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

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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 trophoectoderm 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...
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 × 10⁴ 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 % CO₂. Cells were washed with D-PBS and equilibrated in serum free medium for 45 min at 37 °C, 5 % CO₂. 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 × 10⁸ 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).

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

| Gene bank accession | Product length (bp) | Annealing temperature (°C) | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|---------------------|---------------------|--------------------------|------------------------|------------------------|
| NM_174445           | 359                 | 60                       | CTG AGT ACT TTT GAC TGT GGG AG | CTC TTC CTC TGT TGC CTG AT |
| NM_001075864.1      | 229                 | 56                       | AAA TGT CCA CAA CCC TC | ATG GAG GTG GAA AAG CG |
| NM_001205356.1      | 227                 | 60                       | GAG AGT CAT CAA GGC AAT TGG | CAC CAT CCC TCG CAA TAT CTTG |
| AYS38775            | 234                 | 58                       | AGG GAA GCT CCT ATT TGC TCC | CCG TGG ATG TGG TCC TTC TTC |
| DQ38689.1           | 219                 | 60                       | GGG AGG ACT TCA AGG AGA AGG | CTC CTC AGT AGG AGC GGA TG |
| NM_001205587.1      | 286                 | 60                       | TTC GTG GGA CAG GAG AGA CC | GTG CAC CAA GGG CAA TGT AG |
| U85042              | 266                 | 58                       | CGG TGC CCA TCT ATG AGG | GAT GTG GAT GAC CTG CCC |
|                     | 306                 | 32                       | CCC AGA AGA CTG TGG ATG G | AGT CGC AGG AGA CAA CCT G |

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

| Primer sequence (5’-3’) | bta-miR-106a | bta-miR-99a-5p |
|-------------------------|--------------|---------------|
| RT6-miRNA               | TGT CAG GCA ACC GTA TCC ACC GTG AGT GTC CTG |
| miRNA-rev               | CGT CAG ATG TCC GAG TAG AGG GGG AAG GCC GAA AAG TGC TTA CAG TG |
| RT6-miRNA               | TGT CAG GCA ACC GTA TCC ACC GTG AGT GGC ACA AGA |
| miRNA-rev               | CGT CAG ATG TCC GAG TAG AGG GGG AAG GCC G AAC CGC TAG ATC CGA TCT |
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
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...
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                      | RTGS2 |
| IRF-6   | 1                      | ESR1  |
| 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.

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