Altered Expression of Transforming Growth Factor-Beta Isoforms in Bovine Cystic Ovarian Disease

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Contents

Cystic ovarian disease (COD) is one of the main causes of infertility in dairy cattle. It has been shown that intra-ovarian factors may contribute to follicular persistence. Transforming growth factor-beta (TGFB) isoforms are important paracrine and autocrine signalling molecules that regulate ovarian follicle growth and physiology. Considering the importance of these factors in the ovarian physiology, in this study, we examined the expression of TGFB isoforms (TGFB1, TGFB2 and TGFB3) in the ovary of healthy cows and animals with spontaneous and adrenocorticotrophic hormone (ACTH)-induced COD. In the oestrous-synchronized control group, the expression of TGFB1 in granulosa and theca cells was higher in spontaneous cysts than in atretic or tertiary follicles. When we compared TGFB2 expression in granulosa cells from atretic or tertiary follicles from the oestrous-synchronized control group with that in ACTH-induced or spontaneous follicular cysts, we found a higher expression in the latter. The expression of the TGFB isoforms studied was also altered during folliculogenesis in both the spontaneous and ACTH-induced COD groups. As it has been previously shown that TGFB influences steroidogenesis, ovarian follicular proliferation and apoptosis, an alteration in its expression may contribute to the pathogenesis of this disease.

Introduction

Cystic ovarian disease (COD) is one of the main causes of infertility in dairy cattle and has been defined as the presence in the ovaries of one or more follicular structures with a diameter greater than that achieved at the time of ovulation, which persist in the absence of luteal tissue, interrupting the normal reproductive cycle (Silvia et al. 2002; Peter 2004; Vanholder et al. 2006). Although it is accepted that the main component of the ethio-pathogenesis of COD is related to an altered function of the hypothalamus–pituitary–ovarian axis, the persistence of follicles over time is related to an intra-ovarian component (Silvia et al. 2002).

Folliculogenesis is regulated by paracrine and autocrine growth factors, as well as by steroids and gonadotropic hormones (Hirshfield 1991). An effective communication between granulosa and theca cells and oocytes is vital for follicular growth and reproductive success. An abnormal regulation of cell growth and cell signalling can lead not only to infertility but also to some disease conditions such as cystic ovaries (Homburg 1998; Nilsson et al. 2003). The transforming growth factor-beta (TGFB) superfamily of growth factors is comprised of a large group of important paracrine and autocrine signalling molecules that bind to receptors with serine/threonine kinase activity and thus regulate ovarian follicle growth and physiology (Knight and Glister 2006). Three TGFB isoforms have been detected in mammals: TGFB1, TGFB2 and TGFB3 (Massague 1990).

Several studies have demonstrated that TGFB isoforms regulate physiological activities in the ovarian follicles of numerous species like human, rats, mice, sheep and cattle (Magoffin et al. 1989; Benahmed et al. 1993; Liu et al. 1999; Nilsson et al. 2003). Members of the TGFB superfamily have profound inhibitory and/or stimulatory effects on the growth and differentiation of many cell types such as granulosa, luteal and thecal cells (Benahmed et al. 1993). Transforming growth factor-beta biology can appear confusing, as similar types of experiments often generate observations that suggest that these growth factors have opposite effects. Lin and Lodish (1992) and Benahmed et al. (1993) have suggested that the myriad effects of the different members of the TGFB isoforms become less confusing if these effects are classified into three categories: effects on the cell cycle, effects on the extracellular matrix and effects on other peptide growth factors and their receptors. In this context, these authors suggested that the effects on the progression of cells through the mitotic cycle (i.e. interruption of the cell cycle in the mid-to-late G1 phase, preventing induction of DNA synthesis and progression into the S phase) are immediate, while the effects mediated by the extracellular matrix and by other growth factors and their receptors are secondary. Interestingly, the latter effects of the different TGFB isoforms may sometimes counteract the direct growth inhibitory effects of these growth factors, leading to apparent stimulatory actions (Benahmed et al. 1993).

Transforming growth factor-beta has been characterized as a strong growth suppressor in epithelial cells (Roberts et al. 1990), and in this sense, it inhibits the growth of granulosa cells in cattle (Skinner et al. 1987) and pigs (May et al. 1988; Mondschein et al. 1988). Transforming growth factor-beta also inhibits bovine theca cell growth (Roberts and Skinner 1991) and induces rat theca/interstitial cell apoptosis (Foghi et al. 1997). In addition, TGFB increases oestrogen production by granulosa cells in rats (Zachow et al. 1999) and mice (Liu et al. 1999). Similarly, TGFB increases the production of progesterone by theca cells in cattle (Roberts and Skinner 1991). In rat granulosa cells,

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TGFB increases the expression of FSH receptors (Dunkel et al. 1994).

Transforming growth factor-beta 1, TGFB2 and TGFB3 can be differentially expressed in various tissues during development and in adults, and have been localized to specific cell types in the ovaries of several species (Nilsson et al. 2003). In cows, TGFB1, TGFB2 and TGFB3 protein is localized on oocytes of all sizes of follicles (Nilsson et al. 2003). Transforming growth factor-beta 1 protein is also localized to the granulosa cells of primordial through small antral follicles but not to granulosa cells of larger follicles or theca cells of any follicular size. In contrast, TGFB2 and TGFB3 are present in granulosa cells of all sizes of follicles and in theca cells from the early antral stage and thereafter (theca cells of pre-antral follicles were not examined; Nilsson et al. 2003).

Considering the importance of these factors in folliculogenesis, we hypothesized that an alteration in the expression of TGFBs may result in ovarian alterations such as follicular persistence that could contribute to the pathogenesis of COD. Therefore, in this study, we examined the immunolocalization and expression of TGFB1, TGFB2 and TGFB3 in the ovary of oestrous-synchronized control bovines and bovines with adrenocorticotrophic hormone (ACTH)-induced and spontaneous COD.

Materials and Methods

Induction and detection of cysts

All procedures were evaluated and approved by the Institutional Ethics and Security Committee (*Facultad de Ciencias Veterinarias – Universidad Nacional del Litoral, Argentina;* Protocol number: 44/10) and are consistent with the 'Guide for the Care and Use of Agricultural Animals in Research and Teaching' (Adam 2010).

Groups

Spontaneous COD: ovaries from ten lactating Argentinean Holstein cows showing a single cyst of 20 mm or more in diameter that persisted for at least 10 days in the absence of corpus luteum (CL) were studied.

Adrenocorticotrophic hormone-induced COD: five nulliparous Argentinean Holstein heifers (18–24 months old; 400–450 kg body weight; maintained under standard husbandry conditions) with regular oestrous cycles were used for the ACTH-induced COD protocol as detailed below.

Oestrous-synchronized control group, five heifers with regular oestrous cycles, was used as control animals.

Induction of COD by adrenocorticotropin (ACTH) administration

Ten nulliparous Argentinean Holstein heifers (18–24 months old; 400–450 kg body weight; maintained under standard husbandry conditions) with regular oestrous cycles were used. Oestrous cycles were synchronized using the Ovsynch protocol as follows: animals were injected with a gonadotropin-releasing hormone (GnRH)

analogue (Buserelin acetate, Gonaxal®; Biogénesis-Bagó, Buenos Aires, Argentina, 10 μg/animal) on day 9, a Prostaglandin F$_{2\alpha}$ analogue (D+Cloprostenol, Enzaprost D-C®; Biogénesis-Bagó, 150 μg/animal) on day 2 and a GnRH analogue (Buserelin acetate, Gonaxal®; Biogénesis-Bagó, 10 μg/animal) on day 0. The time of ovulation was monitored by transrectal ultrasonography and was designated as day 1 of the oestrous cycle, because ovulation occurs 24–32 h after the second injection of GnRH (Pursley et al. 1995).

The model of ACTH-induced ovarian follicular cysts used in this study has been previously described (Dobson et al. 2000; Ortega et al. 2008; Salvetti et al. 2010; Amweg et al. 2013). Briefly, beginning on day 15 of a synchronized oestrous cycle, five heifers received subcutaneous injections of 1 mg of a synthetic polypeptide with ACTH activity (Synacthen Depot®; Novartis, Basel, Switzerland), every 12 h for 7 days (ACTH-induced COD group). Other five heifers received saline solution (1 ml; oestrous-synchronized control group).

Ovarian ultrasound examinations were performed in all animals, using a real-time, B-mode scanner equipped with a 5 MHz, linear-array, transrectal transducer (HS101V; Honda, Tokyo, Japan; Sirois and Fortune 1988). The growth and regression of follicles >5 mm, corpora lutea and follicular cysts were monitored. Daily ovarian ultrasonography was performed through a complete oestrous cycle in the oestrous-synchronized control heifers (21–23 days) and from day 14 (day 0 = day of ovulation) until ovariectomy in ACTH-treated heifers. Follicular cysts detected by ultrasonography were defined as any follicular structure with a diameter equal to or greater than 20 mm present for 10 days or more, without ovulation or luteinization or CL formation (Dobson et al. 2000). The first day of follicular cyst formation was the day a follicle attained 20 mm or more in diameter, and the ovaries were removed 10 days later by ovariectomy (Amweg et al. 2013). In the oestrous-synchronized control group, ovariectomy was conducted when the dominant follicle reached a diameter >10 mm, in the absence of an active CL, to obtain vpre-ovulatory follicles (approximately day 18).

Blood samples were obtained daily throughout the entire experiment to test the corresponding hormone concentrations. These data have been published previously (Ortega et al. 2008; Amweg et al. 2013).

Spontaneous COD

Ovaries with spontaneous cystic follicles were evaluated (Bartolome et al. 2005). Following initial diagnosis of COD, ovaries were examined daily using transrectal ultrasonography (5 MHz linear transducer, HS101V; Honda). Blood samples were obtained before ovariectomy to test the corresponding hormone concentrations. These data have been published previously (Amweg et al. 2013).

Ovariectomy, tissue sampling and processing and classification of follicles

Follicular fluid was aspirated before ovariectomy to prevent the rupture of the follicular cysts during surgery.

For this, a digital ultrasound system 8300vet Chison equipped with a micro-convex transducer of 5.0 MHz mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Limited®, San Pablo, Brazil) was used. The follicular fluid was transported to the laboratory at 4°C for processing and then stored at −20°C until use.

The ovaries were removed by transvaginal ovariectomy and macroscopically examined. Small samples from ovarian tissues were immediately frozen at −80°C until used in Western blotting for the determination of the specificity of the antibodies used in immunohistochemistry. The health status of the follicles was confirmed by the hormonal concentrations measured in the follicular fluid (Ortega et al. 2008; Amweg et al. 2013).

The ovaries were fixed in 4% buffered formaldehyde for 8–10 h at 4°C and then washed in phosphate-buffered saline (PBS). Fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. Thick sections of 4 μm were mounted on slides previously treated with 3-amin-opropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and primarily stained with haematoxylin-eosin for a preliminary observation of the ovarian structures (Amweg et al. 2013). Follicles were classified into primary, secondary, tertiary, atretic follicles (Priedkalns 1998) and follicular cysts (Silvia et al. 2002). Only cystic follicles with a complete granulosa cell layer and without signs of luteinization were analysed.

Immunohistochemistry

The details, suppliers and concentrations of antibodies used are reported in Table 1. Each antibody was assayed in a minimum of five sections of each ovary from each heifer or cow. A streptavidin-biotin immunoperoxidase method was performed as previously described (Salvetti et al. 2010). Briefly, after deparaffinization, antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0) in a domestic microwave oven at 800 W. The endogenous peroxidase activity was inhibited with 3% (v/v) H_2O_2 in methanol, and non-specific binding was blocked with 10% (v/v) normal goat serum. Slides were incubated with polyclonal rabbit antihuman antibodies against each of the three TGFB isoforms (SC-146, SC-90 and SC-83; Santa Cruz Biotechnology, Dallas, TX, USA)

for 18 h at 4°C and then for 30 min at room temperature with biotinylated secondary antibodies selected specifically for polyclonal antibodies. The antigens were visualized by the ExtrAvidin®-Peroxidase method (Sigma-Aldrich), and 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Invitrogen, Camarillo, CA, USA) was used as the chromogen. Finally, the slides were washed in distilled water and counterstained with Mayer's haematoxylin, dehydrated and mounted.

To verify the immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibodies with rabbit and mouse non-immune sera. The specificity of the secondary antibodies was tested by incubation with a primary antibody raised against human antigens with a proven negative reaction to tissues of cattle: anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako, Carpinteria, CA, USA). To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB alone.

Western blotting

The specificity of the above-mentioned primary antibodies to TGFB1, TGFB2 and TGFB3 has been previously demonstrated (Nilsson et al. 2003). In addition, to test the specificity of the antibodies, complete walls of tertiary follicles from the control group were homogenized in a radio-immunoprecipitation assay lysis buffer consisting of 1% v/v IGEPAL CA630 (octylphe-nyl-polyethylene glycol), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM sodium fluoride (all from Sigma-Aldrich Corp., St. Louis, MO, USA), 0.1 M PBS and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany). Follicle homogenates were centrifuged at 14 000 g for 20 min, and the supernatant stored frozen at −80°C. Proteins (40 μg) were separated by SDS–PAGE (12% resolving gel), transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK), blocked for 1 h in 2% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (Sigma-Aldrich Corp.) and then incubated overnight at 4°C with the specific primary antibodies (Table 1). Following washing, membranes were treated for 1 h with the corresponding secondary peroxidase-conjugated antibody (Table 1). The immunopositive bands were detected by chemiluminescence, using the

Table 1. Antibodies, suppliers and dilutions used for immunohistochemistry (IHC) and Western blot (WB)

Antibodies	Source	Dilution
Primary antibodies		
TGFB1	Rabbit polyclonal IgG. SC-146 Santa Cruz Biotechnology (Dallas, TX, USA)	IHC 8 μg/ml
		WB 4 μg/ml
TGFB2	Rabbit polyclonal IgG. SC-90 Santa Cruz Biotechnology	IHC 1 μg/ml
		WB 1 μg/ml
TGFB3	Rabbit polyclonal IgG. SC-83 Santa Cruz Biotechnology	IHC 1 μg/ml
		WB 1 μg/ml
Secondary antibodies		
Biotinylated anti-rabbit IgG (IHC)	Goat Polyclonal. 65-6140-Zymed (San Francisco, CA, USA)	6 μg/ml
Anti-rabbit IgG peroxidase (WB)	Goat-anti-rabbit IgG peroxidase (Amersham, Buckinghamshire, UK)	1 : 1000

TGFB1, Transforming growth factor-beta 1; TGFB2, Transforming growth factor-beta 2; TGFB3, Transforming growth factor-beta 3.

ECL-plus system (GE Healthcare) on hyperfilm-ECL film (GE Healthcare).

Image analysis

Images were analysed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA).

For immunohistochemistry, images were digitized using a CCD colour video camera (Motic 2000; Motic China Group, Xiamen, China) mounted on a conventional light microscope (Olympus BH-2; Olympus Co., Tokyo, Japan), using an objective magnification of ×40. The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections by replacing the primary antibody by non-immune serum. The positive controls were used as interassay controls to maximize the levels of accuracy and robustness of the method (Ranefall et al. 1998). The image analysis score was calculated separately in each follicular wall layer (granulosa and theca interna) from at least 50 images of the secondary, tertiary, atretic and cystic follicles from ovaries of all the groups. The percentage of immunopositive area was calculated as the percentage of the total area evaluated through the colour segmentation analysis, which extracts objects by locating all objects of the specific colour (brown stain). The brown stain was selected with a sensitivity of 4 (maximum 5), and a mask was then applied to separate the colours permanently. The images were then transformed to a bi-level scale TIFF format. The methodological details of image analysis as a valid method for quantification have been described previously (Ortega et al. 2009).

Statistics

A statistical software package (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL, USA) was used to perform the statistical tests. The dependent variable was the expression of each marker (TGFB1, TGFB2 and TGFB3). The independent variable was the follicular category (primary, secondary, tertiary, atretic, cystic follicles) or group (Control, Spontaneous COD and ACTH-induced COD). Similar follicular structures (primary, secondary, tertiary, atretic and cystic follicles) were compared both between groups and throughout follicular development within each group. The differences between the groups of data were assessed by one-way ANOVA, followed by Duncan's multiple range tests.

Further comparison between the following structures corresponding to different groups was performed by t-test:

Spontaneous cysts vs cysts induced by ACTH.
Spontaneous cysts vs control atretic follicles.
Cysts induced by ACTH vs control atretic follicles.
Spontaneous cysts vs control tertiary follicles.
Cysts induced by ACTH vs control tertiary follicles.

p values <0.05 were considered significant. Results are expressed as mean ± standard deviation (SD).

Results

Histological description of ovaries from control, ACTH-induced and spontaneous COD groups

Successful induction of COD was confirmed by the ovarian morphology. In addition, successful induction of cysts by ACTH was confirmed by serum and follicular fluid hormone analysis (Amweg et al. 2013).

In animals with ACTH-induced and spontaneous COD, healthy developing follicles, follicles showing different degrees of atresia and one large follicular cyst (in one ovary) with a complete granulosa cell layer were observed. Active corpora lutea (CL) were absent in all cases.

Ovaries from control animals exhibited follicles in various stages of development including primary, secondary and tertiary follicles, as well as atretic follicles and CL in regression.

Antibody specificity

The results from the Western blotting of ovarian homogenate are summarized in Figs 2c, 3c and 4c. The Western blotting analysis detected intense positive bands of appropriate sizes for each of the molecules studied by immunohistochemistry.

Localization and expression of TGFB1

Transforming growth factor-beta 1 was localized in the cytoplasm of granulosa and theca interna cells of all follicles and groups analysed.

In the control animals, a moderate expression of TGFB1 was observed in the granulosa and theca interna of all follicular categories, with the highest expression in granulosa cells of secondary follicles (Figs 1a and 2b).

In the ACTH-induced COD group, the expression of TGFB1 was higher in granulosa cells of primary and secondary follicles than in tertiary, atretic and cystic follicles, and the lowest expression was in the theca of tertiary, atretic and cystic follicles (Figs 1d and 2b).

In the spontaneous COD group, TGFB1 expression was higher in the granulosa of cysts than in tertiary and atretic follicles, without differences between the other follicular categories. The theca showed low levels of TGFB1 expression (Figs 1i and 2b).

When each specific follicular structure was compared, no differences were observed for primary, secondary, tertiary or atretic follicles in granulosa and theca cells between groups. The theca of ACTH-induced cysts showed lower expression than spontaneous cysts ($p < 0.05$; Fig. 2a).

However, an increased expression of TGFB1 was observed in the spontaneous cysts when compared with tertiary follicles in the control group and atretic follicles in the control group ($p < 0.05$; Fig. 2a).
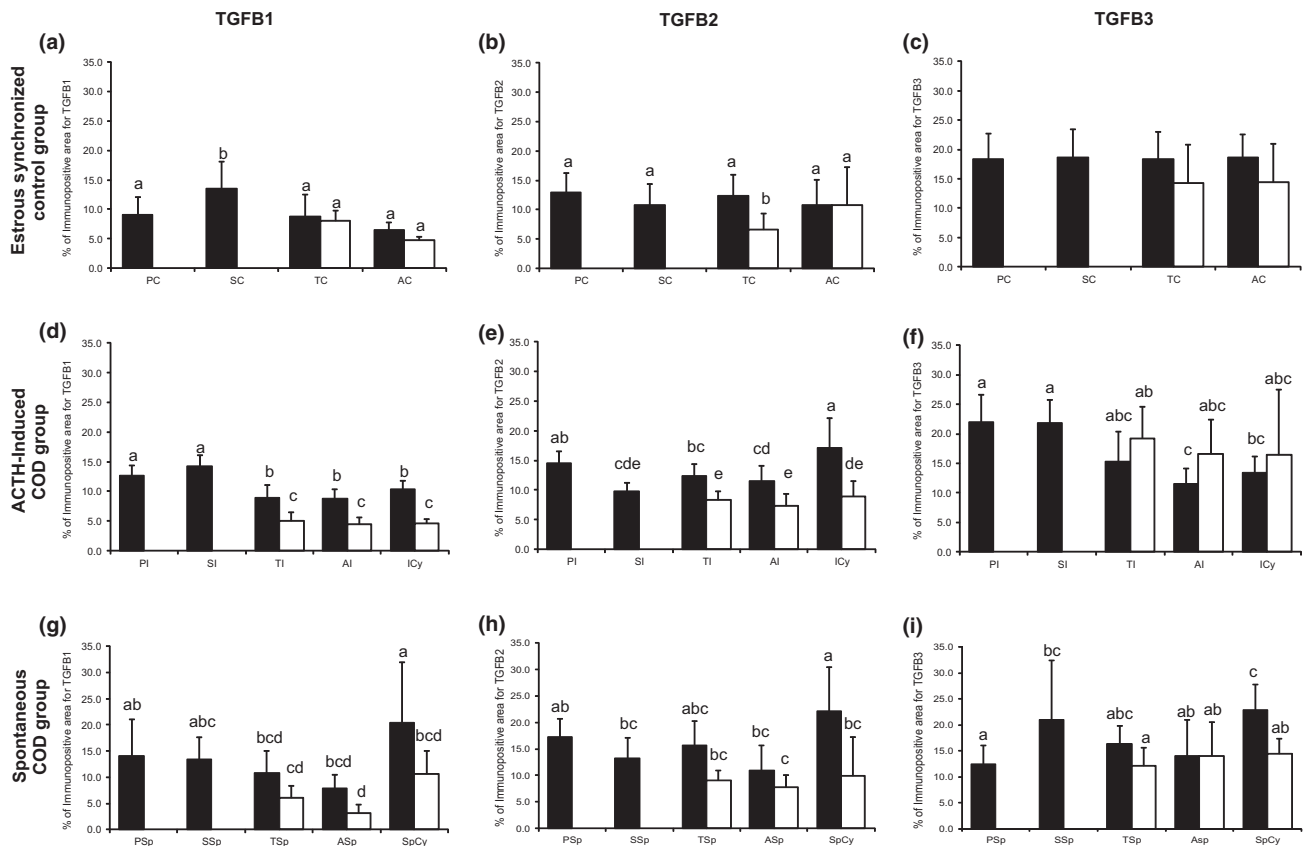
Fig. 1. Relative expression (measured as percentage of immunopositive area) of Transforming growth factor-beta 1 (a,d,g), Transforming growth factor-beta 2 (b,e,h) and Transforming growth factor-beta 3 (c,f,i) in granulosa (black columns) and theca interna (white columns) of primary (only granulosa), secondary (only granulosa), tertiary and atretic follicles (all groups) and cystic follicles (adrenocorticotrophic hormone-induced and spontaneous cystic ovarian disease (COD) groups) within each group. Values represent mean ± SD. Bars with different letters are different (p < 0.05)

Localization and expression of TGFB2

Transforming growth factor-beta 2 was localized in the cytoplasm of granulosa and theca interna cells of all follicles and groups analysed.

In the control animals, a moderate expression was observed in the granulosa and theca interna of all follicular categories, with the lowest expression in theca cells of tertiary follicles (Figs 1b and 3b).

In the ACTH-induced COD group, TGFB2 expression in granulosa cells of primary and cystic follicles was higher than that in the other follicular categories. In theca cells, the expression was similar to that observed in granulosa cells of secondary follicles (Figs 1e and 3b).

In the spontaneous COD group, the pattern was similar to that observed in the ACTH-induced COD group, with the highest expression in granulosa of primary, tertiary and cystic follicles (Figs 1h and 3b).

When each specific follicular structure was compared between groups, no differences were observed (Fig. 3) for granulosa and theca cell layers.

However, an increased expression of TGFB2 was observed in the spontaneous cysts when compared with tertiary follicles in the control group and atretic follicles in the control group (Fig. 3a).

Localization and expression of TGBF3

Transforming growth factor-beta 3 was localized in the cytoplasm of granulosa and theca interna cells of all follicles and groups analysed.

In the control animals, a moderate to high expression was observed in the granulosa and theca interna of all follicular categories, without differences between follicular structures (Figs 1c and 4b).

In the ACTH-induced COD group, granulosa cells showed a similar expression in primary, secondary and tertiary follicles, and a lower expression in atretic and cystic follicles (p < 0.05), while theca cells showed expression levels similar to those found in the granulosa (Figs 1f and 4b).

In the spontaneous COD group, granulosa cells of primary follicles showed the lowest expression, whereas secondary, tertiary and cystic follicles showed similar high levels of expression. In theca cells, TGFB3 expression was similar to that of granulosa cells of primary follicles (Figs 1l and 4b).

The comparison between groups for equivalent follicular structures showed differences in granulosa of primary, atretic and cystic follicles. Transforming growth factor-beta 3 expression in primary follicles was lower in the spontaneous COD group than in
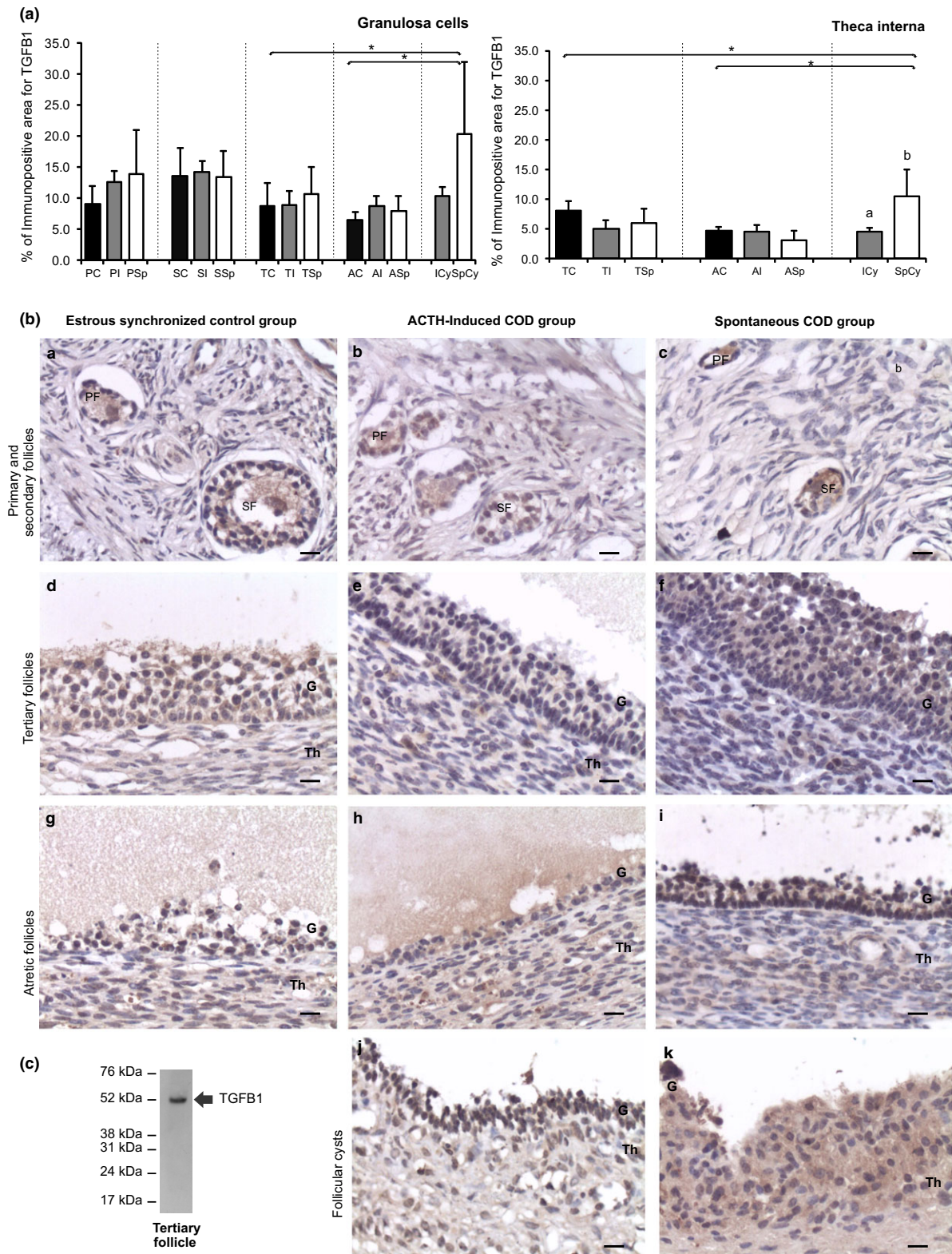
Fig. 2. Transforming growth factor-beta 1 (TGFB1) localization and expression in different follicular categories from the control, adrenocorticotrophic hormone (ACTH)-induced cystic ovarian disease (COD) and spontaneous COD groups. (a) Relative expression (measured as percentage of immunopositive area) of TGFB1 in the granulosa and theca interna of primary (P), secondary (S), tertiary (T) and atretic (A) follicles from the control group (C, black columns), ACTH-induced COD group (I, grey columns) and spontaneous COD group (Sp, white columns); ACTH-induced cysts (ICy) and spontaneous cysts (SpCy). Values represent mean ± SD. Bars with different letters within a category are significantly different (p < 0.05). (b) Representative images of TGFB1 immunostaining in primary, secondary, tertiary and atretic follicles from the oestrous-synchronized control group and cystic follicles from the spontaneous and ACTH-induced COD groups. Granulosa (G), Theca Interna (Th). Bars = 20 μm. (c) Verification of antibody specificity by Western blotting analysis of ovarian homogenate is shown
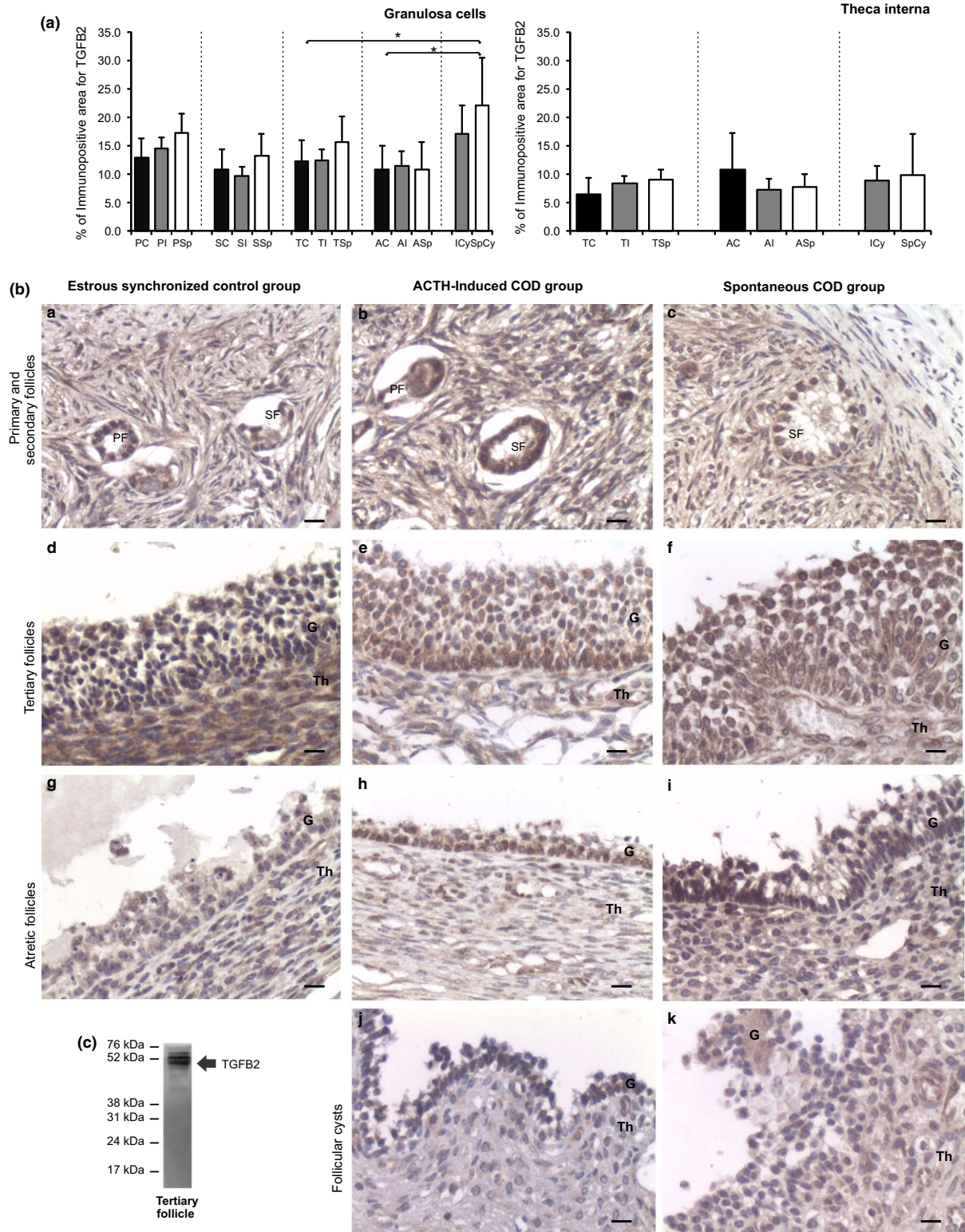
Fig. 3. Transforming growth factor-beta 2 (TGFB2) localization and expression in different follicular categories from the control, adrenocorticotrophic hormone (ACTH)-induced cystic ovarian disease (COD) and spontaneous COD groups. (a) Relative expression (measured as percentage of immunopositive area) of TGFB2 in the granulosa and theca interna of primary (P), secondary (S), tertiary (T) and atretic (A) follicles from the control group (C, black columns), ACTH-induced COD group (I, grey columns) and spontaneous COD group (Sp, white columns); ACTH-induced cysts (ICy) and spontaneous cysts (SpCy). Values represent mean ± SD. Bars with different letters within a category are significantly different ($p < 0.05$). Differences of TGFB2 expression in ACTH-induced or spontaneous cystic follicles related to control tertiary or atretic follicles are also indicated by an asterisk ($p < 0.05$). (b) Representative images of TGFB2 immunostaining in primary, secondary, tertiary and atretic follicles from the oestrous-synchronized control group and cystic follicles from the spontaneous and ACTH-induced COD groups. Granulosa (G), Theca Interna (Th). Bars = 20 μm. (c) Verification of antibody specificity by Western blotting analysis of ovarian homogenate is shown
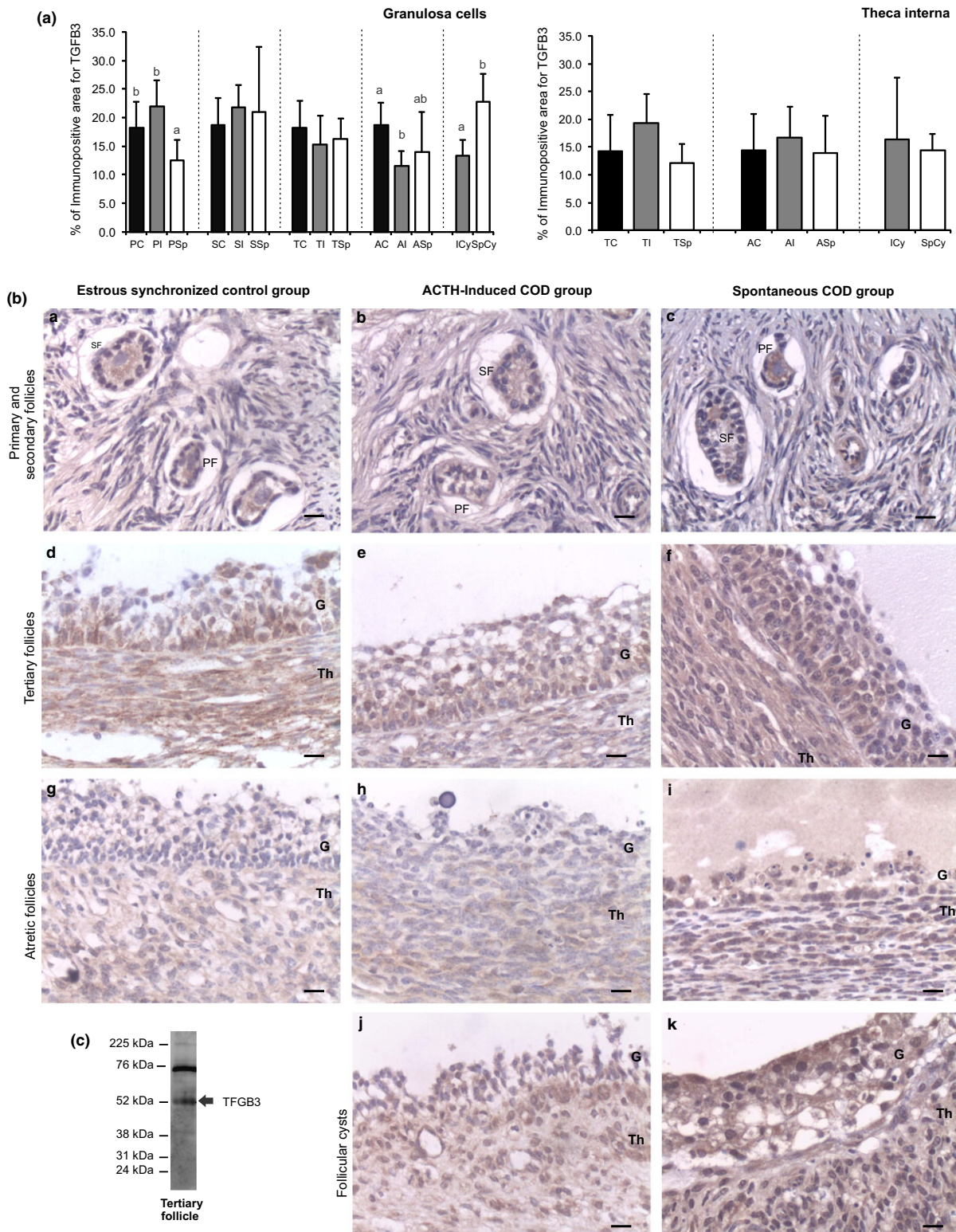
Fig. 4. Transforming growth factor-beta 3 (TGFB3) localization and expression in different follicular categories from the control, adrenocorticotrophic hormone (ACTH)-induced cystic ovarian disease (COD) and spontaneous COD groups. (a) Relative expression (measured as percentage of immunopositive area) of TGFB3 in the granulosa and theca interna of primary (P), secondary (S), tertiary (T) and atretic (A) follicles from the control group (C, black columns), ACTH-induced COD group (I, grey columns) and spontaneous COD group (Sp, white columns); ACTH-induced cysts (ICy) and spontaneous cysts (SpCy). Values represent mean ± SD. Bars with different letters within a category are significantly different (p < 0.05). (b) Representative images of TGFB3 immunostaining in primary, secondary, tertiary and atretic follicles from the oestrous-synchronized control group and cystic follicles from the spontaneous and ACTH-induced COD groups. Granulosa (G), Theca Interna (Th). Bars = 20 μm. (c) Verification of antibody specificity by Western blotting analysis of ovarian homogenate is shown

control group. Transforming growth factor-beta 3 expression in granulosa cells of atretic follicles was lower in ACTH-induced COD group than the control group. Spontaneous cysts showed higher TGFB3 expression than ACTH-induced cysts (Fig. 4a).

Discussion

In the current study, we observed differences in the expression of all three isoforms of TGFB in the follicular structures studied between control animals and those with spontaneous or ACTH-induced COD. We also noted a higher expression of TGFB1 and TGFB2 in cysts than tertiary and atretic follicles in the control group.

The results for the protein expression of TGFB1 isoform in the normal ovary (control animals) differed from those previously described in cattle, probably due to methodological differences. Nilsson et al. (2003) found no TGFB1 protein expression in the theca interna and found expression only in the granulosa of tertiary follicles up to 2 mm in diameter. Although the antibody used was the same, differences in the fixation process and in the tissue fixatives used are likely the cause of these discrepancies. However, the same authors found mRNA expression for all TGFB isoforms in all follicular structures studied in both the granulosa and theca interna, which would support the results of our study. On the other hand, the results previously obtained by Nilsson et al. (2003) in relation to TGFB2 and TGFB3 are consistent with those here presented, with high expression in granulosa and theca cells in all structures studied in control animals.

Regarding the regulation of the expression of TGFBs, the experiments performed by Nilsson et al. (2003) determined that TGFB1 expression is regulated by gonadotropins in the bovine ovary. These authors showed that FSH treatment decreased *TGFB1* mRNA expression in granulosa cells, without changes when the treatment was performed with hCG, and observed no effects on the expression of the other isoforms. Results obtained by real-time PCR have shown that the mRNA for the FSH receptor in granulosa cells is lower in follicular cysts than in small, medium and large normal tertiary follicles (Marelli et al. 2014). These lower levels would indicate that this hormone has less influence, thus leading to a deregulation at the intracellular level that could be impacting on the expression of several growth factors and hormones, including TGFB1. It is logical to suggest that an altered endocrine environment could lead to an alteration in the local ovarian milieu and thus in the expression of important ovarian regulators like TGFBs.

The actions of TGFB on granulosa cell function are known to vary between species. In rodents, TGFBs are potent stimulators of granulosa cell proliferation (Dorrington et al. 1988; Roy 1993; Saragueta et al. 2002). However, in species of zootechnical interest, such as cattle (TGFB2), sheep (TGFB1 and TGFB2) and pigs (TGFB1 and TGFB2), these growth factors have only mild stimulatory or even inhibitory effects on granulosa

cell proliferation/survival (Skinner et al. 1987; May et al. 1988; Gangrade and May 1990; Gilchrist et al. 2003; Juengel et al. 2004; Juengel and McNatty 2005). Also, under *in vitro* conditions, TGFBs stimulate progesterone synthesis from rodent granulosa cells (Dodson and Schomberg 1987; Hutchinson et al. 1987; Knecht et al. 1987), whereas they show inhibitory effects in granulosa cells collected from cattle, sheep (TGFB1 and TGFB2) and pigs (Mondschein et al. 1988; Kubota et al. 1994; Gilchrist et al. 2003; Juengel et al. 2004; Juengel and McNatty 2005). Using an *in vitro* model of FSH-stimulated granulosa cell culture, Zheng et al. (2008, 2009) observed differences in steroidogenesis when adding TGFB1. These authors showed that in FSH-stimulated granulosa cells, TGFB1 inhibits estradiol and progesterone secretion and that in the absence of FSH, TGFB1 stimulates estradiol secretion and inhibits progesterone secretion (Zheng et al. 2008, 2009). In contrast to the effects of TGFB on granulosa cells, those on theca/interstitial cells with respect to steroidogenesis appear to be similar between species. Transforming growth factor-beta suppresses LH or forskolin-stimulated androgen production in rat (Magoffin et al. 1989; Hernandez et al. 1990), porcine (Caubo et al. 1989; Engelhardt et al. 1992), bovine (Demeter-Arlotto et al. 1993) and human (Attia et al. 2000) theca/interstitial cells (Juengel and McNatty 2005). Expression of both CYP17A1, a key protein controlling androgen production that catalyses the conversion of progestagens to androgens, and steroidogenic acute regulatory protein (STAR), which facilitates the transport of cholesterol into mitochondria for steroid synthesis, is downregulated by TGFB in theca cells, indicating some common underlying mechanisms in the regulation of androgen production between species (Demeter-Arlotto et al. 1993; Attia et al. 2000; Juengel and McNatty 2005). Animals with spontaneous and ACTH-induced COD have low intrafollicular levels of progesterone in cysts, without differences in 17 β-estradiol concentrations when compared with preovulatory follicles, and increased levels of testosterone in spontaneous cysts (Amweg et al. 2013). Considering that in the present work, both TGFB1 and TGFB2 were increased in cystic follicles when compared to similar structures in the control group as along folliculogenesis in the same group, it could be suggested that these growth factors may be influencing some aspects of steroidogenesis.

In *in vitro* studies, Zheng et al. (2009) showed that in granulosa cells, TGFB1 promotes apoptosis and reduces entry into the proliferative phase of the cell cycle, independently of FSH stimulation. It has been confirmed both by us and other researchers that the proliferation/apoptosis balance is altered in cystic follicles in animals with spontaneous and ACTH-induced COD (Isobe and Yoshimura 2007; Salvetti et al. 2010). While many factors influence this delicate balance, the TGFB system could play an important role by adding its antiproliferative actions to other systems such as the insulin-like growth factor (IGF) system. It has been observed that the expression and bioavailability of IGF1, a strong inducer of proliferation, is lower in cysts than in analogous structures in the ovary (Ortega

et al. 2008; Rodríguez et al. 2013). However, it has also been observed that follicular cyst proliferation is also decreased, with low apoptosis levels related to control follicles (Salvetti et al. 2010). Such unusual circumstances favour the persistence of these structures, which continue to secrete altered levels of growth factors and hormones and thus contribute to the pathogenesis of this disease.

In summary, in the present study, we found an altered expression of TGFB isoforms during folliculogenesis, particularly in follicular cysts of bovines with spontaneous and ACTH-induced COD. It has been previously shown that TGFB influences steroidogenesis, ovarian follicular proliferation and apoptosis. Thus, an alteration in its expression may contribute to the pathogenesis of COD. Further studies are needed to determine the mechanism of action of TGFB isoforms in ovaries of cows with COD and to determine whether other components of the system that interact with this growth factor, such as TGFB receptors and associated proteins (SMADs), could be also altered, supporting the results here presented.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

VM and MLS carried out the immunohistochemistry studies and participated in the data analysis. MLS, PUD and EH participated in the design of the study and helped to collect and process the samples. FR and HHO participated in the design of the study and helped to draft the manuscript. NRS and HHO conceived of the study, and participated in its design and coordination, participated in the data analysis and drafted the manuscript. All authors read and approved the final manuscript.

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