In vitro effects of relaxin on gene expression in porcine cumulus-oocyte complexes and developing embryos

Jean M Feugang1*, Jonathan M Greene1, Scott T Willard2, Peter L Ryan1,3*

Abstract

Background: Relaxin hormone peptide is found in porcine follicular and utero-tubal fluids, but its possible actions during early embryo development are still undetermined. Here, we investigated the effects of porcine relaxin during oocyte maturation and embryo development, and gene expression in the pig.

Methods: Immature cumulus-oocyte complexes (COCs) were obtained from ovarian follicles of sows. In experiment 1, COCs were matured in the presence of 0, 20, or 40 ng relaxin/ml, or 10% (v/v) porcine follicular fluid. In experiment 2, COCs were in vitro matured, fertilized and resulting embryos were cultured in the presence of 0, 20, or 40 ng relaxin/ml. In experiment 3, COCs were matured in the presence of 40 ng relaxin/ml, fertilized and zygotes were cultured as indicated in experiment 2. We evaluated the proportions of matured oocytes in experiment 1, cleaved and blastocysts on Day 2 and Day 7 post insemination in all experiments. The total cell number of blastocysts was also evaluated. In parallel, transcription levels of both relaxin and its receptors (RXFP1 and RXFP2), as well as a pro- (Bax) and anti- (Bcl2-like 1) apoptotic-related genes were determined. All data were analyzed by ANOVA and significant differences were fixed for P < 0.05.

Results: In experiment 1, relaxin significantly increased the proportions of matured oocytes and cleaved embryos, as well as the expression level of RXFP2 mRNA compared to RXFP1 (P < 0.05). There was no effect on endogenous expression of relaxin and Bcl2-like1/Bax ratios. In all experiments, relaxin did not affect the proportions of blastocysts, but did significantly increase their total cell numbers (P < 0.05). Furthermore, no effect of relaxin was observed on Bcl2-like1/Bax expression ratios, which were similar between groups.

Conclusions: Exogenous relaxin influences its own receptors expression, improves oocyte nuclear maturation. Its beneficial effect on total cell number of blastocysts appears to be through a Bcl2-like1/Bax-independent mechanism.

Background

Gene expression is one of the major events occurring during oocyte maturation and early embryo development, and its perturbation in culture system may contribute to the limited production of high quality blastocysts [1-3]. Numerous strategies such as low oxygen tension during culture, co-culture, and utilization of simple or complex media supplemented with various molecules have been developed to improve embryo culture efficiencies [1,4-6]. These molecules such as relaxin are generally found in the vicinity of oocytes and embryos. Relaxin is a peptide hormone found at higher levels in follicular fluids and genital tract secretions of various species [7-11]. Despite its pleiotropic roles reported in various reproductive and non-reproductive tissues [11-13], little is still known on embryonic effects of relaxin during early pregnancy.

In livestock, the pig is the primary animal model used for the study of relaxin effects [11,14,15] due to the important production of relaxin in both pregnant and non-pregnant pigs. Indeed, pigs exhibit high levels of relaxin in ovarian follicular fluids [8,14], and its secretions by corpora lutea and uterine cells significantly increase during the post-ovulatory period [11,14,16-18].
Numerous studies have reported various actions of relaxin on pig reproductive tissues such as cervical and uterine tissues, including modulation of embryo implantation during pregnancy in rats [19–21]. Despite these physiological conditions, it is not known whether relaxin influences oocyte maturation and/or embryo development in the pig.

Relaxin hormone peptide exerts autocrine, endocrine, and paracrine effects through membrane receptors known as RXFP1 and RXFP2 [22–24]. These receptors have been detected in porcine reproductive tissues [25,26], including oocytes and embryos [27]. But, their potential activation by relaxin and further implications on oocyte maturation and embryo development are still unknown.

From this background, the present study aims to bring more insight into the biological functions of relaxin during early pregnancy using pigs as experimental models. We evaluated the effect of relaxin on the proportion of oocytes resuming nuclear maturation and on embryo development, as well as the total cell number of blastocysts. We determined the effects of relaxin on endogenous relaxin, RXFP1, RXFP2, and pro- (Bax) and anti- (Bcl2-like1) apoptotic-related genes expressions in porcine oocytes and in vitro produced embryos.

Methods

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA) for embryo production or Invitrogen Co. (Carlsberg, CA, USA) for gene expression. Lyophilised relaxin hormone (pRLN) produced from pregnant sow ovaries was a gift from Dr. C Bagnell [15]. It was dissolved in deionized water (10 μg/ml), aliquoted, and stored as stock solution at -80°C. Pre-warmed solutions of NaCl (0.9%; w/v) and Hepes-buffered Tyrode Lactate solution (Vigro-Bioniche, Pullman, WA, USA) supplemented with polyvinyl alcohol (PVA: 0.1%; w/v) and pyruvate (100 μM) were used to wash the ovaries, and the oocytes and the embryos, respectively. Oocytes were matured in TCM199+L-glutamine medium supplemented with PVA (1%; w/v), glucose (2.8 mM), pyruvate (0.91 mM), cysteamine (0.57 mM), EGF (10 ng/ml), and FSH (0.4 μg/ml) and fertilized in the modified Tris-buffered medium (mTBM) containing caffeine (2 mM) and BSA-fraction V (0.1%; w/v). Embryos were cultured in NCSU-23 medium (Millipore, Billerica, MA, USA) supplemented with BSA-FAF (0.4%; w/v). All media contained 10 μl/ml antibiotics (gentamycin and penicillin G/Streptomycin sulphate) and were pre-incubated for at least 2 h before use. Oocytes and embryos were placed in 4-well dishes (Nunc, Roskilde, Denmark) for incubations under a humidified atmosphere of 39°C and 5% CO₂ in air.

Cumulus-oocyte complex collection, in vitro maturation (IVM), fertilization (IVF) and embryo culture (IVC)

Sow (Yorkshire-Landrace) ovaries were harvested at a local abattoir (Southern Quality Meats, Pontotoc, MS, USA) and washed in the laboratory. Immature cumulus-oocyte complexes (COCs) were aspirated from follicles of 3-6 mm diameter. Oocytes with homogenous ooplasm and surrounded by cumulus cells were selected for maturation per groups of 50-75 COCs in 500 μl of medium. After 44 h incubation, COCs were collected and transferred into 250 μl of fertilization medium. High motile spermatozoa of pooled semen (Prestage Farms, West-Point, MS, USA) were purified through a discontinuous percoll gradient (90%-45%) and 250 μl of suspended sperm cells (12 × 10⁵ spermatozoa/ml) was used to fertilize the COCs. Both gametes were co-incubated (Day 0 post-insemination or Day 0pi). Presumptive zygotes were harvested at 18 hpi and mechanically separated from the cumulus cells followed by the transfer of cumulus free-zygote into drops of cultured medium (1 embryo/1-2 μl) overlaid with mineral oil. Embryos were incubated for up to 6 days (Day 7pi).

Experiment 1: Developmental and gene expression effects of pRLN added during IVM

Cumulus-oocyte complexes were matured in the presence of 0, 20 or 40 ng/ml pRLN, or 10% (v/v) pFF. They were subsequently fertilized, and the presumptive zygotes were cultured until the blastocyst stage. All steps were processed as described above.

In a preliminary study aimed at evaluating our culture conditions, a total of 550 COCs (in 4 independent replicates) were matured in the presence of 10% pFF. Proportions of 44 ± 4% (mean ± SD) cleaved embryos and 17 ± 3% (mean ± SD) blastocysts (per cleaved: 40/229) were obtained.

Evaluation of oocyte maturation

COCs were collected after maturation (44 h) followed by a mechanical separation of oocytes and cumulus cells. Denuded oocytes were fixed in acetic acid: ethanol mix (1:3, v/v; 24 h at 4°C), stained with 1% aceto-orcein and mounted onto slides for nuclear status evaluation under a phase-contrast microscope (Nikon Instruments Inc.; Melville, NY). Oocytes with germinal vesicle, metaphase I, or metaphase II nucleus features were classified as immature (GV), resuming meiosis (MI), or mature (MII) oocytes.

Evaluation of embryo development

Proportions of cleaved and blastocyst stage embryos were evaluated on Day 2pi and Day 7pi, respectively.

Sample collection for gene expression

Groups of 30 immature and 30 mature COCs were mechanically separated from their respective cumulus cells under a stereomicroscope. Isolated cumulus cells
and cumulus free (or denuded) oocytes and other groups of 10 cleaved embryos (2-4 cells) and 3 blastocysts (Day 7pi) were separately selected under microscope based on morphological criteria (homogenous cytoplasm) for the gene expression study. All samples were stored at -80°C until analysis.

Experiment 2: Developmental and gene expression effects of pRLN added during IVM and IVC
Cumulus-oocyte complexes were maturated in the presence of 0, 20 or 40 ng pRLN/ml followed by their fertilization. Resulting zygotes were cultured for up to 6 days (Day 7pi) in the presence of 0, 20 or 40 ng/ml of pRLN. All steps were processed as described above and embryo production was done in duplicate for developmental and gene expression studies.

Developmental evaluation of relaxin effects
Cleaved and blastocyst stage embryos were recorded on Day 2pi and Day 7pi, respectively. Day 7pi-blastocysts were placed on microscope slides, air dried, fixed (for 24 h in absolute alcohol), and stained with the Hoechst 33342 dye. Blastocyst cell numbers were evaluated under an Epifluorescence microscope (Nikon Inc., Melville, NY, USA).

Sample collection for gene expression
Groups of 10 zygotes, 10 cleaved (2-4 cells) and 3 blastocyst stage embryos were selected based on their morphology under a stereomicroscope and stored per groups at -80°C until analysis.

RNA isolation and RT-PCR
Total RNA were isolated from frozen-thawed samples (RNeasy Micro Kit, Qiagen Inc., Valencia, USA), reverse-transcribed into cDNA (Superscription III Platinum® Two-Step qRT-PCR Kit) which were used for detecting the expression levels of relaxin and its receptors (RXFP1 and 2) and apoptotic related genes (Bax and Bcl2-like1). Expression levels of β-actin in each biological sample were determined for normalization. To this end, semi-quantitative (Taq DNA polymerase kit) and real-time (SYBR® GreenER qPCR SuperMixes for iCycler) PCR techniques were employed. Table 1 summarizes the primer pairs’ characteristics. The PCR conditions were as followed: 5 min at 95°C; 45 cycles of [30 sec at 95°C, 30 sec at the optimal annealing temperature (Table 1) and 30 sec at 72°C]; 10 min at 72°C. Real time PCRs using tested (Bax, Bcl2-like1, Relaxin, RXFP1 and RXFP2) and internal control (β-actin) genes’ primer pairs were performed, and the comparative Ct method was used to determine the transcript levels as previously described [28]. Relaxin, RXFP1 and RXFP2 gene expression data are indicated as relative expression to β-actin, while individually normalized Bax and Bcl2-like1 to β-actin are shown as Bcl2-like1/Bax ratios. All PCR products were further resolved on 1.5% agarose gels to verify of the amplification quality given the size of the amplicon products.

In parallel, sections of electrophoresis gels containing relaxin, RXFP1 and RXFP2 PCR products generated from porcine neonates uterine cells (Ut) and ovarian corpora lutea (CL) samples were excised to test their authenticity. Agarose gel sections were purified and submitted for sequencing (BigDye terminator V1.1 cycle sequencing kit; Applied Biosciences Inc., Foster City, USA) followed by their BLAST on pig genome (NCBI repository database). Bax, Bcl2-like1 and β-actin primer sequences published by Wang et al. were used in this study [28].

Statistical analysis
All experiments were repeated at least three times and RNA samples obtained from each experimental replicate. One-way ANOVA (SYSTAT, Systat software Inc., Chicago, IL, USA) followed by the Fisher’s Least Square Difference test for pairwise comparisons were used to analyze the pRLN effects. The Student’s t-test was used to compare the expression levels of Bax, Bcl2-like1,

Table 1 Primer sequence characteristics

| Gene symbols | GenBank Acc.#, (NCBI) | Primer sequences (5’-3’) | AT (°C) | Amplicon sizes (bp) |
|--------------|----------------------|--------------------------|--------|---------------------|
| RXFP1        | CA994862.1           | S: AGGCTGACGAGGAACACT    | 52.5   | 132                 |
|              |                      | AS: CAGAACGAACCAAGCTT    |        |                     |
| RXFP2        | CA997681.1 (XM_001927967.1) | S: CATCTGCTGATTCCTGGAT | 55     | 117                 |
|              |                      | AS: TTCAAGCCTGGTCGTAC   |        |                     |
| RELAXIN      | NM213872.1           | S: TTGAGCTGCTCTCTCTCTCT | 55     | 164                 |
|              |                      | AS: GTAGGTCAGTCTCTCTCT   |        |                     |
| BCL2-like1   | NM214285.1           | S: TGAATCGAGGAGGAGACCTAG | 60     | 416                 |
|              |                      | AS: GCATCTGTGATGGATGGAGCTA |        |                     |
| BAX          | AJ606301             | S: TTCTGACGAGGAATCTGCACTG | 60     | 238                 |
|              |                      | AS: AGGCAACAAGATGGTCTGCTT |        |                     |
| β-ACTIN      | U07786               | S: ACGGATGTGAGGTGTCTC    | 60     | 397                 |
|              |                      | AS: AGTGAAGGTTGTTGATGAT  |        |                     |

S, sens; AS, AntiSens.
RXFP1, and RXFP2 mRNA within the sample type (MCC: mature cumulus cell, or MII: mature oocyte). Results are expressed as mean (± SD) for gene expression or (± SEM) for developmental data, and P < 0.05 are fixed for significant differences.

Results

Experiment 1: Development and gene expression effects of pRLN added IVM

Developmental effects

The presence of pRLN during IVM did not affect the cumulus cell expansion (data not shown); however, it did significantly increase the proportion of oocytes that resumed meiosis (79% ± 4%, 87% ± 3%, and 91% ± 3% for 0, 20 and 40 ng pRLN/ml, respectively, P < 0.05), and, subsequently, increased the proportions of oocytes that reached metaphase II (68% ± 5%, 80% ± 4% or 88% ± 4% for 0, 20 or 40 ng pRLN/ml, respectively, P < 0.05; ANOVA; Table 2).

Furthermore, we evaluated the effect of relaxin on embryo development by maturing a total of 1,169 COCs in the presence of 0, 20 and 40 ng pRLN/ml (4 to 7 independent replicates, Table 3). Only 40 ng pRLN/ml significantly increased the cleavage and blastocyst rates in comparison to the control group (51 ± 5% and 10 ± 3% vs. 37 ± 4% and 12 ± 3%, respectively; P < 0.05). There were no significant differences between the 20 ng pRLN/ml treatment and the control. Moreover, the mean cell number of blastocysts was significantly higher in the 40 ng pRLN/ml group (38 ± 3) compared to others (control: 31 ± 4 and 20 ng pRLN/ml: 32 ± 6; P < 0.05), which appeared similar.

Gene expression effects

We evaluated the effect of relaxin on Bax, Bcl2-like1, relaxin, RXFP1 and RXFP2 gene expression in both cumulus cells and oocytes. With exception of relaxin, all other gene transcripts were detected in both cell types (Figure 1, 2 and 3). Their expression levels in cumulus cells and oocytes. With exception of relaxin, all other gene transcripts were detected in both cell types (Figure 1, 2 and 3). Their expression levels in cumulus cells were always lower than that in oocytes (P < 0.05). The concentration of 40 ng pRLN/ml significantly increased RXFP2 mRNA transcript levels in oocytes and cumulus cells (Figure 1A; P < 0.05), but had no effect on Bcl2-like1/Bax ratios in both cell types (Figure 2). The presence of 10% porcine follicular fluid (pFF) significantly increased RXFP1 mRNA amounts in mature cumulus cells and oocytes, as well as the Bcl2-like1/Bax ratio in mature oocytes (Figure 1B and 2; P < 0.05). However, relaxin transcripts were not detected in oocytes, and neither pRLN nor pFF were able to induce its de novo expression (Figure 3A).

Table 2 Effect of relaxin on porcine oocyte maturation

| pRLN during IVM (ng/ml) | Total Oocytes (N) | GV% (n) | MI% (n) | MII% (n) |
|-------------------------|-------------------|---------|---------|---------|
| 0                       | 451               | 21 ± 4 (93)abc | 11 ± 5 (51)ab | 68 ± 5 (307)bc |
| 20                      | 472               | 13 ± 3 (61)abc | 7 ± 2 (31)abc | 80 ± 4 (380)abc |
| 40                      | 331               | 9 ± 3 (30)abc | 3 ± 3 (9)abc | 88 ± 4 (291)abc |

Table 3 Developmental effects of relaxin added during oocyte maturation

| pRLN during IVM (ng/ml) | Total Zygotes (N) | % of cleaved at Day 2pi (n) | Blastocyst formation at Day 7pi |
|-------------------------|-------------------|-----------------------------|--------------------------------|
|                         | Total (T) | % (T/N) | Cell number (n) |
|-------------------------|-----------|---------|----------------|
| 0                       | 383       | 37 ± 4 (141)ab | 45 | 12 ± 3 a | 31 ± 4 (160)ab |
| 20                      | 344       | 40 ± 4 (135)ab | 23 | 8 ± 4 a | 32 ± 6 (122)ab |
| 40                      | 442       | 51 ± 5 (226)ab | 46 | 10 ± 3 a | 38 ± 3 (12)ab |

Experimental study conducted with a total of 1,343 zygotes cultured in culture media containing 0 (control), 20, or 40 ng pRLN/ml. We found no significant effects of relaxin on the proportions of cleaved embryos and blastocysts. However, the mean cell numbers of blastocysts were significantly increased in the presence of pRLN (control: 31 ± 4, 20 ng/ml: 49 ± 12, 40 ng/ml: 38 ± 8; P < 0.05, ANOVA). Based on these results, we evaluated the effect of pRLN added during oocyte maturation (0 or 40 ng/ml) and embryo culture (0, 20, or 40 ng/ml).

Oocytes matured in the presence of 40 ng pRLN/ml followed the culture of resulting zygotes in the presence of 20 or 40 ng pRLN/ml (Treated-1 and Treated-2, respectively). Embryos produced without any exposure to pRLN served as the control group. A total of 949 were used in at least three independent replicates to evaluate the effect of pRLN added during both maturation (Treated-1) and embryo culture (Treated-2). Results summarized in Table 4 revealed a negative effect of pRLN on the cleavage rate in group Treated-1 (40-20) compared to Treated-2 (40-40) and control (33 ± 5% vs. 51 ± 8% and 48 ± 3%, respectively; P < 0.05), whereas no differences in term of blastocyst rates were observed. However, the mean cell number of blastocysts was significantly increased in the Treated-2 group (43 ± 6) compared to the control group (31 ± 3; P < 0.05).
Figure 1 Effect of porcine relaxin (pRLN) and follicular fluid (pFF) on RXFP1 and RXFP2 gene expression in 
in vitro matured oocyte 
porcine. Figures represent real-time PCR results of RXFP1 and RXFP2 gene expression in oocytes matured in the presence of 0 or 40 ng/ml 
pRLN (A), and 0 or 10% (v/v) pFF (B). Data are relative expression to their corresponding internal control, β-actin, and are shown as mean ± SD of at least three independent replications. Letters (a,b) represent significant differences between immature oocytes and cumulus for both genes, while asterisks (*) indicate significant differences between treated groups and their corresponding controls within the same gene (P < 0.05, ANOVA). Immature oocyte and cumulus cells are labelled as GV and ICC, while those that are matured in the presence of pRLN are indicated as MII-40 and MCC-40, or MII-pFF and MCC-pFF for those matured in the presence of pFF.
Gene expression effects

We evaluated the effect of 40 ng pRLN/ml added during oocyte maturation (IVM), embryo culture (IVC), or both periods (IVM/IVC) on Bax and Bcl2-like1 gene expression in cleaved and blastocyst stage embryos. Figures 3B and 3C show that the Bcl2-like1/Bax ratios were significantly higher in cleaved embryos compared to blastocysts (P < 0.05, t-test). However, the moment of relaxin exposure (IVM or IVC) had no significant effect on these ratios (ANOVA).

Discussion

Numerous physiological roles have been attributed to relaxin [10,11]; but its effects during embryo development are still unknown despite its presence in gamete and embryo environments. Here, we could not detect the expression of relaxin in porcine COCs but found an accumulation of its receptors RXFP1 and RXFP2 mRNA in COCs which appeared to be modulated by exogenous relaxin. In addition, relaxin moderately influenced embryo development but significantly increased the total cell number of blastocysts irrespective of the moment of its addition in the media (oocyte maturation and/or embryo culture).

The present study reports developmental effects of relaxin used at concentrations similar to those found in the literature [29-31]. Indeed, variable but large amounts of relaxin are found in follicular fluids surrounding porcine oocytes, while pre-implantation embryos are suspected to be exposed to unknown amounts of relaxin during their utero-tubal progression and development (corresponding to the ovarian luteal phase). Beneficial roles of relaxin have been demonstrated during follicle growth and ovulation [7,14], uterine receptivity and activity process in various species such as pigs [14], primates [32,33] and rodents [21,34,35]. To our knowledge, the current study is the first to report developmental
Figure 3 Effect of porcine relaxin (pRLN) on relaxin, pro- (Bax) and anti- (Bcl2-like 1) apoptotic genes expression in porcine embryos. Figure represent representative agarose gel of relaxin amplification through end-point PCR (A) and real-time PCR results of Bax and Bcl2-like1 amplifications (B). COCs were collected immature (GV) and matured in the presence of 0 (0), 40 ng pRLN/ml (40) or 10% pFF (pFF). Cleavage embryos and blastocysts were obtained from various experiments: exposure to 40 ng pRLN/ml during oocyte (IVM) or embryo culture (IVC), or during both periods (IVM/IVC). The control group corresponds to embryos produced without any exposure to pRLN. Embryos were analyzed per groups in three independent replicates. Data were expressed as relative to their corresponding internal control, β-actin, and shown as ratios (mean ± SD) of individually normalized Bcl2-like1/Bax gene expression. There was no effect of pRLN compared to the control group, and values in blastocysts were always significantly lower than that of cleaved embryos within the same treatment group (*, P < 0.05, ANOVA). Neonate porcine uterine RNA and water were used as positive control (PC) and negative control (NC), respectively.
effects of exogenous relaxin during both oocyte maturation and early embryo development.

These effects are results of putative interactions between relaxin and its receptors located on plasma membranes of target cells [22,24,36]. In previous studies, we reported the presence of these receptors on porcine oocytes and embryos [27]. Here, we found that the addition of porcine relaxin in a follicular fluid-free maturation medium induces an accumulation of RXFP2 mRNA in COCs. This effect is surprising given that relaxin is the specific ligand of RXFP1, although it can bind with low affinity to RXFP2. This observation suggests a possible role of RXFP2 receptors during mammalian oocyte maturation process in the pig, as also suggested in rhesus macaque [37] and rat [38] oocytes expressing RXFP2 mRNA. In-depth studies of INSL3-RXFP2 complex during oocyte maturation and embryo development will definitely contribute to better characterize the full developmental effects of this complex.

Nevertheless, given the complexity of molecular interactions existing within the follicular fluid, a likely activation of RXFP1 receptors during in vivo oocyte maturation cannot be ruled out; and seems to be highlighted in the current study by the significant increase of RXFP1 mRNA in oocytes matured in the presence of porcine follicular fluid alone. The existence of such interactions may also explain our contradictory results with a recent study comparing a lack of relaxin effect on nuclear maturation of pig oocytes [39]. These authors tested the effect of recombinant human relaxin added in maturation medium already containing porcine follicular fluid (10%) and whose high relaxin contents [8,40] and beneficial effect on oocyte maturation [41,42] could have interfered with their results. In our study, oocytes were matured in the absence of follicular fluid, and the supplementation of the basal medium with relaxin significantly increased the proportions of oocytes resuming and accomplishing meiotic maturation. This beneficial effect of relaxin could be explained by its interactions with its receptors leading to an inhibition of oocyte meiosis blocking factors such as cyclic adenosine monophosphate (cAMP) as previously suggested [38].

Most interesting, the developmental effects of relaxin suggest both direct and indirect effects on embryos and oocytes, respectively. While embryos were freed of the surrounding cumulus cells after fertilization, oocytes were matured together with their surrounding cumulus cells. Using rodent oocytes, Kawamura et al. have proposed an indirect effect of relaxin on its targets in a paracrine way [38]. Irrespective of the moment of its addition, the increased total cell number of blastocysts appears as the hallmark of relaxin during early embryo development. Indeed, beneficial effects of relaxin were limited at the cleavage stage (high proportions) following oocyte maturation in the presence of relaxin, while any effects were observed on blastocyst formation compared to the control groups.

Developmental effects of relaxin in our study indicate possible perturbations of molecular mechanisms associated with cell cycle, and specifically cell proliferation. A recent study has demonstrated a positive effect of relaxin on glutathione (GSH) synthesis in in vitro matured porcine oocytes [39]. Indeed, GSH is a routine indicator of reduced-oxidative (REDOX) status of cells leading to potential modifications of cellular functions such as cell cycle (incidence of apoptosis) [49]. Therefore, we suggest that the increase of blastocyst cell number in our study is probably associated to an increase of GSH in oocytes and/or developing embryos.

In addition, production of GSH induces an imbalance of reduced-oxidative (REDOX) status of cells leading to potential modifications of cellular functions such as cell cycle (incidence of apoptosis) [49]. Therefore, we anticipated an activation of apoptotic-related genes by relaxin. Apoptosis is an important biological process during blastocyst formation of which fine-tuned balances between pro-apoptotic (Bax) and anti-apoptotic (Bcl2-like1) genes are critical for the survival of embryos [50]. Our study revealed that Bcl2-like1/Bax ratios were always in direction of Bcl2-like1 transcripts which is favorable to embryo survival. The presence of relaxin during culture did not significantly affect this ratio which remained comparable between groups.

Conclusions

In summary, our findings suggest that the increase in the total cell number in blastocysts is the hallmark effect of exogenous relaxin. Given that the cell number of

### Table 4 Effects of relaxin added during both oocyte maturation and embryo culture

| pRLN (ng/ml) during | Total Zygotes (N) | % of cleaved at Day 2pi (n) | Blastocyst formation at Day 7pi |
|---------------------|-------------------|-----------------------------|--------------------------------|
|                      | IVM               | IVF                         | Total (T) | % (T/N) | Cell number (n) |
| 0                   | 0                 | 324                         | 48 ± 3 (156)<sup>a</sup> | 24       | 8 ± 2<sup>a</sup> | 31 ± 3 (24)<sup>a</sup> |
| 40                  | 20                | 272                         | 33 ± 5 (91)<sup>b</sup>  | 19       | 7 ± 2<sup>b</sup>  | 33 ± 3 (12)<sup>b</sup> |
| 40                  | 40                | 353                         | 51 ± 8 (172)<sup>a</sup> | 33       | 10 ± 2<sup>a</sup> | 43 ± 6 (10)<sup>b</sup> |

<sup>a,b</sup>Different superscripts within the same columns indicate significant difference (P < 0.05; ANOVA). Data are mean values (± SEM) of at least 5 independent replicates.
bластокysts is an important gauge for in vitro-produced embryos, the supplementation of porcine embryo culture media with relaxin could be considered. However, the effects of relaxin on the expression of developmentally important genes should be investigated.

Acknowledgements
The authors thank the Southern Quality Meats (Pontotoc, Mississippi, USA) for facilitating the collection of ovaries. This work was supported by the USDA-ARS Biophotonics Initiative project # 58-6402-3-0120 and the Mississippi Agricultural and Forestry Station (MAFES).

Author details
1Department of Animal & Dairy Sciences, Mississippi State University, 4025 Wise Center Dr, Mississippi State, MS 38762, USA. 2Department of Biochemistry & Molecular Biology, Mississippi State University, 402 Dorman Hall, Mississippi, MS 38762, USA. 3Department of Pathobiology & Population Medicine, Mississippi State University, 240 Wise Center Dr, Mississippi State, MS 38762, USA.

Authors' contributions
JMF conceived and performed all the experiments, and wrote the first draft of the manuscript. JMG contributed to the in vitro embryo production. STW and PLR contributed to the design of the study, revised and approved the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 14 December 2010 Accepted: 27 January 2011
Published: 27 January 2011

References
1. Kitakawa Y, Suzuki K, Yoneda A, Watanabe T: Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology 2004, 62(7):1196-1197.
2. Persson RM, Wells KD: Culture of pig embryos. J Reprod Fertil Suppl 1993, 48:61-73.
3. Bydheera LR: In vitro production of embryos in swine. Theriogenology 2002, 57(1):236-273.
4. Webb R, Gansswothy PC, Campbell BK, Hunter MG: Intra-ovarian regulation of follicular development and oocyte competence in farm animals. Theriogenology 2007, 68:522-529.
5. Bagnell CA: Production and biologic action of relaxin within the ovarian follicle: an overview. Steroids 1991, 56(5):242-246.
6. Matsumoto D, Chamley WA: Identification of relaxins in porcine follicular fluid and in the ovary of the immature sow. J Reprod Fertil 1980, 58(2):363-375.
7. Sepalwa M, Tittinen A: Endometrial responses to corpus luteum products in cycles with induced ovulation: theoretical and practical considerations. Hum Reprod 1995, 10(suppl 2):67-75.
8. Hirasawa S: Experimental relaxation of the pubic ligament of the guinea pig. Proc Soc Exp Biol Med 1926, 23:661-663.
9. Sherwood OD: Relaxin’s physiological roles and other diverse actions. Endocr Rev 2004, 25(2):203-234.
10. Gunner P, Crawford DJ, Tregear GW: Expression of the relaxin gene in rat tissues. Mol Cell Endocrinol 1995, 110(1-2):53-64.
11. Dichtl et al: Relaxin-a pleiotropic hormone and its emerging role for experimental and clinical therapeutics. Pharmacol Ther 2006, 112(1):38-56.
37. Hanna C, Yao S, Patta M, Jensen J, Wu X: Expression of insulin-like 3 (INSL3) and differential splicing of its receptor in the ovary of rhesus macaques. Reprod Biol Endocrinol 2010, 8(1):150.

38. Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Monta H, Toppari J, Fu P, Wade JD, Bathgate RA, et al: Paracrine regulation of mammalian oocyte maturation and male germ cell survival. Proc Natl Acad Sci USA 2004, 101(19):7323-7328.

39. Kim HS, Jeong YI, Lee JY, Jeong YW, Hossein MS, Hyun HS, Hwang WS: Effects of Recombinant Relaxin on In Vitro Maturation of Porcine Oocytes. J Vet Med Sci 2010, 72(3):333-337.

40. Ohleth KM, Zhang Q, Bagnell CA: Relaxin protein and gene expression in ovarian follicles of immature pigs. J Mol Endocrinol 1998, 21(2):179-187.

41. Avery B, Strobech L, Jacobsen T, Bogh B, Greve T: In vitro maturation of bovine cumulus-oocyte complexes in undiluted follicular fluid: effect on nuclear maturation, pronucleus formation and embryo development. Theriogenology 2003, 59(3-4):987-999.

42. Marchal R, Feugang JM, Penneau C, Venturi E, Terqui M, Memillod P: Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 2001, 56(1):17-29.

43. Luberda Z: The role of glutathione in mammalian gametes. Reprod Biol 2005, 5(1):5-17.

44. de Matos DG, Furnus CC: The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. Theriogenology 2000, 53(3):761-771.

45. Feugang JM, de Roover R, Moens A, Leonard S, Dessy F, Donnay I: Addition of beta-mercaptoethanol or Trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. Theriogenology 2004, 61(1):71-90.

46. Furnus CC, de Matos DG, Picco S, Garcia PP, Inda AM, Mattioli G, Errecaleal AL: Metabolic requirements associated with GSH synthesis during in vitro maturation of cattle oocytes. Anim Reprod Sci 2008, 109(1-4):88-99.

47. Van Soom A, Yuan YQ, Peelman LJ, de Matos DG, Dewulf J, Laven H, de Kruif A: Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. Theriogenology 2002, 57(5):1453-1465.

48. Bing YZ, Hira Y, Iga K, Che LM, Takenouchi N, Kuwayama M, Fuchimoto D, Rodríguez-Martínez MN: In vitro maturation and glutathione synthesis of porcine oocytes in the presence or absence of cysteamine under different oxygen tensions: role of cumulus cells. Reprod Fertil Dev 2002, 14(3):125-131.

49. Ozawa M, Nagai T, Fahnudin M, Karja NWK, Kaneko H, Noguchi J, Ohnma K, Kikuchi K: Addition of glutathione or thioredoxin to culture medium reduces intracellular redox status of porcine IVF embryos, resulting in improved development to the blastocyst stage. Mol Reprod Dev 2006, 73(8):998-1007.

50. Yang M, Rajamahendran R: Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced in vitro. Anim Reprod Sci 2002, 70(3-4):159-169.

doi:10.1186/1477-7827-9-15

Cite this article as: Feugang et al.: In vitro effects of relaxin on gene expression in porcine cumulus-oocyte complexes and developing embryos. Reproductive Biology and Endocrinology 2011 9:15.
Author/s:
Feugang, JM; Greene, JM; Willard, ST; Ryan, PL

Title:
In vitro effects of relaxin on gene expression in porcine cumulus-oocyte complexes and developing embryos

Date:
2011-01-27

Citation:
Feugang, J. M., Greene, J. M., Willard, S. T. & Ryan, P. L. (2011). In vitro effects of relaxin on gene expression in porcine cumulus-oocyte complexes and developing embryos. REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY, 9 (1), https://doi.org/10.1186/1477-7827-9-15.

Persistent Link:
http://hdl.handle.net/11343/256124

File Description:
Published version

License:
CC BY