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Three-Dimensional Nuclear Telomere Architecture Changes During Endometrial Carcinoma Development

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Abstract

Endometrioid or type-I endometrial carcinoma (EC) develops from hyperproliferative glandular pathologies. Inactivation of the tumor suppressor gene PTEN is frequently associated with type-I EC. Using a previously characterized Pten heterozygous (Pten^{+/-}) mouse model, this study investigates the three-dimensional (3D) telomere profiles during progression from hyperplastic lesions to EC to test the hypothesis that altered 3D telomere profiles can be detected prior to Pten loss in early hyperproliferative lesions. We used immunohistochemistry and 3D-telomere fluorescent in-situ hybridization to investigate Pten expression, telomere length and signal distribution, average number and spatial distribution of telomeres and formation of telomere aggregates in uterine glandular epithelial cells from wildtype and Pten^{+/-} mice. Pten showed nuclear and cytoplasmic localization in WT, predominantly cytoplasmic staining in simple hyperplasia (SH) and was markedly reduced in atypical hyperplasia (AH). Telomere length in glandular epithelial cells does not shorten with age. The average number of telomeres per nucleus was not different in WT and Pten^{+/-} mice indicating the lack of substantial numeric chromosome aberrations during EC development. We observed telomere aggregates in lesions of AH and EC. SH lesions in Pten^{+/-} mice differed from normal glandular epithelium by an increased relative number of shorter telomeres and by a telomere signal distribution indicative of a heterogeneous cell population. Our study revealed that alterations in the nuclear 3D telomere architecture are present in early proliferative lesions of mouse uterine tissues indicative of EC development. The changes in telomere length distribution and nuclear signal distribution precede the loss of Pten. © 2013 Wiley Periodicals, Inc.

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Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Endometrial carcinoma (EC) is the most common gynecological cancer, and accounts for more than 40,000 new cases in the US (Jemal et al., 2010). Endometrial precancerous lesions, as endometrial hyperplasia, may undergo malignant transformation (Koul et al., 2002; Lacey et al., 2008a, 2010). The cases where this initial hyperplasia is diagnosed consist of various histology types that may or may not progress into EC (Bergeron et al., 1999; Hecht et al., 2005). To overcome the diagnostic uncertainty, pathologists are using computerized morphometric analysis and molecular genetics classification that improve but do not entirely solve this problem (Hecht et al., 2005). Endometrioid carcinoma, or EC type I, represents 80% of the cases of EC and it is associated with unbalanced estrogen stimulation in the endometrium (Koul et al., 2002). Mutations in the *PTEN* gene and microsatellite instability (MI) are common genetic changes in EC type I (Latta and Chapman 2002). *PTEN* inactivation occurs in 35–50% of all cases of EC type I (Lax, 2004). This occurs through mutations, loss of heterozygosity or promoter methylation (Bonneau and Longy, 2000; Stambolic et al., 2000). Furthermore, *PTEN* mutations play an early role in this type of cancer by altering normal cell growth, apoptosis, metabolism and migration via negatively regulating the AKT pathway (Chow and Baker, 2006; Di Cristofano and Pandolfi, 2000; Kanamori et al., 2001; Levine et al., 1998; Mutter et al., 2000a). Endometrial glands that lack *PTEN* proliferate excessively in an estrogen independent way (Saito et al., 2011). We have previously demonstrated the role of *PTEN* in normal development and carcinogenesis by creating *Pten* deficient mice (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 2000; Suzuki et al., 1998). Homozygous *Pten* mutant mice are embryonic lethal (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998), demonstrating the essential role of *Pten* in normal development. Heterozygous mice spontaneously develop tumors of the breast, thyroid, or endometrium similar to Cowden's syndrome in humans which is associated with *PTEN* mutations (Blumenthal and Dennis, 2008; Gustafson et al., 2007). Biallelic inactivation of *Pten* occurs early in the progression of the disease (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 2000; Suzuki et al., 1998). The development of malignant tumors in *Pten* heterozygous mice starts in the endometrial glands with simple hyperplasia (SH) followed by atypical hyperplasia (AH) showing abnormal cell polarity with dilation of the glandular lumen, atypical nuclei, and an increased gland-to-stroma ratio. EC develops from these premalignant lesions in older *Pten* heterozygous mice (Vilgelm et al., 2006).

Tumor initiation and progression is accompanied by genomic instability (Bakhoum and Compton, 2012; Mai and Mushinski, 2003). Telomeres play an essential role in preserving chromosome stability and in controlling cell proliferation (Blackburn, 2001; de Lange, 2002). Telomeres are complex structures where tandem repeats of 5'-TTAGGG-3' associate with a specialized group of proteins (shelterin proteins) which act to safeguard the chromosome ends (Blackburn, 2001; de Lange, 2002). Telomere length is generally maintained by telomerase, an enzyme with reduced expression in somatic cells (Greider and Blackburn, 1989). The loop conformation (T-loop) of telomere ends masks the single stranded terminal DNA and prevents it from being recognized as DNA breaks, enables their protection against chromosome end-to-end fusions or degradation (Griffith et al., 1999).

Dicentric chromosomes (with telomeric end-to-end fusions) initiate breakage-fusion-bridge (BFB) cycles (McClintock, 1941; Verdun and Karlseder, 2007).

The genome is highly organized in chromosome territories and chromatin domains (Cremer et al., 2000, 1993) and three-dimensional (3D) positioning of chromosomes inside the nucleus dictates genome function (Cremer et al., 2000; Schneider and Grosschedl, 2007). Changes of this non-random organization can lead to genomic instability and cancer (Chuang et al., 2004; Mai, 2010; Mai and Garini, 2005). The analysis of the 3D nuclear architecture of telomeres in interphase nuclei is a technique to study genomic stability in normal and cancer cells (Chuang et al., 2004; Klonisch et al.; Mai 2010). Combined fluorescence microscopy and quantitative image analysis enable us to characterize the 3D organization of telomeres inside the nucleus (Chuang et al., 2004). We have previously shown that in normal lymphocytes the 3D telomere organization in the nucleus is cell-cycle dependent and telomeres assemble in one plane to form a disk in the G2-phase of the cell cycle (Vermolen et al., 2005). Telomeres in cancer cells showed changes in the 3D architecture showing a clustering into aggregates (Mai and Garini, 2006). We demonstrated the formation of telomere aggregates in cancer cells in primary head and neck cancer, primary mouse plasmacytoma, human neuroblastoma, colon carcinoma cell lines (Chuang et al., 2004; Mai and Garini, 2006), human Burkitt lymphoma, glioblastoma (Gadji et al., 2010), Hodgkin cells (Knecht et al., 2010b), and Reed-Sternberg cells (Knecht et al.), myelodysplastic syndromes and acute myeloid leukemia (Gadji et al., 2012). Following the formation of telomeric aggregates, chromosomes can fuse and form anaphase bridges that can break and generate unbalanced translocations and terminal deletions causing the genomic instability characteristic of cancer cells (Mai and Garini, 2006; Knecht et al., 2010a; Guffei et al., 2010; Klonisch et al., 2010). Telomere instability is a hallmark of cancer causing structural and numerical chromosomal aberrations in cancer cells (Murnane, 2012).

In this study, we employ our previously characterized mouse model of progressive development of endometrial cancer (EC) with full penetrance between the ages of 5–9 months (Vilgelm et al., 2006) to investigate changes in the spatial 3D distribution of telomeres in early hyperplastic and precancerous lesions of EC development. We have utilized 3D imaging and quantitative 3D analysis of telomere hybridization signals in interphase nuclei to determine the onset of detectable changes in the telomere architecture during endometrial carcinoma development in this heterozygous *Pten* mouse model.

MATERIALS AND METHODS

Uterine Section Histology

Mouse uteri were cut in half and uterine tissues were fixed in buffered 10% formalin for 24 hrs and subsequently embedded in paraffin. A total of 59 paraffin embedded uterine tissue samples were collected for this project. Of these, 28 samples were collected from WT mice and 31 samples were collected from *Pten* heterozygous mice ranging from 3 months to 13 months of age. All mice had the same 129Sv genetic background. Uterine tissues were sectioned at 5µm. Haematoxylin and Eosin (H&E) staining was performed for morphological analysis. After deparaffinization in xylene, and rehydration in descending

alcohol baths, sections were stained with Meyer's haematoxylin for 8 minutes, washed in running tap water and in 1% acid alcohol. Next, the slides were washed in running tap water and placed in saturated lithium carbonate solution. After being rinsed in alcohol, slides were counterstained in eosin-phloxine B solution for 2 min before dehydration in alcohol and mounting with xylene-based mounting medium.

PTEN Immunohistochemistry

PTEN expression was detected by immunohistochemistry. Slides were kept at 60°C, followed by dewaxing in xylene rehydration in descending alcohol baths. Slides were washed with double distilled water and then equilibrated in TBS (pH = 7.4) buffer at room temperature (RT). After blocking the endogenous peroxidase in 3% hydrogen peroxide in methanol, slides were washed in TBS (pH = 7.4) and incubated in 10 mM citrate buffer (pH 6.0) for 30 min at 90°C for antigen retrieval. Tissue sections were blocked with 10% NGS (normal goat serum) in TBST and incubated overnight at 4°C with the primary antibody (PTEN polyclonal, Abcam, Toronto, Canada) using a 1:100 antibody dilution. The following day, the slides were washed in TBS (pH = 7.4) buffer and incubated with biotinylated secondary antibody (goat anti-rabbit), diluted at 1:200 for 1 hr at RT. After washes, ABC complexes (Vectastain ABC kit, Vector Laboratories, Burlington, ON, Canada) were applied for 30 min at RT room temperature. To visualize specific antibody binding we used the HRP-substrate DAB (DAB, Thermo Scientific) for 10 min. Sections were counterstained with Harris's haematoxylin, dehydrated in ethanol and coverslipped with xylene-based Permount™ for imaging. Rabbit polyclonal IgG (Abcam) was used as an isotype control antibody. Images were taken with the Axio Imager A1 microscope coupled to an AxioCam ICc3 camera and the AxioVision software version 4.8 (Carl Zeiss, Canada).

Telomere Fluorescence In-situ Hybridization (FISH)

Slides were deparaffinized and dehydrated in 100% ethanol, then incubated in 1 M NaSCN for 30 min at 80°C in a water bath. After rinse in double distilled water, slides were incubated in 3.7% buffered formalin solution (Sigma-Aldrich, Oakville, ON, Canada) in 2x SSC buffer (pH 7.6) for 10 min at RT and washed two times in 2xSSC for 5 min each. Totally 50 µg/ml pepsin was added to 0.01 M 37°C prewarmed HCL and slides were incubated for 6 min followed by two washes in 2xSSC for 5 min each. After exposure to 3.7% buffered formalin solution (Sigma-Aldrich, Oakville, ON, Canada) in 2x SSC buffer (pH 7.6) for 10 min at RT and two washes in 2xSSC, tissues were dehydrated in ethanol. 5 µl of Cy3-labeled peptide nucleic acid telomere (PNA) probe (Dako, Glostrup, Denmark) was applied to each slide to detect (T2AG3)_n repeats. Slides and probe were incubated at 80°C for 3 min to denature DNA, followed by hybridization at 30°C for 2 hr using the Hybrite chamber (Vysis, Abbott Diagnostics Mississauga, ON, Canada). Next, slides were washed twice in 70% formamide in 10 mM Tris (pH 7.4) followed by successive washings in PBS at RT for 1 min, in 0.1 × SSC at 55°C for 5 minutes and in 2 × SSC in 0.05% Tween 20 twice at RT. Slides were counterstained with 4',6-diamino-2-phenylindole (DAPI, Invitrogen, Burlington, ON, Canada) (0.1 µl/ml), washed in double distilled water and dehydrated in ethanol. Before imaging, slides were mounted to coverslips using Vestashield antifade mounting medium (Vector Laboratories, Burlington, ON, Canada).

Image Acquisition

Fluorescent images were captured using the AxioImager Z1 microscope, an AxioCamMR3 camera and a Plan-Apochromat 63x/1.40 Oil DIC M27 lens (all from Carl Zeiss, Canada). Acquisition time was 300 msec for Cy3 (telomeres signals) and 100 msec for DAPI. For each nucleus, images were acquired from 60 optical planes (*z*-stacks) through the nucleus with a sampling distance in the *xy* axis of 107 nm and *z* axis of 200 nm using AxioVision 4.8 software (Carl Zeiss, Canada). Images were deconvolved using a constrained iterative algorithm (Schaefer et al., 2001) available in the Axiovision software (Zeiss).

Image Analysis

Analysis was performed with TeloView™ (Chuang et al., 2004; Vermolen et al., 2005) with the Cy3 signal intensity providing the relative fluorescent intensities of telomere signals that are proportional to telomere size (Poon et al., 1999). TeloView™ calculates the center of gravity and the integrated intensity of each telomere, the latter determines the length of the telomere. Telomere aggregates (TA) show a larger volume and an increased integrated fluorescence intensity compared with normal telomeres which are nonoverlapping and not aggregated (Chuang et al., 2004). TAs represent telomere clusters which cannot be further resolved as separate signals at the Abbe resolution limit of 200 nm. TeloView determines the spatial distribution of telomere signals within an ellipsoid nuclear shape (Vermolen et al., 2005) where the two main axes *a* and *b* are equal and referred to as “*a*”, and the third axis “*c*” is of different length. We showed previously using mouse lymphocytes that telomeres arrange in a telomeric disc in G2 phase of the cell cycle with high *a/c* ratio. For each histological type of endometrial pathology, such as normal, simple hyperplasia, atypical hyperplasia, and carcinoma for individual slides, 30 nuclei were marked, coded and selected for image processing and analysis. In this study, we analyzed the glandular epithelial cells of WT and *Pten* heterozygous mice. Based on DAPI images of the nuclei in conjunction with the corresponding H&E serial sections, we selected nuclei of glands presenting clear characteristics of normal morphology or a particular pathology such as SH, AH, or EC. Nuclei that overlapped other nuclei or that lacked nuclear integrity were excluded from further analysis. We did not consider other criteria for rejecting a cell for analysis. Constrained iterative deconvolution (Schaefer et al., 2001) was performed by using the AxioVision 4.8 software (Carl Zeiss, Canada). Next, the images were converted to TIFF files for three-dimensional analysis using the TeloView software (Vermolen et al., 2005). Among many other features, TeloView calculates the intensities of the telomere signal in the 3D image and defines the positions where telomeres are located in the nucleus (Vermolen et al., 2005). We analyzed 30 glandular endometrial cell segmental nuclei per animal and a total of 28 wildtype mice and 31 *Pten* ± mice. The graphs in Figures 4–7 present the average values per nucleus per sample (30 nuclei) for the respective parameters analyzed with TeloView. A total number of 28 samples from WT mice and 48 samples from *Pten* heterozygous mice: 8 samples with SH pathology, 22 samples with AH pathology and 18 samples with EC pathology were considered.

Statistical Analysis

We determined the following: (1) telomere length and telomere signal distribution, (2) average number of telomeres, (3) *a/c* ratio of telomere distribution, and (4) telomere aggregates. An association between age and intensity was assessed with Pearson correlation coefficients using a linear regression model. Telomere aggregates, *a/c* ratio, average telomere signal intensities were analyzed with a nested analysis of variance, followed by a least square means pairwise comparisons when the group effects were found to be significant. Significance level was set at $P < 0.05$.

RESULTS

Reduction of PTEN Protein Expression Is Associated With Atypical Hyperplasia in Endometrial Glands

We analyzed a total number of 28 samples from WT mice and 48 samples from *Pten* heterozygous mice. Among the *Pten* heterozygous mice, we identified 8 samples with SH pathology, 22 samples with AH pathology and 18 samples with EC pathology (Table 1). Uterine tissues from *Pten* heterozygous mice showed progressive alteration of endometrial glandular architecture, from SH to AH and EC, with increasing age (Fig. 1). Furthermore, in some cases different pathologies coexisted in the same endometrial tissue such as SH and AH or AH and EC. As we have described earlier (Vilgelm et al., 2006), *Pten* heterozygous mice develop endometrial carcinoma in a multistep process via hyperproliferative stages very similar to the human EC type I (Vilgelm et al., 2006). In accordance with our previous findings in this EC model (Vilgelm et al., 2006), the onset of the appearance of endometrial carcinoma lesions varied between mice and could be detected in mice as young as 6 months of age.

We observed a nuclear and cytoplasmic localization of PTEN in the endometrial glands of WT mice (Fig. 2). In contrast, endometrial glands of early proliferative lesions in *Pten* heterozygous mice showed a predominant cytoplasmic PTEN expression (Fig. 2B). The cellular localization for *Pten* in the IHC sections of five WT and five SH samples of the 3–5 months age groups were quantified and revealed that 85% of WT, but only 18% of SH glandular epithelial cells showed nuclear *Pten* (Fig. 2H). PTEN-immunostaining was strong in endometrial glandular epithelial cells of WT mice and in SH lesions of *Pten* heterozygous mice. In AH and EC pathology, PTEN protein expression was drastically reduced to background levels. Decreased expression of PTEN was an early event in endometrial carcinoma development as all AH lesions showed only very faint PTEN-immunostaining. An almost complete loss of PTEN immunoreactivity was observed in EC lesions (6- to 13-month-old mice). Twelve animals with EC pathology showed signs of carcinoma cell invasion into the surrounding stroma.

Telomere Length Does Not Shorten With Age in Endometrial Glandular Epithelial Cells

We have performed telomere-FISH on 5 μ m serial sections consecutive to the H&E stained and *Pten* immunostained sections of each animal. The glandular morphology was assessed on the Dapi-stained nuclei using a 20x objective and compared with the H&E stained sections. Single Dapi-stained nuclei from the telomere-FISH hybridizations were numbered

consecutively and chosen for TeloView analysis (Fig. 3). To assess if age played a role in the telomere length distribution in mouse endometrial glandular tissue, we investigated 28 samples from WT mice of 129Sv genetic background between the age of 3 and 12 months (Supporting Information Table 1). A Pearson correlation coefficient was calculated to assess the relationship between the telomere length changes and aging. There was no linear correlation between the two variables (1) age and (2) telomere length with $r = -0.005$, $n = 1,189$, and $P = 0.85$. Linear correlation assessment found that telomere length within glandular endometrial epithelial cells in this strain of mice it is not influenced by age. Our investigations selectively determined the telomere length in glandular epithelial cells of the mouse endometrium and showed no telomere shortening with age in these cells. Consequently, we analyzed the different histopathologies independent of age.

Average Telomere Numbers and a/c Ratios of Telomere 3D Distribution Are Similar Between Histo-Pathologies

The average number of telomere signals in nuclei from each histopathologic category was determined for all animals combined and compared with the telomere numbers of other pathologies and the WT animals. There were no significant differences among the four categories: WT, SH, AH, and EC (Fig. 4). For our analysis, we did not investigate cultured cells, but 5 μm tissue sections. We determined a nuclear diameter of 7–8 μm for mouse endometrial glandular epithelial cells indicating that nuclei in tissue sections are not complete, but represent a 62–71% segment of the total nucleus. Thus, the graph in Fig. 4A shows the average segmental telomere number per analyzed nucleus in the respective histopathologies. A variance component analysis was performed to break down the random variability proportions for the detected segmental telomere numbers. 71% of the random variances derive from the cells within the sample (animal tissue) and 29% of the random variances derive from the samples (individual animal tissues) (Supporting Information Table 2).

The space occupied by telomeres inside the nucleus appears as a spheroid and can be defined by three axes: a , b , and c , with a and b having the same length. Previous data on isolated mouse lymphocytes demonstrated that during the cell cycle the a/c ratio changes with only the c axis varying in length (Vermolen et al., 2005). The a/c ratio is higher in proliferative cells when telomeres align at the center of the cell forming a disk (Chuang et al., 2004). Our analysis failed to detect significant differences in a/c ratio values in segmental nuclei of endometrial epithelial cells of WT, SH, AH, and EC (Fig. 5A). Determining the a/c ratio in nuclei of paraffin sections of 5 μm thickness one has to consider that nuclei may be cut and the a/c ratio assessed here is based on the segmental nuclear volume as shown in Fig. 5B. There was a trend for the segmental nuclear volume to decrease from SH to EC but no significance was found.

Atypical Hyperplasia (AH) Is Associated With Increased Number of Telomere Aggregates

Telomere aggregates (TAs) represent regions of associated telomere signals that cannot be resolved as separate objects by the 200 nm Abbe resolution limit. Thus, telomere aggregates may be the result of telomeres fused together or the effect of close proximity of neighboring telomeres. Telomere aggregates were identified in cancer cells and are an indicator of

genomic instability (Mai 2010; Mai and Garini 2006). The 3D telomere architecture of the reconstructed images showed a significantly higher number of telomere aggregates already in AH when compared with the WT ($P = 0.007$) and to the SH ($P = 0.0002$) categories (Fig. 6). We confirmed the expected presence of telomere aggregates to be higher in EC when compared with WT ($P = 0.0002$) and to SH ($P = 0.0263$). The mean number of telomere aggregates in WT and SH was very low ($<0.2\%$).

Simple Hyperplasia (SH) Is the Earliest Pathology Showing Changes in Telomere Length and in Telomere Signal Distribution

The Cy3 signal intensity provides the relative fluorescent intensities of telomere signals that are proportional to telomere size (Poon et al., 1999). Low intensity signals correspond to shorter telomeres and the high intensity signals corresponded to longer telomeres. According to the telomere profile, four intensity groups were used for statistical analysis: $<6,000$ arbitrary fluorescent intensity units (a.u.); $6,000\text{--}10,000$ a.u.; $10,000\text{--}20,000$ a.u.; $>20,000$ a.u. (Fig. 7). SH had a significantly higher number of short telomeres in the $<6,000$ group and a significantly lower number of telomeres in the $10,000\text{--}20,000$ group compared with WT. There were no significant differences in the telomere intensity distribution of AH and EC compared with WT, except in the $6,000\text{--}10,000$ group where AH had a significantly lower number of short telomeres compared with WT (Fig. 7B). The standard deviation and SEM values for the telomere length distribution in the four intensity categories are presented for all histopathologies in Supporting Information Table 3. Separate graphs showing the SEM values for the telomere length distribution for all histopathologies are presented in Supporting Information Fig. 1 for each intensity group.

To better characterize the telomere signal distribution in cells of the same endometrial pathology, we calculated the number of cells showing a defined number of telomere signals within the 30 cells analyzed per animal and pathology. This analysis depicts the distribution of telomere signals in 30 nuclei within a defined endometrial glandular pathology of an individual animal. The cells of *Pten* heterozygous mice more often showed a heterogeneous population of cells when compared with cells in WT control mice (Fig. 8). We found this pattern in all pathological categories including simple hyperplasia, atypical hyperplasia and carcinoma.

The overall 3D quantitative and qualitative telomere changes are summarized in Table 2.

DISCUSSION

We have shown previously that the 3D telomere architecture can be evaluated in the interphase nucleus using a semiautomated method on archived paraffin-embedded tissues (Klonisch et al., 2010; Mai and Garini, 2006; Vermolen et al., 2005). Telomere length is differently maintained in various cell types by telomerase-dependent or alternative telomere lengthening mechanisms (ALT) (Cesare and Reddel, 2010; Shay and Wright, 2011). Telomere length is different even between mouse strains (Coviello-McLaughlin and Prowse, 1997) and in different tissues of the same strain. As an example, brain and spleen mouse tissue were reported to develop significant changes in telomere length with age in contrast to liver, testis and kidney that do not show length changes when measuring the terminal

restriction fragment (TRF) (Coviello-McLaughlin and Prowse 1997). Since endometrial type-I cancer develops over time through stages of hyperplasia we needed to know whether telomere length changes in endometrial cells are age-dependent. To our knowledge, there is no information available on age-related telomere length changes in mouse endometrial glandular epithelial cells. Our data demonstrate for the first time that the telomere length in uterine glandular epithelial cells of wild-type mice in the 129Sv genetic background does not change with age. Consequently, we were able to compare telomere profiles between different glandular histopathologies independent of age.

Our goal was to investigate if changes in the 3D telomere architecture of interphase nuclei can be identified prior to Pten loss and to histological evidence for the presence of endometrial carcinoma. In contrast to the terminal restriction fragment assay (TRF) which is based on Southern blot analysis 3D-telomere-FISH is capable of quantitative telomere length measurements in individual epithelial cell nuclei in-situ, thus excluding contamination by surrounding stromal, vascular or immune cells. In addition, our analysis in the *Pten*^{+/-} mouse model with 100% penetrance for EC development (Vilgelm et al., 2006) allows for telomere observations during different stages of precancerous hyperplasia until the presence of carcinomas.

The telomere length in tumor cells can be shorter or longer than in normal cells (Meeker et al., 2004b) and telomere shortening was shown to occur in both cancer and precancer stages (Meeker et al., 2004a,b). Using telomere-FISH techniques on histological specimens significant telomere shortening has been demonstrated to be an early event in human epithelial cancer development, such as those from prostate, pancreas, bladder, large intestine, esophagus, oral cavity or uterine cervix (Meeker et al., 2002; van Heek et al., 2002; Meeker et al., 2004b; Maida et al., 2006). Short telomeres have also been described in breast ductal carcinoma in situ and in normal breast secretory cells (Raynaud et al., 2010). Our results showed that there are no changes in the average telomere length between epithelial cells in normal glands versus those in hyperplastic lesions or in EC. This confirms earlier reports in human endometrial samples: contrary to cervical carcinogenesis, where cervical intraepithelial neoplasias (CINs) already showed progressive telomere shortening and chromosome instability, telomere length was found not to be different in endometrial hyperplasia and endometrial carcinoma compared with normal endometrium when comparing the mean telomere signal intensities (Maida et al., 2006). When compared with human type-II EC type-I EC did not show significant telomere shortening nor major chromosomal abnormalities (Akbay et al., 2008). In fact, several cell lines derived from human type-I endometrial carcinoma revealed a nearly diploid karyotype (Kuramoto et al., 1972; Yodate et al., 1995). This confirms our data in the *Pten*^{+/-} mouse model showing no significant changes in telomere length or telomere numbers in progressive hyperplastic stages during endometrial carcinoma development. However, TeloView enables us to determine average telomere intensities as well as the telomere intensity distribution in 3D. We observed differences in the relative telomere length distribution when telomere intensities were categorized in groups. We observed that only simple hyperplasia (SH) was different from normal glands in wildtype (WT) mice with a relative increase in short telomeres (<6,000 a.u. signal intensity) and a relative decrease in longer telomeres (10,000–20,000 a.u. signal intensity). The observed relative telomere shortening in glandular cells

from SH may suggest telomere dysfunction (DePinho and Polyak, 2004) to be present already during this early stage of EC development prior to the loss of immunoreactive Pten (Fig. 2) and before histological characteristics of EC or even atypical hyperplasia was evident. The relative telomere length distribution in AH and EC was similar to WT suggesting an acquired stabilization process against telomere attrition.

In addition, we discovered that the telomere signal distribution of 30 analyzed cells within one individual gland showed a predominantly two-curved pattern in all hyperplastic pathologies and in EC when compared with the one-curved pattern in normal glands. In a highly regenerative tissue such as the human endometrium a clonal lineage of glandular cells was demonstrated (Tanaka et al., 2003). Our finding indicates that glands with SH pathology already have two populations of cells with different numbers of telomeres. Thus, a potential competitive clonal selection process may occur early during the malignant transformation of endometrial tissue, but may also prevail during late stages of EC. Taken together, the relative shortening of telomeres and the presence of more than one cell population in SH glandular epithelial cells represent the first detectable sign of telomere dysfunction indicating chromosomal instability to be present as early as in simple hyperplasia pathologies. This telomere dysfunction may lead to the observed appearance of telomere aggregates in more advanced lesions of AH and in EC.

The 3D position of telomeres in the nucleus is cell-cycle and cell-type dependent (Chuang et al., 2004; Ermler et al., 2004) but telomeres in normal cells do not overlap (Chuang et al., 2004). We have previously shown the presence of telomere aggregates (TAs) to be a specific signature of tumor cells (Chuang et al., 2004; Mai and Garini, 2006) and to be independent of telomerase activity and telomere length (Louis et al., 2005). Telomere aggregates are larger than single telomeres and therefore show a stronger intensity of the hybridization signal. They represent telomere clusters which cannot be further resolved as separate signals at the Abbe resolution limit of 200 nm. TAs occur independent of telomere length and have been observed with critically short telomeres and with telomeres of normal length (Louis et al., 2005; Mai, 2010). The presence of TAs indicates dysfunctional telomeres and likely represent end-to-end telomere fusions which cause breakage-fusion-bridge cycles (Mueller, 1938; McClintock, 1941; Louis et al., 2005) with resulting numerical and structural chromosomal aberrations such as unbalanced translocations, terminal deletions, duplications, dicentric chromosomes. These chromosomal aberrations drive malignant transformation. In noncancerous cells, TA formation was observed to correlate with binding of telomeres to structures of the nuclear lamina in senescent mesenchymal stem cells (MSC) (Raz et al., 2008). This publication also describes TAs to colocalize with γ H2AX DNA-damage foci suggesting TAs to mark sites of DNA-damage during senescence.

In our mouse model we have identified a significantly increased number of TAs in AH and EC compared with WT and SH. The fact that TAs are present in endometrial AH lesions demonstrates that AH lesions already show telomere dysfunction similar to carcinoma. Evidence from our earlier observations confirm the presence of telomere aggregates in precancerous lesions such as in human cervical intraepithelial neoplasia and in early plasmacytotic foci in a mouse model of plasmacytoma (Mai and Garini 2006). Our present study supports the idea that alterations in the organization of the 3D nucleus may promote

tumorigenesis. The “telomere-driven genome-instability” can occur due to the close proximity of telomere territories that increases the risk of recombination especially in subtelomeric regions (Linardopoulou et al., 2005; De Vos et al., 2009).

Our data confirmed our previously published data in this mouse model (Vilgelm et al., 2006) that *Pten* expression decreases already in precancerous lesions of atypical hyperplasia (AH) prior to morphological appearance of endometrial carcinoma (EC). In women, 43% of normal premenopausal endometria contain PTEN-negative glands which persist as hyperproliferative lesions (Mutter et al., 2000b). PTEN loss associated with somatic *Pten* mutations or deletions was suggested to present the earliest sign of nonfamilial endometrial carcinogenesis (Mutter et al., 2000a). It is not known whether the described somatic *Pten* mutations and deletions would prevent PTEN translation and consequently these somatic mutations would select for a PTEN-null phenotype in these glands. Among the known epigenetic mechanisms which regulate PTEN protein expression, *Pten* promoter methylation, and microRNAs represent transcriptional and post-transcriptional regulation, respectively (Fata et al., 2012). In addition, post-translational regulations have been described to decrease PTEN protein expression such as phosphorylation and ubiquitinylation (Fata et al., 2012). In this study, we show in our mouse model that a shift in the subcellular localization of *Pten* occurs as early as in simple hyperplastic lesions (SH) where *Pten* is localized predominantly in the cytoplasm compared with the combined nuclear and cytoplasmic localization of *Pten* in the normal glandular epithelium and in WT endometrial glands. A shift from nuclear to cytoplasmic PTEN was previously described in actively dividing breast cancer cells (Ginn-Pease and Eng, 2003). Hereditary nonpolyposis colon cancer syndrome (HNPCC) patients with EC were shown to have absent or weak (68%), normal (27%), or mixed populations with weak or absent (5%) PTEN expression. Of the somatic PTEN mutations detected in 85% of HNPCC patients more than 50% involved frameshift mutations affecting exons 7 and 8 of *Pten* (Zhou et al., 2002). Exons 7 and 8 encode the C-terminus of PTEN. A C-terminal mutant of PTEN identified in patients with Cow-den syndrome (K289E mutant) was shown to be excluded from the nucleus due to a lack of monoubiquitinylation (Trotman et al., 2007). Although the balance between PTEN protein stability and degradation affecting its cytoplasmic and nuclear localization are cell-type specific (Planchon et al., 2008) PTEN mutations affecting exons 7 and 8 may be responsible for the nuclear exclusion and weaker *Pten* expression in a subset of patients with EC.

The mutation in our *Pten*^{+/-} model replaces *Pten* exons 4 and 5 with a neomycin-resistance gene cassette (Di Cristofano et al., 1998) which not only replaces the phosphatase domain, but completely abolishes expression of the targeted allele. What is detected in the very early hyperplasia (SH) is the protein product of the remaining wild type allele, which might be excluded from the nucleus as a result of the constitutive activation of PI3K signaling. Activated PI3K induces the ubiquitinase NEDD4-1 (Ahn et al., 2008). Although monoubiquitinylation of PTEN by NEDD4-1 induced shuttling of PTEN to the nucleus (Trotman et al., 2007), polyubiquitinylation of PTEN causes its proteasomal degradation (Wang et al., 2007). Thus, the constitutive activation of the PI3K-AKT pathway observed in our *Pten*^{+/-} mouse model (Vilgelm et al., 2006) may shift the balance away from nuclear localization towards proteasomal degradation due to a higher ubiquitinylation status of the

Pten protein from the remaining allele. In later lesions loss of heterozygosity (LOH) completely eliminates Pten in the mouse glandular lesions, and the extremely weak cytoplasmic staining reflects the background staining with this particular antibody on mouse tissues.

A functional C-terminal domain was found to determine its nuclear localization (Trotman et al., 2007). Nuclear Pten was shown to bind to centromeres in mesenchymal stem cells, to mediate correct segregation of chromosomes during mitosis and to maintain chromosome stability (Shen et al., 2007). Contrary to these observations, a different research group was unable to detect any colocalization or interaction between PTEN and centromeres or telomeres or the centromeric protein CENPC in human MSC or prostate cancer cells (Gupta et al., 2009). Instead, PTEN-deficient human cells were shown to have increased structural and numerical chromosome aberrations due to multiple defective cell cycle checkpoints (Gupta et al., 2009). Further studies are needed to investigate a potential direct or indirect interaction of Pten with telomeric proteins of the shelterin complex (de Lange, 2002).

Aneuploidy is common in many human cancers such as acute myeloid leukemia, desmoids tumors or breast adenocarcinomas (Jonsdottir et al., 2011; Gordon et al., 2012), but it is rarely observed in endometrioid cancer when analyzed by flow cytometry (Rosenberg et al., 1989). The majority of investigated human endometrial cancer specimens were found to be diploid, but aneuploidy served as independent prognostic factor for recurrences in EC patients (Song et al., 2011; Takai et al., 2010). A significant variation in average telomere number is usually specific to aneuploid tumors. In this study, we detected a similar average number of telomere signals between normal endometrial glandular cells of control mice and endometrial glandular cells of Pten heterozygous mice with SH, AH, or EC pathology indicating that in this mouse model of EC development, and similar to human EC type-I, aneuploidy is not a prominent feature of endometrial pathology.

TeloViewTM software allows us to determine the distribution of telomeres throughout the 3D nuclear space (*a/c* ratio). We have previously observed that in mammalian cells, telomeres are widely distributed throughout the nucleus in G0/G1 and S-phase, but gather in a telomeric disk during G2 phase of the cell cycle (Chuang et al., 2004) as determined by an *a/c* ratio larger than 1. This demonstrated that the three-dimensional position of telomeres in the nucleus is cell-cycle dependent. However, we were unable to detect any significant differences in the *a/c* ratio in the mouse endometrial glandular cells between WT, SH, AH, and EC cells. Considering the turnover rate in a highly regenerative tissue such as the endometrium, the proliferation of endometrial glandular cells in the normal endometrium may already be high. It was shown in human endometrium, that Ki67 expression levels were higher in proliferative phase normal endometrium than in hyperplastic endometrium or endometrioid carcinoma (Kosmas et al., 2011). As a note of caution it needs to be mentioned that it is difficult to assess the *a/c* ratio of telomere distribution in tissue samples where we often measure a section of the cell in contrast to cultured cells where we can assess the entire cell.

CONCLUSIONS

This study reveals that significant changes in the 3D telomere architecture occur in all steps of malignant transformation in a mouse model of EC development. The relative shortening of telomeres and the presence of more than one cell population in SH glandular epithelial cells represent the first detectable sign of telomere dysfunction indicating chromosomal instability to be present as early as in simple hyperplasia pathologies. This telomere dysfunction may lead to the observed appearance of telomere aggregates in more advanced lesions of AH and in EC. Our data show that 3D telomere architectural changes in endometrial lesions of *Pten* heterozygous mice occur prior to complete loss of Pten protein expression and before histological characteristics of endometrial carcinoma or even atypical hyperplasia are evident. The detection of telomere aggregates in hyperproliferative lesions showing atypical nuclei definitively classifies these lesions as precancerous.

Although loss of PTEN is associated with early stages of endometrial carcinoma development in women, clinical follow-up studies on 129 patients over 25 years have shown that the loss of PTEN protein expression in endometrial glands is not a predictive parameter for the development of endometrial carcinoma in women (Lacey et al., 2008b). Our novel findings of distinct alterations in the 3D telomere profiles in early hyperplastic EC precursor lesions therefore describe a promising parameter for testing in human samples in the future. A systematic investigation of the 3D telomere profiles in human samples was beyond the scope of the present study. But recently, 3D analysis of telomere profiles in interphase nuclei based on 3D-telomere-FISH using an automated scanner and a specific TeloScan software was shown to identify individual tumor cells in large samples (Klewes et al., 2011) making the study of human archived endometrial tissues feasible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Pten heterozygous mice develop endometrial carcinoma (EC) in a multistep process similar to EC development in the human. The images illustrate representative examples of H&E stained uterine sections of WT (A) and Pten heterozygous mice representing SH (B), AH (C) and EC (D). Normal glandular morphology in WT contrasts with the altered organization of uterine glands in SH, AH, and EC. Magnification x400. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2.

Reduction of PTEN protein expression is associated with AH and EC in endometrial glands. The images illustrate representative examples of the Pten immunostaining of uterine sections of normal endometrium (WT mouse) and SH, AH, and EC (Pten heterozygous mice). Normal endometrial glands (WT; A,C,E,G) show PTEN nuclear and cytoplasmic immunostaining (A,C,E). Pten heterozygous mice show SH with cytoplasmic and nuclear PTEN localization in glandular epithelial cells (3 months, B). Pten heterozygous mice (6 months, D) featuring AH and Pten heterozygous mice (12 months, F) with EC show almost complete loss of PTEN protein expression in hyperproliferative and carcinoma lesions. The negative control shows lack of staining using an isotype control antibody (G). Magnification x400. The percentages of Pten-positive and Pten-negative nuclei were quantified in WT and SH (Pten+/-) glands of 3- to 5-month-old animals (n = 5 for each genotype) (H). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3.

Selection of individual nuclei for telomere FISH analysis in SH, AH, and EC. A–C: DAPI fluorescence images acquired after telomere FISH were compared with H&E stained serial sections and provide a visual opportunity to mark and number nuclei to be analyzed with TeloView™ software. This image illustrates examples of single nuclei (D–F) within a gland showing SH (A), AH (B), and EC (C). Two-dimensional (2D) image reconstruction visualizes the telomere hybridization signals within the single nucleus (D–F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4.

Average telomere number is similar in WT, SH, AH, and EC. The Fig. 4A illustrates the average number of segmental telomere signals detected per nucleus per sample (30 nuclei/sample). A total number of 28 samples from WT mice and 48 samples from Pten heterozygous mice: 8 samples with SH pathology, 22 samples with AH pathology, and 18 samples with EC pathology were considered for this graph. Error bars represent stdev of the mean. Figure 4B shows the average values for the telomere number and the standard deviation for each histopathology. The differences in WT, SH, AH, and EC were not significant. For the coefficient of variation we determined the range, the average (mean) values and the stdev for each histopathology (Fig. 4C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5.

Telomere Distribution inside the 3D Nuclear Space (a/c ratio) is similar in WT, SH, AH, and EC. This figure illustrates the a/c ratio (A), an indicator of the spatial placement of telomere signals inside the calculated nuclear volume; when the cell is more flat, this ratio increases. Error bars represent SEM. The differences among WT, SH, AH and EC were not significant. The calculated nuclear volume (B) revealed a progressive trend towards smaller segmental nuclear volumes from SH to EC. Error bars represent SEM. The differences among WT, SH, AH, and EC were not significant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 6.

AH and EC are associated with an increased number of telomere aggregates. The average segmental number of telomere aggregates per nucleus was calculated for a total number of 28 samples from WT mice, 8 samples with SH pathology, 22 samples with AH pathology, and 18 samples with EC pathology from *Pten* heterozygous mice. The number of aggregates were found to be significantly higher in AH ($P = 0.0071$) and EC ($P = 0.0002$) compared with normal glands in WT, and in EC ($P = 0.0263$) compared with SH. Error bars represent SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 7.

SH is the earliest pathology showing changes in telomere signal intensity distribution. This figure summarizes the telomere length measurements (expressed by telomere signal intensity) calculated in a total number of 28 samples from WT mice and 48 samples from Pten heterozygous mice: 8 samples with SH pathology, 22 samples with AH pathology and 18 samples with EC pathology. For each sample 30 nuclei were analyzed and the sample average is shown in the graph (A). SH is the earliest pathology showing changes in telomere length distribution. Telomere signals were categorized in 4 intensity groups (fluorescent arbitrary units of <6,000; 6,000–10,000; 10,000–20,000; >20,000) and the percent distribution of telomere signal intensity within these groups is depicted in the table for WT, SH, AH, and EC histopathologies (B). Chi-square analysis was performed and $P < 0.05$ was considered significant. The table highlights the significant differences between telomere intensity groups of normal glands and respective histopathologies. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 8.

Representative examples of telomere length and telomere signal distribution show earliest changes in SH. The image illustrates representative examples of telomere intensity distribution (from 30 nuclei of a specific pathology in one animal) in the endometrial glandular cells of WT (A), SH (B), AH (C), and EC (D) mice. The number of telomere signals (y axis) are plotted against the specific telomere intensity (arbitrary units, x axis) corresponding to telomere length. The inserts depict a representative example for endometrial glandular epithelial cells of WT, SH, AH, and EC histopathology showing the distribution of the number of nuclei (y axis) having a specific number of telomere signals (x axis). The single-curve pattern is the most frequently encountered in WT (A), indicating a homogenous population of cells regarding the number of telomere signals per nuclei. In contrast, SH, AH, and EC (B–D) predominantly show a double-curved pattern indicating a heterogeneous population of cells within these glands. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1**Mouse Endometrial Tissues**

Pathology/Age	3–5 mo	6–9 mo	10–13 mo	Total n
Wt	n = 7	n = 11	n = 9	n = 27
SH	n = 5	n = 3	n = 0	n = 8
AH	n = 5	n = 8	n = 9	n = 22
EC	n = 0	n = 5	n = 13	n = 18

Summary of the samples from normal and pathological mouse endometrial tissues. We analyzed a total number of 27 Wt and 31 Pten (+/–) animals between the ages of 3 – 13 months of age. A number of 16 Pten (+/–) mice had two different coexisting pathologies. The endometrial pathology observed in the mutant mice was classified in SH (simple hyperplasia), AH (atypical hyperplasia) and EC (endometrial carcinoma).

TABLE 2

Summary of 3D Telomere Distribution Signatures in EC Development Stages

Pathology	WT (PTEN +/+)	SH (PTEN +/-)	AH (PTEN +/-)	EC (PTEN +/-)
Telomere Length Distribution	no difference	↑ Short Telomeres ↓ Medium Telomeres ↓ Long Telomeres	↓ Medium Telomeres	no difference
Telomere Signal Distribution	one curve/population	two curves/populations	two curves/populations	two curves/populations
Telomere Distribution in the 3D Nuclear Space	no difference	no difference	no difference	no difference
Telomere Aggregates	no difference	no difference	↑ Telomere Aggregates	↑ Telomere Aggregates
Average Number of Telomere Signals/ Nucleus	no difference	no difference	no difference	no difference

The main findings of our investigation were the early increase of short telomeres in SH and the existence of heterogeneous cell population regarding the telomere signal distribution already in glands of SH which remained detectable at AH and EC stages in Pten heterozygous. An increased number of telomere aggregates was found in AH and EC. Thus, SH is the earliest endometrial pathology with detectable 3D telomere architectural changes, and the presence of telomere aggregates in AH depicts a definitive cancer characteristic at this stage.