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Evidence for an association between Wnt-independent β-catenin intracellular localization and ovarian apoptotic events in normal and PCO-induced rat ovary

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The association of secreted frizzled related protein type 4 (Sfrp4) as an antagonist of Wnt molecules in apoptotic events has been reported previously. Moreover, its increased expression has been reported in the ovary of women with polycystic ovary (PCO). We have demonstrated increased Sfrp4 in PCO-induced rat ovary related to an increased number of apoptotic follicles showing nuclear βcatenin subcellular localization. The aims of present study were twofold 1) to ascertain nuclear βcatenin presence with apoptosis by using immunolocalization of Bax and active cleaved casapase-3, and 2) to elucidate whether Sfrp4 could be an inducer of apoptosis by using isolated rat granulosa cell culture in the presence of recombinant human SFRP4. To this end, immuno-expression of two key molecules in Wnt signaling, GSK3β and β-catenin and apoptotic markers were investigated in normal and PCO-induced rat ovary by daily administration of testosterone propionate (TP) for four weeks. We showed that in PCO-induced as well as in normal ovaries there was nuclear or cytoplasmic subcellular localization of GSK3β and a weak pGSK3β^{ser9} immuno-staining in apoptotic granulosa cells. Interestingly, intracellular βcatenin localization was observed in Bax and active caspase-3 positive granulosa cells in normal as well as in PCO-induced rat ovary. Treatment of granulosa cells with rhSFRP4 showed co-localization of nuclear subcellular β-catenin and active caspase-3 as revealed by double immuno-fluorescence. Our results suggest that rhSFRP4 induces apoptosis and that there is an association between Wnt-independent β-catenin nuclear subcellular localization and apoptotic events of rat ovary.© 2011 Progress in Biological Sciences. Vol. 1, No. 2, 1-10.

KEY WORDS**:** β-catenin, apoptosis, Sfrp4, GSK3β, polycystic ovary

Introduction

The Wnt and Frizzled (Fzd) families of signaling molecules impact ovarian cell function and follicular organization (Pangas, 2007). Wnts are secreted extracellular signaling molecules that bind to the Fzd receptors (Miller, 2001). In the canonical or Wnt/β-catenin signaling pathway, the second messenger of the pathway, β-catenin, is stabilized by Wnt binding to its receptors, leading to increased levels of β-catenin in cytoplasm. Subsequently, β-catenin translocates to the nucleus where it acts as a transcriptional co-activator with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family. In the absence of ligand, β-catenin is targeted for degradation by a cytoplasmic complex that includes Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β) (Novak and Dedhar, 1999). In non-canonical Wnt signaling, some Wnts activate Fzd receptors that signal via intracellular calcium, protein kinase C (PKC), calmodulin-dependent kinases (CAMK), or the JNK-signaling cascade, which are involved in the reorganization of cytoskeleton and cell motility (Veernan et al., 2003).

Wnt/Fzd activation of the β-catenin pathway is

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further modulated by proteoglycans and/or arrow/LRP-5/LRP-6 as co-receptors (Mi and Johnson, 2005) and by antagonists such as secreted frizzled-related proteins (sFRPs) (Bovolenta et al., 2008). The sFRPs are the largest family of Wnt inhibitors and comprise five members in humans, sFRP1 to sFRP5. Biochemical studies have established that Wnt proteins and SFRPs interact through cysteine-rich domains (CRD) postulated to be the binding domain because of its homology with the Wnt-binding region on Fz receptors (Rattner et al., 1997; Wang et al., 1997). It has been proposed that this interaction impedes Wnt binding to the Fz receptor and, thus, prevents signal transduction.

Wnt4 is important in the reproductive tract for formation of the ovary during embryogenesis. Although Wnt4-null mice have a perinatal lethal phenotype, it was discovered that newborn Wnt4-null female mice exhibit sex-reversed ovaries depleted of germ cells and containing male sex-cord-like structures (Vainio et al., 1999). Multiple Wnts are expressed in the postnatal and adult rodent ovary (Hsieh et al., 2002; Ricken et al., 2003). Wnt4 and the Fzd receptors, Fzd1 and Fzd4, show dynamic changes in expression during folliculogenesis. Wnt4 is expressed in granulosa cells of primary, pre-antral, and pre-ovulatory follicles, as well as in corpus luteum (CL) (Hsieh et al., 2003). Fzd1 is expressed in theca and granulosa cells in ovulating follicles, with expression decreasing as follicles luteinize. It is not expressed in CL (Hsieh et al., 2003). Rather, CL of hormone-stimulated and pregnant mice expresses Fzd4. Mice null for Fzd4 are infertile. Although folliculogenesis and ovulation proceeds, the CL does not develop normally, and implantation does not occur (Hsieh et al., 2005). It has been demonstrated that Sfrp4 (the rat ortholog of human sFRP4) is highly expressed in luteinized granulosa cells and its subcellular localization can be modulated by the presence of prolactin (Hsieh et al., 2003). Sfrp4 has also been reported to be associated with apoptotic events in CL regression (Guo et al., 1998) and ovarian surface epithelial cell apoptosis after ovulation takes place (Drake et al., 2003). It is not yet clear if the apoptotic effects mediated by Sfrp4 represent an activity that is independent of Wnt activity.

Recently, gene expression profiling data demonstrated that Wnt antagonists Sfrp4 and DKK1 are up-regulated, and Wnt-5A is down-regulated in theca cells of women with PCO (Wood et al., 2004). This observation may suggest that disruption of the Wnt signaling cascades participates in the phenotypes of the PCO.

In our previous reports we have shown increased expression of Sfrp4 in PCO-induced immature female rats, related to apoptotic and luteinization events in PCO (Jarooghi et al., 2008; Jannessari-Ladani et al., 2009). We also showed for the first time nuclear subcellular localization of β-catenin in apoptotic granulosa cells, as revealed by TUNEL assay (Jarooghi et al., 2008). Here, we sought to investigate nuclear β-catenin-associated apoptosis by using Bax and cleaved casapase-3 as specific markers of apoptosis. Further investigation was performed to determine whether recombinant human sFRP4 could be an inducer of apoptosis by using rat granulosa cell culture and assessment of co-localization of active β-catenin, which is specifically located in the nucleus and cleaved caspase-3.

Material and methods

Reagents and antibodies

Nutrient Mixture F-12 (DMEM+F12), penicillin-streptomycin, bovin serum albumin (BSA) and DAPI (Sigma-Aldrich, Co.), fungizonem, and fetal bovine serum (FBS) from Gibco and trypsin-EDTA (Fluka). Pregnant mare serum gonadotropin (PMSG) was provided by Intervet/Schering-Plough Animal Health, Netherlands. Recombinant human SFRP4 (rhSFRP4) was obtained from R&D Systems Inc., Minneapolis, MN.

Rabbit polyclonal anti-Bax antibody (Sigma-Aldrich); rabbit polyclonal anti pan β-catenin (a kind gift from Dr. Arab-Nadjafi, Department Cellular and Molecular Biology, University of Tehran, Iran), mouse monoclonal anti-active β-catenin (specific against nuclear β-catenin) and goat anti-mouse IgG rhodamine were from Millipore; rabbit polyclonal rabbit anti-cleaved caspase-3 antibody (Calbiochem, EMD Chemicals,

Inc.); rabbit polyclonal anti-GSK3β and anti-pGSK3β^{ser9} (Santa Cruz Biotechnology., Inc.), goat anti-rabbit IgG-FITC conjugated (Razi Biotech.). Vectastain ABC complex, diaminobenzoide (DAB) reagents were obtained from Vector laboratories, Ltd, UK.

Animals

Eight immature female Wistar rats aged 23-25 days weighing 40-50 g were provided by the School of Public Health and Institute of Public Health Research animal house, University of Tehran or from the Iranian Pasteur Institute. Rats were kept in a 12:12 L:D photoperiod and fed an ordinary laboratory diet *ad libitum*. All experimentation was approved by the Animal Ethics Committee of the School of Biology, University of Tehran and was done in accordance with the NIH guide for the care and use of laboratory animals.

Rats were subcutaneously injected daily for four weeks with TP 1 mg per 100 g body weight dissolved in sesame oil (Beloosesky et al., 2003). Aged-match control animals were injected with sesame oil. Four control animals and four TP-treated animals were examined. All animals were killed by decapitation at the end of treatment, and one ovary from each animal was fixed with 4% paraformaldehyde in PBS (pH 7.2), dehydrated, and paraffin embedded followed by serial sectioning at 7 μm thickness and heat-drying at 42^{o}C overnight.

Immunohistochemistry

Random serial sections from the center of the ovaries were analyzed. Tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0) at 90^{o}C for 20 min, and cooled at room temperature for 20 min, then washed in PBS. Non-specific binding sites on serial ovarian sections were blocked with 4% BSA in PBS for 1 h at room temperature and incubated overnight at 4^{o}C with anti-pan β-catenin antibody (1:300), anti-GSK3β antibody (1:75), anti-pGSK3β^{ser9} antibody (1:75), anti-Bax antibody (15 μg ml^{-1}), and anti-cleaved caspase-3 (20 μg ml^{-1}) all diluted in 4% BSA in PBS, then washed three times in PBS. Negative controls included substitution of primary antibody with rabbit IgG or goat IgG (Zymed Laboratories, Inc.). Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 10 min at room temperature. Sections were washed in PBS and incubated with biotinylated anti rabbit IgG (1:300) in PBS + 4% BSA for 1 h at room temperature. Immunodetection was performed by incubating sections with Vectastain AB reagent (1:10) diluted in AB buffer (10 mM sodium phosphate, pH 7.6, 0.9% saline) for 30 min at room temperature, washing in PBS, and visualizing using diaminobenzoide (DAB) reagent. Sections were counterstained with hematoxylin, dehydrated in an ethanol series, cleared in xylene, and mounted with N-thalene glue.

Slides were analyzed by light microscopy (Zeiss axiophot, Carl Zeiss Inc., Germany). Digitized photographs (DeltaPix 460 camera and software, Maalov, Denmark) were made and white balance was assessed (measurement in a tissue-free area of the section thus representing 100% transmission). Randomly selected regions of interest containing immunopositive structures such as follicular groups, stroma, and cysts were defined.

Preparation of type I rat tail collagen

A component of extracellular matrix such as type I collagen, fibronectin, or laminin is required for granulosa cell adherence and culture. For preparation of type I collagen tails were taken from mature rats and stored in 70% ethanol for 45 min. Under sterile conditions the skin was slit and each tendon was separated using a scalpel and teased apart with a blade to separate the fibers. The preparation was washed repeatedly with sterile distilled water until clean. Tendons were transferred into 1:500 acetic acid solution plus 100 IU penicillin/100 μg ml^{-1} streptomycin and 100 IU fungizone g^{-1} tendon. The mixture was agitated at 4°C on a magnetic stirrer for 9 days in a cold room, after which it was centrifuged at 3000 x g for 15 min. The supernatant was centrifuged in a Beckman 50.2Ti rotor at 35,000 × g for 1 h. The supernatant as type I collagen was stored at 4°C until use. Collagen

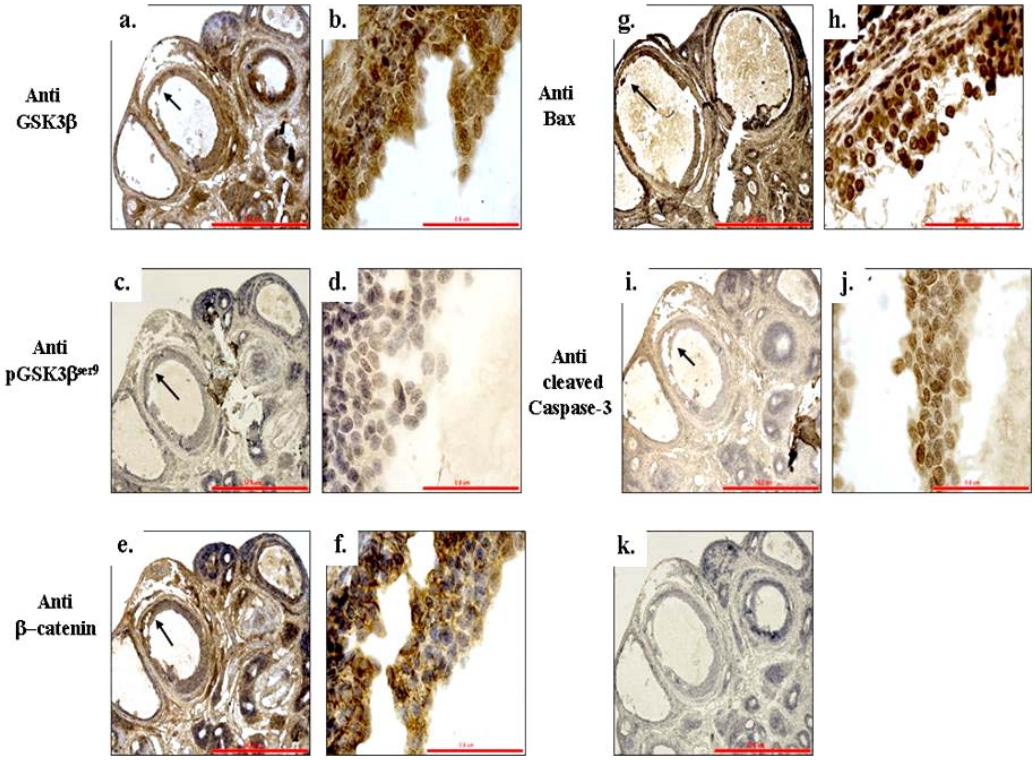

Fig. 1. Immunolocalization of GSK3β, pGSK3β, β-catenin, Bax and active caspase-3 in 28 days TP-treated rat ovary. a-b, GSK3β immunolabelling in the ovarian follicles; c-d, pGSK3β^{ser9} immunolabelling; e-f, β-catenin immunolabelling. Note that GSK3β and β-catenin staining is co-localized with strong Bax immunoreactivity g-h, active caspase-3 i-j. and k, negative control. Arrows in column 1 and 3, indicate the area shown at higher magnification in column 2 and 4.

solution concentration was measured by the Bradford assay (43).

Granulosa cell isolation and culture

Immature female Wistar rats were injected intraperitoneally with 10 IU PMSG to induce follicular growth to the pre-ovulatory phenotype in order to harvest enough granulosa cells. After 48 h, rats were killed and ovaries were harvested for mechanical granulosa cell isolation and culture. Ovaries were punctured with 30 gauge needles and cells were collected by centrifugation at 400 g, the pellet was re-suspended in DMEM:F-12 medium with 10% FBS, 100 IU penicillin, and 100 µg ml^{-1} streptomycin. Cell viability was examined by trypan blue exclusion test, and 20×10^3 cells well^{-1} were seeded into a 96 well plate pre-coated with type I collagen at 37°C in 5% CO_2 and 95% air. After overnight incubation, cells were maintained with or without 0.5 ng ml^{-1} rhsFRP4 for two days prior to double immunofluorescence assay.

Double immunofluorescence staining

Granulosa cells were fixed in 3% glutaraldehyde for 15 min at room temperature, washed with PBS to remove fixative, permeabilized in methanol at -20°C, then washed with PBS and non-specific binding sites were blocked with PBS containing 4% BSA for at least 1 h at room temperature. All antibodies were diluted in PBS containing 4% BSA as blocking buffer, all washing procedures were performed three times with PBS for 5 min each with shaking. Anti-active β-catenin (1:200) detection was performed overnight at 4^0C and revealed by using goat anti-mouse IgG rhodamin (1:200) for 1 h at room temperature.

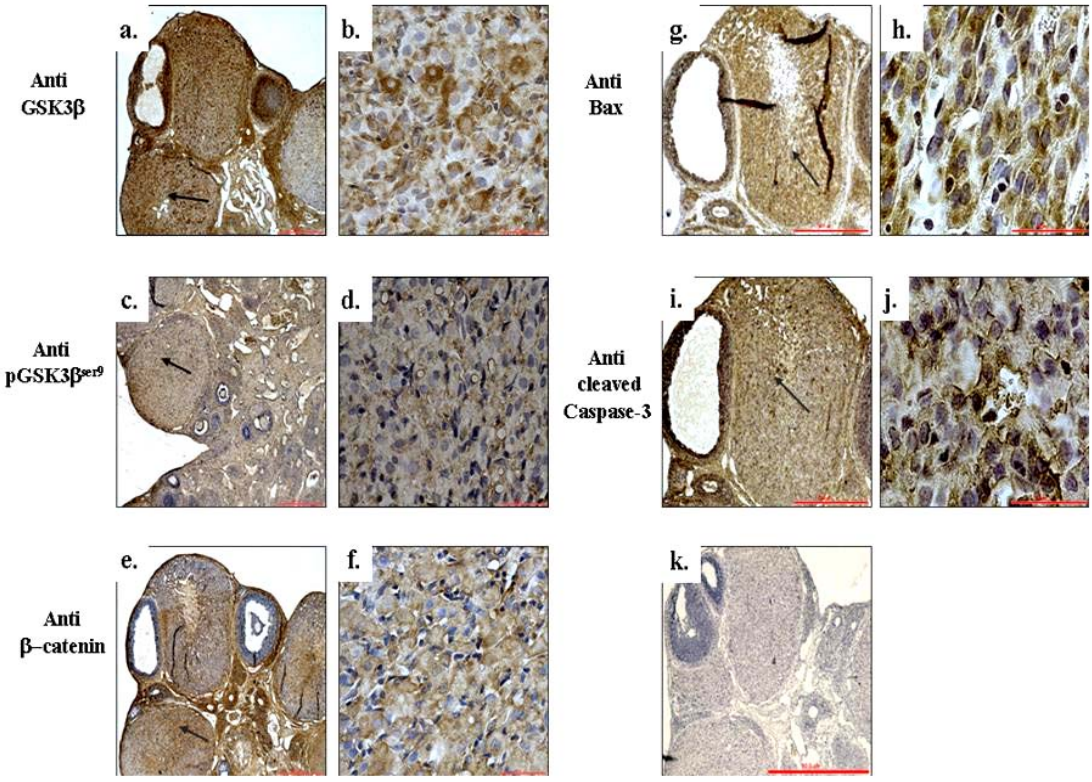

Fig. 2. Immunolocalization of GSK3β, pGSK3β, β-catenin, Bax and active caspase-3 in control cycling rat ovary. a-b, GSK3β immunolabelling in the ovarian follicles; c-d, Images of weak pGSK3β^{ser9} immunolabelling; e-f, β-catenin immunolabelling. Note that GSK3β and β-catenin staining is co-localized with strong Bax immuno reactivity g-h, active caspase-3 i-j. and k, negative control. Arrows in column 1 and 3, indicate the area shown at higher magnification in columns 2 and 4.

For the second immunolabeling, after washing three times with PBS, cells were incubated with blocking buffer, as mentioned, then incubated with anti-active caspase 3 (1:20) overnight at 4°C and revealed by using goat anti-rabbit IgG FITC conjugated (1:20) for 1 h at room temperature. Finally, cells were washed with PBS and nuclear staining was conducted with 1 µg ml^{-1} DAPI for 1 min in darkness. Cells were again washed with PBS and observed with a Zeiss inverted fluorescence microscope (Bonyakhte Laboratories).

Results

Based on our previous reports regarding increased Sfrp4 and apoptosis in PCO-induced rat ovary (Jarooghi et al., 2008; Jannesari-Ladani et al., 2009), we sought to confirm subcellular nuclear localization of the β-catenin relationship with apoptosis by using immunohistochemical localization of specific apoptotic markers such as Bax and cleaved caspase-3 in serial ovarian sections. In addition, as GSK3β is a key molecule in the Wnt/β-catenin pathway, the immune reactivity of this molecule and its inactive form, pGSK3β^{ser9}, were also evaluated in serial ovarian sections.

Fig. 1a shows multiple follicular cysts as a characteristic of PCO-induced rat ovary (Fig. 1a) with extensive GSK3β immunostaining of cytoplasm or nuclei of granulosa cells facing antrum in cysts (Fig. 1b). In addition, oocytes of the primordial follicles showed intense GSK3β immunostaining (data not shown). pGSK3β^{ser9} immunoreactivity was very weak (Fig. 1c) and was barely detected in granulosa cells (Fig. 1d). A strong cytoplasmic or nuclear β-catenin was detected in apoptotic granulosa cells of cystic follicles (Fig. 1e-f). To determine if GSK3β

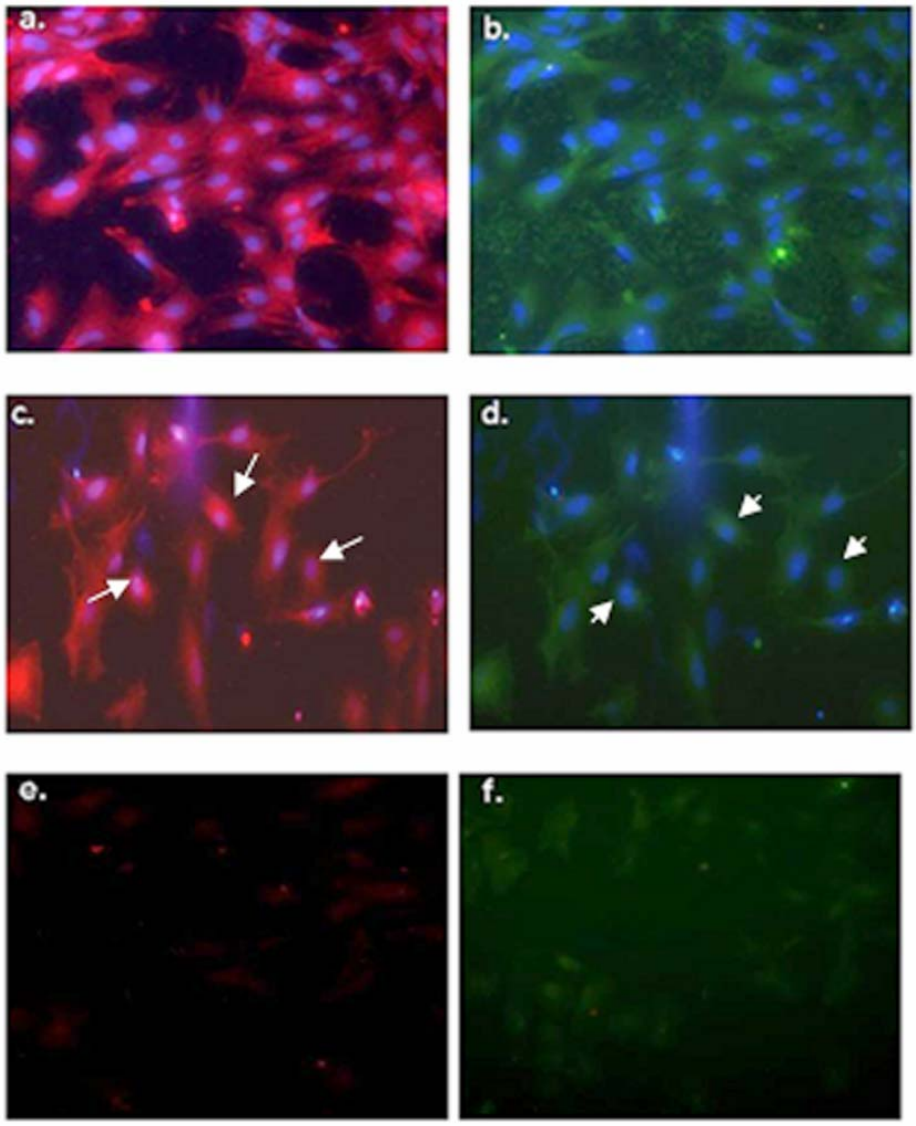

Fig. 3. Co-localization of active β-catenin and active caspase-3 in rat granulosa cell culture in the absence or presence of rhsFRP4. Cells were immunostained with anti active β-catenin (red) and anti active caspase-3 (green) subsequently. DNA was stained by using DAPI (blue). a-b, cytoplasmic β-catenin and active caspase-3 co-localization in the absence of rhsFRP4; c-d, nuclear subcellular co-localization of β-catenin (arrows) and active caspase-3 (arrow heads) localization in the presence of 0.5 ng ml^{-1} rhsFRP4; e-f, represent negative controls, primary antibodies were omitted and cells were only incubated with secondary anti mouse IgG-rhodamine and anti rabbit IgG-FITC, respectively. Image magnification 20x.

could be involved in events related to the apoptotic pathway, we used anti-Bax immunostaining which showed a very strong nuclear immunoreactivity co-localized with GSK3β positivity in follicles (Fig. 1g-h). To confirm these findings, we performed anti-active caspase-3 immunohistochemical analysis which showed strong active caspase-3 positivity co-localized with GSK3β and Bax immunoreactivity in apoptotic granulosa cells (Fig. 1i-j) and oocytes of primordial follicles in animals treated for four weeks with TP (data not shown). No staining was detected in negative controls (Fig. 1k).

Control age-matched cycling rat ovary contained few antral follicles and multiple corpora lutea which showed strong GSK3β staining in large luteal cells (Fig. 2a-b), while pGSK3β^{ser9} was mainly localized in capillaries (Fig. 2c-d). A faint cytoplasmic β-catenin localization was detected in large luteal cells (Fig. 2e-f). The immunostaining was co-localized with strong Bax (Fig. 2g-h) and active caspase-3 (Fig. 2i-j) in few antral follicles and large luteal cells of the CL. No staining was observed in negative controls (Fig. 2k).

As mentioned, increased expression of Sfrp4 was observed in long term TP treated rat ovary (Jarooghi et al., 2008). Until now, the question of whether Sfrp4 is associated with apoptotic events or could be a direct inducer of apoptosis remained unanswered. We sought to resolve this question by treating isolated rat granulosa cells with rhsFRP4 and by using anti active β-catenin which specifically recognizes nuclear β-catenin. In the absence of rhsFRP4 there was no staining for active β-catenin (Fig. 3a), and cytoplasmic active caspase-3 (Fig. 3b) was observed. Interestingly, clear presence of active β-catenin was observed in granulosa cells treated with rhsFRP4 co-localized with nuclear active caspase-3 (Fig. 3c and d, respectively). No staining occurred in negative controls (Fig. 3e-f)

Discussion

Carefully regulated cell death by apoptosis is crucial in the development and homeostasis of ovary. Wnt signaling alteration has been shown in women with PCO (Wood et al., 2004), but whether the molecules involved in Wnt/β-catenin signaling could be associated with apoptotic events occurring in PCO-induced or normal rat ovary remains unknown. The present study was designed to examine whether there is a relationship between Wnt/β-catenin pathway and nuclear subcellular localization of β-catenin with apoptosis in PCO-induced (Beloosesky et al., 2004) and untreated aged-matched control rats.

We have previously shown a decreased number of growing follicles in long term TP-treated rats which may be explained by decreased primordial follicle recruitment and apoptosis (Jannessari-Ladani et al., 2009). Our finding corroborates with the pro-apoptotic effect of testosterone in rodent ovary (Billig et al., 1993), which was prominent following long term testosterone treatment.

sFRPs are the largest family of Wnt inhibitors, which can inhibit both canonical and non-canonical Wnt signaling pathways (Bovolenta et al., 2008). An increased expression of Sfrp4 by long term testosterone treatment was related to luteinization and apoptotic events taking place in PCO-induced rat ovary (Jarooghi et al., 2008; Jannessari-Ladani et al., 2009). This finding agrees with Hsieh et al. (2003) who showed high Sfrp4 expression in luteinized granulosa cells, which was postulated to have a non-apoptotic function, although apoptosis was not examined in this study. Also, Fzd4 knockout mice showed impairment of CL function associated with reduced expression of Sfrp4 (Hsieh et al., 2005). The physiological significance of up-regulated expression of Sfrp4 during luteinization events remains to be determined, however several studies establish an association between Sfrp4 expression and apoptotic events.

We showed that apoptotic follicles were strongly positive for GSK3β, while faint immunolabeling of pGSK3β^{ser9} was observed. This could be because increased levels of Sfrp4 in PCO-induced rat ovary (Jarooghi et al., 2008) may inhibit the Wnt/βcatnin pathway and lead to decreased levels of pGSK3β^{ser9}. However, this study showed nuclear subcellular localization of β-catenin in Bax and cleaved caspase-3 positive follicles. This suggests the existence of a Wnt-independent β-catenin nuclear localization related to granulosa cells apoptosis.

Earlier studies showed that Sfrp4 overexpression in mouse mammary gland *in vivo* induced apoptosis (Lacher et al., 2003). It was postulated that sFRP4-mediated apoptosis involves the suppression of the phosphoinositide 3-kinase (PI3K)/Akt and the protein kinase B (PKB)/Akt survival pathways, possibly through a mechanism independent of canonical Wnt/□-catenin signaling (Lacher et al., 2003). We showed β-catenin nuclear subcellular localization in rat

granulosa cells in the presence of rhsFRP4, which was co-localized with perinuclear activated caspase-3. Although it has been widely accepted that procaspase-3 is cleaved to generate the active form in the cytoplasm, the enzymatic activity of capase-3-like proteases can be found in the nuclear fraction of apoptotic cells (Takemoto et al., 2003). Moreover, Kamada et al. (2005) showed that the nuclear translocation of effector caspases is specific for caspase-3, which requires its proteolytic activation, and that the nuclear translocation of caspase-3 is an active process not simply entailing diffusion after disruption of the nuclear-cytoplasmic barrier. The precise nuclear subcellular localization of active caspase-3 and active β-catenin requires the use of confocal microscopy which was not available.

A key target of PKB/Akt is multifunctional protein GSK3β, which is a constitutively active serine/threonine kinase in resting cells (Cross et al., 1995). GSK3β activity is tightly controlled by several mechanisms such as phosphorylation of serine-9 in GSK3β by both Wnt (Logan and Nusse, 2004) and phosphoinositide 3-kinase/Akt (Pap and Cooper, 1998) signaling pathways. We detected strong GSK3β immunostaining in apoptotic follicles, which seems to be greater following long term TP treatment. This finding corroborates the study of Constantinou et al. (2008) which showed that Sfrp4 directly affects PKB/Akt signaling by inhibiting its activity, leading to increased apoptosis of mammary gland epithelial cells. Mechanisms in addition to phosphorylation are needed to regulate GSK3β, including control of its intracellular localization and of its association in protein complexes, mechanisms that serve to selectively direct its activity towards specific substrates (reviewed by Grimes and Jope, 2001). The present study showed nuclear subcellular localization of GSK3β in granulosa cells of apoptotic follicles. Moreover, following long term TP treatment, oocytes of non-growing follicles showed GSK3β immunoreactivity. It has been demonstrated that GSK3$^{\beta}$ in the nucleus is more active than cytosolic GSK3$^{\beta}$ (Bijur and Jope, 2003) and may regulate many transcription factors, such as nuclear factor-κB (NF-κB), cyclic AMP response element-binding protein (CREB), p53, AP-1, Myc, and others (reviewed by Grimes and Jope, 2001) that exert widespread effects on cellular functions by regulating the gene expression. Moreover, increased GSK3β in *Hydra* was associated with nurse cell apoptosis during oogenesis (Rentzsch et al., 2005). Therefore, GSK3β seems to have a conserved role in the ovarian apoptotic pathway during evolution and could be an important regulator of genes involved in ovarian cell cycle regulation.

It was interesting that there was strong cytoplasmic and weak nuclear β-catenin immunostaining in apoptotic granulosa cells and in luteal cells of the CL. The Wnt/β-catenin pathway required inhibition of GSK3β, but following long term TP treatment, pGSK3β^{ser9} was barely detected in apoptotic follicles, which could suggest that increased free β-catenin in these follicles is not related to Wnt signaling. Disturbances in ovarian cell adhesion *in vivo* are linked to increased atresia, insufficient luteal function, and cyst formation (Sundfeldt et al., 2000; Peluso et al., 2000 and 2001; Khan-Dawood et al., 1996). Thus, a possible result of cytoplasmic β-catenin localization may be disruption of E-cadherin in apoptotic granulosa cells. E-Cadherin binds β-catenin tightly and stabilizes it at the membrane. Studies have indicated a function for E-cadherin as an anti-apoptotic molecule, since E-cadherin was found to be present in ovine granulosa cells of healthy follicles, whereas expression was not detected in atretic follicles (Ryan et al., 1996), and rat granulosa cells of apoptotic follicles did not express E-cadherin (Sundfelt et al., 2000). It has been demonstrated that E-cadherin-mediated cell contact, either directly or indirectly, promotes PKB/Akt kinase activity, which in turn, inhibits caspase-3 activation and thereby maintains spontaneously immortalized granulosa cell (SIGCs) viability (Peluso et al., 2001). The β-catenin subcellular localization in SIGCs altered in response to serum depletion within the cytoplasm and nucleus (Peluso et al., 2001), and accumulation of β-catenin in the nucleus of the rat ovarian surface epithelial cells stimulated the expression of genes required for these cells to

undergo apoptosis (Peluso et al., 2000). However, it is not known if β-catenin has the same effect in granulosa cells. Moreover, the E-cadherin–catenin complex is also present in the primate CL in which E-cadherin is expressed primarily during the early luteal phase and was not detected in the regressing CL (Khan-Dawood *et al*., 1996). β-catenin showed a different pattern compared to E-cadherin and was expressed in high concentrations in the mid- and late-luteal phases. According to this finding, the immunostaining results of the present study indicate cytoplasmic β-catenin immunohistochemical localization in luteal cells, which may be associated with regression of the CL. Since sFRP4 has been reported to inhibit AKT activation, and based on the key role of the PKB/Akt pathway in granulosa cell survival (Johnson et al., 2001), we investigate whether rhsFRP4 could be a direct inducer of granulosa cells apoptosis and whether active β-catenin, known as nuclear β-catenin, could be modulated by this molecule. This study showed that rhsFRP4 could induce apoptosis, which was related to the presence of active β-catenin.

Although Sfrps have generally been described as antagonists of Wnt signaling, recent studies provide evidence for positive regulation. It has been suggested that sFRP1 has low-affinity and high-affinity binding sites for Wg (ortholog of Wnt in *Drosophila*). Binding to the high-affinity site would promote Wg signaling whereas binding to the low-affinity site would inhibit it (Uren et al., 2000). Moreover, Wawrzak et al. (2007) reported that, although sFRP4 binds directly to canonical Wnt3a with affinities in the nanomolar range, it could not supress Wnt3a induced β-catenin accumulation in L cells. On the basis of our results and other reports, it is possible that Sfrp4 has no antagonistic effect on the Wnt/β-catenin signaling pathway in the rat ovary. However, future molecular studies should clarify how Sfrp4 activity is integrated into granulosa cell cellular signaling pathways.

Acknowledgments

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