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Promoter-2-derived *Cyp19* expression in bovine granulosa cells coincides with gene-specific DNA hypo-methylation

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Abstract

Cyp19, the key gene of oestrogen biosynthesis, is expressed at very different concentrations and from different promoters in bovine granulosa cells (GCs) and in pregnant corpora lutea (CL), respectively. The present study was aimed to investigate if DNA methylation and thus epigenetic mechanisms might play a potential role in the regulation of *Cyp19* expression and promoter-specific activity in GCs of cycling versus CL of pregnant cows.

It was demonstrated that GCs express high concentrations of promoter-2-derived *Cyp19* transcripts whereas CL samples isolated before and after implantation, and at the end of the first trimester, showed very low *Cyp19* transcript concentrations, all of them derived from promoter 1.1. Two genomic regions including promoter 1.1 and promoter 2 were largely unmethylated in GCs but methylated in all CL samples. The data suggest that promoter-2-derived high-level expression but not promoter-1.1-derived low-level expression of *Cyp19* might be controlled by cell-type-specific DNA methylation.
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1. Introduction

Expression of the *Cyp19* gene that encodes the key enzyme of oestrogen biosynthesis, aromatase cytochrome P450 ($P450_{arom}$), is considered as an important milestone during follicular development (Bao and Garverick, 1998). In cattle, recruitment starts with a cohort of up to seven follicles of about 4 mm in diameter, which continue to grow to 8–9 mm (Webb et al., 1999). This is followed by selection and rapid divergence whereby one follicle grows rapidly and becomes the dominant follicle (Bao et al., 1997; Webb et al., 1999). Immediately after recruitment granulosa cells (GCs) of healthy follicles start expressing *Cyp19* transcripts (Xu et al., 1995). Expression increases with growing diameter (Lenz et al., 2004) culminating in large dominant follicles (Xu et al., 1995). Therefore GCs of large dominant follicles are the most abundant source of oestrogen. Following the luteinising hormone (LH) surge, a rapid decrease of oestradiol production and expression of $P450_{arom}$ was observed in preovulatory follicles (Komar et al., 2001). However, *Cyp19* expression is not completely switched off after luteinisation. It was demonstrated that bovine luteal cells are still able to locally produce oestradiol (Okuda et al., 2001). As published recently, *Cyp19* expression is directed by different promoters in GCs of cycling ovaries and CL during pregnancy. Whereas promoter P2 is responsible for high-level expression of *Cyp19* in GCs (Lenz et al., 2004), P2-derived transcripts are virtually absent in CL. Instead, the low level, residual expression in luteal tissue is maintained only by promoter P1.1 (Vanselow et al., 2004). This suggests that the dramatic downregulation of *Cyp19* expression during luteinisation is regulated by switching off promoter P2. P1.1 may escape this LH-induced de-activation and thus is responsible for residual *Cyp19* expression in CL. Besides P2- and P1.1-derived transcripts, low concentrations of P1.5 transcripts were also found in GCs isolated from medium-sized follicles. However, the contribution of P1.5 to *Cyp19* expression in large follicles (>9 mm) and

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CL was either very small or absent, respectively (Lenz et al., 2004).

In vertebrates methylation of cytosines (C) that does occur only in the context of CpG dinucleotides plays an important role in the local organisation of the chromatin structure and thus the long-term regulation of gene expression via epigenetic mechanisms. In recent years, it became evident that transcriptional silencing that is associated with DNA methylation plays a role for protection against intragenomic parasites (Walsh et al., 1998) and in carcinogenesis (Jones and Baylin, 2002). But also essential regulatory processes during mammalian development as genomic imprinting or X-chromosome inactivation (Li, 2002) are closely associated with DNA methylation of CpG-rich regions called CpG islands. However, the potential role of DNA methylation in tissue-specific gene expression or in the regulation of CpG-poor promoters is less well established. In the bovine it was demonstrated that P1.1 of *Cyp19* as well as the distal promoter of the oxytocin gene show expression correlated DNA methylation and chromatin structure in placenta and ovary, respectively (Fürbass et al., 2001; Kascheike et al., 1997).

LH triggers irreversible molecular, physiological and morphological changes in preovulatory follicles. A well-established effect of the LH surge on the molecular level is the dramatic downregulation of *Cyp19* expression during luteinisation (Voss and Fortune, 1993). However, the signal transduction pathways and responsive elements on the DNA level that are responsible for *Cyp19* downregulation and maintained repression of this gene are not yet fully understood.

The present study was aimed to investigate if the very different levels of *Cyp19* expression and the activity of different *Cyp19* promoters in GCs of cycling ovaries and pregnant CL coincide with different levels of gene-specific DNA methylation. This would provide first hints that *Cyp19* expression and possibly also tissue-specific promoter selection before and after luteinisation are controlled and stabilised by DNA methylation and thus by local changes of the chromatin structure.

Cyp19 transcript concentrations and the DNA methylation status of *Cyp19* were comparatively analysed in GCs isolated from medium to large bovine follicles of cycling ovaries and in CL from pregnant cows. CL samples were collected before and after implantation (nidation), and at the end of the first trimester. Promoter-specific transcript concentrations were measured individually by an established real-time PCR system (Lenz et al., 2004; Vanselow et al., 2004). In order to analyse the DNA methylation status of the corresponding promoter regions, a quantitative assay system based on bisulfite genomic sequencing (Frommer et al., 1992) was established for *Cyp19* and for the α-s1-casein gene (*CSN1S1*) as a control. Caseins are the major milk proteins of mammals but so far were not found in other tissues. Among the caseins, α-s1-casein is the most abundant, exceeding a concentration of 13 mg/ml of milk (Jennes, 1970; Koczan et al., 1991). The status of CpG methylation from the bisulfite-treated and PCR-amplified DNA was determined by direct PCR sequencing. This technique requires only small amounts of genomic DNA, does not depend on restriction enzyme specificities, and enables the analysis of the methylation status of all CpG in the target DNA irrespective of nearest-neighbour compositions.

2. Materials and methods

2.1. Tissue collection, DNA and RNA preparation

Ovaries were collected and pooled from randomly cycling German Holstein cows at a local abattoir during two different days. GCs were aspirated and flushed from medium to large follicles (>5 mm, 1–5 follicles per ovary) with a syringe under visual control. For the first sample (GC1), granulosa cells from 14 ovaries and for the second sample (GC2) granulosa cells from 11 ovaries were pooled prior to RNA preparation thus representing at least 7 and 6 animals, respectively. To obtain CL of defined stages of pregnancy, three individual German Holstein cows were slaughtered before and after implantation, and at the end of the third trimester (4, 20 and 100 days post-artificial insemination, respectively). Ovaries were removed, corpora lutea dissected and freed from surrounding tissues. From one of these animals, a liver sample was also collected as control tissue. Mammary gland (MG) was obtained from a cow slaughtered during lactation. Liver, CL and MG were chopped into chunks of about 0.5 cm diameter, immediately transferred to appropriate volumes of RNAlater solution (Qiagen, Hilden, Germany), incubated for 24 h at 4 °C and subsequently stored at −20 °C. All procedures involving animals were approved by the Landesveterinär- und Lebensmitteluntersuchungsamt Mecklenburg-Vorpommern according to the German law for animal protection (TierSchG).

Total RNA and DNA were extracted by the standard Trizol procedure (Invitrogen, Karlsruhe, Germany) and quantified in a GeneQuant II instrument (Pharmacia, Freiburg, Germany). Quality of nucleic acid preparations were monitored from randomly selected samples by denaturing (RNA) or non-denaturing (DNA) agarose (1%) gel electrophoresis stained with ethidium bromide.

2.2. Reverse transcription and real-time PCR

Quantitative real-time RT-PCR assays for *Cyp19* transcripts derived from promoters P1.1, P1.5 and P2 (T1.1, T1.5 and T2) were performed as described earlier (Lenz et al., 2004; Vanselow et al., 2004). Primers (purchased from Sigma-Genosys, Steinheim, Germany) are shown in Table 1. Briefly, for cDNA synthesis M-MLV reverse transcriptase, RNase H Minus, Point Mutant (Promega, Mannheim, Germany) was used according to the standard protocol. The freshly synthesised cDNA samples were cleaned with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and eluted in 50 μl elution buffer.

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