REPRODUCTION

Molecular aspects of bovine cystic ovarian disease pathogenesis

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Abstract

Cystic ovarian disease (COD) is one of the main causes of reproductive failure in cattle and causes severe economic loss to the dairy farm industry because it increases both days open in the *post partum* period and replacement rates due to infertility. This disease is the consequence of the failure of a mature follicle to ovulate at the time of ovulation in the estrous cycle. This review examines the evidence for the role of altered steroid and gonadotropin signaling systems and the proliferation/apoptosis balance in the ovary with cystic structures. This evidence suggests that changes in the expression of ovarian molecular components associated with these cellular mechanisms could play a fundamental role in the pathogenesis of COD. The evidence also shows that gonadotropin receptor expression in bovine cystic follicles is altered, which suggests that changes in the signaling system of gonadotropins could play a fundamental role in the pathogenesis of conditions characterized by altered ovulation, such as COD. Ovaries from animals with COD exhibit a disrupted steroid receptor pattern with modifications in the expression of coregulatory proteins. These changes in the pathways of endocrine action would trigger the changes in proliferation and apoptosis underlying the aberrant persistence of follicular cysts. Free Spanish abstract: A Spanish translation of this abstract is freely available at http://www.reproduction-online.org/content/149/6/R251/suppl/DC1.

Introduction

Cystic ovarian disease (COD), which is an important cause of infertility in dairy cattle, has been defined as the presence of one or more follicles of more than 20 mm in diameter in the ovaries, persisting for up to 10 days without luteal tissue, interrupting the normal reproductive cycle (Silvia *et al.* 2002). The incidence of COD in dairy herds has been reported to vary from 5 to 30% (Vanholder *et al.* 2006, Cattaneo *et al.* 2014) and this condition generates significant economic losses to the dairy industry because it increases the calving-to-conception and inter-calving intervals (Peter 2004).

The etiopathogenesis of COD in dairy cattle is a complex process that involves dysfunctions in various physiological processes, including folliculogenesis, steroidogenesis, and ovulation, and many factors, such as stress, herd management, nutritional status, body condition, and infectious disease, can coexist (Silvia *et al.* 2002). Although it is accepted that the central

component of the etiopathogenesis of COD is associated with an altered function of the hypothalamus–pituitary–ovarian axis, the persistence of follicles over time is linked to an important intraovarian component (Silvia *et al.* 2002). The ovulatory failure leads to cyst development and persistence in the ovary, interfering with the normal ovarian function (Vanholder *et al.* 2006). Some authors define persistence as a temporal stage of the life span of the cyst (Cook *et al.* 1990, Hamilton *et al.* 1995), whereas others define it as a separate follicular pathology (Mihm *et al.* 1994).

Intraovarian alterations, as contributors to follicular persistence, have not yet been clearly established. However, several studies have contributed to a better understanding of specific aspects related to the pathogenesis of COD. In relation to endocrine signaling pathways, some of these studies have demonstrated altered expression of steroid and gonadotropin receptors (Salvetti *et al.* 2007, 2012, Marelli *et al.* 2014). Other studies have postulated and tested the intrafollicular

roles of steroids in regulating follicular development (Berisha *et al.* 2002, Gümen & Wiltbank 2005, Salvetti *et al.* 2007, 2012).

In addition, it has been previously demonstrated that the proliferation/apoptosis balance in follicles from animals with COD is altered. These follicles show diminished cell proliferation and apoptosis *in situ* and decreased expression of pro-apoptotic proteins relative to antiapoptotic proteins (Salvetti *et al.* 2009, 2010). The proliferation of granulosa cells and the fate of follicles (degeneration by follicular atresia or cystic development) are specifically related to steroid hormone and gonadotropin receptors. The imbalance between proliferation and apoptosis found in follicular cysts could explain the development of cystic follicles and the preservation of a static condition without atresia, which leads to their persistence. These alterations may be due to structural and functional changes that could be related to the hormonal milieu and take place in the follicles of animals with COD. In relation to cellular changes, it has been proposed that follicular cysts represent a distinctive stage of follicular differentiation, with a characteristic protein and gene expression profile in ovarian cells that differs from that found in dominant follicles or other follicular categories (Ortega *et al.* 2007, Salvetti *et al.* 2010, 2012, Velázquez *et al.* 2010, 2011, Matiller *et al.* 2014).

This review examines evidences for the role of altered gonadotropin and steroid signaling systems and the proliferation/apoptosis balance in the ovary from animals with COD. These data suggest that changes in the expression of ovarian molecular components associated with these cellular mechanisms could play a fundamental role in the pathogenesis of the disease.

Expression of gonadotropin receptors

Follicular growth and steroidogenesis depend on the coordinated interaction between gonadotropins and their receptors in granulosa and theca cells. The cellular mechanisms regulating folliculogenesis, ovulation, and follicular regression in cows are not fully defined. However, a key role of gonadotropins in the regulation of follicular development has been well established (Nogueira *et al.* 2007, Nimz *et al.* 2009). In normal ovaries, the interactive system of follicular growth and steroidogenesis suggests that granulosa and theca cells are involved in the secretion of steroid hormones through the two-cell/two-gonadotropin model (Fortune & Quirk 1988). In this model, granulosa cells possess membrane receptors to follicle-stimulating hormone (FSHR) and theca cells contain receptors to luteinizing hormone/choriogonadotropin (LHCGR) during the earlier stages of follicular development (Bao & Garverick 1998). As follicular development progresses, changes occur within the dominant follicle that is preparing for ovulation. Among these changes, the acquisition of LHCGR by the granulosa cells of the dominant follicle and the increase

in the aromatization of androgens provided by the theca cells in response to LH and FSH are some of the most important changes (Wiltbank *et al.* 2002).

As mentioned earlier in this review, neuroendocrinological dysfunction of the hypothalamic–pituitary–gonadal axis is the most accepted hypothesis related to the formation of cystic follicles (Liptrap & McNally 1976, Kesler & Garverick 1982, Garverick 1997, Vanholder *et al.* 2006). It has been demonstrated that gonadotropin regulation of bovine FSHR and LHCGR protein content during follicular growth, ovulation, and luteinization is associated with analogous changes in their respective receptor mRNA levels (Soumano *et al.* 1998). Gonadotropin receptors are structurally related members of the seven transmembrane domain G protein-associated receptor superfamily (Vassart *et al.* 2004). The gene encoding LHCGR contains 11 exons (Segaloff *et al.* 1990, Segaloff & Ascoli 1993), while that encoding FSHR contains ten exons (Rajapaksha *et al.* 1996, Hillier 2001). It has also been demonstrated that *LHCGR* and *FSHR* mRNAs are highly alternatively spliced (Figs 1 and 2; Rajapaksha *et al.* 1996, Simoni *et al.* 1997, Robert *et al.* 2003, Kawate 2004, Nogueira *et al.* 2010). However, there are no investigations of splice variants in ovarian follicular cysts.

The distribution pattern and expression levels of gonadotropin receptors in healthy ovaries have been evaluated by several methods, including conventional and real-time RT-PCR, northern blot assays, and *in situ* hybridization (Ireland & Roche 1983, Xu *et al.* 1995, Bodensteiner *et al.* 1996, Rajapaksha *et al.* 1996, Bao *et al.* 1997, Evans & Fortune 1997, Bao & Garverick 1998, Soumano *et al.* 1998, Odore *et al.* 1999, Calder *et al.* 2001, Manikkam *et al.* 2001, Robert *et al.* 2003, Braw-Tal & Roth 2005, Luo & Wiltbank 2006, Mihm *et al.* 2006, Nogueira *et al.* 2007, Nimz *et al.* 2009). In recent studies conducted in our laboratory, we analyzed the relative expression levels of LHCGR and FSHR in follicular cysts of cows with COD and antral follicles of healthy animals by real-time RT-PCR and, in agreement with that found by other authors, we found that mRNA
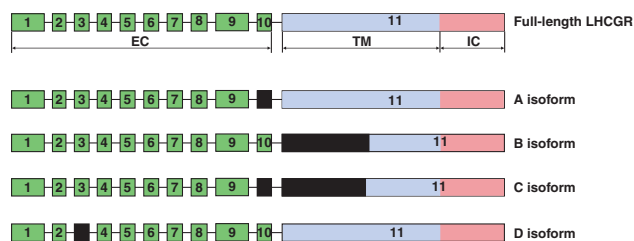

Figure 1 Schematic diagram of the alignment for the different isoforms of the luteinizing hormone/choriogonadotropin receptor (LHCGR). Exons 1 through 10 code for the extracellular ligand-binding domain (EC), while exon 11 codes for the transmembrane (TM) and intracellular (IC) domains. On the different isoforms, solid black boxes represent missing portions.

Figure 2 Schematic diagram of the alignment for the different isoforms of the follicle-stimulating hormone receptor (FSHR). Exons 1 through 9 code for the extracellular ligand-binding domain (EC), while exon 10 codes for the transmembrane (TM) and intracellular (IC) domains. On the different isoforms, solid black boxes represent missing portions.
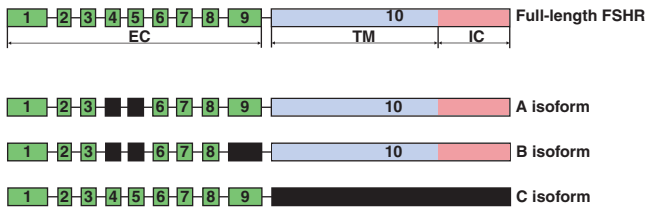
expression of FSHR in antral follicles was exclusively localized to granulosa cells and it decreased as follicle size increased, and that *LHCGR* mRNA was detected in theca cells of antral follicles of all sizes and in the granulosa cells of large antral follicles, being theca cells of medium antral follicles, the ones that showed the highest expression (Marelli *et al.* 2014). In cattle, Xu *et al.* (1995) detected *FSHR* mRNA in follicles with less than three layers of granulosa cells, and Beg *et al.* (2001) demonstrated that *LHCGR* mRNA was higher in granulosa cells from the largest follicle than in those from the second largest follicle of the follicular wave, before morphological deviation, suggesting that the capacity of granulosa cells to respond to LH is part of the deviation process.

The results of our analysis of the mRNA expression of gonadotropin receptors (Marelli *et al.* 2014) agree with previous studies demonstrating that LHCGR expression in granulosa cells from bovine follicles smaller than 8 mm in diameter is absent or not detectable (Ginther *et al.* 1996, Sartori *et al.* 2001). These studies have demonstrated that follicles smaller than 8 mm in diameter are independent of gonadotropin support, whereas follicles larger than this diameter require endogenous LH to develop. Therefore, follicles are considered to be FSH dependent until dominance occurs, after which they become LH dependent (Fortune *et al.* 2001, Ginther *et al.* 2001, Garverick *et al.* 2002). In this sense, LHCGR expression in granulosa cells has been detected by *in situ* hybridization in follicles of more than 9 mm in diameter that become dominant (Xu *et al.* 1995, Bao *et al.* 1997, Garverick *et al.* 2002), together with higher binding of hCG to granulosa cells in dominant follicles (Ireland & Roche 1983). It must also be considered that *LHCGR* mRNA levels in granulosa cells of the dominant follicle are higher than those of subordinate follicles (Beg *et al.* 2001, Evans *et al.* 2004). Moreover, in agreement with Mihm *et al.* (2006), our findings (Marelli *et al.* 2014) support the hypothesis that the dominant follicle presents a decrease in FSH dependence and an increase in LH dependence as it grows during the low FSH environment of follicular waves.

Several studies have evaluated the possible alterations in the expression of gonadotropin receptors as a component of COD etiopathogenesis, but, currently, there is controversy concerning this issue. Hormonal binding assays suggest that the amounts of FSHR and LHCGR in granulosa cells of cysts are lower than those in normal antral follicles (Kawate *et al.* 1990). However, studies using the same binding assay, but with a different sample preparation, have shown that LHCGR and FSHR concentrations in the follicular cysts are similar to those observed in control follicles (Odore *et al.* 1999). Calder *et al.* (2001) compared *LHCGR* and *FSHR* mRNA expression by *in situ* hybridization in ovaries from cows with dominant and non-dominant ovarian follicular cysts and in healthy dominant follicles and found that *LHCGR* mRNA expression was higher in granulosa cells of dominant follicular cysts than in dominant follicles, without differences in theca cells, and without differences in *FSHR* mRNA expression. Discrepancies between studies may be partly explained by differences in the methodology used, such as the determination of the receptor itself or its mRNA, and the classification of cysts into estrogen-active and estrogen-inactive (Vanholder *et al.* 2006). These differences may also be because some authors study the whole follicular wall, while others analyze the granulosa and theca cells separately.

Stimulation of the hypothalamic–pituitary–adrenal/ovarian axis by stress disrupts the reproductive function and could be associated with bovine COD pathogenesis (Moberg 1987). Altered folliculogenesis, reduced ovulation rates, and follicular cyst development have been reported in association with adrenocorticotropic hormone (ACTH) administration and increased levels of glucocorticoids (Liptrap & McNally 1976, Liptrap 1993, Kawate *et al.* 1996, Dobson *et al.* 2000, Amweg *et al.* 2013). In this sense, Kawate *et al.* (2001) proposed stress as a potential mechanism for the development of bovine follicular cysts. First, ACTH stimulates the release of cortisol and progesterone, and then an increased secretion of progesterone inhibits the release of gonadotropin-releasing hormone. Enhanced secretion of cortisol decreases estradiol secretion and LHCGR content in antral follicles. As a result of these hormonal imbalances, the positive feedback action of estradiol on the hypothalamus and pituitary is worsened and the LH surge is suppressed. Finally, ovulation does not occur and the follicle becomes cystic. This proposed mechanism agrees with the decreased expression of LHCGR reported both by Kawate (2004) and our group (Marelli *et al.* 2014).

The intraovarian steroid pathway

Steroid receptors

Steroid hormones play a critical role in folliculogenesis as well as in ovarian development and differentiation.

The main hormones involved in these processes are androgens, estrogens, and progesterone (Rosenfeld *et al*. 2001, Drummond *et al*. 2002, Schams & Berisha 2002, Brosens 2004, Drummond 2006, Kimura *et al*. 2007, Ortega *et al*. 2009). Steroid hormones act through specific receptors that are members of a superfamily of ligand-dependent transcriptional activators, which directly regulate the expression of specific gene complexes involved in regulating the differentiation and growth of reproductive tissues, as well as other metabolic processes (Brosens 2004). In mammals, two subtypes of the estrogen receptor (ESR) have been identified, ESR1 and ESR2. These are related structurally but encoded by two distinct genes (Kuiper *et al*. 1996). The progesterone receptor (PGR), instead, has different isoforms that originate from the same gene (PGRA, PGRB, and PGRC) (Wei *et al*. 1990, Bramley 2003). Finally, androgens perform their actions by binding to the androgen receptor (AR), which is presented in at least two isoforms (ARA and ARB) originated from the same gene (Takeo & Yamashita 1999, Brinkmann 2001).

The location of steroid hormone receptors in the ovarian follicle has been evaluated by many authors and in different species (Manikkam *et al*. 2001, Cassar *et al*. 2002, Jo *et al*. 2002, Schams & Berisha 2002, Van den Broeck *et al*. 2002*a*,*b*, Hampton *et al*. 2004, D'Haeseleer *et al*. 2005). It has been previously demonstrated that a subtle imbalance in the expression of the two subtypes of ESR in the components of the ovarian follicle could be involved in the pathogenesis of follicular cysts in cattle (Garverick 1997, Salvetti *et al*. 2007, 2012, Alfaro *et al*. 2012), sheep (Ortega *et al*. 2009), humans (Shushan *et al*. 1996, Jakimiuk *et al*. 2002), and rodents (Salvetti *et al*. 2009).

In addition, studies on cows with COD induced by the administration of ACTH have evidenced changes only in the expression of ESR2 in cystic follicles compared with control follicles, partially coinciding with the findings in animals with spontaneous disease (Salvetti *et al*. 2007, 2012, Alfaro *et al*. 2012). Studies have also demonstrated an increase in ESR1 expression in animals with spontaneous COD and not in the experimental model, differences that are probably due to the persistence time of the cysts (Salvetti *et al*. 2012). Other authors have found analogous modifications in the expression of ESR2 in cysts from women with polycystic ovarian syndrome related to normal-size follicles (Jakimiuk *et al*. 2002) and in prenatal testosterone-treated sheep characterized by an abnormal follicular persistence (Ortega *et al*. 2009).

ESR1 and ESR2 bind to 17β-estradiol with high-affinity dimerization between them, forming hetero- or homodimers. In this context, estrogen-dependent transcriptional activity varies according to the cell type, the promoter, and the types of dimers formed (McInerney *et al*. 1998, Pettersson *et al*. 2000). Therefore, expression of ESR2 in specific cells could regulate the responsiveness to estrogens in certain target genes in

a cell-dependent mode (O'Brien *et al*. 1999). Previous studies have demonstrated that ESR2/ESR1 heterodimers repress ESR1 activity and the affinity to 17β-estradiol (Hall & McDonnell 1999). Recently, it has been suggested that the main determinants of the transcriptional activity of ESR1 and ESR2 are their individual concentrations in target cells as well as the structure of the estrogen ligand and not their binding ability (Gougelet *et al*. 2007, Bhavnani *et al*. 2008). Thus, a specific ligand could exert various activities according to the ESR subtypes expressed in cells, leading to the fact that small changes in the ESR1/ESR2 ratio could alter folliculogenesis and ovulation (Mosselman *et al*. 1996, Pettersson *et al*. 1997) by altering cellular proliferation and apoptosis, the expression of hormonal receptors, and steroidogenesis, and all molecular aspects of COD (Fig. 3; Isobe & Yoshimura 2000*a*, Calder *et al*. 2001, Salvetti *et al*. 2010, Marelli *et al*. 2014).

In relationship to AR, Hampton *et al*. (2004) showed that this receptor is expressed in ovarian follicles of bovines, and that its expression is increased throughout folliculogenesis, whereas Alfaro *et al*. (2012) found that *AR* mRNA expression is significantly increased in granulosa cells of bovine follicular cysts. This increase would be associated with the role of androgens in follicular differentiation and growth (Hillier & Tetsuka 1997, Vendola *et al*. 1998, Walters *et al*. 2008). Furthermore, there are discrepancies between AR mRNA and protein expression, which could be explained by post-translational regulation (Sette *et al*. 2010, Salvetti *et al*. 2012). Finally, changes in the delicate balance between the ESR subtypes, coupled with alterations in the expression of AR, may contribute COD development especially to the maintenance over time of the cystic structures (Ortega *et al*. 2008, Salvetti *et al*. 2010, 2012).

Previous studies have demonstrated cell-specific expression of PGR in the bovine ovary (Jo *et al*. 2002, Van den Broeck *et al*. 2002*b*). In our laboratory, we analyzed the expression of PGR in ovaries from cattle with COD and found different results according to the group of animals tested. In cattle with spontaneous COD from abattoirs, we detected increased mRNA levels of PGRB in theca cells (Alfaro *et al*. 2012), but found no differences in protein expression (Salvetti *et al*. 2007). On the other hand, in animals with ACTH-induced COD, we detected decreased PGR protein expression in granulosa cells of cysts, and levels similar to those of controls in theca cells (Salvetti *et al*. 2012). In addition, specific technical factors such as the sensitivity of the PCRs and the post-transcriptional regulation of gene expression should be considered (Anderson *et al*. 1992, Garverick 1997, Sette *et al*. 2010).

Taking into account the influence of gonadotropin and steroid hormones on PGR expression and that these hormones are altered in animals with COD (Vanholder *et al*. 2006, Amweg *et al*. 2013), it is likely that there will
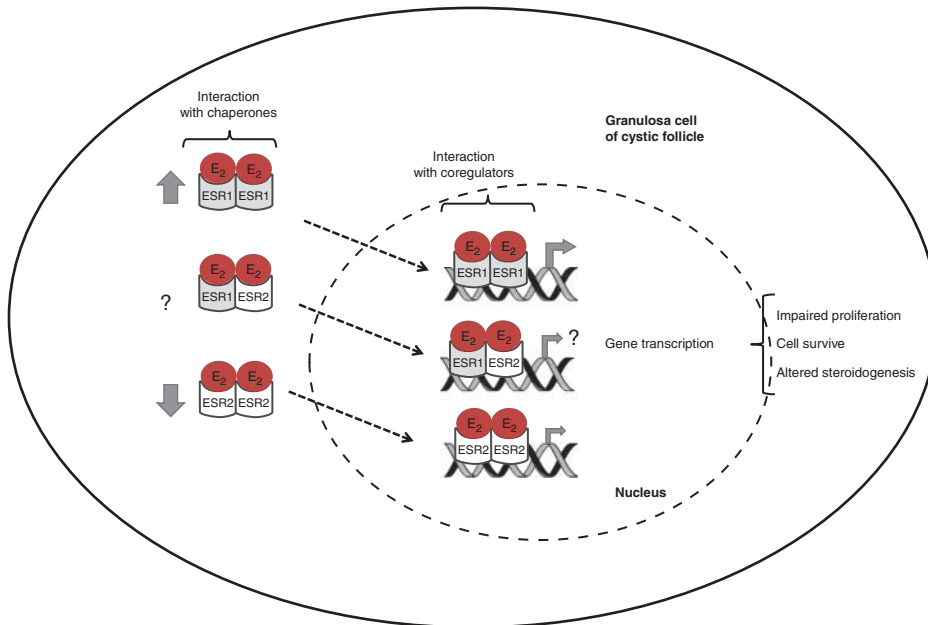
Figure 3 Estrogen and estrogen receptor (ESR) dimer interaction in granulosa cells of experimentally induced cystic follicles. Interaction between endogenous 17β-estradiol (E$_2$) and chaperones allows binding to ER type α (ESR1) or β (ESR2) with similar affinity and forms homo- and heterodimers. Higher levels of ESR1 and lower levels of ESR2 were detected in granulosa cells of induced cysts. Dimers are able to go through the nuclear envelope and, in the nucleus, they interact with different coregulators that modulate differential transcriptional activities in a cell- and promoter context-dependent manner.

be changes in the expression of PGR or the predominance of one isoform over another. It has been widely reported that the coexpression of PGRA or PGRC in the same kind of cell as PGRB modulates its activity (Vegeto *et al*. 1993, Wei *et al*. 1997). Moreover, the genomic actions of PGR isoforms are affected by their association with nuclear coactivators and molecular chaperones (Bramley 2003). Both PGRA and PGRB may activate different sets of genes, even within the same cell, revealing the enormous complexity of progesterone-dependent activation in the target cell (Graham & Clarke 2002, Alfaro *et al*. 2012). This suggests that changes in the expression of PGR isoforms could regulate the biological activity of progesterone, resulting in functional hormone withdrawal at the ovarian level, under unchanged progesterone serum concentrations (Schams *et al*. 2003, Amrozi *et al*. 2004, Goldman *et al*. 2005).

Steroid receptor coregulators

The biological activity of steroid hormone receptors is determined by ligand-binding proteins and also by the relative activities of nuclear receptor-associated coregulators (Mussi *et al*. 2006). Moreover, the equilibrium between them and the nature of the ligand define the state of nuclear receptor activation (Park *et al*. 2005). Among the large number of coactivators, the most important ones are the nuclear receptor coactivator (NCOA) or steroid receptor coactivator (SRC) family, which includes NCOA1 (SRC1), NCOA2 (SRC2/GRIP1/TIF2), and NCOA3 (SRC3/pCIP/ACTR/AIB1/RAC3/TRAM1) (McKenna *et al*. 1999). Mostly, NCOA3 acts as a coregulator for steroid receptors and affects a large

number of signaling systems that are essential for normal cell physiology (Wu *et al*. 2002, Yang *et al*. 2006). In contrast to the function of NCOAs, corepressors generally suppress or silence gene transcription (Auger & Jessen 2009). Nuclear receptor corepressor 2 (NCOR2/SMRT), which was discovered through its interaction with thyroid and retinoid hormone receptors, is one of the most widely studied corepressors (Chen & Evans 1995, Hörlein *et al*. 1995). Another important corepressor is the repressor of ESR activity (REA), which interacts with ESR among others (Montano *et al*. 1999, Delage-Mourroux *et al*. 2000). These corepressors are thought to decrease gene transcription by attenuating steroid receptor activity by recruiting class I and II histone deacetylases, which modify chromatin into a transcriptionally silent status (Kurtev *et al*. 2004), and by competing with coactivators for binding to receptors in the presence of ligands (Martini *et al*. 2000).

Although the expression of coregulators has been scarcely studied in the ovary (Hlaing *et al*. 2001, Zhang *et al*. 2003, Hussein-Fikret & Fuller 2005, Chen *et al*. 2008), follicular development can be influenced by an altered expression of coregulators that may lead to differential transcriptional activation of steroid receptors (Fig. 4; Delage-Mourroux *et al*. 2000). Under these circumstances, some hormone-dependent organs or tumors exhibit over-expression of the NCOA family members that would be directly involved in the increase in cell proliferation and differentiation, indicating their important physiological role (Sarvilinna *et al*. 2006, Mukherjee *et al*. 2007).

Considering that any change in the steroid receptor equilibrium is correlated with follicular health and the
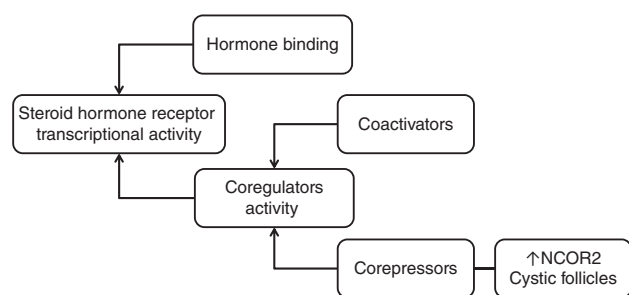
Figure 4 Transcriptional activity of steroid hormone receptors is determined by hormone binding and by the relative activities of nuclear receptor-associated coregulators, which are able to increase (coactivators) or suppress or silence gene transcription (corepressors). Higher levels of NCOR2, which could play a role as corepressors of estrogens, androgens, and progesterone, modulating the response to steroid hormones in the microenvironment affected by the imbalance in steroid receptor expression, are detected in cystic follicles.

stage of development, modifications in intrafollicular activity of steroid hormones determine the fate of a specific follicle (Rosenfeld *et al.* 2001, Drummond *et al.* 2002, Walters *et al.* 2008).

Studies determining the expression levels of steroid receptor coregulators in reproductive tissue are limited mainly to rodents (Misiti *et al.* 1998, 1999, Xu *et al.* 1998, Nephew *et al.* 2000). In domestic animals, Hlaing *et al.* (2001) analyzed the mRNA expression of several coregulators in the ovary of sheep, cows, and pigs by northern blot and found mRNA expression of NCOA1, NCOA2, NCOA3, p300, RIP140, SPA, and NCOR2 in follicles of the three species studied. By *in situ* hybridization, they localized NCOA1, RIP140, and SPA in granulosa and theca cell layers and stroma of the ovine ovary (Hlaing *et al.* 2001).

In previous studies of our group, we have described the localization of several nuclear receptor coregulators in normal bovine ovarian follicles as well as in follicles of animals with COD. We have demonstrated that induced COD in cattle is concurrent with alterations in the expression of steroid receptors and coregulators in ovarian follicles (Salvetti *et al.* 2012). We also found intense immunostaining of NCOA3 in granulosa cells of different follicle categories and an increase in its expression in theca cells of follicular cysts. Furthermore, we found a similar pattern for REA in granulosa cells. Notably, REA differs from other coregulators, in that it is highly specific for ESR, acting as a negative regulator of control ESR-dependent gene expression in normal cells (Mussi *et al.* 2006). On the other hand, we found that NCOR2 shows a gradual increase in its expression while folliculogenesis progresses, with greater expression in cystic follicles (Salvetti *et al.* 2012). These findings suggest that NCOR2 could have a role as a corepressor of sexual steroids in the ovary of animals with COD, modulating the response to these hormones in an environment of altered nuclear steroid receptor expression.

Several studies have highlighted the role of coregulators in total transcriptional activity of steroid hormone receptors (Evers *et al.* 2014*a*, Feng & O'Malley 2014). However, most of these studies have been carried out in mammary and uterine normal tissues or tumor tissues (Evers *et al.* 2014*a*,*b*, Feng & O'Malley 2014, Szwarc *et al.* 2014). To date, there are few studies evaluating the role of these proteins and their interactions in the ovary, and their function (Hlaing *et al.* 2001, Hussein-Fikret & Fuller 2005). Overexpression of coactivator proteins may lead to increased transcriptional activation of nuclear steroid receptors, leading to an enhanced response in hormone-dependent tissues. Despite the lack of information about normal expression, an amplification of NCOA3 has been observed in ovarian tumors and has been correlated with cell differentiation and tumor growth (Bautista *et al.* 1998, Tanner *et al.* 2000). On the other hand, in breast cancer, amplification of NCOA3 has been correlated with high expression levels, enhanced positivity to ESR and PGR, and an increase in tumor size, supporting the hypothesis that NCOA3 plays an important role in estrogen-dependent tumor development and progression (Anzick *et al.* 1997, Bautista *et al.* 1998). Although the information is limited, steroid receptor-associated coregulators may be involved in multiple ovarian functions such as folliculogenesis, steroidogenesis, and ovulation, and thus their altered expression adds another piece in the intricate pathogenesis of COD in cattle. However, these changes could be the consequence of follicle persistence, rather than a cause of it.

Cell proliferation and survival mechanisms in follicular cysts

During folliculogenesis, ovarian cells must follow two essential steps in order to complete follicular development: proliferation and differentiation. After follicles are established, a continuous process of proliferation and differentiation allows some of them to reach the preovulatory size (maximum differentiation level) and ovulate, but most ovarian follicles undergo a process of regression and death known as follicular atresia (Robker & Richards 1998*a*,*b*, Adams *et al.* 2008).

Cell cycle kinase cascades are in charge of controlling cell cycle proliferation and progression by a complex signaling system involving positive and negative regulators (Robker & Richards 1998*a*,*b*). Cyclins have been recognized as positive regulatory components of a class of protein kinases designated as cyclin-dependent kinases (CDKs). These protein kinases have been shown to be important regulators of major cell cycle transitions in diverse eukaryotic systems. In mammals, the process involving activation of cyclin proteins, followed by activation of their CDK partner and the phosphorylation of target proteins, plays an essential role in cell cycle transitions (Zwijsen *et al.* 1997).

Table 1 Cell proliferation in cystic follicles of different species and models.

Species	Spontaneous disease/model	Cell types evaluated	Methods	Results	Reference
Rat	Continuous light-induced PCOS rat model	Granulosa, theca interna, and theca externa cells of tertiary, atretic, and cystic follicles and complete ovaries	PCNA and Ki-67 immunohistochemistry expression (proliferation index)	Granulosa and theca interna of cystic follicles presented lower proliferation index when compared with control tertiary follicles. The theca externa also had reduced proliferation index in atretic and cystic follicles	Salvetti *et al.* (2009)
Human	Spontaneous PCOS	Granulosa cells	Ki-67 immunocytochemistry expression (proliferation index)	Ki-67 was significantly higher in the PCOS group	Das *et al.* (2008)
Bovine	Spontaneous COD	Granulosa and theca cells of healthy antral follicles, early and late atretic ones, and early and late cystic ones	PCNA immunohistochemistry expression	Lower frequencies of PCNA-positive cells in cystic follicles compared with healthy follicles in the granulosa layer from subapical to basal regions	Isobe & Yoshimura (2000*b*)
Bovine	Spontaneous COD	Granulosa and theca cells of follicular cysts	PCNA immunohistochemistry expression	Low frequencies of PCNA-positive cells in cystic follicles	Isobe & Yoshimura (2007)
Bovine	Adrenocorticotropin (ACTH)-induced COD model	Granulosa and theca cells of secondary, tertiary, atretic, and cystic follicles in ACTH-induced COD model and synchronized control cows	Ki-67 expression by immunohistochemistry and RT-PCR of cyclins E and D	Greater proliferation index in granulosa cells of tertiary follicles from the control group than in tertiary, atretic, and cystic follicles from COD group. Secondary follicles from the COD group exhibited an elevated proliferation index	Salvetti *et al.* (2010)
Swine	Spontaneous COD	Granulosa and theca cells of normal, atretic, and cystic follicles	PCNA immunohistochemistry, western blot and real-time PCR	Lower proliferation activity was detected in granulosa and theca cells from cystic follicles, and a lesser number of PCNA-positive cells were found in cystic follicles	Sun *et al.* (2012)

PCOS, polycystic ovary syndrome; COD, cystic ovarian disease.

The progression through the G1 phase of the cell cycle requires the action of one specific group of cyclins, D-type cyclins. In humans as well as in cows, three D-type cyclins (cyclins D1, D2, and D3) have been identified (Zwijsen *et al.* 1997, Yamauchi *et al.* 2003). Although they have different functions according to the cell type, all of them appear to act by activating CDK4 and CDK6 (Zwijsen *et al.* 1997, Robker & Richards 1998*a*, Yamauchi *et al.* 2003). According to Robker & Richards (1998*a,b*), the expression of cyclin D2 and CDK4 in the ovary is confined to the granulosa layer, whereas cyclin D1 and cyclin D3 are expressed in both granulosa and theca layers, with higher expression levels in theca cells. Cyclin E, another cyclin that seems to play a role in the ovary, acts as a positive regulator of cell cycle progression through activation of CDK2 (Reed 1996, Robker & Richards 1998*a*).

Follicular development is an inefficient process, whereby more than 99% of the follicles present at birth are destined to degenerate during lifetime via atresia (Tilly *et al.* 1991). It is well documented that follicular atresia occurs by apoptosis (Hughes & Gorospe 1991). Two of the main players involved in the apoptosis of follicular cells are the FAS system and BCL2 family members (Kim *et al.* 1999, Roughton *et al.* 1999). According to Krammer (1999), the rate of apoptosis might be defined by the interactions between these two proteins. The binding between the FAS receptor (FAS/CD95), a member of the TNF family, and its ligands (FASLG) leads to the formation of a death-induced signaling complex (Krammer 1999, Slot *et al.* 2006). The BCL2 family might be divided into two main groups, according to their proapoptotic (BAX, BAD, BIM, BclxS, and BOK) or antiapoptotic function (i.e., BCL2, BclxL, and BCL2L2) (Slot *et al.* 2006). All of them are regulatory proteins whose actions occur at the mitochondrial level. The antiapoptotic effect is achieved through the blockage of caspase 3, caspase 6, and caspase 7, which transduce the apoptotic signals (Tilly 1996). The penultimate stage of cell death requires caspases (Das *et al.* 2008). DNA repair enzymes and cytoskeletal and nuclear scaffold proteins are activated by caspase 3 (Scaffidi *et al.* 1998, Krammer 1999, Slot *et al.* 2006), which is required for apoptosis in follicular atresia. Granulosa cells, oocytes, and theca cells undergo apoptosis as part of atresia (Hsueh *et al.* 1994, Markstrom *et al.* 2002), to which early antral follicles are more sensitive (Markstrom *et al.* 2002).

In cows, the expression of FAS and FASLG during the first follicular wave is lower in dominant follicles than in subordinate follicles (Porter *et al.* 2000, 2001).

Table 2 Apoptosis determination in cystic follicles of different species and models.

Species	Spontaneous disease/model	Cell types evaluated	Methods	Results	Reference
Rat	DHEA-treated PCOS rat model	Granulosa cells of cystic follicles	TUNEL	Apoptosis in the granulosa cells of the cumulus and those lining the antral cavity of cysts but not in the outer mural resting on the basement membrane	Anderson & Lee (1997)
Rat	Continuous light-induced PCOS rat model	Granulosa, theca interna, and theca externa cells of tertiary, atretic, and cystic follicles	TUNEL, activated caspase 3, and Bcl2 family member's expression by immunohistochemistry	Lower expression of DNA fragmentation, activated caspase 3, and BAX protein in all layers of tertiary and cystic follicles from COD rats than in normal atretic follicles from both groups. Protein expression of Bcl2, BclxL, and Bclw was high in healthy and cystic follicles in both groups	Salvetti *et al.* (2009)
Rat	DHEA-treated PCOS rat model	Granulosa cells of follicular wall of cysts	Immunohistochemistry and western blot of Bax/Bcl2	Bax/Bcl2 ratio significantly higher in the ovaries from the PCOS group than in control group	Bas *et al.* (2011)
Mouse	DHEA-treated PCOS mouse model	Complete ovarian follicles	TUNEL	Increased ovarian apoptosis and larger follicle size, thereby producing a characteristic cystic and atretic appearance in the mouse ovary	Kim *et al.* (1999)
Human	Spontaneous PCOS	Granulosa cells of control, ovulatory, and anovulatory PCOS patients	Flow cytometry, propidium iodide non-viable (apoptotic) cell detection	Most of the granulosa cells of polycystic ovaries are healthy and non-apoptotic	Almahbobi *et al.* (1996)
Human	Spontaneous PCOS	Granulosa cells	Immunocytochemistry of activated caspase 3 and inhibitor of apoptosis proteins (IAP), TUNEL, and Real-time RT-PCR of IAP and Bcl2 families in the granulosa cells. The IAP family included cIAP1,cIAP2, XIAP, and Survivin. Bcl2 family members: Bax, Bcl-Long, and Mcl1	Decreased expression of DNA fragmentation, activated caspase 3, and BAX protein	Das *et al.* (2008)
Bovine	Spontaneous COD	Granulosa and theca cells of healthy antral follicles, early and late atretic ones, and early and late cystic ones	TUNEL	Apoptosis occurs in the granulosa and theca interna cells of cystic as well as atretic follicles, but the frequency of apoptosis in theca interna cells decreases in late cystic follicles	Isobe & Yoshimura (2000a)
Bovine	Spontaneous COD	Granulosa and theca cells of follicular cysts	Immunocytochemistry of activated caspase 3	Decreased expression of activated caspase 3	Isobe & Yoshimura (2007)
Bovine	Adrenocorticotropin (ACTH)-induced COD model	Granulosa and theca cells of secondary, tertiary, atretic, and cystic follicles in ACTH-induced COD model and synchronized control cows	TUNEL, active caspase 3, FAS/FASLG, and members of the BCL2 family by immunohistochemistry and multiplex PCR	DNA fragmentation, caspase 3, FASLG, and BAX protein were significantly less in layers of tertiary and cystic follicles from COD cows than in normal atretic follicles of both groups, whereas BCL2 was greater in growing and cystic follicles in both groups. A relatively greater BCL2 than BAX in cystic follicles when compared with tertiary follicles, as indicated by multiplex analysis	Salvetti *et al.* (2010)
Swine	Spontaneous COD	Granulosa and theca cells of normal, atretic, and cystic follicles	TUNEL, expression of apoptosis regulators (XIAP, bax, bc12, and caspase 3) by immunohistochemistry, western blotting, and real-time quantitative PCR	Apoptosis levels were high in granulosa of atretic follicles, but low in normal and cystic follicles. In theca layer, the apoptosis was low in all follicular categories	Sun *et al.* (2012)

PCOS, polycystic ovary syndrome; COD, cystic ovarian disease.

Additionally, granulosa cells are able to activate endogenous apoptosis pathways in the absence of survival factors (Quirk *et al.* 2004). For instance, *in vitro* studies have indicated that in the presence of serum in the culture media, granulosa cells express FAS but are resistant to be killed by exogenous FASLG (Porter *et al.* 2000, Quirk *et al.* 2000, 2004, Vickers *et al.* 2000).

In previous studies conducted in our laboratory, we observed that the immunoexpression of FAS in cyst and atretic follicles is similar, whereas FASLG is only expressed in atretic follicles (Salvetti *et al.* 2010). These results suggest that, in follicular cysts, reduced apoptosis (which means a delay in atresia) could take place due to the absence of the ligand. On the other hand, dominant follicles are characterized by the presence of survival factors, such as insulin-like growth factor (IGF), which prevent the activation of the FAS pathway and reduce the expression of FAS and FASLG. Furthermore, the ability of growth factors such as IGF1 to facilitate the progression through the cell cycle seems to be a key event to prevent apoptosis. *In vitro* studies by Quirk *et al.* (2004) demonstrated that FASLG-induced apoptosis as well as proliferation in granulosa cells can be stimulated by IGF1, basic fibroblast growth factor, and epidermal growth factor. Conversely, the treatment with gonadotropins seems to decrease the expression of death-inducer genes and stimulate the expression of death repressor genes. Specifically, gonadotropins inhibit cell apoptosis and follicular atresia probably via a reduction in BAX in granulosa and maintenance of the constitutive levels of antiapoptotic factors such as BCL2 and BclxL (Tilly 1996).

Ovarian signaling, mainly mediated by steroid hormones and local growth factors, might also play an important role in the balance between proliferation and apoptosis of granulosa cells via regulation of *BCL2* and *BCL2L1* gene expression (Tilly *et al.* 1991, Johnson 2003). Regarding BAX, a protein with pro-apoptotic effects, it has been shown that *Bax* knockout mice develop an excessive number of granulosa cells in their abnormal follicles (Knudson *et al.* 1995).

Cows with COD show a decrease in the index of proliferation in granulosa and theca cell layers of cystic follicles, similar to that observed in atretic follicles. Previous studies found that the mRNA levels for cyclins D1 and E in samples of the follicular wall are lower in cystic follicles than in healthy tertiary follicles (Isobe & Yoshimura 2007, Salvetti *et al.* 2010). Similarly to the results found in induced follicular cysts in rats in different experimental models (Table 1; Baravalle *et al.* 2006, 2007, Salvetti *et al.* 2009), tertiary follicles of cows show an intense proliferation in the basal area of the granulosa layer, whereas in atretic and cystic follicles proliferation declines (Isobe & Yoshimura 2007, Salvetti *et al.* 2010).

In addition, results from different studies performed in our laboratory suggest that the expression of different markers of apoptosis, such as BAX, FASLG, caspase 3,

and DNA fragmentation, is significantly higher in normal atretic follicles than in cystic and tertiary follicles. Conversely, the expression of BCL2 (antiapoptotic) is higher in cysts and growing follicles than in atretic ones (Salvetti *et al.* 2010). Similar results about apoptosis have been found in ovarian cysts of bovines (Isobe & Yoshimura 2000*b*, 2007), as well as in those of rats and other species used as models of COD (Table 2; Anderson & Lee 1997, Shirwalkar *et al.* 2007, Salvetti *et al.* 2009).

In summary, follicular persistence in cystic follicles appears to be related both to reduced apoptosis and to a reduced proliferation rate. The expression of apoptotic markers in follicular cells is related to activation of both the exogenous pathway through death receptors and the endogenous pathway by the *BCL2* gene family (Salvetti *et al.* 2010). However, the poor levels of apoptosis observed in bovine follicular cysts suggest that the activation of both apoptosis pathways is significantly reduced in COD. On the other hand, the balance between proapoptotic and antiapoptotic factors, along with FAS/FASLG, could be influenced by the concentration of different hormones. In this regard, changes in these factors might be induced as a result of altered hormonal levels that characterize COD, and, consequently, cell proliferation and apoptosis might be affected. For example, a reduction in caspase 3 expression could be induced, resulting in decreased apoptosis (Salvetti *et al.* 2010). All these data support the assumption that, in COD, the expression of proteins related to follicular cell proliferation and apoptosis might be influenced by the hormonal changes characteristic of this disease, contributing to the persistence of cysts.

Conclusions

Cumulative evidence suggests the existence of multi-factorial causes in the pathogenesis of COD. Owing to the lack of a distinct cause of COD, it has been difficult to understand its origins and thus develop an effective treatment. In this sense, considering that ovulation is a complex process where, after initiation by LH, cascades of several pathways interacting within cell types and between cell compartments are involved, any alteration in the multiple links can inhibit it. These processes are essential for the successful establishment of pregnancy, and involve changes in gene expression with overlapping control and interdependent consequences in the theca and granulosa compartments of the ovarian follicle. Systemic and local inputs coordinate with signals from the follicle; thus, ovulation is under multipartite control, facilitating synchronization of oocyte maturation. In this context, interpretation of alterations in endocrine signals through gonadotropins and in paracrine and autocrine signals through steroids is essential for understanding the pathogenesis of COD. A delay in follicle regression after ovulation (by alteration in the proliferation/apoptosis balance) is

an alternative component in the pathogenesis of cysts because preovulatory follicles that can neither be ovulated nor become atretic will affect the normal ovarian function, being the first step in follicular persistence and establishment of follicular cysts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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