Expression of estrogen receptor coregulators in benign and malignant human endometrium

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Expression of Estrogen Receptor Coregulators in Benign and Malignant Human Endometrium

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INTRODUCTION

Endometrial carcinoma (EC) is the most common cancer of the female genital tract (Cotran et al., 1999). It is the fourth most common cancer in women and the eighth leading cause of cancer death in women (2002). The average age of onset is 60 yr and only 25% of cases occur before menopause (Hoskins et al., 2000). Endometrial cancers are often divided into two groups. Type I, estrogen-dependent EC usually arise from hyperplasias due to unopposed estrogen (Inoue, 2001; Berek and Hacker, 2000). These cancers have a better prognosis than the Type II estrogen-independent tumors that arise from atrophic and non-hyperplastic endometrium (Inoue, 2001; Berek and Hacker, 2000).

Estrogen receptor (ER) is a type I receptor of the nuclear receptor superfamily and consists of five domains. An A/B domain containing a ligand-independent activation function-1 (AF-1) region, a DNA binding domain, a hinge region, a ligand binding domain containing a ligand-dependent AF-2 region, and an F domain (Lonard and Smith, 2002). There is also a recently discovered ERβ (Mosselman et al., 1996), which contains several regions that are similar to the original ERα (Lonard and Smith, 2002). The highest homology is found in the DNA binding domain (84%), while the hinge region (12%) and F domain (12%) have the least homology (Lonard and Smith, 2002). The AF-1 region of ERβ has absent or reduced function (Kushner et al., 2000; Speroff, 2000).

In the Classical Pathway of ER activation, unliganded ER in the nucleus is bound to heat-shock proteins (Hall et al., 2001). Estrogen binds the ER and causes a conformational change (Hall et al., 2001). Estrogen receptor then homodimerizes
(Kumar and Chambon, 1988), binds to an estrogen response element (Kumar and Chambon, 1988), and recruits coactivators which help to initiate transcription through chromatin remodeling and their interaction with general transcription factors (Hall et al., 2001; McKenna and O'Malley, 2002).

Coregulators are proteins which bind to nuclear receptors and either up-regulate (coactivators) or down-regulate (corepressors) their transcriptional activity. Coactivators have an LXXLL motif, also known as a nuclear receptor (NR) box (Warnmark et al., 2002), where L is leucine and X can be any other amino acid (Heery et al., 1997). The NR box binds to the hydrophobic groove on the nuclear receptor ligand-binding domain (Shiau et al., 1998). The three members of the p160 family, SRC-1, SRC-2, and SRC-3, are coactivators which recruit histone acetyltransferases (HAT) such as CREB-Binding Protein (CBP) (Leo and Chen, 2000; Bannister and Kouzarides, 1996). In addition, SRC-1 (Spencer et al., 1997) and SRC-3 (Chen et al., 1997) have been shown to have intrinsic HAT activity. Acetylation of histones increases access of transcription factors to the promotor (Aranda and Pascual, 2001).

In addition, there is an LXX(I/H)IXXX(I/L) motif which is found in certain corepressors (Perissi et al., 1999). In this motif, L is leucine, I is isoleucine, H is histidine, and X is any amino acid (Perissi et al., 1999). It is thought that there is an extra helical turn in this sequence, which keeps corepressors out of the hydrophobic pocket when the nuclear receptor is bound with estrogen (Rosenfeld and Glass, 2001). Corepressors such as nuclear corepressor (N-CoR) and silencing mediator for retinoic and thyroid receptors (SMRT) repress transcription by recruiting histone deacetylases
(HDAC). Deacetylation causes chromatin to adopt a more compact structure, which represses transcription (Burke and Baniahmad, 2000).

Estrogen causes the endometrium to proliferate and stimulates gland formation and vascular growth (Ryan et al., 1999). It also induces growth in breast tissue (Wilson et al., 1998), which may lead to an increased risk of breast cancer (Rossouw et al., 2002; Lonard and Smith, 2002). The Selective Estrogen Receptor Modulator (SERM), tamoxifen, was developed in 1966 and approved by the FDA in 1978 (Goldstein, 2001). It is the most prescribed anti-neoplastic in the world (Goldstein, 2001), and it is most effective in ER-positive breast cancer (1998). While tamoxifen has been shown to increase bone mineral density, it also is associated with thromboembolic events, vasomotor symptoms, cataracts, and an increased risk of endometrial cancer (Morello et al., 2002; Fisher et al., 1998).

There are several theories as to why tamoxifen seems to have different effects based on the target tissue. One common theory is that the ratio of coactivators to corepressors may determine tamoxifen action. A tissue with a higher level of coactivators may be expected to have a greater agonist effect with tamoxifen, while a tissue expressing a high level of corepressors may have the opposite. It has been shown that changing the level of coactivators and corepressors alters both estrogen and tamoxifen activity (Delage-Mourroux et al., 2000; Martini and Katzenellenbogen, 2001; Oesterreich et al., 2000a; Smith et al., 1997; Jackson et al., 1997). Changing the level of coactivators and corepressors also has been shown to directly affect cell growth (Cavarretta et al., 2002; Chien et al., 1999; List et al., 2001a; Tai et al., 2000). Thus, it is
reasonable to speculate that there is a higher coactivator to corepressor ratio in endometrial cancer as compared to normal endometrium.

Coregulators also may be used as biomarkers. In breast cancer, ER/PR positive tumors have a better prognosis (Osborne, 1998), and SRC-3 expression was correlated with a higher grade and absence of ER and PR (Bouras et al., 2001). Patients with low levels of N-CoR had shorter disease free survival times than patients with intermediate and high levels (Girault et al., 2003). In prostate cancer, tumors that had a poor response to endocrine therapy had significantly higher levels of SRC-1 than tumors with a good response (Fujimoto et al., 2001). Identifying coregulators that are associated with poor outcomes in endometrial cancer may help clinicians in determining the prognosis of their patients.

OBJECTIVES

1. To identify estrogen receptor coregulators important in the normal endometrium and in the progression from benign to malignant.
   - Measure mRNA expression of the estrogen receptor coregulators SRC-1, SRC-2, SRC-3, N-CoR, and SMRT in benign and malignant endometrium using real-time RT-PCR;
   - Measure expression of estrogen receptor-α protein in benign and malignant endometrium using western blot and digital quantification;
• Measure expression of progesterone receptor mRNA in benign and malignant endometrium using real-time RT-PCR;
• Determine if the expression of coregulators and the ratio of coactivators to corepressors changes during the progression of endometrial cancer.

2. To correlate the endogenous expression of estrogen receptor coregulators with clinicopathologic parameters.
• Correlate estrogen receptor coregulator expression with grade, stage, age, depth of myometrial invasion and ER/PR expression.
Estrogen Receptor

Estrogen receptor (ER) is a type I receptor of the nuclear receptor superfamily (Olefsky, 2001; Hall et al., 2001). The ERα protein was discovered in 1960 and the gene was cloned in 1986 (Greene et al., 1986). It is approximately 595 amino acids in length (Greene et al., 1986), has a molecular weight of 64 kDa (Speroff, 2000) and consists of 5 domains (Figure 1).

The A/B domain contains a constitutively active, activation function-1 (AF-1) region, that is involved in ligand-independent activation of ER (Speroff, 2000; Webb et al., 1998). It also contains four major phosphorylation sites at Serines 104, 106, 118, and 167 (Lannigan, 2003). The C domain, also known as the DNA binding domain (DBD), has a conserved core of 66 amino acids (Mader et al., 1993) containing two zinc fingers (Green et al., 1988). The first zinc finger is responsible for the specificity of target gene binding, while the second finger stabilizes the interaction between the ER and DNA (Green et al., 1988). The D domain, or hinge region, is important for the ER’s localization to the nucleus (Picard et al., 1990) and its conformational changes (Speroff, 2000). It also contains a phosphorylation site at Serine 236 (Chen et al., 1999b). The E domain, or ligand binding domain (LBD) is responsible for ligand binding, dimerization, and binding to heat-shock proteins (HSP) (Speroff, 2000). It contains 12 conserved helices and a ligand-dependent activation function-2 (AF-2) region (Lonard and Smith, 2002). In addition, there are phosphorylation sites at Serine 305 (Wang et al., 2002),
The estrogen receptor is a Type I nuclear receptor composed of 5 subunits. An A/B domain containing a ligand-independent AF-1 region, a DNA-binding domain (C), a hinge region (D), a ligand-binding domain (E) containing a ligand-dependent AF-2 region, and an F domain.
Threonine 311 (Lee and Bai, 2002), and Tyrosine 537 (Arnold et al., 1995). There also is an F domain; and while it is not required for an estrogen response, it does affect the magnitude of the response (Speroff, 2000).

The ERβ gene was first cloned and sequenced in 1996 (Mosselman et al., 1996). It was found to be 530 amino acids long (Fuqua et al., 1999), have a molecular weight of 58-60 kDa (Fuqua et al., 1999), and bind estrogen with the same affinity as ERα (Pace et al., 1997). It contains several regions that are similar to ERα, but the highest homology is found in the DNA binding domain (84%) (Lonard and Smith, 2002). Homology of the DBD is followed by the LBD (58%), the A/B domain (26%), the hinge region (12%), and the F region (12%) (Lonard and Smith, 2002). The AF-1 region of ERβ is structurally different from the AF-1 of ERα (Speroff, 2000), and shows no constitutive activity (Kushner et al., 2000). In mice, ERα is found in higher concentrations than ERβ in the uterus, oviducts, and mammary glands (Couse and Korach, 1999). However, in the mouse ovary, ERβ has higher expression (Couse and Korach, 1999).

The Estrogen Response Element (ERE) has an inverted repeat structure with the nucleic acid sequence, AGGTCA(N₃)TGACCT (Aranda and Pascual, 2001). It is usually found in the promotor of estrogen-responsive genes (Lonard and Smith, 2002), and is located upstream of the TATA box where general transcription factors (GTF) and RNA polymerase II bind (Aranda and Pascual, 2001). The promotor also may contain other transcription factors, such as the promotor for the pS2 gene, which contains both ERE and activator protein-1 (AP-1) sites (Barkhem et al., 2002).
Mechanisms of ER Activation

Estrogen receptor is believed to be activated by three major mechanisms. The Classical Pathway, the Ligand-Independent Pathway, and the ERE-Independent Pathway. In the Classical Pathway (Figure 2A), unliganded ER in the nucleus is bound to heat-shock proteins (HSP) (Hall et al., 2001). Estrogen (E2) binds the ER and causes a conformational change (Hall et al., 2001). The ER protein then homodimerizes (Kumar and Chambon, 1988), binds to an ERE (Kumar and Chambon, 1988), and recruits coactivators which help to initiate transcription through chromatin remodeling and their interaction with GTFs (Hall et al., 2001; McKenna and O'Malley, 2002).

Estrogen receptor activation in the absence of ligand is known as the ligand-independent pathway (Figure 2B). In this mechanism, growth factors and kinases are responsible for ER activation. Mitogen-activated protein kinase (MAPK), epidermal growth factor (EGF) and insulin-like growth factor (IGF), have been shown to phosphorylate Ser 118 on the A/B domain of ER (Kato et al., 1995). Also, phosphorylation of coregulators may be as important as ER phosphorylation in this pathway (Schiff et al., 2003). It has been shown that EGF mimics the effect of E2 on the reproductive tract (Ignar-Trowbridge et al., 1992), so this pathway may allow ER activation when E2 concentration is low (Hall et al., 2001).

The ERE-Independent Pathway is the initiation of transcription by ER through non-ERE sites after activation with E2 or growth factors (Figure 2C). It has been shown that both ERα and ERβ can bind transcription factors such as AP-1 (Webb et al., 1995) and specificity protein-1 (SP-1) (Porter et al., 1997). Binding of Jun/Fos proteins to the
Figure 2. Mechanisms of ER Activation and Repression

A. Estrogen binds ER and causes a conformational change. ER then recruits coactivators and general transcription factors to the TATA box to initiate transcription. Coactivators such as SRC-1 and CBP acetylate DNA and upregulate transcription.

B. Growth factors and kinases phosphorylate the ER and activate transcription in the absence of ligand. Coregulators can also be phosphorylated.

C. Initiation of transcription through non-ERE sites. ER activates transcription at AP-1 sites indirectly through Jun/Fos proteins.

D. SERMs bind to ER creating a unique conformation. Corepressors and HDACs are then recruited and downregulate transcription.
AP-1 site is needed for ER activation, and ER increases the intrinsic transcriptional activity when it is bound (Kushner et al., 2000). E2 bound to ERα requires both the AF-1 and the AF-2 to activate an AP-1 site (Hall et al., 2001), and the ERβ, which lacks a functional AF-1, cannot activate AP-1 sites when bound with agonist (Kushner et al., 2000).

There is also evidence that ER has non-genomic actions. Estrogen receptor has been found in the membranes of several cell types (Levin, 2002), however, it is unclear whether it is different from the nuclear ER (Hall et al., 2001), or if it is the same nuclear ER translocated to the membrane (Pappas et al., 1995). It has not been isolated and sequenced, but it is possible that signaling from the membrane may amplify the effect of the nuclear receptor (Levin, 2002).

Coregulators

Coregulators are proteins which bind to nuclear receptors and either up-regulate (coactivators) or down-regulate (corepressors) their transcriptional activity (Table I). Coactivators have an LXXLL motif, also known as a nuclear receptor (NR) box (Warnmark et al., 2002), where L is leucine and X can be any other amino acid (Heery et al., 1997). The NR box is an amphipathic α-helix, which binds to the hydrophobic groove on the nuclear receptor formed by helices 3, 4, 5, and 12 (Shiau et al., 1998). Mutations in these helices diminished AF-2 function and coactivator binding (Kushner et al., 2000; Shiau et al., 1998).
<table>
<thead>
<tr>
<th></th>
<th>Other Nomenclature</th>
<th>Mechanism</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>COACTIVATORS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRC-1</td>
<td>NcoA1</td>
<td>Intrinsic HAT activity and recruitment of HATs</td>
<td>(Leo and Chen, 2000; Gregory et al., 2002; Spencer et al., 1997; Rowan et al., 2000b)</td>
</tr>
<tr>
<td>SRC-2</td>
<td>TIF2/GRIP1</td>
<td>Recruitment of HATs</td>
<td>(Leo and Chen, 2000; Gregory et al., 2002; Warnmark et al., 2002; Lopez et al., 2001)</td>
</tr>
<tr>
<td>SRC-3</td>
<td>RAC3/ACTR/pCIP/AIB1/TRAM-1</td>
<td>Intrinsic HAT activity and recruitment of HATs</td>
<td>(Leo and Chen, 2000; Gregory et al., 2002; Font and Brown, 2000; Chen et al., 1997)</td>
</tr>
<tr>
<td>CBP</td>
<td>p300</td>
<td>Intrinsic HAT activity</td>
<td>(Leo and Chen, 2000; Kurebayashi et al., 2000; Martinez-Balbas et al., 1998; Bannister and Kouzarides, 1996)</td>
</tr>
<tr>
<td>pCAF</td>
<td></td>
<td>Intrinsic HAT activity</td>
<td>(Leo and Chen, 2000; Kurebayashi et al., 2000; Yang et al., 1996)</td>
</tr>
<tr>
<td>DRIP205/TRAP220</td>
<td>PAF400</td>
<td>Helps recruit RNA polymerase II</td>
<td>(Burakov et al., 2000; Coulthard et al., 2003; Kang et al., 2002; Warnmark et al., 2001)</td>
</tr>
<tr>
<td>CARM1</td>
<td></td>
<td>Methylate histones</td>
<td>(Leo and Chen, 2000; Koh et al., 2001; Chen et al., 1999a)</td>
</tr>
<tr>
<td>PRMT1</td>
<td>HRMT1L2</td>
<td>Methylates histones</td>
<td>(Scorilas et al., 2000; Koh et al., 2001)</td>
</tr>
<tr>
<td>PTMA</td>
<td></td>
<td>Recruits REA away from ER</td>
<td>(Martini et al., 2000; Martini and Katzenellenbogen, 2001)</td>
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</tr>
<tr>
<td><strong>COREPRESSORS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCOR</td>
<td>RIP13</td>
<td>Recruits HDAC complex</td>
<td>(Kurebayashi et al., 2000; Huang et al., 2000; Nagy et al., 1997; Smith et al., 1997)</td>
</tr>
<tr>
<td>SMRT</td>
<td>TRAC2</td>
<td>Recruits HDAC complex</td>
<td>(Kurebayashi et al., 2000; Huang et al., 2000; Nagy et al., 1997; Smith et al., 1997)</td>
</tr>
<tr>
<td>REA</td>
<td>BAP37/D-Prohibitin</td>
<td>Competes with SRC-1 for ER binding.</td>
<td>(Delage-Mourroux et al., 2000; Martini et al., 2000; Simon et al., 2000; Murphy et al., 2000)</td>
</tr>
<tr>
<td>SAF-B</td>
<td>HET</td>
<td>Recruits HDAC. Crosslinks ER to nuclear matrix.</td>
<td>(Oesterreich et al., 2000a; Oesterreich et al., 2000b; Townson et al., 2000; Samuel et al., 1998)</td>
</tr>
<tr>
<td>MTA1</td>
<td></td>
<td>Recruits HDAC complexes</td>
<td>(Mazumdar et al., 2001; Talukder et al., 2003)</td>
</tr>
<tr>
<td>MTA1s</td>
<td></td>
<td>Sequesters ER in the cytoplasm and enhances its non-genomic actions</td>
<td>(Kumar et al., 2002)</td>
</tr>
<tr>
<td>FKHR</td>
<td></td>
<td>Regulates apoptosis and cell-cycle arrest</td>
<td>(Schuur et al., 2001)</td>
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In addition, there is an LXX(I/H)XXX(I/L) motif, also an amphipathic $\alpha$-helix (Aranda and Pascual, 2001), which is found in certain corepressors (Perissi et al., 1999). In this motif, L is leucine, I is isoleucine, H is histidine, and X is any amino acid (Perissi et al., 1999). It is thought that there is an extra helical turn in this sequence, which keeps corepressors out of the hydrophobic pocket when the nuclear receptor is bound with agonist (Rosenfeld and Glass, 2001).

**Coregulator Mechanisms of Action**

Histones have electrostatic contacts between lysine side-chains and DNA phosphate groups (McKenna and O'Malley, 2002). Acetylation disrupts these bonds by neutralizing the positive charge on the lysines and increasing access of transcription factors to the promotor (Aranda and Pascual, 2001). Activated ER recruits SWI/SNF complexes and HATs to the ERE for chromatin remodeling and acetylation (Moggs and Orphanides, 2001).

Members of the p160 family (Figure 3A) are coactivators which recruit HATs such as Creb-Binding Protein (CBP) (Leo and Chen, 2000; Bannister and Kouzarides, 1996). The first member identified, steroid receptor coactivator-1 (SRC-1), contains a basic loop-helix-loop domain (bHLH) of unknown function, 3 LXXLL motifs in its receptor interacting domain and separate regions for interaction with CBP, CBP/p300-associated factor (pCAF), and coactivator-associated arginine methyltransferase (CARM1) (Leo and Chen, 2000). The three members of the p160 family, SRC-1, SRC-2, and SRC-3, show a sequence similarity of 40% (Aranda and Pascual, 2001). They can
Members of the p160 family are 160 kD proteins that have a basic helix-loop-helix domain (bHLH) of unknown function, a receptor interacting domain (RID) containing three LXXLL motifs, and activation domains containing HAT activity and interaction sites for CBP, p/CAF, and CARM1.

N-CoR and SMRT are 250-350 kD proteins that have repressor domains containing interaction sites for mSin 3 and HDACs, and a receptor interacting domain containing two LXX(I/H)XXX(I/L) motifs.
interact with the AF-1 and AF-2 of ER simultaneously (Benecke et al., 2000), however, binding to AF-1 does not involve an LXXLL motif (Bevan et al., 1999). SRC-1 (Spencer et al., 1997) and SRC-3 (Chen et al., 1997) have been shown to possess intrinsic HAT activity in addition to their recruitment of HATs.

SRC-1 knock-out (KO) mice show reduced reproductive tract growth in response to hormones (Xu et al., 1998). SRC-2 KO mice were hypofertile but showed no difference in uterine or prostate growth in response to hormones (Gehin et al., 2002). SRC-3 KO mice show growth retardation, delayed puberty, and impaired female reproduction (Xu et al., 2000).

It has been proposed that after ER binds to the promoter, it recruits a p160 HAT complex (made up of p160, CBP, and pCAF) and the vitamin D receptor interacting protein/thyroid receptor associated protein (DRIP/TRAP) complex (Leo and Chen, 2000; Shang et al., 2000). The HAT complex may be composed of more than one p160 coactivator (McKenna et al., 1998) and the DRIP/TRAP complex may enhance recruitment of RNA polymerase II to the promoter (Rosenfeld and Glass, 2001). Members of the p160 family may also recruit the histone methyltransferase CARM1 to regulate ER activity (Leo and Chen, 2000; Chen et al., 1999a).

Corepressors such as nuclear corepressor (N-CoR) and silencing mediator for retinoic and thyroid receptors (SMRT) repress transcription by recruiting histone deacetylases (HDAC). Deacetylation causes chromatin to adopt a more compact structure, which represses transcription (Burke and Baniahd, 2000). N-CoR and SMRT both have repressor domains which interact with HDACs (Li et al., 1997), and a
receptor interacting domain containing two LXX(I/H)IXXX(I/L) motifs (Perissi et al., 1999). It has been shown that N-CoR is associated with HDAC-1 or HDAC-2 and mSin3 (Nagy et al., 1997), a protein that may form the base for the deacetylase complex (Ayer, 1999). Both N-CoR and SMRT also can associate with Class II HDACs such as HDAC-4 and HDAC-5 without the presence of mSin3 (Huang et al., 2000). Estrogen receptor can also recruit HDACs through the growth factor-induced expression of metastasis-associated protein 1 (MTA1) (Mazumdar et al., 2001).

**Regulation of Coregulators**

Coregulators control their expression and action in cells through shuttling, phosphorylation, proteolysis, and acetylation. Nucleocytoplasmic shuttling may control how much of a certain coregulator is available to interact with a nuclear receptor and SRC-3 has been shown to shuttle between the cytoplasm and the nucleus (Wang et al., 2000). Another mechanism of regulation is phosphorylation of coregulators, which has been shown to increase acetyltransferase activity (McKenna and O'Malley, 2002). Phosphorylation of SRC-1, -2, and -3, by MAPK increased their transcriptional activity (Font and Brown, 2000; Rowan et al., 2000b; Lopez et al., 2001), while phosphorylation of the corepressor SMRT by MAPK/extracellular signal-regulated kinase-1 (MEK-1) caused a decrease in its activity (Hong and Privalsky, 2000). Mutations of phosphorylation sites on SRC-1 reduced the interaction between SRC-1 and CBP (Rowan et al., 2000a). The proteosomal degradation of N-CoR is regulated by mSiah2, whose expression is dependent on the cell type (Zhang et al., 1998). Acetylation of SRC-3 by
CBP decreased the association of the coactivator complex and the ER (Chen et al., 1999c), and acetylation of coregulators also has been shown to influence nucleocytoplasmic shuttling (Soutoglou et al., 2000).

**Estrogen and SERMs**

Selective Estrogen Receptor Modulators (SERM) is a term that arose when it was found that ER antagonists sometimes displayed partial agonist activity (Lonard and Smith, 2002). They can act as pure antagonists or partial agonists depending on cell type and promoter context (Aranda and Pascual, 2001), and ER binds corepressors only in the presence of SERMs (Smith et al., 1997). Pure anti-estrogens inhibit dimerization, inhibit nucleocytoplasmic shuttling, and increase degradation (Speroff, 2000).

Estrogen regulation begins in the hypothalamus. Pulsatile Gonadotropin Releasing Hormone (GnRH) from the hypothalamus signals the release of Follicle-Stimulating Hormone (FSH) and Leutinizing Hormone (LH) from the anterior pituitary (Ryan et al., 1999). These hormones cause the release of androstenedione from the theca cells of the ovaries, which then is converted to estradiol by the granulosa cells of the ovaries (Ryan et al., 1999). In a 28 d menstrual cycle, menses occurs for the first 4-7 followed by a proliferative phase (Ryan et al., 1999). During the proliferative phase, estradiol stimulates gland formation and vascular growth in the endometrium until an LH surge on day 14 causes ovulation (Ryan et al., 1999). As the progesterone levels rise, the endometrium differentiates into secretory phase epithelium. At day 28, the epithelium is sloughed off and the cycle begins again (Ryan et al., 1999). One study showed no
change in the expression of SRC-1, -2, and -3 during the normal menstrual cycle except for an increase in glandular SRC-3 during the late secretory phase (Gregory et al., 2002). However, another study showed a decrease in SRC-1 during the secretory phase in both glands and stroma (Shiozawa et al., 2003). N-CoR also decreased during the secretory phase in both glands and stroma, while SMRT decreased in the stroma alone (Shiozawa et al., 2003).

Estrogen causes the endometrium to proliferate, but it also induces growth in breast tissue (Wilson et al., 1998), which may lead to an increased risk of breast cancer (Rossouw et al., 2002; Lonard and Smith, 2002). It was previously believed that estrogen had a cardioprotective effect (Low et al., 2002), but a recent study by the Women’s Health Initiative (WHI) has concluded that estrogen plus progestin should not be prescribed for the primary prevention of coronary heart disease (CHD) (Rossouw et al., 2002). Estrogen also was believed to maintain bone mass (Lonard and Smith, 2002), and the WHI data support this theory (Rossouw et al., 2002). It has been shown in cell culture experiments that E2 causes an increase in the degradation of ER (Lonard and Smith, 2002; Lonard et al., 2000; Nawaz et al., 1999), but it increases ER production in the endometrium in vivo during the proliferative phase of the menstrual cycle (Ryan et al., 1999). Estrogen also causes an increase in progesterone receptor (PR) levels (Speroff, 2000; Ryan et al., 1999).

Tamoxifen

Tamoxifen was developed in 1966 and approved by the FDA in 1978 (Goldstein,
It is the most prescribed anti-neoplastic in the world (Goldstein, 2001), and it is most effective in ER-positive breast cancer (Early Breast Cancer Trialists' Collaborative Group, 1998). It also has been shown to be effective for chemoprevention in high-risk women (Fisher et al., 1998). While tamoxifen has been shown to increase bone mineral density (BMD), it is also associated with thromboembolic events, vasomotor symptoms, cataracts, and an increased risk of endometrial cancer (Morello et al., 2002; Fisher et al., 1998). The first report of the association of tamoxifen and endometrial cancer was in 1985 (Killackey et al., 1985), and since then there have been many studies of this relationship (Barakat et al., 2000; Andia et al., 2000; Seoud et al., 1999; Marchesoni et al., 2001; Gerber et al., 2000; Ozsener et al., 1998; Cheng et al., 1997).

Tamoxifen is a type III anti-estrogen (triphenylethylene derivative) and is cytostatic as opposed to cytocidal (Speroff, 2000). As an antagonist, it competitively inhibits E2 binding (Speroff, 2000), and causes helix 12 to interact with the hydrophobic pocket on the LBD of the ER (Shiau et al., 1998). This displacement of the AF-2 allows corepressors such as N-CoR to bind and repress transcription (Shiau et al., 1998) (Figure 2D). Although tamoxifen has been shown to have non-ER mechanisms of action (Mandlekar and Kong, 2001), its ER antagonist action in the breast and agonist action in the uterus is the focus of this literature review.

Theories for the Tissue-Specific Effects of Tamoxifen

There are several theories as to why tamoxifen seems to have different effects based on the target tissue. One common theory is that the ratio of ERα and ERβ in a cell
may determine the tissue-specific response (Hall et al., 2001). It has been shown that ERα and ERβ heterodimerize (Pace et al., 1997), and this heterodimerization may alter the activity of the ER (Moggs and Orphanides, 2001). It also has been shown that ERα and ERβ display differences in p160 coactivator recruitment when bound with various agonists (Routledge et al., 2000; Yi et al., 2002), and that coexpression of ERβ decreased tamoxifen’s agonist activity on ERα in HepG2 cells (liver cancer) (Hall and McDonnell, 1999).

Estrogen receptor conformation may be another reason why SERMs display tissue-specific effects. After ligand binds, the LBD pocket changes shape, allowing coregulators to bind (Speroff, 2000). It has been shown that agonists and SERMs induce unique ER conformations (Hubbard et al., 2000; Yi et al., 2002) and that intermediate conformations also are possible (Speroff, 2000).

Tamoxifen is an antagonist in cells where both the AF-1 and AF-2 are active, but works as an agonist in cells where AF-1 is dominant such as the endometrium, bone, and liver (Speroff, 2000). This dominance is based on factors in the cell environment such as promotor context (Tzukerman et al., 1994), phosphorylation (Chen et al., 2002) and coregulator ratio (Smith et al., 1997).

Tamoxifen displays gene-specific effects and depending on the promotor, it can show different activity. Tamoxifen has been shown to activate AP-1 target genes (Kushner et al., 2000; Webb et al., 1995). Tamoxifen had an antagonist effect on gene expression at ERE sites but an agonist effect on gene expression at AP-1 sites in HeLa cells (cervical cancer) (Paech et al., 1997). Tamoxifen also was shown to have a greater
agonist effect on AP-1 target genes in Ishikawa cells (endometrial cancer) versus MCF-7 cells (breast cancer), while having similar effects on ERE-dependent genes in both cell lines (Shang and Brown, 2002).

Crosstalk between growth factors, ER, and tamoxifen also may contribute to tissue-specific responses. Tamoxifen induced phosphorylation of ERα at Ser 118 in Cos cells (monkey kidney) (Kato et al., 1995; Chen et al., 2002). Tamoxifen also has been shown to activate c-Jun N-terminal kinase 1 (JNK1) and extracellular signal-regulated kinases (ERK2) in HeLa cells (Duh et al., 1997). Incubation of MCF-7 cells with EGF decreased the interaction of N-CoR and tamoxifen-bound ER (Lavinsky et al., 1998). Overexpression of both MAPKK in Cos cells (Kato et al., 1995), and MEKK1 in Ishikawa and MDAH-2774 cells (endometrioid ovarian cancer) (Lee et al., 2000), has been shown to enhance tamoxifen’s agonist effect.

Finally, the ratio of coactivators to corepressors, may determine tamoxifen action. A tissue with a higher level of coactivators may be expected to have a greater agonist effect with tamoxifen, while a tissue expressing a high level of corepressors may have the opposite. It has been shown that changing the level of coactivators and corepressors alters both estrogen and tamoxifen activity. The coactivator prothymosin alpha (PTMA) was shown to enhance estrogen activity in CHO cells (chinese hamster ovary) and MDA-MB 231 (breast cancer), and coexpression of SRC-1 and PTMA in CHO cells increased the effect (Martini and Katzenellenbogen, 2001). Repressor of estrogen activity (REA) reduced both the PTMA (Martini and Katzenellenbogen, 2001) and the SRC-1 (Delage-Mouroux et al., 2000) effect on estrogen-bound ER in MDA-MB 231 cells and CHO
cells, respectively. Tamoxifen’s agonist activity was enhanced by SRC-1 in HepG2 cells (Smith et al., 1997), and suppressed by both N-CoR in HepG2 cells (Smith et al., 1997) and SMRT in Cos cells (Jackson et al., 1997). Coexpression of SRC-1 and SMRT in HepG2 cells reduced the SRC-1 effect on tamoxifen action (Smith et al., 1997). The corepressor HET/SAF-B also reduced tamoxifen’s agonist activity in HepG2 cells (Oesterreich et al., 2000a). MCF-7 cells, implanted into nude (athymic) mice and chronically exposed to tamoxifen, have decreased N-CoR levels and this may be a mechanism in tamoxifen resistance (Lavinsky et al., 1998). It also has been shown that different coregulators are recruited to ER\(\alpha\) depending on the specific ligand (Norris et al., 1999; Schaufele et al., 2000).

Endometrial Cancer

Endometrial Carcinoma (EC) is the most common cancer of the female genital tract (Cotran et al., 1999) and there were an estimated 39,300 new cases and 6,600 deaths in the U.S. in 2002 (American Cancer Society, 2002). It is the 4\(^{th}\) most common cancer in women and the 8\(^{th}\) leading cause of cancer death in women (American Cancer Society, 2002). The average age of onset is 60 yr and only 25% of cases occur before menopause (Hoskins et al., 2000). The probability of invasive cancer increases as age increases, and the probability of acquiring EC throughout an entire lifetime is 2.70\% or 1 in 37 women (American Cancer Society, 2002). Risk factors for EC include being overweight, nulliparity, late menopause, diabetes, hypertension, and unopposed estrogen (Hoskins et al., 2000).
Carcinoma of the endometrium most commonly presents with abnormal vaginal bleeding (Abeloff et al., 2000). The standard test for detecting endometrial disease is a biopsy (Goldstein, 2001), and once the diagnosis has been made, surgical staging must be performed (Benedet et al., 2000). The International Federation of Gynecologic Oncologists (FIGO) recommends that the standard surgical procedure be extrafascial total hysterectomy with bilateral salpingo-oophorectomy (Benedet et al., 2000). Further treatment with surgery, chemotherapy, and radiation also are used when necessary (Benedet et al., 2000).

Classifications of Hyperplasia and Carcinoma

Hyperplasia of the endometrium is thought to arise from prolonged estrogenic stimulation and its gross appearance is similar to the late secretory endometrium of the normal female (DiSaia and Creasman, 2002). It is divided into four categories: simple, complex, simple with atypia, and complex with atypia (Table II). The difference between simple and complex lies in the amount of glandular crowding, the branching of the glands, and the layering of the cells (Silverberg, 2000; Kurman, 1994; Hoskins et al., 2000; DiSaia and Creasman, 2002). The addition of atypia denotes an increased nuclear to cytoplasmic ratio and irregularity to the cells (Silverberg, 2000; Kurman, 1994; Hoskins et al., 2000; DiSaia and Creasman, 2002). The risk of progression to adenocarcinoma increases as the level of complexity and atypia increases (DiSaia and Creasman, 2002), and 17-25% of patients with atypical hyperplasia will have concurrent, well-differentiated carcinoma (Abeloff et al., 2000).
Adenocarcinoma of the endometrium can be classified into several subgroups (Table II) (Abeloff et al., 2000; Hoskins et al., 2000; DiSaia and Creasman, 2002), with endometrioid adenocarcinoma comprising approximately 87.4% of all endometrial cancers (DiSaia and Creasman, 2002). Endometrioid adenocarcinomas tend to be estrogen dependent and most closely resemble normal endometrium (Inoue, 2001; Hoskins et al., 2000). The prognosis of endometrioid and mucinous adenocarcinomas is relatively good, while serous, clear cell, undifferentiated, and squamous cell adenocarcinomas tend to have a poor prognosis (Hoskins et al., 2000). Metastatic endometrial cancer most often arises from ovary, breast, colon, stomach, pancreas, and kidney (Hoskins et al., 2000).

Endometrial adenocarcinoma is graded on a scale of 1 to 3 with 1 being well-differentiated and 3 being poorly differentiated (Table III) (DiSaia and Creasman, 2002; Pecorelli et al., 1999; Abeloff et al., 2000). As the grade increases, the prognosis becomes less favorable (Hoskins et al., 2000). Tumors are graded on both architectural and nuclear criteria and “notable nuclear atypia,” defined as nuclear grade 3 (Zaino et al., 1995), can raise the architectural grade by one (Pecorelli et al., 1999). In some cancers (serous, clear cell, squamous cell) nuclear grading takes precedence (Pecorelli et al., 1999), and these tend to have a poor prognosis (Hoskins et al., 2000). Among patients in hyperestrogenic states, 52% are grade 1 while among patients with normal estrogen, 22.1% are grade 1 (Abeloff et al., 2000).

Stage is determined by surgery (Table IV) (Cavanagh et al., 1999; DiSaia and Creasman, 2002; Pecorelli et al., 1999), and the likelihood of spread outside the uterus is
Table II: Classifications of Hyperplasia and Carcinoma

<table>
<thead>
<tr>
<th>HYPERPLASIA</th>
<th>CLINICAL INFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple</td>
<td>1% progress to carcinoma</td>
</tr>
<tr>
<td>Complex</td>
<td>3% progress to carcinoma</td>
</tr>
<tr>
<td>Simple Atypical</td>
<td>8% progress to carcinoma</td>
</tr>
<tr>
<td>Complex Atypical</td>
<td>29% progress to carcinoma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ADENOCARCINOMA</th>
<th>CLINICAL INFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrioid</td>
<td>87.4% of carcinomas</td>
</tr>
<tr>
<td></td>
<td>Mean age 59</td>
</tr>
<tr>
<td></td>
<td>Prognosis based on grade</td>
</tr>
<tr>
<td>Villoglandular (papillary)</td>
<td></td>
</tr>
<tr>
<td>Secretory</td>
<td></td>
</tr>
<tr>
<td>Ciliated cell</td>
<td>w/ squamous differentiation</td>
</tr>
<tr>
<td>Serous</td>
<td>2.3% of carcinomas</td>
</tr>
<tr>
<td></td>
<td>Median age 63</td>
</tr>
<tr>
<td></td>
<td>Poor prognosis</td>
</tr>
<tr>
<td>Mucinous</td>
<td>0.6%</td>
</tr>
<tr>
<td></td>
<td>Prognosis based on grade</td>
</tr>
<tr>
<td>Clear cell</td>
<td>2.2% of carcinomas</td>
</tr>
<tr>
<td></td>
<td>Mean age 65</td>
</tr>
<tr>
<td></td>
<td>Poor prognosis</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>0.2% of carcinomas</td>
</tr>
<tr>
<td></td>
<td>Mean age 65</td>
</tr>
<tr>
<td></td>
<td>Poor prognosis</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>Poor prognosis</td>
</tr>
<tr>
<td>Mixed</td>
<td>Multiple types</td>
</tr>
<tr>
<td>Metastasis</td>
<td>Most commonly from ovary, breast,</td>
</tr>
<tr>
<td></td>
<td>colon, stomach, pancreas, and kidney</td>
</tr>
</tbody>
</table>
### Table III: Grading of Endometrial Carcinoma

<table>
<thead>
<tr>
<th>GRADE</th>
<th>DEFINITION</th>
<th>5-YEAR SURVIVAL</th>
<th>ER (+)</th>
<th>PR (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5% or less of a nonsquamous or nonmorular solid growth pattern</td>
<td>92%</td>
<td>82%</td>
<td>82%</td>
</tr>
<tr>
<td>2</td>
<td>6-50% of a nonsquamous or nonmorular solid growth pattern</td>
<td>87%</td>
<td>72%</td>
<td>60%</td>
</tr>
<tr>
<td>3</td>
<td>More than 50% of a nonsquamous or nonmorular solid growth pattern</td>
<td>74%</td>
<td>46%</td>
<td>59%</td>
</tr>
</tbody>
</table>

### Table IV: Staging of Endometrial Carcinoma

<table>
<thead>
<tr>
<th>STAGE</th>
<th>DEFINITION</th>
<th>INCIDENCE</th>
<th>5-YEAR SURVIVAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>73%</td>
<td>86%</td>
</tr>
<tr>
<td>IA</td>
<td>Tumor limited to endometrium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>Invasion to &lt;1/2 of myometrium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>Invasion to &gt;1/2 of myometrium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>11%</td>
<td>66%</td>
</tr>
<tr>
<td>IIA</td>
<td>Endocervical glandular involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>Cervical stromal invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>13%</td>
<td>44%</td>
</tr>
<tr>
<td>IIIA</td>
<td>Tumor invasion of serosa and/or adnexa, and/or positive peritoneal cytology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIB</td>
<td>Vaginal metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIC</td>
<td>Metastasis to pelvic and/or para-aortic lymph nodes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>3%</td>
<td>16%</td>
</tr>
<tr>
<td>IVA</td>
<td>Tumor invasion of bladder and/or bowel mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVB</td>
<td>Distant metastasis including intra-abdominal and/or inguinal nodes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
best predicted by the depth of myometrial invasion and grade (Abeloff et al., 2000).

Generally, Stage I represents cancer that is confined to the myometrium; Stage II is spread to the cervix; Stage III is spread beyond the cervix, and Stage IV is spread to the bladder and/or bowel mucosa and beyond (Pecorelli et al., 1999).

The presence of the ER and PR is associated with a more favorable prognosis (Esteva et al., 2002). Among patients in hyperestrogenic states, 67% were ER-positive, while among patients with normal estrogen, 29% were ER-positive (Abeloff et al., 2000). Also, it has been shown that as the grade increases, the proportion of ER-positive and PR-positive tumors decreases (Abeloff et al., 2000).

**Estrogen and Tamoxifen as Promoters**

Carcinogenesis consists of initiation, promotion, and progression from normal to malignant (Inoue, 2001). Endometrial cancer may arise in normal, atrophic, or hyperplastic endometrium (Hoskins et al., 2000), however, Type I, estrogen-dependent EC usually arise from hyperplasias due to unopposed estrogen (Inoue, 2001; Berek and Hacker, 2000). These cancers have a better prognosis than the Type II, estrogen-independent tumors that arise from atrophic and non-hyperplastic endometrium (Inoue, 2001; Berek and Hacker, 2000). Type I tumors tend to be endometrioid, while Type II tumors are usually of the non-endometrioid type (Silverberg, 2000).

Genes known to be associated with EC include oncogenes, tumor suppressor genes, DNA mismatch-repair genes, and genes involving immortality and apoptosis (Inoue, 2001). Overexpression of the tyrosine kinase HER-2/neu (10% of EC) and the
transcription factor c-myc (20-30% of EC), has been shown, and mutations in the G protein K-ras (10-30% of EC), the transcription factor p53 (20% of EC), and the tyrosine phosphatase PTEN (40%), also are common (Berek and Hacker, 2000). PTEN mutations have also been found in 20% of endometrial hyperplasias (Inoue, 2001; Maxwell et al., 1998).

Hyperestrogenic states such as obesity and unopposed estrogen pose the greatest risk for EC (Abeloff et al., 2000; Hoskins et al., 2000) and estrogen has been shown to be a promotor of endometrial lesions in mice (Takahashi et al., 2001). Telomerase may be important in tumor formation (Cotran et al., 1999), and it has been shown that telomerase activity is upregulated by estrogen (Kyo et al., 1999).

Tamoxifen also appears to promote the growth of EC. Tamoxifen use greater than 5 yr has a four times greater risk of EC (Bernstein et al., 1999). Patients found to have benign lesions by transvaginal ultrasound have an eighteen times risk of atypical hyperplasia with tamoxifen use (Goldstein, 2001). In a retrospective study of 700 tamoxifen-treated breast cancer patients, there were 33 cases of uterine cancer (Deligdisch et al., 2000). All 33 of the cases were primarily classified as endometrioid, and 13 of those cancers also contained mixed non-endometrioid components (Deligdisch et al., 2000).

Coregulators as Possible Biomarkers

Knowing the coregulator make-up of a tumor may aid pathologists in classifying tumors. Complex atypical hyperplasia may be difficult to distinguish from well-
differentiated adenocarcinoma (DiSaia and Creasman, 2002), and Kurman et al. suggests using “non-histologic techniques” to help distinguish the two (Silverberg, 2000). Also, upon imaging with transvaginal ultrasound, patients on long-term tamoxifen often show abnormal endometrial thickening in the absence of disease (Berek and Hacker, 2000). Other diagnostic techniques may be useful in these types of cases.

The coregulator make-up also may aid clinicians in treatment planning and predicting survival. In breast cancer, ER/PR positive tumors have a better prognosis (Osborne, 1998), and SRC-3 expression was correlated with a higher grade and absence of ER and PR (Bouras et al., 2001). SRC-3 overexpression also was associated with a worse prognosis in tamoxifen-treated patients versus untreated patients (Schiff et al., 2003). In endometrial cancer, ER-positive and PR-positive tumors also have a favorable prognosis (Esteva et al., 2002). The tumor antigen CA-125 is elevated in the serum of most patients with advanced stage carcinomas (Hoskins et al., 2000). SRC-1, SRC-2, SRC-3, N-CoR, and SMRT have been shown to be expressed in normal endometrium (Gregory et al., 2002; Shiozawa et al., 2003), however, no study to date has examined their role in the progression of endometrium from normal to malignant. Identifying coregulators that are associated with poor outcomes and measuring their expression in the tissue may help determine the invasiveness or metastatic potential of a tumor.
Expression of Estrogen Receptor Coregulators in Normal and Malignant Human Endometrium

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Running Title: Coregulator Expression in the Endometrium

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ABSTRACT

Objectives. To compare the expression of nuclear receptor coregulators in normal and malignant human endometrium and to identify any relationship to grade, stage, age, depth of myometrial invasion, estrogen receptor α (ERα) or progesterone receptor (PR) expression.

Methods. Gene expression of SRC-1, SRC-2, SRC-3, N-CoR, SMRT, ERα, and PR was measured in 26 samples of normal endometrium and 30 primary endometrial carcinomas using real-time RT-PCR. ERα protein expression of each tissue was also measured by Western blot.

Results. All coregulators showed significantly increased mRNA expression in endometrial carcinoma as compared to normal endometrium. The mRNA expression of each coregulator showed a high correlation with ERα mRNA, PR mRNA, and with the other coregulators in both normal and malignant endometrium. In the normal endometrium, SRC-1 mRNA expression was positively correlated with ERα protein expression and SRC-3 mRNA expression was positively correlated with patient age. No relationship was found between coregulator mRNA expression and grade, stage, or depth of myometrial invasion.

Conclusion. The nuclear receptor coregulators SRC-1, SRC-2, SRC-3, N-CoR and SMRT were found to be upregulated in malignant endometrium. Our findings suggest that these proteins may have a role in the development of endometrial carcinoma.
INTRODUCTION

Endometrial cancer is the most common gynecological malignancy and the fourth most common cancer of women in the United States. There were an estimated 39,300 new cases and 6,600 deaths in the U.S. in 2002 [1]. Many of the risk factors for endometrial cancer can be partly explained by the actions of unopposed estrogen [2,3].

Estrogen receptor α (ER) is a Type I receptor of the nuclear receptor superfamily and consists of 5 conserved domains. An A/B domain containing a ligand-independent activation function-1 region, a DNA binding domain, a hinge region, a ligand binding domain (LBD) containing a ligand-dependent AF-2 region, and an F domain [4]. In the classical pathway of ER activation, estrogen binds to the LBD and induces a conformational change, causing release of heat-shock proteins and dimerization with another ER [5,6]. The ER dimer then interacts with promoter regions of estrogen-responsive genes and recruits coactivators which potentiate transcription through chromatin remodeling and their interaction with general transcription factors [5-7]. The p160 family of coactivators, SRC-1/NCoA1, SRC-2/TIF2/GRIP1, and SRC-3/RAC3/ACTR/pCIP/AIB1/TRAM-1, have all been shown to recruit histone acetyltransferases (HAT) which disrupt electrostatic bonds on DNA and increase access of transcription factors to the promoter [8,9]. In addition, SRC-1 [10] and SRC-3 [11] have both been shown to possess intrinsic HAT activity.

In contrast, the antagonist action of selective estrogen receptor modulators (SERM) such as tamoxifen, causes ER to recruit corepressors, which downregulate the transcriptional activation activity of the ER [4]. The corepressors, N-CoR and SMRT,
repress transcription by recruiting histone deacetylases (HDAC) [12]. Deacetylation causes chromatin to adopt a more compact structure, which represses transcription [13].

Changing the level of coregulators in cells has been shown to alter estrogen and tamoxifen activity [14-16], and to directly regulate cell growth [17-20]. Recent studies have examined coregulator expression in cancers of the breast [21-25], ovary [26], and prostate [27,28]. In addition, amplification of the SRC-3 gene has been detected in breast and ovarian cancer [29-31].

To determine if coregulators have a role in the progression of normal endometrium to endometrial carcinoma, we measured the expression of the p160/SRC family coactivators (SRC-1, SRC-2, SRC-3) and the corepressors N-CoR, and SMRT in normal and malignant human endometrium using real-time RT-PCR. These proteins are the best characterized coregulators for the nuclear receptor superfamily, are expressed in endometrium [32-34], and have been shown to interact with and regulate ER action in \textit{in vivo} and \textit{in vitro} studies [16,29,35-37]. We then attempted to correlate the expression with clinicopathologic variables such as grade, stage, depth of myometrial invasion, patient age, ER expression, and progesterone receptor (PR) expression.
MATERIALS AND METHODS

Tissue samples

26 samples of normal endometrium and 30 samples of primary endometrial carcinoma were obtained from the Cooperative Human Tissue Network following approval of a Category #4 Exempted Research protocol by the Medical College of Ohio Institutional Review Board Committee. Tissues were removed by hysterectomy, immediately snap frozen in liquid nitrogen, and stored at -80°C until use. All tissue samples were dissected and reexamined by a pathologist (M.M.D.). Tissues were divided into three sections that were subsequently used for protein extraction, RNA extraction, or H&E staining. Special care was taken to separate endometrium from myometrium and necrotic material. Histologic diagnosis and grade of the tissues was determined from the H&E stained slides. Age, stage, and depth of myometrial invasion were provided by the accompanying pathology reports. Normal endomyometrial tissue samples were classified as proliferative (n=16) or secretory (n=10). Malignant tissues were classified as endometrioid adenocarcinoma (n=23), serous papillary carcinoma (n=4), and clear cell carcinoma (n=3). The median age of all patients in the study was 46 and ranged from 28-85. The median age of patients undergoing hysterectomy for benign disease was 39 and ranged from 28 to 47, while the median age of patients with malignant disease was 71 and ranged from 30 to 85.

RNA extraction and cDNA synthesis

Tissues set aside for RNA extraction were homogenized with a polytron in 1 mL of Trizol (Invitrogen Life Technologies, Carlsbad, CA). 300 μL chloroform was added
and the resulting solution was briefly vortexed and centrifuged for 10 min at 20,000 x g. The top aqueous layer was removed and 1.2 mL isopropanol was added followed by incubation for 10 min at room temperature and centrifugation for 10 min at 20,000 x g. The RNA pellet was washed with 70% ethanol and the pellet was resuspended in 75 µL of DEPC water. RNA concentration was measured by a spectrophotometer. RNA was diluted to 52 ng/µL and reverse transcribed to cDNA using TaqMan Kit reagents (Applied Biosystems) using the manufacturers protocol.

**Real-time RT-PCR**

Primers and probes for ER, PR and coregulators were designed using Primer Express Version 2.0 (Applied Biosystems) (Table 1) and manufactured by Integrated DNA Technologies, Inc (Coralville, IA). The primers and probes for β-actin were designed and manufactured by Applied Biosystems. The probes were dual-labeled with the fluorescent dye 56-FAM at the 5’ end and the quenching dye 3BHQ-1 at the 3’ end. Due to inequalities in primer/probe annealing efficiency, initial titration experiments were performed per manufacturers instructions (Applied Biosystems) to determine the optimal primer and probe concentration for each gene. Three different primer concentrations were titrated against a fixed probe concentration and subsequently, three different probe concentrations were titrated against the optimized primer concentration to identify the lowest concentration of primer and probe required for real-time RT-PCR measurement of each gene. Fwd primer, rev primer, probe, and TaqMan Universal PCR Master Mix (Applied Biosystems) were added to 200 ng cDNA for SRC-1, SRC-2, SRC-3, N-CoR, SMRT, ER, PR and β-actin quantitation. Real-time RT-PCR was performed.
using GeneAmp 5700 Sequence Detection System (Perkin Elmer, Wellesley, MA). PCR conditions were as follows: samples were heated at 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Values were quantified using the Comparative CT method outlined in the GeneAmp 5700 users manual. The original amount of target gene was given by $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is the threshold CT value of the gene of interest, normalized to $\beta$-actin and calibrated to an arbitrarily chosen sample of normal endometrium.

**Western Blot**

Tissues set aside for protein extraction were homogenized with a polytron in 600 µL of lysis buffer (10 mM Tris-HCl pH 8, 0.4 M NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM potassium phosphate, 50 mM sodium fluoride, 10 mM $\beta$-mercaptoethanol, 0.1% triton-x, 0.2% protease inhibitor cocktail (Sigma), 0.1% PMSF) and centrifuged for 10 min at 20,000 x g. Following protein concentration determination (Bradford assay) and addition of 5X Laemmli sample buffer to the supernatant, 100 µg of protein extract was separated using SDS-PAGE and the gel was transferred to nitrocellulose membrane for Western blotting analysis. Purified human recombinant ER$\alpha$ protein (Panvera, Madison, WI) was used as positive control. Western blotting was performed using mouse monoclonal anti-ER$\alpha$ antibody (Nova Castra, Newcastle upon Tyne, UK) and goat polyclonal anti- $\beta$-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, the membranes were blocked with TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween-20) containing 5% nonfat dry milk for 1 hour followed by incubation with 1.5 µg/mL of ER$\alpha$ antibody in TBST containing 5% milk overnight at 4°C. Following washes, the
membranes were incubated with 7.5 µg/mL of biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Membranes were washed and incubated with 5 µg/mL of Horseradish Peroxidase Streptavidin (Vector Laboratories) for 1 hour at room temperature. Following additional washes, the membranes were incubated with 5 mL of enhanced chemiluminescent (ECL) reagent (1.25 mM luminol, 0.2 mM p-coumaric acid, 0.01% hydrogen peroxide, 0.01 M Tris) for 1 min. Membranes were exposed using a Kodak Digital Science 440CT instrument (Eastman Kodak Company, Rochester, NY) and the ERα bands were quantified using the 1D Image Analysis software.

To measure β-actin levels, the membranes were incubated in stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) for 30 min at 55° C to remove ERα antibody. Stripped membranes were probed with 1 µg/mL of anti-β-actin antibody and the signal was quantified as described above. ERα values were normalized to β-actin values.

**Statistical Analysis**

Normalized protein and mRNA expression was reported as median and interquartile range (IQR). Due to the distribution of the data, non-parametric tests were used. Mann-Whitney U tests were used to compare between normal and malignant endometrium and between proliferative and secretory endometrium. Kruskal-Wallis tests were used to compare between grades and between stages. Spearman’s Rank Correlation was used to examine relationships between the variables. P values less than 0.05 were considered significant. All tests were 2-sided. Statistical tests were performed using
SPSS for Windows ver. 11.5.1 (SPSS Inc., Chicago, IL). Graphs were created using Sigma Plot ver. 8.02 (SPSS Inc.).
RESULTS

Coregulator Expression in Normal and Malignant Endometrium

Table 2 shows the median and inter-quartile range (IQR) of mRNA expression for each coregulator measured in normal and malignant endometrium. SRC-1 (p=.022), SRC-2 (p<.001), SRC-3 (p=.001), N-CoR (p<.001), and SMRT (p<.001), all showed increased mRNA expression in malignant endometrium as compared to normal endometrium (Figure 1). The fold increase in median expression was 2.4 for SRC-1, 6.5 for SRC-2, 2.5 for SRC-3, 3.5 for N-CoR, and 4.1 for SMRT. There was no significant difference in the expression of any of the coregulators between the different grades (Table 3) or stages (Table 4). The difference in the mRNA expression of SRC-2 (p=.053) and N-CoR (p=.065) between the grades approached statistical significance where significance was determined at p<.050. There was no significant difference found between normal proliferative and normal secretory endometrium in coregulator mRNA expression (Table 5).

Expression of Steroid Receptors in Normal and Malignant Endometrium

Table 2 and Figure 1 shows the expression of ER\(\alpha\) and PR mRNA in normal and malignant endometrium. Although there was a significant increase in the expression of ER\(\alpha\) mRNA in the malignant endometrium (p=.002), there was no corresponding increase in ER\(\alpha\) protein expression in malignant endometrium (see below). There was no significant difference in PR mRNA expression between normal and malignant endometrium. The difference in both ER\(\alpha\) (p=.052) and PR (p=.079) mRNA expression showed a trend toward significance between the different grades of carcinoma (Table 3).
No statistically significant difference in ERα or PR mRNA expression was detected between the different stages of carcinoma (Table 4). PR mRNA expression significantly decreased during the normal secretory phase (p=.011), while ERα mRNA expression remained unchanged (Table 5).

Figure 2 shows the expression of ERα protein as measured by Western Blot. No statistically significant difference was detected in ERα protein expression between normal and malignant endometrium. There was also no significant difference in ERα protein between normal proliferative and normal secretory endometrium or between the different grades or stages of carcinoma (Table 6).

Relationships of Coregulators, Steroid Receptors, and Clinicopathologic Variables

Table 7 shows the Spearman Correlation Coefficient for all variables of interest in normal endometrium and malignant endometrium. The mRNA expression of each coregulator showed a high positive correlation with the mRNA expression of every other coregulator in both normal and malignant endometrium. Each coregulator also showed a positive correlation with ERα and PR mRNA expression in these groups. Only SRC-1 mRNA expression in normal endometrium showed a significant correlation with ERα protein expression (r=.493, p=.011), although the correlation between SRC-3 and ERα approached statistical significance (r=.349, p=.080). SRC-3 was significantly correlated with age in normal endometrium (r=.442, p=.024) and the correlation between age and SMRT approached statistical significance in malignant endometrium (r=-.352, p=.056). No relationship was found between any coregulator and the depth of myometrial invasion shown by the tumor.
Table 8 shows the relationships of coregulators and steroid receptors in normal proliferative and normal secretory endometrium. The mRNA expression of each coregulator was significantly correlated with PR mRNA expression in both proliferative and secretory endometrium. ERα mRNA expression was significantly correlated with all coregulators except SRC-1 in proliferative endometrium.

Comparison of Endometrioid and Non-Endometrioid Tumors

Serous papillary and clear cell carcinomas were combined to form one group called non-endometrioid. Grade 3 endometrioid and grade 3 non-endometrioid tumors were compared in order to identify any differences (Table 9). No significant difference was found between endometrioid and non-endometrioid tumors in the mRNA expression of ERα, PR, or any of the coregulators. The decreased expression of N-CoR mRNA showed a trend toward statistical significance (p=.086).

Ratio of Coactivators to Corepressors in Normal and Malignant Endometrium

To examine how the relationship between coactivators and corepressors changes during the progression to carcinoma, the ratio of coactivator to corepressor was examined for each possible combination (Table 10). The ratio of SRC-2:N-CoR increased significantly between normal and malignant (p<.001), while the ratio of SRC-1:N-CoR (p=.004) and SRC-1:SMRT (p<.001) decreased. The decrease in the ratio of SRC-3:SMRT approached statistical significance (p=.069). None of the other combinations showed a significant difference.
DISCUSSION

The endometrium is an estrogen-responsive organ and the coregulators examined in this study have all been shown to directly interact with ERα and regulate ER-dependent gene transcription [16,29,35-37]. Previous studies have examined coregulator expression in normal endometrium [32-34] and in endometrial carcinoma [38], but to the best of our knowledge, this is the first study to directly compare normal and malignant endometrium.

Using real-time RT-PCR we have shown that the coactivators SRC-1, SRC-2, and SRC-3, as well as the corepressors N-CoR and SMRT, display increased mRNA expression in malignant endometrium as compared to normal. These results are similar to the findings of Kurebayashi et al who found increased mRNA expression of these same coregulators in breast cancer [21]. SRC-3 protein expression has also been shown to be increased in breast cancer [23,24]. We found no difference in coregulator mRNA expression between the grades or stages, although differences in SRC-2 and N-CoR expression between the grades approached statistical significance. This is in contrast to what has been found in the breast, where SRC-3 expression has been associated with higher grade [22,23]. In the prostate, no significant difference was found in the expression of SRC-1 between benign and malignant tissue samples, however, a significant difference was found between tumors with intermediate Gleason scores and high Gleason scores [27].

Multiple comparisons done between 2 groups increases the probability of a Type I error [39]. If we apply Bonferroni’s adjustment for multiple testing, the increased
expression of SRC-2, SRC-3, N-CoR, and SMRT remains significant, but the difference in SRC-1 expression loses significance. This is notable because Shang et al concluded that SRC-1 is crucial to tamoxifen’s agonist activity in Ishikawa cells, a well differentiated endometrial cancer cell line [40]. If SRC-1 is the most important coregulator for tamoxifen’s agonist activity, then the absolute amounts will most likely already be high. A malignant transformation may not cause the levels of SRC-1 to change as much as the other coregulators.

The coregulators assayed were all highly correlated with each other and with ERα mRNA expression, which may indicate a common regulatory pathway. Loss of control by this pathway may lead to the upregulation of these coregulators as well as other proteins responsible for cell growth and proliferation. All coregulators were also well correlated with PR mRNA in both normal and malignant endometrium, however PR did not increase in malignant tissue, indicating that it may not be regulated in the same manner as the coregulators. Only SRC-1 was positively correlated with ERα protein in normal endometrium. This may indicate that SRC-1 is important for normal ERα action in the endometrium or that ERα protein regulates the transcription of SRC-1. A positive correlation between SRC-3 and ERα protein in normal endometrium showed a trend toward significance. In contrast, a previous study found that SRC-3 protein expression in breast cancer was correlated with the absence of ER and PR [22], while another study found that SRC-3 protein in breast cancer was negatively correlated with PR alone [41].

Endometrial cancer is a disease of post-menopausal women and as expected, there was an age difference between normal and malignant endometrium. Patients with
histologically normal endometrium had a median age of 39 and a range of 28-47, while the patients with endometrial cancer had a median age of 71 and ranged from 30-85. However, when we examined normal and malignant endometrium separately, only SRC-3 was significantly correlated with patient age in normal endometrium. The relationship between age and SMRT expression in malignant tissue showed a trend toward significance. This provides evidence that SRC-1, SRC-2, and N-CoR expression may not change with age, and that once a malignant transformation occurs, SRC-3 expression will not change while SMRT expression may actually decrease.

No difference was found in coregulator mRNA expression between normal proliferative and normal secretory endometrium. A previous study showed that protein expression of SRC-1, SRC-2, and SRC-3 did not change during the menstrual cycle except for an increase in glandular SRC-3 during the late secretory phase [32]. Another study showed a decrease in glandular and stromal SRC-1 and N-CoR and decreased SMRT in the basalis stroma alone, during the early, mid-, and late secretory phase [33].

No difference was seen in ER\(\alpha\) mRNA or protein expression between normal proliferative and normal secretory endometrium or between the different grades of endometrial carcinoma. PR mRNA expression showed no significant difference between the grades, but did show a significant decrease between normal proliferative and normal secretory endometrium, which has been previously shown for PR protein [42-44]. All coregulators were significantly correlated with ER\(\alpha\) and PR mRNA in both proliferative and secretory endometrium with the exception of SRC-1, which was correlated with ER\(\alpha\) in secretory endometrium alone.
An interesting trend toward statistical significance involved the different histological subtypes of endometrial cancer. Type I endometrioid tumors arise from unopposed estrogen exposure and tend to have a better prognosis than the Type II non-endometrioid tumors, such as serous papillary and clear cell carcinomas, that arise in atrophic endometrium [45,46]. While not significant, the level of N-CoR appears greater in endometrioid tumors as compared to the non-endometrioid tumors. Recently, Girault et al. showed that tamoxifen-treated breast cancer patients with low levels of N-CoR had significantly shorter survival times than patients with intermediate and high levels [47]. A different study showed that patients who experienced recurrence of their tumor had lower levels of N-CoR, than patients without recurrence [21]. Low levels of N-CoR may be associated with a worse prognosis in both breast and endometrial cancers.

Changing the levels of coactivators and corepressors can alter the effect of estrogen and tamoxifen on ER transcriptional activity. Increased expression of SRC-1 in cell culture increased tamoxifen’s agonist activity, but the introduction of SMRT decreased the effect [16]. The ratio of coactivator to corepressor may regulate the effect of estrogen and tamoxifen on the growth of human tissue. We found that the ratio of SRC-1:N-CoR and SRC-1:SMRT both decreased in malignant endometrial tissue while the ratio of SRC-2:N-CoR increased. In addition, the decrease in the ratio of SRC-3:SMRT approached statistical significance. Findings by Murphy et al. showed that the ratio of SRC-3 to the corepressor REA (repressor of estrogen activity) increased in breast tumors [25].
The increased expression of these coregulators does not only translate to increased hormone responsiveness. Besides nuclear receptors, coregulators interact with and regulate the activity of a wide variety of unrelated transcription factors including NFκB [48], p53 [49], and AP-1 [50] proteins. Whether or not the interaction of these specific coregulators and other transcription factors is important in the transition from normal to malignant endometrium remains to be determined.

In conclusion, we have shown that the coregulators SRC-1, SRC-2, SRC-3, N-CoR, and SMRT display increased mRNA expression in endometrial carcinoma as compared to normal endometrium. This may indicate a role for coregulators in the malignant transformation of the endometrium.
ACKNOWLEDGEMENTS

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REFERENCES


32. Gregory CW, Wilson EM, Apparao KB, Lininger RA, Meyer WR, Kowalik A, Fritz MA, Lessey BA. Steroid receptor coactivator expression throughout the


ARTICLE PRÉCIS

The nuclear receptor coregulators SRC-1, SRC-2, SRC-3, N-CoR, and SMRT exhibited increased expression in endometrial carcinoma.
### TABLE 1

Primers and Probes for Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Accession No.</th>
<th>Sequence 5'-3'</th>
<th>Final Conc. (nM)</th>
<th>Position in Coding Sequence</th>
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TABLE 2

mRNA Expression in Normal and Malignant Endometrium

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<th>Normal N=26</th>
<th>Malignant N=30</th>
<th>( P^b )</th>
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<tr>
<td>SRC-1</td>
<td>1.85(1.29-2.51)*</td>
<td>4.39(1.59-8.88)</td>
<td>.022</td>
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<tr>
<td>SRC-2</td>
<td>2.68(1.74-6.45)</td>
<td>17.4(6.32-37.3)</td>
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<tr>
<td>SRC-3</td>
<td>1.91(1.18-2.99)</td>
<td>4.76(2.16-15.7)</td>
<td>.001</td>
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<tr>
<td>N-CoR</td>
<td>2.09(1.13-5.17)</td>
<td>7.25(3.01-12.6)</td>
<td>&lt;.001</td>
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<tr>
<td>SMRT</td>
<td>1.69(1.27-2.48)</td>
<td>6.88(2.22-27.9)</td>
<td>&lt;.001</td>
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<tr>
<td>ER( \alpha )</td>
<td>1.25(0.71-2.60)</td>
<td>3.29(1.45-12.47)</td>
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<td>PR</td>
<td>3.50(1.61-4.96)</td>
<td>2.33(1.01-5.43)</td>
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*a Median (IQR)

*b Mann-Whitney U Test
TABLE 3

mRNA Expression Among Different Grades of Endometrial Cancer

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<tr>
<th>Gene</th>
<th>Grade 1 N=8</th>
<th>Grade 2 N=9</th>
<th>Grade 3 N=13</th>
<th>( P^b )</th>
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<tr>
<td>SRC-1</td>
<td>3.13(1.30-5.98)(^a)</td>
<td>5.50(0.84-11.3)</td>
<td>6.19(1.96-12.3)</td>
<td>.576</td>
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<td>SRC-2</td>
<td>15.0(5.84-17.5)</td>
<td>37.3(24.6-81.0)</td>
<td>9.92(5.78-22.9)</td>
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<td>3.69(1.70-10.7)</td>
<td>4.69(1.37-15.7)</td>
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<td>N-CoR</td>
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<td>12.6(10.3-22.2)</td>
<td>4.89(2.89-7.67)</td>
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<td>SMRT</td>
<td>3.62(1.76-13.5)</td>
<td>15.7(3.61-50.2)</td>
<td>4.44(1.99-15.6)</td>
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<td>ERα</td>
<td>4.20(2.06-19.2)</td>
<td>11.39(3.68-13.4)</td>
<td>1.99(1.06-3.32)</td>
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<tr>
<td>PR</td>
<td>2.80(0.71-4.71)</td>
<td>4.03(3.81-8.06)</td>
<td>1.23(1.01-2.10)</td>
<td>.079</td>
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</tbody>
</table>

\(^a\) Median (IQR)

\(^b\) Kruskal-Wallis test
### TABLE 4

mRNA Expression Among Different Stages of Endometrial Cancer

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<thead>
<tr>
<th>Gene</th>
<th>Stage I N=17</th>
<th>Stage II N=3</th>
<th>Stage III N=6</th>
<th>Stage IV N=4</th>
<th>( P^b )</th>
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</thead>
<tbody>
<tr>
<td>SRC-1</td>
<td>3.03(0.94-6.73)(^a)</td>
<td>1.59(0.91-15.0)</td>
<td>6.28(1.97-6.50)</td>
<td>11.8(6.64-12.8)</td>
<td>.374</td>
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<td>SRC-2</td>
<td>14.6(5.35-22.5)</td>
<td>68.1(45.5-74.6)</td>
<td>20.6(8.46-62.2)</td>
<td>23.6(7.85-63.3)</td>
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<td>SRC-3</td>
<td>3.92(1.79-12.5)</td>
<td>4.82(2.85-11.0)</td>
<td>7.92(2.16-17.6)</td>
<td>12.2(4.52-38.9)</td>
<td>.542</td>
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<td>N-CoR</td>
<td>4.89(3.01-10.3)</td>
<td>7.67(7.25-9.90)</td>
<td>8.00(2.53-23.4)</td>
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<tr>
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<td>3.29(2.22-15.7)</td>
<td>4.44(4.02-22.8)</td>
<td>11.6(1.99-27.9)</td>
<td>30.1(5.89-63.4)</td>
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<td>ER(\alpha)</td>
<td>2.14(1.35-13.4)</td>
<td>3.27(2.30-3.48)</td>
<td>7.49(3.32-23.4)</td>
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<td>PR</td>
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<td>2.10(1.57-3.06)</td>
<td>2.75(0.96-8.06)</td>
<td>3.67(1.98-5.25)</td>
<td>.996</td>
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</tbody>
</table>

\(^a\) Median (IQR)

\(^b\) Kruskal-Wallis test
TABLE 5

mRNA Expression in Proliferative and Secretory Endometrium

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<tr>
<th>Gene</th>
<th>Proliferative</th>
<th>Secretory</th>
<th>$P^{b}$</th>
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<td>N=16</td>
<td>N=10</td>
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</tr>
<tr>
<td>SRC-1</td>
<td>1.85(1.17-3.59)$^a$</td>
<td>1.69(1.29-2.20)</td>
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<tr>
<td>SRC-2</td>
<td>3.15(1.78-7.31)</td>
<td>2.12(1.21-4.82)</td>
<td>.399</td>
</tr>
<tr>
<td>SRC-3</td>
<td>1.84(1.18-2.69)</td>
<td>1.94(1.04-2.99)</td>
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<tr>
<td>N-CoR</td>
<td>2.15(1.33-6.52)</td>
<td>1.94(1.08-3.81)</td>
<td>.429</td>
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<td>SMRT</td>
<td>1.69(1.31-2.42)</td>
<td>1.70(1.10-2.48)</td>
<td>.712</td>
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<td>ERα</td>
<td>1.25(0.96-2.85)</td>
<td>1.24(0.42-2.50)</td>
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<td>PR</td>
<td>4.10(3.08-5.31)</td>
<td>1.96(0.60-3.41)</td>
<td>.011</td>
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</table>

$^a$ Median (IQR)

$^b$ Mann-Whitney $U$ test
Table 6

Expression of Estrogen Receptor α Protein in Normal and Malignant Endometrium

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<tr>
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<th>Median (IQR)</th>
<th>P</th>
<th>Median (IQR)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Proliferative</td>
<td></td>
<td>Secretory</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.92(0.22-1.89)</td>
<td>.599a</td>
<td>0.98 (0.20-1.80)</td>
<td>.635a</td>
</tr>
<tr>
<td>Malignant</td>
<td>0.55(0.15-1.61)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>1.59 (0.72-2.46)</td>
<td>.387b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>0.56 (0.22-1.32)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>0.42 (0.14-0.60)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>0.65(0.20-1.61)</td>
<td>.402b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>0.56(0.35-1.66)</td>
<td></td>
<td></td>
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<tr>
<td>Stage III</td>
<td>1.07(0.20-6.32)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>0.18(0.13-0.39)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Mann-Whitney U Test

*b Kruskal Wallis Test
## TABLE 7

Relationships of Coregulators, Steroid Receptors, and Clinicopathologic Variables

<table>
<thead>
<tr>
<th>Group</th>
<th>Gene</th>
<th>SRC-1</th>
<th>SRC-2</th>
<th>SRC-3</th>
<th>N-CoR</th>
<th>SMRT (m)</th>
<th>ERα (m)</th>
<th>PR</th>
<th>ERα (p)</th>
<th>Age</th>
<th>MF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Endometrium</td>
<td>SRC-1</td>
<td>r</td>
<td>.530</td>
<td>.881</td>
<td>.585</td>
<td>.629</td>
<td>.484</td>
<td>.579</td>
<td>.493</td>
<td>.322</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p</td>
<td>.0050</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.001</td>
<td>.012</td>
<td>.002</td>
<td>.011</td>
<td>.109</td>
<td>-</td>
</tr>
<tr>
<td>SRC-2</td>
<td>r</td>
<td>-</td>
<td>.680</td>
<td>-</td>
<td>.922</td>
<td>.812</td>
<td>.819</td>
<td>.609</td>
<td>.043</td>
<td>.198</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>-</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.836</td>
<td>.333</td>
<td>-</td>
</tr>
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<td>SRC-3</td>
<td>r</td>
<td>-</td>
<td>-</td>
<td>.753</td>
<td>.767</td>
<td>.656</td>
<td>.549</td>
<td>.349</td>
<td>.442</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>p</td>
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<td>-</td>
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<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.004</td>
<td>.080</td>
<td>.024</td>
<td>-</td>
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<tr>
<td>N-CoR</td>
<td>r</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.828</td>
<td>.729</td>
<td>.647</td>
<td>.041</td>
<td>.228</td>
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</tr>
<tr>
<td></td>
<td>p</td>
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<td>-</td>
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<td>&lt;.001</td>
<td>.841</td>
<td>.263</td>
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<tr>
<td>SMRT</td>
<td>r</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>.592</td>
<td>.621</td>
<td>.241</td>
<td>.182</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.235</td>
<td>.372</td>
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<td>-</td>
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<tr>
<td>Malignant Endometrium</td>
<td>SRC-1</td>
<td>r</td>
<td>.475</td>
<td>.896</td>
<td>.567</td>
<td>.710</td>
<td>.547</td>
<td>.543</td>
<td>.005</td>
<td>-</td>
<td>.170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p</td>
<td>.0080</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.002</td>
<td>.979</td>
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<tr>
<td>SRC-2</td>
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<td>.774</td>
<td>.715</td>
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<td>p</td>
<td>-</td>
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<td>&lt;.001</td>
<td>&lt;.001</td>
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<td>&lt;.001</td>
<td>.912</td>
<td>-.326</td>
<td>-</td>
<td>-.284</td>
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<tr>
<td>SRC-3</td>
<td>r</td>
<td>-</td>
<td>-</td>
<td>.526</td>
<td>.738</td>
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<td>.459</td>
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<td>p</td>
<td>-</td>
<td>-</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.658</td>
<td>-.284</td>
<td>-</td>
<td>-.382</td>
</tr>
<tr>
<td>N-CoR</td>
<td>r</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.791</td>
<td>.740</td>
<td>.887</td>
<td>-.163</td>
<td>.217</td>
<td>-</td>
<td>-.166</td>
</tr>
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<td>p</td>
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<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.391</td>
<td>.249</td>
<td>-</td>
<td>-.352</td>
</tr>
<tr>
<td>SMRT</td>
<td>r</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.655</td>
<td>.755</td>
<td>.006</td>
<td>-.199</td>
<td>-</td>
<td>-.321</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>.973</td>
<td>.291</td>
<td>-</td>
<td>.056</td>
</tr>
</tbody>
</table>

a Spearman’s Rank Correlation Coefficient

b Significant correlations in bold type

c ERα mRNA expression

d ERα protein expression

e Depth of myometrial invasion
TABLE 8

Relationships of Coregulators and Steroid Receptors

<table>
<thead>
<tr>
<th>Group</th>
<th>Gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SRC-1</th>
<th>SRC-2</th>
<th>SRC-3</th>
<th>N-CoR</th>
<th>SMRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative Endometrium</td>
<td>ERα&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.259</td>
<td>.732</td>
<td>.620</td>
<td>.671</td>
<td>.562</td>
</tr>
<tr>
<td></td>
<td>PR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.333</td>
<td>.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.010</td>
<td>.004</td>
<td>.023</td>
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<td></td>
<td>r</td>
<td>.714</td>
<td>.642</td>
<td>.656</td>
<td>.624</td>
<td>.627</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>.002</td>
<td>.007</td>
<td>.006</td>
<td>.010</td>
<td>.009</td>
</tr>
<tr>
<td>Secretory Endometrium</td>
<td>ERα&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.830</td>
<td>.952</td>
<td>.758</td>
<td>.855</td>
<td>.745</td>
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<tr>
<td></td>
<td>PR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.003</td>
<td>&lt;.001</td>
<td>.011</td>
<td>.002</td>
<td>.013</td>
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<tr>
<td></td>
<td>r</td>
<td>.648</td>
<td>.661</td>
<td>.661</td>
<td>.758</td>
<td>.830</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>.043</td>
<td>.038</td>
<td>.038</td>
<td>.011</td>
<td>.003</td>
</tr>
</tbody>
</table>

<sup>a</sup> ERα and PR mRNA expression

<sup>b</sup> Spearman’s Rank Correlation Coefficient

<sup>c</sup> Significant correlations in bold type
TABLE 9
mRNA Expression in Grade 3 Endometrioid and Non-Endometrioid Tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Endometrioid N=7</th>
<th>Non-Endometrioid N=6</th>
<th>$P^{\text{b}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRC-1</td>
<td>6.73(2.31-18.6)</td>
<td>4.33(1.96-6.39)</td>
<td>.475</td>
</tr>
<tr>
<td>SRC-2</td>
<td>12.1(7.56-45.5)</td>
<td>9.02(5.78-18.5)</td>
<td>.475</td>
</tr>
<tr>
<td>SRC-3</td>
<td>7.94(3.97-18.5)</td>
<td>6.03(4.35-17.6)</td>
<td>.886</td>
</tr>
<tr>
<td>N-CoR</td>
<td>6.82(4.16-9.26)</td>
<td>2.71(1.89-6.68)</td>
<td>.086</td>
</tr>
<tr>
<td>SMRT</td>
<td>4.44(2.93-25.5)</td>
<td>5.65(1.80-15.6)</td>
<td>.568</td>
</tr>
<tr>
<td>ERα</td>
<td>1.35(1.20-2.63)</td>
<td>2.82(0.40-3.58)</td>
<td>.775</td>
</tr>
<tr>
<td>PR</td>
<td>1.46(1.14-2.82)</td>
<td>0.99(0.42-1.68)</td>
<td>.153</td>
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</tbody>
</table>

*a Median (IQR)*

*b* Mann-Whitney *U* test
## TABLE 10

Ratio of Coactivator to Corepressor in Normal and Malignant Endometrium

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Normal</th>
<th>Malignant</th>
<th>( P^{b} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=26</td>
<td>N=30</td>
<td></td>
</tr>
<tr>
<td>SRC-1:N-CoR</td>
<td>0.97(0.89-1.17)(^a)</td>
<td>0.61(0.28-0.88)</td>
<td>.004</td>
</tr>
<tr>
<td>SRC-2:N-CoR</td>
<td>1.30(1.02-1.96)</td>
<td>2.44(1.69-3.32)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>SRC-3:N-CoR</td>
<td>0.95(0.78-1.07)</td>
<td>1.02(0.55-1.73)</td>
<td>.440</td>
</tr>
<tr>
<td>SRC-1:SMRT</td>
<td>1.31(0.97-1.77)</td>
<td>0.37(0.20-0.86)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>SRC-2:SMRT</td>
<td>1.83(1.28-2.71)</td>
<td>1.91(1.07-4.89)</td>
<td>.974</td>
</tr>
<tr>
<td>SRC-3:SMRT</td>
<td>1.31(0.85-1.46)</td>
<td>0.88(0.38-1.43)</td>
<td>.069</td>
</tr>
</tbody>
</table>

\(^a\) Median (IQR)

\(^b\) Mann-Whitney \( U \) Test
FIG. 1.

Coregulator mRNA expression in normal and malignant endometrium was measured using real-time RT-PCR as described in Material and Methods. Median, 25th percentile, and 75th percentile of (A) SRC-1, (B) SRC-2, (C) SRC-3, (D) N-CoR, and (E) SMRT are shown by the box. Whiskers represent the 10th and 90th percentiles respectively. All coregulators showed a significant increase in mRNA expression in malignant endometrium. * $p<.05$, ** $p<.01$

FIG. 2.

ERα protein content in normal and malignant endometrium was measured using Western blot as described in Material and Methods. Example of digital images used to quantify ERα and β-actin. Gels were loaded on every other lane in order to quantify bands more easily. Purified ERα protein was used as a positive control. No statistically significant change was seen between normal and malignant endometrium, normal proliferative and normal secretory endometrium, or between the different grades or stages of endometrial carcinoma.
**Figure 1:**

**A** and **B**: SRC-1 and SRC-2 expression levels in normal and malignant tissues.

**C**: SRC-3 expression levels.

**D**: N-CoR expression levels.

**E**: SMRT expression levels.

**F**: ERα expression levels.

**G**: PR expression levels.
ERα positive control

<table>
<thead>
<tr>
<th>Normal</th>
<th>Normal</th>
<th>Normal</th>
<th>Malignant</th>
<th>Malignant</th>
<th>Malignant</th>
</tr>
</thead>
</table>

**ERα**

**β-actin**
DISCUSSION/SUMMARY

The nuclear receptor coregulators SRC-1, SRC-2, SRC-3, N-CoR, and SMRT have previously been shown to directly interact with ER and regulate its transcriptional activity. Measurement of these coregulators in benign and malignant endometrium has shown that:

1. The mRNA expression of SRC-1, SRC-2, SRC-3, N-CoR, and SMRT is increased in malignant tissue as compared to normal.
2. The mRNA expression of SRC-1, SRC-2, SRC-3, N-CoR, and SMRT does not change between the different grades or stages of endometrial cancer.
3. The mRNA expression of SRC-1, SRC-2, SRC-3, N-CoR, and SMRT is not correlated with the depth of myometrial invasion.
4. The mRNA expression of SRC-1, SRC-2, SRC-3, N-CoR, and SMRT are all positively correlated with each other and with PR mRNA in both normal and malignant endometrium.
5. SRC-1 mRNA expression is positively correlated with ERα in normal endometrium.
6. The mRNA expression of SRC-1, SRC-2, N-CoR, and SMRT do not change with increasing age.
7. SRC-3 mRNA expression is positively correlated with patient age in normal endometrium but not malignant endometrium.
8. The ratio of SRC-1:N-CoR and SRC-1:SMRT decreased and the ratio of SRC-2:N-CoR increased in malignant endometrium.


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Stallcup, M.R. 1999a Regulation of transcription by a protein methyltransferase.
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Chen, D., Pace, P.E., Coombes, R.C., and Ali, S. 1999b Phosphorylation of human
estrogen receptor alpha by protein kinase A regulates dimerization. Mol. Cell

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2002 Phosphorylation of human estrogen receptor alpha at serine 118 by two


Gehin, M., Mark, M., Dennefeld, C., Dierich, A., Gronemeyer, H., and Chambon, P.  


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ABSTRACT

Endometrial carcinoma is the most common neoplasm of the female genital tract and the fourth most common cancer in women overall. The largest risk factor for endometrial carcinoma is exposure to unopposed estrogen. In the classical pathway of estrogen activation, estrogen binds to an estrogen receptor and recruits coactivators that increase its transcriptional activity. In contrast, certain compounds known as Selective Estrogen Receptor Modulators (SERM), cause estrogen receptor to recruit corepressors that decrease its transcriptional activity. The coactivators SRC-1, SRC-2, and SRC-3 showed higher mRNA expression in the malignant tissue as compared to the benign tissue. The corepressors N-CoR and SMRT also showed increased expression in malignant endometrium. Expression levels of coregulator mRNA were highly correlated with each other as well as with progesterone receptor mRNA. There was no correlation between coregulator mRNA expression and known prognostic markers such as grade, stage, and estrogen receptor-alpha protein expression.