Sex hormone regulation of survivin gene expression

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Abstract

Survivin (*BIRC5*) is a cell survival gene that is overexpressed in endometrial cancer and has been implicated to have a physiological role in normal endometrial function. To determine whether survivin gene expression is regulated by reproductive steroid hormones in the human endometrium, RNA was prepared from normal cycling women in the proliferative and secretory phases of the menstrual cycle. RNA was also isolated from 21 endometrial biopsies from premenopausal women at baseline and following 3 months of treatment with depot medroxyprogesterone acetate. Finally, RNA was isolated from endometrial biopsies from ten healthy postmenopausal women participating in a clinical trial of estrogen replacement therapy at baseline and following 6 months of treatment with conjugated equine estrogen. Quantitative RT-PCR analysis was used to determine

survivin, insulin-like growth factor binding protein 1 (*IGFBP1*), *Ki67*, and *IGF1* gene expression levels. Survivin gene expression was highest in the proliferative phase of the menstrual cycle and showed a statistically significant 4-fold increase in expression following chronic treatment with estrogens; this was strongly correlated with increased *Ki67*, a marker of proliferation. Survivin gene expression decreased 4.6-fold following chronic progestin treatment in the human endometrium. These data suggest that survivin transcript is regulated by estrogens and progestins in the disease-free human endometrium. The data also suggest that survivin transcript may be used as a biomarker of estrogen and progestin treatment efficacy, but validation studies must be conducted to support this conclusion.

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Introduction

The human endometrium is a dynamic tissue that lines the uterine cavity. It is composed of both epithelial and stromal cells and is cyclically regenerated. The reproductive cycle in human females is driven through neuroendocrine signaling. Both positive and negative feedback loops within the hypothalamus and pituitary control steroid hormone production in the ovaries. Increased estrogen (E_2) drives the endometrium into the proliferative phase of the cycle. As the cycle progresses, progesterone (P_4) is secreted by the ovary, which inhibits proliferation and restructures the endometrium into a secretory tissue in anticipation of fertilization. If fertilization does not occur, the endometrium is shed and the local drop in hormone levels reactivates the cycle (Knobil 1972, Bischof *et al.* 1974). Several lines of evidence suggest that apoptosis also plays a significant role in the normal cycling of the human endometrium (Kokawa *et al.* 1996, Vaskivuo *et al.* 2000, Harada *et al.* 2004).

Survivin (*BIRC5*) is a member of the inhibitor of apoptosis (*IAP*) gene family. Survivin has received much attention as a mediator of cell survival due to its dual function in both apoptosis and cell cycle regulation. The pattern of survivin gene expression is distinct from the other IAPs. Survivin is highly expressed during embryonic and fetal development and is overexpressed in virtually all tumor types (Ambrosini *et al.* 1997, Li & Altieri 1999, O'Driscoll *et al.* 2003) including endometrial cancer (Lehner *et al.* 2002, Takai *et al.* 2002, Pallares *et al.* 2005, Nabils *et al.* 2009). In contrast, survivin is negligibly expressed in most highly differentiated adult tissues. It is, however, expressed in the cycling human endometrium (Konno *et al.* 2000, Tarkowski *et al.* 2000, Lehner *et al.* 2002, Fukuda & Pelus 2006), in the decidua and villus of early pregnant women (Li *et al.* 2002), and in non-malignant endometrial pathologies such as endometriosis (Tarkowski *et al.* 2001, Ueda *et al.* 2002, Goteri *et al.* 2005, Fujino *et al.* 2006) and endometrial hyperplasia (Tarkowski *et al.* 2000, Chen *et al.* 2009), suggesting that it may play a role in endometrial physiology and pathology.

Information on survivin function in normal tissues has been limited as survivin knockout mice suffer from embryonic lethality. Animal models have implicated a physiological role for survivin in the murine endometrium, but the precise function of survivin in endometrial tissue is unclear. High expression of survivin has been found in the endometrium of mice exhibiting defective implantation and subsequent pregnancy loss (Garcia *et al.* 2007, Li *et al.* 2008). Loss of survivin expression has also been described in the decidua of interleukin-11 receptor α null mice which are infertile due to aberrations in decidualization and trophoblast invasion (Garcia *et al.* 2007). Studies in endometrial cancer-derived Ishikawa cells indicate that silencing of survivin decreases cell proliferation and increases apoptosis via down-regulation of cyclin D1 and phospho-retinoblastoma protein and activation of caspase-3 and caspase-8 (Ai *et al.* 2006).

Since the endometrium is a hormone-responsive tissue, we hypothesized that if survivin is involved in maintaining normal tissue homeostasis, then it may be regulated by reproductive steroid hormones in the normal human endometrium. Studies in hormone-responsive cell lines indicate that survivin transcript and protein levels are increased by E_2 treatment (Nakayama *et al.* 2000, Sayeed *et al.* 2007), and in Ishikawa cells, the induction is reversed upon treatment with the estrogen receptor antagonist Fulvestrant (Chen *et al.* 2009). Also in hyperplastic endometria and in Ishikawa cells, survivin protein is decreased by progestin treatment, and in Ishikawa cells, this decrease is reversed by treatment with the P_4 receptor antagonist RU486 (Chen *et al.* 2009). The effects of estrogen and P_4 administration on survivin expression in disease-free human endometrium have not been reported.

In this study, we measured survivin gene expression levels *in vivo* in cycling human endometrium before and after treatment with progestins and in postmenopausal endometrium after treatment with estrogens.

Materials and Methods

Endometrial samples

All human tissue samples were obtained from patients under protocols approved by Institutional Review Boards at the University of Texas Health Science Center and MD Anderson Cancer Center. Formalin-fixed, paraffin-embedded (FFPE) sections of human endometrial biopsies were obtained from the Department of Pathology, University of Texas MD Anderson Cancer Center (Houston, TX, USA) from 12 women who underwent biopsy for the clinical evaluation of abnormal vaginal bleeding as previously described (McC Campbell *et al.* 2006). The endometrial samples were classified as proliferative ($n=7$) or secretory ($n=5$) according to Noyes criteria (Noyes *et al.* 1975) after microscopic examination of H&E-stained slides. For the proliferative phase endometrial biopsies, women ranged in age from 29 to

50 years (mean of 40.4 years), and body mass index (BMI) ranged from 17.1 to 52.3 kg/m² (mean of 29.5 kg/m²). For the secretory phase endometrial biopsies, women ranged in age from 31 to 46 years (mean of 41.8 years), and BMI ranged from 19.3 to 30.3 kg/m² (mean of 22.6 kg/m²). There was no significant difference in the mean age or BMI between women with proliferative or secretory phase endometrium ($P=0.6$ and $P=0.2$ respectively).

The RNA used for analysis of P_4 regulation of survivin gene expression was obtained from healthy cycling women who were enrolled in a chemoprevention trial of depot medroxyprogesterone acetate (DEPO; 150 mg i.m. DEPO, Pharmacia & UpJohn) for prevention of endometrial cancer. The endometrial biopsies were timed so that they were performed between days 8 and 10 (proliferative phase) of the woman's menstrual cycle. All of the baseline (pre-treatment) endometrial biopsies used in this study were microscopically confirmed by a gynecologic pathologist to be in proliferative phase according to Noyes criteria. The group included women between the ages of 25 and 48 years (mean age = 36.8 years), and mean BMI = 28.4 kg/m² (range 19.3–48.4 kg/m²) with no prior hysterectomy, no history of prior pelvic radiation, no chemotherapy for 2 years, and no use of oral contraceptives or hormones for 4 months prior to initiation of the study. Women also had to have no medical contraindication to use DEPO, including known or suspected pregnancy, undiagnosed vaginal bleeding, active thrombophlebitis or past history of thromboembolic disorders or cerebral vascular disease, gall bladder disease, history of diabetes, coronary artery disease, or a current tobacco smoker age > 35.

The RNA used for the analysis of estrogen regulation of survivin expression in postmenopausal women was obtained from a randomly selected subset ($n=10$) of a large group of healthy postmenopausal women ($n=210$) participating in a clinical trial of estrogen replacement therapy as previously described (Deng *et al.* 2003, 2005). The group included women between the ages of 44 and 59 years (mean age = 50.7 years), mean BMI = 24.4 kg/m² (range 19.3–28.6 kg/m²), mean serum E_2 = 9.7 pg/ml (range 1.4–19.3 pg/ml), mean FSH = 86.3 mIU/ml (range 51.7–148.2 mIU/ml), and mean prolactin = 7.2 ng/ml (range 3.0–24.7 ng/ml). Endometrial biopsies were obtained at baseline and after 6 months of Premarin (0.625 mg/day conjugated equine estrogen, Wyeth-Ayerst) treatment. All endometrial biopsies were microscopically examined to confirm the absence of endometrial pathology (hyperplasia or carcinoma). Tissues were frozen in liquid nitrogen and stored at -80°C . Frozen tissues were homogenized in TRI Reagent, and RNA was precipitated with isopropanol, applied to RNeasy spin columns (Qiagen), eluted, and treated with RNase-free DNase for 30 min at 37°C , followed by heat inactivation at 75°C and storage at -80°C . RNA was isolated from FFPE tissues as previously described (McC Campbell *et al.* 2006).

Quantitative real-time RT-PCR

All TaqMan quantitative real-time RT-PCR (QPCR) assays were designed with Primer Express software. The assays were developed, and all reactions were completed at the Quantitative Genomics Core Laboratory (UT-Houston Medical School, Houston, TX, USA). Forty nanograms of RNA from each sample were assayed in triplicate with a fourth reverse transcriptase negative control as previously described (Xie *et al.* 2007). The results were analyzed using SDS 1.9.1 software (Applied Biosystems, Carlsbad, CA, USA) with SuperROX (BioSearch, Novato, CA, USA) as a reference dye. Transcripts were quantified against a standard curve comprised of five serial log dilutions of known DNA quantity. The mean transcript levels for all assays were normalized to 18s rRNA transcript levels of the housekeeping gene. Data are presented as a median ratio of (transcript/18s rRNA). Survivin and 18s assay sequences have been reported as follows (Nabilsli *et al.* 2009): insulin-like growth factor binding protein 1 (IGFBP1; NM_000596): 620+GGGACGCCATCAG-TACC, 681-CCATTTTTTGTATGTTGGTGAC, 638+FAM-ATGATGGCTCGAAGGCTCTCCA-BHQ1; IGF1 (M27544): 187+TCCAACCCAATTATTTAAGTGCT-GC, 278-ACAGCGCCAGGTAGAAGAGA, 227+FAM-AAGGTGAAGATGCACACCATGTCTCCTC-BHQ1; Ki67 (NM_002417): 3323+AAGTTCACACGGACGT-CAG, 3391-GATGCTCTTGCCATCTCC, 3347+FAM-ACCACGCACACGCACAGAGAG-BHQ1.

Statistical analysis

In all cases, we assumed that our data were not normally distributed, and statistical significance was calculated using either paired or unpaired non-parametric *t*-tests as appropriate. Wilcoxon signed-rank test was used to determine significance in matched (paired) samples for both patient treatment groups. A Mann-Whitney *U* test was used to determine significance from unpaired samples. A Spearman's rank correlation test was used to determine significant correlations between changes in transcript expression. All types of analysis were conducted in GraphPad Prism 5 statistical software (La Jolla, CA, USA), and a *P* value <0.05 was considered significant.

Results

Survivin mRNA is differentially expressed in the cycling human endometrium

The proliferative and secretory phases of the reproductive cycle in the human endometrium are dominated by E₂ and P₄ signaling respectively. Previous reports on survivin gene expression in the cycling human endometrium have been conflicting (Konno *et al.* 2000, Tarkowski *et al.* 2000, Lehner *et al.* 2002). To determine whether survivin transcript is physically expressed in the cycling human endometrium,

QPCR analysis was conducted on RNA isolated from proliferative (*n*=7) and secretory (*n*=5) phase endometrial biopsies. Survivin gene expression levels were nearly 50-fold higher in proliferative endometrial biopsies compared to secretory biopsies (median proliferative=4.08, median secretory=0.08; *P*=0.01; Fig. 1). Since differences between these two phases are largely driven by steroid hormone signaling, we hypothesized that survivin transcript levels are increased in response to E₂ and decreased in response to P₄ in the human endometrium.

Survivin mRNA levels are decreased in response to P₄ treatment in the human endometrium

To determine whether endometrial tissue from women treated for 3 months with DEPO responded physiologically to progestin treatment, post treatment biopsies were examined histologically. The presence of microscopic changes associated with non-proliferative endometrium (presence of endometrial stromal cells that are pre-decidualized, characterized by stromal cells with increased eosinophilic cytoplasm and acquisition of an epithelioid shape; presence of secretory-type or inactive endometrial glands) indicated a good response to progestins. Of the 21 post treatment endometrial biopsies, 19 exhibited features consistent with a good response to progestin treatment (Fig. 2a). Two biopsies showed a poor response indicated by the absence of these changes and/or the presence of mitotic figures in the endometrial glands and/or stroma (Fig. 2b).

To determine whether DEPO treatment induced expression of known P₄-responsive genes, we measured the transcript levels of *IGFBP1*, a gene that is strongly expressed in the secretory endometrium and is known to be induced by

Figure 1 Survivin mRNA is differentially expressed in the cycling human endometrium. RNA samples obtained from endometrial biopsies of women in the proliferative phase (*n*=7) or secretory phase (*n*=5) of the reproductive cycle were analyzed by QPCR for survivin. Data are presented as a median ratio of (survivin/18s rRNA) × 10⁶. Box denotes 25–75% distribution, and whiskers denote 10–90% range. Mann-Whitney unpaired *U* test was used to determine significance.

Figure 2 Survivin gene expression is decreased in the human endometrium after progesterin treatment. Representative photomicrographs demonstrating examples of a good (a) and poor (b) histological response to progesterin treatment. (a) Good progesterin treatment response was characterized by the presence of small, inactive appearing endometrial glands composed of a single layer of glandular epithelial cells that are free of mitotic figures (arrow) and stromal cells undergoing pre-decidualization changes (open circle). The pre-decidualized stromal cells have more eosinophilic cytoplasm and are more epithelioid in shape than the usual endometrial stromal cell, which typically has very little cytoplasm and has poorly defined cell borders. (b) A poor progesterin response was characterized by the presence of proliferative-type endometrial glands composed of multiple layers of glandular epithelial cells and containing darkly stained mitotic figures (arrows). Note that the proliferative-type endometrial glands in (b) are much larger than the inactive-appearing glands in (a). H&E, 200×. RNA samples obtained from endometrial biopsies of 21 women at baseline and after 3 months of DEPO treatment were analyzed by QPCR for IGFBP1 (c) and survivin (d). Data are presented as a median ratio of (c) (IGFBP1/18s rRNA) × 10⁵ or (d) (survivin/18s rRNA) × 10⁶. Box represents 25–75% distribution, and whiskers represent 10–90% range. Wilcoxon signed-rank test was used to determine significance. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0128>.

P₄ in the endometrium (Bell *et al.* 1991, Gao *et al.* 1994a,b). IGFBP1 is a P₄-inducible secreted factor that modulates insulin-like growth factor (IGF) bioavailability and functions in concert with IGF proteins to regulate a variety of reproductive processes including menses, puberty, ovulation, decidualization, implantation, and fetal growth (Lane *et al.* 1994, Richards *et al.* 1995, Fowler *et al.* 2000). We observed negligible expression of IGFBP1 in all of the baseline endometrial samples followed by a 3000-fold increase in the median level of mRNA in post treatment endometrial samples indicating successful drug administration (Fig. 2c). Overall, survivin transcript expression decreased 4.6-fold following treatment compared to baseline levels (median pre = 23.1, median post = 4.1, *n* = 21, *P* < 0.0001; Fig. 2d). Fold change in survivin did not correlate with change in IGFBP1 (*P* = 0.19). If we examine change per individual, the median change in survivin transcript levels following

treatment for the good responders is −3.3-fold (*n* = 19). With only two poor responders, there are insufficient data to draw conclusions regarding survivin in these women (mean fold change = +0.74, *n* = 2).

Survivin levels are increased in response to estrogen treatment in the human endometrium

QPCR analysis of endometrial biopsies taken at baseline and after 6 months of treatment with estrogens showed a significant 4-fold up-regulation of survivin transcript (median pre = 0.65, median post = 3.23, *n* = 10, *P* = 0.013; Fig. 3a). Survivin expression was increased in seven of the ten patients with the remaining three patients showing no change or a small decrease in survivin expression (#2, #5, and #9; Fig. 3b). To confirm induction of estrogen-regulated genes in the endometrial tissue of the women after treatment, we measured the transcript levels of *IGF1*, a well known estrogen-inducible gene involved in estrogen-mediated endometrial proliferation (Rutanen 1998, Moyano & Rotwein 2004, McCampbell *et al.* 2006, Kashima *et al.* 2009), which we have previously shown to be induced by

Figure 3 Survivin mRNA expression is increased in the human endometrium after estrogen treatment. RNA samples obtained from endometrial biopsies of ten women at baseline and after 6 months of estrogen treatment were analyzed by QPCR for survivin (a and b), IGF1 (c and d), and Ki67 (e and f). Data are presented as a median ratio of (transcript/18s rRNA) (a, c, and e) or fold change for each patient post treatment compared to baseline (b, d, and f). Box represents 25–75% distribution, and whiskers represent 10–90% range. Wilcoxon signed-rank test was used to determine significance.

estrogen treatment in the endometrium of postmenopausal women (Westin *et al.* 2009). To confirm that the women responded to estrogen treatment, we measured transcript levels of nuclear antigen Ki67, a standard marker of proliferation (Hurskainen *et al.* 2000, Jaroslav *et al.* 2005, Strick *et al.* 2007). We determined that while all ten of the post treatment endometrial tissues exhibited estrogen induction of IGF1 (Fig. 3d), only nine of the ten patients responded with a proliferative phenotype indicated by increased Ki67 expression. Notably, patient # 9, who showed a decrease in Ki67 transcript, also showed a decrease in survivin transcript (Fig. 3f). The fold change in survivin induction significantly correlated with the fold change in Ki67 ($P < 0.0001$, $r^2 = 0.98$) but not with IGF1 fold change ($P = 0.76$, $r^2 = 0.12$), and IGF1 fold change also did not correlate with fold change in Ki67 ($P = 0.758$, $r^2 = 0.11$). The women's age ($P = 0.73$), BMI ($P = 0.89$), time since last menstrual period ($P = 0.94$), baseline serum E_2 ($P = 0.87$), FSH ($P = 0.47$), and prolactin ($P = 0.97$) levels did not correlate with fold change in survivin expression.

Discussion

Survivin has been shown by our group and others to be overexpressed in several endometrial disease states including endometriosis, endometrial hyperplasia, and endometrial carcinomas. Information on the role of survivin in maintaining normal tissue homeostasis is lacking. The human endometrium is a classically hormone-responsive tissue; therefore, it is susceptible to endocrine-related pathologies as well as treatments. In this study, we show that survivin transcript expression is significantly increased in the E_2 -predominated proliferative phase, and is significantly decreased in the P_4 -predominated secretory phase of the normal endometrium. These data are in agreement with those published by Lehner *et al.* (2002) and suggest that survivin gene expression is hormonally regulated in the human endometrium.

It is enticing to speculate that survivin's anti-apoptotic and cell cycle regulatory functions may be involved in maintaining normal tissue homeostasis; however, it is important to note that our studies examined only survivin gene expression, not survivin protein. Konno *et al.* (2000) published work indicating that survivin transcript and protein were increased in the secretory phase rather than the proliferative phase of cycling endometrium. While our transcript data are contrary to the Konno *et al.* results, there are, to our knowledge, no published works that disagree with the Konno *et al.* protein results. It has been shown that survivin protein is increased by estrogens and decreased by progestins in endometrial cancer-derived cells *in vitro*; however, further studies will be required to determine whether survivin protein expression is similarly hormonally regulated in disease-free human endometrial tissue. We postulated that the survivin transcript may be functional in the endometrium as it is a natural antisense transcript to the effector cell protease receptor 1 (EPR1). EPR1 is the receptor

for factor Xa and is involved in vascular inflammation and blood coagulation. Inverse expression of survivin and EPR1 has been observed in several systems and hematologic malignancies. However, QPCR analysis conducted in our laboratory with an EPR1-specific probe could not detect EPR1 transcript neither in normal proliferative human endometrial tissue nor in endometrial tumors (data not shown). We did not examine secretory tissue for *EPR1* gene expression.

We are the first to report that survivin gene expression is changed in response to administration of E_2 and P_4 in the disease-free human endometrium. A 6-month treatment with E_2 significantly increased the expression of a well-known E_2 -regulated gene, *IGF1*. In addition, survivin gene expression increased and was associated with increased proliferation indices as measured by Ki67 expression. These data suggest that while the IGF1 protein is functionally involved in mediating the proliferative response towards estradiol, the survivin transcript may be more useful than the IGF1 transcript as an indicator of hormone treatment efficacy, specifically as a marker of proliferation in response to E_2 . A larger study including more women will be necessary to validate this finding.

A 3-month treatment with DEPO resulted in a significant increase in a well-known P_4 -regulated gene, *IGFBP1*, and resulted in a significant decrease in the gene expression of survivin. A report published recently indicated that increased survivin protein levels predicted resistance to progestin therapy in patients with endometrial hyperplasia (Chen *et al.* 2009). In our dataset, upon histological examination, two women were determined to be non-responders to progestin treatment, and both patients also exhibited an aberrant survivin response (no change or increased gene expression) as measured by QPCR. A larger study including more non-responders will be necessary to assess the significance of this finding.

Herein, we report preliminary results suggesting that survivin transcript as a biomarker could be helpful to clinicians who prescribe hormone replacement therapies to postmenopausal women or to those who use hormone therapies as chemopreventive agents in high-risk populations. Larger studies will be needed to validate the utility of survivin transcript as a biomarker for hormone treatment efficacy and/or resistance in these populations.

Declaration of interest

D S Loose's work has been funded by the NIH and the John S Dunn foundation. He is a consultant for Wyeth Pharmaceuticals for which he receives compensation. The other authors have no conflicts of interest.

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