

## Research Article

# Loss of Inhibitory Insulin Receptor Substrate-1 Phosphorylation Is an Early Event in Mammalian Target of Rapamycin–Dependent Endometrial Hyperplasia and Carcinoma

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## Abstract

Insulin-like growth factor-I receptor signaling contributes to the development of endometrial hyperplasia, the precursor to endometrioid-type endometrial carcinoma, in humans and in rodent models. This pathway is under both positive and negative regulation, including S6 kinase (S6K) phosphorylation of insulin receptor substrate-1 (IRS-1) at S636/639, which occurs downstream of mammalian target of rapamycin (mTOR) activation to inhibit this adapter protein. We observed activation of mTOR with a high frequency in human endometrial hyperplasia and carcinoma, but an absence of IRS-1 phosphorylation, despite high levels of activated S6K. To explore when during disease progression mammalian target of rapamycin (mTOR) activation and loss of negative feedback to IRS-1 occurred, we used the Eker rat (*Tsc2<sup>EK/+</sup>*) model, where endometrial hyperplasia develops as a result of loss of *Tsc2*, a “gatekeeper” for mTOR. We observed mTOR activation early in progression in hyperplasias and in some histologically normal epithelial cells, suggesting that event (s) in addition to loss of *Tsc2* were required for progression to hyperplasia. In contrast, whereas IRS-1 S636/639 phosphorylation was observed in normal epithelium, it was absent from all hyperplasias, indicating loss of IRS-1 inhibition by S6K occurred during progression to hyperplasia. Treatment with a mTOR inhibitor (WAY-129327) significantly decreased hyperplasia incidence and proliferative indices. Because progression from normal epithelium to carcinoma proceeds through endometrial hyperplasia, these data suggest a progression sequence where activation of mTOR is followed by loss of negative feedback to IRS-1 during the initial stages of development of this disease. *Cancer Prev Res*; 3(3): 290–300. ©2010 AACR.

## Introduction

Endometrial carcinoma is the most common gynecologic malignancy, with 41,100 new cases and 7,470 deaths predicted to occur in the United States in 2008 (1). The progression of the normal endometrium to type I (endometrioid) endometrial adenocarcinoma (EC) involves an intermediary state of abnormal proliferation, complex

atypical hyperplasia (CAH; ref. 2). Numerous epidemiologic studies have shown that obesity has a strong association with the development of EC (3, 4). Whereas an average woman has a 3% lifetime risk of EC, obese women have a 9% to 10% lifetime risk (5). Obesity is associated with the development of insulin resistance and compensatory chronic hyperinsulinemia (6). Increased serum insulin levels lead to reduced hepatic synthesis and blood levels of insulin-like growth factor (IGF) binding proteins and increased bioavailability of IGF-I. Hyperinsulinemia is proposed to increase insulin/IGF signaling in peripheral tissues, such as the endometrium, of obese women to promote tumorigenesis (6).

The effect of IGF-I is mediated primarily by activation of the IGF-I receptor (IGF-IR), a tyrosine kinase receptor expressed in endometrial stromal and epithelial cells that signals via activation of the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway (7). Ligand activation of IGF-IR induces a phosphorylation cascade of the downstream components, including insulin receptor substrate (IRS)-1 and IRS-2, Akt, and mTOR, to promote cell proliferation and protein translation (8, 9). Canonical IRS signaling is defined by

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the binding of IRS-1 or IRS-2, via the conserved pleckstrin homology and phosphotyrosine-binding domains, to ligand-phosphorylated IGF-IR (10). The IRS-1 and IRS-2 adaptor proteins are major substrates of the IGF-IR (11), and signaling via IRS-1/2 leads to activation of phosphatidylinositol 3'-kinase (PI3K) and Akt. Akt in turn signals to other proteins in the PI3K pathway, including tuberlin, the protein product of the tuberous sclerosis complex 2 (TSC2) tumor suppressor gene (9). The TSC tumor suppressor, composed of tuberlin and hamartin, functions to inhibit activation of mTOR, a serine/threonine protein kinase that acts as an amino acid and ATP sensor to control nutrient availability and cell growth (12). mTOR is part of two distinct multiprotein complexes, TOR complex (TORC)-1 and TORC2. TORC1 is a central controller of cell growth, whereas TORC2 signals to the actin cytoskeleton to determine cell shape and polarity (13). The TORC1 mTOR complex is sensitive to rapamycin, which directly inhibits mTORC1 activity by binding of FKBP12-rapamycin to TOR exclusively in TORC1 (14). Phosphorylation of TSC2 by Akt inactivates the TSC tumor suppressor and relieves repression of mTOR. Activated mTOR then signals via mTORC1-dependent phosphorylation of p70 S6 kinase (S6K) and 4EBP1, which in turn activate downstream effectors such as the S6 ribosomal protein (S6) to promote protein transcription, translation, ribosome biogenesis, and metabolism (12, 13, 15).

IRS-1 and IRS-2 have similar molecular structures, tissue distribution, and ligand stimulation, as well as complementary function. IRS-1 and IRS-2 share significant structural homology, with a highly conserved NH<sub>2</sub> terminus with more than 20 common tyrosine phosphorylation motifs and with more than 20 serine phosphorylation motifs in the COOH-terminal that bind many of the same signaling molecules (10). Tyrosine and serine phosphorylation of IRS-1/2 positively and negatively regulate IGF-IR signaling, respectively (16–18). IRS-1/2 contain multiple tyrosine phosphorylation motifs that serve as docking sites for SH2 domain-containing proteins, such as the p85 regulatory subunit of PI3K. Serine/threonine phosphorylation motifs cause dissociation of IRS-1/2 from the IGF-IR and PI3K, thereby inhibiting IGF-IR signal transduction (16–19). In particular, S6K phosphorylates multiple serine residues of IRS-1/2, resulting in inhibition of IRS activity and promoting proteasomal degradation. Specifically, S636/639 of IRS-1 lies close to the Y<sup>632</sup> MPM motif implicated in PI3K binding to IRS-1 on insulin stimulation. Phosphorylation of S636/639 by S6K prevents IRS-1 association with IR/IGF-IR and promotes ubiquitin-proteasome-mediated degradation of IRS-1 (20). This S6K-dependent negative feedback inhibition of IRS-1 attenuates IGF-IR/Akt signaling in normal insulin-responsive cells (16, 18, 21–24). A proposed mechanism of insulin resistance in metabolic tissues, such as white adipose tissue, liver, and skeletal muscle, of obese individuals is increased negative feedback to IRS-1 (25). Therefore, in the scenario of insulin resistance, increased serine phosphorylation of IRS-1 leads to IRS-1 dissociation from the IGF-IR, thereby preventing

insulin signaling through the IGF-IR. S6K-dependent negative feedback inhibition of IRS-1 has been studied primarily in metabolic tissues but has not been well characterized in cancer, particularly those cancers associated with obesity. Here, we present the first report of loss of negative feedback inhibition of IRS-1 during progression of hyperplasia and carcinoma with concomitant activation of mTOR.

We observed in the Eker (*Tsc2<sup>E<sup>kl</sup>/+</sup>*) rat model that endometrial hyperplasia associated with activated mTOR signaling occurred in 100% of aged females. Hyperplasias with activated mTOR signaling exhibited loss of inhibitory IRS-1 phosphorylation, whereas histologically normal-appearing endometrial glands with mTOR activation exhibited inhibitory S636/639 phosphorylation. Similar data were obtained in human endometrial hyperplasia and carcinoma, which also exhibited activation of mTOR and loss of inhibitory IRS-1 phosphorylation. Furthermore, pharmacologic inhibition of mTOR signaling in preclinical studies with the rapamycin analogue WAY-129327 (26) completely abrogated mTOR signaling and significantly decreased the proliferative index and incidence of endometrial hyperplasias, confirming the dependence on mTOR signaling for development and growth of these lesions.

## Materials and Methods

### Endometrial tissue samples

For Western blot analysis, endometrioid endometrial carcinomas ( $n = 22$ ) from hysterectomy surgical specimens were flash frozen and stored at  $-80^{\circ}\text{C}$ . For immunohistochemistry, formalin-fixed paraffin-embedded sections of endometrial complex hyperplasia with atypia (CAH;  $n = 28$ ) and endometrial endometrioid adenocarcinoma grade 1 (EEC grade 1;  $n = 11$ ) were derived from hysterectomy surgical specimens submitted to the Department of Pathology, M.D. Anderson Cancer Center. H&E-stained slides were microscopically evaluated by a gynecologic pathologist (R.R.B.) to confirm the diagnosis.

### Animals

The care and handling of rats were in accord with NIH guidelines in Association for the Assessment and Accreditation of Laboratory Animal Care-accredited facilities. All protocols involving the use of these animals were approved by the M.D. Anderson Animal Care and Use Committee. Fifteen-month-old Eker (*Tsc-2<sup>E<sup>kl</sup>/+</sup>*) rats received i.p. injections of either 10  $\mu\text{g}$  (0.5 mg/kg) of WAY-129327 in 50  $\mu\text{L}$  of TPE (5% Tween 80, 5% polyethylene glycol-400, and 4% ethanol) per rat per day (WAY-129327 treated;  $n = 8$ ) or 50  $\mu\text{L}$  of TPE (vehicle control;  $n = 11$ ) for 2 wk. Animals were euthanized, and right and left uterine horns, ovaries, and vagina were formalin fixed and paraffin embedded for light microscopic examination ( $n = 19$ ). H&E-stained slides of left and right uterine horns collected from all animals were microscopically evaluated for the presence of endometrial hyperplastic lesions. Microscopic criteria for identifying rat endometrial hyperplasia have been previously published by our group (27).

### Protein isolation and Western blot analysis

To prepare lysates, a small portion (0.5 cm<sup>3</sup>) of tumor was isolated and crushed with a mortar and pestle over dry ice and the resulting pulverized tissues were collected with liquid nitrogen and transferred into a microcentrifuge tube. Radioimmunoprecipitation assay buffer (0.5-1.0 mL; 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 mol/L NaCl, 0.01 mol/L NaPO<sub>4</sub>, 2 mmol/L EDTA, 200 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L activated sodium orthovanadate, and 1× Roche complete protease inhibitor) was added and samples were rotated at 4°C for 2 h. After rotations, the samples were centrifuged at 14,000 rpm at 4°C for 10 min. The resulting supernatant was collected, aliquoted, and stored at -80°C for future analysis. Lysate protein concentrations were determined with BCA Protein Assay Kit (Pierce Biotechnology).

Samples of protein from each sample (30 µg) were size separated by SDS-PAGE and transferred overnight at 4°C onto polyvinylidene difluoride membranes (Pierce Biotechnology). Membranes were blocked in a TBST (TBS plus 0.05% Tween 20) + 5% nonfat milk solution for 1 h. Membranes were incubated in a primary antibody solution for 2 h at room temperature with varying antibody (1:500-1:2,000) and milk (3-5%) concentrations. For both Western analysis and immunohistochemistry, primary antibodies were purchased from Cell Signaling for IRS-1, phosphorylated (S636/639) IRS-1, phosphorylated (S235/236) S6 ribosomal protein, and S6 ribosomal protein. Primary antibody for glyceraldehyde-3-phosphate dehydrogenase was purchased from Santa Cruz Biotechnology. Membranes were then washed three times with TBST solution for 10 min each and then incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature. Membranes were washed following the same procedure as previously mentioned and visualized with LumiGLO chemiluminescent reagents (Kirkegaard and Perry Laboratories) or the enhanced chemiluminescence plus kit (Amersham Pharmacia Biotech) for more sensitive detection. Membrane immunoreactivity was detected by X-ray film (BioMax, Eastman Kodak). In our hands, the antibody to IRS-2 was useful in immunohistochemistry. However, for Western analysis, this same antibody resulted in not clearly distinguishable bands; therefore, we believe that this antibody is not acceptable for Western analysis.

### Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were deparaffinized and endogenous peroxidases were quenched by incubation in 1% H<sub>2</sub>O<sub>2</sub>. Antigen retrieval was done by microwaving the slides in 10 mmol/L citrate buffer (pH 6.0). Slides were incubated with primary antibody (1:50) in PBS containing 10% normal goat or horse serum overnight at 4°C. Primary antibody for Ki67 was purchased from DAKO North America, Inc. Primary antibody for terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was purchased from

Calbiochem. A biotin-labeled secondary antibody was conjugated for 30 min at 37°C. The sections were stained using avidin-biotinylated horseradish peroxidase complex from the DAKO Cytomation LSAB2 System according to the manufacturer's instructions. Diaminobenzidine reagent plus chromagen (DAKO) was incubated with the sections for up to 30 min. The sections were counterstained with hematoxylin, dehydrated, and mounted. Controls that lacked primary antibody were incubated in 1× PBS with 10% goat serum in each experiment. Immunostained sections were examined by light microscopy by two investigators (A.S.M. and R.R.B.), one of whom is a gynecologic pathologist (R.R.B.), and scored semiquantitatively according to the intensity of staining on a scale of 0 (no staining) to 3+ (strong staining). Tissues with 2+ or 3+ staining in >10% of cells were considered positive for protein expression. Ki67 and TUNEL labeling indices were scored by assessing the percentage of positive cells per 100 epithelial cells evaluated for vehicle- and WAY-129327-treated rats.

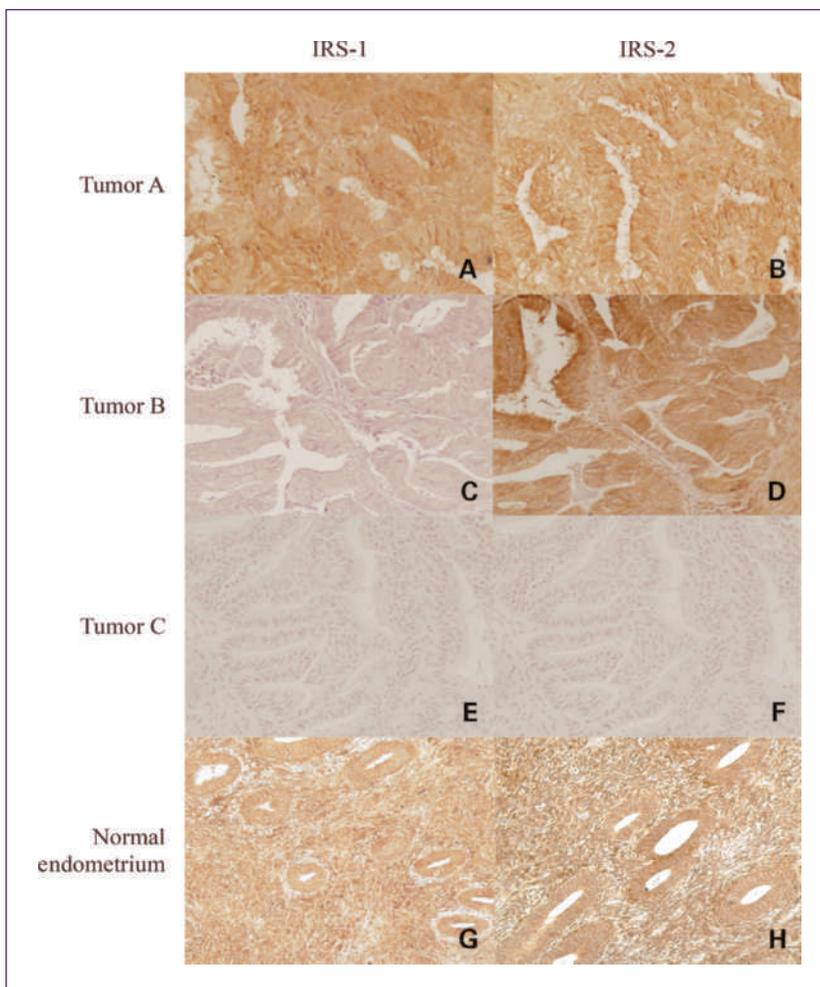
### Statistical analysis

Differences in means were calculated by unpaired Student's *t* tests, ANOVA, or nonparametric Mann-Whitney test.  $\chi^2$  test was used to evaluate the frequency of immunostaining by group. Any correlations were evaluated by Pearson correlation analysis and confirmed by Spearman's and Kendall's tests. Statistical significance was defined as *P* < 0.05.

## Results

### IRS-1 and IRS-2 expression in human endometrial hyperplasia and carcinoma

The development of endometrial hyperplasia and cancer is linked to estrogen exposure (28), and we previously reported that neonatal exposure to a synthetic estrogen, diethylstilbestrol, resulted in IRS-1 overexpression and increased susceptibility to develop endometrial hyperplasias in the rat endometrium (27). To determine if IRS-1/2 were aberrantly expressed in the human disease, we examined the expression of IRS-1 and IRS-2 in normal endometrium (*n* = 6), endometrial CAH (*n* = 28), and grade 1 EC (*n* = 11). In the normal endometrium, there is abundant expression of IRS-1 (5 of 6 biopsies evaluated) and IRS-2 (6 of 6 biopsies evaluated) in both the endometrial stromal cells and glandular epithelial cells (Fig. 1G and H). For endometrial CAH and EC, the majority of the cases were clearly positive for IRS-1 and IRS-2 (25 of 39 positive for IRS-1; 33 of 39 positive for IRS-2; Fig. 1). Importantly, IRS-1 and/or IRS-2 were expressed in 25 of 28 CAH and 10 of 11 ECs, consistent with a molecular mechanism of endometrial proliferation involving dysregulation of the IGF-IR/Akt/mTOR signaling pathway in the majority of tumors. Therefore, for subsequent studies, we focused on CAH and grade 1 EC cases that had positive expression of IRS-1 and/or IRS-2.



**Fig. 1.** Immunohistochemistry of IRS-1 and IRS-2 in normal proliferative phase endometrium and endometrial endometrioid carcinomas from three different patients. Tumor A, positive for both IRS-1 and IRS-2 (A and B). Tumor B, negative for IRS-1 and positive for IRS-2 (C and D). Tumor C, negative for both IRS-1 and IRS-2 (E and F). Normal endometrium, positive for both IRS-1 and IRS-2 (G and H). Original magnification,  $\times 200$ .

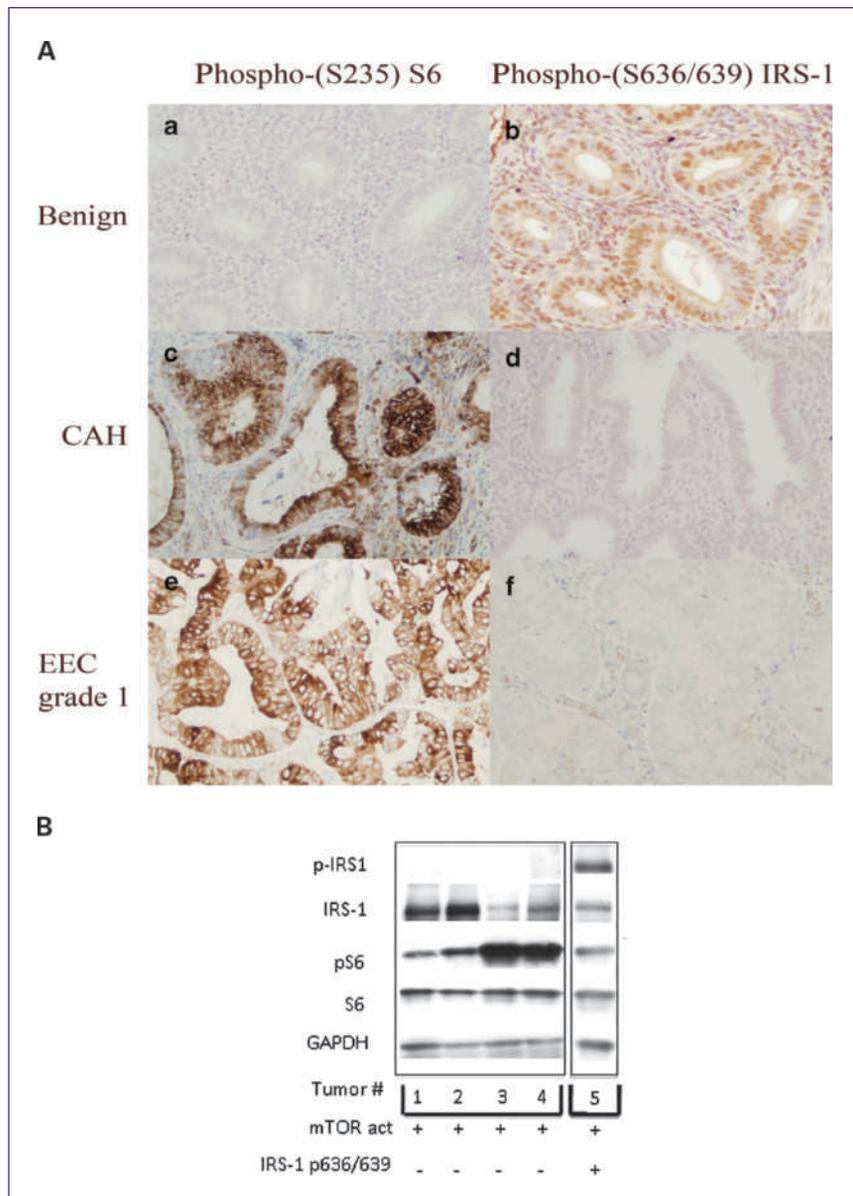
### Loss of inhibitory serine phosphorylation of IRS-1 is associated with progression to hyperplasia

In normal tissues, as a negative feedback mechanism to insulin/IGF stimulation, IRS-1/2 are phosphorylated on multiple serine/threonine residues (e.g., via S6K phosphorylation at S636/639), promoting their degradation (23). As shown in Fig. 2A and Table 1, immunohistochemical expression of serine-phosphorylated IRS-1 was high (3+) in the normal endometrial epithelium and stroma. In contrast, serine-phosphorylated IRS-1 was absent in the majority of endometrial CAH and EC [Fig. 2A (d and f)], despite the fact that these lesions had activated mTOR [Fig. 2A (c and e)], showing that activation of mTOR signaling and loss of inhibitory IRS-1 phosphorylation were negatively correlated ( $P < 0.025$ ; Table 1).

Western blot analysis confirmed the immunohistochemistry findings and showed an absence of IRS-1 serine phosphorylation in the majority of EC (Fig. 2B). Of 20 tumors evaluated by Western, 9 tumors had detectable expression of IRS-1. IRS-2 expression was not evaluated because the antibody to IRS-2 used for immunohisto-

chemistry was unsuitable for Western analysis. Whereas only 2 of 20 tumors exhibited phosphorylation of IRS-1 at S636/639, 18 of 20 tumors exhibited phospho-(S235) S6, indicative of mTOR activation and consistent with the high frequency of PI3K activation that is a hallmark of this disease (29). Therefore, despite activation of mTOR signaling in 18 of 20 tumors examined by Western blot analysis, IRS-1 was phosphorylated at S636/639 in only 2 of 9 IRS-1 expressing tumors, or 10% of all tumors examined, consistent with what was observed in CAH and EC by immunohistochemistry.

TSC2 is a gatekeeper for mTOR activity, and expression of this tumor suppressor is lost in a significant proportion of EC (13%), identifying loss of TSC2 as a mechanism for mTOR activation in this malignancy (15, 30). Eker ( $Tsc2^{Ekr/+}$ ) rats carry a germline defect in the *Tsc2* gene and are susceptible to the development of carcinomas of the kidney and leiomyomas of the reproductive tract (31). To determine if these animals also developed spontaneous endometrial hyperplasia or carcinoma, we examined uteri of aged (16 month) females and observed that 100% of



**Fig. 2.** Activation of mTOR signaling (increased phosphorylated S6 ribosomal protein) and loss of inhibition of IRS-1 in human endometrial carcinomas. A, immunohistochemistry of phosphorylated S6 ribosomal protein (a, c, and e) and serine phosphorylation of IRS-1 (b, d, and f) in normal human endometrium (benign), CAH, and EEC grade 1. Normal endometrium had high expression of phosphorylated (S636/639) IRS-1, with absent expression of phosphorylated (S235) S6. These expression patterns were altered in both CAH and EEC. B, Western blot analysis of expression of IRS-1 and S6 ribosomal protein and phosphorylation of IRS-1 (S636/639) and S6 (S235) in five different grade 1 endometrioid carcinoma lysates. The Western blot shows one tumor expressing phosphorylated (S636/639) IRS-1 and four different tumors with absent expression of phosphorylated (S636/639) IRS-1. All five tumors shown have high expression of phosphorylated (S235) S6. Original magnification,  $\times 200$ .

these animals had histologically detectable endometrial hyperplasia. Endometrial hyperplasia that developed in female Eker rats was characterized by glandular epithelium with papillary-shaped projections, nuclear stratification, and nuclear enlargement. There was also the presence of glandular proliferation and crowding, similar to that seen in human endometrial complex hyperplasia (Fig. 3A).

To determine if, similar to human endometrial hyperplasia, the development of endometrial hyperplasia in the Eker rat model was associated with activation of mTOR signaling, we evaluated S6 phosphorylation in these lesions by immunohistochemistry. The majority of microscopically normal endometrial glands adjacent to foci of endometrial hyperplasia did not express phosphorylated

S6 (Fig. 3E), and all exhibited strong immunoreactivity to phosphorylated S636/639 of IRS-1 (Fig. 3F). The absence of S6 phosphorylation and the presence of IRS-1 serine phosphorylation in the majority of histologically normal-appearing endometrial glands from these rats indicated that the mTOR signaling pathway was not constitutively activated in *Tsc2<sup>+/-</sup>* animals, and inhibition of the IGF-IR pathway via IRS-1 serine phosphorylation was intact. Similar to what was observed in human endometrial lesions, we found that 90% of individual foci of endometrial hyperplasia that developed in Eker rats showed strong (3+) phosphorylated S6 (Fig. 3B) staining and concomitant absent expression of serine-phosphorylated IRS-1 (Fig. 3C). Histologically normal glands were evaluated

**Table 1.** Immunohistochemical expression of phosphorylated S6 ribosomal protein and serine-phosphorylated IRS-1 in normal proliferative endometrium, endometrial CAH, and EEC grade 1

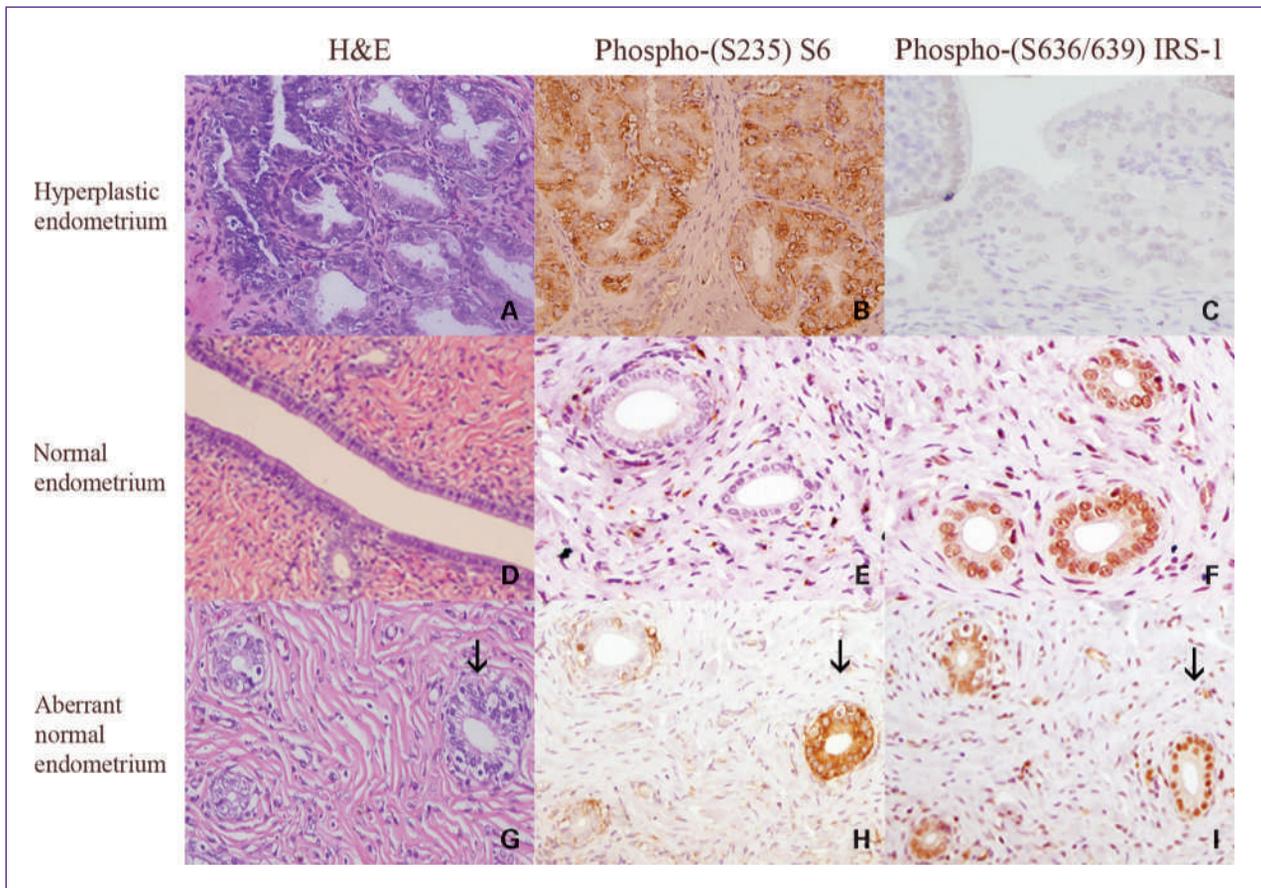
Group	Phospho-(S235) S6, n (%)	Phospho-(S636/639) IRS-1, n (%)
Normal (n = 6)	1 (17)	6 (100)
CAH (n = 25)	24 (96)*	5 (20)
EEC grade 1 (n = 10)	9 (90)*	2 (18)

NOTE: All of these tissues expressed IRS-1 and/or IRS-2. Results are expressed as the number of cases with 2+ or 3+ immunohistochemical expression. Correlation between expression of phospho-(S235) S6 and phospho-(S636/639) IRS-1 was determined by Pearson's correlation ( $P < 0.025$ ).

\*Statistically significant as compared with normal endometrium at the  $P < 0.001$  level by  $\chi^2$  analysis.

for expression of phosphorylated S6 and IRS-1 ( $n = 11$  rats;  $n = 844$  normal glands). Interestingly, whereas all of the histologically normal-appearing glands exhibited inhibitory IRS-1 phosphorylation at S636/639, occasionally

histologically "normal" glands (Fig. 3G) were positive for phosphorylated S6 (Fig. 3H), with phospho-S6 positivity observed in 51 of 844 (6%) of histologically normal glands. This suggests that activation of mTOR precedes



**Fig. 3.** Rat endometrial hyperplasia (A) has increased glandular crowding, increased glandular size, increased glandular complexity, and increased epithelial nuclear atypia compared with normal rat endometrial epithelium (D). Similar to humans, rat endometrial hyperplasia has high immunohistochemical expression of phosphorylated (S235) S6 (B) compared with normal endometrium (E). Similar to humans, rat endometrial hyperplasia has absent immunohistochemical expression of phosphorylated (S636/639) IRS-1 (C), whereas expression is retained in normal endometrium (F). The majority (94%) of normal endometrial epithelium do not express phospho-(S235) S6 (E) and retain negative feedback to IRS-1 (F). Very few (6%) of normal-appearing glands [indicated by the arrow (↓)] express phospho-(S235) S6 (H) and retain phospho-(S636/639) IRS-1 (I), suggesting that these glands are "preneoplastic." Data shown in H and I suggest that activation of mTOR signaling precedes the loss of negative feedback to IRS-1 in the progression to endometrial hyperplasia. Original magnification,  $\times 200$ .

loss of inhibitory IRS-1 phosphorylation, and that loss of IRS-1 inhibition, which is negatively correlated with phospho-S6 positivity in CAH and EC in the human disease, is associated with progression to hyperplasia in this rat model as well.

### Endometrial hyperplasias are dependent on mTOR signaling for growth

To determine whether mTOR activation directly contributes to the development of endometrial hyperplasia, we conducted a series of experiments using an mTORC1 inhibitor, the rapamycin analogue WAY-129327 (14, 26). Endometrial hyperplasia incidence, proliferation and apoptotic indices, expression of IRS-1 and IRS-2, and mTOR signaling pathway components were evaluated in 15-month-old Eker rats following a 2-week treatment with either vehicle or WAY-129327. In the vehicle-treated rats ( $n = 11$ ), endometrial hyperplasias were typically multifocal, with the majority of uterine cross-sections examined displaying foci of hyperplasia [54 hyperplastic foci/89 uterine cross-sections (61%); Table 2]. In approximately half of

the vehicle-treated rats (6 of 11), endometrial hyperplasia was more diffuse, with 50% or more of the uterine cross-sections from these 6 rats displaying involvement. All hyperplastic lesions in the vehicle-treated rat expressed either IRS-1 and/or IRS-2. In 50 of 54 hyperplastic lesions from the vehicle-treated rats, there was a significant correlation ( $P < 0.001$ ) of high expression of phosphorylated S6 and absent expression of IRS-1 serine phosphorylation (Table 3). As shown in Table 2, the incidence of endometrial hyperplasia was significantly decreased ( $P < 0.001$ ) from 100% in vehicle-treated rats to 50% in WAY-129327-treated rats, with the number of hyperplastic foci decreasing to 6 hyperplastic foci/90 uterine cross-sections (7%), with none of the uteri from WAY-129327-treated rats exhibiting diffuse endometrial hyperplasia. Importantly, whereas 100% of endometrial hyperplasias in vehicle-treated animals exhibited strong phosphorylated S6 expression (Fig. 4A), none of the uteri from rats treated with WAY-129327 expressed phosphorylated S6 in the endometrium (Table 3; Fig. 4B). Furthermore, in WAY-129327-treated rats, endometrial epithelial proliferation,

**Table 2.** Endometrial hyperplasia incidence and immunohistochemical expression of phosphorylated S6 ribosomal protein and serine-phosphorylated IRS-1 in vehicle- and WAY-129327-treated Eker ( $Tsc2^{+/-}$ ) rat endometrium

Treatment	No. of hyperplastic foci/no. uterine cross-sections	% Hyperplastic foci (+) for phospho-(S235) S6	% Hyperplastic foci (+) for phospho-(S636/639) IRS-1
Vehicle 1	2 of 2	100	0
Vehicle 2	1 of 1	100	0
Vehicle 3	1 of 8	100	0
Vehicle 4	7 of 10	100	0
Vehicle 5	6 of 12	100	0
Vehicle 6	5 of 6	100	0
Vehicle 7	3 of 12	100	0
Vehicle 8	8 of 9	100	0
Vehicle 9	3 of 10	100	0
Vehicle 10	8 of 9	100	0
Vehicle 11	10 of 10	100	0
Total	54 of 89 (61%)		
WAY-129327 1	1 of 8	0	0
WAY-129327 2	1 of 11	0	0
WAY-129327 3	1 of 10	0	0
WAY-129327 4	0 of 15	N/A*	N/A*
WAY-129327 5	0 of 11	N/A*	N/A*
WAY-129327 6	0 of 11	N/A*	N/A*
WAY-129327 7	0 of 13	N/A*	N/A*
WAY-129327 8	3 of 11	0	30
Total	6 of 90 (7%)		
$P^\dagger$	<0.001	<0.001	NS

NOTE: All rat endometrial sections expressed IRS-1 and/or IRS-2.

Abbreviation: NS, not significant.

\*Not applicable due to lack of endometrial hyperplasia.

†Statistical significance was evaluated between the vehicle- and WAY-129327-treated groups by  $\chi^2$  analysis.

**Table 3.** Association of strong immunohistochemical expression of phosphorylated S6 with absent expression of serine-phosphorylated IRS-1 in hyperplastic Eker (*Tsc2<sup>+/-</sup>*) rat endometrium

Rat	No. of hyperplastic foci with strong phospho-S6 expression and absent phospho-IRS-1 expression/total no. hyperplastic foci
Vehicle 1	2/2
Vehicle 2	1/1
Vehicle 3	1/1
Vehicle 4	6/7
Vehicle 5	6/6
Vehicle 6	4/5
Vehicle 7	3/3
Vehicle 8	6/8
Vehicle 9	3/3
Vehicle 10	8/8
Vehicle 11	10/10
Total	50/54 (93%)*

NOTE: In hyperplastic foci, strong expression of phosphorylated S6 is significantly associated with absent expression of serine-phosphorylated IRS-1.

\*Correlation between phospho-S6 expression and phospho-(S636/639) IRS-1 expression was evaluated by Pearson correlation analysis (statistically significant at the  $P < 0.001$  level).

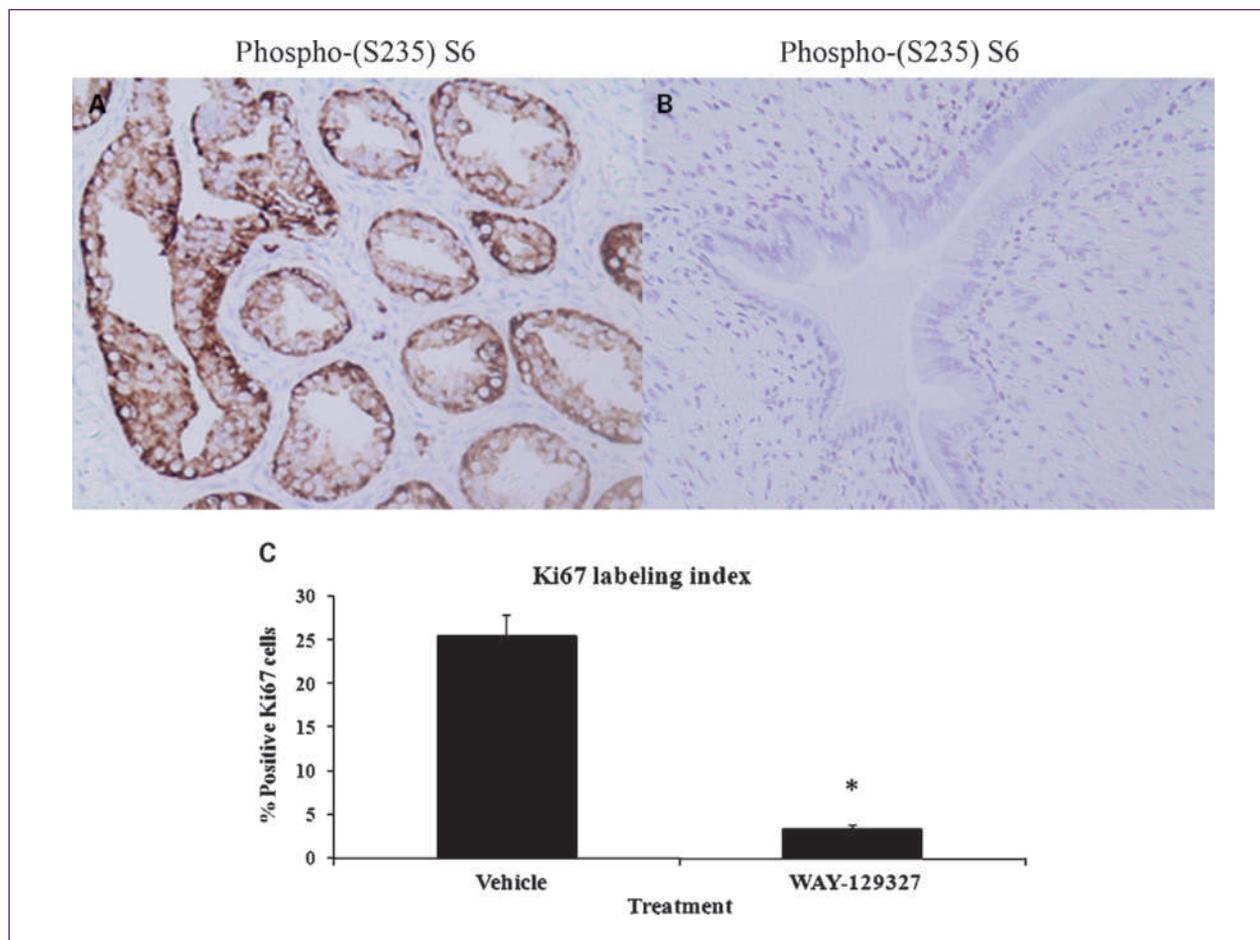
as assessed by the immunohistochemical expression of Ki67 nuclear antigen, was significantly decreased compared with vehicle-treated controls (Fig. 4C). Endometrial apoptotic indices as measured by TUNEL were low in both vehicle- and WAY-129327-treated rats (data not shown). Thus, inhibition of mTOR signaling by WAY-129327 resulted in a significant reduction in endometrial hyperplasia incidence and decreased proliferative indices.

## Discussion

Loss of growth inhibition via receptor tyrosine kinase (IGF-IR) and PI3K activation seems to be an important theme in endometrial cancer pathobiology. PTEN phosphatase negatively regulates IGF-I signaling by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol-4,5-bisphosphate, thereby preventing activation of Akt (32). PTEN activity is decreased in 55% of endometrial CAH and up to 83% of EC (33). In a recent study, our group identified loss of expression or function of LKB1 and TSC2 in ECs that showed frequent activation of mTOR signaling (30). Our group has shown that for human endometrial hyperplasia and grade 1 EC, IGF-IR activation and Akt signaling are critical events (34). We and others have also shown that a large percentage of ECs have defects in PTEN, TSC2, and LKB1 tumor suppressors and a high frequency of activating mutations in PIK3CA (30, 35, 36). These endometrial signaling pathways are also important in rodent models of endometrial hyperplasia and carcinoma, as mice with defects in PTEN (37) and LKB1 (35) exhibit these pathologies. We previously

reported that female rats exposed neonatally to diethylstilbestrol, a xenoestrogen, developed endometrial hyperplasia in 60% of exposed animals by 5 months of age (27). We found that dysregulation in IGF-IR signaling pathway components, including activation of AKT and IRS-1, occurred in the hyperplastic lesions that developed in neonatal diethylstilbestrol-exposed rats (27). It is therefore clear that IGF-IR signaling is an important event in the majority of ECs. Interestingly, in the present study, we found a small number of EC cases in which both IRS-1 and IRS-2 were lost, implying that pathways independent of IGF-IR may be important in a subset of ECs. It is well known that unopposed estrogen is a risk factor for EC and that the ER $\alpha$  and IGF-IR pathways crosstalk (28). In addition to crosstalk with IGF-IR, the ER $\alpha$  is also known to crosstalk with the mitogen-activated protein kinase pathway, and this may constitute an alternative pathway in this disease (38).

The Eker rat is a well-established model of tumorigenesis of the reproductive tract that has been used extensively as a model for the study of uterine leiomyoma (39). In this model, uterine leiomyomas and renal carcinomas develop subsequent to the loss of function of the second TSC2 allele (31). We observed that at 16 months, 100% of Eker rats develop endometrial hyperplasia and that endometrial hyperplasia in these animals is characterized by activation of mTOR signaling. Whether the development of endometrial hyperplasia is due to the loss of TSC2 function is not yet known, but is likely because activation of mTOR signaling and phosphorylation of S6 ribosomal protein were identified in 100% of endometrial hyperplastic foci, and TSC2 is known to be an inhibitor of mTOR



**Fig. 4.** Immunohistochemistry for phosphorylated (S235) S6 ribosomal protein and quantitation of Ki67 labeling indices in vehicle- and WAY-129327-treated Eker (*Tsc2<sup>+/-</sup>*) rats. WAY-129327 treatment significantly decreased endometrial phosphorylated (S235) S6 immunoreactivity (B) compared with vehicle-treated controls (A). Immunohistochemistry,  $\times 200$ . WAY-129327 treatment also significantly reduced the endometrial epithelial Ki67 labeling index (C). Cell proliferation was scored by calculating the percentage of Ki67-positive endometrial epithelial cells per 100 epithelial cells for endometrial hyperplastic lesions from vehicle-treated controls ( $n = 54$  lesions) and from hyperplastic lesions in the WAY-129327-treated rats ( $n = 6$  lesions). Original magnification,  $\times 200$ . Columns, mean; bars, SE. \*,  $P < 0.05$ .

activation (15). Similar to human endometrial CAH, loss of negative feedback inhibition of IRS-1 was identified in nearly all of the Eker rat endometrial hyperplastic foci, indicating that this characteristic of the neoplastic progression from normal endometrium to endometrial hyperplasia is conserved across species, a powerful indicator of its importance in disease progression.

Interestingly, in both the human samples and in the rat model, we occasionally identified endometrial glands that were microscopically normal but expressed phosphorylated S6. This observation was made in both normal human and rat endometrial glands. All of the microscopically normal endometrial glands with positive expression of phosphorylated S6 also retained positive expression for serine-phosphorylated IRS-1. This suggests that mTOR activation can occur early in endometrial glands that are microscopically normal ("pre-endometrial hyperplasia"), whereas loss of serine-phosphorylated IRS-1 was associat-

ed with progression to hyperplasia. Our data suggest a progression sequence in which activation of mTOR (via loss of TSC2 or other events) is followed by loss of the negative feedback to IRS-1 in the early stages of development of endometrial hyperplasia. Mutter et al. (40) have also identified molecular defects in microscopically normal human endometrium, namely, loss of PTEN expression. Such PTEN-null, microscopically normal endometrial glands persist between menstrual cycles (41), but regress with progestin therapy (42). Importantly, loss of PTEN and upregulation of Akt would also lead to phosphorylation and inactivation of TSC2, resulting in loss of mTOR repression by this tumor suppressor. In our study, treatment with the mTOR inhibitor WAY-129327 was not associated with reexpression of serine-phosphorylated IRS-1. This suggests that some early defects in the progression of normal endometrium to endometrial hyperplasia, such as loss of PTEN expression, are potentially reversible, whereas other

molecular defects, such as loss of serine-phosphorylated IRS-1, may not be reversible. Alternatively, other pathways not affected by WAY-129327 treatment, such as mitogen-activated protein kinase signaling, may be regulating IRS-1 phosphorylation.

In normal metabolic tissues such as white adipose tissue, liver, and skeletal muscle of both humans and rats, the IGF-IR/Akt/mTOR signaling pathway is negatively regulated by serine phosphorylation of IRS-1 and IRS-2 (17, 22, 23). This negative feedback to IRS-1/2 via S6K normally prevents rampant activation of insulin signaling (19). Numerous epidemiologic studies have shown that obesity is strongly associated with an increased risk of endometrial hyperplasia and EC; in fact, EC is the cancer most closely associated with obesity (6, 43, 44). Hyperinsulinemia and the insulin-resistant state are closely associated with obesity. Using serum adiponectin as a surrogate marker of insulin resistance, a number of groups have documented that insulin resistance is an independent risk factor for EC (45). Despite the link with obesity and insulin resistance, abnormal physiologic states that are typically associated with increased serine phosphorylation of IRS-1, the majority of endometrial hyperplasias and cancers examined in this study had loss of serine-phosphorylated IRS-1, irrespective of body mass index. The basis for the loss in serine-phosphorylated IRS-1 in endometrial hyperplasia and cancer cells is not clear at this point. However, our results from this work and from our previous studies (34) show that there are at least two different molecular mechanisms for increased signaling via the IGF-IR pathway in endometrial hyperplasia and cancer: upregulation of IGF-IR and downregulation of the negative feedback component, serine-phosphorylated IRS-1.

Preclinical studies using rapamycin and its analogues have shown decreased growth of tumors, particularly in those that have reduced expression of PTEN or overexpression of AKT (46). Neshat et al. compared the growth of PTEN<sup>+/+</sup> and PTEN<sup>-/-</sup> tumors either produced by s.c. injection of murine embryonic stem cells into immunodeficient nude mice or in androgen-dependent

human prostate cancer xenografts in male severe combined immunodeficient mice treated with a rapamycin analogue (CCI-779). The growth of PTEN<sup>+/+</sup> tumors was slowed by treatment with a rapamycin analogue but was completely blocked in PTEN<sup>-/-</sup> tumors in both models, showing an enhanced dependence on mTOR for tumor growth in the absence of PTEN expression (46). In addition, decreased neoplasia incidence with treatment with rapamycin analogues has been identified in endometrial hyperplasia in the PTEN<sup>+/+</sup> mouse model, prostate intraepithelial neoplasia in mice expressing Akt1, alveolar epithelial neoplasia induced by oncogenic K-ras, and in a mouse model of ductal carcinoma *in situ* (47–50). In our study, a 2-week treatment of Eker rats with the rapamycin analogue WAY-129327 resulted in a significant decrease in endometrial hyperplasia incidence, decreased endometrial epithelial proliferative indices, and inhibition of mTOR signaling in the endometrium. These results suggest the possible utility of using analogues of rapamycin as chemopreventive agents for women with endometrial hyperplasia to prevent the development of endometrioid-type endometrial carcinoma.

#### Disclosure of Potential Conflicts of Interest

H.A. Harris, J.S. Crabtree, and R.C. Winneker were employees of Wyeth Research at the time these studies were performed. Studies using the proprietary Wyeth drug WAY-129327 were supported in part by a Sponsored Research agreement from Wyeth Research to MD Anderson. The other authors disclosed no potential conflicts of interest.

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# Cancer Prevention Research

## Loss of Inhibitory Insulin Receptor Substrate-1 Phosphorylation Is an Early Event in Mammalian Target of Rapamycin–Dependent Endometrial Hyperplasia and Carcinoma

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