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Progestins Regulate the Expression and Activity of the Forkhead Transcription Factor FOXO1 in Differentiating Human Endometrium

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Menstruation, or cyclic shedding of nonpregnant endometrial tissue with associated bleeding, occurs only in humans and a few other species. This breakdown of the endometrium in response to falling ovarian progesterone levels is a complex process, characterized by local leukocyte infiltration, expression and activation of matrix metalloproteinases, and apoptosis. Spontaneous decidualization (differentiation) of the stromal compartment precedes the cyclic shedding of the endometrium in various menstruating species but the mechanisms that link these processes are not understood. In this study, we identified FOXO1 as a key transcription factor responsible for mediating apoptosis of decidualized human endometrial stromal cells (HESCs) in response to progesterone withdrawal. We demonstrate that medroxyprogesterone acetate (MPA, a synthetic progestin) enhances the expression of FOXO1 in differentiating HESCs while simultaneously inducing cytoplasmic retention and inactivation of FOXO1. Withdrawal of MPA from decidualized HESCs results in rapid nuclear accumulation of FOXO1, increased *BIM* expression, a proapoptotic FOXO1 target gene, and cell death. Conversely, silencing of FOXO1 expression completely abolishes cell death induced by MPA withdrawal. In summary, the observation that differentiating HESCs become dependent on progesterone signaling for survival through induction and reversible inactivation of FOXO1 suggests a novel mechanism that links decidualization of the endometrium to menstruation. (*Molecular Endocrinology* 20: 35–44, 2006)

DECIDUALIZATION REPRESENTS the process of morphological and biochemical differentiation of the endometrial stromal compartment that occurs in all species in which embryo implantation involves breaching of endometrial surface epithelium (1, 2). Decidualization is essential for the formation of a functional placenta and, in most species, this differentiation process is triggered by signals derived from the implanting blastocyst (3). However, in humans and other menstruating primates, decidual transformation of the upper (functional) endometrial layer occurs in the midsecretory phase of the cycle independently of the presence of a conceptus (2, 4, 5). In the absence of pregnancy, luteolysis and falling ovarian progesterone levels elicit a cascade of events in the endometrium of menstruating species, leading to proteolytic breakdown, shedding of the functional layer, and bleeding. This complex but coordinated process involves local expression of chemokines and proinflammatory cytokines, influx of leukocytes, expression and activation of matrix metalloproteinases, and loss of the decidualized phenotype (6–9). Apoptosis, first apparent in epithelial cells and then in a proportion of the decidualized stromal cells, is another cardinal feature of impending menstruation (10, 11).

The notion that decidualization in the absence of pregnancy is causally linked to menstruation is supported by several other lines of evidence. For instance, spontaneous decidualization also occurs in the few nonprimate species known to menstruate, such as the black mastiff bat (*Molossus ater*) (12). In ovariectomized mice, withdrawal of progesterone support induces tissue breakdown but only if the endometrium has first been artificially decidualized (13). Furthermore, the decidual process in humans starts around the terminal spiral ar-

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Abbreviations: Bim, Bcl-2 interacting mediator; 8-br-cAMP, 8-bromoadenosine-cAMP; HESC, human endometrial stromal cell; MPA, medroxyprogesterone acetate; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol 3-kinase; siRNA, short interfering RNA.

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teries and coincides with expression of various factors implicated in tissue hemostasis. For example, decidualized stromal cells highly express tissue factor, the initiator of the extrinsic coagulation pathway, and plasminogen activator inhibitor-1, which limits fibrinolysis by inhibiting both tissue-type and urokinase-type plasminogen activator (14, 15). Consequently, apoptosis and loss of the decidual phenotype upon progesterone withdrawal promote focal bleeding and menstruation.

High progesterone levels are absolutely necessary for maintaining the decidual phenotype before menstruation and during pregnancy. However, several studies have shown that initiation of this differentiation process requires elevated cAMP levels and sustained activation of the protein kinase A pathway (16–18). We recently reported that activation of the cAMP second messenger pathway in human endometrial stromal cells (HESCs) induces the expression of FOXO1, a member of the FOXO subfamily of Forkhead transcription factors (19). FOXO family members, FOXO1, FOXO3a, and FOXO4, are direct downstream targets of the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway (20, 21). Phosphorylation of FOXO proteins by Akt results in cytoplasmic retention and inhibition of their transcriptional activity. Depending on the cellular context, activated FOXO proteins have been shown to induce expression of genes that encode for proteins involved in cell cycle inhibition, such as $p27^{kip1}$ and p130-Rb2, or proapoptotic proteins including Bim (Bcl-2 interacting mediator), TRAIL (TNF-α-related apoptosis-inducing ligand), and Fas ligand (22–26).

We now demonstrate that, upon decidualization, endometrial cells become dependent upon progesterone signaling for survival through induction, cytoplasmic translocation, and inactivation of FOXO1. Withdrawal of progesterone from differentiated cultures results in nuclear reaccumulation of FOXO1, enhanced Bim expression, and cell death. Together, the data demonstrate a critical role for progesterone as a survival factor for decidualized endometrium and provide novel insights into the molecular pathways that control the onset of menstruation.

RESULTS

cAMP and progesterone signaling differentially regulate the expression of FOXO1 and FOXO3a in HESCs. To investigate the role of FOXO proteins in human endometrium, we first profiled their expression in undifferentiated and decidualizing HESCs. Primary cultures were treated with either 8-bromoadenosine-cAMP (8-br-cAMP), the progestin MPA (medroxyprogesterone acetate), or a combination for 1 or 3 d and harvested for mRNA and protein analyses (Fig. 1, A and B). As expected, 8-br-cAMP induced the expression of FOXO1 mRNA and protein in HESCs (19) but, interestingly, this response was markedly enhanced by MPA. Treatment with MPA alone did not induce FOXO1 expression but up-regulated FOXO3a mRNA and protein levels although not when cells were cotreated with 8-br-cAMP. FOXO4 transcripts were present in HESCs, but we found no evidence of expression at protein level by Western blot analysis (Fig. 1B). The differential regulation of FOXO proteins upon HESC differentiation *in vitro* reflected the expression patterns determined *in vivo*. Analysis of timed endometrial biopsies showed an approximately 4-fold increase in the abundance of FOXO1 transcripts during the midsecretory phase of the cycle (Fig. 1C). Elevated FOXO1 mRNA levels were maintained in late-secretory or premenstrual endometrium. In contrast, the abundance of FOXO3a transcripts appeared to decline before menstruation whereas FOXO4 mRNA levels did not exhibit cycle-dependent regulation (Fig. 1C). Enhanced FOXO1 mRNA levels in mid- and late-secretory endometrium coincided with strong FOXO1 immunostaining in the stromal compartment (Fig. 1D). In contrast, there was little or no FOXO3a or FOXO4 immunoreactivity in the stromal compartment at any stage of the cycle (Fig. 1D).

Progesterone Inhibits BIM Expression

Having established that FOXO1 is the predominant FOXO family member in differentiating human endometrium, we examined whether its expression profile correlated with that of *BIM (BCL2L11)*, a known FOXO target gene that encodes for a proapoptotic Bcl-2 homology 3 domain-only protein (24, 25). Although Bim transcripts were present in endometrial samples throughout the cycle, their abundance increased only in the premenstrual phase of the cycle (Fig. 2A). Immunohistochemical analysis of late-secretory endometrium showed marked Bim staining in endometrial surface epithelium as well as the adjacent stroma (Fig. 2B). In culture, Bim mRNA and protein were induced upon treatment with 8-br-cAMP (Fig. 2, C and D), mirroring the expression of FOXO1. However, whereas addition of MPA to 8-br-cAMP-treated cultures enhanced FOXO1 protein levels, *BIM* expression was inhibited (Fig. 2D). This was paradoxical given that several lines of evidence indicated that *BIM* is regulated by FOXO1 in HESCs. First, transiently transfected FOXO1 activated the wild-type *BIM* promoter coupled to a luciferase reporter in HESCs but not a mutant *BIM* promoter construct in which the FOXO1 consensus sequence, located between $-164/-170$ relative to the transcription start site (27), was altered (data not shown). Second, DNA-pull down experiments demonstrated that endogenous FOXO1 is recruited to the FOXO-binding site of the *BIM* promoter in 8-br-cAMP-stimulated cultures although not in cells cotreated with MPA. No binding was observed to a mutated FOXO-response element (Fig. 2E). Finally, silencing of endogenous FOXO1 expression by small interfering RNA (siRNA) abrogated *BIM* expression in 8-br-cAMP or 8-br-cAMP plus MPA-treated HESCs (data not shown and Fig. 2F).

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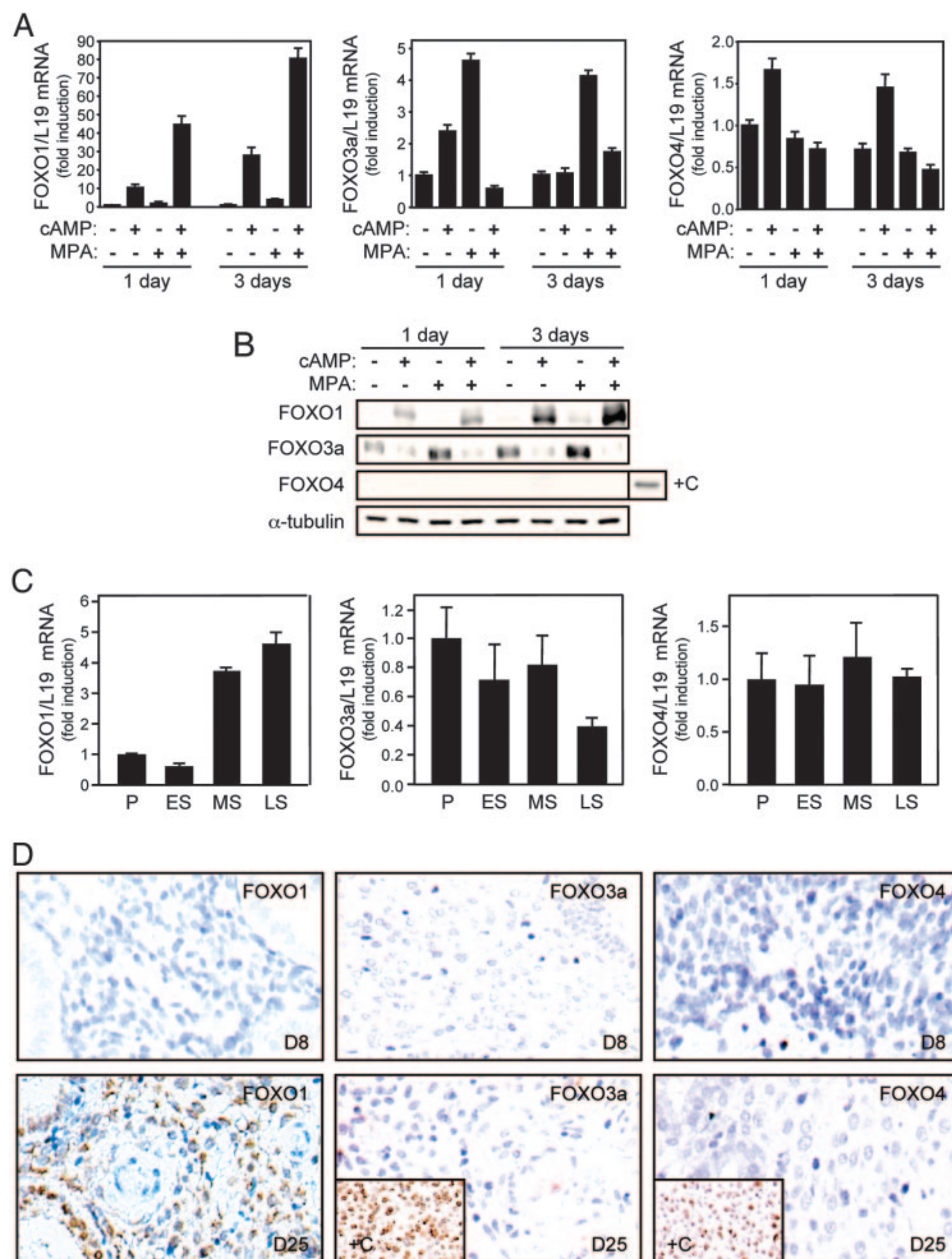

Fig. 1. Expression and Regulation of FOXO Proteins in Differentiating Human Endometrium

A, RTQ-PCR analysis of FOXO1, FOXO3a, and FOXO4 transcript levels in HESCs treated with 8-br-cAMP (cAMP), MPA, or combination for 1 or 3 d. B, Western blot analysis of FOXO proteins in whole-cell lysates obtained from primary HESC cultures treated as indicated. Protein extracts from cells transiently transfected with an FOXO4 expression vector were used as a positive control (+C). α-Tubulin served as a loading control. C, RTQ-PCR analysis of the various FOXO transcripts in endometrial biopsies obtained from different phases of the cycle: proliferative (P; n = 8), early secretory (ES; n = 8), midsecretory (MS; n = 6), and late secretory (LS; n = 7). *Error bars* are ±SD. D, FOXO immunostaining in proliferative (D8) and late-secretory (D25) endometrium (original magnification: ×100). Rhabdomyosarcoma tissue sections were used as a positive control (+C) for FOXO3a and FOXO4 immunostaining (*insets*). D8, day 8; D25, day 25.

Progesterone Induces Cytoplasmic Translocation of FOXO1

Phosphorylation of FOXO proteins, in response to activation of the PI3K/Akt signaling pathway or other kinases, leads to their retention in the cytoplasm and, consequently, transcriptional inactivation (21, 28). We speculated that the opposing effects of MPA on FOXO1 expression and transcriptional activity in 8-br-cAMP-treated HESCs could reflect changes in the subcellular distribution of FOXO1. Confocal microscopy demonstrated that this was indeed the case (Fig.

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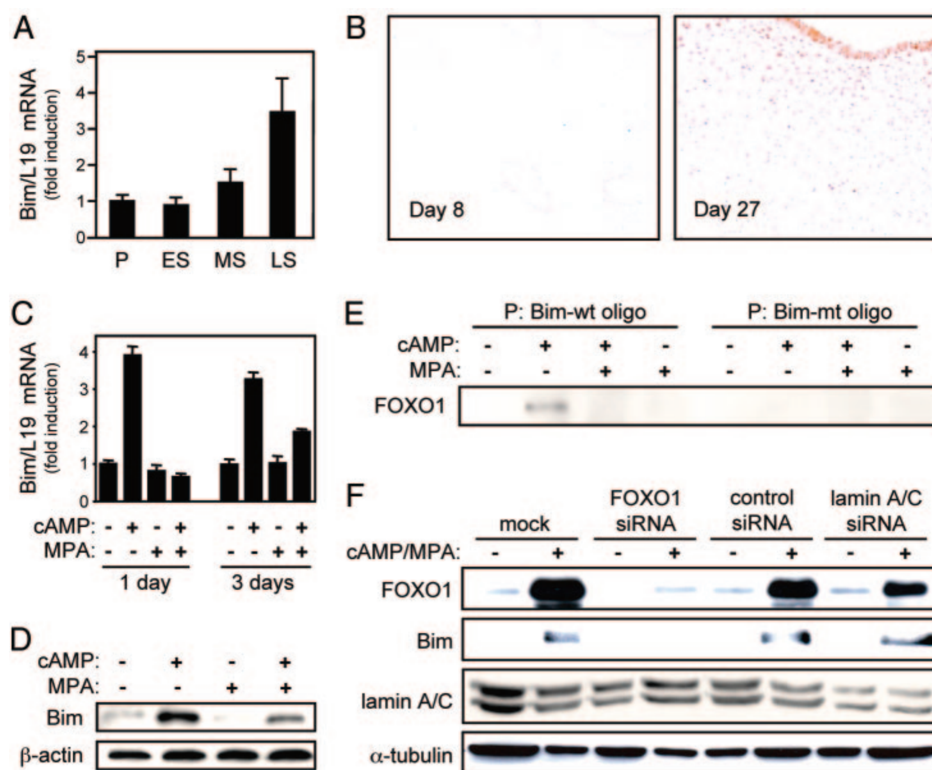

Fig. 2. MPA Inhibits FOXO1-Mediated *BIM* Expression in Decidualizing HESCs

A, Relative level of Bim mRNA expression, measured by RTQ-PCR, in proliferative (P; n = 9), early-secretory (ES; n = 9), midsecretory (MS; n = 6), and late-secretory (LS; n = 7) endometrial biopsies. *Error bars* indicate ±SD. B, Bim immunostaining in proliferative (d 8) and premenstrual (d 27) endometrium. Original magnification: ×100. C, Relative expression of Bim transcripts in undifferentiated HESCs and cells stimulated as indicated for either 1 or 3 d. D, Bim expression in total protein extracts from untreated HESCs or cells treated for 3 d as indicated were analyzed by Western blot analysis. β-Actin expression was used as a loading control. E, Nuclear protein extracts from HESCs treated for 3 d as indicated were incubated with a 5′-biotinylated double-stranded oligonucleotide corresponding to the −139 to −145 region of the BIM promoter (Bim-wt oligo) or biotinylated oligonucleotide in which the FOXO-binding site was mutated (Bim-mt oligo). DNA-bound proteins were pulled down and immunoblotted for FOXO1. F, Mock transfected HESCs or cells transfected with FOXO1 siRNAs, lamin A/C siRNA, or nontargeting (control) siRNAs were treated as indicated for 3 d. Whole-cell lysates were used to examine expression of FOXO1, Bim, and lamin A/C by Western blot analysis.

3A). Endogenous FOXO1 immunoreactivity in 8-br-cAMP-treated cells was confined to distinct punctate nuclear structures, but cotreatment of cells with MPA resulted in cytoplasmic retention of FOXO1. Notably, discrete FOXO1 foci remained apparent in nuclei of cells treated with 8-br-cAMP and MPA (Fig. 3A). Western blot analysis of cytoplasmic and nuclear protein fractions confirmed that MPA induces phosphorylation and cytoplasmic accumulation of FOXO1 in 8-br-cAMP-treated cells, which coincided with elevated levels of activated (phosphorylated) Akt (Fig. 3B). As reported previously, total Akt levels were lower in cells treated with 8-br-cAMP alone (29). Treatment of decidualized cells with the PI3K inhibitor LY294002 abrogated Akt and FOXO1 phosphorylation, indicating that the cytoplasmic translocation of FOXO1 in the presence of MPA is mediated by the PI3K/Akt signaling pathway.

Apoptosis of Decidualized Cells in Response to Progesterone Withdrawal Is Mediated by FOXO1

We investigated whether withdrawal of MPA from decidualized cultures would restore FOXO1 activity and increase cell death. To test this, we first monitored the expression of Bim in primary cultures treated with 8-br-cAMP and MPA for 3 d followed by selective withdrawal of the differentiation stimuli for 24 h (Fig. 4A). Western blot analysis showed that withdrawal of MPA alone substantially increased Bim levels whereas withdrawal of both 8-br-cAMP and MPA from the cultures only marginally increased the expression levels. Surprisingly, withdrawal of 8-br-cAMP for 24 h did not substantially lower Bim protein levels, which may reflect protein stability. Flow cytometry of parallel cultures stained with propidium iodide demonstrated a 2.5-fold increase in the number of dead or dying cells,

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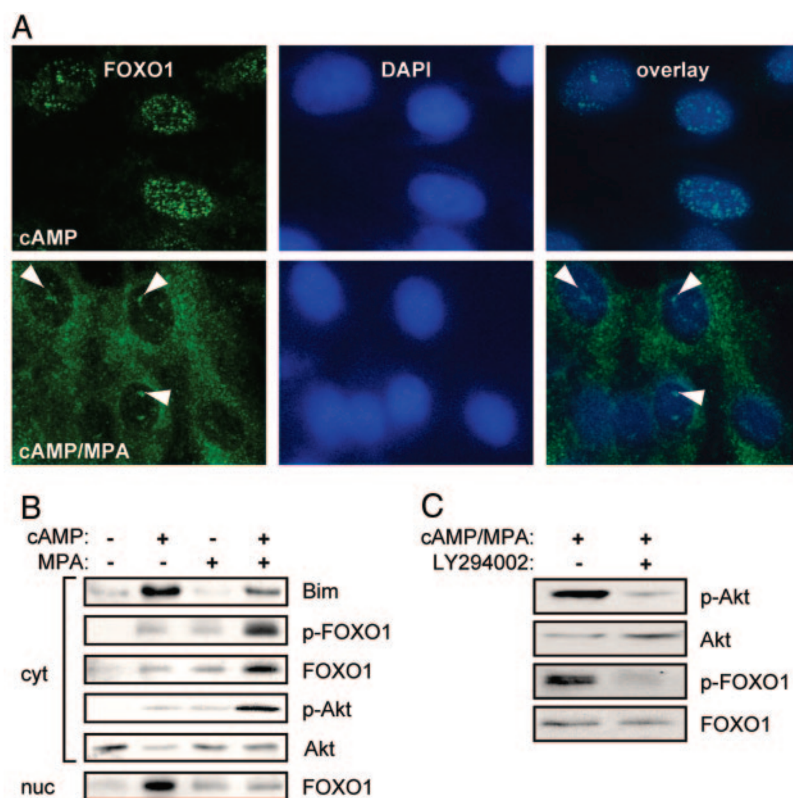

Fig. 3. Progestins Induce FOXO1 Phosphorylation and Cytoplasmic Retention in Differentiating HESCs

A, Confocal microscopy demonstrating endogenous FOXO1 expression in cells treated for 3 d with 8-br-cAMP (cAMP) in the presence or absence of MPA. *Arrowheads* indicate residual nuclear FOXO1. B, Western blot analysis of Bim, FOXO1, phosphorylated FOXO1 (p-FOXO1; Ser256), Akt, and phosphorylated Akt (p-Akt; Ser473) expression in the cytosolic (cyt.) or nuclear (nuc.) compartments of untreated cells or cells treated as indicated for 3 d. C, HESCs were treated with 8-br-cAMP plus MPA (cAMP/MPA) for 3 d. Subsequently, some cultures were treated with LY294002 for 2 h. Cytosolic protein fractions were analyzed by immunoblotting. DAPI, 4′,6-Amidino-2-phenylindole.

containing less than 2 N DNA, upon withdrawal of MPA from decidualized cultures (Fig. 4, B and C). In contrast, withdrawal of 8-br-cAMP had no effect, whereas withdrawal of both 8-br-cAMP and MPA marginally increased the apoptotic fraction. Overall, the level of Bim expression correlated well with the degree of cell death. Two lines of evidence demonstrated that apoptosis of decidualized cells upon progestin withdrawal is mediated by FOXO1. First, MPA withdrawal increased the nuclear FOXO1 levels while simultaneously lowering expression in the cytoplasmic compartment (Fig. 4D). Notably, nuclear reaccumulation of FOXO1 was a rapid event, apparent as early as 1 h after MPA withdrawal (Fig. 4D), and preceded the expression of apoptotic markers such as cleaved poly(ADP-ribose) polymerase (PARP)-1 and cleaved caspase-3 (data not shown). Second, knockdown of FOXO1 expression by siRNA lowered the basal apoptosis levels and completely abolished the ability of differentiated HESCs to undergo programmed cell death in response to progestin withdrawal (Fig. 4, E and F).

DISCUSSION

The three human FOXO proteins are homologs of DAF-16 in *Caenorhabditis elegans*, an important regulator of longevity in this organism (30). In mammalian cells, FOXO proteins are critical mediators of cell fate decisions, such as cell cycle arrest, senescence, and apoptosis, in response to growth factor, and hormonal and environmental cues (31, 32). FOXO proteins are ubiquitously expressed in many cell types. In normal cycling human endometrium, however, FOXO1 is the predominant isoform, and its expression is restricted to the epithelial and stromal compartments during the luteal phase of the cycle. Using primary cultures, we demonstrated that decidualization in response to activation of the cAMP second messenger pathway coincides with the expression of FOXO1. Surprisingly, whereas MPA treatment led to a further increase in FOXO1 levels, FOXO1 transcriptional activity was inhibited as a result of targeted phosphorylation and cytoplasmic retention. FOXO1 phosphorylation in re-

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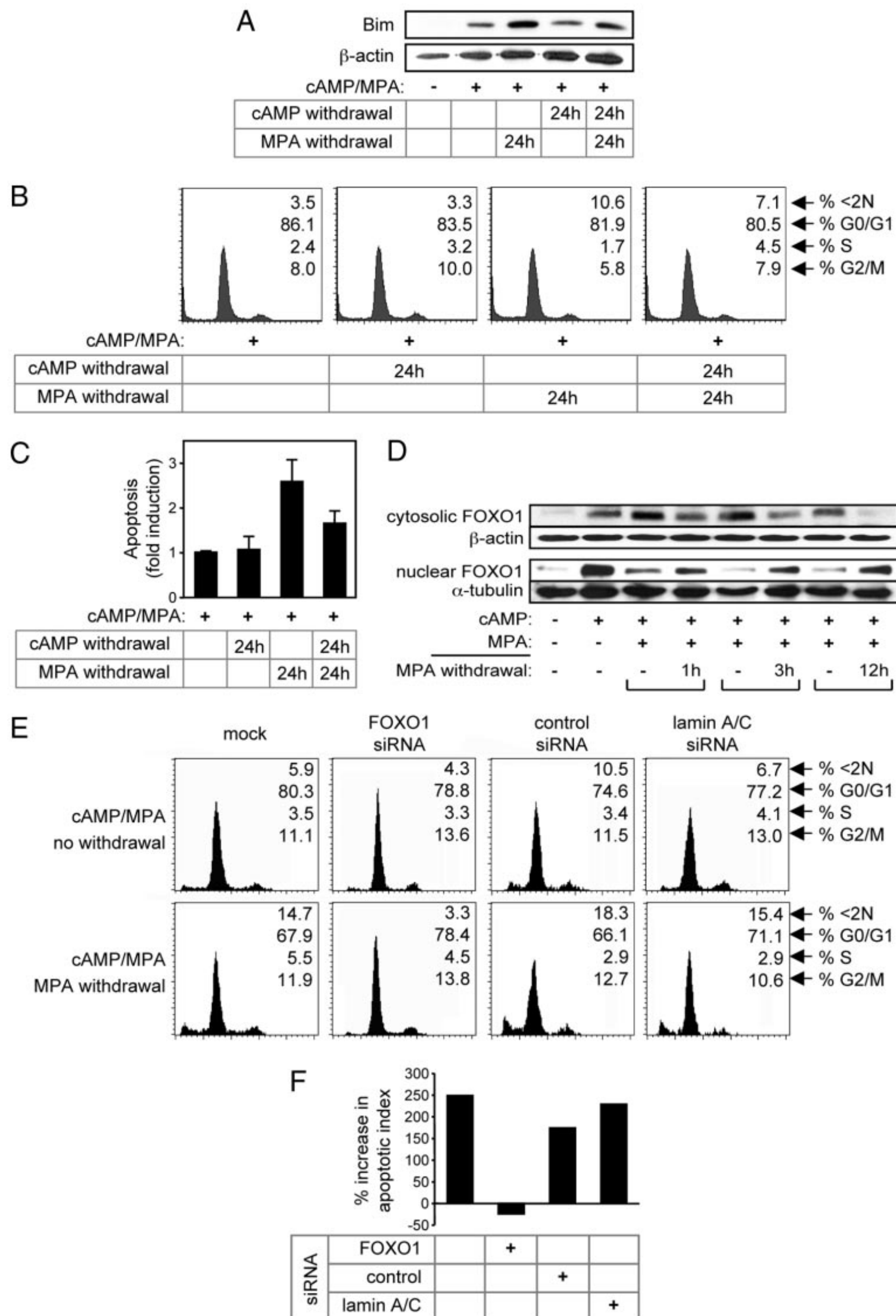

Fig. 4. Progestin Withdrawal from Decidualizing Cells Results in Nuclear Accumulation of FOXO1 and Increased Cell Death

A, Western blot analysis of Bim protein levels in untreated HESCs and in decidualized cells (pretreated with 8-br-cAMP and MPA for 3 d) upon 24-h withdrawal of the differentiation stimuli, as indicated. B, Cell cycle analysis of 8-br-cAMP and MPA-pretreated HESCs upon withdrawal of either 8-br-cAMP, MPA, or both from the cultures for 24 h. The percentage of cells in each phase of the cell cycle (<2 N, G_0/G_1, S, and G_2/M) is indicated. C, Mean fold increase (F.I.) in the apoptotic fraction of four separate decidualizing cultures upon 24-h withdrawal of MPA. *Error bars* are ±SD. D, Western blot analysis demonstrating the relative levels of FOXO1 in cytosolic and nuclear protein fractions of decidualized cells harvested at various time points after MPA was withdrawn from the cultures. E, Mock transfected HESCs or cells transfected with FOXO1 siRNAs, lamin A/C siRNA, or nontargeting (control) siRNAs were treated for 3 d with 8-br-cAMP and MPA (cAMP/MPA) whereupon MPA was withdrawn (−MPA) from some cultures for 24 h. F, Percent change in the apoptotic index of mock transfected HESCs or cells transfected with FOXO1 siRNAs, lamin A/C siRNA, or nontargeting (control) siRNA after 24 h of MPA withdrawal.

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sponse to MPA was dependent upon PI3K/Akt activation. This is most likely an autocrine effect as progesterone is known to enhance the expression of a variety of growth factors in differentiating HESCs, including epidermal growth factor, vascular epithelial growth factor, and IGF-I, capable of activating this signal transduction pathway (33, 34).

PI3K/Akt-mediated phosphorylation and nuclear export of FOXO1 have been shown to trigger proteasomal degradation (35, 36). Recent data suggest that Skp2, an oncogenic subunit of the Skp1/Cul1/F-box protein complex, directs FOXO1 ubiquitination and proteasome degradation (37). In human endometrium, the expression of this E3 ubiquitin ligase is markedly down-regulated during the secretory phase of the cycle (38), suggesting that reduced degradation may contribute to the cytoplasmic accumulation of FOXO1 in decidual cells. Notably, this cytoplasmic translocation was partial, and a discernible amount of FOXO1 remained detectable in the nuclei of cAMP- and MPA-treated cells. Based on expression of recombinant proteins and promoter-reporter systems, it has been suggested that FOXO1, through its ability to bind other transcription factors such as C/EBPβ and HOXA10, regulates the expression of two major decidua-specific genes, *prolactin* (*PRL*) and *IGF- binding protein 1(IGFBP1*) (19, 39). Whether endogenous FOXO1 is indeed involved in multimeric complexes that drive the promoters of these genes is currently under investigation.

Withdrawal of MPA from decidualized cultures elicited reaccumulation of FOXO1 in the nucleus and increased cell death. Previous studies have implicated *Fas ligand (FASLG*), also a FOXO target gene, in regulating HESC apoptosis (40, 41). However, several lines of evidence presented in this study indicate that Bim may also be involved in effecting perimenstrual endometrial apoptosis. First, we demonstrated that *BIM* is a *bona fide* FOXO1 target gene in HESCs. Second, Bim expression is up-regulated in decidualized cultures upon MPA withdrawal and, most relevantly, its expression *in vivo* increases premenstrually when progesterone levels decline. Notably, FOXO1 and Bim are also expressed in the epithelial cells in late secretory endometrium. In contrast to the stroma, progesterone receptor levels are markedly down-regulated in epithelial cells before menstruation, which could account for the earlier onset and higher apoptotic index in this compartment (10).

Cyclic decidualization and menstrual shedding occur on average 400 times during the reproductive years of women in developed countries (8). Consequently, abnormal uterine bleeding is one of the most common disorders in women and a major indication for surgical intervention such as hysterectomy or endometrial ablation (5). The unique ability of FOXO1 to regulate HESC survival and apoptosis suggests that it might be a useful therapeutic target for menstrual disorders. Selective regulators of FOXO expression and nuclear transport, currently being developed for the treatment of a variety of cancers (42), may also be useful in the treatment of other gynecological disorders, including endometriosis. This debilitating condition is characterized by ectopic growth of endometrial tissue and affects 5–10% of women of reproductive age (43). A recent study demonstrated that FOXO1 mRNA levels are lower in secretory endometrium of women with endometriosis when compared with healthy controls (44). Others have reported that apoptosis is markedly reduced in premenstrual endometrium of endometriosis patients as well as in the endometriotic lesions (45, 46). This raises the possibility that dysregulation of FOXO1 expression or activity contributes to implantation, survival, and growth of ectopic endometrium. Furthermore, 50–80% of endometrial cancers harbor a mutation in the *PTEN* tumor suppressor gene, the highest incidence of *PTEN* mutation in any tumor type analyzed to date (47, 48). *PTEN* encodes a lipid phosphatase that specifically dephosphorylates the D3 position of phosphatidylinositol 3,4,5-triphosphate and, in so doing, functionally antagonizes the PI3K/Akt pathway. Whether inactivation of FOXO proteins constitutes the link between loss of PTEN function, constitutive activation of the PI3K/Akt pathway and endometrial tumorigenicity is currently under investigation.

Understanding the mechanisms of menstruation is of paramount importance given the enormous health burden associated with abnormal uterine bleeding. Our study demonstrates how progesterone acquires the role of a survival factor upon decidualization of the human endometrium through the regulation of FOXO1 expression and activity. To what extent this mechanism is perturbed in menstrual disorders remains to be determined. However, the pivotal role of FOXO1 in regulating endometrial apoptosis upon progesterone withdrawal suggests that it could be a useful therapeutic target.

MATERIALS AND METHODS

Tissue Collection and Primary Culture

The Local Research and Ethics Committee approved this study, and patient consent was obtained before tissue collection. Endometrial samples from cycling women without uterine pathology were divided for histological dating, using standard criteria, and for storage in RNAlater (Ambion, Inc., Austin, TX). Endometrial samples were classed as proliferative, early secretory (d 15–19), midsecretory (d 20–23) or late secretory (d 24 or later). HESCs were isolated from fresh biopsies and primary cultures were established, maintained, and decidualized with 0.5 mM 8-br-cAMP (Sigma Chemical Co., St. Louis, MO) and 10^{-6} M MPA (Sigma) as previously described (49, 50). All experiments were carried out before the third cell passage.

Real-Time Quantitative PCR

Total RNA extracted from untreated or decidualizing HESC cultures and from tissue samples stored in RNAlater (Am-

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bion), was reversed transcribed, and the resulting cDNA was amplified using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) (49) and the following gene-specific primer pairs: *FOXO1*-sense (TGG ACA TGC TCA GCA GAC ATC) and *FOXO1*-antisense (TTG GGT CAG GCG GTT CA); *FOXO3a*-sense (CCC AGC CTA ACC AGG GAA GT) and *FOXO3a*-antisense (AGC GCC CTG GGT TTG G); *FOXO4*-sense (CCT GCA CAG CAA GTT CAT CAA) and *FOXO4*-antisense (TTC AGC ATC CAC CAA GAG CTT); *BIM*-sense (ATC TCA GTG CAA TGG CTT CC) and *BIM*-antisense (CAG GCG GAC AAT GTA ACG TA); *L19*-sense (GCG GAA GGG TAC AGC CAA T) and *L19*-antisense (GCA GCC GGC GCA AA). *L19*, a nonregulated ribosomal housekeeping gene, served as an internal control and was used to normalize for differences in input RNA. All measurements were performed in triplicate.

Western Blot Analysis

Whole-cell extracts or nuclear and cytoplasmic protein fractions were immunoblotted as described elsewhere (17, 49). In some experiments, cells were treated with LY294002 (20 μM) for 2 h before harvesting. Antibodies to FOXO1 (Cell Signaling Technology, Beverly, MA), FOXO3a (Upstate Biotechnology, Inc., Lake Placid, NY), FOXO4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Bim (Calbiochem, La Jolla, CA), phospho-FOXO1 (Ser256) (Cell Signaling), Akt (Cell Signaling), phospho-Akt (Ser473) (Cell Signaling), PARP Cleavage site (214/215) (Biosource Technologies, Inc., Camarillo, TX), PARP (F-2) (Santa Cruz Biotechnology), Lamin A/C (636) (Santa Cruz Biotechnology), and α-Tubulin (TU-02) (Santa Cruz Biotechnology) were used at 1:1,000 whereas the antibody to β-Actin (Abcam, Cambridge, UK) was diluted 1:40,000.

Immunohistochemistry, Confocal Microscopy, and Flow Cytometry

Paraffin-embedded, formalin-fixed endometrial specimens, obtained at various stages of the cycle, were examined for *in vivo* FOXO and Bim immunoreactivity. Sections (5 μm), placed on 1% (wt/vol) polylysine slides, were deparaffinized, dehydrated, exposed to 0.3% (vol/vol) H_2O_2 for 15 min, and subsequently microwaved in 0.01 M citrate buffer, pH 6.0. Immunostaining was carried out using antibodies to FOXO1 (1:400), FOXO3a (1:800), or FOXO4 (1:400), biotinylated horse antigoat Ig (1:500), and peroxidase-labeled streptavidin (1:500). Primary antibodies were from Santa Cruz Biotechnology. Vaginal rhabdomyosarcoma tissue sections were used as positive control. Immunostaining for Bim was carried out using antihuman Bim antibody (1:600; Calbiochem) and peroxidase-conjugated goat antirabbit IgG (1:200; Vector Laboratories, Inc., Burlingame, CA). In control slides, the primary antibody was replaced with normal goat or rabbit IgG. Confocal microscopy of primary cultures was performed as described elsewhere (49) using antibodies to FOXO1 (1:200; Santa Cruz Biotechnology) and cleaved caspase-3 (Asp175) (1:100; Cell Signaling). Flow cytometry analysis was used to quantify apoptosis in primary cultures by evaluating the sub-G_1 fraction ($<$2 N) after propidium iodide staining of ethanol-fixed cells (25, 27).

Oligonucleotide Pull-Down Assay

Nuclear extracts were prepared from cultured cells using the high-salt buffer [20 mM HEPES-KOH (pH 7.9), 25% (vol/vol) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol; and 1 mM phenylmethylsulfonylfluoride]. After diluting with 2 vol of low-salt lysis buffer (10 mM HEPES-KOH, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol; and 1 mM phenylmethylsulfonylfluoride), 50 μg of the cell extracts was incubated at 30 C for 10 min with either 0.5 nmol of the 5′-biotinylated double-stranded wild-type (5′-CAGAGTTACTCCGGTAAACACGCCAGGGAC-3′) or mutant (5′-CAGAGTTACTCCGGTAGGCACGCCAGGGAC-3′) oligonucleotides (Invitrogen) previously coupled to streptavidin agarose beads (Sigma). The wild-type oligonucleotide corresponded to the region of −183 to −154 of the human *Bim* promoter (27). After incubation, the biotinylated oligonucleotide-coupled streptavidin beads were washed at least six times with low-salt buffer containing 150 mM NaCl, denatured in sodium dodecyl sulfate-sample buffer, and immunoblotted for FOXO1.

Transient Transfection and siRNA

HESCs cultured in 24-well plates were transiently transfected, using calcium phosphate precipitation as described previously (49), with either wild-type or mutant *BIM* promoter-reporter constructs (27) (400 ng/well) and a FOXO1 expression vector (100 ng/well). Cells were harvested for luciferase assay 24 h later. A β-galactosidase control expression vector was cotransfected to control for transfection efficiency. Transfections were performed in triplicate and repeated at least three times. For gene silencing, undifferentiated or decidualized HESCs were transiently transfected with 50 nM of the following siRNA reagents purchased from Dharmacon (Lafayette, CO): FOXO1 si*GENOME* SMARTpool, si*CONTROL* Non-Targeting siRNA Pool, or si*CONTROL* Lamin A/C siRNA.

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