Palma-Vera and Einspanier *Reproductive Biology and Endocrinology* (2016) 14:22
DOI 10.1186/s12958-016-0156-y

Reproductive Biology and Endocrinology

SHORT COMMUNICATION Open Access

# Experimental and bioinformatic analysis of cultured Bovine Endometrial Cells (BEND) responding to interferon tau (IFNT)

Sergio E. Palma-Vera[1,2] and Ralf Einspanier[1*]

## Abstract

**Background:** In ruminants, embryo implantation depends on progesterone (P4) and interferon tau (IFNT) controlling endometrial function. IFNT antagonizes bovine endometrial cells (BEND) response to phorbol 12,13-dibutyrate (PDBU) through posttranscriptional regulation of gene expression. We have previously described microRNAs (miRNAs) profiles in bovine endometrium, detecting miR-106a, relevant for embryo maternal communication. In this study, we investigated the expression miR-106a and genes for prostaglandin-endoperoxide synthase 2 (PTGS2), phospholipase A2, group IVA (PLA2G4A), estrogen receptor 1 (ESR1) and progesterone receptor (PR) in response to IFNT in BEND cells and searched for interferon responsive factors (IRFs) binding sites in their promoter genomic regions. The aim of this study was to unravel the molecular mechanisms involved in IFNT signalling and its regulation of miR-106a.

**Findings:** *PTGS2* showed increased expression under PDBU, which was antagonized by IFNT. IFNT induced expression of *PR* and miR-106a and downregulation of *ESR1* and *PR*. Bioinformatic analyses detected that *PLA2G4A* was associated to IRF-1 and IRF-6, while *ESR1*, *PR* and *PTGS2* were associated to only IRF-6. All genes exhibit one motif per IRF, except miR-106a that had three binding sites for IRF-6.

**Conclusions:** We report the IFNT regulatory effect on miR-106a expression through IRF-6 in bovine endometrial cells. We identified a set of potential binding sites for IRF-1 and IRF-6 within the bovine genome. A set of candidate gene regions could be characterized where IFNT can act via IRFs to regulate the expression of proteins and miRNAs. Future studies will use these data to detect new IFNT regulatory mechanisms in the endometrium.

**Keywords:** Endometrium, Interferon tau, MiRNA, Promoter region

## Introduction

Failed embryo implantation is one of the main causes of poor reproductive performance in cattle [1]. Implantation in ruminants depends on uterine receptivity derived from ovarian progesterone (P4) and embryonic interferon tau (IFNT) signalling in endometrial cells. Here, both P4 and IFNT are able to regulate the expression of estrogen receptor 1 (ESR1) [2]. Together, they modulate genes involved in endometrial attachment of the trophectoderm and suppress the luteolytic release of prostaglandin F2 alpha (PGF2alpha) by the endometrium [2–4]. The response of endometrial cells to IFNT has been shown to be dependent of IFN regulatory factors (IRFs) [5]. There are nine mammalian IRFs, which share a conserved 115 aminoacid N-terminal DNA binding domain (DBD) that binds to the promoter region of target genes [6].

Bovine endometrial cells (BEND) [7] provide a model to understand prostaglandin (PG) biosynthesis in response to IFNT. Stimulation of PG production in BEND cells leads to an increased expression of the enzymes prostaglandin-endoperoxide synthase 2 (PTGS2) and phospholipase A2, group IVA (PLA2G4A) and production of PGF2alpha, and these responses are diminished by IFNT, through a transcriptional dependent process [8–11].

MicroRNAs (miRNAs) are short non-coding RNA molecules controlling gene expression [12]. Studies in cattle have identified miRNAs within the endometrium regulating subclinical endometritis and fertility [13, 14]. However, studies are missing describing miRNAs involved in embryo maternal communication. The miR-106a is known to

* Correspondence: ralf.einspanier@fu-berlin.de
[1]Institute of Veterinary Biochemistry, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany
Full list of author information is available at the end of the article

BioMed Central

© 2016 Palma-Vera and Einspanier. **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

**Table 1** List of primers used for quantitative RT-PCR amplification

| | Forward primer (5′-3′) | Reverse primer (5′-3′) | Product length (bp) | Annealing temperature (°C) | Gene bank accession |
|---|---|---|---|---|---|
| PTGS2 | CTG AGT ACT TTT GAC TGT GGG AG | CTC TTC CTC CTG TGC CTG AT | 359 | 60 | NM_174445 |
| PLA2G4A | AAA TGT CAG CCA CAA CCC TC | ATG GAG GGT GAA AAG CG | 229 | 56 | NM_001075864.1 |
| PR | GAG AGCT CAT CAA GGC AAT TGG | CAC CAT CCC TGC CAA TAT CTTG | 227 | 60 | NM_001205356.1 |
| ESR1 | AGG GAA GCT CCT ATT TGC TCC | CGG TGG ATG TGG TCC TTC TCT | 234 | 58 | AY538775 |
| SDHA | GGG AGG ACT TCA AGG AGA GG | CTC CTC AGT AGG AGC GGA TG | 219 | 60 | DQ386895.1 |
| SUZ12 | TTC GTT GGA CAG GAG AGA CC | GTG CAC CAA GGG CAA TGT AG | 286 | 60 | NM_001205587.1 |
| ACTB | CGG TGC CCA TCT ATG AGG | GAT GGT GAT GAC CTG CCC | 266 | 58 | AY141970 |
| GAPDH | CCC AGA AGA CTG TGG ATG G | AGT CGC AGG AGA CAA CCT G | 306 | 32 | U85042 |

have roles embryo-endometrial cross talk [15–19]. We have previously characterized the expression of miRNAs in bovine endometrium across the estrous cycle and detected the expression of miR-106a [20]. In this study, we aimed to assess the effects of IFNT on miR-106a expression and to predict the location of genomic binding sites for interferon responsive factors (IRFs) that can regulate the expression of genes involved in endometrial response to embryo implantation.

## Material and methods

### BEND cell culture

Immortalization of BEND cells has been previously described [7]. They are distributed by the American Type Culture Collection (ATCC, Manassas, USA), whose indications for handling were followed. BEND cells are able to respond to phorbol 12,13-dibutyrate (PDBU), an activator of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signalling pathway, increasing the production of prostaglandins. This effect is antagonized by IFNT [8, 10, 11].

### Experimental design

$5 \times 10^4$ cells per mL medium (40 % Ham's F-12 (Biochrom), 40 % EMEM (ATCC), 200 U insulin/L (Sigma–Aldrich), 50 μg gentamicin (Biochrom), 10 % FBS (Biochrom), 10 % horse serum (ATCC)) were plated into wells of a 12 well plate (Greiner Bio-One) and grown to ~ 90 % confluence at 37 °C and 5 % $CO_2$. Cells were washed with D-PBS and equilibrated in serum free medium for 45 min at 37 °C, 5 % $CO_2$. Next, cells were cultured for 6 h with the following treatments: vehicle control, PDBU (100 ng/mL, Sigma–Aldrich), IFNT (50 ng/mL, source see below), P4 (10 ng/mL, Sigma–Aldrich), PDBU + IFNT, PDBU + P4, IFNT + P4, PDBU + IFNT + P4. Doses of IFNT and PDBU were applied as described previously [9], while P4 dose was selected according to the luteal phase levels in cattle [21]. Total RNA was extracted following the instructions of the kit's manufacturer (mirVana™, Life Technologies). The quality and quantity of the resulting RNA was measured by absorbance at 260 nm (NanoDrop 8000, Thermo Scientific). Recombinant ovine IFNT (antiviral activity, $1 \times 10^8$ U/mg) was kindly donated by Dr. F.W. Bazer (Texas A&M University, College Station, TX, USA).

### RT-qPCR for miRNAs and mRNAs

Quantitative RT-PCR was performed as described previously [22] and miRNA was quantified implementing the miR-Q method [23]. For protein coding gene transcripts, primers and annealing temperatures are indicated in Table 1. For miRNAs, primers are indicated in Table 2. All oligonucleotides were purchased from Sigma–Aldrich. Expression levels of mRNA and miRNAs were determined in duplicate and relative gene expression was calculated applying the method described by Livak and Schmittgen [24], correcting for PCR efficiency. Four housekeeper genes (*SDHA*, *ACTB*, *GAPDH*, *SUZ12*) were tested for normalization of protein coding gene expression. The two most stable genes were selected by using the GeNorm algorithm [25]. For miRNA normalization, bta-miR-99a-5p was selected as reference, since its expression was not affected by any of the treatments. All amplicons were validated by DNA sequencing at GATC Biotech AG (Konstanz, Germany).

**Table 2** Oligonucleotides for miR-Q PCR amplification

| | Primer sequence (5′-3′) | |
|---|---|---|
| bta-miR-106a | RT6-miRNA | TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT TAC CTG |
| | miRNA-rev | CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GAA AAG TGC TTA CAG TG |
| bta-miR-99a-5p | RT6-miRNA | TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT ACA AGA |
| | miRNA-rev | CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC G AAC CCG TAG ATC CGA TCT |

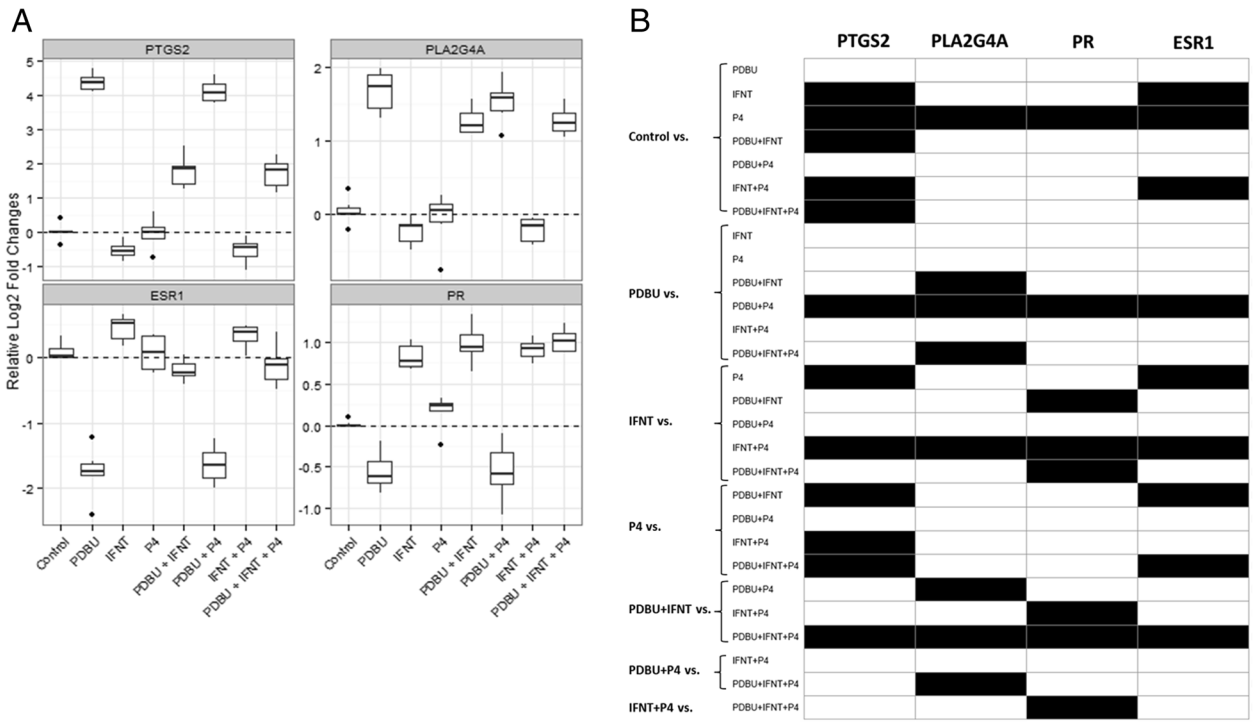


**Fig. 1** Regulation of PTGS2, PLA2G4A, ESR1 and PR expression in BEND cells. **a** Normalized log 2 fold change mRNA expression of characteristic genes for BEND cells in response to treatment with PDBU, IFNT, P4 and their combinations. Transcript expression was normalized to a combination of two housekeeping genes (SUZ12 and SDHA). **b** Matrix of significances (white: $p < 0.05$; black: $p > 0.05$). For each experiment, six biological replicates were used. Outliers are indicated as single dots above or below the whiskers

### Statistical and bioinformatics analysis

Data for gene expression are presented as boxplots. Depending on whether or not data showed normality, analysis of variance (ANOVA) or Kruskal-Wallis rank sum test were applied, followed by the post-hoc tests Bonferroni or Mann-Whitney *U*-test, respectively.

Candidate IRFs binding sites to DNA promoter gene regions were performed in R, applying the corresponding Bioconductor workflow. Binding motifs for IRFs were retrieved from MotifDb and then matched to the promoter regions of protein coding and miRNA coding genes of the bovine genome (UMD3.1.1).

## Results and discussion

### Regulation of PTGS2 and PLA2G4A

Previous studies have described the antagonizing effect of IFNT when PDBU was added to BEND cells. The result was a reduction of the mRNA of *PTGS2* and *PLA2G4A* [8, 9, 11]. In our study, *PTGS2* and *PLA2G4A* were upregulated by PDBU. For *PTGS2*, the PDBU effect was antagonized by IFNT, but this was not observed for *PLA2G4A* (Fig. 1). The lack of *PLA2G4A* regulation implies a stronger effect of IFNT on the expression of *PTGS2* and a reduced effect on the expression of *PLA2G4A*. It has been shown that IFNT antagonizes the effect PDBU on the protein levels of *PLA2G4A* [9]. Thus, it could be possible that at

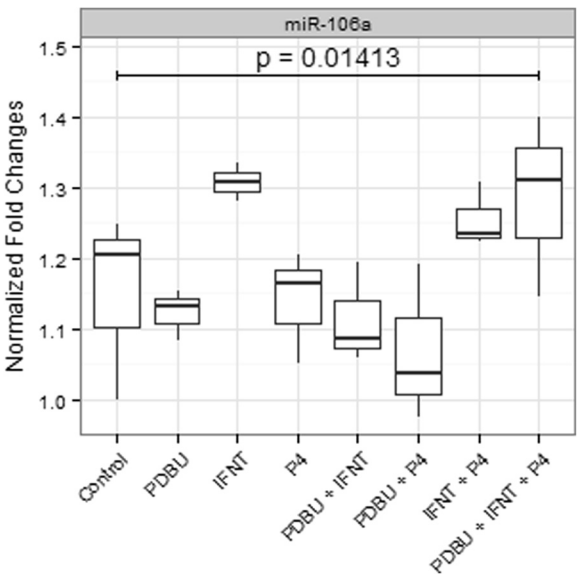


**Fig. 2** Normalized fold change expression of miR-106a for BEND cells. Expression was normalized to a stable unregulated miRNA (bta-miR-99a-5p). For each experiment, six biological replicates were used

the mRNA level, this effect remains inconspicuous. Nevertheless, the downregulation of *PTGS2* corroborates the validity of our assays.

### IFNT upregulates PR and PDBU downregulates estrogen and progesterone receptors

We detected a significant upregulation of progesterone receptor (PR), but not ESR1 transcripts upon IFNT signalling (Fig. 1). To our knowledge, these results have not been reported in the BEND cell model before. From the physiological point of view, upregulation of *PR* by IFNT is reasonable, due to the positive role of P4 in maintaining pregnancy and its permissive effect on IFNT activity. However, in vivo implantation events are preceded by loss of expression of *PR* and *ESR1* [4]. Such discrepancy might be explained by the nature of the BEND cell line, where not all physiological properties are preserved after establishment.

However, IFNT was able to induce a significant increase of PR mRNA expression. This effect remained when IFNT was combined with P4 and PDBU. On the other hand, *ESR1* and *PR* expression was reduced in response to PDBU and this effect was reversed by IFNT in different magnitudes: *ESR1* returned to basal levels and *PR* was 2 folds upregulated. Unlike IFNT, P4 was not able to reverse the effects of PDBU on *ESR1* and *PR* expression.

### Expression of miR-106a is regulated by IFNT

An overall significant effect was detected on the expression of miR-106a (Fig. 2). This effect was most likely due to the activity of IFNT, which increased the expression of miR-106a approximately 30 % when applied alone. Also, when IFNT was applied with P4 and PDBU plus P4, a similar increment was detected. The only treatment group where the regulatory effect of IFNT was not observed when IFNT was added in combination with PDBU. This indicates that PDBU might counter-regulate the activity of IFNT and P4 ameliorates this effect.

Evidence showed that miR-106a responds to IFNT alone and in combination with P4. This is physiologically

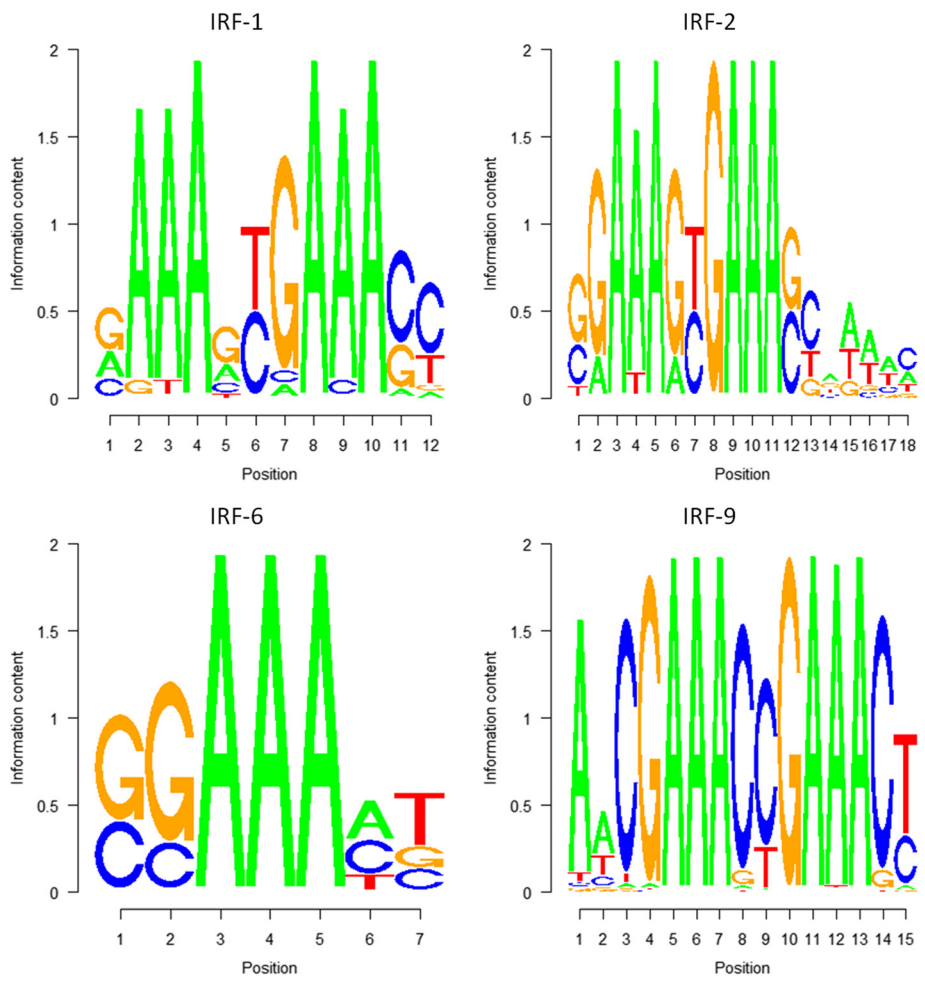


**Fig. 3** Sequence logos for IRF-1, IRF-2, IRF-6 and IRF-9 binding motifs. Sequences were retrieved from MotifDb and searched in the bovine genomic regions corresponding to the promoters of *PTGS2*, *PLA2G4A*, *ESR1*, *PR* and *MIR106A*

relevant, since progesterone is permissive for IFNT activity [4]. On the other hand, when IFNT combined with PDBU were applied, miR-106a expression was not affected, pointing towards a counter-regulation of PDBU over IFNT. Considering that PDBU action is analogous to the activity of oxytocin, e.g. induction of PGF2alpha production, this event parallels the physiology of embryo maternal communication. Therefore, it is possible that miR-106a contributes to the control of endometrial responses to IFNT and oxytocin.

### IRF-1 and IRF-6 are found in the promoter regions of regulated genes

We searched for the binding sites of IRFs in the bovine genome at the promoter site of known genes. Binding sites were determined by the presence of DNA motifs for a specific IRF. These motifs can be visualized as sequence logos in Fig. 3, showing the frequency of nucleotides at each position of the sequence. IRFs binding sites lengths ranged from 7 (IRF-6) to 18 (IRF-2), all having adenines as the most prevalent nucleotides. IRFs were selected based on previous studies, as they are known to be present in the endometrium of ruminants [5]. For protein coding genes, there were severe differences in the number of binding sites: IRF-6 was identified more than 40 thousand times, while IRF-1, 2 and 9 lay far behind (Table 3). A similar pattern was detected for miRNA coding genes. We decided to search for promoter binding sites at genes relevant for BEND function and miR-106a, leaving out thousands of genomic regions where IRFs can bind. These regions may regulate the expression of other genes and miRNAs. Future experimental studies will define what their roles are in order to detect pathways controlled by IFNT in BEND cells.

We found that for all the protein coding genes relevant to BEND cell function, IRF-1 and 6 had binding sites in the promoter regions. Interestingly, miR-106a was 3x enriched for IRF-6 in its promoter region (Table 4). In this context, it has been reported that IRF6 could play a critical role in endometrial gene expression and trophectoderm growth [5]. This can explain the upregulation of miR-106a when BEND cells are treated with IFNT and imply a potential role of this miRNA in embryo maternal communication in cattle.

**Table 3** Number of binding sites per IRF for protein and miRNA coding genes promoter regions

| | IRF-1 | IRF-2 | IRF-6 | IRF-9 |
|---|---|---|---|---|
| Protein coding genes promoters | 2603 | 83 | 40315 | 5 |
| miRNA coding genes promoters | 54 | 2 | 1135 | 0 |

**Table 4** IRF enrichment in promoter regions for *ESR1*, *PR*, *PTGS2*, *PLA2G4A* and *MIR106A*

| IRF | Number of binding sites | Gene |
|---|---|---|
| IRF-1 | 1 | *PLA2G4A* |
| IRF-6 | 1 | *PR* |
| IRF-6 | 1 | *PTGS2* |
| IRF-6 | 1 | *ESR1* |
| IRF-6 | 1 | *PLA2G4A* |
| IRF-6 | 3 | *MIR106A* |

## Conclusions

We present evidence that miR-106a in a bovine endometrial cell culture (BEND) is regulated by IFNT. IFNT might induce binding of IRF-6 to the promoter region of miR-106a inducing its expression. This study shows that bioinformatic methods for detecting IRF binding sites in the genome can explain and support the observed experimental data. In the future, these data sets may be used to search for more candidate genes involved in embryo maternal communication. Finally, the BEND cell model, provides a simple and reliable cell system for discovering key regulators of bovine fertility, such as miRNAs.s

#### Abbreviations

BEND: bovine endometrial cells; ESR1: estrogen receptor 1; IFNT: interferon tau; IRF: interferon responsive factor; MAPK: mitogen-activated protein kinase; miRNA: MicroRNA; P4: progesterone; PDBU: phorbol 12,13-dibutyrate; PG: prostaglandin; PGF2alpha: Prostaglandin F2 alpha; PKC: protein kinase C; PLA2G4A: phospholipase A2, group IVA; PR: progesterone receptor; PTGS2: prostaglandin-endoperoxide synthase 2.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

SP was involved in the study design, acquisition of data, analysis and interpretation of data, and paper drafting. RE was involved in the study design, supervision, paper drafting and contributed to the interpretation of the data. All authors read and approved the final manuscript.

#### Acknowledgements

Recombinant ovine IFNT (antiviral activity, 1 × 10 8 U/mg) was kindly donated by Dr. F.W. Bazer (Texas A&M University, College Station, TX).
This study was supported by the German Academic Exchange Service (DAAD).

#### Author details

[1]Institute of Veterinary Biochemistry, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany. [2]Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany.

Received: 2 February 2016 Accepted: 10 April 2016
Published online: 18 April 2016

#### References

1. Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim. 2008;43:260–7.
2. Bazer FW. Pregnancy recognition signaling mechanisms in ruminants and pigs. J Anim Sci Biotechnol. 2013;4:23.

3. Bazer FW, Spencer TE, Ott TL. Interferon tau: a novel pregnancy recognition signal. Am J Reprod Immunol. 1997;37:412–20.
4. Bazer FW, Burghardt RC, Johnson GA, Spencer TE, Wu G. Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. Reprod Biol. 2008;8:179–211.
5. Fleming J-AGW, Song G, Choi Y, Spencer TE, Bazer FW. Interferon regulatory factor 6 (IRF6) is expressed in the ovine uterus and functions as a transcriptional activator. Mol Cell Endocrinol. 2009;299: 252–60.
6. Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant MJ, LePage C, DeLuca C, Kwon H, Lin R, Hiscott J. Interferon regulatory factors: the next generation. Gene. 1999;237:1–14.
7. Staggs KL, Austin KJ, Johnson GA, Teixeira MG, Talbott CT, Dooley VA, Hansen TR. Complex induction of bovine uterine proteins by interferon-tau. Biol Reprod. 1998;59:293–7.
8. Binelli M, Guzeloglu A, Badinga L, Arnold DR, Sirois J, Hansen TR, Thatcher WW. Interferon-tau modulates phorbol ester-induced production of prostaglandin and expression of cyclooxygenase-2 and phospholipase-A(2) from bovine endometrial cells. Biol Reprod. 2000; 63:417–24.
9. Godkin JD, Roberts MP, Elgayyar M, Guan W, Tithof PK. Phospholipase A2 regulation of bovine endometrial (BEND) cell prostaglandin production. Reprod Biol Endocrinol. 2008;6:44.
10. Guzeloglu A, Michel F, Thatcher WW. Differential effects of interferon-τ on the prostaglandin synthetic pathway in bovine endometrial cells treated with phorbol ester. J Dairy Sci. 2004;87:2032–41.
11. Guzeloglu A, Subramaniam P, Michel F, Thatcher WW. Interferon-tau induces degradation of prostaglandin H synthase-2 messenger RNA in bovine endometrial cells through a transcription-dependent mechanism. Biol Reprod. 2004;71:170–6.
12. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010;11:597–610.
13. Ponsuksili S, Tesfaye D, Schellander K, Hoelker M, Hadlich F, Schwerin M, Wimmers K. Differential expression of miRNAs and their target mRNAs in endometria prior to maternal recognition of pregnancy associates with endometrial receptivity for in vivo- and in vitro-produced bovine embryos. Biol Reprod. 2014;91:135.
14. Hailemariam D, Ibrahim S, Hoelker M, Drillich M, Heuwieser W, Looft C, Cinar MU, Tholen E, Schellander K, Tesfaye D. MicroRNA-regulated molecular mechanism underlying bovine subclinical endometritis. Reprod Fertil Dev. 2014;26:898–913.
15. Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL, Salamonsen LA. Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. PLoS One. 2013;8:e58502.
16. Bidarimath M, Khalaj K, Wessels JM, Tayade C. MicroRNAs, immune cells and pregnancy. Cell Mol Immunol. 2014;11:538–47.
17. Pan Q, Chegini N. MicroRNA signature and regulatory functions in the endometrium during normal and disease states. Semin Reprod Med. 2008;26:479–93.
18. Su L, Liu R, Cheng W, Zhu M, Li X, Zhao S, Yu M. Expression patterns of MicroRNAs in porcine endometrium and their potential roles in embryo implantation and placentation. PLoS One. 2014;9:e87867.
19. Kumar P, Luo Y, Tudela C, Alexander JM, Mendelson CR. The c-Myc-regulated microRNA-17 ~ 92 (miR-17 ~ 92) and miR-106a ~ 363 clusters target hCYP19A1 and hGCM1 to inhibit human trophoblast differentiation. Mol Cell Biol. 2013;33:1782–96.
20. Palma-Vera SE, Sharbati S, Einspanier R. Identification of miRNAs in bovine endometrium through RNAseq and prediction of regulated pathways. Reprod Domest Anim. 2015;50:800–6.
21. Roberson MS, Wolfe MW, Stumpf TT, Kittok RJ, Kinder JE. Luteinizing hormone secretion and corpus luteum function in cows receiving two levels of progesterone. Biol Reprod. 1989;41:997–1003.
22. Scholven J, Taras D, Sharbati S, Schon J, Gabler C, Huber O, Meyer zum Buschenfelde D, Blin N. Intestinal expression of TFF and related genes during postnatal development in a piglet probiotic trial. Cell Physiol Biochem. 2009;23:143–56.
23. Sharbati-Tehrani S, Kutz-Lohroff B, Bergbauer R, Scholven J, Einspanier R. miR-Q: a novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample. BMC Mol Biol. 2008;9:34.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402–8.
25. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002; 3:RESEARCH0034.



Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit