Progesterone Prevents High-Grade Serous Ovarian Cancer by Inducing Necroptosis of p53-Defective Fallopian Tube Epithelial Cells

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In Brief
Ovarian high-grade serous carcinomas (HGSOC) originate mainly from the fallopian tube epithelium and exclusively carry early TP53 mutations. Wu et al. find that progesterone, acting via its receptor, induces necroptosis of p53-deficient tubal epithelial cells. Supplementation of progesterone in diestrus further augments this clearance, suggesting that progesterone may be chemopreventive for HGSOC.

Highlights
- Progesterone (P4) induces necroptosis of the oviductal epithelium of Trp53-null mice
- Necroptosis occurs via the P4 receptor (PR) and the TNF-α/RIPK1/RIPK3/MLKL pathway
- P4 supplementation at diestrus further augments this clearance effect
- Progesterone may be chemopreventative for HGSOC
Progesterone Prevents High-Grade Serous Ovarian Cancer by Inducing Necroptosis of p53-Defective Fallopian Tube Epithelial Cells

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SUMMARY
High-grade serous ovarian carcinoma (HGSOC) originates mainly from the fallopian tube (FT) epithelium and always carries early TP53 mutations. We previously reported that tumors initiate in the FT fimbria epithelium because of apoptotic failure and the expansion of cells with DNA double-strand breaks (DSB) caused by bathing of the FT epithelial cells in reactive oxygen species (ROSs) and hemoglobin-rich follicular fluid (FF) after ovulation. Because ovulation is frequent and HGSOC is rare, we hypothesized that luteal-phase progesterone (P4) could eliminate p53-defective FT cells. Here we show that P4, via P4 receptors (PRs), induces necroptosis in Trp53−/− mouse oviduct epithelium and in immortalized human p53-defective fimbrial epithelium through the TNF-α/RIPK1/RIPK3/MLKL pathway. Necroptosis occurs specifically at diestrus, recovers at the proestrus phase of the estrus cycle, and can be augmented with P4 supplementation. These results reveal the mechanism of the well-known ability of progesterone to prevent ovarian cancer.

INTRODUCTION
Epithelial ovarian cancer (EOC) is one of the most devastating cancers prevalent in women worldwide. High-grade serous ovarian cancer (HGSOC) accounts for the majority of EOCs and the related deaths (Siegel et al., 2013). The clinical outcomes of HGSOC have not changed significantly over the past three decades, mainly because of a lack of knowledge about its etiology and natural history. Given the lack of an effective means for the early detection of EOC, generating effective prevention options is vital.

The model of the fallopian tube (FT) origin of HGSOC (Piek et al., 2001) has gained wide acceptance (Medeiros et al., 2006). Loss of p53 functioning seems to be a necessary and early event in the malignant transformation of tubal epithelium, with the TP53 mutation being identified in virtually all HGSOCs (Ahmed et al., 2010) and in the tubal precursor of serous tubal intraepithelial carcinomas (STICs) (Kuhn et al., 2012).

The maturing follicular fluid (FF) generates reactive oxygen species (ROSs) that are indispensable for ovulation (Shkolnik et al., 2011) and that may act upon the approaching FT fimbria during oocyte pick-up. As found in our previous studies, a subset of preovulatory FF containing high levels of ROS could induce DNA double-strand breaks (DSBs) in cultured human fimbrial epithelial cells and fimbria tissue (Huang et al., 2015). With defective p53, these cells are more resistant to the stress but are still apoptotic upon exposure to FF ROSs. However, the presence of hemoglobin, which is most likely contributed by retrograde menstruations of previous menstrual cycles, rescues the bathed fimbrial epithelial cells from apoptosis and allows the expansion of cell populations carrying DSBs (Huang et al., 2016). Considering that both ovulation and retrograde menstruation are frequent events and HGSOC is rare, an efficient mechanism that prevents the progression of cell transformation must exist.

Many large-scale epidemiological studies and meta-analyses have revealed that oral contraceptive (OC) use can reduce ovarian cancer risk by inhibiting ovulation in a dose-dependent manner (Havrilesky et al., 2013). Lurie et al. (2008) noted that each year of OC use provided a 5% reduction in the risk of EOC. However, the extent of ovarian cancer protection by OC cannot be fully explained by only examining ovulation inhibition when calculating the duration of anovulation (Siskind et al., 2000). A protection effect can also be observed in short-term users (Lurie et al., 2008), who exhibited a greater effect than
predicted according to ovulation inhibition. Clearly, the mechanism of the EOC risk-protection effect of OC use is beyond ovulation prevention.

Progesterone (P4) is one of the two hormone components of OC. Unlike the trend of decreasing estrogen (E2) doses in new OC formulas, the dose of P4 has not changed for decades. However, the EOC protection effect of OCs with different E2 doses has remained steady (Beral et al., 2008). This suggests that the P4 component in OC is responsible for the additional protection activity. We thus hypothesized that P4 has a chemopreventive effect on the initial p53-defective tubal epithelial cells. Here we provide in vitro and in vivo evidence supporting this hypothesis.

RESULTS

Through Progesterone Receptors, P4 Induces Cytolysis and Cell Flaking in the Oviduct Epithelium of Trp53−/− Mice during Diestrus

To investigate the effect of P4 on fimbrial epithelial cells in the menstrual cycle, Trp53−/− or wild-type mice were superovulated with or without P4 supplementation and progesterone receptor (PR) inhibition using mifepristone (RU486; Figure 1A). The oviduct epithelium of Trp53−/− mice sacrificed at diestrus exhibited features of cytolysis and cell flaking with a loss of cytoplasm and increase of bare nuclei (Figure 1B, Trp53−/−/Diestrus). These changes were exacerbated when P4 was supplemented at diestrus (Figure 1B, Trp53−/−/P4/Diestrus) but completely disappeared when the PR inhibitor RU486 was administered before the P4 supplement (Figure 1B, Trp53−/−/P4+RU/Diestrus). When the same treatment of superovulation and P4 supplement was administered to wild-type mice, the oviduct epithelium showed milder changes (Figure 1B, WT/P4/Diestrus). These changes were not observed in oviducts collected at proestrus and estrus, indicating efficient regeneration.

To confirm this result of P4-induced cell flaking, the oviduct epithelium was examined through transmission electron microscopy (TEM). The diestrus P4-treated wild-type oviduct epithelium showed a marked increase in secretory vacuoles and exocytosis (Figure 1C, WT/P4/Diestrus), indicating apocrine secretion (Gesse and Satoh, 2003). In Trp53−/− mice, cell necrosis with cytolysis, bared nuclei, and spilled-out cellular contents were noted in the diestrus oviduct (Figure 1D, Trp53−/−/P4/Diestrus), including a complete absence of cellular structures and membrane lysis. A complete reversal of these phenotypes by RU486 was also confirmed (Figure 1D, Trp53−/−/P4+RU/Diestrus).
P4 Induces Cytolysis of FT Epithelial Cells in a Non-apoptotic Manner

We used a p53-defective human FT fimbrial epithelial cell line (FE25 cells) (Huang et al., 2015) to investigate the P4-induced cytotoxic mechanism. Similar to most steroid hormone receptor-expressing tissues that lose expression after long term in vitro culturing (Yang et al., 2014), FE25 cells do not express PR-A and express low levels of PR-B (Figure 2A) but remain dose-dependently vulnerable to P4, with maximal killing at 100 μM (Figure 2B; Figure S2). Upon transfection with the PR-A gene, we observed a significant increase in P4-induced cytotoxicity that was completely blocked by RU486 (Figures 2A–2C). The same P4-induced cytotoxicity was observed in a p53-defective HGSOC cell line, PEO4, after PR expression was induced by E2 (Figures 2A and 2C). In contrast, TP53-wild-type cells (Pri epi) cultured from human fimbriae were resistant to P4 (Figure 2D). The p53 defect-specific cytotoxicity was verified in two additional human fimbrial epithelial cell lines in which TP53 was dominantly mutated (FT282-V) or suppressed by an SV40 TAg (FT194) (Karst et al., 2014; Sherman et al., 2016). They were as vulnerable as FE25 cells (Figure 2D). Flow cytometry revealed a pattern of necrotic cell death with a simultaneous increase in propidium iodide (PI)-positive cells (Figure 2E) and cell debris (Figure 2F).

P4 Induced Necroptosis of FE25 Cells

The P4-treated FE25 cells exhibited a “ghost cell” appearance with a “shadow” of an empty cell without any structure (Figure 3A). In contrast, similarly treated primary fimbrial epithelial cells did not show any change (Figure 3A). TEM and scanning electron microscopy revealed typical necrotic changes with cytoplasmic swelling, scattered cell fragmentation and debris, and disruptions of plasma membranes (Figures 3B and 3C). As a consequence of cell membrane disruption, lactate dehydrogenase (LDH) was released into the culture medium (Figure 3D). Expression of the late necroptosis marker HMGB1 was also observed (Figure 3E). This marker is specific to primary necroptosis and does not appear during secondary necrosis after apoptosis or partial autolysis (Scaffidi et al., 2002). In contrast, the apoptotic marker caspase 3 was not increased after P4 treatment (Figure 3E).

P4/PR Induces Necroptosis via the TNF-α/RIPK1/RIPK3/MLKL Pathway, and Its Inhibition Results in DSB in Trp53−/− Oviducts

To evaluate the P4-mediated signaling pathway of necroptosis, we analyzed the cytokine profile in the conditioned medium of FE25 cells after P4 treatment. Fourteen cytokines were upregulated, among which tumor necrosis factor α (TNF-α) was predominant (Figure 4A). The mRNA of TNF-α increased soon after P4 treatment (Figure 4B). TNF-α binds to its receptor to activate receptor-interacting protein kinase (RIPK) 1, which binds with and activates RIPK3 to form the necrosome that subsequently activates MLKL to induce necrotic death through the induction of mitochondrial dysfunction and production of ROSs (Vanden Berghe et al., 2014). This necrotic pathway was observed in P4-treated FE25 cells. P4-induced cell death was largely prevented by the RIPK1 inhibitor NEC-1 and the MLKL blocker necrosulfonamide (NSA) (Figure 4C) but not by the caspase inhibitor Z-VAL-FMK (Figure 4C). After P4 treatment, the components of the necrosonome, including RIPK1, RIPK3, and MLKL, were all induced or activated (Figure 4D). In addition, cellular ROSs increased significantly (Figure 4E), and cell viability was completely rescued by the antioxidant Necrox-2 and acetylcysteine (NAC) (Figure 4F).

Furthermore, we confirmed that this P4-induced necroptosis occurs in p53-defective oviduct epithelium in vivo. In the oviducts of the supereovulated Trp53−/− mice, but not wild-type mice, the accumulation of RIPK1 was observed in epithelial cells in the diestrus phase (Figure 4G, Trp53−/−/Diestrus). The same P4 supplementation enhanced this expression (Figure 4G, Trp53−/−/P4/Diestrus), and the same RU486 pretreatment completely blocked accumulation (Figure 4G, Trp53−/−/P4+RU486/Diestrus; Figure S3). In contrast, the induction was not or only barely observed in similarly treated Trp53−/− mice in the estrus or proestrus phase.

Given the findings that ovulation is mutagenic to the oviduct epithelium and P4 signaling is protective, we explored whether long-term superovulation and PR inhibition would induce the transformation of Trp53−/− mouse oviducts. Specifically, Trp53−/− mice were put into 4-day ovulation induction and RU486 treatment cycles for 3 months (Figure S4). DSB accumulation in the diestrus oviduct epithelium was observed in RU486-treated mice (Figure 4H, Trp53−/−/RU486/Diestrus) but not in mice treated with the vehicle control (Figure 4H). However, no pathological lesion was observed.

DISCUSSION

The present study identifies P4 and PRs as natural eradicators of p53-defective FT epithelium, which represents the earliest lesion of HGSOC. Given the repeated exposure of the FT epithelium to ovulatory carcinogens (Huang et al., 2016), this could be the most efficient way to eliminate epithelial cells that initiate transformation. This explains the high frequency (33%) of p53 signature lesions in FT epithelium (Xian et al., 2010) compared with the rare occurrence of locally transformed STIC and HGSOC. Notably, we observed a rapid recovery of the necrotic epithelium after P4 clearance. Within 24 hr (or before entering the proestrus...
Figure 3. Necroptosis Induction of Immortalized p53-Defective Fimbrial Epithelial Cells by P4

(A) FE25 cells and primary fimbria epithelial cells (Pri) were treated with 100 μM P4 or vehicle for 24 hr and viewed under a microscope. A ghost cell appearance with cell swelling, membrane lysis, and cell fragmentation was noted in the FE25 cells.

(B and C) TEM (B) and scanning electron microscopy (C) analyses of FE25 cells treated with P4 (100 μM, 6 h), showing the swelling and disruption of plasma membranes with membrane disruption and lysed cell debris.

(D) LDH levels were measured in a culture medium of FE25 cells at 24 hr after 100 μM P4 treatment. Data are represented as mean ± SD. *p < 0.05 for P4-treated versus vehicle-treated cells.

(E) Western blot analysis of the necroptosis marker HMGB1 and the apoptotic markers caspase 3 and cleaved caspase 3. All results are from three independent experiments.
(legend on next page)
phase of the mouse estrus cycle), the epithelium was re-established. This is compatible with an efficient regeneration system in FT fimbria to repair tissue damage after each ovulation (Ng et al., 2014).

The effective dose of P4 to induce cell death in FE25 cells ranges from 10–100 μM, which is substantially higher than the physiological range of 10–100 nM that clears the oviduct epithelium in Trp53+/− mice. A possible reason for cultured fimbrial epithelial cells to be less vulnerable to P4 is the epigenetic silencing of estrogen receptors (ERs) and PRs after in vitro culturing (Yang et al., 2014). FE25 cells express only limited PR-B and no PR-A, which contrasts with their abundant expression in both the tubal epithelium and stroma (Tone et al., 2011). When FE25 cells were transfected with PR-A or when PRs were induced in PEO4 cells, sensitivity to P4 increased. Another possibility is that P4 also acts through the stroma to affect the survival of the epithelium, similar to the role of stromal ERs/PRs in the developmental and physiological functions of the Mullerian epithelium (Kurita et al., 2001). Moreover, P4 levels in luteinized preovulatory follicles can be thousands of times higher than those in the serum of the luteal phase (Wen et al., 2010) and may well be adequate at eradicating p53-defective lesions when exposed to the FT fimbria after ovulation. Furthermore, serum P4 levels are four times higher during pregnancy than during the luteal phase. This explains the physiological finding of higher protection against EOC by pregnancy than by OC use (Gwinn et al., 1990).

In the context of p53 loss, P4-induced cell death was determined to be necroptosis but not apoptosis. Various sources of cell necroptosis induction (Vanden Berghe et al., 2014) exist. The present study is the first to report that P4 could induce cell necroptosis through the TNF-α-RIPK1/RIPK3/MLKL pathway. P4 induces TNF-α directly and sustainably in the epithelium and stroma of the mouse uterus (Roby and Hunt, 1994), where the TNFR-I and TNFR-II receptors are also expressed specifically at diestrus (Roby et al., 1996). In the oviduct of p53-null mice but not wild-type mice, RIPK1 was readily induced specifically at diestrus. The accumulation of other components of the necrosome complex, such as RIPK3 and phosphorylated MLKL, was also noted.

This study also indicates that the physiological clearance of p53-defective lesions is through PRs. In both in vitro and in vivo experiments, pretreatment with a PR antagonist readily attenuated the signaling pathway and phenotypes of P4-induced necroptosis. PRs are largely downregulated or non-expressed in EOCs (Lau et al., 1999; Tone et al., 2011). In addition, two polymorphisms of PGR were found to contribute to ovarian cancer susceptibility in a meta-analysis (Liao et al., 2015). Accordingly, PRs have a crucial role in protecting against the occurrence of ovarian cancer, and their downregulation is a prerequisite for carcinogenesis.

The long-term consequences of the loss of P4/PR protection were investigated in a repeated superovulation and PR inhibition Trp53−/− mouse model. The accumulation of DSB, but not more severe lesions, was observed in the oviduct epithelium. Therefore, only when P4/PR function is compromised do the tubal epithelial cells have a chance to accumulate DSBs after ovari- tory ROS stress. However, this is not sufficient for the transformation of the oviduct epithelium. Protective mechanisms, such as the efficient DSB repair system, have to be overcome for cell transformation.

The finding that P4 supplements could enhance the eradication of p53-defective tubal epithelium suggests that P4 has a therapeutic effect on HGSOC precursors in the FT. Epidemiological studies have revealed a high efficacy of P4 use for preventing ovarian cancer in long-term or short-term settings (Lurie et al., 2008). Three-year regular use of medroxyprogesterone acetate reduced the ovarian cancer risk by 83% (Wiliaik et al., 2012). The physiological state of high P4 levels in full-term pregnancy is also protective (Gwinn et al., 1990). Our results thus invite clinical trials of periodic, short-term use of P4 as chemoprevention for genetically predisposed women.

**EXPERIMENTAL PROCEDURES**

**Supervolubulation and Treatments of Mice**

The Trp53−/− mice (STOCK Trp53tm1Brd Brca1tm1Aash/J) were purchased from Jackson Laboratory (stock no. 012620). They had a conventional knockout of Trp53 and a conditional floxed Brca1, with no phenotype in the absence of Cre. C57BL/6J mice (the background strain of Trp53−/− transgenic mice) were purchased from the Taiwan National Laboratory Animal Center and served as the Trp53 wild-type control. The superovulation protocol of Fowler and Edwards (1957) was adopted for the 6- to 8-week-old female mice (Figure 1A). Mouse experiments were approved by the Animal Care and Use Committee of Tzu Chi University.

**Primary Culture and Cell Immortalization**

The collection of clinical specimens for this study was approved by the Institutional Review Board of Tzu Chi General Hospital (TCGH-IRB #93-025). The culturing method for the primary fimbrial epithelial cells and the establishment of the human papillomavirus (HPV) E6-E7/hTERT-immortalized human FT fimbria secretory cells (FE25) were described previously (Huang et al., 2015). FT282-V and FT194 are human FT cell lines immortalized by human telomerase reverse transcriptase (hTERT) + mutant TP53 (R175H) and...
Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.049.

AUTHOR CONTRIBUTIONS
Conceptualization, H.H.Z., H.S.H., and T.Y.C.; Methodology, N.Y.W. and H.S.H.; Investigation, N.Y.W., T.H.C., C.F., Y.L., and C.Z.Q.; Writing – Original Draft, N.Y.W., H.S.H., and T.Y.C.; Funding Acquisition & Resources, T.Y.C. and H.H.Z.; Supervision, T.Y.C. and H.H.Z.

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Supplemental Information

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Figure S1. Related to Figure 1
TEM morphology of the normal appearing oviduct epithelial cells of p53-null mice during estrus.
Figure S2. P4 dose-dependently reduces cell viability of FE25, Related to Figure 2

Cell viability was determined using a dye exclusion assay with flow cytometry in FE25 cells treated with different concentrations of P4 for 24 h. The normalized cell viabilities of three independent experiments are represented as the mean ± SD. *P < 0.05.
Figure S3. Related to Figure 4

Semi-quantitative measurement of RIPK1 protein in the oviduct of wild-type (WT) or Trp53−/− superovulated mice treated with or without P4 or P4+RU486 cotreatment, following the protocol showed in Figure 1A. Data are represented as mean ± SD.
Figure S4. Related to Figure 4

The protocol of long-term superovulation with RU486 or vehicle treatment with predicted estrous cycle of diestrus-I (DIE-I), diestrus-II (DIE-II), proestrus (ProE) and estrus (E) phases and accompanied levels of luteinizing hormone and progesterone. Trp53-null mice were superovulated with (5 i.u.) PMSG and (5 i.u.) hCG in a 4-day cycle for 3 months. RU486 (10 mg/kg) or vehicle was given at 24, 48, and 72 h after each HCG injection.
Supplementary Experimental Procedures

Cell viability and inhibitor treatment
Cell proliferation, apoptosis and cell cycle assay were performed as described previously (Huang et al., 2015). Caspase inhibitor Z-VAD-FMK (Selleck), RIPK1 inhibitor necrostatin1 (Nec1) (Selleck), MLKL inhibitor Necrosulfonamide (NSA) (Abcam), antioxidant Necrox-2 (Nec-2) (Enzo Life), Acetylcysteine (NAC) (Selleck), and RU486 (Abcam) were pretreatment 30 min of P4.

Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM)
FE25 cells (after 6 h of 100 μM P4 or vehicle treatment) and FT tissues were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.2) at 4 °C for more than 2 h, washed three times with 0.1 M phosphate, and then fixed in 1% osmium tetroxide for 1 h and washed three more times with phosphate. Cells or tissues were dehydrated in gradient acetone concentrations then soaked in pure acetone with an embedded solution (1:1) at 37 °C for 12 h and embedded in epon 812 resin. Semithin sections were stained with 3% uranyl acetate and lead nitrate. The sections were examined using FEI Tecnai G2 Spirit.
FE25 cells cultured in chamber slides were treated with P4 or vehicle for 6 h, fixed with 4% glutaraldehyde for 24 h, and then further fixed with 1% osmium tetroxide for 2 h. Subsequently, the samples were dehydrated using acetone with the gradient concentration and then in isoamyl acetate. The samples were dried using critical point drying and liquid carbon dioxide, and were coated using an EiKO IB-5-type coater. They were then analyzed using an S-3400N scanning electron microscope.

Hematoxylin-eosin staining, Immunohistochemistry and Immunofluorescence assays
HE staining, DNA DSB assay was performed as in a previous study (Huang et al., 2015). For immunohistochemistry and immunofluorescence, the slides were treated with the first antibody of anti-phospho-γH2AX (Millipore, 1:200 dilution), anti-BRCA1(Abcam,1:200 dilution) anti-RIPK1 (Abcam,1:200 dilution) at room temperature for 1 h after hydrate and block, and then incubate with second antibody 10 min and examined using a light microscope or fluorescence microscope.

Transfection of PGR isoform A
FE25 cells were transfected with 20 μg of PGR isoform A expression vectors (Vigene biosciences, NM-000926) by using ViaFect Transfection Reagent (Promega Corporation).
**Western blotting**
Crude cellular protein was loaded on SDS-PAGE for protein separation and subsequently transferred to nitrocellulose membranes. After incubation in blocking solution (5% nonfat milk), the membranes were probed using primary antibodies against β-actin (Santa Cruz), HMGB1 (GeneTex), RIPK1 (Abcam), RIPK3 (Abcam), phosphor-S358-MLKL (Abcam), MLKL (Abcam), and PRs (Cell Signaling Technology) overnight at 4 °C. All antibodies had a dilution of 1:1,000. And detected using the Bio-Rad imaging system after incubated with horseradish peroxidase-conjugated second antibodies for 1.5h.

**LDH assays and ROS assays**
LDH in culture medium was evaluated using the LDH assay kit (Amsbio). We used an ROS detection kit from Enzo Life Sciences (ENZ-51011) to measure the ROS level. FE25 cells were detached from flasks after P4 or vehicle treatment, immediately incubated in an oxidative stress reagent at 37 °C for 30 min, and analyzed using 488/520 nm fluorescence in an ELISA reader.

**Quantitative real-time polymerase chain reaction**
Total RNA was extracted from cultured cells by using Trizol reagent (Invitrogen), and cDNA was synthesized with oligo(dT) primers by using a cDNA synthesis kit (Invitrogen). The SYBR Premix Dimer Eraser (Perfect Real Time) (Takara) assay kit was added. Real-time polymerase chain reaction (RT-PCR) was performed using the Roche LC480 PCR device.

**Cytokine antibody array**
RayBio Human Cytokine Antibody Array G-Series 5 was used to analyze cytokine expression in the conditioned medium of the cultured cells after 6 h of 100 μM P4 or vehicle treatment. The cutoff criteria for significance were set at a ratio of > 2-fold and an adjusted P value of < 0.05.
Supplemental References

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