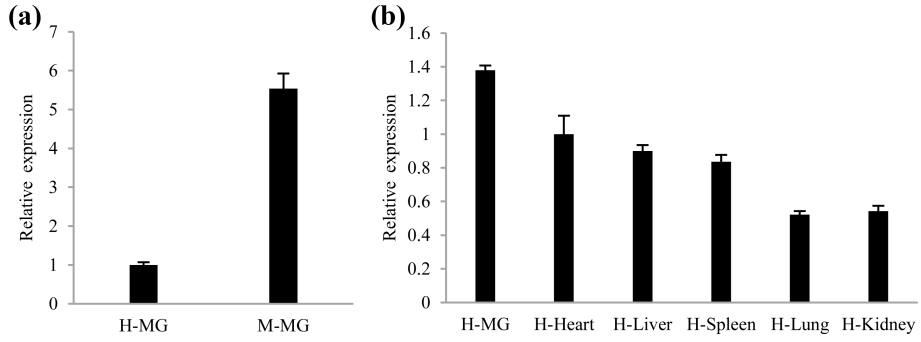

Figure 3. Relative expression of *miR-130b-5p*. **(a)** Relative expression of *miR-130b-5p* in healthy and mastitis-infected mammary gland tissues using qPCR. H-MG denotes healthy mammary gland and M-MG denotes mastitis-infected mammary gland. **(b)** Relative expression of *miR-130b-5p* in a variety of healthy tissues using qPCR. H denotes the healthy cow group. The vertical bar represents the standard error.

3.3 Expression of *miR-144-5p* and *miR-130b-5p* in healthy and mastitic cow tissues

The expression of *miR-144-5p* and *miR-130b-5p* in healthy and mastitis-infected mammary tissues was investigated using stem-loop qPCR. A lower expression of *miR-144-5p* was observed in the mastitis-infected mammary tissues compared to that in the healthy samples (Fig. 2a; $p < 0.01$). The expression of *miR-144-5p* in mammary glands was higher than that in other tissues, including the heart, liver, spleen, lung and kidney in the healthy cows (Fig. 2b). The expression of the *miR-130b-5p* was much higher in the mastitis-infected mammary gland tissues compared to that in the healthy samples (Fig. 3a; $p < 0.01$). The expression of *miR-130b-5p* in mammary glands was higher than that in other tissues, including the liver, heart, lung, kidney and spleen in the healthy cows (Fig. 3b). The findings suggest that *miR-144-5p* and *miR-130b-5p* are highly correlated with mastitis.

4 Discussion

Through sequencing alignment, *bta-miR-144* and *bta-miR-130b* from miRBase are actually *miR-144-3p* and *miR-130b-5p*; the cloned miRNAs in our study are *miR-144-5p* and *miR-130b-5p*. In the present study, *miR-144-5p* and *miR-130b-5p* were identified in mammary, heart, liver, spleen, lung and kidney tissues at different expression levels, which may indicate functional differences.

Twenty potential target genes of *miR-144-5p* and *miR-130b-5p* were predicted, and 10 of them belonged to the chemokine signaling pathway, which plays an important role in orchestrating leukocyte migration under normal conditions and during inflammatory responses (Mellado et al., 2001), such as *CXCR4* (Lapteva et al., 2005; Balkwill, 2004) and *CXCR2* (Acosta et al., 2008). Another important pathway including six target genes is arginine and proline metabolism, which is known to be closely involved in conceptus metabolism (Wu et al., 2008), growth and development. It is also a potential treatment for intrauterine growth restriction (Wu et al., 2009), which is a significant problem in both human medicine and animal agriculture. Four of them belonged to the mRNA surveillance pathway, which assesses the quality of mRNAs to ensure that they are suitable for translation (Vasudevan et al., 2002). mRNA surveillance facilitates the detection and destruction of mRNAs that contain premature termination codons (Wagner and Lykke-Andersen, 2002). Whether these genes are regulated directly

by *miR-144-5p* and *miR-130b-5p* needs to be confirmed in a further study.

5 Conclusions

In summary, the differential expression of *miR-144-5p* and *miR-130b-5p* in healthy and mastitis-infected mammary glands indicates that *miR-144-5p* and *miR-130b-5p* may play important roles in inflammation response. There could be a relationship between *miR-144-5p* and *miR-130b-5p* and mastitis in Chinese Holstein cattle. Our findings suggest that the differential expression of the post-transcriptional regulators *miR-144-5p* and *miR-130b-5p* may bind to complementary sequences of target mRNAs, resulting in different translational bovine repression. *miR-144-5p* and *miR-130b-5p* likely play critical roles in mastitis resistance in dairy cattle.

Data availability. The original data for the paper are available upon request from the corresponding author.

Competing interests. The authors declare that they have no conflict of interest.

Acknowledgements. This study was supported by the National 863 Program of China (2008AAl02144), the"13115" Sci-Tech Innovation Program of Shaanxi Province (2008ZDKG-11) and the Xi'an city science and technology project (NC09049-2). Comments from anonymous reviewers have greatly improved the paper.

Edited by: Steffen Maak
Reviewed by: three anonymous referees

References

Acosta, J. C., O'Loghlen, A., Banito, A., Guijarro, M. V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N., Takatsu, Y., Melamed, J., d'Adda di Fagagna, F., Bernard, D., Hernando, E., and Gil, J.: Chemokine signaling via the CXCR2 receptor reinforces senescence, Cell, 133, 1006–1018, 2008.

Baggiolini, M. and Loetscher, P.: Chemokines in inflammation and immunity, Immunol. Today, 21, 418–420, 2000.

Balkwill, F.: The significance of cancer cell expression of the chemokine receptor CXCR4, Semin. Cancer Biol., 14, 171–179, 2004.

Bannerman, D. D.: Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows, J. Anim. Sci., 87, 10–25, 2005.

Bartel, D. P.: MicroRNAs, genomics, biogenesis, mechanism, and function, Cell, 116, 281–297, 2004.

Bartel, D. P.: MicroRNAs, target recognition and regulatory functions, Cell, 136, 215–233, 2009.

Berezikov, E.: Evolution of microRNA diversity and regulation in animals, Nature Rev. Genet., 12, 846–860, 2011.

Charo, I. F. and Ransohoff, R. M.: The many roles of chemokines and chemokine receptors in inflammation, New Engl. J. Med., 354, 610–621, 2006.

Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., Barbisin, M., Xu, N. L., Mahuvakar, V. R., Andersen, M. R., Lao, K. Q., Livak, K. J., and Guegler, K. J.: Real-time quantification of microRNAs by stem–loop RT–PC R, Nucleic Acids Res., 33, e179, https://doi.org/10.1093/nar/gni178, 2005.

Colangelo, T., Fucci, A., Votino, C., Sabatino, L., Pancione, M., Laudanna, C., Binaschi, M., Bigioni, M., Maggi, C. A., Parente, D., Forte, N., and Colantuoni, V.: MicroRNA-130b promotes tumor development and is associated with poor prognosis in colorectal cancer, Neoplasia, 15, 1218–1231, 2013.

Coussens, L. M. and Werb, Z.: Inflammation and cancer, Nature, 420, 860–867, 2002.

Cullen, B. R.: Transcription and processing of human microRNA precursors, Mol. Cell, 16, 861–865, 2004.

Fu, Y. F., Du, T. T., Dong, M., Zhu, K. Y., Jing, C. B., Zhang, Y., Wang, L., Fan, H. B., Chen, Y., Jin, Y., Yue, G. P., Chen, S. J., Chen, Z., Huang, Q. H., Jing, Q., Deng, M., and Liu, T. X.: Mir-144 selectively regulates embryonic a-hemoglobin synthesis during primitive erythropoiesis, Blood, 113, 1340–1349, 2009.

Gu, Y., Li, M., Wang, T., Liang, Y., Zhong, Z., Wang, X., Zhou, Q., Chen, L., Lang, Q., He, Z., Chen, X., Gong, J., Gao, X., Li, X., and Lv, X.: Lactation-related MicroRNA expression profiles of porcine breast milk exosomes, PLoS One, 7, e43691, https://doi.org/10.1371/journal.pone.0043691, 2012.

Kim, V. N.: MicroRNA biogenesis, coordinated cropping and dicing, Nat. Rev. Mol. Cell Bio., 6, 376–385, 2005.

Lapteva, N., Yang, A. G., Sanders, D. E., Strube, R. W., and Chen, S. Y.: CXCR4 knockdown by small interfering RNA abrogates breast tumor growth in vivo, Cancer Gene Ther., 12, 84–89, 2005.

Li, B. L., Lu, C., Lu, W., Yang, T. T., Qu, J., Hong, X., and Wan, X. P.: miR-130b is an EMT-related microRNA that targets DICER1 for aggression in endometrial cancer, Med. Oncol., 30, 484–484, 2013.

Li, Z. X., Wang, H. L., Chen, L., Wang, L. J., Liu, X. L., Ru, C. X., and Song, A. L.: Identification and characterization of novel and differentially expressed microRNAs in peripheral blood from healthy and mastitis Holstein cattle by deep sequencing, Anim. Genet., 45, 20–27, 2014a.

Li, Z. X., Zhang, H. L., Song, N., Wang, H. L., Chen, L., Zhai, M. X., and Liu, X. L.: Molecular cloning, characterization and expression of miR-15a-3p and miR-15b-3p in dairy cattle, Mol. Cell. Probe., 28, 255–258, 2014b.

Livak, K. J. and Schmittgen, T. D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods, 25, 402–408, 2001.

Mellado, M., Rodríguez-Frade, J. M., Mañes, S., Martínez-A, C.: Chemokine signaling and functional responses: The role of receptor dimerization and TK pathway activation, Annu. Rev. Immunol., 19, 397–421, 2001.

Nash, D. L., Rogers, G. W., Cooper, J. B., Hargrove, G. L., and Keown, J. F.: Heritability of intramammary infections at first parturition and relationships with sire transmitting abilities for somatic

cell score, udder type traits, productive life, and protein yield, J. Dairy Sci., 86, 2684–2695, 2003.

Sangokoya, C., Telen, M. J., and Chi, J. T.: microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease, Blood, 116, 4338–4348, 2010.

Vasudevan, S., Peltz, S. W., and Wilusz, C. J.: Non-stop decay – a new mRNA surveillance pathway, Bioessays, 24, 785–788, 2002.

Wagner, E. and Lykke-Andersen, J.: mRNA surveillance: the perfect persist, J. Cell Sci., 115, 3033–3038, 2002.

Wu, G., Bazer, F. W., Datta, S., Johnson, G. A., Li, P., Satterfield, M. C., and Spencer, T. E.: Proline metabolism in the conceptus: implications for fetal growth and development, Amino Acids, 35, 691–702, 2008.

Wu, G., Bazer, F. W., Davis, T. A., Kim, S. W., Li, P., Marc Rhoads, J., Carey Satterfield, M., Smith, S. B., Spencer, T. E., and Yin, Y.: Arginine metabolism and nutrition in growth, health and disease, Amino Acids, 37, 153–168, 2009.

Zhao, G., Zhang, J. G., Shi, Y., Qin, Q., Liu, Y., Wang, B., Tian, K., Deng, S. C., Li, X., Zhu, S., Gong, Q., Niu, Y., and Wang, C. Y.: MiR-130b is a prognostic marker and inhibits cell proliferation and invasion in pancreatic cancer through targeting STAT3, PLoS One, 8, e73803, https://doi.org/10.1371/journal.pone.0073803, 2013.

Zhou, Q., Li, M., Wang, X., Li, Q., Wang, T., Zhu, Q., Zhou, X., Wang, X., Gao, X., and Li, X.: Immune-related MicroRNAs are abundant in breast milk exosomes, Int. J. Biol. Sci., 8, 118–123, 2012.