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Estradiol Modulation of Hepatocyte Growth Factor by Stromal Fibroblasts in the Female Reproductive Tract

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NARRATIVE ABSTRACT

Primary human uterine, cervical and ectocervical stromal fibroblasts constitutively secrete hepatocyte growth factor (HGF); yet only uterine stromal fibroblasts increase HGF secretion in response to estradiol. Estradiol-induced HGF secretion by uterine stromal fibroblasts may have significant impact on uterine cancer and endometriosis.

Epithelial-stromal cell interactions are essential in facilitating steroid hormone-induced growth and development in the endometrium (1). Cooke *et al* used female reproductive tract (FRT) tissues from estrogen receptor (ER) knockout mice in recombinations to demonstrate that underlying estrogen receptor positive stromal cells regulate the differentiation of the adjacent epithelial cells (1). These studies indicated that estradiol acts on stromal cells to release one or more paracrine factors that then modulate estradiol's effects on epithelial cells for growth and differentiation in the FRT.

Some tumors, including uterine (reviewed in (2, 3), ovarian and cervical cancers (reviewed in (4)), are estradiol-sensitive. Estradiol levels are also elevated in women who have endometriosis, a pathology associated with growth of endometrial tissue outside the uterus (5–8). However, there is still very little known about stromal fibroblast paracrine molecules and indirect estradiol action on epithelial cells of the human FRT.

Hepatocyte Growth Factor (HGF), a pleiotropic agent initially shown to stimulate proliferation of hepatocytes *in vivo* (9), is produced primarily by stromal fibroblasts (10, 11). HGF has been well characterized in terms of its normal physiological roles of increasing epithelial cell motility and proliferation, wound healing (12), angiogenesis (13, 14), epithelial cell scattering and embryogenesis (15) (see review (16)). Using primary human uterine epithelial cells, Sugawara *et al* found that HGF stimulates proliferation, migration,

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and morphological changes in human uterine epithelial cells and concluded that HGF may modulate the cyclic regeneration and development of endometrial lining of the uterus (17).

Recognizing that estradiol and HGF regulate normal physiological functions in the mucosal tissue as well as in certain tumors, we hypothesized that estradiol may have a regulatory effect on HGF secretion by stromal cells in the FRT. The goals of this study were to determine if HGF is secreted by human FRT stromal fibroblasts and, if so, if HGF secretion is modulated by estradiol.

FRT mucosal tissue was obtained following surgery from women who underwent hysterectomies at Dartmouth-Hitchcock Medical Center. Tissues used in this study were distal to the sites of pathology and were determined to be unaffected by disease upon inspection by a trained pathologist. Approval to use tissues was previously obtained from the IRB. Tissues from the uterus, cervix (endocervix), and ectocervix were dispersed into epithelial and stromal cell fractions by enzymatic digestion and differential filtering as described previously (18, 19) and stromal fibroblasts were cultured in 24 well culture plates (Fisher Scientific, Pittsburgh, PA). After 4 days of culture, all cells had the characteristic fibroblast morphology, and the culture was devoid of other cell types (20). Complete media, consisting of DMEM/F-12 without phenol red (Gibco) supplemented with 10% heat inactivated stripped fetal calf serum (Hyclone, Logan, UT), 2 mM fresh L-glutamine, 25 mM HEPES, 50 ng/ml primocin (InvivoGen, San Diego, CA) was replaced at 48-hour intervals. Since cultures were used several days following seeding, the likelihood that macrophages remain is greatly reduced, as macrophages are not viable in this media for very long. Cells were treated with 17β -estradiol (CalBiochem, San Diego, CA) at a concentration of 10^{-8} M. Conditioned media from each well were collected at 48-hour intervals, centrifuged at 10000xg and supernatants were analyzed for HGF via a commercially available ELISA duoset (Quantikine; R & D system, Minneapolis). The limit of sensitivity for this assay was 50 pg/ml. InSTAT Software was used to perform a one-way repeated-measures analysis of variance (ANOVA).

Constitutive HGF secretions by uterine stromal fibroblasts derived from nine patients varied from 240 pg/ml to 2716.7 pg/ml, and continued for at least 8 days (48 hour collections). Patient variables such as age (range 39–65), reason for surgery and menstrual status at time of surgery could not be correlated with HGF secretion. Stromal fibroblasts from cervix and endocervix also secreted HGF constitutively.

As shown in Figure 1, treating uterine stromal fibroblasts with estradiol resulted in significant HGF secretion, and the estradiol-induced secretion of HGF increased with the duration of exposure to estradiol. Cells treated with estradiol for six days secreted three times more HGF than cells exposed for two days. The long-lasting effect of estradiol on HGF secretion by uterine stromal fibroblasts could have implications for cancer or endometriosis where continued HGF secretion could lead to further proliferation of uterine epithelial cells. Cell numbers in the control and estradiol-treated wells did not differ, suggesting that HGF secretion was increased by estradiol treatment and not due to changes in cell numbers. In contrast to uterine stromal fibroblasts, estradiol did not increase HGF secretion by cervical (n=5 patients) and ectocervical stromal fibroblasts (n=6 patients).

The findings of this study indicate that stromal fibroblasts of the uterus, cervix, and ectocervix can secrete HGF constitutively *in vitro*. Estradiol treatment significantly increases uterine stromal fibroblast HGF secretion. Interestingly, HGF secretion by cervical and ectocervical stromal fibroblasts was unaffected by estradiol treatment (not shown), which suggests that estradiol selectively modulates HGF secretion by uterine stromal cells in the FRT. Our studies extend the findings of Sugawara *et al* (17) by demonstrating that estradiol acts directly on uterine stromal fibroblasts to stimulate the secretion of HGF. The importance of HGF secreted by endometrial stromal cells in regeneration of the endometrium during the normal menstrual cycle was suggested by Nasu and colleagues (21). Our finding that estradiol increases the magnitude of HGF secretion over time may also reflect a role for HGF in preparing the endometrium for implantation.

The escalated increase of HGF secretion induced by estradiol over 6 days (with 48 hour collections and complete change of media) suggests that a long-lasting effect of estradiol could have implications for cancer or endometriosis where continued and higher quantities of HGF secretion could lead to further proliferation of uterine epithelial cells. HGF production by stromal fibroblasts in the breast is increased by estradiol stimulation (22), and increased levels of estradiol are also associated with increased HGF secretion in mammary epithelial cell carcinogenesis (22, 23). HGF has been reported to act as a paracrine molecule between stromal fibroblasts and epithelial carcinomas (see review in (23)). Jankowski *et al* reported that increased HGF levels regulate metastatic behavior of rhabdomyosarcoma cells (24). In the mouse, estradiol modulates the expression of HGF in the ovary during ovarian cancer (25). Another critical consideration is that increased HGF levels are associated with increased tumor aggressiveness in uterine squamous cervical cancer (26). HGF secretion by stromal fibroblasts is increased in certain ovarian (27) and cervical tumors (26). Understanding how estradiol affects HGF in breast and FRT tumors is critical to a complete understanding of these pathologies.

Khan *et al* have shown that ovarian steroids may mediate HGF production by macrophages and stromal fibroblasts in women with endometriosis (5). The results of our study extend those of Khan *et al* and show that estradiol treatment of human uterine stromal fibroblasts does increase HGF secretion from normal, non-endometriotic patients, which underscores the likelihood that estradiol modulation of HGF secretion in these cells has important ramifications in normal as well as pathological conditions. Using gene-chip analysis, Talbi and colleagues showed a 2-fold increase in HGF expression of endometrial cells derived from normo-ovulatory women in the mid-secretory phase (28). In addition Liu and associates have shown that the HGF promoter contains classical, functional EREs in the mouse (25, 29).

An unexpected finding of this study was that estradiol treatment of cervical and ectocervical stromal fibroblasts, in contrast to uterine stromal fibroblasts, did not affect HGF secretion from these cells. Since each FRT component has specific functions in normal and pathological conditions, it is reasonable to hypothesize that HGF secretion between compartments might not be regulated identically. Since most of the work done in this area dealt with pathologies such as FRT carcinomas and endometriosis, there is little information on how HGF is regulated in normal, non-pathological tissues. Our finding suggests that

HGF secretion is regulated differentially in the FRT, which may have significant impact on our understanding of hormonal regulation of the FRT as well as FRT carcinomas and endometriosis. To the best of our knowledge, this is the first investigation of HGF secretion by normal stromal fibroblasts from three FRT compartments and its regulation by estradiol.

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Figure 1.

Treatment of uterine stromal fibroblasts with estradiol over 6 days shows that HGF secretion increases with increased exposure to estradiol (10^{-8} M). Conditioned media was completely removed at 48-hour intervals and replaced with fresh media with or without estradiol. The mean $\pm$ SEM of HGF secreted in four replicates per treatment is shown. Representative of 4 donors with similar results. ** $p < 0.01$, *** $p < 0.001$ compared to control.