

## Molecular Identification of Latent Precancers in Histologically Normal Endometrium<sup>1</sup>

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### Abstract

Discovery of somatically mutated cells in human tissues has been less frequent than would be predicted by *in vitro* mutational rates. We analyzed the *PTEN* tumor suppressor gene as an early marker for endometrial carcinogenesis, and we show that 43% of histologically normal premenopausal endometria contain rare glands that fail to express *PTEN* protein because of mutation and/or deletion. These persist between menstrual cycles. Histopathology of *PTEN*-null glands is initially unremarkable, but with progression, they form distinctive high-density clusters. These data are consistent with a progression model in which initial mutation is not rate limiting.

### Introduction

The endometrium is a highly proliferative and cyclically regenerative tissue in which loss of *PTEN* tumor suppressor gene function heralds the beginning of multistep carcinogenesis. Loss of *PTEN* function occurs in approximately 50% (1) of all endometrial carcinomas, increasing to 83% (2) for tumors with adjacent premalignant lesions. An association between *PTEN* mutation and endometrial cancer risk is further supported by animal studies. Heterozygote *pten* mutant mice uniformly (100%) develop endometrial "hyperplasia," and 21% of these progress to carcinoma (3). E1N<sup>3</sup> lesions of humans, which are monoclonal (4) and frequently *PTEN* mutation-bearing (2), are thought to be precursors of endometrioid carcinoma but cannot be diagnosed in routine histological sections until localized clusters of neoplastic glands have reached a critical dimension (generally over 1 mm) characterized by a crowded architecture and altered cytology (5). *PTEN* mutation appears to be coincident with initiation of these clones, in the manner of a gatekeeper for endometrial carcinogenesis (6).

We previously showed acquisition of *PTEN*-null endometrial glands in 29% of a very small series of endometria exposed to unopposed estrogens, a known epidemiological risk factor for endometrial adenocarcinoma (2). It is unlikely that these glands acquired mutations within the narrow interval of unopposed estrogen exposure typical of a single abnormal menstrual cycle. More likely, *PTEN*-null clones are generated over a lifetime of menstrual cycles and retained for a long interval. This was posed as a testable hypothesis: sporadic somatic endometrial mutation of the *PTEN* gene is frequent in the histologically unremarkable endometrium of women of reproductive

age, and, once generated, *PTEN*-null clones are retained or regenerated between menstrual cycles.

From our previous work, we know that immunohistochemically *PTEN*-null glands are likely to harbor *PTEN* mutations and/or deletions (2). Therefore, we used highly sensitive *PTEN* immunohistochemistry to screen histologically normal proliferative endometrium of endogenously cycling (not on replacement hormones) young premenopausal women (<40 years) for *PTEN*-null glands, and then analyzed these glands for *PTEN* mutation and deletion. Continued presence of *PTEN*-null glands was evaluated in another series of patients who had paired proliferative endometrial samples separated by at least one menstrual cycle. The prevalence and morphology of *PTEN*-null glands in persistent proliferative (endometria exposed to a protracted nonphysiological estrogen interval) and E1N endometria, was also determined by immunohistochemistry. E1N (7) is used to describe readily diagnosable lesions (5) that have been shown by computerized morphometric analysis (4) to have diagnostic features that increase risk for concurrent (8) or future (9) endometrial adenocarcinoma.

### Materials and Methods

**Case Selection.** One hundred thirty-eight paraffin-embedded endometrial biopsies and curettings obtained in 1998–2000 (Department of Pathology, Brigham and Women's Hospital) were allocated to proliferative, persistent proliferative, or E1N diagnostic classes based on slide review consensus of two gynecological pathologists (G. L. M. and T. A. I.). "Normal" proliferative endometria all came from premenopausal women <40 years of age (average age, 34.0 ± 4.5) who were not taking supplemental hormones. Persistent proliferative endometria (mean age, 45.2 ± 9.3) had mitotically active but cytologically uniform glands with occasional cystically dilated glands, and were ascribed either to endogenous (anovulation) or exogenous (pharmacological) estrogen sources based on clinical history. Endometrial polyps disqualified a case from the proliferative and persistent proliferative categories. E1N diagnosis (mean age 54.1 ± 7.8) was made visually, according to published criteria (5). We have not used the WHO endometrial hyperplasia classification system in our studies because of its poor reproducibility, and discordance with discrete biological groups defined by genetic analysis (4).

Ninety repeat biopsies were retrieved by diagnostic review from 45 individual women with proliferative endometrium on more than one occasion. Most repeat biopsies were symptomatically indicated (usually bleeding), but some were incidental to unrelated findings such as uterine fibroids or polyps.

**Immunohistochemistry.** Dewaxed rehydrated 4- $\mu$ m paraffin sections underwent microwave antigen retrieval before adding primary anti-*PTEN* antibody 6H2.1 (Cascade Biosciences, Winchester, MA) at 1:300 dilution. Anti-estrogen receptor antibody ER-1D5 (Dako), and anti-progesterone receptor antibody IA6 (Dako) were used at 1:300 and 1:100 dilutions, respectively. Primary antibody was incubated overnight at 4°C, washed, incubated with appropriate secondary biotinylated immunoglobulin (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) and signal detected by sequential addition of avidin peroxidase and 3,3'-diaminobenzidine. Epithelial staining was scored by two pathologists (G. L. M., T. A. I.) using endometrial stroma and/or normal endometrial epithelium as an internal positive control and negative run controls without addition of primary antibody. All of the tissue fragments were

Received 3/9/01; accepted 4/12/01.

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<sup>1</sup> Supported by the American Cancer Society (RPG98-211-01CCE) and the United States Army Breast Cancer Research Program (to C. E.), 28-1203 from the Health Research and Development Council of The Netherlands (to J. P. A. B.) and P30CA16058 from the National Cancer Institute (to the Ohio State University Comprehensive Cancer Center).

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<sup>3</sup> The abbreviations used are: E1N, endometrial intraepithelial neoplasia; DGGE, denaturing gradient gel electrophoresis.

Table 1 Clinicopathological features of endometria by PTEN immunohistochemistry and slide diagnosis

|  | % PTEN-null by diagnosis (fraction) |                          |              |               |
|--|-------------------------------------|--------------------------|--------------|---------------|
|  | Proliferative                       | Persistent proliferative | EIN          | Total         |
| Sample   |                                     |                          |              |               |
| Curettage  | 42.9 (6/14)                         | 54.5 (6/11)              | 75.0 (6/8)   | 54.5 (18/33)  |
| Biopsy   | 42.9 (18/42)                        | 56.7 (17/30)             | 59.3 (16/27) | 51.5 (51/99)  |
| Clinical indication                              |                                     |                          |              |               |
| Bleeding   | 42.9 (12/28)                        | 56.3 (18/32)             | 55.0 (11/20) | 51.3 (41/80)  |
| Infertility/recurrent abortion                   | 41.7 (5/12)                         | 0.0 (0/0)                | 100.0 (1/1)  | 46.2 (6/13)   |
| Prior hyperplasia                                | 0.0 (0/1)                           | 0.0 (0/0)                | 71.4 (5/7)   | 75.0 (6/8)    |
| Anatomic (fibroids, septum, polyp, thick stripe) | 50.0 (3/6)                          | 100.0 (1/1)              | 100.0 (1/1)  | 62.5 (5/8)    |
| Other (pain, endometriosis)                      | 20 (1/5)                            | 33.3 (1/3)               | 0.0 (0/2)    | 20.0 (2/10)   |
| Unspecified                                      | 75.0 (3/4)                          | 60.0 (3/5)               | 100 (4/4)    | 76.9 (10/13)  |
| Hormones   |                                     |                          |              |               |
| Endogenous                                       | 42.9 (24/56)                        | 55.9 (19/34)             | 64.3 (18/28) | 51.7 (61/118) |
| Exogenous  | 0.0 (0/0)                           | 57.1 (4/7)               | 57.1 (4/7)   | 57.1 (8/14)   |
| Menopause  |                                     |                          |              |               |
| Pre  | 42.9 (24/56)                        | 57.1 (20/35)             | 66.7 (14/21) | 51.8 (58/112) |
| Post   | 0.00 (0/0)                          | 50.0 (3/6)               | 57.1 (8/14)  | 55.0 (11/20)  |
| Total  | 42.9 (24/56)                        | 56.1 (23/41)             | 62.9 (22/35) | 52.3 (69/132) |

examined, and individual glands were scored as PTEN null when signal was absent in the nuclear and cytoplasmic compartments of most cells in that gland. Hormone receptors were scored by signal intensity in the nuclear compartment.

**Genomic Analysis.** Matched PTEN expressing and nonexpressing proliferating endometrial epithelial cells were sampled using laser capture microdissection directed by PTEN immunohistochemistry of flanking serial sections. Approximately 10–50 ng of DNA per sample was PCR amplified using primers which define the coding region and flanking introns of all 9 *PTEN* gene exons. PCR products were subjected to DGGE, which in our hands is virtually 100% sensitive and specific in detecting sequence-confirmed PTEN mutations (10). DNA samples that show DGGE variants are resubjected to PCR and semi-automated direct sequencing (ABI377a or PE3600).

For each patient, DNA from PTEN expressing and nonexpressing epithelial cells was subjected to PTEN deletion analysis by PCR using 5'-tagged fluorophor primers, which amplify microsatellites flanking and within the *PTEN* gene, D10S579, D10S2491, and D10S541, and then electrophoresed through an ABI377a gel and analyzed with GeneScan software (11). Marker heterozygosity manifests as two peaks on a GeneScan gel, representing two different alleles present at that marker. Matched sets of DNA samples from PTEN expressing and nonexpressing glands are compared at each marker, and if one peak is reduced by at least one-third, loss of heterozygosity has occurred, which represents deletion of one of the alleles and, usually, that chromosomal region.

**Morphometry.** A 1-mm circular window (surface area, 0.785 mm<sup>2</sup>) containing 100 randomly distributed points was superimposed on digitized photomicrographs of PTEN immunohistochemically stained endometria, and points over the fragment of interest (*PTS100*) tallied by composition of underlying tissue [stroma (*STROMA100*); PTEN-expressing or "positive" glands (*POS100*); PTEN-null glands (*NULL100*). Excluded were seven fragmented or small (<1/2 sample window) samples; four cases diagnosed on H&E slides as EIN, in which the targeted PTEN-null glands did not involve the EIN focus; and one PTEN-null EIN focus, which was distorted by tangential sectioning on recut. Surface area assigned to glands included combined epithelial and luminal compartments. Geometric centroids of each gland profile were marked, and the number of PTEN-null gland centroids (*NULLCT*) and PTEN-expressing gland centroids (*POSCT*) that were within the window were counted. Variables were calculated as follows: (a) volume percentage stroma (*VPS*) = 100 × (*STROMA100/PTS100*); (b) volume percentage PTEN-null gland (*VPNULL*) = 100 × (*NULL100/PTS100*); (c) volume percentage PTEN-positive gland (*VPPOS*) = 100 × (*POS100/PTS100*); (d) density of PTEN-null glands (*DENNULL*) = (*NULLCT/PTS100*) × (100 points in window/0.785 mm<sup>2</sup> window size); (e) density of PTEN-positive glands (*DENPOS*) = (*POSCT/PTS100*) × (100 points in window/0.785 mm<sup>2</sup> window size); (f) size of PTEN-null glands (*SZNULL*) = (*NULL100/NULLCT*) × (0.785 mm<sup>2</sup>/100 points in window); and (g) size of PTEN-expressing glands (*SZPOS*) = (*POS100/POSCT*) × (0.785 mm<sup>2</sup>/100 points in window).

## Results

PTEN-null endometrial rates were 43, 56, and 63% in proliferative, persistent proliferative, and EIN diagnostic categories, respectively

(Table 1; Fig. 1; all of the histological images are available online).<sup>4</sup> There was a linear trend by decade of age for increasing PTEN-null rates in older women (Coachman's test of linear trend, *P* = 0.014). Average age of women with and without PTEN-null glands was 43.8 ± 9.7 and 40.2 ± 11.6 years, respectively. PTEN-null glands in the three diagnostic groups are present in women biopsied for a variety of reasons; therefore, these results are applicable to a broad range of women seeking routine medical care (Table 1).

The occurrence of PTEN-null glands in 43% (24 of 56) of histologically normal proliferative endometrium (confirmed by staining two sections in each case) was unexpectedly high. In general, only a few histologically unaltered glands were PTEN-null among hundreds of proliferating glands in these otherwise unremarkable endometria. Because PTEN expression responds to the hormonal environment (12), estrogen and progesterone receptor immunohistochemistry were performed on flanking serial tissue sections and showed in all cases that the PTEN-null and -expressing glands in proliferative endometria retained comparable receptor quantities. Nineteen of 24 proliferative endometria with PTEN-null glands had sufficient material for microdissection. Matched DNA from PTEN-expressing and -nonexpressing glands from the same patient were coprocessed for direct comparison of PTEN mutation and deletion.<sup>5</sup> All of the PTEN-expressing matched control glands had a wild-type (normal) genotype, whereas 84% (16 of 19) of nonexpressing glands had a mutation (*n* = 8) and/or loss of at least one 10q23 heterozygous marker (*n* = 13) in the region of the *PTEN* locus.

The appearance of rare histologically normal glands harboring *PTEN* mutations would be inconsequential if they are completely shed with normal menstruation. We, thus, performed PTEN immunohistochemistry on 34 premenopausal women (no hormonal therapy; average age, 42.3 ± 6.2 years) with unremarkable proliferative endometrium on two separate occasions (interval averaged 400 days; range, 26–1167 days). Twelve of 34 women had PTEN-null glands initially (Table 2), scattered throughout varying depths of the endometrial thickness, and 83% (10 of 12) of these continued to be present on follow-up. PTEN status of paired biopsies in Table 2 is highly associated with initial phenotype (Fisher's exact test, *P* = 0.01; odds ratio, 10.71). A woman with PTEN-null glands in her endometrium is five times more likely to have PTEN-null glands on repeat biopsy than not. A separate series of 11 postmenopausal women (mean age, 58.1 ± 2.6 years) with two proliferative endometria separated by an

<sup>4</sup> Internet address: [www.endometrium.org](http://www.endometrium.org).

<sup>5</sup> Detailed genotype table, and additional supplemental data, are available at Cancer Research Online [<http://cancerres.aacrjournals.org>].

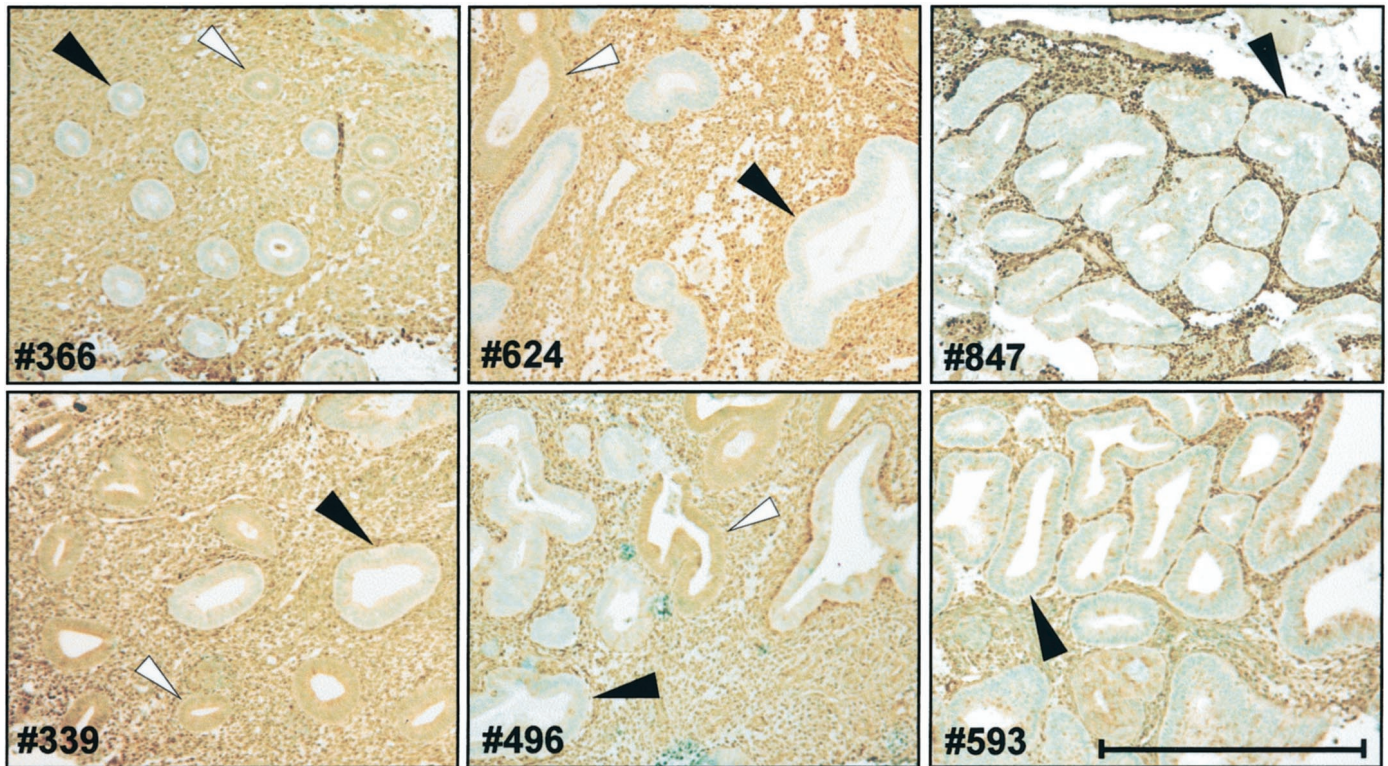


Fig. 1. PTEN immunohistochemistry in endometrium. PTEN-null glands (black arrowheads) appear pale green against dark brown staining of surrounding stroma in proliferative (left, cases 339, 366), persistent proliferative (center, cases 624, 496), and EIN (right, cases 847, 593) endometria from six different patients. Brown PTEN-positive glands (white arrowhead) are present in proliferative and persistent proliferative examples for comparison. Scale bar, 500  $\mu$ m.

average of 494 days (range, 142–985 days) and sampled during the estrogenic phase of sequential estrogen/progestin replacement therapy were used for PTEN immunohistochemistry. Two had PTEN-null glands initially, retained by both on follow-up. 11% (1 of 9) with PTEN-expressing first biopsies developed PTEN-null glands in the second biopsy. These 11 patients are not shown in Table 2. Combined statistical analysis of all repeat biopsies studied (34 premenopausal, 11 post-menopausal) also shows a high association of PTEN status between first and second biopsy (Fisher's exact test,  $P < 0.001$ ; odds ratio, 17.3).

Changes in the histological structure of PTEN-null clones were documented by morphometric analysis of PTEN-immunostained normal proliferative, persistent proliferative, and EIN endometria (Fig. 2).

## Discussion

Insights into the earliest stages of human carcinogenesis are limited by our ability to identify precursor lesions *in vivo*. Estimates of the rate of sporadic mutagenesis in human cells, on the order of  $10^{-7}$  mutations per gene per cell division (13), suggest that the number of cells with "first hits" of a multistep carcinogenesis (14) pathway may number in the hundreds for every gram ( $10^9$  cells/gram) of proliferative tissue. Although it has been possible *in vitro* to directly observe such events using sensitive model systems, complex primary tissues

present many confounding factors including sampling errors, insensitive methods of ascertainment, and dynamic fluxuations in the selection of altered clones.

Initiation of *PTEN*-mutant clones is common in endometria of premenopausal women and, once acquired, is stably maintained by a cell population that is incompletely shed during menses. These findings were possible because of the unique suitability of endometrium for discovery and surveillance of acquired somatic mutations. Easily sampled superficial endometrium is regenerated cyclically from a deeper basal layer; therefore, the resultant biopsy genotype reflects that of the functional stem cells in this tissue and can be nondestructively monitored on multiple occasions. Loss of PTEN protein in proliferative glands cannot be attributed to local estrogen unresponsiveness, because they retain estrogen and progesterone receptors at normal levels. Rather, mutation and/or deletion of the *PTEN* gene in 84% of PTEN-null glands microdissected from histologically normal proliferative endometrium confirms that this is a primary event capable of being perpetuated in the mutant clones. In fact, PTEN-null cells that we observed had undergone some clonal expansion, forming entire glands devoid of PTEN protein that contrast clearly with surrounding stroma and unaffected glands. Mutations in these "normal-appearing" tissues are found only after immuno-directed microdissection. In bulk tissue, they would be easily overlooked because of dilution, and this may be the reason for underestimation of mutations in normal-appearing tissue in earlier studies (2). Our data show that mutational events that are rare for a single cell may achieve a high prevalence if the tissue contains a large number of dividing cells (the average woman grows about 1 kg of endometrial tissue in her reproductive years) with strong positive selection and/or retention factors.

PTEN immunohistochemistry is able to bring out what is perhaps the earliest stage of nonfamilial carcinogenesis yet identified in any human tissue, before histological change is manifest and in patients

Table 2. *PTEN* status in repeat biopsies of premenopausal women with endogenously cycling proliferative endometrium<sup>a</sup>

|                            | Second sample<br>PTEN-positive | Second sample<br>PTEN-null | Total |
|----------------------------|--------------------------------|----------------------------|-------|
| First sample PTEN-positive | 15                             | 7                          | 22    |
| First sample PTEN-null     | 2                              | 10                         | 12    |
| Total                      | 17                             | 17                         | 34    |

<sup>a</sup> Initial (first sample) and repeat (second sample) endometrial samples scored as PTEN-nonexpressing (null) or having only PTEN-expressing glands (positive).

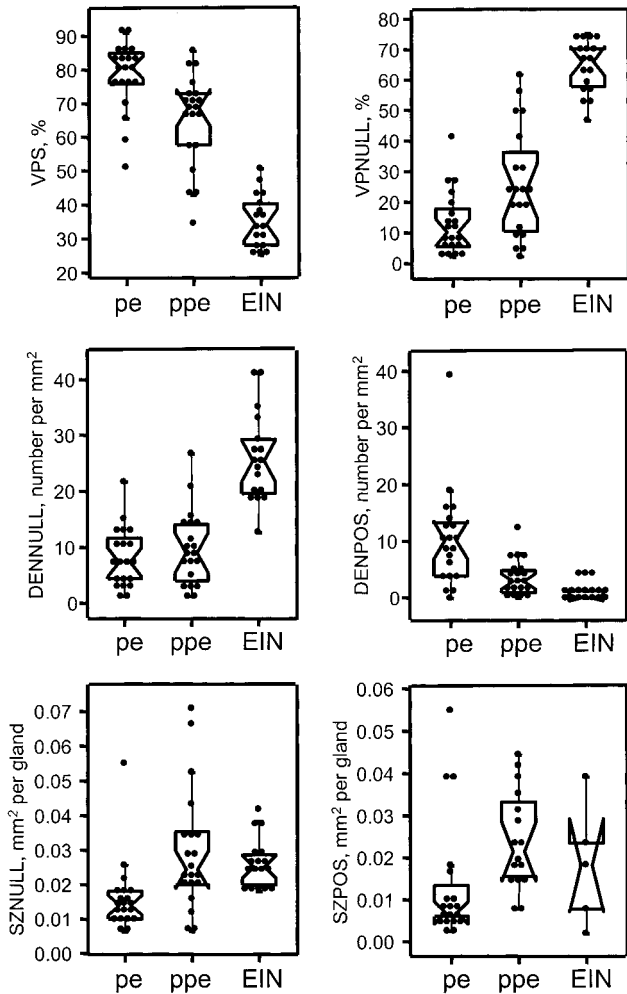


Fig. 2. Morphometric progression of PTEN-null glands. Box-plot of PTEN-null gland morphometric variables (Y axis), X axis arranged from left to right in order if a putative progression sequence of proliferative (PE,  $n = 20$ ), persistent proliferative (PPE,  $n = 20$ ), and EIN ( $n = 17$ ) endometria stained for PTEN protein. Volume percentage stroma (VPS) declines steadily because of increasing PTEN-null gland volume (VPNULL). Two elements sequentially contribute to rising VPNULL. The first, in the proliferative-to-persistent-proliferative transition, is enlargement of individual PTEN-null glands (SZNULL). The density of PTEN-null glands (DENNULL) then increases between persistent proliferative and EIN endometria. PTEN-expressing glands increase in size (SZPOS) in the proliferative-to-persistent-proliferative transition but rapidly decline in density.

without concurrent carcinoma. Almost all of the well-known examples of mutation-bearing premalignant disease, including EIN, colonic polyps, and *in situ* carcinomas, have morphological and cytological changes recognizable by light microscopy, which indicates a significant change in phenotype. Precedents in the colon (15) and breast (16) in which histologically normal epithelium may bear genetic changes as seen in adjacent concurrent adenocarcinomas have not yet been demonstrated in patients without cancer. The fact that >80% of PTEN-null glands in proliferative endometrium have *PTEN* mutations seen in endometrial cancer suggests that it is this subpopulation of glands that preferentially will give rise to EIN.

The fate of PTEN-null endometrial clones throughout the life cycle and under varying hormonal conditions will provide new insights into endometrial carcinogenesis. Although ~40% of premenopausal women acquire PTEN-null glands, only 2.4% (17) of them will ever get endometrial cancer, most following menopause. This suggests that acquiring an initial mutation is not the rate-limiting step in endometrial carcinogenesis. Once initiated, ~15% (4) of premalignant endometrial lesions have microsatellite instability, expected to confer a

mutator phenotype that may accelerate subsequent progression to carcinoma. Expansion or contraction of mutant clones would also be expected to modify the likelihood that additional genetic mutations will occur in a cell already having a first mutation (18). Hormonal factors are attractive as putative modulators of this process, because mutant clones lose their normal ability to increase *PTEN* expression (12) in response to estrogens. Unopposed estrogens increase endometrial cancer risk for several years after exposure (19), and this may be reduced or erased by subsequent administration of oral contraceptives (20). We present limited, repeat biopsy data from 11 postmenopausal women on combinatorial hormonal replacement therapy, which shows a lower prevalence (18%; 2 of 11) and acquisition rate (11%; 1 of 9) of PTEN-null glands compared with endogenously cycling premenopausal women (35% or 12 of 34 and 32% or 7 of 22, respectively). This may indicate either an age-related phenomenon or a suppression of PTEN-null clones by pharmacological means. Distinguishing between these possibilities is of great interest in defining a mechanism whereby endogenous and exogenous hormonal factors modify endometrial cancer risk.

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# Cancer Research

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*Cancer Res* 2001;61:4311-4314.

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