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# Progesterone and estrogen receptor expression and activity in human non-small cell lung cancer

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ABSTRACT

Lung cancer is the most common cause of cancer mortality in male and female patients in the US. Although it is clear that tobacco smoking is a major cause of lung cancer, about half of all women with lung cancer worldwide are never-smokers. Despite a declining smoking population, the incidence of non-small cell lung cancer (NSCLC), the predominant form of lung cancer, has reached epidemic proportions particularly in women. Emerging data suggest that factors other than tobacco, namely endogenous and exogenous female sex hormones, have a role in stimulating NSCLC progression. Aromatase, a key enzyme for estrogen biosynthesis, is expressed in NSCLC. Clinical data show that women with high levels of tumor aromatase (and high intratumoral estrogen) have worse survival than those with low aromatase. The present and previous studies also reveal significant expression and activity of estrogen receptors (ERα, ERβ) in both extranuclear and nuclear sites in most NSCLC. We now report further on the expression of progesterone receptor (PR) transcripts and protein in NSCLC. PR transcripts were significantly lower in cancerous as compared to non-malignant tissue. Using immunohistochemistry, expression of PR was observed in the nucleus and/or extranuclear compartments in the majority of human tumor specimens examined. Combinations of estrogen and progestins administered *in vitro* cooperate in promoting tumor secretion of vascular endothelial growth factor and, consequently, support tumor-associated angiogenesis. Further, dual treatment with estradiol and progestin increased the numbers of putative tumor stem/progenitor cells. Thus, ER- and/or PR-targeted therapies may offer new approaches to manage NSCLC.


## 1. Introduction

Lung cancer is the most common cause of cancer mortality in female and male patients in the US. In contrast to that in men, lung cancer mortality among women has reached epidemic proportions, increasing 600% since 1950 and accounting for 25% of all female cancer deaths [1]. About 1.75 times more women in the United States are expected to die from lung cancer than from breast cancer in 2010 [2]. Non-small cell lung cancer (NSCLC) is the predominant type of lung cancer and includes major histologic types such as adenocarcinoma, squamous carcinoma and large cell carcinoma. Of these, adenocarcinoma is the most common lung cancer. Survival rates from NSCLC are unacceptably low (about 15% five-year survival), and new approaches to treat and prevent this disease are urgently needed. Although there is clear evidence that tobacco smoking is a major cause of lung cancer, about 53% of all women with lung cancer worldwide are never-smokers [3]. Despite a decline of the smoking population, the incidence of NSCLC, particularly adenocarcinoma subtypes, is rapidly increasing [1,2]. Adenocarcinoma represents three-fourths of primary lung cancers in women and is also the most frequent histologic type of NSCLC in nonsmokers and young people [2,3]. Such data suggest that etiologic factors other than tobacco may also have a role in development and progression of lung cancer.

Emerging evidence shows that female sex hormones, especially endogenous and exogenous estrogens (E), are key contributors to NSCLC progression in women [4–12]. Aromatase, a cytochrome P-

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450 enzyme that mediates the final, rate-limiting step in estrogen synthesis, catalyzing conversion of androstenedione and testosterone to estrone and estradiol, respectively, is expressed in both primary and metastatic NSCLC. Clinical data now show that women with high levels of lung tumor aromatase expression and activity (and consequently high intratumoral estrogen levels) have significantly worse survival than those with low levels of this enzyme [4,5,8]. In both male and female patients, about 73% of NSCLCs show higher levels of intratumoral estradiol in cancer tissue than in paired non-neoplastic lung tissue. Such results confirm that estradiol is locally produced in NSCLC by aromatase.

Despite earlier conflicting data on the presence of estrogen receptors (ERs) in lung, numerous studies now confirm significant expression and activity of ERα and ERβ in most NSCLC, particularly in adenocarcinoma [4,6–12]. In the epithelium of the lung, ERβ is abundant while ERα tends to be relatively lower. Further, levels of ERβ and ERα mRNA expression are noted to be increased in lung cancer as compared to normal epithelia [13,14]. ERα and ERβ proteins occur in nuclei and extranuclear sites. In lung, as in the breast, extranuclear ERs derive from the same transcript as nuclear ER [7,15–17]. Nuclear and extranuclear ERs appear to act in concert to promote cell growth [15,18]. In normal lung, ERs are involved in important physiologic functions, including alveolar formation in development, activation of alveolar regeneration and maintenance of pulmonary diffusion capacity in the adult [19–21]. In lung malignancy, estrogens stimulate rapid cellular effects on MAPK and AKT kinases and phosphorylation of steroid receptor coactivators (SRC-3/AIB1) that appear to correlate with later stimulation of NSCLC cell proliferation, angiogenesis and tumor progression, and these actions in lung tumors are inhibited by the pure antiestrogen fulvestrant and by aromatase inhibitors [4–8,22–27]. Estrogen-induced transcription is mediated by ERα and ERβ in cell nuclei and is augmented by protein–protein interactions of ER forms with other transcription factors or extranuclear complexes (MAPK and AKT kinases) that, in turn, modulate ERα and ERβ and downstream gene transcription [15]. Recent work offers additional proof that estradiol significantly increases NSCLC proliferation in the presence of either ERα or ERβ [26,27].

Several decades ago, the Coronary Drug Project identified men who had a previous myocardial infarction and randomly assigned them, as part of a multi-component clinical trial, to conjugated equine estrogen at 2.5 mg/day or to placebo, anticipating a reduction in future cardiac events in the estrogen treatment arm. However, this intervention was stopped for primary endpoint futility when increased lung cancer mortality was observed in the estrogen therapy group [28]. Data from more recent randomized, prospective trials suggest that hormone replacement therapy with estrogen plus progestins increases both the incidence of and the mortality from lung cancer in postmenopausal women [29,30], although conflicting data based on earlier retrospective studies are reported [31]. Of special note, similar prospective trials suggest that hormone replacement therapy with estrogen alone is not sufficient to enhance mortality from lung cancer in women [32]. The reason for this apparent contradiction is not known and requires further investigation of the role of progestins in lung cancer progression.

Effects of progestins are mediated by progesterone receptor (PR), and reported PR expression in lung tumors is variable, with some studies reporting a high expression frequency of 39–63% [25,33–35] and others showing little or no expression [36–38]. Low PR expression is a prognostic factor for poor clinical outcome in some studies of NSCLC [33,35] but other survival studies with PR showed no correlation with outcome [25]. Progesterone supplementation has also been shown to inhibit the growth of PR-positive lung tumors in mice [33]. In contrast, treatment of mice with an antiprogestin (mifepristone; RU-486) reduced the progression of spontaneous lung tumors [39]. In embryonic lung cells, combined treatment with estrogen and progesterone increased expression of vascular endothelial growth factor (VEGF) mRNA and protein [40]. Pretreatment with antiestrogen ICI 182,780 and antiprogestin RU-486 completely abolished the sex steroid-induced effects. Thus, estrogen and progestins appear to cooperate in promoting expression of VEGF in primary embryonic lung cells and are also involved in regulating expression of key molecules for prenatal lung development and postnatal lung function [40]. Support for this notion also comes from recent reports showing that progestins and estrogen can promote the expansion of stem/progenitor cells in mammary tissues [41,42].

Cancer progression is also dependent on the development of a rich vascular network, a process regulated by a number of potent growth factors, particularly VEGF [43,44]. It has been clearly established that VEGF is produced by many tumor cells, including NSCLC cells [43–46], and the VEGF content of malignant cells has been shown to correlate with the prognosis of patients with lung cancer [45,46]. It is also reported that VEGF produced by tumor cells is essential for the expansion of lung cancer, largely by increasing proliferation of endothelial cells from neighboring blood vessels through interactions with VEGF receptors present on these cells. Only limited information is currently available on the roles of estrogens and progestins in regulating angiogenic growth factors and tumor-associated angiogenesis in human lung cancer [7,19,47]. Although much emphasis has been placed on the role of estrogens in NSCLC progression, this work reports further on the expression of PR and the potential activity of progestins in this malignancy.

## 2. Experimental

### 2.1. Cell culture and reagents

Human NSCLC cells (A549, H23, H1975, H3255, HCC827) were obtained from the American Type Culture Collection (Manassas, VA). Lung cancer cell lines were routinely maintained in RPMI 1640 media with 10% fetal bovine serum (FBS; Invitrogen/Life Technologies) and antibiotic-antimycotic solution (Mediatech). For steroid-free conditions, medium was changed 48 h before studies to phenol-red free RPMI 1640 with 0.1% dextran-coated, charcoal-treated (DCC)-FBS [48,49]. Lung tumor cells were characterized previously for ER (ERα, ERβ) expression using ligand binding, immunoassay and immunofluorescence methods [6,7,27]. Human umbilical vein endothelial cells (HUVEC; Clonetics/BioWhittaker) at passages 4–6 were grown on attachment factor-coated tissue culture flasks in MEM containing 10 ng/mL basic-FGF and 15% FBS [50–53]. All cultures were maintained in a humidified incubator at 37 °C under 5% $CO_2$, 95% air and free of *Mycoplasma* and pathogenic murine viruses. Recombinant human VEGF-165 (rhVEGF), anti-VEGF antibody, IgG antibody control and a Quantikine VEGF ELISA kit were acquired from R&D Systems, Inc. (Minneapolis, MN). Progesterone, medroxyprogesterone acetate (MPA), RU-486 and estradiol-17β were purchased from Sigma–Aldrich Corp. (St. Louis, MO).

### 2.2. VEGF secretion

Secretion of VEGF, a primary proangiogenic factor, was quantitated in the extracellular media of NSCLC cells by ELISA assays using established methods as before [50–52]. NSCLC cells were grown in maintenance medium containing 10% FBS in 100-mm tissue culture dishes and allowed to reach 60–70% confluence. Cells were washed twice with PBS, and the medium was changed to phenol red-free, serum-free medium and incubated for 24 h. The serum-free medium was replaced, and the cells were treated with or without 10 nM progesterone or MPA for 18 h. Condi-