Chemotherapy has been associated with premature ovarian failure and infertility in women with cancer. It is well known that anticancer drugs reduce the primordial follicle pool and harm the ovarian blood vascularization leading to ovarian atrophy. However, their mechanism of injury still remains unclear. The aim of this study was to identify the cellular mechanisms involved in the toxicity of chemotherapy drugs belonging to different classes on human ovarian luteinized granulosa cells (LGCs). Treatment with doxorubicin (DXR), paclitaxel (PC), and cisplatin (CP) affected LGCs viability by inducing apoptosis and downregulating both estrogen receptor β and follicle-stimulating hormone receptor in a dose-dependent manner. Several members of the WNT signaling pathway are expressed in granulosa cells where they regulate follicle development, ovulation, and luteinization. Here we show that treatment with DXR, PC, and CP induced upregulation of WNT4 expression, whereas WNT3 expression was downregulated by DXR and PC and upregulated by CP. Analysis of the WNT3 downstream signaling pathway showed that total β-catenin protein levels were reduced upon treatment with DXR and PC. Additionally, restoration of β-catenin signaling by lithium chloride protected LGCs from the injury induced by chemotherapy. The in vitro LGC toxicity model described might represent a tool to identify components of specific signaling pathways, such as the Wnt pathway, that can be targeted in order to limit the follicular damage caused by chemotherapy.

Key Words: chemotherapy; reproductive toxicity; granulosa cells.

Novel approaches in early detection and effective management strategies have led to increased rates of cancer survivors throughout the past 3 decades (Jemal et al., 2008), thus yielding an additional focus on preservation of an optimal quality of life after cancer treatment. This has led to an increasing research of long-term side effects, including the impact on fertility. The influence of anticancer treatments on female fertility depends on the woman’s age and ovarian reserve at the time of treatment, the chemotherapy protocol, the duration, and total cumulative dose administered (Anderson and Cameron, 2007; Wallace and Kelsey, 2010). Chemotherapy-related amenorrhea occurs in over 90% of patients treated with high-dose chemotherapies, induction therapies before bone marrow transplantation, or total body irradiation (TBI). Virtually all women undergoing induction chemotherapy and TBI before transplantation are irreversibly sterilized (Lobo, 2005).

Chemotherapeutic drugs have been categorized into 3 risk categories according to their ovarian failure odds ratio adjusted for age: high risk, medium risk, and low risk (Meirow and Nugent, 2001). The most severe damage is induced by alkylating agents that have been shown to cause destruction of oocytes, follicular depletion, and blood vessel damage leading to ovarian cortical fibrosis. Low-risk agents include antimetabolites and antibiotics. Medium-risk agents such as plant alkaloids, taxanes, and platinum agents have been shown to be toxic to germ cells (Meirow et al., 2010).

Despite an extended literature on clinical data regarding the long-term adverse effects of anticancer treatments, the understanding of the biological mechanisms responsible for gonadotoxicity still lag behind. Indeed, the cellular machinery of the ovarian follicle has an inherent high susceptibility to the apoptosis induced by many chemotherapeutic agents (Perez et al., 1997). Several signaling pathways have been shown to be involved in follicular development and ovulation. Among them are the transforming growth factor family, the protein kinase C (Varnold et al., 1990), and the mitogen-activated protein kinase/extracellular-signal-regulated pathways (Fan et al., 2009). Also, many components of the Wnt signaling pathway have been shown to play a role in different developmental processes, including cell differentiation, proliferation, and apoptosis, have been shown to be implicated in follicular development.
and ovulation (Hsieh et al., 2002). Wnts are evolutionarily conserved secreted proteins that couple to various receptors and thereby activate different downstream pathways known to contribute to ovarian steroidogenesis (Lapointe and Boerboom, 2011). Wnt-activated downstream signaling pathways are classified as either “canonical” (β-catenin dependent) or “noncanonical” (β-catenin independent). In the first case, in absence of Wnt stimulation, a destruction complex—containing the proteins adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), and axin—phosphorylates and targets β-catenin for ubiquitination and proteasomal degradation. In the absence of β-catenin, members of the TCF/LEF family of high-mobility-group transcription factors associate in a repressive complex with transducin-like enhancer (TLE) corepressor proteins, which promote the recruitment of histone deacetylases to repress β-catenin target genes. The binding of Wnts, such as Wnt3A and Wnt1, to frizzled and LRPS or LRPS coreceptors transduces a signal across the plasma membrane that results in the activation of the Dishevelled (DVL) protein. Activated DVL inhibits the destruction complex, resulting in the accumulation of β-catenin, which then enters the nucleus where it can act as a coactivator for TCF/LEF-mediated transcription. β-Catenin acts therefore as a transcriptional switch, as the presence of β-catenin reduces the association of TLE with TCF/LEF, while recruiting various transcriptional cofactors (Taurin et al., 2006).

Some Wnts, such as Wnt4, Wnt5A, and Wnt11, fail to stabilize β-catenin. Instead, they regulate signaling pathways that are associated with cell polarity and migration. In some contexts, β-catenin-independent Wnts regulate small GTPases, such as RhoA, Rap, and Cdc42, in a DVL-dependent manner. Wnt5A and Wnt11 can also induce a calcium flux, which results in the activation of various signaling pathways, such as protein kinase C (PKC), calcium/calcmodulin-dependent protein kinase II (CAMKII), and Jun N-terminal kinase (JNK). In addition, β-catenin-independent Wnts also regulate the planar cell polarity (PCP) signaling pathway that also requires the intact function of DVL and numerous other cytosolic factors.

This study was designed to identify the Wnt signaling pathways that are affected by in vitro treatment of human luteinized granulosa cells (LGCs) with medium-risk chemotherapeutic drugs. In particular, we focused on platinum agents (cisplatin, CP; carboplatin, CB), anthracycline antibiotics (doxorubicin, DXR; epirubicin, EP), and taxanes (paclitaxel, PC; docetaxel, PC) because of their frequent use in protocols of the most common female cancers, such as breast cancer.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Among platinum agents, CP (Sandoz Pharmaceuticals GmbH, Holzkirchen, Germany) and CB (Teva Pharmaceuticals Industries Ltd, Petah Tikva, Israel) were used. Anthracyclines employed were DXR (Adriablastina, Pfizer, New York) and EP (Farmorubicina, Pfizer, New York). Finally, two taxanes were used: PC (Teva Pharmaceuticals Industries Ltd) and docetaxel (Taxotere, Sanofi-Aventis, Paris, France). All drugs were diluted in RPMI plus 0.5% dimethyl sulfoxide (control medium).

**Human LGCs collection.** Human LGCs were obtained by ultrasound-guided follicle aspiration from women receiving assisted reproduction treatment at the Infertility Center of our institution. The cells were by-products of the in vitro fertilization procedure that are normally discarded. LGCs obtained from all the follicles obtained from the same woman were pooled. Women gave their written informed consent, and the institutional Ethical Committee approved the study protocol.

**LGCs’ isolation and culture.** Cells were prepared by initial centrifugation followed by layering onto a 40% Percoll gradient (Sigma). Following centrifugation, 3 layers could be distinguished: a top layer containing the follicular fluid, a bottom layer containing erythrocytes, and a middle ring-like layer containing the cells present in the follicular fluid. This middle layer was collected, washed by centrifugation for 5 min at 600 x g, and resuspended in RPMI 1640 medium. In order to deplete immune cells in the cell suspension, follicular fluid–derived cells obtained by the density gradient were collected from the interface and incubated in 10 ml medium at 37°C for 15 min. LGCs were recovered and incubated in a petri dish at 37°C for 24 h. Leukocytes adhered to the plastic whereas LGCs did not, thus allowing easy segregation of the 2 cell types (Ferrero et al., 2012).

**MTT cell viability assay.** Cells were plated at 15 × 10⁴ cells/well in a 96-well plate and cultured in RPMI 1640 medium supplemented with 1% penicillin G 100 UI/ml and 100 µg/ml streptomycin (Invitrogen, UK) plus 10% fetal calf serum. Chemotherapeutic drugs or control medium was added to LGCs. After 48 h, cells were treated with the Cell Titer AQueus (Promega, UK) and cultured for 3 h in a CO₂ incubator. Absorbance was measured with a microplate spectrophotometer reader at λ = 570 nm.

**Apoptosis assessment with annexin V staining.** LGCs were cultured in 35-mm plates as described above in the presence or absence of drugs. The cells were then washed with PBS and with annexin buffer and incubated in 0.5 ml annexin buffer with 4 µg/ml annexin V-APC for 15 min. Cells were then washed and resuspended in PBS and 0.6 µg/ml Propidium iodide (PI). Samples were analyzed by flow citometry by FACS Canto II flow cytomter (Beckton Dickinson, Mountain View, California). Apoptotic cells were annexin V positive, whereas necrotic cells were PI positive and annexin V negative.

**Real-time quantitative PCR.** mRNA was extracted with RNasey Micro Kit (Qiagen, UK), and mRNA was reverse transcribed using the High Capacity cDNA Reverse Transcript kit (Applied Biosciences). Real-Time PCR amplification was performed with SYBR Master Mix kit (Applied Biosciences) on a AB 7700 Fast Real-Time PCR System and SDS software version 1.4 (Applied Biosystems). The initial denaturation step was 95°C for 15 s followed by 40 cycles of amplification at 95°C for 10 s and 60°C for 30 s. Melting curves were evaluated for each gene. All analyses were performed in triplicates, and the relative amount of the target was normalized with the housekeeping gene 18S. Reaction conditions included 10 µl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl of primers and probes mixture, and 50 ng of template cDNA and nuclease-free water. The total reaction volume was 20 µl. The cycling conditions were as follows: 10 min at 95°C and 40 cycles of 15 s at 95°C followed by 1 min at 60°C. The data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The Δ cycle threshold (ΔCt) values from each sample were obtained by subtracting the values for the reference gene from the sample Ct. For each experimental sample, the ΔCt was calculated, and data have been graphically reported as Relative Expression. The sequences of the primers used in the real-time RT-PCRs are the following:

- **BAX:** FW5′ CC CGGAGAGGTCCTTTTTCGAG3′, RV5′ CCAGCAGCATGA TG TCTCCCTGAT3′, BCLXL: FW5′ CCCCGCTTCTCCGAAATG3′, RV5′ CTTCTCCCGACCTGTTGATA3′, WNT4: FW5′ CATGCAAAACAGACGTCCA AG3′, RV5′ AACGACCACAGTGGAATT3′; WNT1: FW5′ GGTGGGGTAT TGTGAACTAG3′, RV5′ CGTATCAGACGCCGCTGT3′, WNT3: FW5′ GAGAGGGACCCTGTCATC3′, RV5′ CTGGTGGCAAAAGGACCGCT3′; BMP4: FW5′ ATGATTCTGGTAAACGGAATGC3′, RV5′ CCCGGTGTCAG TATCAAACT3′; ERLBETA: FW5′ AGACGCGGCCTCCATATACAT3′, RV5′ TGGCTGTGATATCCATGGTCGC3′; FSHR: FW5′ CTGTGACTGCTTCAA
CHEMOTHERAPICS AFFECT Wnt SIGNALING IN LGCs

CAGGG3, RV5'TGCACCTTTTGTAGACTCG3'; 18S: FW5'AAACCGCTACCAACACCTAAG3, RV5'CGTCCCAAGATCCACTAC3'.

Protein extraction and Western blot. Cells were collected by gentle scraping in lysis buffer (20mM Hepes, pH 7.4, 1mM EDTA, 400mM NaCl, 25% glycerol, 0.1% NP40) containing protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride) and disrupted during 30 min at 4°C. The extracts were centrifuged at 8000 × g for 15 min, and the supernatants were collected. Before electrophoresis, the samples were boiled for 5 min at 100°C, the amounts of protein were determined, and 20 μg of protein/lane was applied. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis at 120V for 3–4 h. Proteins were transferred onto PVDF membranes (Bio-Rad, Hercules, California) by electroblotting in a buffer containing 25mM Tris, 192mM glycine, and 15% methanol. After protein transfer, the membranes were blocked for 1 h with 5% nonfat dry milk in PBS and incubated with anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, California), anti-phospho β-catenin (5525) (Cell Signaling Technologies, Danvers, Massachusetts), anti-β-catenin (Cell Signaling Technologies), anti-phospho-PAN-Akt (Cell Signaling Technologies), anti-total Akt (Cell Signaling Technologies), anti-phospho-ERK1/2 (Cell Signaling Technologies), anti-total ERK1/2 (Cell Signaling Technologies), and anti-phospho-GSK3β (59) (Cell Signaling Technologies) overnight at 4°C. Afterwards, the membranes were washed and then incubated for 1 h with a secondary horseradish peroxidase–conjugated antibody and developed with ECL chemiluminescent substrate (Millipore, Billerica).

17-β-Estradiol measurement. Samples were tested for estradiol level in a Tosoh AIA fluorometric system with ST-AIA-PACK immunassay (Tosoh Corporation), which has a sensitivity of 25 pg/ml. Besides the internal quality control checks performed daily by the institutional laboratory, the assays were calibrated whenever a new reactive batch was used or whenever an outcome outside the normal range was observed. Furthermore, external quality control assessment was performed bimonthly at the National Research Committee; no actions of correction had to be performed during the study period.

Statistical analysis. Data are presented as the mean ± SD of the results obtained in at least 3 independent experiments. Statistical comparisons among groups were made with a Student’s t test, and the difference was considered to be statistically significant when the p value was <.05.

RESULTS

Viability of LGCs Treated In Vitro With Chemotherapeutic Drugs

To evaluate the toxic effects of chemotherapeutic agents exposure on viability of LGCs, isolated human LGCs obtained from women undergoing follicle aspiration for oocyte retrieval were in vitro stimulated with the indicated escalating doses of the following chemotherapeutic agents: CP and CB, DXR and EP, and PC and docetaxel, belonging to the platinum derivatives, anthracyclins, and taxanes families, respectively. After 48-h incubation, cell viability was measured by MTT assay (Fig. 1). The results show that the 2 members of the platinum derivatives family CP and CB showed similar dose-response curves and both induced cell death in 20% of the treated cells at a concentration of 10μM. A more toxic effect was observed when LGCs were treated with either DXR or EP, both belonging to the anthracyclins family. For both drugs, cell viability was reduced to 50% at the concentration of 10μM. A similar reduction of cell viability was also detected with the 2 members of the taxane family; however, 10μM PC was slightly more toxic than 10μM docetaxel inducing a 40% vs 50% reduction of cell.

Because drugs belonging to the same family showed a similar effect on LGCs viability, 1 representative agent per class (DXR, PC, and CP) was selected for further experiments.

Chemotherapeutic Drugs Promote Apoptosis in LGCs In Vitro

To examine the gene expression of apoptosis-related mediators in LGCs treated for 24h with DXR, PC, and CP, RT-PCR analysis of the mitochondrion outer membrane proteins Bcl-xL and Bax was undertaken. The expression of Bcl-xL was significantly reduced in LGCs treated with the 3 agents at equitoxic dose (60μM CP: p < .05; 10μM DXR: p < .01; 10μM PC: p < .01), whereas the expression of Bax was significantly increased (60μM CP: p < .01; 10μM DXR: p < .05; 10μM PC: p < .01) (Fig. 2A).

We then studied the mechanism underlying apoptosis induction by DXR, PC, and CP. After 24-h treatment with 10μM DXR or 60μM CP, we observed an increase of both apoptosis and necrosis, whereas treatment with 10μM PC induced a predominant increase of necrosis compared with apoptosis (Fig. 2B). The apoptotic cell death induction was confirmed by Western blot detection of the cleaved form of caspase-3, an event that is commonly used as an apoptotic hallmark (Fig. 2C).

Chemotherapeutic Drugs Induce a Dysregulated Expression of Follicle-Stimulating Hormone and Estrogen Receptor in LGCs

Estrogen plays a pivotal role as an intrafollicular modulator, stimulating granulosa cell proliferation and facilitating the differentiative actions of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) on these cells. In response to FSH, LGCs produce estrogens, mainly 17-β-estradiol (E2), that preferentially binds to estrogen receptor β (ERβ) stimulating follicle development in an autocrine fashion. Hence, we analyzed the expression of ERβ and FSH receptors on LGCs treated with different doses of chemotherapeutic drugs. We found that all drugs induced a dose-dependent downregulation of both ERβ and FSHR transcripts. At equitoxic doses, DXR, PC, and CP induced a significant reduction of both ERβ and FSHR expression as indicated by the percentage of transcript expression compared with control (10μM DXR: ERβ, 59.26±±4.94 and FSHR, 30.48±±19.28; 10μM PC: ERβ, 58.38±±17.6 and FSHR, 62.77±±16.89; 60μM CP: ERβ, 10.19±±1.58 and FSHR, 17.4±±16.85) (Figs. 3A and B).

Interestingly, the amount of E2 in the LGC culture supernatant was significantly decreased upon DXR and CP treatment, whereas no significant changes in E2 levels were observed upon PC treatment (Fig. 3C).

Chemotherapeutic Drugs Induced a Differential Expression of WNT Family Members in LGCs

Many components of the Wnt signaling pathway are known to play a role in follicular development and oocyte maturation (Hsieh et al., 2002). Although several Wnt family members preferentially activate either β-catenin-dependent or β-catenin-independent pathways, they cannot be rigorously subdivided.
according to the pathway they induce (Niehrs, 2012). Because Wnt1 and Wnt3 are commonly encountered in β-catenin-dependent signaling, and Wnt4 is predominantly involved in β-catenin-independent signaling, we analyzed their gene expression in LGCs treated with DXR, PC, and CP. We found that DXR, PC, and CP significantly upregulated WNT4 expression in a dose-dependent manner, whereas WNT3 expression was significantly upregulated by CP and downregulated by DXR and PC (Figs. 4A and B). No changes were observed in WNT1 gene expression (data not shown).

Chemotherapeutic Drugs Induce a Dysregulation of Signaling Pathways in LGCs

Sustained Erk1/2 signaling is essential for oocyte maturation and cumulus cell-oocyte complex (Fan et al., 2009), whereas the phosphatidylinositol 3-kinase (PI3k)/Akt signaling
pathway serves to transduce the biological signals of key hormones, including FSH (Hunzicker-Dunn and Maizels, 2006). Levels of both Erk1/2 and Akt phosphorylated proteins were reduced by DXR, PC, and CP, further emphasizing their toxic effect on LGCs differentiation and proliferation. Both DXR and PC at equitoxic doses strongly reduced phosphorylated Ser552 β-catenin, and this correlated with reduced total β-catenin protein levels. On the contrary, CP did not affect the levels of total β-catenin and its phosphorylated form (Fig. 5A).

One of the known β-catenin target genes is bone morphogenetic protein-4 (BMP4) (Kim et al., 2002). The BMP family of growth factors is involved in the folliculogenesis where they inhibit granulosa cell apoptosis via different pathways (Shimizu et al., 2012).

In agreement with the observed downregulation of β-catenin, our results showed that DXR and PC but not CP reduced BMP4 gene expression in LGCs in a dose-dependent manner (Fig. 5B).

It is well known that lithium chloride (LiCl) dose dependently inhibits GSK3β, stabilizes free β-catenin, and inhibits β-catenin degradation (Rao et al., 2005). In order to address the role of β-catenin in the survival of the LGCs upon chemotherapeutic drug treatment, we performed a MTT analysis of DXR- and PC-treated LGCs in the presence or absence of 20mM LiCl. We observed
that LiCl restored β-catenin pathway activation and GSK3β phosphorylation in LGCs treated with DXR and PC, leading to a statistically significant increase of cell viability (Fig. 5C).

**DISCUSSION**

Chemotherapy can result in subfertility or infertility due to a reduction in primordial follicle stockpiles, diminished ovarian weight, and ovarian atrophy. However, it is not easy to draw clear conclusions regarding the gonadotoxic potential of various anticancer treatments. The effects of chemotherapy on fertility depend on the patient’s age, chemotherapeutic regimens, dose and duration, and pretreatment fertility status of the patient.

Chemotherapeutic agents have been reported to damage the ovary via multiple mechanisms in a cell context manner. DXR can cause double-strand DNA breaks in a topoisomerase II–dependent manner or induce oxidative stress depending on the cell type and drug dose. The cellular response to the DXR insult is also cell type and dose dependent. It has been demonstrated that within the acute phase of DXR insult, granulosa cells of the secondary and antral follicles are the predominant sites of

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**FIG. 3.** Treatment with chemotherapeutic drugs reduced ERβ and FSH receptor gene expression and 17-β-estradiol secretion by human LGCs. Cells were treated with different doses of DXR, PC, and CP for 48h. ERβ (A) and FSHR (B) mRNA levels were normalized relative to 18S mRNA and expressed as percent of mRNA expression in drug-treated versus control untreated cells. Data are presented as mean ± SD and represent a minimum of 3 independent experiments. *p < .05. **p < .01. C) LGCs were treated with 10µM DXR, 10µM PC, and 60µM CP. Supernatants were collected after 48h, and 17-β-estradiol levels were measured by ELISA. Levels of 17-β-estradiol (pg/mg) were normalized to total protein concentration. Data are presented as mean ± SD of a minimum of 3 independent experiments. Abbreviations: DXR, doxorubicin; PC, paclitaxel; CP, cisplatin; LGCs, luteinized granulosa cells; n.a., sample not available; FSHR, follicle-stimulating hormone receptor; ERβ, estrogen receptor β.
induced apoptosis (Ben-Aharon et al., 2010; Roti Roti et al., 2012). PC primary effect in granulosa cells is to cause abnormal stabilization of the dynamic microtubule polymerization, leading to the failure of mitosis (Herman et al., 1983). In addition, PC, in combination with perifosine, leads to inhibition of Akt, induction of early reactive oxygen species production, and apoptosis in ovarian cancer cells (Sun et al., 2011). CP binds with DNA to form intrastrand crosslinks and adducts that affect DNA replication, induce mitochondrial damage, decreased ATPase activity, altered cellular transport mechanisms, and cell cycle arrest in the G2 phase leading to apoptosis (Galluzzi et al., 2012). Our results show that DXR, PC, and CP affect LGCs viability in a dose-dependent manner by increasing expression of Bax that permeabilizes the mitochondrial outer membrane to promote apoptosis and reduces levels of the prosurvival Bcl-xl, therefore inhibiting retrotraslocation of Bax into the cytoplasm (Edlich et al., 2011).

A variable proportion of LGCs (20%–50%) survived the chemotherapy damaging insult. However, these cells were likely to be severely affected in their ability to respond to hormonal stimulation, as demonstrated by downregulation of ERβ and FSH receptors by CP, DXR, and PC. LGCs produce estrogens, principally E2 from androgens via aromatization, in response to FSH. ERβ, expressed predominantly by the ovarian LGCs, is required for antrum formation, preovulatory follicle growth, oocyte maturation, expression of genes involved in ovarian differentiation, and follicle rupture during ovulation (Richards et al., 1980). It is at the antral stage that the quota of dominant follicle(s) is selected from the cohort of growing follicles and become critically dependent upon FSH for continued growth and survival. Any follicle(s) that is exposed to factors that augment LGCs responsiveness to FSH has a greater propensity than cohort follicles to achieve dominance. Hence, downregulation of ERβ and FSHR expression on LGCs upon chemotherapeutic treatment represents critical factors impeding the successful development of an ovulatory follicle.

FIG. 4. Deregulation of WNT family member gene expression after treatment with DXR, PC, and CP. LGCs were treated with DXR, PC, and CP at different doses or with control medium for 48h. WNT4 (A) and WNT3 (B) transcript levels were normalized relative to 18S mRNA and expressed as percent of mRNA expression in drug-treated versus control untreated cells. Data are presented as mean ± SD and represent a minimum of 3 independent experiments. *p < .05, **p < .01. Abbreviations: DXR, doxorubicin; PC, paclitaxel; CP, cisplatin; LGCs, luteinized granulosa cells; n.a., sample not available.
Gonadotropin receptors signaling pathways interact with the Wnt signaling pathways in female fertility in a cell context-dependent and developmental stage-specific manner. Several Wnt signaling pathway components are expressed in mouse granulosa cells, where they have been shown to direct female gonadal development, and play a potential role in follicle formation, growth,
and ovulation/luteinization (Boyer et al., 2010). FSH and Wnt signaling pathways can cooperate to activate cytosolic β-catenin in mouse granulosa cells to enhance FSH action and promote preovulatory follicular growth and survival (Fan et al., 2010).

Multiple extracellular, cytoplasmic, and nuclear regulators intricately modulate Wnt signaling. At least 2 distinct pathways transduce Wnt signals: the well-established β-catenin-dependent pathway and the β-catenin-independent pathway. Our results show that CP preferentially upregulated the expression of WNT3, a common activator of the β-catenin-dependent pathway, whereas DXR and PC preferentially upregulated expression of WNT4 that is predominantly involved in the β-catenin-independent pathway. In cancer cells, the activation of the β-catenin-independent pathway can antagonize the β-catenin-dependent signaling pathway by promoting β-catenin degradation in a GSK3β-independent manner (Yuan et al., 2011). Similarly, the observed upregulation of WNT4 expression and downregulation of WNT3 induced by DXR and PC on LGCs suggest that DXR and PC might induce cell death by overactivation of the β-catenin-independent pathway that blocks the β-catenin-dependent signaling pathway, leading to degradation of β-catenin and/or translocation of cytosolic β-catenin to the cell surface. This hypothesis deserves further investigation.

As a result of the downregulation of the Wnt3 signaling pathway, both DXR and PC reduced total β-catenin levels and phosphorylation of β-catenin at Ser552 leading to inhibition of β-catenin accumulation in the nucleus. Indeed, we found that LiCl that blocks β-catenin proteosomal degradation by inhibiting the activity of GSK3β partly restores LGCs viability after treatment with DXR and CP.

Akt downregulation by DXR and PC could also contribute to follicular atresia by reducing β-catenin phosphorylation at Ser552. β-Catenin-dependent FSH gene transcription is indeed essential for follicular growth as it has been demonstrated in a mouse model in which β-catenin was constitutively activated in granulosa cells (Fan et al., 2010).

CP seems to induce LGC damage via a different mechanism compared with DXR or PC. In fact, we observed upregulation of both WNT3 and WNT4 transcripts and no changes in total β-catenin protein levels. CP toxic effect seems to be independent of the β-catenin pathway and could be mediated at least in part by inhibition of the Akt and Erk1/2 pathways whose sustained signaling in granulosa cells is essential for oocyte maturation (Fan et al., 2009). These results therefore suggest that targeting of the β-catenin-dependent pathway might not be useful in preventing LGCs death upon CP treatment.

Our data provide the basis for further investigations of novel targets for fertility preservation purposes. Indeed, an optimal intervention for the preservation of fertility in young women with cancer is not available. Temporary ovarian suppression using GnRH agonists during adjuvant chemotherapy has been proposed to prevent premature ovarian failure, but its efficacy in preserving ovarian function is doubted (Munster et al., 2012). Alternative options to protect fertility in women preparing to undergo chemotherapy include embryo or oocyte cryopreservation, for which a woman must undergo ovarian hyperstimulation for oocyte harvest. However, the time required for ovarian hyperstimulation renders this option not applicable to all patients.

The broad involvement of Wnt signaling in diseases had driven extensive research efforts aimed at targeting the Wnt pathway with small molecules. Inhibitors of Wnt/β-catenin signaling are under development for the treatment of cancers (Anastas and Moon, 2013). In the case of fertility preservation, however, a strong concern is represented by the possibility that a dysregulation of the Wnt/β-catenin pathway could lead to ovarian granulosa tumors, as it has been reported in a mouse strain in which β-catenin was constitutively activated in granulosa cells (Boerboom et al., 2005). The dysregulation of the Wnt signaling pathway in granulosa cells contributes to ovarian depletion as part of a series of multiple events including ovarian stroma demise and remodeling, increased recruitment of primordial follicles following burn out of growing follicles, and a delayed damage response in the primordial follicles (Meirow et al., 2010; Roti et al., 2012). Further research in mouse models is needed to expand our understanding of the involvement of the Wnt signaling pathway in the chemotherapy-induced damage in the pregranulosa cells of the dormant primordial follicle pool.

In conclusion, we suggest that the in vitro LGC toxicity model could be suitable for a rapid and easy screening of the gonadotoxicity of both current chemotherapeutic regimens and novel agents. This information, together with the pretreatment fertility status and other patient-based factors, might help to gauge the posttherapy reproductive window. Moreover, the model allows the identification of the triggered molecular pathways, such as the Wnt signaling pathway, and of compounds able to interfere with such pathways, thus preventing follicular damage induced by chemotherapy.

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