Expression of transforming growth factor-ß3 (TGF-ß3) in the porcine ovary during the oestrus cycle

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Summary. Transforming growth factor-ß (TGF-ß) proteins are growth factors that have been shown to be involved in regulation of ovarian follicular development. Ovarian expression, activity and functional significance of TGF-ß1 and TGF-ß2 isoforms were extensively studied in most species. However, little is known about the biological role of TGF-ß3 previously shown to be expressed independently of the other two isoforms. Therefore, expression of TGF-ß3 mRNA and protein was evaluated by RT-PCR and immunohistochemistry, respectively, in porcine ovaries collected during different phases of the oestrus cycle. Results of RT-PCR analysis showed that TGF-ß3 mRNA is expressed throughout the oestrus cycle. The level of TGF-ß3 mRNA expression was found to be higher at metoestrus and dioestrus. Weak TGF-ß3 immunoreactivity was present in follicular epithelial cells and oocytes of preantral follicles in all stages examined. TGF-ß3 protein expression was exclusively present in theca interna cell layer of antral follicles, and was particularly prominent in large antral follicles. Immediately after ovulation, almost all theca cells outside of the granulosa cell layer were intensively stained with anti-TGF-ß3. Immunostaining of TGF-ß3 in theca lutein cells rapidly decreased during corpus luteum development. It is suggested that TGF-ß3 may play an important role in modulating theca cell function of pre- and postovulatory follicles of the pig.

Key words: TGF-ß3, Ovary, Pig, Oestrus cycle, Immunohistochemistry, RT-PCR

Introduction

In the mammalian ovary three structurally and functionally similar transforming growth factor-ß, (TGF-ß) isoforms, termed TGF-ß1, TGF-ß2, and TGF-ß3, have been shown to be important regulators of follicle development (Juengel and McNatty, 2005). Studies in different species have demonstrated that cell-type specific expression of TGF-ß is highly dependent on the species studied and the stage of the follicle differentiation. Generally, in rodents and humans, TGF-ß appears to be produced from both theca and granulosa cells, whereas in sheep, cow, and pig it is mainly derived from theca cells (Juengel and McNatty, 2005). Qualitative differences in TGF-ß mRNA subtype expression could contribute to quantitative differences in TGF-ß mRNA bioactivity. For example, porcine granulosa cells are known to produce very low levels of TGF-ß-like activity, probably due to absent expression of TGF-ß2 mRNA and corresponding protein product (Mulheron et al., 1992). Consequentially, porcine theca cells were identified as the major source of follicular TGF-ß, whereas TGF-ß1 was suggested to be the predominant subtype in porcine follicles, affecting both growth and differentiation of follicle cells and stromal reorganization, respectively (May et al., 1996). Most of our knowledge of the biological role of TGF-ß in the regulation of follicle development has been derived from in vitro experiments predominantly performed in cultured granulosa cells treated with TGF-ß1. Despite the difficult interpretation of in vitro results TGF-ß appears to participate directly or indirectly in important ovarian cell events, including steroidogenesis (Kubota et al., 1994; Ford and Howard, 1997), follicle growth and differentiation (May et al., 1996), follicular atresia (May et al., 1996; Sirotkin et al., 2004), and probably corpus luteum function (Gangrade and May, 1990).

While cellular localization of TGF-ß1 and TGF-ß2 isoforms has been extensively studied in most species (summarized by Nilsson et al., 2003), there is little

information about ovarian expression of TGF-ß3, especially in the pig. In murine and bovine ovaries TGF-ß3 expression was found to be present in all major cell types of preantral and antral follicles (Schmid et al., 1994; Nilsson et al., 2003). In contrast, TGF-ß3 mRNA in the sheep was exclusively localized in cells associated with the vascular system of the ovary (Juengel et al., 2004). Collectively, these data suggest that expression of TGF-ß3 is species-specific and independently regulated of TGF-ß1 and TGF-ß2 (Nilsson et al., 2003). In this study we analysed the expression of TGF-ß3 in the porcine ovary during the oestrus cycle, using both immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) procedures. Our results show in detail the specific localization of TGF-ß3 in theca interna cells of antral follicles, as well as changes in protein expression during the pre- and postovulatory phase. A preliminary report was already presented at the 40th Annual Conference of Physiology and Pathology of Reproduction, Berlin, Germany, February 22, 2007.

Materials and methods

Animals and tissue preparation

A total of 12 pigs of the German Landrace were obtained from the experimental station of animal husbandry, animal breeding, and small animal breeding of the University of Hohenheim. Oestrus detection was performed twice daily in the presence of a boar. The animals were slaughtered at metoestrus (days 1-3, n=4), dioestrus (days 5-13, n=3), prooestrus (days 18-20, n=3), and oestrus (n=2). The day the female pig accepts the boar for mating was determined as day 0 of the oestrus cycle. Ovaries were immediately fixed in methanol/glacial acid (2:1, 24 h) and paraffin embedded according to standard procedures. For immuno-histochemistry, ovaries were cut into 5 µm thick serial sections, and mounted on Superfrost® glass slides.

Immunohistochemistry

After deparaffinization and antigen retrieval in a microwave oven in 10 mM sodium citrate buffer at pH 6.0 (3x5 min at 700 W), sections were processed for immunohistochemistry using the rabbit anti-human TGF-ß3 antibody (Lab Vision, Newmarket, UK) diluted 1:200 in PBS (pH 7.4). This antibody does not cross-react with TGF-ß1 or TGF-ß2, as indicated by the manufacturer.

Briefly, before incubation with the primary antibody at 5°C overnight in a humid chamber, sections were treated with 1% hydrogen peroxide in double-distilled water for 10 min at room temperature (RT) to block endogenous peroxidase activity and 10% normal rabbit serum (Dako, Hamburg, Germany) for 30 min at RT to prevent non-specific protein binding, respectively. The next day, sections were incubated with the biotinylated

goat anti-rabbit IgG (diluted 1:400 in PBS; Dako) for 30 min at RT. Afterwards, Strept-ABC kit (Dako) was performed according to manufacturer's instructions. Each incubation step was followed by 3x5 min rinsing with PBS. The reaction product was visualized with 3,3'-diaminobenzidine-hydrogen-peroxide reagent (DAB) (Biotrend Chemicals, Köln, Germany). Finally, sections were counterstained with Mayer's haematoxylin, dehydrated, cleared with xylene, and mounted in Entellan (Merck, Darmstadt, Germany). Pictures were taken using a brightfield light microscope (DMRBE, Leica, Bensheim, Germany) and a video camera (ProgRes, Kontron Instruments, Watford, UK) coupled to a Pentium PC.

Immunohistochemical controls were performed by (1) replacing the primary antibody with non-immune serum, (2) omitting the secondary antibody, and finally (3) incubation with DAB solution alone to ensure specificity of staining. Specimens from porcine adrenal gland served as positive controls. Immunoreactive TGF-ß was previously found in the cortex of adrenal glands (Keramidas et al., 1991).

RNA extraction, reverse transcription and polymerase chain reaction

Total RNA was extracted from the ovary at different stages of the oestrus cycle (days 0, 3, 13, and 20) using the Invisorb reagent (Invitek, Berlin, Germany) according to the manufacturer's protocol and treated with RNAse-free DNAse (Promega, Mannheim, Germany) to exclude any possible contamination by genomic DNA. The isolated RNA was dissolved in Diethylpyrocarbonate (DEPC)-treated water and quantified by means of spectrophotometry. For each sample 1.5 µg of total RNA was reverse transcribed using Reverse-iT 1st Strand Synthesis kit (Abgene, Epsom, UK) and oligo(dN10) primer.

PCR was performed with 105 ng of the obtained cDNA product using the following primers: TGF-ß3 (sense: 5'-TGGAGGAGAACTGCTGTGTG-3', antisense: 5'-GGTCCTCCCGACGTAGTACA-3'). Primer sequences were derived from porcine gene sequences found in GenBank. The annealing temperature was 65°C and 35 cycles of PCR were performed. Expression of the housekeeping gene GAPDH (Glycerinaldehyde-3-phosphate dehydrogenase) (sense: 5'-AAGTGGACATTGTCGCCATC-3', antisense: 5'-TCACAAACATGGGGGCATC-3') served as a control for RNA content. The annealing temperature was 61°C and 35 cycles of PCR were used.

The PCR amplification products were visualized after electrophoresis on a 20% polyacrylamide gel using a Silver-Staining kit (Amersham, Freiburg, Germany).

PCR-control reactions were performed without cDNA as a template and with all used primer and also with total RNA instead of cDNA as a template, to exclude any unspecific amplification of genomic DNA. All controls have shown negative results.

Results

Immunohistochemistry

In paraffin sections of porcine ovaries, immunohistochemical procedure revealed cell-type-specific and follicle stage-specific staining of TGF-ß3. Expression of TGF-ß3 protein was detected in oocytes and follicle epithelial cells of preantral follicles in all cycle stages examined (data not shown). Staining intensity was consistently weak from the primordial to secondary stage. TGF-ß3 was first observed in the theca interna cell layer at the time of antrum formation. In early to late antral follicles, strong staining of TGF-ß3 was exclusively found in theca interna cells, but not in granulosa cells. Staining intensity of TGF-ß3 did not change within the different antral follicles. However, differences were seen in the number of positively stained theca interna cells as follicles matured. TGF-ß3-positive cells were less frequently observed in theca interna of early- and mid-antral follicles (Fig. 1a). As follicles reached the preovulatory size, almost all theca interna cells stained positive for TGF-ß3 throughout the cytoplasm (Fig. 1b). Immediately after ovulation, strong staining of TGF-ß3 was observed in all theca cell layers surrounding granulosa cell lobules (Fig. 1c). During early luteogenesis, numerous TGF-ß3-positive cells were distributed in the interlobular connective tissue of the theca interna layer and neighbouring blood vessels in this layer of the new developing corpus luteum (CL) (Fig. 1d). Both number of stained cells and staining intensity decreased rapidly during the course of CL formation. TGF-ß3 immunostaining largely disappeared in the CL of the mid-luteal phase with rather unspecific staining in some luteal cells and blood vessels (data not shown). Furthermore, we found variable staining of TGF-ß3 in theca cells of atretic follicles and in the wall of blood vessels distributed in the ovarian stroma (data not shown). Additionally, sections of adult porcine adrenal glands were stained in parallel as positive controls (Fig. 1 e). As clearly demonstrated, TGF-ß3 immunoreactivity was present in adrenal cortex. Immunostaining of TGF-ß3 was restricted to the zona fasciculata/reticularis and largely absent in the zona glomerulosa. No immunostaining was found in the capsule or adrenal medulla. The absence of detectable staining of tissue elements in negative control sections verified the specificity of the applied antibody (Fig. 1f).

RT-PCR analysis

The expression of TGF-ß3 mRNA in porcine ovaries at different stages of the oestrus cycle (see material and methods) was shown in figure 2. RT-PCR products of the expected size (254 bp) were clearly detected in all stages of the oestrus cycle (Fig. 2A). Additionally, RT-PCR analysis resulted in the anticipated 254-bpTGF-ß3 PCR product in porcine adrenal gland. GAPDH (resultant PCR product was 318 bp) was used as a housekeeping gene (Fig. 2B). The semi-quantitative analysis of the PCR products revealed that the level of TGF-ß3 mRNA expression was low at oestrus, markedly up-regulated at metoestrus, and slightly decreased during prooestrus, respectively (Fig. 2A).

Discussion

Members of TGF-ß family have been shown to be closely associated with important ovarian functions, including follicular development, proliferation, steroidogenesis, ovulation, and corpus luteum formation (Knight and Glister, 2006). Collected data from mRNA and protein expression studies have demonstrated that TGF-ß isoforms are species-specifically expressed and that each of the three mammalian isoforms is involved in a unique subset of ovarian cellular and developmental activities (Nilsson et al., 2003). Unlike TGF-ß1 and TGF-ß2, expression of TGF-ß3 in porcine ovaries during the follicular development is largely uncharacterised. In this study we have shown that porcine ovaries express TGF-ß3 mRNA and protein throughout the oestrus cycle. Semi-quantitative PCR analysis revealed that the level of TGF-ß3 mRNA is up-regulated at metoestrus, as compared to the other cycle stages. Furthermore, we found that in antral follicles TGF-ß3 protein is exclusively localized to theca interna cells.

This is the first report showing the specific localization of TGF-ß3 in theca interna cells of porcine antral follicles. In previous studies, porcine theca cells were already identified as the major source of follicular TGF-ß, whereas TGF-ß1 was suggested to be the predominant subtype in antral follicles (Gangrade and May, 1990; May et al., 1996). However, it should be noted that the TGF-ß subtype was determined under *in vitro* conditions (May et al., 1996) which may not accurately reflect those *in vivo*. It is known that both theca cells (Engelhardt et al., 1991) and granulosa cells (Garrett et al., 1991) of pigs undergo luteinization in vitro, therefore such experiments should be interpreted with caution. Mulheron and colleagues (1992) reported that TGF-ß3 mRNA is expressed in cultured porcine granulosa cells isolated from 1-3 mm size follicles, but protein production was not observed. In our studies no immunoreactivity of TGF-ß3 was found in granulosa cells of antral follicles, indicating that mRNA detection does not appear to be correlated with translation. Similar phenomena were reported in the human placenta, where negative staining for TGF-ß1 and TGF-ß2 in immunohistochemical and Western blotting analyses, but presence of TGF-ß1 and -2 mRNA by RT-PCR analysis of the same sample was found (Simpson et al., 2002).

One of our most striking findings in the current study was the level of TGF-ß3 staining in theca interna cells before ovulation. As follicles reached the preovulatory size the theca interna cell layer was completely stained with TGF-ß3 protein. We strongly suggest that in the pig ovary TGF-ß3 may be involved in steroidogenic activity of the theca interna cell layer
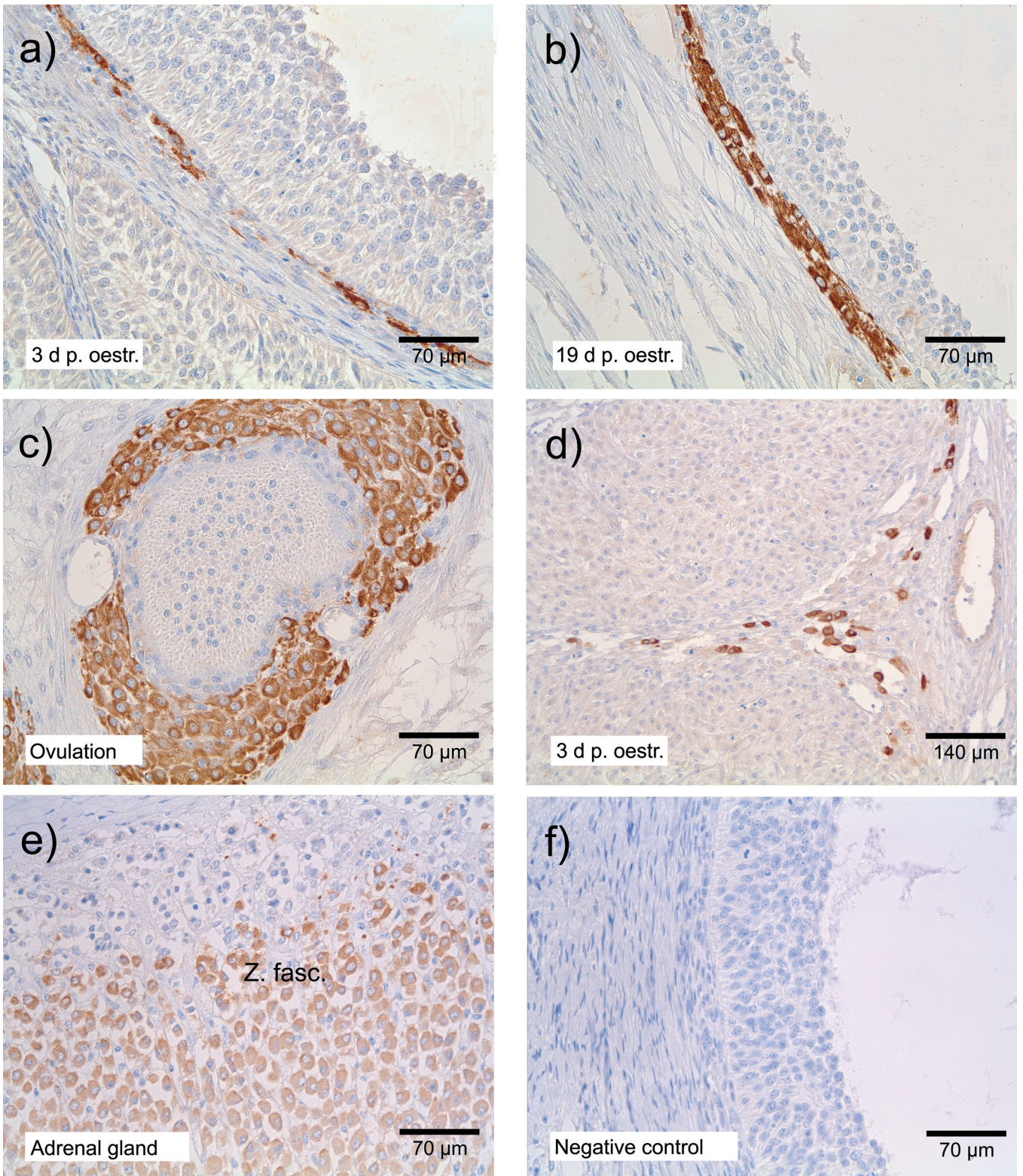
Fig. 1. Immunohistochemical detection of TGF-ß3 in porcine ovary (**a-d, f)** and adrenal gland (**e**). Strong immunostaining of TGF-ß3 is detected in numerous theca interna cells of mid-antral follicles **(a)**. In late-antral follicles TGF-ß3 immunostaining is present in the cytoplasm of almost all theca interna cells **(b)**. Immediately after ovulation TGF-ß3 immunoreactivity is massively found in theca interna and externa cells surrounding the collapsed follicle **(c)**. During early luteogenesis TGF-ß3-positive cells are also found to be distributed in interlobular connective tissue of the theca interna and neighbouring blood vessels in this layer. Number of stained cells and staining intensity is increasingly reduced **(d)**. In positive control tissue adrenal cortex is stained with anti-TGF-ß3 antibody (Z. fasc.: Zona fasciculata) **(e)**. Negative control sections on which the primary antiserum was not applied never showed immunoreactivity **(f)**.

during the final stages of follicle development. Both autocrine effects and paracrine interactions with granulosa cells and cells of the stroma, respectively, are possible routes of action of theca cell-derived TGF-ß in the porcine ovary (Engelhardt et al., 1992; Kubota et al., 1994; May et al., 1996). The role of TGF-ß as a potent autocrine regulator of thecal steroidogenesis in the pig by alternating inhibition or stimulation of local steroid hormone production was already described (Caubo et al., 1989; Engelhardt et al., 1992). Furthermore, it has been demonstrated in the pig that the theca interna cell layer may be the primary steroidogenic compartment of the porcine follicle during its final stages of preovulatory development (Conley et al., 1994). While granulosa cells of the preovulatory follicle only aromatize theca-derived androgens, theca cells are capable of synthesizing oestrogen independent of the granulosa cell layer (Conley et al., 1994). Interestingly, specific localization of TGF-ß3 in the porcine ovary (theca interna layer) and adrenal gland is consistent with cellular expression of steroidogenic enzyme cytochrome P-450 17α-hydroxylase/17,20-lyase (P-450$_{17\alpha}$) in the same tissues (Suzuki et al., 1992). P-450$_{17\alpha}$ is very important for the entire system of steroid hormone biosynthesis, especially androgen biosynthesis. These observations emphasize our suggestion that TGF-ß3 may modulate steroidogenesis in porcine theca interna cells, especially around the time of ovulation. Further studies are required to elucidate the specific molecular targets of TGF-ß3 at this time point of follicle development.

Besides autocrine functions, TGF-ß proteins are known to act on granulosa cells. The regulatory action of TGF-ß on porcine granulosa cells may be mediated through type II and I receptors identified in this cell-type (Goddard et al., 1995). TGF-ß type I and II receptors are essential for signal transduction, whereas TGF-ß type III receptor regulates the access of TGF-ß isoforms to the signalling receptors (Wang et al., 1991). In several studies the paracrine interaction between theca cell-derived TGF-ß and granulosa cell-derived factors has been proven. For example, it was shown that granulosa cells can synthesize and secrete parathyroid hormone-related peptide (PTH-rp) (Garmey et al., 2000) and connective tissue growth factor (CTGF) (Harlow et al., 2002) when treated with TGF-ß1. Granulosa cell-derived PTH-rp increases intracellular free calcium ion concentration in porcine theca cells and may mediate calcium-dependent steroid hormone producing events (Garmey et al., 2000). These and other unknown TGF-ß-mediated factors may promote steroidogenesis, tissue reorganization, and extracellular matrix deposition during follicular growth and atresia (Huet et al., 1997;
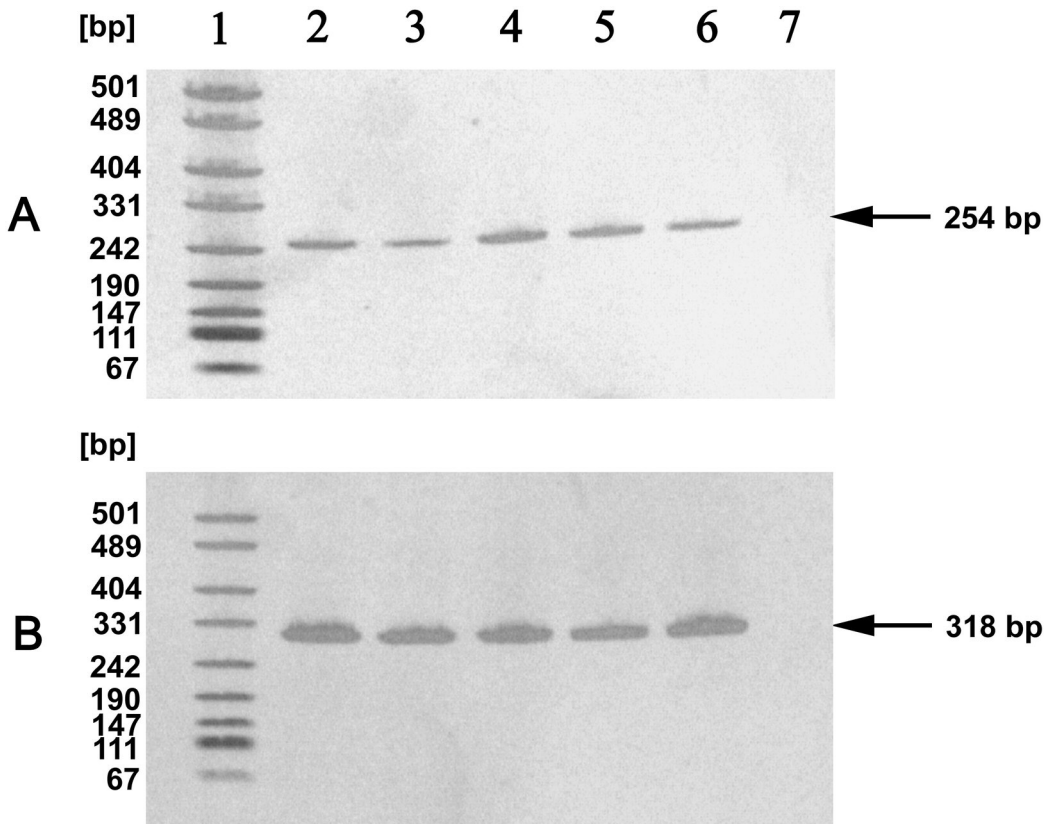

Fig. 2. Expression of TGF-ß3 mRNA in porcine ovaries and adrenal gland at different stages of the oestrus cycle determined by RT-PCR. Porcine GAPDH was used as housekeeping gene. The PCR amplification products demonstrate the corresponding fragments of 254 bp for TGF-ß3 **(A)** and 320 bp for GAPDH **(B)**. Lane 1: molecular size marker, lane 2: adrenal gland, lane 3: ovary, day 0, lane 4: ovary, day 3, lane 5: ovary, day 13, lane 6: ovary, day 20, lane 7: negative control with TGF-ß3 or GAPDH primer, respectively.

Wandji et al., 2000). Whether TGF-ß3 may be a paracrine regulator of granulosa cell function is currently not clear.

Although we did not perform cell-type-specific and follicle stage-dependent mRNA analyses, our RT-PCR results indicate that the ovarian expression of TGF-ß3 mRNA is higher at metoestrus and dioestrus as compared to other cycle stages. One possible explanation may be that following ovulation follicular tissue is remodelled to form a corpus luteum (CL). Therefore, it is conceivable that massive tissue reorganization after the ovulation process requires enhanced TGF-ß3 expression. Our hypothesis is supported by immunohistochemical data and considerations by others (May et al., 1996). Immediately after ovulation, strong staining of TGF-ß3 in almost all theca cell layers was found, and TGF-ß3 expressing cells appear to migrate into the luteal tissue. Afterwards, immunostaining of TGF-ß3 in theca lutein cells successively decreased during CL formation. This pattern of TGF-ß3 protein localization coincides with the rapid migration and proliferation of vascular endothelial cells following ovulation. This hypothesis is supported by the fact that TGF-ß3 has been shown to promote mesenchymal cell proliferation and angiogenesis in other tissues (Muraoka et al., 2005). Furthermore, TGF-ß3 is known to have a potent stimulatory effect on collagen synthesis during the wound healing process (Kinbara et al., 2002). In this context, it is not surprising that following ovulation, a wound-like process, TGF-ß3 expression is up-regulated in the whole theca layer, probably to stimulate collagen synthesis for formation of the new CL. Together with the detection of TGF-ß activity previously shown in porcine corpus luteum (Gangrade and May, 1990), we propose that TGF-ß3 may be a good candidate involved in stimulating angiogenic factors and tissue remodelling events during early luteal development.

In conclusion, this is the first report showing the specific localization of TGF-ß3 during pre- and postovulatory follicle development in the pig. Intraovarian functions of TGF-ß3 remain to be proved, but the pattern of protein localization in theca cells predicts different roles for this growth factor during follicular development and luteinization. Collectively, the role and significance of different TGF-ß subtypes in the regulation of ovarian events is very complex and difficult to evaluate, due to species-dependent differences in expression pattern, subtype-specific divergent effects regarding stimulation and inhibition (Gitay-Goren et al., 1993), different methodical approaches and limitations, and in vitro results which may not reflect relationship *in vivo*. Nevertheless, since a role for TGF-ß isoforms has been established in several pathological conditions, including ovarian cancer (Inan et al., 2006), TGF-ß appears to be a very attractive target for therapeutic intervention. This requires identification of targets of single isoforms in different ovarian cell-types and their mechanism of regulation.

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