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Cellular Expression and Localization of Estrogen Receptor α and Progesterone Receptor mRNA in the Bovine Oviduct Combining Laser-Assisted Microdissection, Quantitative PCR, and In Situ Hybridization

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Summary

The importance of using techniques that allow the study of pure populations of cells has been increasingly recognized. The authors used laser-assisted microdissection (LAM) in combination with quantitative real-time PCR (qPCR) to assess the relative expression of mRNAs encoding estrogen receptor α (ERα) and progesterone receptor (PR) in the different compartments of the bovine oviduct (epithelium, stroma, smooth muscle coat) during the follicular and mid-luteal phases of the estrus cycle. The localization of receptor mRNA was further studied using non-radioactive in situ hybridization (NISH). A special focus was on whether formalin fixation and paraffin embedding influence the quality and quantity of mRNA obtained from microdissected material. Distinct cyclic changes of the mRNA in the bovine oviduct were observed with elevated levels of PR mRNA transcripts in the epithelium and smooth muscle coat during the follicular phase. The expression of PR mRNA did not vary significantly in the stroma of the bovine oviduct during follicular and mid-luteal phases. In conclusion, the authors found that LAM with qPCR can precisely locate and accurately quantify mRNA expression in specific cell populations from formalin-fixed and paraffin-embedded oviductal tissue. (J Histochem Cytochem 59:312–327, 2011)

Keywords

oviduct, bovine, steroid receptors, laser-assisted microdissection (LAM), qPCR

Gene expression in complex organs is currently mainly analyzed by Northern blots, RNase protection assay, microarrays, or real-time PCR after homogenization of the tissues. These methods do usually not allow the precise estimation of physiologically relevant mRNA alterations in different tissue compartments of an organ, such as parenchyma and stroma. Advanced morphological techniques, such as in situ hybridization or in situ PCR, are more precise with respect to cell type–specific characterization of gene expression, but changes in expression levels can only be described semiquantitatively.

Recently, microdissection of distinct and well-characterized cell populations in combination with real-time PCR has been described to overcome the mentioned methodological disadvantages. Laser-assisted microdissection (LAM) allows cell type–specific mRNA isolation without contamination of adjacent cells within a complex tissue. For this purpose, use of cryosections from unfixed frozen tissues has been recommended because molecules to be analyzed remain intact (Takagi et al. 2004). However, precise LAM from unfixed frozen sections is inconvenient and difficult because of the

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restricted accuracy of observation in this material, which frequently prevents the exact identification of cells and tissue structures, which foils the superior contact and contamination-free isolation of cells offered by LAM (Li et al. 2008). In addition, it is usually extremely difficult to cut cryosections under RNase-free conditions. Fixation of cryosections only partly resolves this problem and usually results in a considerable degradation of the RNAs. Therefore, formalin-fixed paraffin-embedded (FFPE) material appears as an alternative, especially as archival FFPE tissue represents an abundant source of experimental and clinical specimens (Ben-Ezra et al. 1991; Li et al. 2008). Their use, however, is limited to applications involving analysis of gene expression due to RNA degradation and modification during fixation. It therefore would be of great interest to develop reliable and reproducible protocols for combining LAM with quantitative PCR (qPCR). Accurate quantification of mRNAs from precisely defined cell types of heterogeneous organs such as the oviduct could significantly contribute to a better understanding of the gene expression in the different compartments of an organ.

Morphological changes of the bovine oviduct under the influence of steroid hormone and alterations in its secretory activity have been well documented both in vivo (Wijayagunawardane et al. 1996) and in vitro (Abe et al. 1995; Reischl et al. 1999). The effects of estrogen and progesterone are mediated through intracellular receptors (estrogen receptor alpha [ERα], estrogen receptor beta [ERβ], and progesterone receptor [PR]) that are members of the nuclear receptor family, which regulate the expression of a wide variety of genes on a transcriptional level. In this way, estrogens induce significant compositional changes of the oviductal fluid with greatest protein secretion during the follicular phase of the cycle.

The aim of our study was to investigate the expression of ERα and PR in the bovine oviduct during the follicular and mid-luteal phase of the estrus cycle at the mRNA level, using LAM followed by qPCR. The localization of the respective mRNA has been done by non-radioactive in situ hybridization (NISH), with the corresponding proteins localized by immunohistochemistry. A focus was put on the interesting question, which cannot be solved by qPCR of tissue homogenates from the oviduct, of whether there are qualitative and quantitative differences in the expression of mRNA for steroid hormone receptors (ERα and PR) in the different cellular compartments of the oviduct (epithelium, stroma, smooth muscle layer) during different phases of the estrus cycle (follicular and mid-luteal phases).

Material and Methods

Animals and Tissue Preparation

Bovine oviducts of 20 healthy German Fleckvieh cows from different cycle stages were collected at the local Munich abattoir within 10 min postmortem. They were grouped depending on the cycle stage, referring to Ireland et al. (1980) by examining the color, consistency, number, size of follicle, and corpora lutea: follicular phase (days 19–21, proliferation cycle phase) and mid-luteal phase (days 6–12, secretory cycle phase).

Ampulla samples of the oviduct (0.5 cm side length) were either directly immersed in RNAlater (Ambion; Applied Biosystems, Darmstadt, Germany) or fixed in 3.7% buffered formol (Tris-HCl buffer, pH 7.4) for different times (3, 6, 12, 18, 24, 32, and 48 hr) and embedded in paraplast (Roth, Karlsruhe, Germany) using a Shandon Citadel 2000 tissue processor. Paraffin sections of defined thickness (5, 10, 20, 30, 40, 60, and 80 µm) were cut using a rotary microtome (Microm, Walldorf, Germany). Polyethylene naphthalate (PEN) membrane-covered glass slides (PALM Membrane Slides; PALM Microlaser Technologies, Zeiss, Munich, Germany) were exposed to ultraviolet light for 30 min before use to obtain better adherence and to cross-link any contaminating DNA sources. Sections (5–80 µm thick) from FFPE oviductal samples were cut using a rotary microtome HM 340 E (Microm, Munich, Germany) under RNase-free conditions. Sections were dewaxed (xylene, 2×, for 5 min each) and transferred into 100% isopropanol for 2 × 5 min. Nuclei were stained with hematoxylin (Mayer's hematoxylin; Sigma, Steinheim, Germany) for 1 min and rinsed in RNase-free blueing solution (DEPC-treated tap water) for 30 sec. Eosin (Accustain Eosin Y Solution aqueous; Sigma) was used for counterstaining, followed by a short dip into distilled water. Finally, the sections were air-dried at 37°C for a minimum of 30 min.

Laser-Assisted Microdissection (LAM)

LAM was performed using a PALM MicroBeam Laser System (Zeiss). For histological evaluation, sections (5, 10, 20, 30, 40, 60, and 80 µm) were stained with hematoxylin and eosin (Mayer's hematoxylin; Sigma). The PALM-LAM system (PALM MicroBeam; Zeiss) consisted of an inverted microscope (Zeiss Axiovert 200m) with a motorized stage and a "cold" nitrogen UVA laser. The microscope used in our experiments was fitted with 5×, 10×, 20×, 40×, and 63× objectives. Viewing of tissue was possible with all objectives, and microdissection was done using 10× or 20× magnification. Regions of interest were then identified and manually delineated on the computer screen using the software program. The microscope was then instructed to collect delineated regions. A fine-tuned 377-nm pulsed nitrogen laser was used to dissect the cells of interest from the surrounding tissue. The isolated specimens and the underlying PEN membrane were then retrieved in a non-contact fashion by catapulting into an overlying receptacle (cap of a 0.5 ml Eppendorf tube that contained a depressed lid; PALM MicroBeam, Zeiss). The collected areas of the

different oviductal tissue compartments (epithelium, stroma, smooth muscle) added up to an area of ~10^6 μm^2 for each compartment of the oviduct. Images of tissue sections before and after cell capture and of captured cells attached to the lid of the caps were taken using a Zeiss Axiocam.

RNA Isolation

RNA isolation from RNAlater immersed tissue was performed using the Tri Reagent isolation kit (Sigma-Aldrich, Munich, Germany) according to the manufacturer's protocol.

Total RNA from FFPE microdissected and laser-catapulted tissue (epithelium, lamina propria, and tunica muscularis of the bovine oviductal ampulla) was extracted using the RNeasy FFPE Kit (Qiagen, Hilden, Germany), starting the protocol by adding proteinase K to the tubes. RNA extraction from FFPE material without LAM was done according to the following protocol: Freshly cut paraffin sections (5, 10, 20, 30, 40, 60, and 80 µm) were dewaxed in 1 ml xylene for 10 sec and centrifuged (Eppendorf 5451 C) at 15,000 × g for 2 min (20°C) to form a pellet. The supernatant was removed by pipetting; 1 ml of 100% biograde ethanol was added to clear residual xylene from the sample. After centrifuging at 15,000 × g again, the supernatant was carefully removed. The tube was then left to dry at 37°C for 15 min. It was very important to wait until all alcohol had evaporated, as any residual alcohol reduced RNA yield. The pellet was resuspended in PKD buffer containing 10 µl proteinase K (Qiagen). To evaluate the optimal extraction procedure, different incubation times with proteinase K (15 min, 3 hr, 6 hr, 18 hr, and 24 hr at 55°C) followed by incubation at 80°C for 15 min were tested. A short terminal incubation at a higher temperature partially reversed formalin cross-linking of the released nucleic acids, improving RNA yield and quality as well as RNA performance in downstream assays. As result of these preliminary experiments, an optimal proteinase K incubation time of 24 hr at 55°C was found and used in the later experiments. The lysate was then thoroughly mixed with RBC buffer (Qiagen), pipetted onto a gDNA Eliminator spin column (Qiagen), and centrifuged for 30 sec at 8000 g. The samples were then transferred to an RNeasy MinElute spin column (Qiagen) placed in a 2-ml collection tube where the total RNA binds to the membrane and contaminants are efficiently cleared.

RNA Quality Control

Quality and quantity of isolated total RNA were evaluated using the microfluid-based automated electrophoresis system Experion (Bio-Rad, Munich, Germany). After extraction of RNA from the microdissected samples, an aliquot (1 µl) of the total RNA was taken to check quality and RNA yield. All RNA samples were analyzed in triplicates (n = 60). We used the Standard Sense analysis chip (Bio-Rad) for RNAlater immersed material and complete FFPE tissue sections and a HighSense analysis (Bio-Rad) chip for microdissected FFPE following the manufacturer's protocol. The resulting electropherograms were used to determine RNA integrity and concentration.

Three different parameters were used to assess total RNA quality (Kerman et al. 2007) : (1) 28S/18S rRNA subunit ratio (28S/18S), which was calculated by dividing the area under the 28S peak by that of the 18S peak; (2) 18S/baseline peak ratio (18S baseline), which was calculated by dividing the height of the 18S peak by the height of the highest baseline peak preceding the 18S peak; and (3) RNA Quality Indicator (RQI) method, which is based on a proprietary Bio-Rad algorithm (Denisov et al. 2008) and is calculated by the Experion software (Bio-Rad).

cDNA Synthesis from RNAlater Immersed, Tri-Reagent Extracted Material

Each RNA sample of bovine ampulla in follicular and midluteal phases was dispensed into two tubes. For control, in one of the tubes, reverse transcriptase was omitted (RT–): In this tube, it was expected that no PCR products were formed. This test was done to confirm the absence of genomic DNA in RNA samples.

First-strand DNA synthesis was carried out using the iScript cDNA Synthesis Kit (Bio-Rad). iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided preblended with RNAse inhibitor.

The reaction setup was as follows (volume per reaction): 5× iScript Reaction Mix 4 µl, iScript Reverse Transcriptase 1 µl, RNA template (100 fg to 1 µg Total RNA) × µl and × µl RNAse-free water. The RNA elute was previously measured by a spectrophotometer (Smart Spec 3000; Bio-Rad), and up to 2 µl was added to the iScript Reverse Transcription reaction, always considering that the amount of total RNA should not exceed 1 µg. The extracted RNA was thus reverse-transcribed at a final volume of 20 ml in the heat block of an iCycler (Bio-Rad) using the following reaction protocol: 5-min incubation at 25C, followed by 30 min at 42C and an additional 5 min at 85C. Finally, the cDNA was diluted with RNase-free aqua dest 1:8 to obtain our previously tested concentration.

cDNA Synthesis from FFPE-Fixed, RNeasy FFPE Kit Extracted Material

The total RNA elute extracted with the RNeasy FFPE Kit (Qiagen) was basically processed in the same way as above except for slight changes of the reaction setup. The setup was adjusted to the following: 5× iScript Reaction Mix 4 µl, iScript Reverse Transcriptase 1 µl, RNA template 12 µl, and 3 µl RNAse free water.

Table 1. PCR Primer Sets Used for Quantitative PCR of Laser-assisted Microdissected Tissue from the Ampulla of the Bovine Oviduct in Follicular and Mid-Luteal Phases

Primers	Forward Primer	Reverse Primer	Nucleotide Product Size, bp	Gen-Bank Accession No.
Estrogen receptor α	GAAGTGGGCATGATGAAAGG Exon 5	AAGGTTGGCACGTCTCATGT Exon 6	135	AY_538775
Progesterone receptor	TCGAGCTCACAGCGTTTCTA Exon 7	CCCGGGACTGGATAAATGT Exon 8	106	XM_583951
Glyceraldehyde-3-phosphate dehydrogenase	AGATGGTGAAGGTCGGAGTG Exon 2	GAAGGTCAATGAAGGGGTCA Exon 3	117	NM_001034034
Polymerase II	TTCTATCCGGAGGGTCTTCA Exon 2	CGTCACTGGTGAGAGGGATT Exon 4	134	NM_001034212
β-Actin	GATCATTGCTCCTCCTGAGC Exon4	ACTCCTGCTTGCTGATCCAC Exon5	101	NM_173979

PCR Primer Design

Genomic DNA and mRNA sequences were downloaded from NCBI Locus Link at http://www.ncbi.nlm.nih.gov/Locuslink for the following genes: bovine ERα, bovine PR, 18S rRNA, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and polymerase II (Pol II). Primer pairs were designed using the Ensemble (http://www.ensembl.org/index.html) databank, showing the exon constellation, and Primer3 (http://primer3.sourceforge.net/releases.php). Primer pairs were chosen to generate PCR products between 100 and 140 bp, as RNA recovered from FFPE-laser microdissected material was expected to be considerably fragmented. Bovine and human sequences used for primer design are shown in Table 1. Primer pairs were constructed to amplify products for ERα, PR (the primer pair was designed to detect both the A- and B-isoform), and the reference genes GAPDH, β-actin, Pol II, and 18S ribosomal protein. To assess the reference genes for these investigations, we used the software GeNorm (included in GenEx Professional software, Version 4.3.5; http://www.multid.se).

18S was used only as an internal system control and not as a housekeeping gene for normalizing the receptor results. All primer pairs were designed in an intron flanking fashion to limit the possibility of amplifying genomic DNA. The primers were synthesized by MWG-Biotech (Ebersberg, Germany). For qPCR, the concentration of the primer sets was adjusted to 1.2 pmol/µl.

Quantitative Real-Time RT-PCR (qPCR)

qPCR reactions were performed using the iCycler SYBR Green protocol (Bio-Rad) in a 96-well plate (Bio-Rad). We used a SYBR Green PCR Master Mix (Bio-Rad). The PCR reaction setup contained the following components: iQ SYBR Green Supermix (Bio-Rad [for each well, 10 µl]), cDNA template (5 µl of each template), and the different primer sets (0.4 mM primer forward and reverse each, 10 µl). In all cases, a non-template control (NTC) was performed as follows: 4.6 µl H_2O + 5.4 µl primer + 10.0 µl iQ SYBR Green Supermix (Bio-Rad) of each tissue was run simultaneously, and also a doublet of all templates was used.

The PCR thermal cycling program was accomplished by a two-step temperature protocol with following conditions:

- Phase 1: 95°C for 1.45 min for enzyme activation
- Phase 2: 50 cycles at 95°C for 15 sec (denaturizing) and 60°C for 30 sec (annealing and extension)

To identify unspecific PCR products, melt curve analysis was carried out immediately after the amplification protocol: One initial cycle at 95°C for 1 min was followed by 70 cycles (10 sec) starting from 95°C with a regular decrease of 0.5C per cycle. To reduce effects of pipetting errors, all oviductal samples were amplified in triplicates. Parallel reactions with total RNA, which were not reverse transcribed (–RT), and reactions without templates (NTC) were used as negative controls. The optical data obtained by qPCR were analyzed using the iCycler software Version 3.0a (BioRad). The threshold cycle value (Ct) was defined as the cycle number required for the SYBR Green fluorescence intensity to exceed the 10× standard deviation of the baseline fluorescence. By plotting the Ct values obtained by amplification of the standard samples versus the log values of the starting copy numbers, a standard curve was generated and a correlation coefficient was calculated.

For analysis of gene expression levels, GenEx Professional software, Version 4.3.5, was used.

Identification of PCR Products

To identify and validate the different PCR amplification products, we used a melting curve analysis gel electrophoresis (gels

stained with ethidium bromide) and sequencing of the PCR products. Melting curve analysis was accomplished in the iCycler (Bio-Rad) and confirmed the expected product identity. The predicted size of the amplicons was check by running them in a 1.5% ethidium bromide–stained agarose gel (Biozym Phor Agarose, Biozym Scientific GmbH, Oldendorf, Germany). The position of the bands was compared with a DNA Sizer XII marker (Peqlab, Erlangen, Germany).

In addition, the amplicons were sequenced by *GEN*terprise (*GEN*terprise GENOMICS, Mainz, Germany). The specificity of the sequence data was verified by a *GEN*terprise own tool named FINCH, linked to BLAST search (www.ncbi.nlm.nih.gov/BLAST).

Standard Curves

To monitor amplification efficiency and to calibrate qPCR, we prepared concentration standards of ERα and PR as well as the reference genes GAPDH, β-actin and Pol II transcript fragments by PCR amplification from pooled total bovine cDNA (pool of epithelium, lamina propria and tunica muscularis of the used ampullae). For each transcript fragment, a dilution series from 10 pg/µl in a 10-fold dilution was used to generate the standard curves. The amplification efficiency was estimated from the slope of the regression line using the following formula: PCR efficiency = $10^{1/-\text{slope}} - 1$. The efficiency of each primer pair based on the standard curves was expressed as a percentage. The software GenEx Professional software, Version 4.3.5, includes correction for variation in efficiency of the different primer sets.

Statistical Analysis

For all statistical analyses, we used the GenEx Professional software, Version 4.3.5. The data are expressed as mean and standard error of the mean (SEM). Differences between groups were examined for statistical significance using the Student *t*-test, and $p < 0.05$ denoted a statistically significant difference.

Non-radioactive In Situ Hybridization (NISH)

For in situ hybridization, small pieces of oviductal tissue (side length: 5 mm) were fixed in 3.7% formalin for 12 hr and embedded in paraffin. Sections (5 µm) were mounted on amino propylene ethoxysilane-coated slides and dried at 50°C. All steps during in situ hybridization were conducted under strict RNase-free conditions, and all solutions for RNA in situ hybridization were prepared using diethylpyrocarbonate (DEPC)–treated water. The glassware was sterilized at 200°C. Sections were deparaffinized with xylene (3 × 10 min), immersed in absolute ethanol (2 × 5 min), and air-dried. Immediately after drying, the sections were dipped in 2% saline sodium citrate (SSC) prewarmed in a water bath (80°C) for 10 min, followed by cooling off for 20 min at room temperature. Slides were then washed in distilled water (2 × 5 min) and Tris buffer (pH 7.4; 2 × 5 min) and incubated for 20 min with 0.05% proteinase E (VWR, Ismaning, Germany) in Tris buffer at room temperature. Sections were subsequently washed in Tris buffer (2 × 5 min) and distilled water (2 × 5 min) and postfixed for 10 min in freshly prepared 4% paraformaldehyde/PBS (pH 7.4). After further washing in PBS (2 × 5 min) and distilled water, slides were dehydrated in an ascending graded series of ethanol and air-dried. Oligonucleotide probes labeled with biotin were diluted with RNA hybridization buffer (DAKO, Munich, Germany) to a final concentration of 5 pmol/ml. Hybridization was carried out by overlaying the dry sections with 40 µl of the hybridization mixture and incubation was done under a cover slip in a humidified chamber at 38C overnight. Then, slides were washed in 2 × SSC (2 × 15 min) prewarmed to 38C, distilled water (2 × 5 min), and Tris Buffer (2 × 5 min). Detection of transcripts was performed using a streptavidin-biotin peroxidase complex kit and diaminobenzidine (DAKO) according to the manufacturer's instructions. For negative controls, parallel sections were hybridized either with the sense oligonucleotide probe or with buffer alone. Tissue from bovine uterus was used as a positive control. The used primers for ERα and PR are shown in Table 2.

Immunohistochemistry

For immunohistochemical studies, 5-µm serial sections of each oviduct segment were collected on slides (SupraFrost Ultra Plus, Menzel-Gläser, Raunschweig, Germany) that were coated with APES (aminopropyltriethoxysilane). Sections were preincubated with proteinase XXIV 0.1% (P8038; Sigma) for 15 min and washed with PBS buffer (pH 7.4). Endogenous peroxidase activity was blocked with 0.1% H_2O_2 at room temperature for 10 min.

Antigen localization was achieved using the avidin-biotin-complex (ABC) technique (Hsu et al. 1981) according to the following protocol. Paraffin sections (5 µm) were dewaxed and subjected to the following immunohistochemical staining schedule: (1) elimination of endogenous peroxidase activity with 0.5% H_2O_2 in PBS for 15 min at 20°C, (2) elimination of nonspecific protein binding by incubation at 20°C with 10% normal goat serum for 1 hr, (3) incubation overnight at 4°C with one of the following primary antibodies against ERα (rabbit; Santa Cruz Biotechnology, Santa Cruz, CA) and PR (mouse; Beckmann Coulter, Krefeld, Germany) (diluted 1:500 [vol/vol] in PBS and 1% BSA), (4) incubation with a corresponding secondary biotin-conjugated antibody (for ERα, anti-rabbit IgG-BIOT pig [DAKO]; for PR, anti-mouse IgG-BIOT rabbit [Sigma]) for 2 hr at 20°C (diluted 1:150 [vol/vol] in PBS and 1% BSA),

Table 2. Probes Sets Used for In Situ Hybridization from the Ampulla of the Bovine Oviduct in Follicular and Mid-Luteal Phases

	Sense	Antisense
Estrogen receptor α	5′gaa gtg ggc atg atg aaa gg3′	5′cct ttc act atg ccc act tc3′
Progesterone receptor	5′tcg agc tca cag cgt ttc ta3′	5′tag aaa cgc tgt gag ctc ga3′

(5) incubation with horseradish peroxidase–conjugated ABC for 1 hr at 20C (diluted 1:150 [vol/vol] in PBS and 1% BSA), and (6) treatment with 0.05 mg/ml of diaminobenzidine in PBS. All incubations were performed in a humidified chamber. Sections were left unstained or counterstained in Mayer's hematoxylin, dehydrated, and mounted with DePeX Eukitt (Riedel de Haen, Seelze, Germany).

After incubation with the secondary antibody, the sections were washed three times with PBS (3 × 5 min), and development of the staining with DAB (diaminobenzidine) for 5–10 min was performed. To stop the reaction, sections where then washed in distilled water. The slides were then counterstained with hematoxylin (30 sec).

As negative control, buffer (DAKO) was used instead of the specific primary antibody. As positive controls, bovine ovary was used, which is known to show specific immunostaining with the antibodies used in this study.

Results

Using RNAlater immersed material and whole paraffin sections, we could demonstrate the presence of ERα mRNA and PR mRNA in the bovine ampulla during the proliferative (follicular) and secretory (mid-luteal) phases of the estrus cycle. Subsequently, qPCR was performed to detect expression changes during the cycle. At first, we investigated the expression of ERα and PR in RNAlater immersed and also FFPR bovine ampulla without separating the different tissue cell types. The expression changed throughout the estrus cycle as detected by qPCR. In both studies, we detected a decrease of PR expression during the mid-luteal phase. Also, ERα mRNA was expressed always at a higher rate during the follicular phase.

To evaluate and quantify the distribution of steroid hormone receptors between the three major tissue compartments of the oviduct (epithelium, stroma, and smooth muscle), LAM of FFPE material from the bovine oviduct was used. This approach allows obtaining clearly separated samples of the three different tissue compartments in a contact-free manner (Fig. 1A-E) for downstream applications (check of RNA quality and quantity using the Experion System [Bio-Rad], relative quantification of ERα and PR with qPCR).

To enhance recovery and quality of totalRNA from FFPE microdissected oviductal tissue and to conserve the morphology of the tissue, we tried to optimize various steps of the tissue preparation, fixation, and totalRNA extraction. It was very important to process the tissue samples as fast as possible to maintain the morphology and RNA integrity. In our study, we collected our oviductal samples within less than 10 min after slaughter. We used 3.7% formalin as a fixative, which provided acceptable preservation of both parameters. In preliminary studies, we tested also the influence of different fixation times (3–48 hr), section thickness (5–80 μm), and different proteinase K incubation time on the quality and quantity of totalRNA extracted from FFPE oviductal material. Our preliminary studies showed that in sections of 20 μm, most details of the oviduct could be still clearly identified for LAM, if the sections were covered with Liquid Cover Glass spray (Zeiss). Thicker sections (40–80 μm) increased the amount of catapulted material into the caps of the microtubes, but the exact identification of the boundaries between the different compartments of the oviduct became more difficult. In addition, thicker sections (>20 μm) showed a decreased adherence to the PEN-membrane-coated slides, which made LAM more time-consuming, as it appeared more difficult to maintain a constant focusing plane for the cutting UV-laser of the MicroBeam system of Zeiss.

The digestion time of the microdissected FFPE material with proteinase K also had influence on the subsequent extraction efficiency with the RNase kit (Qiagen). Best quality and quantity of the retrieved totalRNA (checked by the Experion system [Bio-Rad]) were obtained after 24 hr of incubation with proteinase K.

Compared to RNAlater immersed material, which showed a RQI between 8 and 9 (Fig. 2A–C), the quantity and quality of total RNA procured from FFPE laser-captured material (RQI 5-8) had decreased (Fig. 2D-F). Nevertheless, we were able to demonstrate the specific expression of mRNA of ERα and PR in all three compartments. The dissociation curves for the amplified products from the laser microdissected FFPE material showed a single peak, which corresponds to a specific melting temperature. Furthermore, the respective amplicons for ERα and PR were detected on an ethidium bromide–stained agarose gel and showed a single band at the expected molecular weight (Fig. 3).

We could clearly demonstrate that the expression of mRNA for the PR receptor varies distinctly within the three compartments during the follicular and mid-luteal phases. In the epithelium, the PR mRNA was expressed

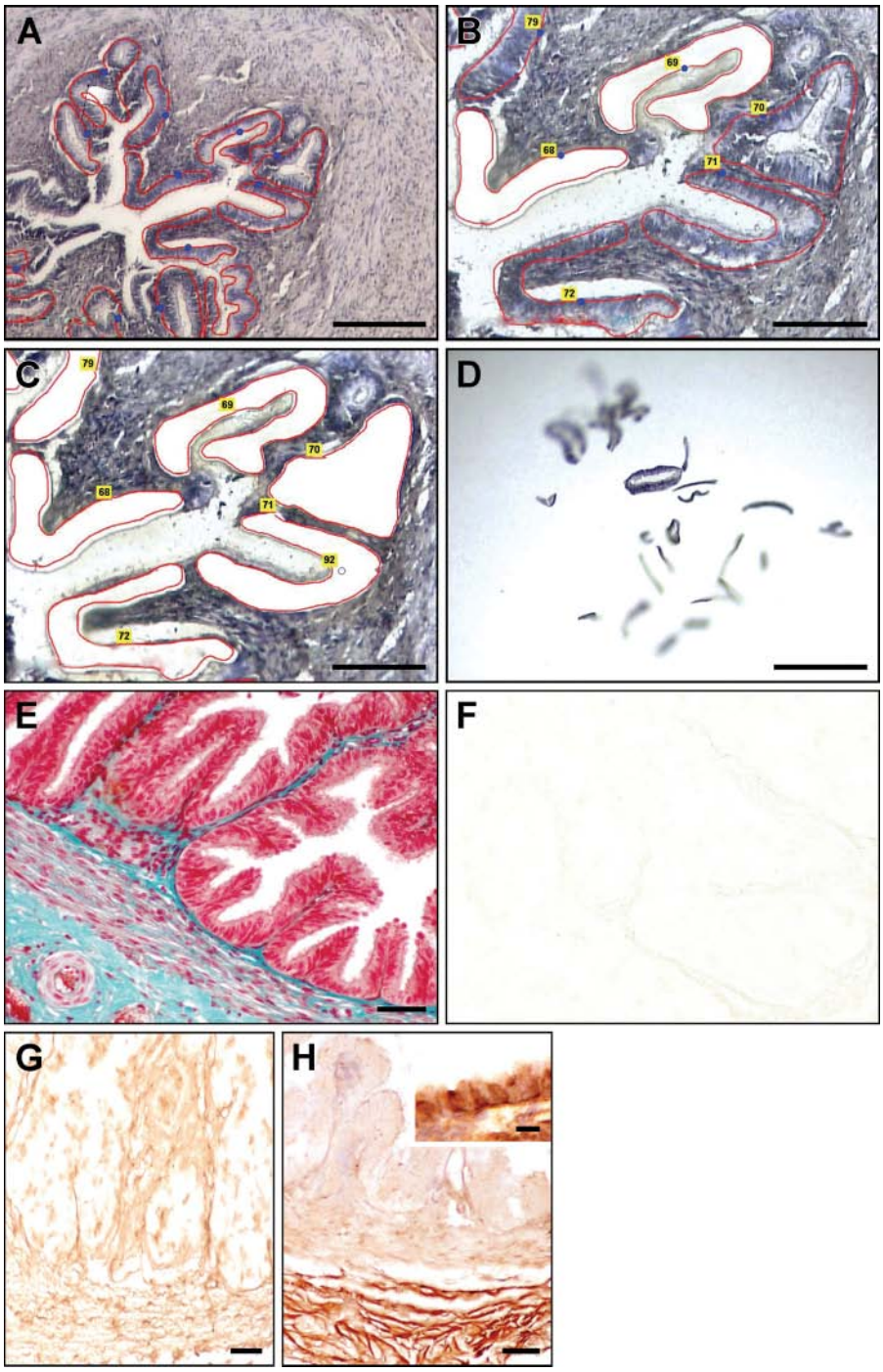

Figure 1. Microdissection and in situ hybridization of the bovine ampulla. (A–D) Demonstration of laser-assisted microdissection (LAM) in bovine ampulla. The 20-µm thick formalin-fixed paraffin-embedded (FFPE) section of bovine oviduct is stained with hematoxylin and eosin (H&E). (A) The epithelium of the bovine ampulla was marked. (B, C) Then the epithelium cells of interest were cut with a fine-tuned laser. (D) The isolated cell groups were catapulted into a tube. (E) The ampulla consisted of three main compartments: the epithelium, the lamina propria, and the tunica muscularis. Goldner, sb = 50 µm. (F) Negative (Sense) control for the progesterone receptor (PR) in situ hybridization. (G) In the tunica muscularis, the PR expression does not increase during the mid-luteal phase. sb = 50 µm. (H) In the tunica muscularis, the PR expression increased during the follicular phase. sb = 50 µm; inset: In the epithelium we detected a stronger expression for PR mRNA in the follicular phase than in the epithelium of the mid-luteal phase. sb = 15 µm. sb, scale bar.

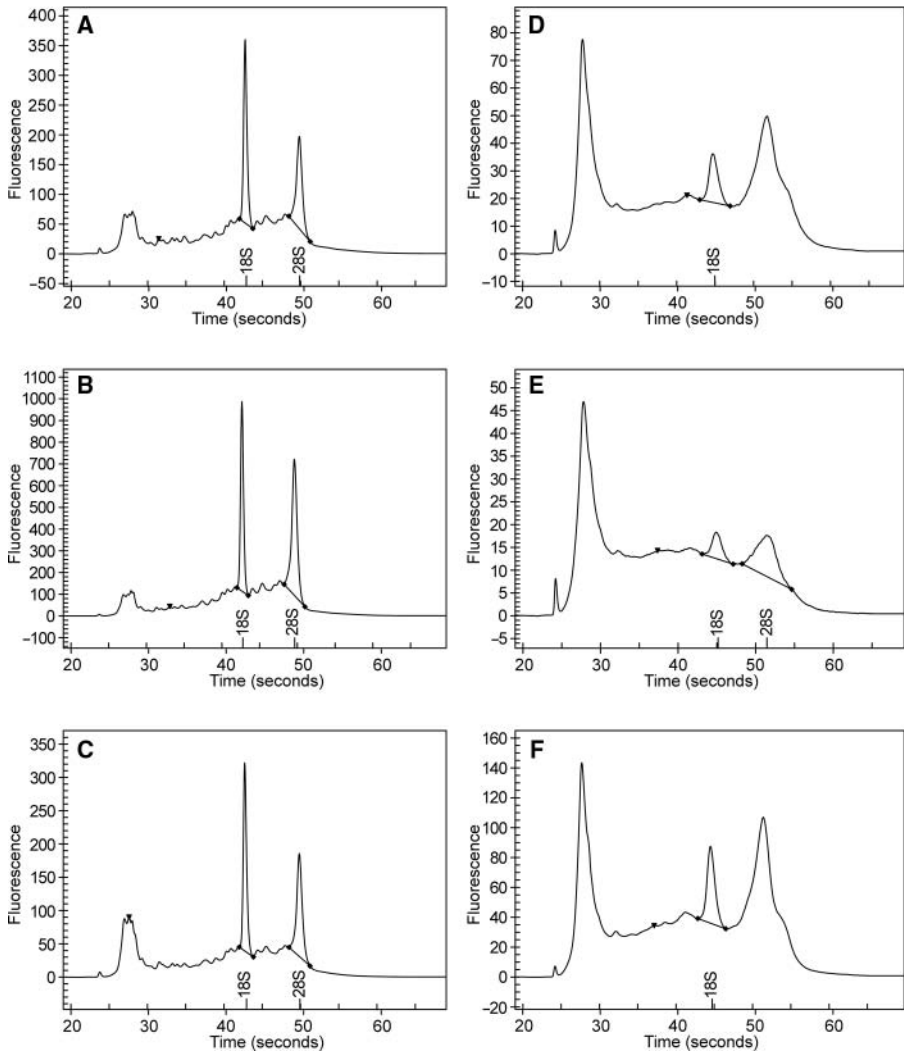

Figure 2. Comparison of extracted RNA of RNAlater immersed and formalin-fixed paraffin-embedded (FFPE) oviductal cell populations using the Experion system. The extracted RNA of RNAlater immersed (A–C) and FFPE (D–F) oviductal cell populations were compared.

significantly higher ($p < 0.05$) during the follicular phase compared to the mid-luteal phase. No significant difference in the expression of PR mRNA was found in lamina propria in the bovine ampulla during the investigated cycle stages. In the smooth muscle layer, a significant increase of PR mRNA expression ($p < 0.05$) occurred during the follicular phase. The expression of ERα mRNA in the epithelium of the follicular phase was higher than in the epithelium of the oviduct during the mid-luteal phase. Furthermore, we observed a distinct upregulation of ERα mRNA in the stromal cells of the follicular phase. No significant differences were seen in the expression of ERα mRNA in the smooth muscle layer of the ampulla during both phases of the cycles.

Using NISH with biotin-labeled oligonucleotides, a distinct signal for PR mRNA was found in many smooth muscle cells of the tunica muscularis of the oviduct during the follicular phase (Fig. 1F–H). In the epithelium, we observed a stronger expression for PR mRNA in the follicular phase (mainly in the secretory cells) than in the epithelium of the mid-luteal phase (H). In the stroma, only weak signals for PR mRNA were seen throughout the cycle. For ERα mRNA, in situ hybridization revealed transcripts in the epithelial cells and the stromal cells predominantly during the follicular phase, whereas during the mid-luteal phase, the hybridization signals appeared weaker. No significant change was seen in the smooth muscular layer of the oviduct, where a weak staining was seen throughout the cycle.

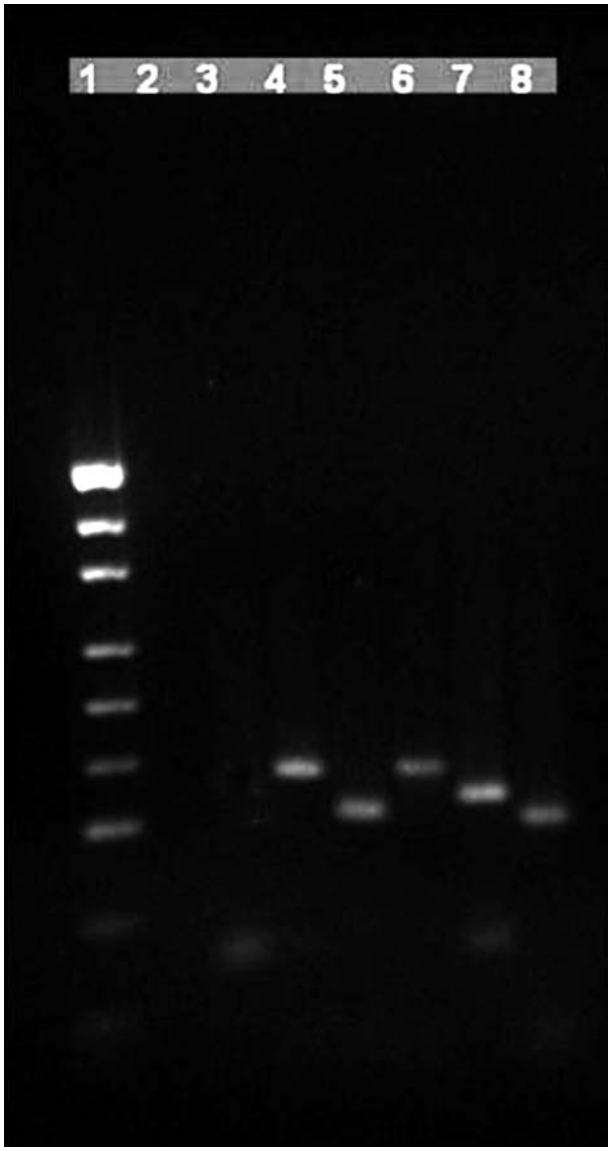

Figure 3. Gel electrophoresis of amplification products. Lane 1: marker (Peqlab, Erlangen, Germany), lane 2: water, lane 3: water, lane 4: estrogen receptor α (ERα; 135 bp), lane 5: progesterone receptor (PR; 106 bp), lane 6: POLR2C (134 bp), lane 7: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 117 bp), lane 8: β-actin (101 bp).

Protein expression of ERα in the ampulla of the oviduct during the follicular and mid-luteal phases of the cycle is shown in Figure 4A–H. Specific positive immunostaining was detected in the nuclei of the epithelial cells, stromal cells, and smooth muscle cells of the tunica muscularis (Fig. 4A,B).

Nuclei of epithelial and stromal cells showed a more distinct ERα staining intensity during the follicular stage compared to the mid-luteal stage. Staining intensity for ERα in basal nuclei and in the epithelial protrusions (including ERα-positive nuclei) appears to be confined to the secretory cells and was more intense in the follicular phase than in the mid-luteal phase (Fig. 4A,B). Also, nuclei found in apical protrusions of the epithelium were distinctly positive, whereas the nuclei of the ciliated cells were only weakly positive or completely negative throughout the cycle (Fig. 4B). Strong positive stromal cells were located in the apical region of the primary and secondary folds, whereas in the more basal and circular stroma areas, only a few ERα-positive fibroblasts could be observed. The positive stromal cells of the folds were mostly located adjacent to the epithelium. Nuclei of smooth muscle cells showed a weaker ERα immunostaining compared to the other cell populations, and no changes in the immunostaining were observed during the cycle (Fig. 4G,H). Immunostaining for PR was exclusively localized to the cell nuclei of the oviductal epithelium, the stromal cells, and the smooth muscle cells of the ampulla (Fig. 4C–F). No cytoplasm staining was observed in any of these cell types. During the follicular phase, the immunostaining for PR in the nuclei of the epithelium cells and muscle cells was intense (Fig. 4C,E). During the follicular phase, the nuclei of the muscle layer generally appeared more intensely immunostained for PR compared to the nuclei of the oviductal epithelium. During the mid-luteal phase, the nuclei of the muscle cells showed only a faint content of PR or were completely negative (Fig. 4F). In the epithelium, mostly the basal or central located nuclei of the secretory cells showed a moderate to strong positive expression for PR in the follicular phase, whereas the basally located nuclei during the mid-luteal phase stained only weakly with the antibody against PR (Fig. 4D). The nuclei of the ciliated cells in the follicular phase displayed weak staining for PR and were mostly completely negative during the mid-luteal phase. The nuclear immunostaining for PR in the stromal cells showed no significant changes between the follicular and the mid-luteal phase. The immunohistochemical results are summarized in Table 3.

Discussion

The use of homogenates of whole tissue samples for quantitative measurements by molecular methods such as qPCR (Bustin et al. 2009) usually results in average values only, determined by the varying contribution of all the heterogeneous cellular elements of the sample, whose proportion is unknown (Fink et al. 2000; Pinzani et al. 2006). Prerequisite for meaningful results of gene expression studies on heterogeneous organs are samples of high purity of the cells under investigation without contamination from unwanted cells and the separation of characteristic cell populations. Gene expression profiling of material isolated by LAM has become an important method for analyzing cellular behavior at a micro scale (Gjerdrum and Hamilton-Dutoit 2005a). During the past few years, LAM techniques have been

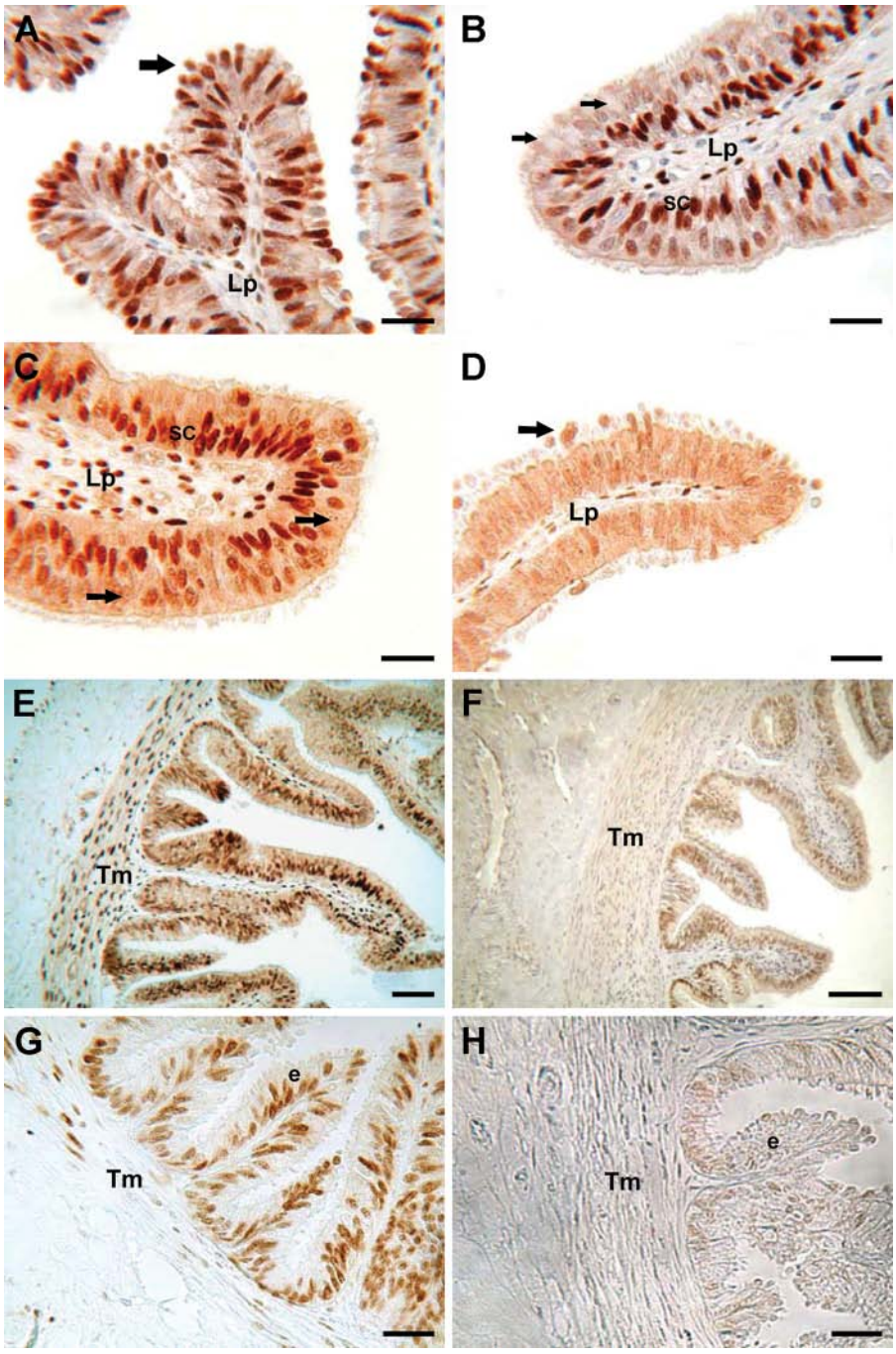

Figure 4. Immunohistochemical localization of estrogen receptor α (ERα) and progesterone receptor (PR) in the bovine ampulla. (A) The nuclei of the secretory cells, which are located in the basal and central areas of the oviductal epithelium, and also the epithelial protrusions (arrow), which contain nuclei, express ERα during the follicular phase. Lp, lamina propria; scale bar (sb) = 30 µm. (B) During the mid-luteal phase, only the more basally located nuclei of the secretory cells show distinct immunostaining for ERα. Lp, lamina propria; sc, secretory cells; arrows, ciliated cells; sb = 30 µm. (C) The nuclei of the secretory cells, which are located in the basal and central areas of the oviductal epithelium, show a moderate expression of immunoreactive PR in the follicular phase. The nuclei of the ciliated cells are usually only weakly stained for PR (arrow). Lp, lamina propria; sc, secretory cells; sb = 30 µm. (D) The nuclei of the secretory cells show only a weak immunostaining for PR in the mid-luteal phase. Lp, lamina propria; arrow, epithelial protrusion; sb = 30 µm. (E) During the follicular phase, the smooth muscle cells are intensively stained for PR. Tm, tunica muscularis; sb = 50 µm. (F) During the mid-luteal phase, the smooth muscle layer of the oviduct shows only a weak staining for PR. Tm, tunica muscularis; sb = 50 µm. (G) In the follicular phase, the immunostaining for ERα in the smooth muscle layer was weaker than in the other cell populations. Tm, tunica muscularis; sb = 50 µm. (H) During the mid-luteal phase, most of the basally located nuclei of the secretory cells were weaker immunostained for ERα than in the follicular phase e, epithelium; Tm, tunica muscularis; sb = 50 µm.

Table 3. Immunohistochemistry for ERα and PR

Immunostaining for ERα				
Epithelium	Nuclei Secretory cells			Nuclei, Ciliated cells
	Basal	Central	Protrusions	
Follicular	++/+++	+/++	++/+++	−/−+
Mid-luteal	−/++	−+/+	−+/++	−/−+
	stroma cells			
Follicular	++	−	−	−
Mid-luteal	+	−	−	−
	smooth muscle cells			
Follicular	++	−	−	−
Mid-luteal	+/++	−	−	−
Immunostaining of PR				
Epithelium	Nuclei Secretory cells			Nuclei Ciliated cells
	Basal	Central	Protrusions (nuclei)	
Follicular	++/+++	+/++	−	+
Mid-luteal	+	−	+	−
	Stroma cells			
Follicular	++	−	−	−
Mid-luteal	++	−	−	−
	Smooth muscle cells			
Follicular	++/+++	−	−	−
Mid-luteal	+	−	−	−

-,negative; +-, weak positive; +, moderate positive; ++, distinct positive; +++, strong positive;

developed, which have allowed the precise and rapid procurement of target cells (Murray 2007). In recent studies, LAM has been successfully combined with qPCR (Burbach et al. 2004; Ehrig et al. 2001) and other semiquantitative or quantitative molecular transcriptomic and proteomic downstream techniques (Gjerdrum and Hamilton-Dutoit 2005b). In our study, we tried to isolate RNA from minute amounts of LAM-isolated FFPE tissue from the bovine oviduct (ampulla) and compared the expression of ERα mRNA and PR mRNA in the different histological compartments during the follicular and mid-luteal phases of the estrus cycle.

To optimize recovery of nucleic acids (RNA) from microdissected tissue, particular attention to initial tissue processing, choice of fixative, and duration of fixation time is required. These three factors cannot be influenced in retrospective studies and often represent unknown variables when archival material is used, resulting in uncertainty when interpreting the results. For the study of homogenates of tissue samples, the specimens are usually immersed in RNAlater or frozen in liquid nitrogen. These techniques usually ensure high RNA quality and facilitate extraction and amplification of RNA for following analysis. However, snap freezing by direct immersion in liquid nitrogen can generate ice crystals within the tissue that destroy histological details (Huang et al. 2002). Further cryosections from unfixed frozen tissue have been shown to be not optimal for LAM for logistic reasons and the considerable skill required for the subsequent microdissection. Therefore, in many cases, tissue embedding after fixation appears preferable for LAM if an acceptable yield and quality of the RNA can be obtained (Goldsworthy et al. 1999; Takagi et al. 2004). Also, several recent studies have shown that paraffin-embedded archival material is in principle suitable for the analysis of mRNA (Abrahamsen et al. 2003). Several useful protocols have been established for the retrieval of mRNA from routinely processed paraffin-embedded tissues (Lehmann and Kreipe 2006; Specht et al. 2001). RNA yield and quality after LAM play a decisive role in the generation of accurate quantitative results from gene expression analysis experiments (Bustin and Nolan 2004; Denisov et al. 2008; Fleige and Pfaffl 2006; Masuda et al. 1999; Srinivasan et al. 2002). cDNA made from degraded RNA is usually not amplified or labeled to the same degree as cDNA from intact RNA. However, for many studies, the isolation of high-quality totalRNA is not possible, for instance, from FFPE clinical samples of archival material. The exact

determination of totalRNA quality is therefore of utmost importance during the quantitative gene expression analysis workflow (Gingrich et al. 2006). Until recently, the traditional approach of determining totalRNA quality was agarose gel electrophoresis. This approach is usually not possible for LAM-retrieved tissue, as it requires relatively large amounts (5–10 mg) of totalRNA. In our study, the Experion system from Bio-Rad provided effective methods for determining both quantity and quality of totalRNA from small amounts of FFPE tissue.

To analyze the quantitative expression pattern of ERα mRNA and PR mRNA in the different compartments of the bovine oviduct (epithelium, lamina propria, and tunica muscularis), we used GenEx Professional software, Version 4.3.5. For the comparison of mRNA concentrations between different samples, normalization of the mRNA data is necessary. The use of reference genes as internal control is the most common method for normalization, although the use of reference genes is generally accepted as the most appropriate normalization strategy (Huggett et al. 2006). Normalization using a single reference gene has been criticized recently unless there is clear evidence that the gene is invariantly expressed under all conditions of the study. In our study, we used therefore three reference genes (Pol II, β-actin, and GAPDH) to normalize the expression of ERα mRNA and PR mRNA in RNAlater and FFPE material using the GeNorm program included in GenEx Professional software, Version 4.3.5.

In this study, we not only depicted the presence of ERα mRNA and PR mRNA in the bovine ampulla but also found clear differences in mRNA and protein expression of ERα and PR in that region of the bovine oviduct during the follicular and the mid-luteal phases of the estrus cycle. Steroid hormones exert their influence in target cells through interactions with specific protein receptors. Lipophilic steroids pass easily through the cell membrane (Giorgi 1980) and bind to specific cytosolic receptors (Chan and O'Malley 1976), which are translocated to the cell nucleus, interact with the chromatin, and induce the transcription of specific hormone-dependent genes (Grody et al. 1982). Previous studies have shown that during normal estrous cycles, 17β-estradiol (E2) stimulates the production of ERα and PR, whereas in some species, progesterone (P4) causes an inhibitory effect on the synthesis of its own receptor (Meikle et al. 2001; Reinhart et al. 2003; Wu et al. 1996; Valle et al. 2007).

Our data demonstrate distinct differences in the expression pattern of transcripts for PR mRNA and ERα mRNA in the bovine ampulla during the follicular and mid-luteal phases. During the estrogen-dominated follicular phase, an increase of PR mRNA could be detected in both RNAlater and formalin-fixed tissue homogenates from bovine oviductal ampulla. In both cases, an increase of PR expression was observed during the follicular phase, which is in agreement with the results of Ulbrich et al. (2003), obtained from bovine oviductal tissue homogenates and cell cultures.

Using laser microdissection and pressure catapulting, we could show that PR mRNA is differentially expressed in the epithelium, lamina propria, and smooth muscle coat of the oviduct during the estrus cycle. In the epithelium, the PR mRNA was expressed significantly higher during the follicular phase than in the mid-luteal phase, whereas there was no significant difference of PR mRNA expression in the lamina propria in the bovine ampulla between the follicular and mid-luteal phases. In the smooth muscle layer, we found a significant higher expression of PR mRNA in the follicular phase. The qPCR data were supported by our in situ hybridization results, which showed a distinct reduction of mRNA expression of PR in the epithelium and smooth muscle coat during the mid-luteal phase. Like Ulbrich et al. (2003), we also observed a distinct upregulation for PR RNA transcripts during the estrogen-dominated follicular phases in bovine epithelium. Results of immunostaining indicate that in secretory cells of the oviduct, the nuclear expression was significantly more intense during the follicular phase compared to the secretory phase. Ciliated cells, on the other hand, showed only a weak staining for PR or were completely negative. In stroma cells, a moderate expression of PR was found throughout the estrus cycle. Nuclei of smooth muscle cells displayed an intense immunostaining for PR during the follicular phase.

In agreement with our results, Ulbrich et al. (2003) have demonstrated that E2 stimulates the expression of PR mRNA. P4 stimulation, on the other hand, results in a reduction of transcript numbers of PR mRNA, which indicates that the oviductal PR mRNA is suppressed during P4 dominance. The same authors (Ulbricht et al. 2003) mentioned that P4 leads to a reduction of PR transcript numbers. With declining peripheral progesterone levels during luteolysis, the inhibition diminishes, causing a strong upregulation of PR expression. A reduced PR mRNA expression after exogenous P4 stimulation was also reported in rabbits (Hyde et al. 1989). In addition, the results of Valle et al. (2007) obtained from studies in heifers suggest a negative effect of P4 on the expression of its own receptor. Results from Garcia-Palencia et al. (2007) indicate that sheep estrus synchronized with P4 show a reduction of ERα and PR protein expression in most oviductal and uterine cells. Shao et al. (2006) suggest that PR expression increases by estradiol stimulation and P4 inhibition. Behind the physiological role of PR in the uterus, Shao et al. (2007) described cellular events leading to cell death or survival that are mediated by modulation of PR expression in different cell types in the mouse oviduct.

From our results, we can conclude that the smooth muscle layer of the oviduct is the compartment that is most affected by changes in progesterone concentrations during the cycle, and

oviductal PR mRNA in the tunica muscularis appears to be suppressed during progesterone dominance. An increased PR expression in the muscle layer in the follicular phase was also reported in women (Amso et al. 1994). Bennet et al. (1988) delineate a maximum increase of muscular contractility in the isthmus during estrus. In contrast, tubal motility is decreased by the P4-induced reduction in the beat frequency of ciliated cells in human oviducts (Mahmood et al. 1998). Our results support the findings of Mwanza et al. (2000) and Bage et al. (2002) that the postovulatory increase in blood P4 and decrease of PR lead to reduced intraluminal pressure and decreased motility in the luteal phase.

Our results from oviductal tissue homogenates of RNAlater or FFPE material demonstrate that the number of ERα transcripts during the follicular phase is higher than in the mid-luteal phase. Using LAM material, we could prove that the stronger expression of ERα mRNA is restricted to the oviductal epithelium and lamina propria, whereas no significant changes occur in the smooth muscle layer. Our immunohistochemical results clearly demonstrate that ERα protein staining of the secretory cells nuclei was more intense in the follicular phase. The nuclei of the ciliated cells appeared to be negative during the estrus cycle. Stroma cell staining was more intense in the follicular phase compared to the mid-luteal phase. The nuclei of the muscle cells, however, displayed no significant staining differences between the two stages of the cycle.

Previous qPCR investigations of Ulbrich et al. (2003) described an increase of ERα expression in cell cultures of bovine oviductal epithelial cells after in vitro stimulation with E2. In vivo, the peripheral blood plasma estrogens levels are highest during the follicular phase of the bovine estrus cycle (Amso et al. 1994; Wijayagunawardane et al. 1996). Upregulation of mRNA for ERα during the follicular phases is probably directly linked to the specific changes in the composition of the oviductal fluid during the estrogen-dominated follicular phase (Wijayagunawardane et al. 1996). Reports from Stanchev et al. (1985) on the porcine oviduct indicated that high levels of plasma estradiol cause an increase of ER, which is first seen in the cytoplasm and later in the nuclei. Also, in primates (Amso et al. 1994; Brenner and Slayden 1994) and in the bovine (van de Leemput et al. 2001), an enhanced nuclear ERα expression has been observed in the oviductal epithelium during estrus. Rodríguez-Piñón et al. (2005) recently demonstrated that E2 treatment of prepubertal ewes affects ERα and PR transcription in the oviduct and cervix. In addition, they found that E2 injection to prepubertal lambs stimulated the steroid receptor transcription in the oviduct and cervix after the first injection. Interestingly, the increments of ERα and PR after E2 injection in the oviduct occurred earlier compared to the cervix, where the increase was higher and was maintained over a longer period. The E2 effect on the oviductal ERα and PR concentrations showed a biphasic course: An initial reduction of receptors was followed by restoration of the receptor concentration. In contrast, Valle et al. (2007) found no correlation between the decrease and increase of ERα and the E2 concentration in the circulation.

Our immunohistochemical results showed that the nuclei of the ciliated cells were mostly negative or only weakly stained for ERα. In contrast, strongly ERα-positive stained stromal cells could be located in the apical region of the primary and secondary folds. The positive stromal cells of the folds were usually located directly beneath the epithelium. Our results confirm the findings of Brenner and Slayden (1994), who assume that steroid hormone receptors in the stromal cells of the primate oviduct contribute to the regulation of the activity of the oviductal ciliated cells. On the other hand, Valle et al. (2007) found no different immunostaining of ciliated and secretory cells, and they maintain that steroid hormone receptors are present in both cell types. Furthermore, we found a weak positive expression for PR in the nuclei and cytoplasm of ciliated cells of the follicular phase, whereas they were negative in the mid-luteal phase.

Nutu et al. (2009) detected PRβ within the cilia and PRγ at the base of the ciliated cell of the mouse and human oviductal cells. Studies using knockout mice (either PRα or PRβ knockout) described that PRβ does not affect ovarian and uterine response to P4 (Conneely et al. 2002). In addition, Ulbrich et al. (2003) observed that PRα mRNA undergoes distinct changes during the estrus cycle, whereas PRβ expression seem to be unaffected by peripherical steroid hormones. As our antibody could not distinguish between the isoform of the PR, a specific localization of PRα, PRβ, and PRγ was not possible. In accordance with Nutu et al. (2009), we assume that the weak positive staining of PR in the oviduct observed in our study could be due to the PRβ or PRγ isoform in ciliated cells. The only weak expression of PR in the epithelium and smooth muscle cells during the mid-luteal phase in bovine ampulla supports the results of Vegeto et al. (1993), who postulate that PRα acts as the dominant repressor of transcription of progesterone-sensitive genes, whereas PRβ is an activator of transcription. Wessel et al. (2004) described a nongenomic ciliary motility as reactions after exposure to progesterone. In agreement with Ulbrich et al. (2003), we think that the presence of PRβ in the mid-luteal phase and the absence of PRα during the same period may enhance the P4-mediated action on the bovine oviduct. Okada et al. (2003) described a lack of ERα and PR (both isoforms) in ciliated cells and therefore think that both receptors play no direct role in the regulation of ciliogenesis. They postulate an interaction between ERα-positive epithelial and stromal cells via intermediate molecules. Okada et al. (2003) further hypothesize that ERα- and PR-positive non-ciliated progenitor cells differentiate into ciliated cells with concomitant loss of ERα and PR.

In conclusion, our study demonstrates distinct changes in the expression of ERα mRNA and PR mRNA in the three different histological compartments of bovine ampulla during the follicular and luteal phases of the cycle. Combining LAM techniques with qPCR, we were able to demonstrate for the first time that the previously observed downregulation of PR in the ampulla of the bovine oviduct during the mid-luteal phase (Ulbrich et al. 2003) is due to significant changes in the mRNA content of the tunica muscularis and not only to downregulation of PR expression in the epithelium or lamina propria, as previously assumed (Ulbrich et al. 2003). Our results also show that during the mid-luteal phase, PR expression appears to be inhibited by P4, whereas the increase of ERα/PR mRNA and protein is stimulated by E2. We could further demonstrate that quantification of steroid hormone receptor mRNA is possible using microdissected FFPE tissue, although the quality and quantity of the RNA appear to be considerably decreased compared to RNAlater-immersed tissue. Nevertheless, if certain precautions concerning tissue collection, fixation, tissue digestion with proteinase K, and size of the amplicons are observed, meaningful results can be gained from FFPE tissue sections, which provide new, interesting data that cannot be gained from tissue homogenates.

Declaration of Conflicting Interests

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