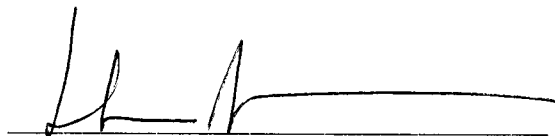


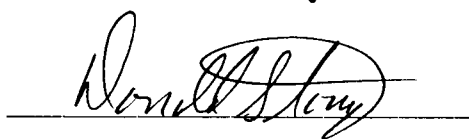
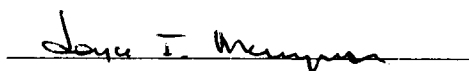
To the Graduate Council:

I am herewith submitting a dissertation by Robert Lindsey Donnell entitled "Platelet-Derived Growth Factor: A Regulatory Link to Endometrial Epithelial Growth." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.



Linda Munson, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor and
Dean of The Graduate School

**Platelet-derived growth factor:
a regulatory link to endometrial
epithelial growth**

A Dissertation
Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

Robert Lindsey Donnell
December, 1997

Dedicated to

Patricia Gail Smith

(1953-1996)

wife, companion, inspiration

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Abstract

Platelet-derived growth factor (PDGF) is a potent mitogenic polypeptide which has been implicated as a common pathway for numerous developmental, physiologic, and pathologic processes. Mediation of estrogenic activity in cyclic endometrial growth and pathologic endometrial proliferation is one of the potential roles of PDGF.

The possible role of PDGF as a mediator for estrogen driven endometrial proliferation was examined in culture and by tissue immunohistochemical methods. Treatment of cultured human endometrial epithelial cells (hEEC) with PDGF caused an increase in proliferation. Sequential exposure of the hEEC to the estrogen, diethylstilbestrol, and PDGF resulted in synergistic growth of the hEEC.

Immunohistochemical staining for PDGF receptors α and β revealed immunoreactivity in human and murine endometrium which varied between tissue compartments with the stage of the estrous (menstrual) cycle. Proliferation of the endometrium was gauged by endometrial morphology and expression of the proliferating cell nuclear antigen (PCNA). A strong positive compartmental association between proliferation of glandular endometrial epithelium and the immunoreactivity for the PDGF receptors was found in both human and murine endometrium.

Endometrial proliferation in endometrial hyperplasia, endometrial adenocarcinoma and endometriosis may reflect proliferative pathways similar to the normal endometrium. Immunohistochemical examination of the endometrial epithelial component of specimens of these conditions revealed positive

immunoreactivity for the PDGF receptors in the epithelium. Association of proliferation with expression of the PDGF receptors was limited to focal areas in these hyperproliferative conditions.

Endometriosis is an incompletely understood disorder of ectopic growth of endometrium in women and menstruating non-human primates. Peptide growth factors, including PDGF, have been suggested as mediators of endometriosis. An inexpensive, reliable animal model of endometriosis would aid in the investigation of this disease. An *in vivo* model, using human endometrial epithelial cells transplanted into the abdominal cavity of severe-combined-immuno-deficient (scid) mice, was developed and compared with the natural disease and existing models. Survival of the hEEC for up to 6 weeks, implant neovascularization, and hEEC recapitulation of rudimentary glandular structures, indicate the scid model is an excellent tool for further investigation of endometriosis and endometrial proliferative disorders.

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Part I

Literature Review

Introduction

The adult mammalian uterus is remarkable in regard to the regular cyclical nature of its growth and regression. Any questions, pertaining to the initiation, progression, control and treatment of pathologic states associated with the uterus, must take into account the cyclicity and plasticity of growth of the uterine components. Embryologically the uterus is derived from the urogenital ridges. Developmental migration and differentiation of these dorsolateral, abdominal, mesodermal evaginations give rise to both the reproductive and urinary tracts^[1]. Differentiation and morphogenesis of the uterine epithelia is dependent on interaction with the stromal elements^[2].

Uterine histology

Histologically the uterus is comprised of smooth muscular, vascular, mesenchymal (stromal), and epithelial components which may be morphologically and functionally divided into the myometrium (muscular) and endometrium (stromal-epithelial). The myometrium is histologically discrete and relatively simple, composed of closely apposed layers of perpendicularly oriented smooth muscle separated by a vascular channel (the vasorum muscularis). The endometrium, in contrast, is a much more complex structure. A single layer of simple, variably villous, cuboidal epithelium lines the luminal surface. The luminal lining is continuous with the cuboidal to columnar epithelium of the uterine glands. Three-dimensional, computer generated, topography has shown that what appear on cross section to be adjacent but

separate glands are instead highly arborized and ramified, with multiple connections between numerous proximate glands[3]. Epithelial stem cells are located in the deep glands within the stratum basalis. Migration of maturing and differentiating epithelial cells proceeds luminally. Although derived from the same cells and morphological continuous with them, uterine epithelium is considered to be comprised of at least three functionally separate subpopulations: luminal epithelium, epithelium of the glands in the functionalis region and the epithelium of the basal glands[4].

The glands are invested in the stroma, a supportive layer of loose to moderately dense, spindle to stellate shaped, mesenchymal cells. Coursing luminally from the muscular layer are arcades of ramifying vessels. Various segmental nomenclature of the endometrium has been assigned by depth, morphology, and functional state. For purposes of this review and the succeeding experimental chapters luminal refers to the epithelial lining of the lumen, superficial may be regarded as synonymous with the glands and surrounding stroma of the functionalis layer which varies greatly in thickness, and deep is used to refer to the basalis or compact zone of the endometrium adjacent to the myometrium.

Each of the uterine components are capable of undergoing remarkable remodeling under normal cyclical / physiologic conditions. The smooth muscle exhibits marked hypertrophy during pregnancy[5]. Mild to marked endometrial neovascularization and arteriolar tortuosity occurs in the cycling and pregnant uterus[6]. Morphologically and biochemically, the stromal component of the endometrium exhibits a range of changes which are species dependent. Stromal changes are most pronounced and best characterized in humans. As

the estrus cycle progresses from estrogenic to progestational dominance, the stromal cells undergo decidualization. Decidualization in women is characterized by a decrease in stromal cell proliferation, an increase in cell size and cell appendages, evolution from spindle shaped to polygonal morphology, vacuolation of the cytoplasm and the storage of numerous secretional products (e.g. glycogen)[7]. Progressive changes in the endometrial epithelium mirror those observed in the stroma. With the change to progesterone dominance there is a decrease in the rate of cell proliferation, the epithelial cells become increasingly columnar and vacuolated with stage specific vacuolar and nuclear localization, orientation and tortuosity of the glandular structures are modified, and apical blebbing due to secretion is evident[7-9].

Role of ovarian steroids in uterine growth

Growth, involution, menstrual shedding and receptivity of the human uterus are controlled by the cyclic release and interplay of the ovarian steroid hormones, estrogen and progesterone. The morphological state of the human uterus reflects the ovarian steroid influences in three phases: proliferative, secretory and menstrual. The proliferative phase is associated with high plasma estrogen levels, the secretory phase with increasing levels of progesterone, and the menstrual phase with a decrease in plasma levels of both estrogen and progesterone[10]. In adult animals, differences exist between species in the compartmental mitogenic influence of the ovarian steroids. For example, in the human endometrium only estrogen is necessary for mitogenic activity in both the epithelia and the stroma, whereas in mice

estrogen alone stimulates epithelial proliferation, but concurrent exposure to progesterone is needed for stromal proliferation[11].

Steroid activity is mediated through specific nuclear receptors. Estrogen receptors (ER) and progesterone receptors (PR) are members of the steroid-thyroid receptor super family (including thyroid, glucocorticoid, retinoic acid, and dioxin receptors) which share structural homology with conservation of tertiary zinc finger motifs[12, 13]. Binding of receptor by its respective steroid results in conformational change, homodimerization and localization of the steroid/receptor complex to its respective steroid response element leading to initiation of steroid responsive genes[12, 14]. The patterns of localization for the steroid receptors are indicative of the responsiveness of the cells to the activity of the steroids. In humans the greatest expression of ER in the uterus occurs in the epithelia and stroma at the time of ovulation while PR expression peaks following ovulation[15].

Exposure of the endometrial tissue to the ovarian steroids, estrogen and progesterone, *in vivo* and *in vitro* affects a wide range of cellular mRNA and protein expression. The endometrial expression of the growth factors, insulin-like growth factor(IGF)[16], transforming growth factor α [17] epidermal growth factor (EGF)[18, 19], and platelet-derived growth factor (PDGF)[20] have been shown to be affected by estrogen levels. Estrogens increase while progestins increase epidermal growth factor receptor (EGF-R), insulin-like growth factors receptor (IGF-R) levels in the human uterus[21-24] and estrogens increase platelet derived growth factor receptors (PDGF-R) in the mouse uterus[20]. Estrogen increases progesterone receptors in endometrial epithelial and stromal cells[25]. Numerous proteins in mouse and human uteri[26-28] and

cell markers on human endometrial epithelium exhibit changes in expression with the predominant steroid milieu[29]. There is upregulation of prostaglandin $F_{2\alpha}$ in endometrial epithelium in response to estrogen[30]. The enzymes cathepsin D[31] and alkaline phosphatase[32] are induced by estrogen in human endometrial cell cultures. Progesterone increases the endometrial production of enkephalinase [33] , plasminogen[34] , glycogen[35], and prolactin in the human endometrium[36]

Endometrial hyperplasia

The responsiveness of human endometrial tissue to the proliferative effects of estrogen and the inhibitory effects progesterone extends to varying degrees to pathological conditions originating from endometrial tissues. The steroid responsiveness of endometrial hyperplasia, endometrial carcinomas, and endometriosis is well established[37, 38].

When the normal, cyclic rhythm of estrogenic / progestational antagonism is disrupted by prolonged, unopposed estrogenic stimulation, such as is seen in anovulatory cycles or polycystic ovarian disease, endometrial hyperplasia is frequently the outcome. Other predisposing factors for endometrial hyperplasia include nulliparity, obesity, high fat diet, ovarian neoplasia and exogenous estrogen administration. The degree of hyperplasia can range dramatically, from minimal to bordering on frank neoplasia[7-9, 39].

Descriptively, endometrial hyperplasia has been divided into cystic and adenomatous (glandular) types[8] . In cystic hyperplasia numerous dilated glands, lacking the secretory progestational changes, are the most obvious finding. Accompanying the glandular ectasia is proportionate stroma

proliferation, occasional squamous metaplasia, and a blurring of the distinction between the deep and superficial glands. Adenomatous hyperplasia which occurs more frequently in premenopausal women is characterized by glandular proliferation without proportionate stromal proliferation. Glands are enlarged and crowded with inter glandular bridging, glandular convolution and pseudo papillary projections. Although morphologically dissimilar, cystic and adenomatous hyperplasia are related to prolonged, unopposed, estrogen dominance. Foci of both cystic and adenomatous hyperplasia may be concurrently present within the same uterus[7, 8, 39]. Some pathologists group cystic and adenomatous hyperplasia into the category of simple hyperplasia both to distinguish them from pre malignant (atypical) hyperplasia and because of their similar response to treatment[9]. Treatment of endometrial hyperplasia is by curettage and / or progestational therapy[1, 8, 39]. Expression of ER and PR is high in epithelium of endometrial hyperplasia[40].

Endometrial carcinoma

Bokhman has postulated two types of endometrial carcinomas based on epidemiology, plasma estrogen levels, behavior, and morphological subtypes[41]. Progression of endometrial hyperplasia through atypical hyperplasia or carcinoma *in situ* and frank endometrial carcinoma has been documented in up to 23% of type I endometrial carcinomas[42]. This low grade type has stable behavior and is associated with concurrent hyperplasia due to unopposed serum estrogen in pre- and peri-menopausal Caucasian women. Type II carcinomas are high grade and progressive, predominantly arising in the atrophic endometrium of post menopausal women. Morphologic

subtypes of endometrial carcinomas include papillary, secretory, ciliated (type I), and mucinous, serous, clear cells and squamous (type II). Predisposing factors for type I endometrial carcinoma are similar to those noted in endometrial hyperplasia. Expression of ER and PR is highly variable, both between and within endometrial carcinomas[40, 43]. The degree of expression is used as both a prognostic indicator and a selective factor in therapeutic regimens. Surgery, progestational therapy, combination progestational / antiestrogenic therapy, radiation, and a range of non-steroidal, anti-neoplastic agents have all been employed in therapy for endometrial carcinomas with variable success, dependent on tumor type and grade[1].

Endometriosis

Endometriosis is defined as the ectopic presence of endometrium outside the uterine cavity and musculature[38]. Naturally occurring endometriosis is confined to females of species which experience periodic menstrual shedding of the uterine lining, (e.g., humans and a limited number of non-human primates). The histogenesis and etiology of endometriosis is incompletely understood and remains controversial[1, 38]. The reported incidence, clinical signs, lesional distribution, and therapeutic responsiveness of endometriosis are variable. Indeed, the "clinical" definition of endometriosis is debatable. Some investigators would limit the use of the term to only those cases with symptoms directly relating to deeply infiltrating ectopic endometrium[44].

Although endometriosis has been estimated to affect approximately 7-10 % women of child bearing age in the US[45, 46], the true incidence of

endometriosis is unknown because the clinical signs are nonspecific and clinical diagnosis requires visualization of the lesions by laparoscopy, laparotomy or biopsy. In women undergoing laparotomy the incidence for documented endometriosis ranges from 18-38 % and the incidence of endometriosis in women with infertility or pain has been reported to be as high as 60 % in some countries[47]. Furthermore the discordance of clinical signs and histologically documented presence of ectopic endometrium has led to debate about the clinical definition of endometriosis[44, 48].

The majority of women with endometriosis are in the 25-40 year age range[1] with a mean age at diagnosis of 29 years[38]. Approximately 10-15 % of cases are reported in adolescents. Less than 5% of the cases occur in post menopausal women. It is generally accepted that the population at greatest risk is women in the upper socioeconomic brackets of developed nations. Women who delay pregnancy are at increased risk within this group[49]. Other proposed factors including length of menstrual cycle[50], smoking[50], exposure to toxins (dioxin)[47, 51, 52] may play roles in the development of endometriosis. Immunologic factors such as specific deficiencies in lymphocyte mediated cytotoxicity, generalized immunosuppression, and B-cell mediated auto immunity have been proposed as possible factors[53-55].

Clinical signs of endometriosis include dysmenorrhea, pain in the lower abdomen, pelvis or back, dyspareunia, irregular bleeding and infertility[38]. The occurrence and degree of these signs vary, and in fact may be absent. The gross and histologic appearance of endometriosis is variable. Descriptive terminology of the gross appearance includes powder burns, chocolate cysts and white plaques. The lesions may be flat, raised, nodular, or cystic. Sites

may be solitary or multiple, and the degree of fibrous adhesion can be absent to severe[9, 38, 56].

Microscopic diagnosis of endometriosis requires the presence of endometrial epithelium in association with endometrial stroma[56]. Typically the lesions consist of glandular to cystic structures lined by flattened to cuboidal epithelium, invested by stroma. The lumen may be empty, contain cellular debris, or be filled by blood[9, 56].. Local infiltration of leukocytes, predominantly macrophages can occur in the surrounding stroma, within the epithelial lining and into the lumen. The macrophages may contain hemosiderin, resulting from phagocytosis of the hemorrhagic red blood cells[1, 9]. There is variable deposition of collagen within and surrounding the supporting stroma. Over time the degree of collagen deposition and fibrosis may increase relative to the epithelium and stroma. The lesion may eventually grossly resemble a white plaque, in which the remaining lesion is composed entirely of dense fibrous tissue[1].

The reported sites of endometriotic lesions are numerous and widespread. The incidence of lesions is greatest on the peritoneal surface and associated structures proximate to the uterus and fallopian tubes: ovaries, uterine ligaments, recto-vaginal septum, cul-de sac, utero-vesicle fold, peritoneal surface of the uterus, fallopian tubes, bladder, and recto-sigmoid flexure[1, 57]. Less common sites include soft tissue sites such as the cervix, vagina, bladder, ureter, omentum, skin, pelvic lymph nodes and inguinal tissues. Endometriosis has been reported to occur on a rare basis in the lungs, pleura, bowel, cecum, skeletal muscle, kidney, stomach, pancreas, sciatic nerve, subarachnoid space of the spine, breast and within bone[1, 57]. The

incidence of distribution may reflect both the histogenesis and the mode of spread of endometriotic tissue.

Several animal models of endometriosis have been developed to study the initiation and progression of endometriosis. The majority of the models rely have involved surgical transposition of autologous uterine tissue to the mesometrium or peritoneal wall. Included among these models are rats[58], rabbits[59] and primates[60]. Because only the primate models experience naturally occurring endometriosis, the primate models have been the most relevant, however cost considerations limit their experimental use. The problems inherent in transplantation of xenographic tissue have limited each of these models to the use autologous tissue. Attempts to use immunologically privileged sites such as intraocular transplantation of human endometrium in the rabbit have still resulted in immune rejection[61]. Development of animal models employing subcutaneous transplantation of human endometrium into immunodeficient recipients such as athymic (nude)[62] and severe-combined-immunodeficient (scid)[63] mice have been successful in terms of xenograft tissue survival. Modification of these immuno-suppressed models may offer the best potential model for study of the initiation and progression of endometriosis.

Several theories have been expounded regarding the initiation of endometriosis. The metastatic theory was first proposed by Sampson in 1940. Central to the metastatic theory is the observation that only menstruating species are at risk for endometriosis. Briefly, it is proposed that during the normal menstrual sloughing of the endometrium retrograde flux of endometrial tissue and cells may occur. Some of this retrograde material escapes into the

peritoneal cavity via the fallopian tubes, a patent passage between the uterine lumen and the peritoneum. Once free within the peritoneum the cells / tissue fragments implant on the peritoneal wall . The metastatic explanation is supported by the high incidence (up to 90 %) of peritoneal endometrial reflux in normally cycling women, viability of menstrual endometrium in subcutaneous implants[63], the relative distribution of peritoneal endometriotic lesions[57], immunocytochemical staining of endometriotic tissue and peritoneal fluid with monoclonal antibodies specific to the endometrial epithelium[64], and successful intraperitoneal injections of autologous endometrial tissue in baboons[65]. Other metastatic pathways including hematogenous, lymphatic and intra-operative have been recognized or proposed[1, 66].

An alternative theory of the histogenesis of endometriosis is grounded in the common mesodermal origin of the genital tract and the mesothelium[1]. In other words the epithelial as well as the stromal components of both the uterus and the mesothelium are derived from the embryonic mesodermal cells. According to this hypothesis no movement of existing cells or tissue from the endometrium occurs, but rather there is a change in the pre-existing multipotential cells in the peritoneal lining. The mesothelium is induced by local and/or systemic factors to differentiate toward uterine endometrial-like cell and tissue morphology. The metaplastic theory gains some support from the rare occurrence of endometriosis in men and post menopausal women on pharmacologic estrogen therapy[66] and the finding of mRNA for the gonadal steroid receptors in the pelvic peritoneum[67, 68]. A third theory combining the endometrial origin of the cells with the interplay of local inductive factors has also been proposed.[69]

Regardless of the ultimate resolution of the metaplastic vs. metastatic argument, the mesodermal derivation of the uterus may be of import in the initiation and progression of reproductive tract associated pathology, such as endometriosis. Tissues derived from the mesoderm, such as the peritoneal and pleural mesothelium can give rise to biphasic neoplasia (mesothelioma), containing both stromal and epithelial components and these components may co-express cytokeratin and vimentin intermediate filaments which in the adult are typically associated with different specific cell lineages[1]. Coexpression of specific types of intermediate filaments may affect the responsiveness of the cells to their environmental signals such as extracellular matrix and cytokines[70]. The common mesodermal lineage of the endometrial and peritoneal tissue may affect their responsiveness to growth factors.

A common method of identification of the tissue / cell lineage in poorly differentiated tumors is the use of immunohistochemistry to evaluate the presence of the cell lineage markers such as Factor VIII-related antigen and cytoskeletal intermediate filaments (IF). As a general rule, epithelial cells contain cytokeratin intermediate filaments, mesenchymal (fibroblasts, white blood cells, lipocytes) cells are vimentin positive, desmin is confined to muscle, neurofilaments are exclusive to neurons and neuroendocrine cells, and the glial fibrillary acidic proteins are present in astrocytes and glial cells of ependymal derivation[71]. Immunohistochemical staining for IF in the uterus reveals an unusual set of staining characteristics in the endometrial epithelial cells. Antibodies to both vimentin and cytokeratin IFs may bind to the cytoskeleton of the uterine epithelium. This characteristic has been noted in

both cell culture and fixed sections of the normal endometrium and in endometriosis[10, 64, 72, 73].

Endometriotic lesions like the normal endometrium, endometrial hyperplasia and some endometrial carcinomas retain some degree of steroidal responsiveness[74] and expression of the gonadal steroid receptors[68, 75]. Medical treatment of endometriosis has exploited this steroid responsiveness using drugs to mimic or create pseudo-pregnant (progestins), pseudo-menopause (gonadotropin-releasing hormone analogs) , or anovulatory (danazol) conditions[38, 76]. All of these treatments are ultimately designed to remove the proliferative effects of estrogen on the ectopic endometrium. However, because of the variability of expression of estrogen and progesterone receptors by the endometriotic cells[75], not all cases of endometriosis are steroid responsive.

Estrogenic modulation of growth factors

Estrogen's proliferative activity on the endometrium and endometrial derived tissues has been interpreted as both direct shortening of the G1 phase of the cell cycle[77] and / or indirectly mediated through the up regulation of growth factors and their receptors[78]. This mediation may be through up regulation of growth factors and their receptors by estrogen, or it may be a coordinated interplay of the activities of estrogen and growth factors[79]. Sekeris and others contend that the epigenetic, tumorigenic and hyperproliferative effects of hormonal steroids are mediated through their modulation of oncogene / peptide growth factor pathways[80]. Oncogenes which are regulated by estrogen directly via estrogen response elements

(described above) or indirectly by trans activation through other oncogenes have been identified in normal and pathologic endometrial tissue[81]. An increase in uterine mRNA expression for the c-myc protein, which is involved in the proliferative response of cells to mitogenic agents, has been demonstrated after estrogen exposure in ovariectomized rats[16]. Increases in mRNA levels of c-fos and c-jun transcription factors, have also been reported in estrogen stimulation in the rat[82, 83]. These cellular oncogenes can mediate the expression of multiple growth factors and their receptors[84]. Among the growth factors or their receptors reported to be increased in the uterus by the administration of estrogen are epidermal growth factor EGF, EGF-R, and IGF and IGF-R[16, 21-24]. The estrogen responsive protein expression of the EGF-R, EGF and IGF IGF-R suggests an role for EGF and IGF in estrogenic stimulation of uterine proliferation[24, 85]. However, no cyclical modulation of expression has been noted with EGF, EGF-R or IGF[86, 87].

Platelet-derived growth factor

Platelet-derived growth factor (PDGF) has also been found in uterine tissue and has been proposed as a possible factor in the progression of endometriosis[20, 88]. Recently Gray et al have demonstrated upregulation of mRNA for both of the PDGF receptors and the PDGF A and B isoforms by diethylstilbestrol in the mouse uterus and vagina[20]. This along with Chegini's earlier findings of correlation of uterine menstrual stage with immunohistochemical localization of the PDGF receptor beta (PDGF-R β) suggests a role for PDGF / PDGF receptor pathway in mediating the estrogen driven cyclic uterine proliferation[87].

Platelet-derived growth factor (PDGF) is a pleuripotent, mitogenically active polypeptide[89]. Initially identified as a serum growth factor derived from the α -granules of platelets, it is now recognized as an interactively complex triad of isoforms produced by a number of cells, with temporally differential expression and variable effects dependent on the target cell population[90]. Since its initial characterization numerous cell types including monocytes / macrophages, smooth muscle cells, endothelial cells, fibroblasts, uterine epithelia, and cytotrophoblasts have been shown to produce PDGF[89].

Biological activities of PDGF are extensive. It is recognized as a competency factor in cell proliferation[89]. It participates in wound repair by increasing cell proliferation, chemotactic recruitment of inflammatory and fibroblasts and modulation of the extra cellular matrix [91]. Activation of neutrophils and monocytes occurs upon exposure to PDGF[92]. PDGF affects cellular ion fluxes, amino acid transport and protein synthesis[93]. PDGF may affect the cellular response to other growth factors (e.g., increasing or decreasing EGF receptor expression and phosphorylation)[94]. Embryonic development and migration are influenced by PDGF[95-98].

PDGF is expressed as two distinct chains (A & B) which are encoded on separate regions of the genome (chromosomes 7 & 22, respectively)[99, 100]. The B chain of PDGF has been shown to be highly related to the transforming protein of simian sarcoma virus (v-sis)[101, 102]. The A and B chain share approximately 60 % identity with conservation of the 8 cysteine residues which participate in their tertiary structure[90]. Both isoforms have approximately equal mitogenic activity in fibroblasts[94]. Biologically active PDGF is a dimeric protein with three isoforms: AA, AB & BB. Expression of the isoforms is

regulated in a temporal and spatial manner[98, 103-105]. The AA dimer is considered the primary isoform occurring in embryonic and neonatal tissue while the AB and BB isoforms are considered the predominant types present in the adult[95].

Activity of the PDGF isoforms is mediated via binding to specific transmembrane receptors belonging to the tyrosine kinase family of receptors[106]. There are two discrete receptor types: PDGF-R α and PDGF-R β . Similar to the situation of the ligand chain types, the receptor types are encoded in distinct regions of the genome(chromosomes 4 and 5 respectively)[107] and the receptors associate by both homo and hetero-dimeric pairing. There is close homology of the overall amino acid sequences of each receptor type between species (e.g., mouse and human 91%) and between the kinase regions of the α and β receptors (84 %)[108]. Expression of the PDGF receptors, like the ligand is controlled in a temporo-spatial manner in multiple species examined[95, 96, 98, 103, 105].

The biological activity of the platelet-derived growth factor peptide is mediated through binding to the transmembrane receptors. Binding of the receptors and ligands exhibit differences in specificity and affinity. The α receptor binds both the A and B chains. β receptor will only bind the B chain. Binding of the dimeric ligand to the transmembrane induces receptor dimerization. Therefore it is possible to have $\alpha\alpha$ and $\alpha\beta$ receptor dimers which interact with all three of the dimeric ligand isoforms. Although the $\beta\beta$ homodimeric receptor binding apparently occurs with both the BB homodimer and the AB hetero-dimeric ligands it has greater affinity and specificity for the BB homodimer[90].

Binding of the PDGF ligand to its respective receptor results in tyrosine phosphorylation of the receptor at two sites (Y751 & Y857 in the β receptor)[109]. This phosphorylation initiates receptor conformational changes and a series of downstream phosphorylation events for sequential activation of multiple intracellular signaling pathways. Gene transcription is mediated via SH2 domain and along the Janus kinase (JAK/STAT) phosphorylation cascade[110]. The SH2 domains may also regulate binding to phosphatidylinositol-3 kinase[111]. The phospholipase C γ 1, GTPase activating protein (GAP), and phosphatidylinositol-3 kinase signaling cascades may each be initiated by the receptor phosphorylation[84]. Each of these systems presumptively mediate one or more of the biological activities of PDGF (e.g., actin reorganization, intracellular calcium fluxes) depending on the cell type[109]. Phosphorylation of both receptor sites is necessary to elicit the full mitogenic effect of PDGF[112]. After ligand binding there is ubiquitination, internalization and lysosomal degradation of the receptor / ligand complex[113].

Although initially considered a serum peptide, PDGF exerts most of its activity by paracrine or autocrine pathways[114]. Autocrine interaction of the PDGF ligands with their respective receptors may not be confined to the cell membrane. The AA and AB isoforms are rapidly secreted from the cell after processing of the precursors, however most of the BB isoform is retained within the cell[115]. This raises the possibility that some of the autocrine activity of PDGF may not require transmembrane binding, instead partially relying on intracellular binding of ligand and receptor[116].

Based on studies done using embryonic fibroblasts PDGF is classified as a competence factor. It was found that sequential addition of PDGF and platelet poor plasma allowed quiescent 3T3 cells to enter S phase 12 hours post exposure to PDGF, and that the percentage of cells entering S phase was dependent on PDGF and plasma concentrations[117]. PDGF made the cells competent to move from the G0 to the G1 stage of the cell cycle , while other factors were necessary for progression through the remainder of the cycle. Further work showed that this same effect could be produced with sequential administration of PDGF, EGF and IGF-I[93]. However, differences have been found regarding competence and progression requirements in cell types of various lineage and stages of differentiation[118]. Upregulation of PDGF or PDGF receptor expression by a great variety of stimuli and agents, through both cis and trans acting promoting elements, imply that PDGF ligand / receptor signaling is common to numerous cell signaling pathways[100].

Cell and tissue activity of the PDGF isoforms and expression of the PDGF receptors were initially described almost exclusively in mesenchymal types of cells. A great deal of attention focused on the role of PDGF in glial cells[119, 120], endothelial cells[121], atherosclerosis, glomerular mesangial cells[99], hepatic Ito cells[121, 122], and dermal fibroblasts[123] in pathologic proliferative and inflammatory conditions. PDGF is considered an important factor in the early dermal wound healing and inflammation[91, 124]. Review of an increasing body of evidence has shown that the PDGF isoforms or receptors can be produced by normal, injured and neoplastic epithelium (pulmonary carcinoma, hepatoma, prostatic carcinoma)[125]. PDGF has been shown to play a role in both normal development and healing in the lenticular and

corneal epithelium of the eye[126] . Given the role that PDGF plays in early wound healing, its mitogenic effect on multiple cell types, its ability to promote cellular chemotaxis and migration, and its ability to induce extracellular matrix production, a role(s) for PDGF in the regeneration of the female reproductive tract might be anticipated. Indeed, the reproductive tract, and accessory or secondary organs have come under greater scrutiny as potential targets for the biological activity of PDGF.

PDGF and / or PDGF-R have been recognized in numerous carcinomas or carcinoma derived cell lines of both the male and female reproductive systems. PDGF-like proteins have been demonstrated in prostatic carcinoma[127]. PDGF B chain produced by mammary gland carcinomas may have both paracrine and autocrine functions in the mammary epithelium[128-130].

In non-neoplastic tissues associated with the reproductive tract, Watson et al found PDGF-R α (by RT PCR) in bovine oviductal cells and pre implantation stages of the bovine embryo[96]. PDGF-R β has been isolated from porcine uterine tissue[131, 132]. Human and bovine endometrial epithelial cells in culture are stimulated by PDGF[73, 133]. Regulation of PDGF-R β expression by administration of diethylstilbestrol in the endometrial epithelium of neonatal mice has been described by Gray et al[20].

Immunohistochemical localization of expression of PDGF A, PDGF B and the PDGF-R β in cycling human endometrium has been described by Chegini et al[87]. PDGF-like substances have been identified in the peritoneal macrophages of patients with endometriosis[134].

Further investigation regarding the role of PDGF in normal and pathologic endometrial tissue is warranted. Estrogenic involvement with proliferation of the uterine endometrium is unquestionable, however other factors are able to replace estrogen or are required cofactors with estrogen for proliferation in reproductive organ systems. The proliferative effects of PDGF on epithelial endometrium *in vitro*, the upregulation of PDGF and PDGF receptors by diethylstilbestrol, and the correlation of PDGF R β to the proliferative phase of the menstrual cycle all suggest a role for PDGF as a mediator of estrogenic activity and as a potential factor in the progression and maintenance of pathologic conditions involving endometrial proliferation when steroidal responsiveness is lost.

Extension of the *in vitro* examination of the effects of PDGF on endometrial epithelial cells to include concurrent and sequential treatment with estrogens would shed light on the potential interaction of estrogens and PDGF. Comparison of endometrial expression of the PDGF receptors to recognized markers of proliferation in the endometrium would confirm and extend the earlier observations of PDGF-R β in the proliferative phase endometrium. Comparison of proliferative markers and expression of the PDGF receptors in conditions of pathologic endometrial growth, such as hyperplasia, carcinoma and endometriosis would address questions regarding estrogen dependent (or independent) growth in these conditions and PDGF's role in the progression of these conditions. Development of animal model in which to examine the growth of separate endometrial cell types in the peritoneal cavity would aid in the examination of the initiation and progression of endometriosis and

investigation of the direct *in vivo* effects of growth factors on normal and pathologic endometrial epithelium.

Part II

Experimental investigations

Chapter 1

***In vitro* response of cultured human endometrial epithelial cells to platelet-derived growth factor**

Introduction

Platelet-derived growth factor (PDGF) is a multi-isoform polypeptide with mitogenic activity in numerous tissues and cell types. PDGF was initially considered to be almost exclusively mitogenic for cells of mesenchymal lineage, however recent findings have extended its proliferative effects to some epithelial cells. Many of these epithelial cells are in organs (mammary, uterus, prostate) which respond to gonadal steroid influence by increases in secretory or proliferative activity[87, 135, 136]. Numerous growth factors, (including PDGF) and their respective receptors have been isolated in uterine tissue[20, 87, 131, 137]. Increased replicative DNA synthesis has been reported in rat uterine cells in response to PDGF[138]. Proliferation of bovine and human endometrial epithelial cells has been reported in response to the PDGF isoforms[133]. Because the proliferative response of PDGF is mediated through its receptors, the proliferative response of the endometrial cells to PDGF is presumptive evidence for the presence of PDGF receptors in these epithelial cells.

Increasingly, there is consensus that the effects of steroidal hormones are mediated through pathways involving peptide growth factors[23, 78, 80]. Steroid hormone exposure may be endogenous or exogenous. Numerous

natural (e.g. phytoestrogens) and synthetic (e.g., diethylstilbestrol [DES]) environmental estrogens have been documented[139]. Exposure to DES during fetal development is associated with congenital abnormalities and multiple reproductive tract neoplasias[140]. DES has been demonstrated to stimulate endometrial cell proliferation in culture[141]. Gray *et al* , have shown an upregulation of mRNA for PDGF receptors in the uteri of immature mice given the synthetic estrogen diethylstilbestrol[20]. The expression of PDGF-R β is known to have increased expression in proliferative phase endometrium[87]. This suggests that PDGF may mediate estrogen's action in endometrial proliferation. Examination of the response of cultured endometrial epithelial cells to PDGF, ovarian steroids and combinations of the ovarian steroids and PDGF will shed light on their interactions as stimulators of endometrial proliferation.

Experimental overview

The purpose of this study was to assess the effect of steroidal hormones in combination with platelet-derived growth factor on the *in vitro* proliferation of human endometrial epithelial cells. Human endometrial epithelial cells (hEEC) were isolated and characterized by morphology and confluent culture behavior. Additional characterization was performed using immunocytochemistry for intermediate filaments expression and platelet-derived growth factor receptor expression. Multiple cell lines were exposed to platelet-derived growth factor isoform AB (PDGF AB) and the steroid hormones 17- β estradiol (E2), progesterone (P4) and diethylstilbestrol (DES) alone or in combination. Uptake of tritiated [^3H] thymidine was measured, as a indicator of replicative DNA

synthesis, the values normalized, and standard indices of proliferation were compared among the different treatment groups.

Materials and methods

Development of cell lines

Endometrial tissue was obtained from women undergoing elective ovario-hysterectomy at the University of Tennessee Medical Center, Knoxville (Institutional Review Board protocol # 0291). Tissue sections of 0.5-1 cm diameter were obtained post-surgically, then transported at 4°C in Dulbecco's modified Eagle's medium / Ham's F12 (DMEM /F12) media (Sigma, St. Louis MO). The techniques used for isolating the hEEC were modified from previously described techniques as follows[73, 142-144]. Under sterile conditions the 1-2 mm thick superficial endometrial sections were cut into approximately 1 mm³ segments. The segments were subjected to digestion in 10% collagenase (Boerrhinger Mannheim, Indianapolis, IN) in DMEM-F12 for 10 minutes in a 37 °C shaking water bath. Two basic methods were used to obtain the purest cultures of the uterine epithelial cell component. The initial method employed differential sedimentation and attachment to take advantage of the greater mass and slower attachment time of epithelial cells compared to stromal cells. After collagenase digestion the tissue suspension was allowed to sit at room temperature for 10 minutes. The upper 80 % of the suspension (consisting primarily of stromal & endothelial cells) was removed and the remaining 20 % was resuspended in DMEM-F12. This procedure was repeated three additional times. The cells were then placed in tissue flasks and incubated at 37°C for 15 minutes to allow for differential (more rapid)

attachment of stromal cells. The suspension (now comprised of primarily epithelial cells) was then transferred to a second flask. The procedure was repeated three times. Sizable numbers ($>1 \times 10^7$) of relatively pure hEEC (90-95 %) could be quickly and easily obtained after collagenase digestion and mechanical disruption followed by repeated differential sedimentation and attachment separations.

A second method was also employed using microscopic identification, separation and isolation of glands from the partially digested endometrium. After collagenase digestion and mechanical disruption, single glands were isolated in a sterile laminar flow hood using a phase contrast microscope (10 x magnification) and placed in 12-well Corning culture plates in the minimum amount of media necessary to cover only the explant. The plates were inverted for 30 minutes, righted, and the wells filled with media to cover the explant. The explants were then incubated in media for 48-72 hours before further manipulation.* {Color Plate 1: photomicrograph 1.1} Based on the previously described morphological appearance of the endometrial explant outgrowth, the cultures in each well were continued or terminated within 8 days. Media for the cell cultures was composed of DMEM /F12 with 10% fetal bovine serum (FBS), insulin (5 μ g/ml), transferrin (5 μ g/ml), selenium (5ng/ml), epidermal growth factor (EGF) (10ng/ml), penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin (0.25 μ g/ml)[73]. Media was changed every 72 to 96 hours unless noted otherwise.

* Color Plates are located in Appendix C

Primary cell cultures were grown to confluence and then further subcultured three times by dissociation with trypsin (0.25 %) before, based on morphology and contact inhibited growth behavior, they were selected for characterization of epithelial lineage markers. {Color Plate 1: photomicrograph 1.2} In cultures with evidence of stromal cell growth, either direct removal of stroma was accomplished by scraping and suction of stromal foci, or differential trypsinization was used to remove more extensive stromal growth. Differential trypsinization takes advantage of the more rapid detachment of stromal (fibroblast) cells compared to epithelial cells. Briefly, media is removed from the flask and the cell monolayer rinsed with a calcium and magnesium-free buffer. Trypsin (0.25 %) is then placed on the cells and the monolayer observed (by phase microscopy) for cell detachment (approximately 30-90 seconds). The plate is then rinsed twice to remove cells which have lifted from the surface and the media replaced. Cultures with epithelial characteristics were cryopreserved in 20% FBS and 10 % dimethylsulfoxide by step down freezing[145]. For epithelial characterization, the selected cultures were subcultured into 8-well Lab Tek permanox chamber slides (Nunc, Naperville, IL) allowed to reach 80-90% confluence, fixed with cold 100 % methanol for 10 minutes, and air dried prior to immunocytochemical procedures.

Characterization of cell lines

Intermediate filament expression in the selected cells was examined by immunocytochemistry using commercially available antibodies in a biotin-streptavidin-enzyme system (Biogenex Laboratories, San Ramon CA). Primary

antibodies employed were a monoclonal antibody to vimentin (V9), a polyclonal pan-cytokeratin antibody, a cocktail of monoclonal antibodies to cytokeratins 8,18,19, a monoclonal antibody to cytokeratin 8, a polyclonal antibody to Factor VIII antigen, and a polyclonal antibody to desmin. All antibodies were obtained from Biogenex Laboratories. Pre-diluted non-immune mouse and rabbit sera (Biogenex) were used for negative controls. Chromagens used for these investigations were diaminobenzidine (DAB)(Sigma, St. Louis MO), 5-bromo-4-chloro-3-indolyl-phosphate-nitro blue tetrazolium (BCIP/NBT)(Sigma), or fast red (Biogenex).

After fixation and drying, the attached cells were rehydrated in PBS (2 x 3 min.), treated with 3 % hydrogen peroxide in 50 % methanol to quench endogenous peroxidase (10 min. at room temperature), and rinsed in PBS (2 x 3 min.). Each of the chambers of the slides was incubated at room temperature (RT) with either the selected antibodies or non-immune sera according to the manufacturer's recommendation. The slides were rinsed (PBS, 2 x 3 min.) and incubated with a secondary biotin-labeled antibody to the primary antibody host IgG (20 min., RT). After rinsing (PBS, 2 x 3 min.) the slides were incubated with an enzyme-labeled streptavidin solution (20 min., RT). After a final PBS rinse (2 x 3 min.), the chromagen solution was applied and color development was gauged by microscopic (10 x) observation comparing to negative controls. The slides were washed in running water to stop color development, counter stained with Harris' hematoxylin, and cover slipped with Crystal Mount (Biomed Corporation, Foster City, CA)

Subcultures of the primary cell lines were also assessed for expression of PDGF-R β and PDGF-R α by immunocytochemistry using the methods

described above. Polyclonal (rabbit-derived) anti-human PDGF-R β antisera (2 μ g IgG/ μ l: Upstate Biotechnology, Lake Placid, NY) was diluted 1:1000 in a commercially buffered solution (Common Antibody Diluent, Biogenex). Polyclonal (rabbit-derived) anti-human PDGF-R α antisera (250 μ l serum: Upstate Biotechnology Lake Placid, NY) was diluted 1: 500 of its supplied concentration as described above

Proliferation assays

Six lines of the primary hEEC (subcultured from 5 to 12 times) were selected for proliferation studies: M92-2, M92-5, DH 95-28, DH 95-33, DH 95-43, DH 96-R1. The M92-2 and M92-5 cell lines (isolated and characterized by L Munson) had been used in previous studies[73]. They were included in this study to confirm previous data. During the course of these studies equipment became available which decreased potential radioisotope exposure and solid radioactive waste. Cells were plated either at 1×10^5 cells per well in 48-well tissue culture plates (Corning Glass Works, Corning NY) or 1×10^4 cells per well in 96-well tissue culture plates (approximately 60 % confluence). For all assays, cells were incubated for 24 hours in DMEM / F12 with 10 % FBS to allow acclimation to the plates. The media was then removed, the cultures rinsed and the cells cultured in DMEM /F12 without serum or growth factors for 24 hours to obtain cell quiescence. The plates were rinsed and the cells incubated with the treatment media.

Treatment levels (with the exception of DES) were chosen to reflect reported physiological serum preovulatory serum values[10]. The DES treatment level was equivalent on a molar basis with that of estradiol and is

reported to have approximately the same activity as estradiol[139]. Treatments consisted of phenol red-free DMEM/F12 medium supplemented with the following: PDGF AB (10 ng/ml), 17- β estradiol (E2) (350 pg/ml), progesterone (P4) (10 ng/ml), epidermal growth factor (EGF) (10 ng/ml), diethylstilbestrol (DES) (3×10^{-9} M), PDGF AB with E2, PDGF AB with P4, PDGF AB with E2 and P4, EGF with E2, DES with PDGF, or PDGF with EGF. Eight hours after applying the treatment media, the cultures were pulsed with 1 μ Ci of ^{3} H thymidine. Sixteen hours later the cultures were harvested. Cultures from the 48 well plates were harvested by cell lysis with sodium hydroxide. Cultures from the 96 well plates were harvested by freeze fracture lysis (-20°C). [^{3} H] Thymidine uptake was measured with a Matrix direct beta counter (Packard Instrument Company, Downer's Grove, IL). Cell proliferation was measured by tritiated [^{3} H] thymidine uptake in cells incubated in the treatments and were compared with uptake in cells treated with phenol red free DMEM / F12 alone.

The treatments described above used combinations of steroids and peptide growth factors in which the cells were cocultured with the treatments. To further examine the possibility of estrogenic regulation of PDGF responsiveness a second study in which the treatments were given sequentially was done to see if prior exposure to one substance affected the responsiveness to a second. The cells were treated with one agent for 24 hours and the cells rinsed. The cells were then exposed for 24 hour exposure to a second treatment. The second series of proliferation assays were performed using three of the cell lines: M92-2, DH 95-43, and DH 96-R1. Subcultures of the primary cell lines were trypsinized and transferred to 96 well plates at 1×10^4 cells per well. The wells were treated as above through the serum deprivation

step. The cells were treated by sequential exposures to the following: DMEM/F12 followed by DES ($2 \times 10^{-7}M$), DES followed by DES, DES followed by PDGF AB (10 ng/ml) and PDGF AB followed by PDGF AB. Because of the lack of stimulatory activities of estradiol and DES in the above studies, the DES level in this study was increased to pharmacological values reported to have been effective in stimulating endometrial cell proliferation[141]. Wells were rinsed twice between the first and second treatment. Eight hours after applying the second treatment media the cultures were pulsed with 1 μ Ci of [^3H] thymidine. Sixteen hours later the cultures were harvested from the 96-well plates by freeze fracture lysis ($-20^{\circ}C$). [^3H] Thymidine uptake was measured by Beta Matrix counting.

Statistics

Because these mitogen studies were performed using multiple cells lines and two similar but technically different protocols, the raw data obtained was not directly comparable. However, the effect of the treatments could be compared by converting the raw data to a standard index of proliferation. Within each assay the mean value of the controls was calculated. Each data point within that assay was then divided by this mean and a standard index derived. After the data obtained for each assay were converted to a standard index of proliferation, the results of similar treatments from different assays were pooled. The pooled data consisted of a minimum of two replicates of at least three cell lines. All replicate experiments were performed using at least a triplicate of each treatment. One way analysis of variance (ANOVA) was used for multiple

comparisons in each study. Differences among specific treatment groups were assessed by the Bonferroni method.

Results

Development of cell lines

Both methods of isolation of hEEC primary cultures proved effective. The differential separations employed in the isolation of hEEC facilitated elimination of endometrial stromal cells, lipocytes, endothelial cells, and fibroblasts. However, over time and after repeated passages, residual stromal cells would emerge and repeated differential trypsinization was required to maintain the purity of the epithelial population. Although the gland isolation method for isolation of hEEC was time consuming initially and resulted in low initial yield numbers of cells, the purity of the cell populations approached 100%, virtually eliminating the necessity of later differential trypsinization purification.

Characterization of cell lines

Based on the morphology, behavior and cell-lineage staining, 10 primary cell lines were isolated and cryopreserved. The cell lines were composed of polygonal to elongated cells that exhibited contact inhibition upon reaching confluent growth. These characteristics were maintained up to 6 months in selected lines. No positive staining was noted for desmin (a cell lineage marker for smooth muscle cells) or Factor VIII-related antigen (a marker for endothelial cells) in the cell lines selected. Immunocytochemical staining with antibodies against the cytokeratin intermediate filaments, markers of epithelial cell lineage, had variable results. Moderate to strong staining was

observed for pan cytokeratin markers in the cell lines. {Color Plate 1: photomicrograph 1.3} Variably strong staining in occasional, and mild staining in the majority of cells was noted for the combination of cytokeratins 8, 18 & 19 (markers for some glandular epithelial cells). {Color Plate 1: photomicrograph 1.4 }

Positive immunocytochemical staining for both the PDGF-R α & β was present in greater than 90 % of the cells in all cell lines examined (M92-2, M92-5, DH 95-43 and DH 96-R3). The staining was granular and diffuse within the cytoplasm with variable concentration of staining along the cell membrane. {Color Plate 1: photomicrograph 1.5 & 1.6}. Staining for vimentin (a marker of mesodermal cells, and some epithelial cells in culture) was consistently moderate to strong in all cells of all cell lines. {Color Plate 1: photomicrograph 1.7} Co-expression of both pan-cytokeratin and PDGF-R β could be demonstrated by dual staining. {Color Plate 1: photomicrograph 1.8}

Proliferative responses

Treatment with PDGF AB alone or in combination with the other treatments stimulated proliferation in all the assays.* (Table 1 & Figure 1.1) The levels of PDGF AB mediated proliferation was significantly greater than the levels in cultures treated with E2, EGF, E2 & EGF, DES or DMEM / F12 alone ($p < 0.001$). (Figure 1.2) Treatment of the cell lines with PDGF AB in combination with E2, E2 & P4, EGF, or DES also resulted in significantly greater [^3H] thymidine uptake in comparison to uptake in cells which did not receive any PDGF AB treatment. The combination of treatment of EGF with PDGF AB had significantly greater ($p < 0.01$) [^3H] thymidine uptake when compared to all other

treatments. The increase in proliferation with PDGF AB and EGF above the non stimulated control was greater than the increase of PDGF AB alone plus EGF alone above the nonstimulated control (Figure 1.3). Confirmation of cellular proliferation was done for selected assays using cell counts in ten, 10x fields of the control cultures vs PDGF stimulated cultures in parallel cultures on Lab Tech chamber slides..

In the proliferation assays of sequential 24 hour treatments of endometrial epithelial cells, all treatments resulted in significant ($p < 0.001$) [^3H] thymidine uptake compared with controls (DMEM / F12 for 48 hours). Proliferation in the cells treated with PDGF AB following incubation with DMEM / F12 was increased, but not significantly, over that in cultures treated with DES following incubation with DMEM / F12 or DES following treatment with DES. Treatment of the cells with PDGF AB after prior DES exposure caused significantly greater [^3H] thymidine uptake than in all other treatment groups ($p < 0.001$). The increase observed with the sequential treatment of DES followed by PDGF AB was greater than the additive increase of DES above control and PDGF AB above control. (Figure 1.4)

Discussion

The primary human cell lines isolated by both methods exhibited characteristics consistent with those previously reported for endometrial epithelium[73, 144-146]. Positive staining for vimentin intermediate filaments, which is traditionally considered a marker of mesodermal derivation[71], has

* (Tables are located in Appendix A, Figures are located in Appendix B)

been previously reported in both cultured and endometrial epithelium *in vivo* [72, 73, 147]. The expression of vimentin by these cells is in keeping with their mesodermal origin[148].

Positive immunocytochemical staining for platelet-derived growth factor receptors in cultured endometrial epithelial cells has not been previously reported, although PDGF-R β has been immunohistochemically localized in the human and murine uterus[20, 87]. That both receptors can be identified in cultured hEEC is not surprising in light of previous work describing the proliferative effects of both the PDGF AA and PDGF BB isoforms in hEEC cultures[73]. These findings show that PDGF receptor expression is retained by the endometrial epithelial cells under culture conditions, and imply a critical role for PDGF in control of hEEC proliferation.

[^3H] Thymidine uptake is used as a measurement of the incorporation of thymidine in replicative DNA synthesis[138]. Comparison of increase in cell numbers in parallel cultures confirmed the use of the ^3H thymidine as a reliable measurement of proliferation. The significant proliferative effect of PDGF AB isoform in subconfluent hEEC cultures as measured by ^3H thymidine uptake confirms Munson *et al* 's prior findings. Kiesel and Mappes have also reported increased human uterine cellular proliferation in response to PDGF BB, although the lineage of the cell types were not determined to be epithelial[149]. Results reported in endometrial epithelial cell cultures with mouse endometrial epithelial cells grown on collagen gels in media with low calcium concentrations conflict with results from other investigations using mouse or human cells[150]. Differences in specie's sensitivity and culture conditions may explain the apparent conflicts regarding PDGF activity *in vitro* .

Given the regulatory roles of the ovarian steroid hormones on the cyclic growth of the uterus, their lack of significant direct effects on proliferation of hEEC in culture seems discordant. The lack of significant proliferative activity by physiological levels of the estrogens on cultured endometrial cells has been noted by multiple investigators[27, 141, 151, 152]. The lack of proliferative activity of E2 and DES (at physiologic serum values) on endometrial epithelium in culture may be indicative of a necessity for secondary factors, such as growth factors (lacking in these *in vitro* models), to mediate estrogen's mitogenic activity.

Likewise, EGF has been reported to be a potent mitogen for multiple epithelial cell lines. EGF is a necessary intermediary for estrogen stimulation of growth in the mammary gland[153]. EGF and its receptors are expressed in the uterus and are considered to be regulated by the gonadal steroids[21, 87]. EGF can induce estrogenic like stimulatory effects on uterine tissue in ovariectomized rats [154]. Yet in these investigations EGF failed to stimulate significant proliferation above that of the control. The findings of other investigators regarding the effect of EGF on endometrial cultures have been conflicting; some have reported decreased proliferation while others have reported increased proliferation. These conflicts may reflect responses affected by such factors as cell density, media composition, species and anatomic origin of the cells (e.g. glandular vs. luminal epithelium)[138, 150]. EGF is considered a progression factor in the cell cycle, allowing cells to proceed from the G1 phase through the remainder of the cycle. Absence of prior competence factor activity (e.g., PDGF) in cultures challenged with EGF may result in inadequate numbers of cells in the G1 phase for EGF alone to have strong proliferative

effect. The significantly increased, mildly synergistic proliferative response observed with the combination of EGF & PDGF AB supports the necessity of prior exposure to a competence factor for EGF to have its proliferative effects.

The proliferative effects of PDGF AB in all treatments was consistent and significant. Despite increased [³H] thymidine uptake by combination treatments (DES with PDGF AB, E2 & P4 with PDGF AB), the lack of significant difference between those combinations and PDGF AB alone implies that PDGF AB is the dominant mitogen among these factors. Implicit in these findings is that if (as other investigators have proposed)[20] estrogen does upregulate PDGF receptor expression, it takes longer than the 24 hour treatment time used in these assays.

Increases in uterine proliferation treated with DES have been reported previously in *in vitro* and *in vivo* [20, 141]. Significantly increased proliferative effects in cell cultures treated with DES prior to PDGF AB compared to the PDGF AB treatment following incubation with DMEM / F12 suggested that pretreatment with DES sensitizes hEEC to the action of PDGF.

Activity of the PDGF isoforms are mediated through their ligand-receptor binding. The increased proliferation in response to PDGF in cells pretreated with DES may be via an increase in PDGF-R α and / or β protein expression in hEEC stimulated by DES. Endometrial upregulation of expression of multiple growth factors, receptors, and other proteins by estrogen has been well documented[16, 20, 30, 149]. Increase in [³H] thymidine uptake (an indicator of DNA synthesis) is significant only after at least 24 hours of previous exposure to DES. In other words, it takes at least that length of time for PDGF receptor transcription, translation, receptor expression, ligand-receptor binding and

movement of the cell cycle from G0 to S phase for proliferation to occur at significant levels. Similar time frames have been reported by Beck and Garner for estradiol induced production of insulin-like growth factor[138]. Significant increases in the expression of the EGF receptor occurred 12 to 24 hours after *in vivo* administration of estradiol[155]. More specific time frame analysis for DES upregulation of PDGF receptor protein will require additional testing.

In summary, the earlier findings of Munson, Van Meter and Upadhyaya regarding the proliferative response of cultured human endometrial epithelial cells to platelet-derived growth factor was confirmed and extended to other cell lines. The presence of both PDGF-R α & β in the hEEC was demonstrated immunohistochemically. The response of the hEEC to the pharmacological dosage of DES confirms that hEEC in culture retain responsiveness to estrogenic stimulation. In light of the response of the hEEC to the pharmacological DES dosages, the lack of responsiveness by the hEEC to physiological dosages of estradiol and DES implies either a refractory phenomena of culture or the lack of a second factor(s) necessary for mediation of estrogenic activity that can be partially overcome by increased levels of estrogenic substances. The significantly increased [^3H] thymidine uptake observed with PDGF AB treatment preceded by DES suggests PDGF may serve a role in the mediation of some of estrogen's proliferative activity in the uterus and that the mediation may occur through an estrogen stimulated upregulation of the PDGF receptors.

Chapter 2

Identification of PDGF-R in human endometrium and association with proliferating cell populations.

Introduction

PDGF is a multi-isoform, pleiotrophic serum growth factor with potent mitogenic activity on multiple cell types. PDGF activity is mediated by two distinct transmembrane receptors (α & β). It has been demonstrated previously by Northern blot analysis that both PDGF-R α & β are present in the human uterus. Localization of PDGF-R β in the human uterus has been accomplished by immunohistochemistry[87]. However, reliable immunohistochemical labeling of the PDGF-R α has proved elusive.

The proliferative response of human endometrial epithelial cells *in vitro* to the PDGF isoforms[73], and the modulating effect of estrogenic steroids on PDGF activity[20] imply an estrogen modulated, proliferative role for PDGF in endometrial epithelium. The isolation of PDGF isoform and PDGF receptor mRNAs from uterine tissue[132, 137], and immunohistochemical localization of PDGF-R β in the human and mouse endometrium[20, 87], reinforce a biological role for PDGF in the cyclical growth of the uterus. PDGF's proliferative activity has been most strongly associated with mesenchymal tissue[156], however, an increasing number of epithelial tissues have been recognized to respond to PDGF[73, 157, 158]. The mesodermal / Mullerian derivation of the uterine epithelium[148], and its expression of the

mesenchymal cytoskeletal intermediate filament vimentin[10], may impact the responsiveness of the epithelial component of the endometrium to growth factors. Correlation of the localization of the PDGF receptors with vimentin expression in the endometrial epithelium and sites of proliferation during the menstrual cycle would confirm or refute these hypotheses and extend the knowledge of the role of PDGF in endometrial growth and remodeling .

Experimental overview

The aim of this study was to develop immunohistochemical methodologies to visualize PDGF receptor α in the human endometrium and to examine relationships between expression of the PDGF receptors and the proliferative state of the endometrium. Cyclical uterine expression of PDGF-R α , PDGF-R β , proliferating cell nuclear antigen (PCNA) and vimentin was evaluated by immunohistochemical staining of uterine tissue samples obtained at the time of ovariectomy from mature women. Intensity and localization of PDGF-R α , PDGF-R β , PCNA and vimentin staining were compared using correlation analysis.

Materials and methods

Partial to full thickness uterine segments were collected from adult women undergoing routine hysterectomy at the University of Tennessee Medical Center Knoxville under UTMCK Institutional Review Board protocol #0291. The specimens were placed in 4% paraformaldehyde (4°C) for 24 hr., then transferred to 70 % methanol (4°C). Tissue sections were processed for paraffin embedding, cut at 4-6 μ m and mounted on non-charged glass

microscope slides. Histological examination of hematoxylin and eosin-stained sections of the specimens was employed to stage the specimens under the guidance of Dr. Stewart Van Meter UTMCK, Department of Pathology) according to the recognized criteria[8, 39] {Color Plate 2: photomicrographs 2.9, 2.17, 2.25}. Specimens from 17 women were selected. The specimens included proliferative (8), secretory (6), and inactive (3) uteri. Additionally, formalin-fixed, paraffin embedded specimens of inactive (1) or secretory stage (1) endometrium, were obtained from the archives of the UTMCK pathology department.

Immunohistochemical staining with each antibody investigated was performed on duplicate sets of slides from each of the 19 specimens . The commercially available biotin-streptavidin system from Biogenex was used for all immunohisto-chemical procedures. Briefly the system employs the binding of a biotinylated secondary antibody to the species IgG of the primary antibody. The binding of the secondary to the primary antibody was then amplified by the binding of a streptavidin-conjugated enzyme substrate. The tissue bound chains of antigen, primary antibody, secondary antibody, biotin, streptavidin and enzyme was visualized by the addition of a chromagen reacting with the enzyme and precipitating in the tissue at the site of the reaction. Control slides incubated with non-immune autologous serum (corresponding to the primary antibody host species) were run with each procedure to recognize and correct for any nonspecific immunoglobulin, streptavidin, biotin or chromagen staining.

Slides were heated in a dry oven for 30 minutes at 65°C and stored at room temperature until use. For immunohistochemical staining the sections

were deparaffinized in xylene (3 x 5 min.) and step rehydrated with 100 (3 x 3 min.), 95, 90, 80 and 70 % ethanol (1 x 3 min.) and PBS (2 x 3 min.) at room temperature. Tissue sections were pretreated with 3% hydrogen peroxide in 50% methanol for 10 minutes at room temperature (RT), and rinsed with PBS (3x 5 min. RT), prior to pretreatment with normal goat serum (Biogenex, San Ramon, CA) for 10 minutes at RT to decrease nonspecific binding of the primary antibodies.

Sections to be stained for the presence of PCNA were subjected to antigen retrieval procedures prior to the protein block. Antigen unmasking or tissue permeabilization by various methods has been shown to be effective in immunohistochemical procedures for a variety of antigens[159]. Citra buffer antigen retrieval (Biogenex) was modified from the manufacturer's recommended protocol for improved stain quality and to decrease tissue loss during the procedure. The slides were transferred from PBS to a Citra buffer solution (Biogenex), microwave heated to 199°F for 10 minutes and allowed to cool to room temperature (5 - 10 minutes). The slides were rinsed in PBS (2 x 3 min.), marked with a PAP pen (Binding Site, San Diego CA), and incubated in 4N HCl for 30 minutes at room temperature. The slides were rinsed in PBS (2 x 3 min.) and then incubated with 3% hydrogen peroxide for 10 minutes at room temperature. After rinsing in PBS, the slides were incubated with the protein block as described above.

After incubation with the protein block, the block was removed and the appropriate primary antibodies were applied without an intervening PBS rinse. The slides were incubated in a humidified chamber with the primary antibodies for 18 hours at 4°C. Commercially available primary antibodies

used in this study were PDGF-R α , PDGF-R β , vimentin, and PCNA. The PDGF-R α , PDGF-R β antibodies (Upstate Biotechnology Inc., Lake Placid, NY) were polyclonal rabbit antibodies recommended for use in Western blot analysis and immunoprecipitation. It was necessary to optimize immunohistochemical procedures for the PDGF receptor antibodies through repeated serial dilutions of the antibodies at multiple incubation times and temperatures. The most satisfactory conditions were determined to be a 1:500 dilution of the supplied concentration. The prediluted vimentin (V9) and PCNA (P10) monoclonal mouse antibodies (Biogenex) were applied at the supplied concentration.

After incubation with the primary antibodies (or negative control sera), the slides were rinsed (2 x 3 min.) in PBS, and biotinylated secondary (or linking) antibody was applied for 20 minutes (RT). After rinsing with PBS, the strepavidin-enzyme (or labeling) solution was applied for 20 minutes (RT). For the slides on which either of the PDGF receptors or the vimentin antibodies were used the labeling solution had strepavidin conjugated to calf intestinal alkaline phosphatase. A horseradish peroxidase conjugated strepavidin-label was used on the slides receiving the PCNA as the primary antibody. The slides were rinsed and the chromagen solution was then applied. Progression of the color development was monitored by microscopy at 5X magnification. Development was compared to the negative controls and was stopped by immersion in distilled water. Typical development times were 2-3 minutes for PCNA; 3-5 minutes for PDGF-R β ; 5-7 minutes for PDGF-R α ; and 2-3 minutes for vimentin. The brown chromagen diaminobenzadine (DAB) (Sigma, St. Louis, MO) was used for the slides

labeled with HRP and the red chromagen Vector Red (Vector Laboratories) was employed on slides labeled by AP. The slides were counter stained with toluidine blue or Harris' hematoxylin, dehydrated through a step alcohol gradient (reverse of above step rehydration) and xylene, and cover slipped.

The positive tissue control used for the PDGF receptor α stains was archival formalin fixed human prostatic carcinoma obtained from UTMCK Department of Pathology. {Color Plates: photomicrographs 2.1-2.2} The PCNA positive control was human intestinal crypt epithelium. Arterioles and endometrial epithelium served as internal positive controls for PDGF-R β [87, 160, 161] Neutralization of positive staining for PDGF-R β in the tissue sections via pre-incubation of the PDGF-R β antibody (1:1000) with 10 mM receptor binding protein (Upstate Biotechnology Inc.) protein was used to show antibody specificity. {Color Plates: photomicrographs 2.3-2.8} Replacement of each of the antibodies employed in the immunostaining by non-immune normal sera (Biogenex) was used for negative controls for the tissues examined.

Slides were evaluated by light microscopy. The uterine sections were examined by the following compartments: luminal epithelium; superficial glands; deep glands; superficial stroma, deep stroma muscle and vessels. The intensity of the staining for the PDGF receptor(s) and vimentin antibodies in each compartment was graded on a scale of 0-3. No staining was graded as 0, mild staining as 1, moderate staining as 2, and strong staining as 3. Multiple fields were examined in each segment to insure consistency of the grading. In segments with variable or localized staining, an average intensity was assigned for each compartment. PCNA staining was evaluated on either

a positive or negative basis. A percentage of PCNA positive cells per total cells (100 cells minimum) was derived for each tissue compartment.

Pearson correlation analysis was employed to measure the relationship of the mean intensity (or positivity for PCNA) of the staining patterns between the various antibodies. Correlation can be used to determine whether two data sets move together. Briefly, correlation analysis allows the comparison of two independent variables scaled independent of unit of measurement. An r value (which has no units), ranging from -1.0 to 1.0 is calculated based on the covariance of the data sets divided by the product of their standard deviations. If large values of one set tend to accompany large values of the comparison set then the r value (correlation) is positive. When the small values of one set accompany large values of the other set the r value (correlation) is negative. If there is a weak correlation between the sets the r values tend toward zero. In the program used for these calculations (Microsoft Excel 4.0 for Macintosh, Microsoft Corporation, Redmond WA) alpha values and p values were defaulted to specified (>0.05) values. (P values of 0.05 or less were confirmed by Student's T-test: two sample assuming unequal variances which also generated a Pearson correlation calculation for the data sets.)

Results

PCNA immunoreactivity

The greatest positive staining for the PCNA antibody was present during the proliferative phase for almost all tissue compartments (as listed above) evaluated. {Color Plate 2: photomicrograph 2.16 } The exception to

this was in the myometrium, in which the secretory phase had the greatest percentage of PCNA positive nuclei. Surprisingly, the luminal epithelium of the inactive endometrium had virtually the same degree of positive staining for PCNA as the proliferative phase luminal epithelium. Within the stroma (deep and superficial) there was only a minimal reduction in the percentage of PCNA positive nuclei in the secretory phase compared with the proliferative phase. (Table 2.1)

PDGF-R α immunoreactivity

PDGF-R α staining was variable in localization in the cells. {Color Plate 2: photomicrographs 2.12, 2.20, 2.28}. In the luminal epithelium and the majority of the superficial glandular epithelium, staining was predominantly in the apical cytoplasm. In the deep glandular epithelium, the staining was more diffuse in the cytoplasm. Staining of the endometrial stroma (except for inflammatory cells) was noted primarily in fine stromal cell cytoplasmic extensions. Staining for the PDGF-R α receptor antibody was weak in the luminal epithelium and superficial glands of proliferative and secretory phase endometrium, was not detectable in the luminal epithelium of inactive endometrium and was very weak in the glandular epithelium. The deep glands of the proliferative phase sections stained moderately. Mild staining was observed in the deep glands of the secretory phase sections. Proliferative and inactive endometrium had more intense (mild to moderate, respectively) stromal staining in the superficial portion. Mild to moderate staining intensity of the deep stroma was greatest in the secretory endometrial stromal compartment. Staining of arteriolar PDGF-R α was

consistently strong in all specimens. Staining of the myometrium varied from mild in the inactive uteri, to moderate in the proliferative and secretory phase uteri. (Table 2.2)

PDGF-R β immunoreactivity

Cellular localization of PDGF-R β was similar to that observed with PDGF-R α . {Color Plate 2: photomicrographs 2.11, 2.19, 2.27} PDGF-R β staining of the epithelium of proliferative stage endometrium was moderate in the cytoplasm of the deep glands, mild in the luminal lining and weak in the superficial glands. Secretory endometrium had weak luminal, and mild glandular staining. Mild staining was present in all epithelial compartments in inactive endometrium, but decreased in intensity from the luminal epithelium to the deep glands. The endometrial stromal staining pattern for PDGF-R β was similar to that observed with PDGF-R α . The superficial stroma stained more intensely in the proliferative and inactive endometrium. The reverse staining pattern occurred in the secretory endometrial stroma. Endometrial stromal staining for PDGF -R β was strongest in the inactive uteri and weakest in the proliferative uteri. Arteriolar staining was strong in all sections. The uterine smooth muscle stained mildly for PDGF-R β in the proliferative and secretory phases, and weakly in the sections with inactive endometrium. (Table 2.3)

Vimentin immunoreactivity

Staining for vimentin was undetectable in the luminal epithelium of the proliferative and secretory endometrium. {Color Plate 2: photomicrographs

2.13, 2.22, 2.23 } Very weak staining was seen in the luminal epithelium of inactive endometrium. {Color Plate 2: photomicrograph 2.32}, and weak staining was present in the superficial glands. The deep glands of all endometrial phases stained moderately for vimentin. Superficial endometrial stroma of the proliferative and secretory phase endometrium had mild staining. No detectable vimentin staining was noted in the superficial stroma of the inactive uteri. Staining intensity of the deep stroma was considered weak to mild in all sections, with the inactive endometrial stroma having the strongest positive staining. Staining was robust in the arterioles of all sections and very weak to mild in the myometrial compartment. (Table 2.4)

Comparison of intensity and localization of antibody immunoreactivity

There was a strong and consistent association between the percent of PCNA-positive cells and PDGF-R β staining intensity in each of the epithelial compartments: luminal ($r=0.98$), superficial glandular ($r=0.68$) and deep glandular ($r=0.76$). (Figures: 2.1, 2.2 & 2.3) PDGF-R α had a weakly negative correlation with PCNA in the luminal and superficial glandular epithelium, but a strongly positive association between the percent of PCNA positive cells and the staining intensity for PDGF-R α in the deep glands ($r=0.96$). Expression of the PDGF-R α and β also correlated strongly in the deep glands ($r=0.89$). Photomicrographs 2.5 & 2.16 demonstrate the co-localization of vimentin and PDGF-R β in the deep glands. There was no correlation between vimentin intermediate filament staining and PCNA in the glandular epithelium ($r=0.2$) and only a mild positive correlation in the luminal epithelium ($r=0.5$).

In the stromal compartment, only the staining intensity of PDGF receptors exhibited a strong positive correlation ($r=0.97$). There was weak positive correlation of the percentage of PCNA-positive nuclei and vimentin intensity ($r=0.32$). Correlation between the expression of the PDGF receptor α and PCNA, and PDGF-R β and PCNA ($r = -0.15$; $r = -0.32$, respectively), and the PDGF receptors α and β , and vimentin ($r = -0.62$; $r = -0.54$, respectively) were all weakly to moderately negative. (Table 2.5, 2.6 & 2.7)

When the comparisons were limited to proliferative and secretory stages, a positive shift occurred in the association of immunoreactivity between PCNA and PDGF-R β , vimentin and the PDGF receptors, and vimentin and PCNA, in the endometrial epithelial compartments (when compared to that seen in proliferative, secretory and inactive endometrium). Association of PDGF-R α to PCNA in the endometrial epithelium declined slightly. A mild negative shift in the association between immunoreactivity of all the antibodies occurred in the endometrial stromal compartment when only proliferative and secretory phase endometrium were compared.

Discussion

These findings supported and extended earlier findings that PDGF α and β receptors are present in the uterus [20, 87, 132]. Distribution of the PDGF receptors in the uterus was widespread and varied in each compartment with the stage of the menstrual cycle. Immunoreactivity to PDGF receptors in the endometrial epithelium was associated with replicating cell populations (as indicated by PCNA staining) and the morphological criteria for proliferative phase endometrium.

The epithelial and stromal distribution of immunostaining for PDGF-R β partially agrees with that observed previously by Chegini *et al.*, who reported the strongest endometrial immunostaining in the proliferative phase with a considerable reduction in secretory phase luminal epithelium and stroma[87]. A similar reduction in immunohistochemical staining intensity was observed in this study between proliferative and secretory luminal and deep glandular epithelium. In contrast to Chegini's observation, these investigations found negligible reduction of superficial endometrial stromal staining and increased staining in the deeper portions (stratum basalis) of the stroma. This discrepancy may be attributable to the different tissue fixation methods employed. Quite remarkable differences in immunohistochemical staining quality and intensity may be seen between tissues fixed in Bouin's solution (employed by Chegini) and other fixatives (e.g., formalin) [162]. Differences in the antibodies employed in this study (rabbit, polyclonal, anti-human PDGF-R α and PDGF-R β) vs Chegini's study (monoclonal anti-human PDGFR- α and β and antiporcine PDGF-R β) may also have affected the results. Even within Chegini's study, differences were noted between the staining intensity of the antibodies to human and porcine PDGF-R β [87].

A staining pattern similar in distribution and relative intensity was noted for both the PDGF receptors antibodies used in this study. The one divergence of receptor α staining from receptor β staining occurred in the luminal epithelium of inactive uteri in which there was a nearly complete absence of positive staining for PDGF-R α .

The vimentin staining observed in the endometrium confirms previous findings on vimentin intermediate filament expression in endometrial epithelial cells.[72]. The predominance of the immunoreactivity in the deep glands and the consistency of the staining between the difference menstrual phase specimens is of interest. Indeed in the proliferative and secretory phases all sections had the most intense staining for all antibodies in the deep glands. Both the consistent intensity of the stain and its localization to the deep glands may reflect a relative lack of differentiation in the cells and the high percentage of stem cells in this region regardless of the stage of menstrual cycle or predominant hormonal milieu[163]. The increase in staining intensity within the stroma from the proliferative to secretory phase is consistent with Nisolle, et al's findings.

Numerous methodologies are available to measure cell proliferation in tissue: Mitotic indices, [^3H] thymidine radioautohistography, antibody staining for incorporation of bromo-deoxyuridine (BrdU), antibody staining PCNA or Ki67, and silver staining for nucleolar organizing regions. PCNA, a cell cycle associated protein (cyclin D), that is expressed during the G1 through S phase of the cell cycle, was chosen because of its staining consistency and high correlation to other methods. PCNA has been deemed the most reliable of the proliferation markers for use in formalin fixed tissues[164] to have a strong correlation with the mitotic index[165] and to exhibit a linear relation to radioautohistographic labeling in formalin fixed tissue[166]. Although the degree of PCNA labeling may vary, depending on the fixative used, double labeling studies have shown a linear relationship with ^3H -thymidine labeling in formalin-fixed tissue[167].

Because attempts at double immunohistochemical staining were rarely successful, the number of tissue sections stained was large and precise matching of serial sections was difficult; multiple sections from a minimum of three samples in each category were used to compare patterns of antibody immunoreactivity. The mean staining intensities of the immunoreactivity for each antibody used (PDGF-R α , PDGF-R β , PCNA and vimentin) were compared to one another. Correlation analysis allows comparison of relationships between two data sets that have independent or dissimilar units of measure. As such, it can be used to determine if the changes in the staining intensity of the various antibodies and percentages of PCNA-positive cells in the endometrium change in parallel during the menstrual cycle.

Review of the correlation analysis revealed a number of trends. There was strong association of the intensity of the PDGF-R β with the percentage of PCNA positive nuclei in each compartment of the epithelium in analysis of proliferative, secretory and inactive endometrium. (Table 2.5) This implies a relationship between endometrial epithelial PDGF-R β expression and cell proliferation. Within the endometrium, proliferation is associated with increased estrogen levels and the persistence of estrogen receptors correlates with increased mitotic activity in the epithelial cells of the basal zone of the endometrium[163].

Numerous growth factors and their receptors have been implicated in mitogenic and differential pathways in the endometrium[78]. PDGF/PDGF-R interaction may serve as a common pathway for numerous hormones and cytokines[20]. PDGF is considered a competence factor allowing cells to enter and move through the G1 phase of the cell cycle[89]. PCNA is initially

expressed in the G1 phase. PDGF has been shown to upregulate PCNA mRNA expression compared to G0 phase levels[168]. The strong positive correlation of PDGF-R β and PCNA, and the in vitro DES / PDGF AB findings (Part II, chapter 1) supports the hypothesis that PDGF / PDGF receptor interaction mediates estrogen activity in the endometrium.

No other strong association with proliferation (as measured by PCNA expression) in the proliferative, secretory and inactive endometrium was noted in each separate compartment. However, the correlation of PDGF-R α to overall epithelial proliferation was moderately strong (0.76). The near complete lack of staining for PDGF-R α in the epithelium of inactive uteri (Table 2.2), which presumptively lack any estrogenic stimulation implies that PDGF-R α expression may also be related to estrogen levels. A similar trend was also seen in the relationship of vimentin expression to PDGF-R α and to PCNA. When the inactive uteri were removed from the correlation analysis there was a moderate positive correlation between vimentin and PCNA, and a strong positive correlation between vimentin and PDGF-R α . The acknowledged (although atypical for epithelial cell lineage) coexpression of cytokeratin and vimentin by endometrial epithelial cells is presumptively due to their embryonic mesodermal / Mullerian derivation. Cytoskeletal intermediate filaments play a role in the interaction of the cell with its surrounding environment and are associated with expression of cell surface adhesion molecules. Epithelial coexpression of the cytokeratin and vimentin intermediate filaments in mammary neoplasia correlated to increased proliferative and invasive ability of the neoplastic epithelial cells[70]. The endometrium routinely undergoes cyclic proliferation and remodeling.

Vimentin expression in the endometrial epithelium may reflect the proliferative readiness of these cells and / or serve an instructive role in cyclic endometrial remodeling.

Within the stroma of all specimens there was no apparent correlation between either of the PDGF receptors staining and the PCNA staining. When the inactive uteri were excluded there was moderate negative correlation between PDGF-R β and PCNA (-0.67) and a mild negative correlation between PDGF-R α and PCNA (-0.40). This suggests a cyclic hormonal relationship, albeit an inverse relationship to that observed in the epithelial component. This finding would seem to imply that the apparent mediation of estrogen driven proliferation by PDGF in the epithelial cells cannot be extended to the stromal compartment. However, complicating factors such as lymphoid proliferation (which could not be differentiated from stromal cell proliferation in this study) in the stromal compartment during the secretory phase[169] may render the observed stromal cyclical relationship less meaningful.

The strong correlation of PDGF-R α & β in the stroma and the deep glandular epithelium imply a similar regulating pathway for their expression in those compartments. The expression of the PDGF-R β in the luminal epithelium of the inactive endometrium suggests that other signaling pathways or factors besides the ovarian steroids may be contributing to the regulation of their expression. Those other influences may be more pronounced in the inactive uteri compared to the proliferative and secretory uteri given the lack of regular cyclic hormonal activity in the inactive endometrium.

In summary, this study demonstrated PDGF-R α in the endometrial epithelium of proliferative, secretory and inactive endometrium. Staining intensity for the PDGF receptors correlated with the proliferative state of the endometrium. The association of PDGF receptor immunoreactivity to the proliferative state of the endometrium suggested that PDGF may be involved in the endometrial proliferation initiated by the cyclical hormonal milieu in the uterus. The expression of vimentin by endometrial epithelial cells was associated with proliferation and PDGF receptor expression in the proliferative and secretory endometrium. Whether this relationship indicates common regulatory pathways or is a coincidental state of these cells during periods of proliferation remains to be elucidated.

Identification of PDGF-R in endometrial hyperplasia, endometrial carcinoma and endometriosis.

Introduction

Uterine hyperplasia, endometrial adenocarcinoma and endometriosis represent disorders of uterine growth affecting the health and reproductive status of millions of women. These diseases involve endometrial epithelial elements that have escaped the regulatory mechanisms controlling their normal growth (carcinoma), are subject to deranged or excessive hormonal stimulation (hyperplasia), or are ectopic (endometriosis). The *in vitro* proliferative response of human endometrial epithelial cells to the PDGF isoforms[73]; the affect of synthetic estrogens on PDGF receptor mRNA and protein expression in the uterus of immature mice[20]; isolation of PDGF its receptor mRNA from porcine uterine tissue[132, 137]; immunohistochemical localization of PDGF-R β in the human and mouse endometrium[20, 87] and the positive correlation of PCNA to the sites of PDGF receptors expression (chapter 2) all imply a role for PDGF in the cyclical growth of the uterus and endometrial epithelium.

PDGF B has very close homology to the transforming viral oncogene, *v-sis* [170]. There is evidence for intracellular PDGF ligand / receptor binding as a possible autocrine pathway[116, 171]. PDGF may also function via paracrine pathways[90]. PDGF B has been localized to endometrial stromal

and epithelial cells[87]. Changes in the degree or localization of expression of PDGF receptors may affect the growth characteristics of the endometrial epithelium in hyperproliferative and ectopic conditions.

Localization of vimentin to the more proliferative compartments of the endometrial epithelium suggests that its expression may be related to the proliferative state of those compartments. If this is the case, then vimentin expression may impact the growth and responsiveness of the epithelial component of hyperproliferative and ectopic endometrial diseases.

Although coexpression of vimentin and cytokeratin appears to be the normal state in at least some subsets of endometrial epithelial cells, there are suggestions that in some other epithelial malignancies, coexpression of these intermediate filaments may result in increased invasiveness and proliferative capacity[70]. This may reflect a lack of differentiation or an interconversion between cell types. Correlation of the site of the PDGF receptor expression with vimentin expression and proliferation in hyperproliferative lesions could confirm or refute these implications and extend the knowledge of the potential role of PDGF in these diseases.

Experimental overview

The aim of this study was to examine PDGF receptor expression in ectopic, hyperplastic, and neoplastic endometrial tissue, and to further examine the relationship of the expression of PDGF receptors to the proliferative state of those conditions. Immunohistochemical staining for PDGF-R α , PDGF-R β , PCNA, and vimentin was performed on tissue sections of cystic hyperplasia, adenomatous hyperplasia, endometrial carcinoma and

endometriosis from women. Expression of PDGF-R α , PDGF-R β , PCNA and vimentin was evaluated in regard to site and intensity of staining. Intensity and localization of PDGF-R α , PDGF-R β , PCNA and vimentin staining in the hyperplastic tissue were compared to that in normal proliferative and secretory phase endometrium.

Materials and methods

Archival blocks of formalin-fixed, paraffin-embedded tissue were obtained from the Department of Pathology at the University of Tennessee Medical Center, Knoxville under UTMCK Institutional Review Board protocol #0291. The specimens had been diagnosed previously (confirmed by Dr. S. Van Meter, UTMCK, Dept. Pathology) as endometrial carcinoma (3), adenomatous hyperplasia (4), cystic hyperplasia (4), and endometriosis (5) {Color Plate 3: photomicrographs 3.1, 3.9, 3.17, 3.25, 3.31, 3.39}. Additionally, a single case of cystic hyperplasia fixed in 4% paraformaldehyde (4°C) for 24 hr. and then transferred to 70 % methanol (4°C) was available from the previous study. Tissue sections were cut at 4-6 μ m thickness, and mounted on Probe-on slides (Fisher) . The slides were heated in a dry oven for 30 minutes at 65°C to optimize tissue / slide adhesion and stored at room temperature until use. The same protocols were used for immunohistochemical staining as were employed in the previous section (Part II, chapter 1) The commercially available primary antibodies used in this study were PDGF-R α (Upstate Biotechnology Inc.), PDGF-R β (Upstate Biotechnology Inc.), vimentin (Biogenex Corp.), and PCNA (Biogenex Corp.). Negative controls included incubation of tissues

with a non-immune serum dilution (Biogenex Corp.) and were performed with each procedure. {Color Plate 3: photomicrographs 3.2, 3.10, 3.18, 3.26, 3.34, 3.40}

Slides were evaluated by light microscopy. The intensity of the staining for PDGF receptors was graded on a scale of 0-3. No staining was graded as 0, mild staining as 1, moderate staining as 2, and strong staining as 3. Multiple fields were examined in each segment to insure consistency of the staining. In segments with variable or localized staining an average intensity was assigned. PCNA staining was evaluated on a positive or negative basis. A percentage of PCNA positive cells per total cells (100 cells minimum) was derived for each tissue compartment: luminal epithelium, superficial glandular epithelium, deep glandular epithelium, hyperplastic glandular epithelium, superficial stroma, deep stroma, neoplastic epithelium, and endometriotic epithelium.

To determine if PDGF receptor α or β , or vimentin expression was related to sites of proliferation, Pearson correlation analysis (as described in chapter 1) was employed to measure the relationship of the mean intensity of the staining patterns of PDGF-R α , PDGF- β and vimentin and the percentage of PCNA positive cells.

Results

Cystic hyperplasia

In cases of cystic hyperplasia, the epithelium had the highest percentage of positive nuclei in the deep and cystic glands. The percentage of positive cells declined in the glands as they neared the luminal surface.

Staining for PCNA was slightly more pronounced in the deep (basalis) stromal compartment than in the epithelium. {Color Plate 3: photomicrograph 3.8 } PDGF-R α stained weakly in the luminal and superficial glandular epithelium. Deep glands had the most intense PDGF-R α staining in the endometrium.{Color Plate 3: photomicrographs 3.3-3.5 } Cystic glands stained for PDGF-R α at an intermediate intensity between the deep and superficial glands. The stromal compartment had mild staining with a mild increase in intensity in the deep (or basalis) portion. Staining for PDGF-R β antibody was almost identical to PDGF-R α in both the epithelial and stromal compartments.{Color Plate 3: photomicrographs 3.4-3.6 } Vimentin staining exhibited a similar pattern of staining intensity in the epithelium as the PDGF receptors. {Color Plate 3: photomicrograph 3.7 } The stromal pattern for vimentin was reversed compared to the PDGF-receptors with the strongest staining occurring in the more superficial (functionalis) portion of the stroma. (Tables: 3.1, 3.2, 3.3 & 3.4)

Adenomatous hyperplasia

PCNA staining of the adenomatous hyperplasia cases had the greatest percentage of positive nuclei in the deep glands and adenomatous glands, slightly reduced in the superficial glands, and a moderately reduced percentage of positive nuclei in the luminal epithelium. {Color Plate 3: photomicrograph 3.14} The stromal compartment had approximately half the positive nuclei as the deep glands. Staining for PDGF-R α antibody was negligible in the luminal epithelium, weak in the superficial glands and mild in the deep and adenomatous glands. {Color Plate 3: photomicrograph 3.11-

3.12)} Stromal staining was mild to moderate, more pronounced than the epithelial staining, and more intense in the deep stroma. The luminal epithelium had weak PDGF-R β staining. {Color Plate 3: photomicrograph

3.13} Staining was mild in the superficial and adenomatous glands, and moderate in the deep glands. Although weaker, the stroma had a PDGF- R β staining pattern similar to that seen with PDGF-R α (weak to mild in the superficial stroma and mild in the deep stroma). Vimentin antibody staining intensity mirrored that seen with PDGF-R β . (Tables: 3.1, 3.2, 3.3 & 3.4) {Color Plate 3: photomicrographs 3.15-3.16}

Endometrial carcinoma

The three cases of endometrial adenocarcinoma examined in this investigation were well differentiated. All had accompanying stromal or epithelial hyperplasia of adjacent uterine tissue. One of the carcinomas had squamous metaplasia. Staining for each of the proteins could be seen in all of the sections, ranging from diffuse to intermittent and scattered. {Color Plate 3: photomicrographs 3.19-3.30} Some areas exhibited high rates of proliferation, as measured by PCNA (>50%), while other areas were quiescent (<10%). PCNA labeling was not greater than the hyperplastic deep glands. Consistent, mild to moderate staining for PDGF-R α & β was present within two of the carcinomas. There was considerable variation of staining intensity for PDGF receptors within one carcinoma. Occasional areas were present in which the staining for only one of the receptors was discernible. Vimentin staining was strong in most areas of neoplastic growth. The strongest staining for all the antibodies (PCNA, PDGF receptors and

vimentin) was present at the leading edges of invasive foci and at the outer edges of centrally necrotic foci. These areas consistently stained at least as intensely as deep glands in adjacent hyperplastic tissue. Focal areas of squamous metaplasia did not stain for either of the PDGF receptors or vimentin, and had low percentages of PCNA-positive nuclei.

Endometriosis

Of the five endometriotic samples examined in this investigation, two were ovarian, one was uterine, one was mesenteric, and one was colonic in location. The normal ovarian morphology was partially disrupted in all the cases of ovarian endometriosis. No active follicles were present in the sections examined. Corpora albicans and atretic corpora lutea were present within the hilar region. There was mild to moderate, focal decidualization of the stroma of the endometriotic lesions within those specimens. Multifocal, mild to moderate hemorrhage and moderately hemosiderin pigment-laden histiocytes were present in the stroma. There was moderate endometriotic glandular cystic expansion with luminal cellular debris and flattened to cuboidal epithelium. The majority of the endometriotic epithelium and stroma had few PCNA positive nuclei (<5%), although occasional glands and foci had up to 30-40 % positive nuclei in the epithelium. PDGF-R β , vimentin and PDGF-R α staining was absent from the majority of endometriotic epithelium with the exception of the focal areas with high percentages of PCNA positive cells. Ovarian endometriotic stroma had mild to no staining for the PDGF receptors or vimentin. {Color Plate 3: photomicrographs 3.33-3.38}

In the uterine specimen there was invasion of the muscular wall from the mesothelial aspect to a depth of approximately 350 μm . The endometriotic lesions were composed of compact stroma surrounding mildly cystic to minimally branched coiled glands with cuboidal to columnar epithelium. Epithelial nuclei were basal to pseudo-pallisading to central with occasional sub nuclear vacuoles . PCNA staining was moderate (35 %), and evenly distributed in the epithelium and the stroma. Vimentin staining was moderate in the epithelium, and mild to negligible in the stroma. Immunoreactivity for both PDGF receptors was mild and diffuse in the epithelium and not detectable in the stroma.

The mesenteric endometriotic lesion had multiple, markedly cystic glands lined by low cuboidal epithelium with compact stroma. PCNA staining varied considerably within the endometriotic epithelium. Vimentin was almost exclusively limited to mild staining of the stroma. PDGF-R α & β were minimally present in the epithelium. A single focal area (< 5 % of total) of relation between PDGF receptors and vimentin occurred in the epithelium.

{Color Plate 3: photomicrographs 3.41-3.46}

The case of colonic endometriosis had mural invasion by endometriotic glandular and stromal components with minimal reactive fibrosis. PCNA positive nuclei were evenly and moderately distributed in both the stroma and the epithelium. PDGF-R β staining was mild to moderate in the endometriotic stroma. Epithelial staining was mild to moderate and intermittent, but more consistent and pronounced in cuboidal than flattened cells. Intensity of the stain appeared to be inversely proportional to the size

of the glandular lumen. PDGF-R α staining was minimal and confined exclusively to the epithelium of the smallest glands and subjacent stroma.

Correlation of immunoreactivity in hyperplasia

There was strong correlation between the presence of positive PCNA nuclei and the intensity of staining for both PDGF-R α ($r=0.89$) and PDGF-R β ($r=0.87$) in the epithelium compartment of specimens of adenomatous hyperplasia. Similarly, strong relationships were observed in the epithelium between vimentin and PDGF-R α ($r=0.93$), and vimentin and PDGF-R β ($r=0.86$). Correlation of PCNA percentages to vimentin were moderately positive ($r=0.69$). Expression of the PDGF receptors closely coincided ($r=0.96$). (Figure 3.1)

In the cases of cystic hyperplasia similar, though less pronounced, trends were noted in the epithelium between PCNA and PDGF-R α ($r=0.79$), PDGF-R β ($r=0.63$), and vimentin ($r=0.56$). The strongest correlations were between the PDGF receptors ($r=0.94$), PDGF-R β and vimentin ($r=0.99$), and PDGF-R α and vimentin ($r=0.89$). (Figure 3.2)

The rate of proliferation as measured by the percentage of PCNA antibody-positive endometrial epithelial cells in hyperplastic endometrium was less than in normal proliferative phase uteri. Evaluation of the distribution of the PCNA positive nuclei within the non-lesional epithelial and stromal endometrial compartments showed a strong correlation of PCNA compartmental localization between adenomatous and cystic hyperplasia ($r=0.96$), adenomatous hyperplasia and normal proliferative uteri ($r=0.97$), and cystic and proliferative hyperplasia ($r=0.99$). Strong positive correlation

was present between all the antibodies examined in the epithelial compartment of the adenomatous, cystic and proliferative uteri. (Figures 3.3-3.6). Only PDGF-R α showed a slightly stronger positive correlation between the hyperplastic and secretory uteri than between the hyperplastic and proliferative uteri in the epithelial compartment.

Discussion

This study demonstrated that PDGF receptors could be identified by immunohistochemistry in the epithelial component of endometrial carcinoma, endometrial hyperplasia and endometriosis. In light of the immunohistochemical localization of PDGF receptors in the normal cycling endometrium (Chapter 2), and the reported distribution of PDGF-R β in the endometrium[87] this is not an unexpected finding.

The steroid-responsiveness of endometrial carcinomas, endometrial hyperplasia, and endometriosis is well established[37, 38]. The epigenetic, tumorigenic and hyperproliferative effects of steroids are thought to be mediated through their modulation of oncogene / peptide growth factor pathways[80]. A variety of oncogenes which are regulated by steroid hormones have been identified in normal and pathologic endometrial tissue[24, 81, 172]. Numerous neoplasias have been reported to express PDGF, PDGF receptors or both[125, 127, 130, 173, 174], however the finding of PDGF receptors in endometrial carcinomas has not been previously reported. The close homology of PDGF B to the v-sis (simian sarcoma viral oncogene), and the finding of intracellular co-localization of the PDGF ligand and receptor has led to speculation that over expression or derangement of

the PDGF signaling pathway may have a role in malignant transformation via autocrine mechanisms[101, 170, 175]. However, in the uterine carcinoma sections examined in this study, the intensity of staining for the PDGF receptors did not differ significantly from that observed in normal and hyperplastic uterine sections. Ligand-receptor co-localization and comparison of normal to neoplastic mRNA expression might resolve those concerns.

The expression of PDGF receptors and vimentin in areas of high proliferation (indicated by percentages of PCNA positive nuclei) is consistent with findings showing strong correlation between the receptors, vimentin, and PCNA-positivity in proliferative uteri (see chapter 1). The degree of variation of receptor expression among regions of the tumors may be indicative of subclonal populations of neoplastic cells that have lost some cell regulatory mechanisms. Lack of PDGF receptors, vimentin expression, and decreased numbers of proliferating cells in areas of squamous metaplasia is suggestive of a relationship between their expression and terminal differentiation. The similarity of intensity of staining for PDGF receptors and vimentin in the neoplastic cells and the deep glands in the hyperplastic areas of the sections may be indirect evidence of estrogen responsiveness.

Immunohistochemical staining revealed the presence of receptors for PDGF in endometriotic epithelium and stroma irregardless of anatomic location. However, their expression was less (both in distribution and intensity) than that found in normal, hyperplastic and neoplastic endometrial epithelium. Consistent coexpression of high percentages of PCNA positive nuclei with PDGF receptors and / or vimentin was not apparent in the same

areas, although epithelial vimentin and PDGF receptors did appear strongly associated. Compact glands with cuboidal epithelium, which were reminiscent of proliferative phase glands, had the greatest (although still limited) degree of staining for all antibodies. The diminished epithelial cell staining for vimentin in the endometriotic lesions compared to its expression in eutopic endometrial epithelium is consistent with Nisolle's finding of stronger vimentin staining in eutopic endometrial epithelium than in endometriotic epithelium [72].

With the exception of the colonic lesion, endometriotic foci in each specimen had stromal decidual characteristics suggestive of progestational dominance. These observations are not inconsistent with the prior findings of stronger staining for PDGF -R β in proliferative (estrogen-dominated), normal endometrial epithelium and compared with the staining of the glandular epithelium in secretory (progestational) epithelium. (Chapter 2) The apparent progestational status of the lesions might explain the generalized weak staining in the majority of the endometriotic tissues. Unfortunately, the endocrine status of the women from whom the limited number of specimens in this study were procured was not available. Further examinations between the relationship of estrogenic dominance and PDGF receptor expression in endometriotic epithelial cells, will require knowledge of both the endocrine status and estrogen receptor expression of the endometriotic epithelium.

Although morphologically dissimilar, adenomatous and cystic hyperplasia are both hyperproliferative conditions related to prolonged estrogen dominance. Foci of both cystic and adenomatous hyperplasia may be present concurrently within the same uterus[8]. The staining for the PDGF

receptors, vimentin and PCNA in the hyperplastic endometrium was consistent with those in proliferative uteri. Although staining intensity was somewhat diminished, strong correlation between the hyperplastic and proliferative epithelium staining distribution suggest common regulatory factors. Diminished staining intensity in the hyperplastic endometrium compared to the proliferative endometrium may reflect a down regulation of the PDGF receptors secondary to chronic estrogen exposure or inhibition by other factors.

In summary, immunohistochemical techniques were used to demonstrate the presence and distribution of both PDGF-R α , PDGF-R β and vimentin in neoplastic and hyperplastic endometrium, and in endometriotic lesions. The positive staining observed in this investigation confirms and extends previous findings regarding the expression of the PDGF receptors and vimentin in normal cycling endometrium. The presence of PDGF receptors in hyperplastic, neoplastic and endometriotic endometrial epithelium partially mimics their expression in normal endometrium. The distributional relationships of staining and similar staining intensities in hyperplastic and proliferative endometrial epithelium suggest that PDGF may have a role in endometrial proliferation, and may imply that estrogen regulates the expression of PDGF receptors. Poor correlation of staining between the PDGF receptors with proliferation (PCNA) in the endometriotic lesions may reflect a loss of steroid receptors by the endometriotic epithelium, and thus loss of regulation of PDGF receptor expression by estrogen. From these limited studies PDGF receptor expression appears to be strongly associated with areas of epithelial proliferation in endometrial

hyperplasia and carcinoma. More extensive studies correlating patient endocrine status, and the expression of epithelial cell steroid receptors to PCNA, PDGF receptors and vimentin are needed to clarify these limited initial findings in endometriotic, hyperplastic and neoplastic lesions.

Chapter 4

Identification of platelet-derived growth factor receptors and their

association with proliferating cell nuclear antigen in the murine endometrium

Introduction

PDGF and PDGF receptors have been reported in the female reproductive tract of a number of mammalian species[20, 87, 96, 132, 135, 137]. Immunohistochemical localization of PDGF receptors and their correlation to the nuclear PCNA expression during the menstrual cycle of the human uterus raises the question of similar spatial and temporal expression of PDGF receptors in the uteri of other species. The findings of Gray, *et al* of increased uterine PDGF receptor mRNA expression in immature mice given diethylstilbestrol (DES) demonstrates a relationship between estrogens and PDGF receptor expression in immature murine uteri. These findings suggest the possibility that an estrous cycle-dependent expression of the receptors may occur in the adult cycling uterus of mice in a manner similar to that observed in human uteri. Comparison of expression of proliferation markers and PDGF receptor expression in the adult mouse endometrium would shed light on the possibility PDGF modulates endometrial epithelial growth during the estrous cycle.

Experimental overview

The purpose of this study was to examine the expression of PDGF receptors in the normal cycling uterus of mice, and to compare PDGF receptors expression with sites of proliferation in the endometrium. Further immunohistochemical staining for PDGF-R α , PDGF-R β , and PCNA were performed on the uteri of young adult, female mice during the normal stages of the estrous cycle. Cyclical endometrial immunoreactivity for antibodies to PDGF-R α , PDGF-R β and PCNA were observed. Expression of immunoreactivity for PCNA and the PDGF receptors was compared.

Materials and methods

Eighteen, 8 week old, 25 gram, female BALB-C mice were purchased from Charles River Laboratory (Jacksonville, FL) under an addendum to UT Animal Care and Use Committee (ACUC) protocol # 668. The mice were held in the UTCVM laboratory animal facility for a 7 day acclimation period in a 12 hour light - 12 hour dark cycle. Upon completion of the acclimation period each of the mice was given 5 IU of human chorionic gonadotropin by intraperitoneal injection (IP) followed 72 hours later by 5 IU of pregnant mare serum gonadotropin IP in order to synchronize the estrous cycles of the subjects[176]. The three subjects were sacrificed by cervical dislocation 27 hours later. Thereafter, paired subjects were sacrificed at twenty four hour intervals over the following eight days.

Upon sacrifice each mouse was examined for gross abnormalities of the abdomen or reproductive tract. The ovaries, uterus, vagina, spleen,

thymus (if identifiable). and multiple sections of the small intestine from each animal were removed within 5 minutes of euthanasia, and immersed in 4 % paraformaldehyde at 4°C overnight. Tissues were then transferred to 70 % ethanol for 24-48 hours. Organ segments from each of the collected tissues were then routinely processed by the UTCVM Department of Pathology histology laboratory and embedded in paraffin. Block sections were cut at 4-6 μ m and adhered to Probe On (Fisher) anion-charged glass slides. All slides were dry heated at 65°C for 30 minutes to insure adequate tissue adhesion to the slide.

Immunohistochemical staining for PDGF-R α , PDGF-R β and PCNA were performed twice on slides from each mouse. Rabbit polyclonal antibodies against mouse PDGF-R α and human PDGF-R β were obtained from Upstate Biotechnology Inc. (Lake Placid, NY), and diluted 1:500 with Biogenex (San Ramon, CA) common antibody diluent. Mouse-derived monoclonal antibody (P10) to the proliferating cell nuclear antigen (PCNA) was obtained from Biogenex and used at manufacturer's provided concentration.

The slides for each procedure were deparaffinized in xylene (3 x 5 min.) and step rehydrated by serial immersion in 100% ethanol (3 X 3 min.), 95%, 90%, 80%, and 70% ethanol (1 x 3 min. each), and phosphate buffered saline (PBS) for 2 x3 min. After rehydration, the slides to be examined for PDGF-R α or PDGF-R β localization were marked with a PAP pen to limit reagent loss, then incubated in a goat serum protein block (Biogenex) for 20 minutes at room temperature. The slides used for PCNA staining were subjected to antigen retrieval procedures prior to PAP pen marking and the

protein block. (Chapter 1, Materials and Methods) A commercially available biotin-streptavidin system (Biogenex) was used for all immunohistochemical procedures. (Chapter 1, Materials and Methods)

Slides were evaluated by light microscopy. The intensity of the staining for PDGF receptor was graded on a scale of 0-3. No staining was graded as 0; mild staining as 1; moderate staining as 2; and strong staining as 3. Multiple 20x fields were examined in each segment to insure consistency of the grading. In segments with variable or localized staining, an average intensity was assigned across fields for each of the following tissue compartments: luminal epithelium; glandular epithelium; endometrial stroma, and myometrium. PCNA staining was evaluated as either positive or negative. A percentage of PCNA positive cells per total cells (100 cells minimum) was derived for each of the tissue compartments. Sections of mouse small intestine, co-incubated with each slide, were used as positive controls for PCNA staining.

The uterine endometrium of each mouse was designated as proestrus, estrus, metestrus or diestrus stage of the estrous cycle according to the accepted histological characteristics of the vagina and uterus[176-178]. The proestrus vagina has superficial mucin cells, thick stratified squamous epithelium, cornifying stratum granulosum cells, active cell division, and occasional stromal leukocytes. The uterus has mild endometrial hyperemia and edema, glandular and luminal epithelial proliferation, and occasional leukocytic infiltrates . Estrus is characterized by decreased vaginal mitoses, thickened superficial cornified epithelial layer, some luminal cellular debris, and a paucity of leukocytes. The scarcity of leukocytes is also noted in the

edematous endometrium which has active glandular and luminal epithelial proliferation. Metestrus has increasing leukocyte infiltrates in the epithelium transmigrating luminally, cornified epithelium desquamation, decreasing layers of vaginal epithelium, and rare mitoses. The uterus has increasing leukocytic infiltrates with minimal glandular mitotic activity. In diestrus, there is rebuilding of the vaginal epithelial lining, with mitotic figures present in the basal epithelial layer, and a continued epithelial / luminal transmigration of leukocytes. Uterine stromal and glandular mitoses start to increase.

The relationship between the percentage of cells staining positively for PCNA and the average staining intensity of the cells for PDGF receptors was examined using correlation analysis of the means of the observed staining intensity in the luminal epithelium, glandular epithelium, and stroma.

Results

From a total of 18 mice, 3 were designated in diestrus, 6 in proestrus, 4 in estrus and 5 in metestrus. {Color Plate 4: photomicrographs 4.1, 4.9, 4.17, 4.25}. In cases where there was transition between stages of the cycle, the dominant phase was used to designate the stage. The stages of metestrus I and II were combined as a single grouping.

PCNA immunoreactivity

Positive nuclear staining for PCNA was present in each tissue compartment during all stages (Table 4.1) {Color Plate 4: photomicrographs 4.7-8, 4.15-16, 4.23-24 & 4.31-32}. The average nuclear staining for PCNA in the luminal epithelium was greatest during metestrus, estrus and proestrus

stages, and was markedly reduced during the diestrus stage. The percentage of PCNA-positive nuclei in the glandular epithelium was highest in the proestrus and estrus stages and was reduced during both the diestrus and metestrus stages. The stromal compartment had increased positive nuclear staining during diestrus in comparison with proestrus, estrus and metestrus stages. There was no significant change in PCNA-positive staining within the muscle compartment (25-36 %) among estrous stages. Vascular PCNA was most pronounced during diestrus.

Positive staining for both PDGF receptors was recognized in all of the compartments, at each stage of the estrus cycle. The staining within the tissue compartments varied from locally diffuse to focal nests of positive cells and scattered positive cells. Within the luminal epithelial cells the most consistent staining occurred near the glandular ostia. Neither length of the endometrial glands nor depth of endometrial stroma had any consistent impact on intensity of the PDGF receptor stains. The stroma subjacent to the glandular epithelium was the most consistently positive portion of the stromal compartment.

PDGF-R β immunoreactivity

Positive cytoplasmic staining for PDGF-R β was present in each tissue compartment during all stages {Color Plate 4: photomicrographs 4.3-4, 4.11-12, 4.19-20 & 4.27-28}. The luminal epithelium had consistent staining of the cytoplasm throughout the entire estrus cycle. Immunoreactivity for PDGF-R β was greater in the luminal epithelium than in either the glandular or stromal compartments in all stages (Table 4.2). PDGF-R- β staining of the

glandular epithelium was most intense in the proestrus and estrus-stage endometrium, and least intense in diestrus. Stromal staining for PDGF-R β was consistently weaker than the epithelial staining. The strongest stromal staining for PDGF-R β occurred during metestrus. Arterioles and muscle exhibited robust staining throughout the entire cycle (data not shown).

PDGF-R α immunoreactivity

Similar to PDGF-R β , positive staining for PDGF-R α was present in all the tissue compartments (Table 4.3) {Color Plate 4: photomicrographs 4.5-6, 4.13-14, 4.21-22 & 4.29-30}. In the luminal epithelium, the proestrus and estrus stages had the strongest, and metestrus stage the weakest staining for PDGF-R α . The luminal epithelial staining intensity was consistently, but not significantly greater than that of the glandular epithelium. PDGF-R α staining in the glandular epithelium was strongest during the estrus stage, and weakest during diestrus. Epithelial staining was more pronounced than stromal staining in the estrus and metestrus stages, but not in the diestrus or proestrus stages. Stromal staining was the most intense during diestrus, and then decreased throughout the remainder of the estrus cycle. Positive staining was strongly present in the stromal and muscular arterioles, and moderately positive in the myometrium throughout the estrous cycle

Comparison of localization and intensity of immunoreactivity

Over the course of the estrus cycle, there was no correlation ($r=0.17$) between PCNA positive staining and the intensity of the staining for PDGF-R α in the luminal epithelium. Expression of PDGF-R β was negatively

associated (-0.58) with the degree of proliferation (as measured by PCNA). The association between the expression of PDGF-R β and PDGF-R α was also negative ($r=-0.33$) (Figure 4.1)

In the glandular epithelium, a moderately strong, positive relationship ($r=0.74$) was observed between the percentage of PCNA positive cells and PDGF-R β immunoreactivity in all stages of the estrous cycle. A positive ($r=0.58$), though weaker, association was found between PCNA and expression of PDGF-R α in the glandular epithelium. A strong relationship ($r=0.91$) in the glandular epithelium was present between the intensity of the stains for PDGF α and β receptors (Figure 4.2)

Correlation analysis in the stroma revealed a mild positive relationship between PDGF-R α and PCNA expression ($r=0.46$). (Figure 4.3) PDGF-R β expression correlated negatively with both PCNA ($r=-0.72$) and PDGF-R α ($r=-0.33$) in the stromal compartment. No relationship was noted between PCNA, PDGF-R α or PDGF R β staining in the muscular or vascular compartments.

Discussion

This study demonstrated immunoreactive PDGF-R α and β in the cycling mouse endometrium, and determined that PDGF-R α and β expression correlated with the expression of PCNA and the morphological stages of proliferation in the glandular epithelia of the endometrium.

PDGF-R β has been localized previously by immunohistochemistry in the human endometrium[87], and in the endometrium of immature mice challenged with supraphysiological dosages of DES, a synthetic

estrogen[20]. The above findings extend the immunohistochemical localization of PDGF-R β to the endometrium of the adult mouse. PDGF-R β was widespread in the mouse uterus. It was present to variable degrees in all compartments of the uterus throughout the estrous cycle. The muscular, vascular, and stromal components all exhibited a basal level of expression of PDGF-R β that did not vary over the course of the estrous cycle. The luminal and glandular epithelium had recognizable variations in the intensity of staining for PDGF-R β throughout estrous.

Gray,*et al* demonstrated the expression of PDGF-R α in prepubescent mice by [^{125} I] affinity-labeling[20]. Reliable receptor localization by immunohistochemical methods has been more difficult. Because of the ability of the α receptor to bind both the A and B isoforms of PDGF, and the potential mitogenic stimulus of ligand / α receptor binding, immunohistochemical identification of the α receptor in the endometrium and examination of its relationship to proliferation in the uterus were undertaken. Optimization of immunohistochemical procedures allowed application of a commercially available antibody for localization of PDGF-R α in the mouse uterus. PDGF-R α was widespread in the uterus, and exhibited variation in immunoreactivity in the luminal, glandular and stromal compartments over the course of the estrous cycle.

Comparisons of the immunoreactivity for PDGF receptors and the degree of proliferation (as measured by percent of PCNA positive nuclei) revealed different relationships between the expression PDGF R α and PDGF-R β to proliferation in the endometrial tissue compartments. Overall expression of PDGF-R α did not correlate with epithelial proliferation and was

only weakly associated with stromal proliferation ($r=0.46$). Significant correlation of PDGF-R β expression with endometrial proliferation was limited to the glandular epithelium ($r=0.76$). A weaker relationship was observed with PDGF-R α and the glandular epithelium ($r=0.58$). In the stroma and luminal epithelium, the relationships of PDGF-R β expression to both proliferation and PDGF-R α expression were negative.

In both the mature rodent and human uteri, the epithelium is the most responsive endometrial compartment to the proliferative action of estrogen[77]. Because the highest proliferation indices in the glandular epithelium were present during the increasingly estrogen-dominated proestrus and estrus stages, these findings suggested that in the mouse, cyclical expression of the PDGF receptors may be closely linked to estrogen levels. In light of the findings of Gray, *et al* that single pulse dosages of DES upregulate PDGF receptor mRNA expression in the uteri of immature mice, PDGF receptors and their ligands may well be part of an estrogen-initiated mitogenic cascade in the endometrial glandular epithelium of mature mice. The Gray, *et al* findings of greater expression of the PDGF-R β mRNA and protein in the stroma than in the epithelium, contrasts with this study's finding of greater epithelial than stromal staining intensity for the PDGF-R β antibody. These seemingly contradictory findings may relate to the age differences between the mice in the respective studies. Gray's findings were in prepubescent mice, whereas the current study was performed using mature mice. Furthermore, when prepubescent (presumptively estrogen naive) mice were given bolus injections of estrogen the expression of receptors

corresponded to findings in mature cycling mice of more epithelial than stromal staining.

The negative association of PDGF-R β expression, and the weak association of PDGF-R α with proliferation in the stroma is similar to that seen in the human endometrium (chapter 2). In ovariectomized mice, treatment with estrogen alone causes marked proliferation in the glandular and luminal epithelium, but is insufficient to stimulate stromal proliferation[11]. Addition of progesterone reverses this pattern of proliferation. The negative association of stromal PDGF-R α and β expression with stromal proliferation further supports the hypothesis that proliferation of the endometrial stromal cells is mediated by different signaling pathways than those employed by glandular endometrium. Combined with the positive association of proliferation and PDGF-R β and α expression in the glandular epithelium, there is strong implication that in the uterus the PDGF / PDGF-R signaling pathway is predominantly, if not exclusively, associated with estrogen driven endometrial glandular epithelial growth.

The intensity of staining for PDGF-R α in the stroma lagged behind the stromal proliferation. In light of the role of PDGF AA / PDGF-R α in developmental migration and differentiation of the embryo[95], perhaps PDGF-R α may fulfill a nonproliferative role in the regulation of stromal cells in the mouse endometrium or may be under the control of different factors than in the epithelium. Mismatching of PDGF receptor expression with proliferation in the luminal epithelium appears to contradict the hypothesis that estrogenic proliferation is mediated through PDGF ligand / receptor pathway. However, primary discordance of proliferation with PDGF-R

expression occurred in the metestrus phase when estrogen levels are lowest and progesterone was the dominant gonadal hormone. The expression of PDGF receptors was lowest during the metestrus stage, which is what would be expected if the hypothesis that estrogen associated proliferation was mediated via PDGF/ PDGF receptors were valid. The finding of continued high percentages of PCNA positive cells in the metestrus luminal epithelium would indicate that control of the proliferation of luminal epithelium may be regulated by multiple factors.

In summary, immunohistochemical techniques optimized for use in formalin-fixed human tissues were used to demonstrate the presence of PDGF-R α & β in paraformaldehyde-fixed uteri of mature mice. Moderate steady levels of both receptors were present in uterine muscle and vessels throughout the estrous cycle. There was variation in the expression of PDGF-R α in the epithelial and stromal compartments during the estrus cycle. Estrous stage variability of PDGF-R β was limited to the epithelial compartment. Staining intensity for PDGF-R β and α was associated positively with proliferation of the glandular epithelium and negatively associated in the stroma. The findings in this study corresponded to previously reported expression of PDGF-R β in proliferative human endometrium[87], and the association noted between expression of PDGF receptors and endometrial proliferation described earlier in this study (Chapters 2 and 3). PDGF has been shown to have a proliferative effect on human and bovine endometrial epithelial cells[73, 133]. The upregulation of PDGF receptors by DES in immature mice [20], and the positive effect of DES pretreatment on PDGF-treated cells (Chapter 1) suggested that estrogenic

activity is strongly associated with PDGF signaling. The findings of this study add further support to the hypothesis that PDGF receptor-mediated signaling pathways may play a key role in the endometrial glandular epithelium's proliferative response to cyclic estrogen levels.

Chapter 5

Development of a scid mouse model of endometriosis

Introduction

Non-neoplastic growth of endometrial tissue outside the uterus is termed endometriosis. Endometriosis represents a major medical problem in women and captive subhuman primates. Endometriosis can result in a range of clinical signs, including dysmenorrhea, intraperitoneal adhesion, pain and infertility. Endometriosis has been estimated to affect approximately 7-10 % women of child-bearing age in the United States[45, 46]. Etiology, histogenesis, and progression factors in the disease are unresolved[66].

Platelet-derived growth factor (PDGF) has been implicated as one of the possible mediators of endometriosis. Its proliferative effect on human endometrial cells in culture, the presence of PDGF receptors in the uterine epithelium and stroma, the increased production of mitogenic growth factors by peritoneal macrophages from patients with endometriosis, and PDGF production by macrophages which are frequently associated with endometriotic lesions all support a potential role for PDGF in the progression of endometriosis[73, 87, 134, 179].

Experimental examination of the progression of endometriosis has been limited because of its restriction to menstruating species. Spontaneous endometriosis has been reported in macaques and baboons[180]. In captive baboons the rate of endometriosis is between 12 and 17%[181]. Although

primates are the optimal models, cost and humane considerations limit their use. Most other animal models of the disease rely on autologous tissue transplantation in species which do not naturally experience the disease. Development of a less costly animal model which accepts human endometrial tissues would allow more extensive investigation of the response of ectopic human endometrium to growth factors.

The first model successful subcutaneous transplantation of human endometrial tissue used nude mice, which have congenital thymic aplasia and therefore cannot mount a T-cell lymphocyte response to xenografts[62]. This model was further refined using the severe-combined-immuno-deficient (scid) mouse as the recipient of subcutaneously placed human endometrium[63]. Scid mice have a defective VJD recombinase system that results in a lack of both functional T and B cell lineage lymphocytes, and decreases their ability to reject xenografts.[182]. Human endometrial tissue transplanted into scid mice had a substantially greater rate of survival than that observed in athymic mice[63]. Subcutaneous transplantation of thyroid follicular cells within Matrigel into scid mice has been reported to allow partial follicular reconstitution[183]. The scid mouse may allow *in vivo* investigation of human conditions in an animal model when an isolated type or combination of types of human cells are required[184].

The nude and scid mouse models of endometriosis are based on subcutaneous implantation that does not reflect the microenvironment of the peritoneal cavity. Intraperitoneal implantation of human tissue would appear to be morphologically and physiologically more relevant to the disease state in women.

These aforementioned nude and scid mouse models examined whole tissue transplants. The histogenesis and role of the various cell types (i.e., epithelial vs. stromal) in the initiation of endometriosis is still in question. Development of a system which would allow examination of the role of the endometrial epithelium alone in endometriosis may help to clarify the initiation of disorder. The potential role of PDGF in the proliferation of endometrial epithelial cells has been explored in the preceding chapters. PDGF or PDGF-like substances have been suggested as potential factors in the progression of endometriosis. An *in vivo* system such as the scid mouse may be potential model for examining the progression of endometriosis in women, and elucidating the role PDGF and other growth factors play in normal and pathological endometrial proliferation.

Experimental overview

The purpose of this study was to determine the best method for implantation of human endometrial epithelium in the peritoneal cavity of scid mice, assess the transplanted endometrial epithelial cells viability and determine if PDGF receptor expression was maintained over the course of matrix subculture and transplantation. Subcultured primary cell lines of human endometrial epithelium were transferred to multiple support matrices. Growth characteristics and the expression of cell lineage markers and PDGF receptors in the matrix-supported cells were examined and compared among matrix types. Human endometrial cells were transplanted into the peritoneal cavity of scid mice for periods of up to 6 weeks. Gross and histological

assessment of the transplants was done and compared to characteristics of ectopic endometrial tissue in women with endometriosis.

Materials and methods

Implant preparation

Support matrices for culture of human endometrial epithelial cells were prepared in advance of transfer to the cells. Millicell CM membranes (Millipore Corp., Bedford, MA) were thin-coated with 0.33 mg/ml rat tail collagen, Type 1 (Becton Dickinson Labware, Bedford, MA), air dried for 12 hr. and rinsed with DMEM / F12. Human amniotic membranes were incubated 0.1% NH_4OH , vigorously scraped with a rubber policeman to disrupt and remove any amniotic epithelial cells and then frozen[185]. A three dimensional support matrix using Gelfoam (Upjohn Laboratories, Kalamazoo, MI) {Color Plate 5: photomicrograph 5.7}, a porous agar sponge coated with Matrigel (Collaborative Research, Lexington, MA) was prepared at the time of cell transfer. Matrigel is an extracellular matrix derived from the basement membrane of the Engelberth-Holm-Swarm (EHS) transplantable mouse sarcoma. The Matrigel matrix contains the PDGF, insulin-like growth factor, epidermal growth factor, nerve growth factor and transforming growth factor β elaborated by the EHS tumor cells[183].

Subcultured, human endometrial epithelial cells from 3 primary cell lines (M 92-2, DH 95-43 & DH 96-R1) were transferred by trypsinization (0.025 %)[73] from monolayer culture in flasks onto the collagen-coated Millicell membranes, the human amniotic membranes {Color Plate 5: photomicrograph 5.1} or into Matrigel supported by Gelfoam {Color Plate 5: photomicrograph

5.8-5.9). The subcultured cells (passaged 5-12 times) had been previously confirmed as epithelial by their morphology, long-term culture behavior and expression of cell lineage markers (Part II, chapter 1).

The trypsinized, suspended endometrial epithelial cells were seeded onto 12 mm diameter collagen coated Millicell membranes (surface area 1.1 cm²) wells at 1×10^5 cells / well. Suspended cells were seeded onto 10-15 mm diameter pretreated human amniotic membranes at 1×10^5 cells / well. Pre-cooled, cells suspended in DMEM / F12 (1×10^6 cells/ml) were admixed with an equal volume of chilled Matrigel solution. The resulting Matrigel cell suspension was slowly absorbed onto 2.5 cm² x 3 mm thick segments of Gelfoam {Color Plate 5: photomicrograph 5.1}. Typically 0.2 to 0.3 ml of the Matrigel cell suspension was sufficient to saturate and cover each gel foam segment. Seeded membranes and the seeded Matrigel suspension in Gelfoam were placed at 37°C for thirty minutes to allow cellular adhesion and gelling of the Matrigel solution before the matrices were submerged in media. All cell cultures were incubated in DMEM / F12 with 10% fetal bovine serum , insulin (5µg/ml), transferrin , selenium , epidermal growth factor (10ng/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin (0.25 µg/ml)[73]. Media was changed every 72 to 96 hours unless otherwise noted. Cells were grown on these membranes and within the Matrigel / Gelfoam matrix for 3 days, at which point the membranes and the Matrigel / Gelfoam supports were formalin-fixed for 24 hours and then paraffin embedded for routine histologic cross sectioning at 6 µm. Parallel cell seeded membranes were cultured for periods of up to 3 weeks to observe the viability, morphology and growth behavior of the cells.

Immunohistochemistry

Slides were heated in a dry oven for 30 minutes at 65°C and stored at room temperature until use. For immunohistochemical staining the slides were deparaffinized by treatment with xylene (3 x 5 min.) and step-rehydrated with 100 (3 x 3 min.), 95, 90, 80 and 70 % ethanol (1 x 3 min.) and PBS (2 x 3 min.) at room temperature. Tissue sections were pretreated with 3 % hydrogen peroxide in 50% methanol for 10 minutes at room temperature (RT), rinsed with PBS (3x 5 min. RT), and pretreated with normal goat serum (Biogenex Laboratories, San Ramon, CA) for 10 minutes at RT to decrease nonspecific binding of the primary antibodies.

After incubation with the protein block, the block was removed and the appropriate primary antibodies were applied, without an intervening PBS rinse. The slides were incubated in a humidified chamber with the primary antibodies for 18 hours at 4°C. Primary antibodies used in this study were 1:500 dilutions of PDGF-R α and PDGF-R β (Upstate Biotechnology Inc., Lake Placid, NY), and vimentin (Biogenex) at the manufacturers supplied concentration.(see Chapter 2). The vimentin antibody is immunoreactive to human vimentin intermediate filaments. Human endometrial epithelial cells have been reported to express the vimentin intermediate filament[72, 73]. Non-immunoreactivity to mouse vimentin was reported by the manufacturer and no positive staining of mouse tissue was observed in slides examined. As such it could be used to positively identify the transplanted human endometrial epithelial cells.

After incubation with the primary antibodies (or nonimmune autologous sera), the slides were rinsed (2 x 3 min.) in PBS, and biotinylated secondary

(or linking) antibody was applied for 20 minutes (RT). After rinsing with PBS the streptavidin-enzyme (or labeling) solution was applied for 20 minutes(RT). The slides were rinsed, and the chromagen solution was then applied. Progression of the color development was observed by microscopy at 10X objective magnification. Development was compared to the negative controls and was stopped by immersion in distilled water.

Transplantation in scid mice

For the transplant studies 70, eight week old, female, scid mice were obtained from Charles River Laboratories (Wilmington, MA) under UT Animal Care and Use Ccommittee protocol # 0668. Upon arrival the mice were held for one week for acclimation and confirmation of health status. Each mouse was individually housed within sterilized filtered cages in the BioClean unit of the UTCVM Laboratory Animal Facility. Sterile food and water were dispensed according to standard aseptic protocols.

Cell cultures on each of the above matrices were prepared as described above and maintained in culture for 3-5 days prior to surgical transplantation into recipient scid mice. Additional confluent monolayer cell cultures in tissue flasks were maintained as a source of trypsinized, suspended cells.

Because the objective of this study was to determine the optimum type of implant for support and viability of the endometrial epithelial cells, the animals were divided into seven groups: 1) controls which received non-seeded implants; 2) mice receiving cell suspensions injected intraperitoneally; 3) mice receiving sub capsular renal and intramesometrial cell suspensions; 4) mice receiving cell-seeded Millicell membranes; 5) mice receiving cell-

seeded amniotic membranes; 6) mice receiving cell-seeded Matrigel / Gelfoam implants, and 7) mice receiving combinations of the implants. All groups of mice were maintained for 2 weeks. The portions of the seventh group of mice were maintained for more extended periods (4 and 6) weeks to determine long term viability of the cells and implants

All pre-surgical, surgical and post-surgical handling of the mice was done in an aseptic manner. A cocktail of ketamine (Fort Dodge Laboratory Inc., Fort Dodge, IA) (100 mg/kg) and xylazine (Bayer Corporation, Shawnee Mission, IA) (2.5 mg/kg) injectable anesthesia was administered subcutaneously during the initial 2 week portion of the study (30 mice). Isoflurane gas anesthesia (Abbott laboratories, North Chicago, IL) was used for induction and maintenance of anesthesia thereafter. All mice were restrained on a heated surgical field to decrease the incidence of hypothermia. A 2-4 cm long midline abdominal incision allowed access to the peritoneal cavity. Two layer (muscle and skin) closure of the abdominal incision was done in a continuous pattern using 5-0 vicryl suture (Ethicon, Somerville, NJ). An injectable subcutaneous dose of butorphenol (Fort Dodge) (5.4 mg/kg) was administered postoperatively for analgesia. Mice receiving transplanted cells were recovered and left undisturbed (except for routine housekeeping purposes) for periods of 2, 4 and 6 weeks.

Two week implantation studies

Six categories of implantation were examined in the initial 2 week studies. Non-seeded segments of Millicell membrane, human amniotic membrane and Matrigel / Gelfoam implants were placed in five mice to serve

as controls. Cell suspensions of trypsinized hEEC (1×10^6 cells / mouse) in DMEM / F12 were slowly dripped into the peritoneal cavity of five mice. 0.1 ml cell suspensions (1×10^6 cells / ml) were injected under the capsule of the left kidney and into the mesometrium of five mice. Five mice were implanted with cell-seeded Millicell membranes only. Cell-seeded human amniotic membrane was implanted into five of the mice. Five mice were implanted with seeded Matrigel / Gelfoam implants. At least two sets of implants were placed in all mice receiving implants. None of the implants in these groups were surgically attached to any peritoneal surface, rather they were placed free floating in the abdomen. These five groups were terminated 14 days post implantation.

All mice were euthanatized by cervical dislocation, the abdominal cavities examined for gross lesions and the position of the implants noted. Samples of the implants, adjacent tissues and multiple segments of the caudo-lateral body wall, and abdominal mesentery and organs were sampled from each mouse. Multiple sections of the left kidney and mesometrium were sampled from those mice receiving directed injections of cells. The tissues were fixed in 4 % paraformaldehyde for 24 hours, transferred to 70 % methanol (4°C) for 24 to 48 hours and routinely processed for paraffin embedding. Sections were examined by light microscopy. Immunohistochemical staining for the vimentin intermediate filament was performed on selected slides from all mice. Further immuno-histochemical characterization using antibodies for pan cytokeratin and the PDGF receptors was done on selected slides using the protocols outlined above.

2 and 4 week studies of combined implants

Based on the results of the initial studies, 20 mice were each implanted with combinations of two segments of cell seeded human amniotic membrane and two segments of the seeded Matrigel / Gelfoam matrix. Seeded amniotic membranes were positioned bilaterally, but not attached to the lateral body wall of each mouse. The Matrigel /Gelfoam segments were loosely positioned on the mesentery in the ventro-lateral aspect of peritoneal cavity. Ten members of this group were terminated two weeks post-implantation and 10 individuals were terminated at four weeks. Tissues samples were processed and immunohistochemistry performed as outlined above.

4 and 6 week studies of combined implants

Paired seeded amniotic membrane segments were placed in 15 mice. The membranes were positioned bilaterally against the lateral body wall immediately caudal to the kidney, and attached to the peritoneum by 3-5 single 5-0 vicryl sutures. In addition Matrigel / Gelfoam implants were loosely positioned on the mesentery in the ventro-lateral aspect of peritoneal cavity. Seven of the mice were terminated four weeks post-surgery, and the remaining eight were terminated after six weeks. Tissues samples were processed and immunohistochemistry was performed as outlined above.

Results

Cell culture on implants

Cell adhesion was observed on both the collagen-coated Millicell CM and the treated human amniotic membranes. Growth of the cells on the

Millicell membranes was confined to the surface of the membrane. Cells maintained a flattened to low cuboidal appearance, and spread of the cells was limited to subconfluence on membranes cultured up to 3 weeks. Cells grown on the human amniotic membranes had flattened to cuboidal morphology. {Color Plate 5: photomicrographs 5.2-5.6}, and growth and polarization of the hEECs were more robust on the amniotic membranes. Confluence of growth was reached within 5-7 days. The majority of cells were confined to the surface of the membrane; however, some areas exhibited migration of cells into the membrane. Cells admixed with Matrigel and coated in the Gelfoam were moderately to densely present on the surface of the Matrigel and within minimally coated trabecular "lumens" within the Gelfoam. Similarly to the amniotic membrane, the cells displayed an admixture of flattened to cuboidal morphology.

Immunohistochemical staining of the hEEC grown on the various matrices was similar to that observed in cells grown in monolayer culture in polystyrene flasks. (Chapter 1) There was mild to moderate staining for pan-cytokeratins with marked staining in some cells.{Color Plate 5: photomicrograph 5.11 } Vimentin staining was positive in all cells, regardless of matrix.{Color Plate 5: photomicrograph 5.3 } PDGF-R α and PDGF-R β staining was present in some cells on all matrices. {Color Plate 5: photomicrographs 5.4-5.5 & 5.12-5.13} Overall, staining intensity for all antibodies was considered to be best in the Matrigel / Gelfoam matrix compared with the Millicell and amniotic membranes.

Transplantation studies

Transplantation of subcultured primary cell lines into the abdominal cavity of scid mice had varied outcomes. No human endometrial cells could be identified after two weeks in mice receiving either cell suspensions dripped into the peritoneal cavity, or cell suspensions injected beneath the left renal capsule or in the mesometrium. One of the mice that received 10^6 cell suspension in the abdominal cavity never recovered fully post-surgically, and died 2 hours post operatively. Individual and clumped cells attached to the peritoneal walls and mesenteric surfaces could be identified in tissue sections.{Color Plate 5: photomicrograph 5.17} Similarly, two mice that received injections of cells in the mesometrium and beneath the renal capsule had extended post operative recoveries and died between 3 and 10 hours post-operatively. Tissue sections were unsuitable for meaningful evaluation regarding cause of death or disposition of the injected cells.

No gross abdominal abnormalities were observed in any of the mice. At the time of necropsy, all implants were visible and attached to the peritoneal wall, abdominal organs or entrapped within the mesentery.{Color Plate 5: photomicrograph 5.14} All implants had evidence, either grossly or microscopically, of neovascularization across the surface of the membranes or within the Matrigel / Gelfoam matrix.{Color Plate 5: photomicrographs 5.15-5.16}

Distribution of implants:

The majority (> 88 %) of the free floating implants were located in the caudal one third of the abdominal cavity.{Color Plate 5: photomicrograph

5.14} Of these, roughly 35% (n=46) of the implants were located predominantly between the lateral aspects of the rectal and / or uterine walls and the left caudo-lateral body wall, 20 % (n=26) were in the contra-lateral position, 30 % (n=39) were ventral, and the remaining 15 % (n=22) were entrapped centrally within the mesentery and mesometrium. Adhesion of the implants to the midline surgical incision site was present in all implants spanning the ventral portion of the abdomen. Of the 19 implants (12%) which localized in the cranial two thirds of the abdomen, sites of adhesion included the caudal aspect of the right liver lobe (5) {Color Plate 5: photomicrograph 5.15}, the spleen (3), the small intestine and mesentery (8){Color Plate 5: photomicrograph 5.18}, and peri-renal (2). In the mice with amniotic membranes sutured to the lateral body wall, 2 died post surgically. Three of 32 sutured membranes in the remaining mice were at sites distal to their initial placement. There was no difference in the distribution of membranes versus Matrigel / Gelfoam implants.

Transplanted cell distribution:

Immunohistochemical staining for vimentin was used to identify positively the transplanted hEEC in the recipient mice. The confluency, distribution and morphology of vimentin positive cells differed with type of implant, but not with the length of transplantation period. Positive staining for vimentin was present in some membrane or matrix-associated cells in all sections examined. {Color Plate 5: photomicrographs 5.20-5.21}

The subconfluent growth of flattened to low cuboidal cells on the implanted Millicell membranes was similar to the growth and morphology of

the cells observed in culture. Vimentin-positive cells were few and scattered, with predominantly flattened to low cuboidal morphology. The positive cells were almost exclusively confined to areas of adhesion or growth of within the mesothelium, and neovascularization over the implanted membranes.

The amniotic membranes free-floating in the abdominal cavity (in the initial portion of the study) adhered predominantly to the ventral midline (5/8). Occasional, vimentin positive cells of flattened to low cuboidal morphology could be identified at the periphery of the membranes; however, no positive cells could be identified at points of adhesion or overgrowth by wound-associated fibroplasia and inflammation. Six of the membranes were partially scrolled and adhered to the caudolateral body walls. Occasional vimentin-positive cells could be identified at the free end of the scrolls, and between layers of the scroll. Minimal to no inflammatory response was noted to either the Millicell or amniotic membranes.

Placement of the membranes against the lateral body wall (in order to decrease scrolling) in for 2-4 week periods was largely unsuccessful. Although the membranes almost remained exclusively in the caudolateral abdominal area, tight scrolling was present in 16 of 20 membranes. Cell morphology and distribution of cells on the membranes were similar to that noted in the initial group. Only rare cells were present adjacent to points of adhesion or entrapment by mesothelium on the amniotic membranes that did not scroll.

Amniotic membranes sutured to the body wall had few, widely separated, vimentin-positive cells on the peritoneal cavity side of the membrane, and no identifiable cells on the membrane side apposed to the

peritoneal surface. Rare aggregates, and occasional single cells were present within folds of the membranes and in the membranes. The majority of the membranes had nearly complete coverage of the luminal surface by mesothelium and/or adherent mesentery at the 4 and 6-week time periods.

The cell-seeded Matrigel / Gelfoam implants had vimentin-positive cells in all sections reviewed. Nearly all positive cells were confined within the implant. Only rare positive cells were located on the surface of the implants. Within the implants, as in cell culture Matrigel / Gelfoam matrices, the majority of cells were confined to minimally Matrigel-coated trabecular "lumens" within the Gelfoam, although individual and aggregates of cells could be seen within the Matrigel. Rare "glandular" arrangement of the cells, completely surrounding the trabecular "lumens" were found in 6 of 95 sections. {Color Plate 5: photomicrograph 5.19, 5.21 & 5.22 } There was neovascularization within the Matrigel / Gelfoam implants present from two weeks through six weeks. Mild, multifocal infiltrations of histiocytic and polymorphonuclear inflammatory cells occurred within the matrix at all time frames.

Immunohistochemical evaluation of selected Matrigel / Gelfoam sections (15) from the 4 and 6 week periods for cytokeratin intermediate filaments and expression of both PDGF receptors revealed that cells within the same areas of the implants stained positively for pan-cytokeratin, PDGF-R α and PDGF-R β . {Color Plate 5: photomicrographs 5.22-5.24}. Attempts at dual staining for vimentin and the other proteins were unsuccessful due to lifting of the Matrigel / Gelfoam implants from the slide surface with the prolonged handling required by the dual staining protocols.

Discussion

Transplantation of human endometrial epithelial cells in Matrigel / Gelfoam implants into the abdomen of scid mice may serve as a useful model for endometriosis. Transplanted hEEC survive and maintain expression of the intermediate filaments cytokeratin and vimentin, and expression of the PDGF receptors for at least six weeks. There was evidence of rudimentary gland-like formation in the Matrigel and along the trabecular structures of the Gelfoam. When placed free in the peritoneal cavity, the implants attach to the same sites as those associated with endometriosis. Vascularization of the implants occurred, insuring potential, long-term cell viability, and the availability of serum levels of growth factors and hormonal steroids. Given the correlation of PDGF receptor expression with the proliferative state of the uterus (Chapter 2), the expression of PDGF receptors by the transplanted cells may indicate these cells are capable of proliferating in response to PDGF.

Stromal elements are a consistent and integral finding in endometriosis, even when only small portions of the lesion can be identified as epithelial. This model purposefully lacked a stromal component, instead substituting an artificial substrata to support already differentiated epithelial cells. Exclusion of stromal cells from this study was done in order to gauge the ability of the epithelial cell component of endometriotic lesions to survive and to form recognizable endometrial structures after transplantation. If these first two conditions could be achieved, then it might be possible ascertain if the epithelium was solely responsible for the formation of the lesions or if concurrent seeding with stromal cells is a necessity for the initiation and progression of endometriotic-like lesions.

Cell survival, and at least partial recapitulation of glandular morphology were recognized for up to six weeks. No other additional features of endometriosis were apparent (cyst formation, fibrosis, hemorrhage). These might develop if the course of these experiments were extended. In baboons, implantation and survival of intraperitoneal injections of autologous endometrial tissues is present at 8 weeks, but progression of endometriosis could not be confirmed until 5 months post injection[65]. No induction of stroma-like cells was observed in the scid mice. This could be a factor of time, a failure of induction between cells of different species, or most likely, the necessity for interactive, original heterologous populations of cells (e.g., epithelial and stromal) to result in the full range of lesional morphology.

The morphology, function, polarity, and growth of uterine epithelial cells in culture has been shown to be affected by the substrata on which the cells are placed[27, 152, 185-189]. Matrix-coated (e.g., collagen, Matrigel) membranes, floating or supported at the air-fluid interface, allows the cells a semi-permeable basolateral surface on which to orient and interact with some basement membrane components[190]. Because the use of some type of solid support was anticipated (indeed, proved necessary) for transplantation of hEEC to scid mice in this study, identification of appropriate substrata for implantation was undertaken.

Morphology and growth of the cells on the submerged, floating, collagen-coated Millicell membrane, the human amniotic membranes, and the surface of Gelfoam-supported Matrigel used in this study was similar to that of cells cultured on coated membranes in previous reports[152, 185, 191]. The collagen-coated Millicell membranes had the poorest growth and cells rarely

obtained a cuboidal morphology. Of the three submerged, floating substrata systems examined *in vitro*, the amniotic membrane appeared to support the most robust growth of the hEEC.

Ingrowth of endometrial epithelial cells into the amniotic membrane was unexpected. This behavior has not been previously reported and is not typical of epithelial cells, although *in vitro* invasive behavior by endometriotic epithelial cells lacking E-cadherin, a gene which mediates cell to cell interaction, has been reported[192]. This behavior was not observed on the collagen-coated Millicell membranes. Freezing and thawing of the amniotic membranes may have caused fractures of the membranes along which the proliferating cells could extend.

Matrigel alone, as a support for the hEEC proved ineffective because of its tendency to disintegrate over 5 - 10 days in culture. Application of the Matrigel to the Gelfoam agar sponge overcame this limitation. The Matrigel-coated Gelfoam support could be considered a three-dimensional substrata. The rigid sponge-like qualities of Gelfoam not only increases resistance to dissolution, but also dramatically increases the surface area for growth in a trabecular pattern which supports cell migration and creates potential glandular-type configurations. Additionally, it acts as a wick, allowing diffusion of nutrients and metabolites, while allowing the longer exposure to paracrine / autocrine factors than in fluid media[193, 194]. Because of the growth factors incorporated into the Matrigel at the time of its production by EHS cells, robust growth of the hEEC might be expected. However, due to irregularities of the surface and the three dimensional nature of the construct, growth of the cells on the surface and within the Matrigel / Gelfoam substratum was difficult to

evaluate {Color Plate 5: photomicrograph 5.8 }. The morphology of the cells and the density of cell growth on sectioning was nearly equivalent to that on the amniotic membrane. The presence of cells deep within the Matrigel matrix was not interpreted as invasive behavior, since the seeding method used involved suspending the cells within the fluid phase Matrigel.

Immunohistochemical staining of the cells grown on the different matrices for pan-cytokeratin was positive, although less intense than that of vimentin (used to confirm the human origin of the cells). Optimal staining with the cytokeratin antibodies employed in this study involved microwave antigen retrieval (per manufacturer's recommendation). Weak adherence of the membranes and the Matrigel / Gelfoam substratum to the slides prevented use of that technique. Positive staining for both PDGF receptor types was also present in the cells grown on all the matrices {Color Plate 5: photomicrographs 5.4-5.5 & 5.12-5.13}.

Survival of the transplanted endometrial cells was instructive. Although suspended cells were observed to have attached in the peritoneal cavity within two hours, long term survival of the hEEC transplanted by this method did not occur. The intact mesothelial lining may have prevented the long term survival of these cells. There is a probable relationship between peritoneal defects and the occurrence of endometriosis[195]. The inability of suspended endometrial epithelial cells to survive and establish themselves raises the question of a co-requirement for stromal cells in the establishment of endometriosis. Differentiation of endometrial tissue is considered dependent on cell signaling between stromal and epithelial cells[196]. Stromal elements are a consistent and integral finding in endometriosis[195].

Although no inflammatory response directed at the heterotopic cells was noted in any of the transplants, mesothelial cells, which have phagocytic ability, may have participated in the demise of suspended cells. The reason for lack of survival of the suspended cells placed beneath the renal capsule or within the mesometrium is not clear.

The scant growth of the transplanted cells on the collagen-coated Millicell membranes and the amniotic membranes appeared at least partially inhibited by the mesothelium. Virtually no transplanted hEE cells were identifiable in the areas covered by mesothelium. Whether this mesothelial covering represented adherent, pre-existing mesothelium, or new mesothelial growth could not be ascertained. The absence of identifiable transplanted cells on the surface of the Matrigel / Gelfoam implants further supports a role for the mesothelium in the demise of the transplanted cells.

Release of growth factors and cytokines by inflammatory cells has been implicated in the pathogenesis of endometriosis[197]. There was moderate, post-surgical inflammatory reaction associated with the ventral abdominal surgical wounds; however, by two weeks post-surgery, the nidus of inflammation (sutures) in all mice were compartmentalized beneath the mesothelium or in the body wall. The mesothelium adjacent to and covering implants appeared quiescent. The scant inflammatory reaction to the collagen-coated Millicell and amniotic membranes contrasted with the moderate, focal suture reactions of the incisional closure site or sites of tacking of the membranes to the body wall.

Of the implants used, only the Matrigel / Gelfoam implants had substantial populations of identifiable human cells remaining at all time

periods. The Matrigel matrix is a solubilized basement membrane preparation from the transplantable EHS mouse sarcoma, and contains PDGF, insulin-like growth factor, epidermal growth factor, nerve growth factor, and transforming growth factor β . Some of these growth factors have been postulated to play a role in the development of endometriosis. The Matrigel / Gelfoam implants had evidence of neo-vascularization at 2 weeks post-transplantation, and were well vascularized at the later time periods. Diffusion of these growth factors may have occurred once vascularization was established. The initial presence of the growth factors in the matrix, and the probable circulatory contribution of serum growth factors may be critical to the survival of the transplanted cells in the peritoneal cavity of the scid mice.

Transplantation of human endometrial epithelial cells in Matrigel / Gelfoam implants into the abdomen of scid mice may serve as a useful model for endometriosis. Transplanted hEEC survive and maintain expression of the intermediate filaments pan-cytokeratin and vimentin, and PDGF receptors for at least six weeks. There was evidence of rudimentary gland-like formation in the Matrigel, and along the trabecular structures of the Gelfoam. When placed free in the peritoneal cavity, the implants attached to the same locales as those associated with endometriosis. Vascularization of the implants occurred, insuring potential, long-term cell viability and the availability of serum levels of growth factors and hormonal steroids.

In summary, the subcultured human endometrial epithelial cells could be successfully transferred to multiple matrices. The cells continued to express the intermediate filaments (pan-cytokeratin and vimentin), and the PDGF receptors. Transplantation of the cells on the matrices revealed that the

Matrigel / Gelfoam implants had the best cell survival rate, that partial recapitulation of glandular morphology and vascularization of the implants occurred, and that the cells continued to express PDGF receptors. Continued refinement of the model is anticipated. Comparison of co-cultured stromal and epithelial cells in the Matrigel /Gelfoam matrix, primary culture of endometrial fragments in the Matrigel / Gelfoam, and direct implantation of human endometrial fragments in the peritoneal cavity of scid mice are considered to be the next logical steps in investigating the usefulness of this model.

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Appendices

Appendix A

Tables

Table 1: Standard index of proliferation (SIP) for treatments on hEEC.

	SIP	SEM	SD
SFM	1	0.021	0.183
PDGF AB	1.795*	0.078	0.54
E2	0.956	0.033	0.266
DES	1.179	0.639	0.35
EGF	1.158	0.071	0.37
E2 & EGF	1.39	0.187	0.649
DES & PDGF	2.066*	0.105	0.491
E2 & PDGF	1.761*	0.109	0.611
E2, P4 & PDGF	1.958*	0.173	0.713
EGF & PDGF	2.304*	0.208	0.895

Subconfluent cultures of hEEC treated with the steroidal hormones 17- β estradiol (E2), diethylstilbestrol (DES), progesterone (P4) alone or in combination with the growth factors platelet-derived growth factor AB (PDGF) and epidermal growth factor (EGF). The standard index of proliferation (SIP), standard error of mean (SEM), and standard deviation (SD) of the treatments represent pooled values of a minimum of 2 replicates of at least 3 cell lines with triplicate treatments for each replicate. Controls (SFM) were incubated with DMEM-F12 without growth factor additives. Astericks denote values of treatments which are significantly greater than control (SFM).

Table 2.1: Mean proportion of PCNA positive nuclei in human uterus

	Proliferative	Secretory	Inactive
Luminal epithelium	30 %	20.8 %	28.9 %
Superfical glands	60.7 %	18.3 %	25 %
Deep glands	75 %	62 %	10 %
Superficial stroma	52.8 %	42.5 %	30 %
Deep stroma	47.1 %	45 %	25 %
Muscle	28.6 %	48.3 %	30 %
Arterioles	42.1 %	32.5 %	15 %

Values represent the mean of the combined percentages of PCNA positive cells from a minimum of three cases for each endometrial stage: proliferative, secretory and inactive. Two slides were immunostained for each case. Percentages were derived from the number of positive staining nuclei out of one hundred cells in each tissue compartment of each slide examined..

Table 2.2: Mean intensity of PDGF-R α immunoreactivity in human uterus

	Proliferative	Secretory	Inactive
Luminal epithelium	0.45	0.41	0
Superficial glands	0.3	0.45	0.06
Deep glands	1.8	1.1	0.18
Superficial stroma	1	0.75	1.6
Deep stroma	0.3	1.3	0.6
Muscle	1.59	1.67	1
Arterioles	2.36	2.67	2.56

Values represent the mean staining intensity from a minimum of three cases for each endometrial stage: proliferative, secretory and inactive. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

Table 2.3: Mean intensity of PDGF-R β antibody staining in human uterus

	Proliferative	Secretory	Inactive
Luminal epithelium	1	0.25	1.21
Superficial glands	0.8	0.77	0.78
Deep glands	1.8	0.87	0.7
Superficial stroma	1.14	1.1	2
Deep stroma	0.3	1.4	0.86
Muscle	1.3	1.17	0.57
Arterioles	2.8	2.75	3

Values represent the mean staining intensity from a minimum of three cases for each endometrial stage: proliferative, secretory and inactive. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1= in each mild staining, 2 = moderate staining, 3 = strong staining.

Table 2.4: Mean intensity of Vimentin antibody staining in human uterus

	Proliferative	Secretory	Inactive
Luminal epithelium	0	0	0.2
Superfical glands	0.25	0.58	0.3
Deep glands	2	1.9	2
Superficial stroma	0.75	1	0
Deep stroma	0.5	0.41	0.7
Muscle	0.17	0	0.5
Arterioles	2.8	3	2.9

Values represent the mean staining intensity from a minimum of three cases for each endometrial stage: proliferative, secretory and inactive. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1= in each mild staining, 2 = moderate staining, 3 = strong staining.

Table 2.5: Correlation of proportion of PCNA positive nuclei with mean immunoreactivity of PDGF-R β , PDGF-R α and vimentin in human endometrium

	PDGF-R α	PDGF-R β	Vimentin
Luminal epithelium:	-0.43 *	0.97	0.5
Superficial glands	-0.02	0.98	-0.73
Deep glands	0.98	0.75	-0.33
Stroma	-0.15	-0.30	0.32

* r values were derived by Pearson correlation analysis . Each r value (e.g., PCNA correlation with PDGF-R α) represents a comparison of the change in the mean values of the immunoreactivity for PCNA with the change in the immunoreactivity of the other antibodies examined (PDGF-R α , PDGF-R β and vimentin) in compartments between the proliferative, secretory and inactive endometrial stages examined. Positive values approaching 1.0 indicate a strong association of values, values approaching 0 indicate a lack of correlation and values approaching -1.0 indicate a negative regional correlation between the immunoreactivity of the antibodies in the endometrium.

Table 2.6: Correlation of mean immunoreactivity of PDGF-R α with mean immunoreactivity of PDGF-R β , vimentin and proportion of PCNA positive nuclei in human endometrium.

	PCNA	PDGF-R β	Vimentin
luminal epithelium:	-0.43*	-0.61	-0.99
superficial glands	-0.02	-0.19	0.70
deep glands	0.98	0.90	-0.08
stroma	-0.15	0.97	-0.62

* r values were derived by Pearson correlation analysis . Each r value (e.g., PDGF-R α correlation with PCNA) represents a comparison of the change in the mean values of the immunoreactivity for PCNA with the change in the immunoreactivity of the other antibodies examined (PDGF-R α , PDGF-R β and vimentin) in compartments between the proliferative, secretory and inactive endometrial stages examined. Positive values approaching 1.0 indicate a strong association of values, values approaching 0 indicate a lack of correlation and values approaching -1.0 indicate a negative regional correlation between the immunoreactivity of the antibodies in the endometrium.

Table 2.7: Correlation of mean immunoreactivity of PDGF-R β with mean immunoreactivity of PDGF-R α , vimentin and proportion of PCNA positive nuclei in human endometrium.

	PCNA	PDGF-R α	Vimentin
luminal epithelium:	0.98*	-0.61	0.67
superfical glands	0.98	-0.19	-0.84
deep glands	0.76	0.90	0.67
stroma	-0.32	0.97	-0.54

* r values were derived by Pearson correlation analysis . Each r value (e.g., PDGF-R α correlation with PCNA) represents a comparison of the change in the mean values of the immunoreactivity for PCNA with the change in the immunoreactivity of the other antibodies examined (PDGF-R α , PDGF-R β and vimentin) in compartments between the proliferative, secretory and inactive endometrial stages examined. Positive values approaching 1.0 indicate a strong association of values, values approaching 0 indicate a lack of correlation and values approaching -1.0 indicate a negative regional correlation between the immunoreactivity of the antibodies in the endometrium.

Table 3.1: Mean proportion of PCNA positive nuclei in hyperplastic human endometrium

	adenomatous hyperplasia	cystic hyperplasia
Luminal epithelium	13 %	16 %
Superficial glands	37 %	27.5 %
Deep glands	40 %	34 %
Superficial stroma	20 %	29 %
Deep stroma	20 %	41 %

Values represent the mean of the combined percentages of PCNA positive cells from a minimum of three cases of each of the morphologic types of endometrial hyperplasia (adenomatous and cystic). Two slides were immunostained for each case. Percentages were derived from the number of positive staining nuclei out of one hundred cells in each tissue compartment of each slide examined..

Table 3.2: Mean intensity of PDGF-R α immunoreactivity in hyperplastic human uteri

	adenomatous hyperplasia	cystic hyperplasia
Luminal epithelium	0.04	0.3
Superficial glands	0.4	0.45
Deep glands	0.92	1.85
Superficial stroma	1	0.7
Deep stroma	1.5	0.85

Values represent the mean staining intensity from a minimum of three cases for each of the morphologic types of endometrial hyperplasia (adenomatous and cystic). Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

Table 3.3: Mean intensity of PDGF-R β immunoreactivity in hyperplastic human uteri

	adenomatous hyperplasia	cystic hyperplasia
Luminal epithelium	0.14	0.13
Superficial glands	0.8	0.47
Deep glands	1.7	1.85
Superficial stroma	0.5	0.77
Deep stroma	1	0.93

Values represent the mean staining intensity from a minimum of three cases for each of the morphologic types of endometrial hyperplasia (adenomatous and cystic). Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

Table 3.4: Mean intensity of vimentin immunoreactivity in hyperplastic human uteri

	adenomatous hyperplasia	cystic hyperplasia
Luminal epithelium	0.1	0
Superficial glands	0.13	0.5
Deep glands	2.1	2.2
Superficial stroma	0.2	1.6
Deep stroma	1.75	0.9

Values represent the mean staining intensity from a minimum of three cases for each of the morphologic types of endometrial hyperplasia (adenomatous and cystic). Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

Table 4.1 Mean proportion of PCNA positive nuclei in murine endometrium

	Luminal epithelium	Glandular epithelium	Stroma
Diestrus	45.0	62.5	61.2
Proestrus	72.2	73.9	36.7
Estrus	67.5	73.8	35.6
Metestrus	74.4	51.3	23.1

Values represent the mean of the combined percentages of PCNA positive cells from a minimum of three cases of each of the estrous cycle stages: diestrus, proestrus, estrus and metestrus. Two slides were immunostained for each case. Percentages were derived from the number of positive staining nuclei out of one hundred cells in each tissue compartment of each slide examined..

Table 4.2: Mean intensity of PDGF-R β immunoreactivity in murine endometrium

	Luminal epithelium	Glandular epithelium	Stroma
Diestrus	1.5	0.88	0.5
Proestrus	1.55	1.45	0.48
Estrus	1.67	1.58	0.58
Metestrus	1.36	1.1	0.67

Values represent the mean staining intensity from a minimum of three cases for each of the estrous cycle stages: diestrus, proestrus, estrus and metestrus. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

Table 4.3: Mean intensity of PDGF-R β immunoreactivity in murine endometrium

	Luminal epithelium	Glandular epithelium	Stroma
Diestrus	1.04	0.38	1.63
Proestrus	1.21	0.91	1.34
Estrus	1.63	1.34	1.19
Metestrus	0.92	0.88	0.29

Values represent the mean staining intensity from a minimum of three cases for each of the estrous cycle stages: diestrus, proestrus, estrus and metestrus. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

Appendix B

Figures

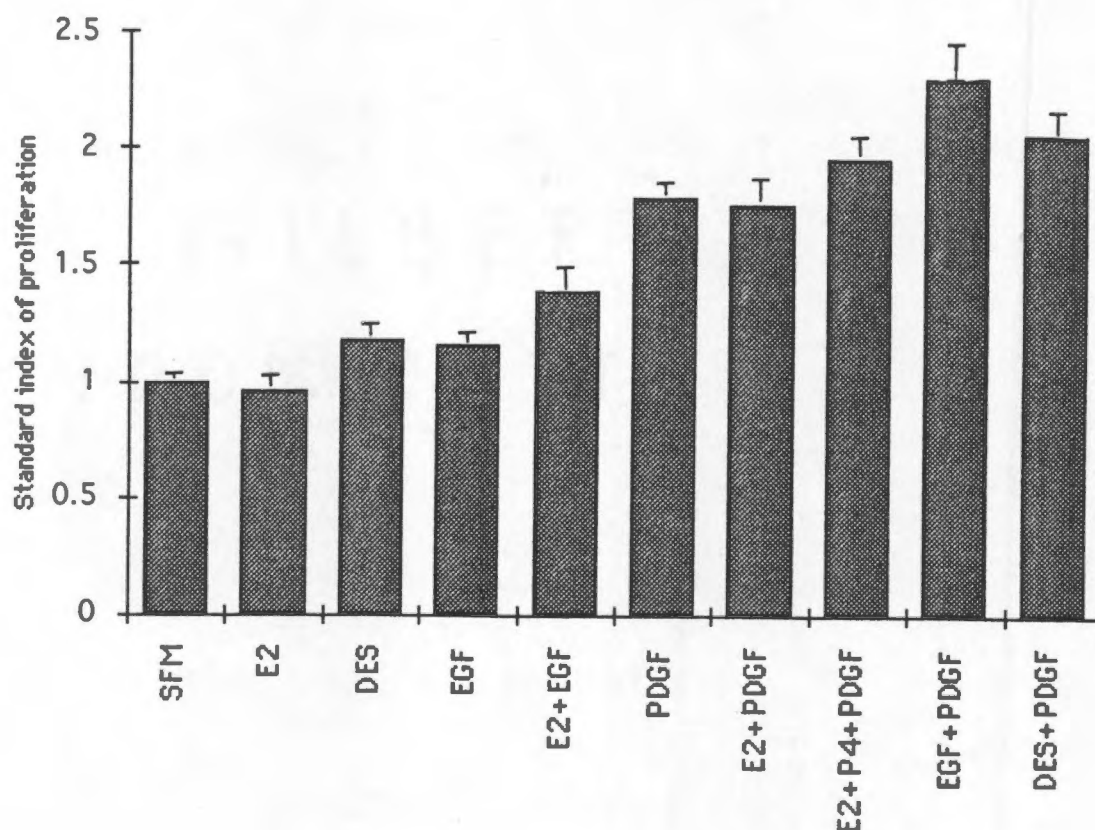


Figure 1.1: Proliferative response of cultured human endometrial epithelial cells to steroidal hormones and growth factors.

Subconfluent cultures of hEEC were treated with the estrogens 17- β estradiol (E2) (350 pg/ml) & diethylstilbestrol (DES) (2×10^{-7} M), the growth factors epidermal growth factor (EGF) (10 ng/ml) & platelet derived growth factor AB (PDGF AB) (10 ng/ml) or combinations of these hormones and growth factors for 24 hours and pulsed with ^3H thymidine (1 μCi). Uptake of ^3H thymidine was standardized to the controls and data from multiple cell lines combined. Each bar represents the sum of triplicate assays of at least three different cell lines. Error bars represent the standard error of the mean. Single asterisks signify a significant ($p < 0.001$) proliferative response compared to media without growth factor or steroid additives (SFM).

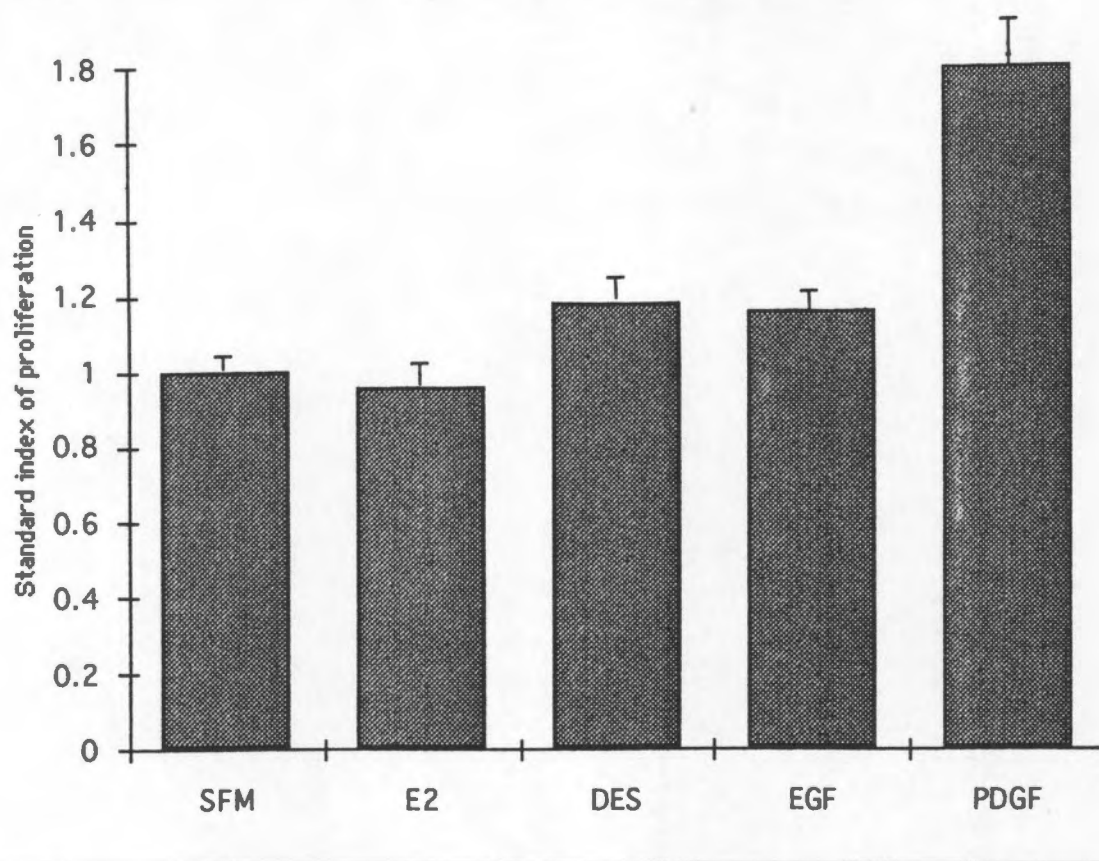


Figure 1.2: Proliferative responses of cultured human endometrial epithelial cells to estrogens, EGF and PDGF AB.

Subconfluent cultures of hEEC were treated with the estrogens 17- β estradiol (E2) (350 pg/ml) & diethylstilbestrol (DES) (2×10^{-7} M), or the growth factors epidermal growth factor (EGF) (10 ng/ml) & platelet derived growth factor AB (PDGF AB) (10 ng/ml) for 24 hours and pulsed with ^{3} H thymidine (1 μ Ci).

Uptake of ^{3} H thymidine was standardized to the controls. Each bar represents the sum of triplicate assays of at least three different cell lines. Error bars represent the standard error of measurement. Only treatment with PDGF elicited a significant ($p < 0.001$) proliferative response compared to control (SFM).

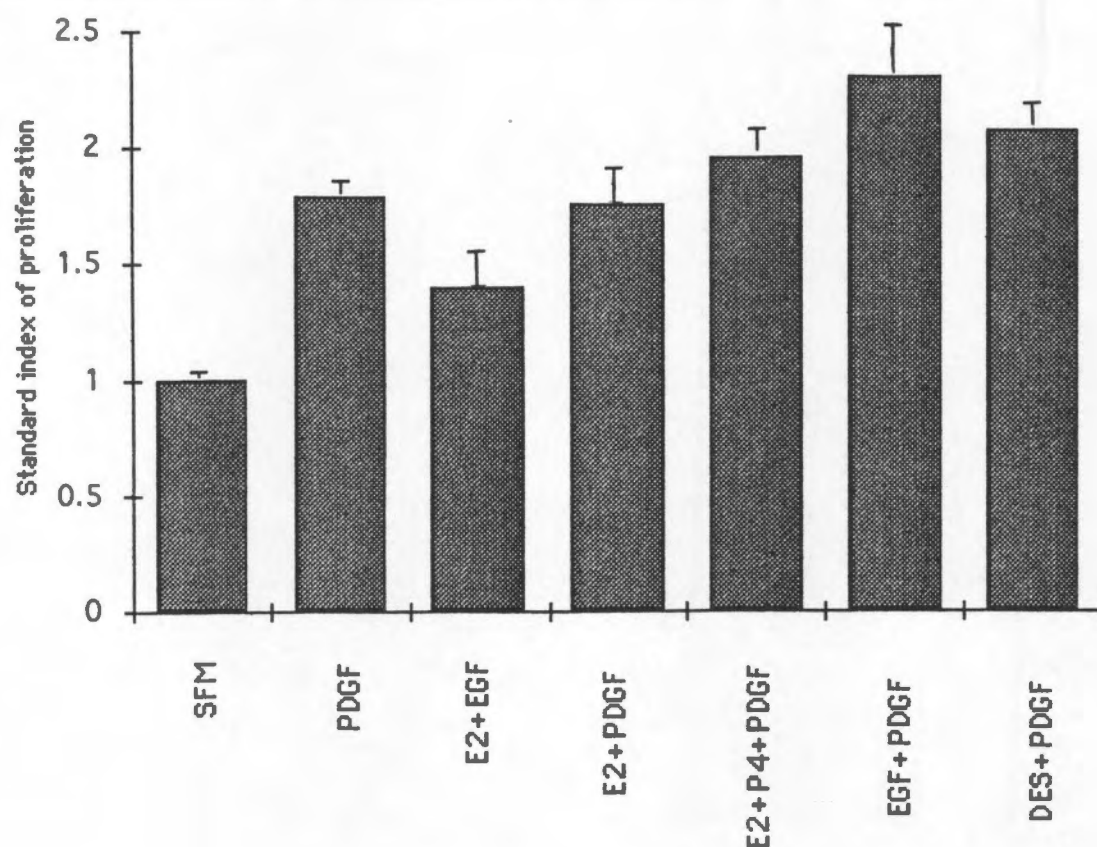


Figure 1.3: Proliferative responses of cultured human endometrial epithelial cells to combinations of estrogens, EGF and PDGF AB.

Subconfluent cultures of hEEC were treated with the estrogens 17- β estradiol (E2) (350 pg/ml) & diethylstilbestrol (DES) (2×10^{-7} M), or the growth factors epidermal growth factor (EGF) (10 ng/ml) & platelet derived growth factor AB (PDGF AB) (10 ng/ml) or combinations of these hormones and growth factors for 24 hours and pulsed with ^3H thymidine (1 μCi). Uptake of ^3H thymidine was standardized to the controls. Each bar represents the sum of triplicate assays of at least three different cell lines. Error bars represent the standard error of measurement. Single asterisks signify a significant ($p < 0.001$) proliferative response compared to control (SFM). Double asterisks signify a significant ($p < 0.01$) proliferative response compared to PDGF AB alone.

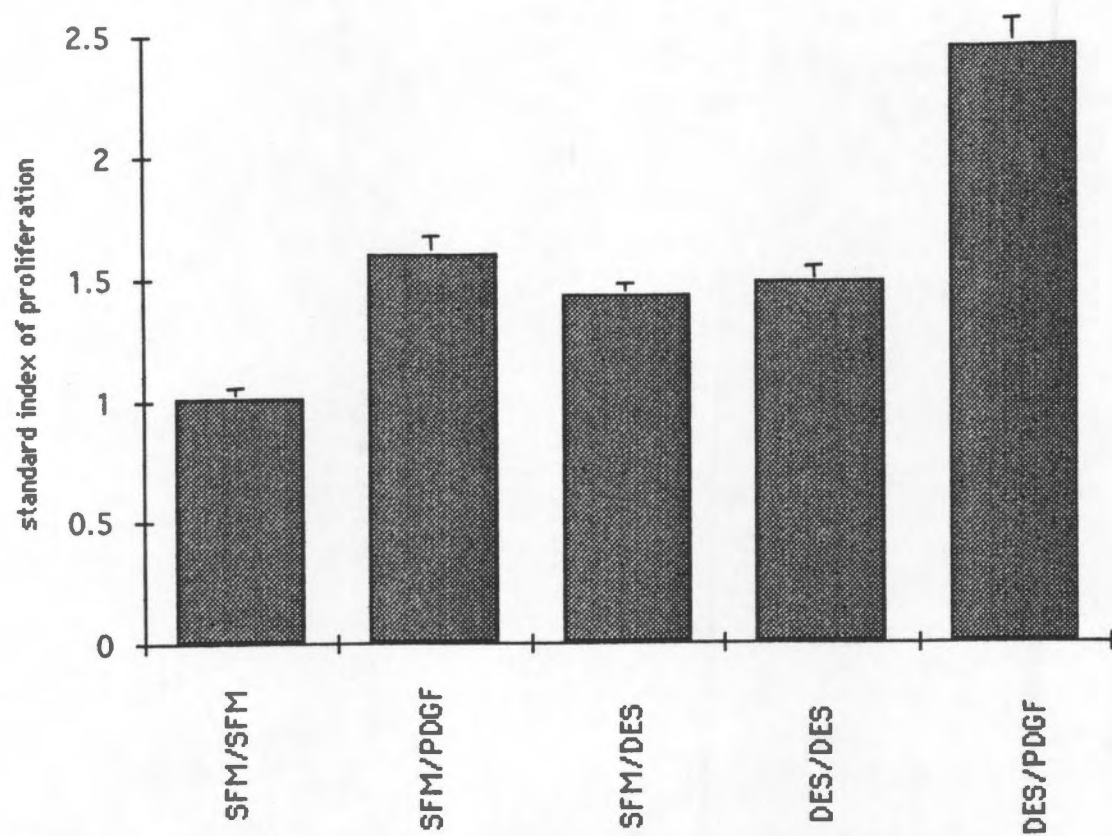


Figure 1.4: Proliferative response of human endometrial epithelial cells to DES and PDGF AB.

Subconfluent cultures of hEEC were treated with SFM or DES ($2 \times 10^{-7}M$) for 24 hours, followed by exposure to DES or PDGF AB (10 ng/ml). All treatments resulted in significant ($p < 0.001$) proliferation above control (SFM/SFM). The combination of DES followed by PDGF AB (DES/PDGF) elicited a significantly ($p < 0.001$) higher proliferative response than any other treatment.

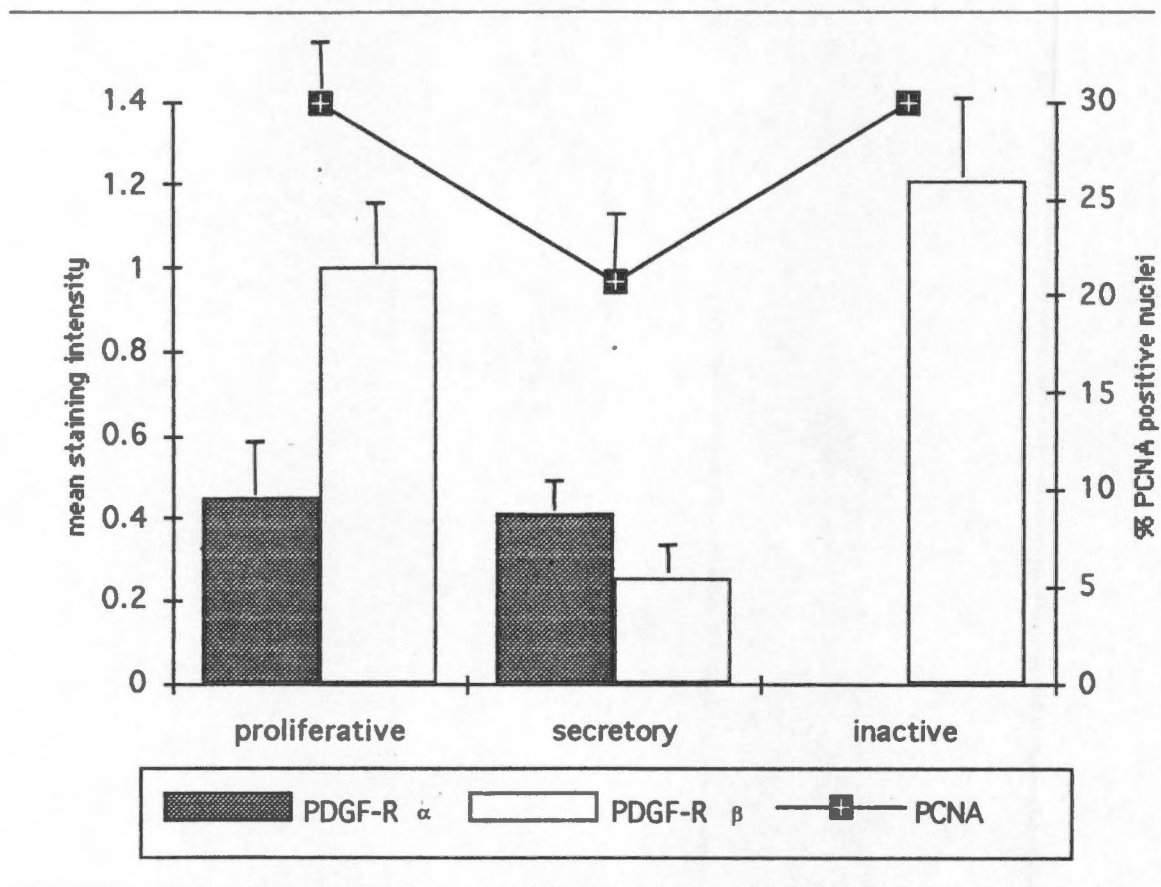


Figure 2.1: Association between PCNA, PDGF-R α and PDGF-R β immunoreactivity in human endometrial luminal epithelium.

Association of PCNA, PDGF-R α and PDGF-R β expression is illustrated in this overlay graph of the luminal epithelium in proliferative, secretory and inactive endometrium. Results are represented as mean values for a minimum of 3 samples in each morphologic group.

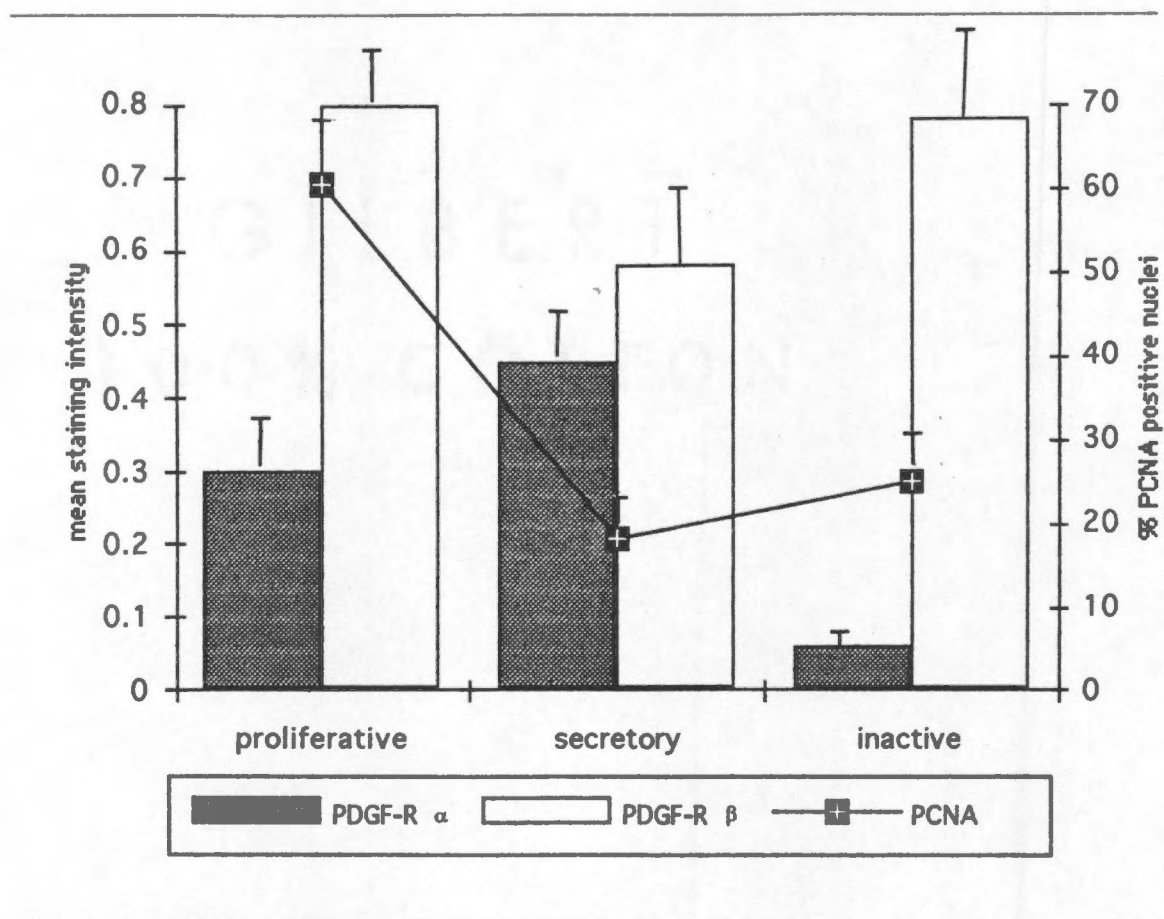


Figure 2.2: Association between PCNA, PDGF-R α and PDGF-R β immunoreactivity in human endometrial superficial glandular epithelium.

Association of PCNA, PDGF-R α and PDGF-R β expression is illustrated in this overlay graph of the luminal epithelium in proliferative, secretory and inactive endometrium. Results are represented as mean values for a minimum of 3 samples in each morphologic group.

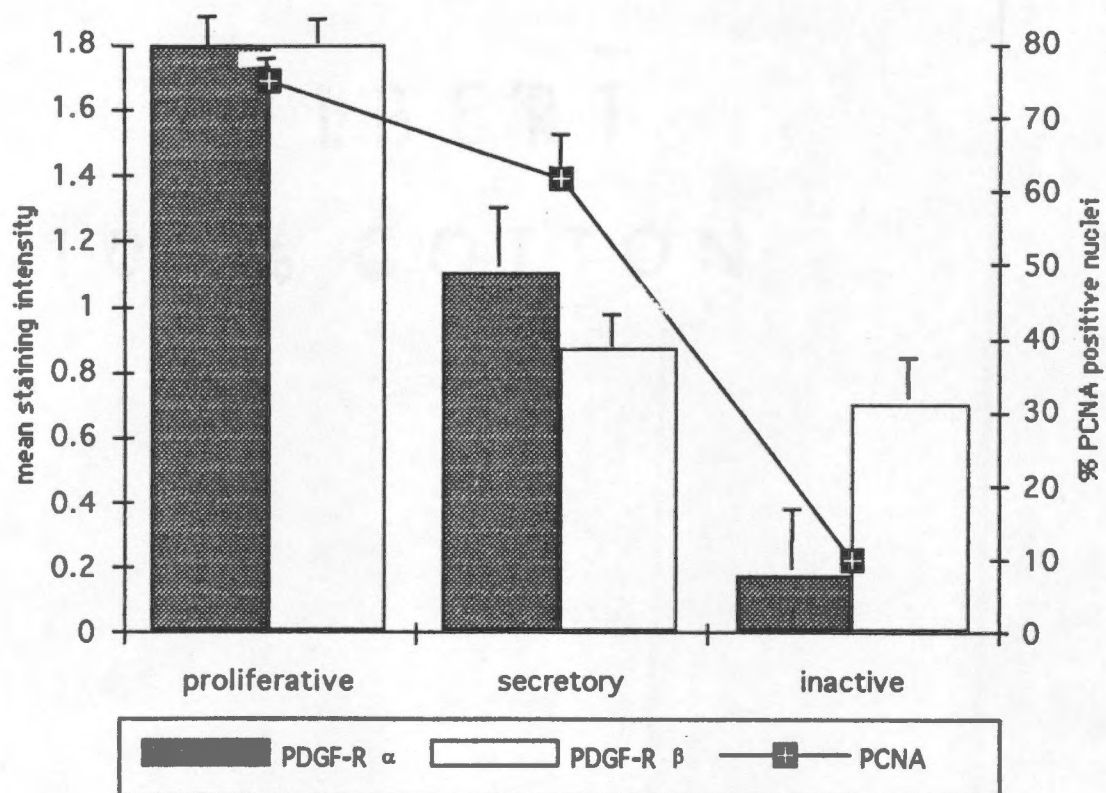


Figure 2.3: Association between PCNA, PDGF-R α and PDGF-R β immunoreactivity in human endometrial deep glandular epithelium.

Association of PCNA, PDGF-R α and PDGF-R β expression is illustrated in this overlay graph of the luminal epithelium in proliferative, secretory and inactive endometrium. Results are represented as mean values for a minimum of 3 samples in each morphologic group.

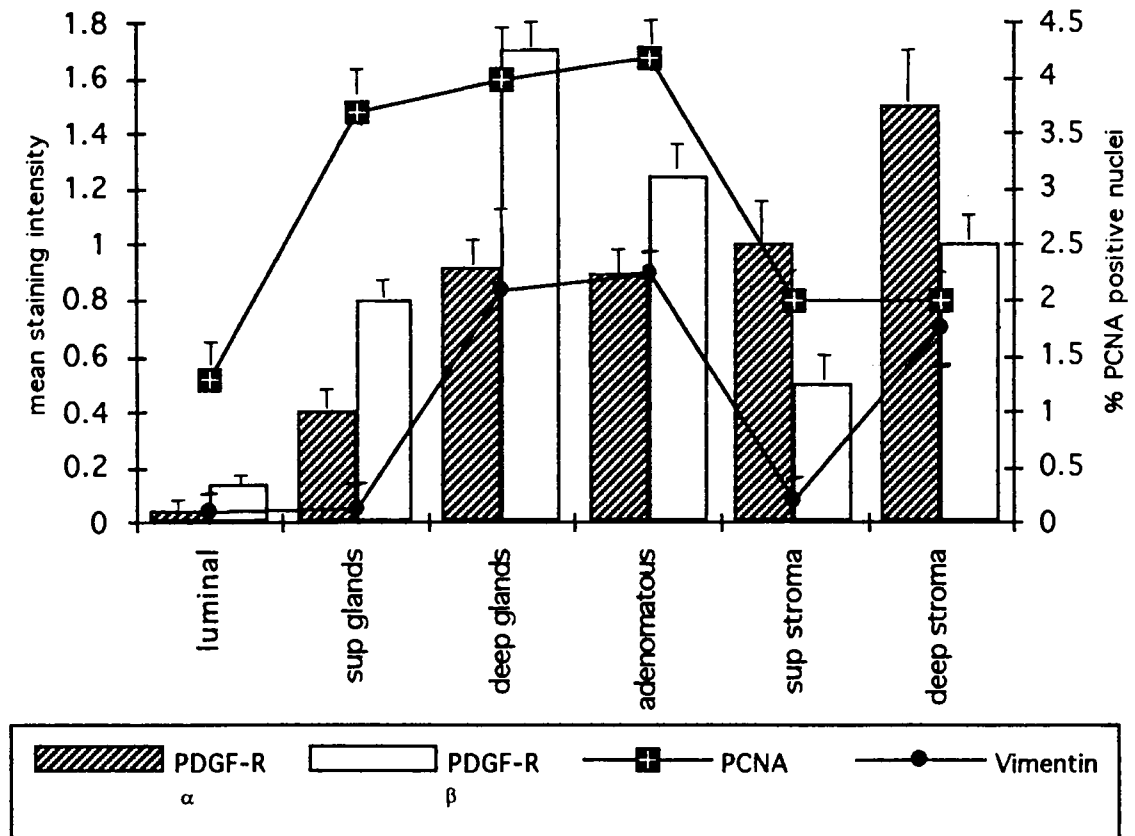


Figure 3.1: Association between PCNA, PDGF-R α , PDGF-R β and vimentin immunoreactivity in endometrial adenomatous hyperplasia of women.

Association of PCNA, PDGF-R α , PDGF-R β and vimentin expression is illustrated in this overlay graph of the endometrium from women with adenomatous endometrial hyperplasia. Results are represented as mean values for a minimum of 3 samples in each morphologic group.

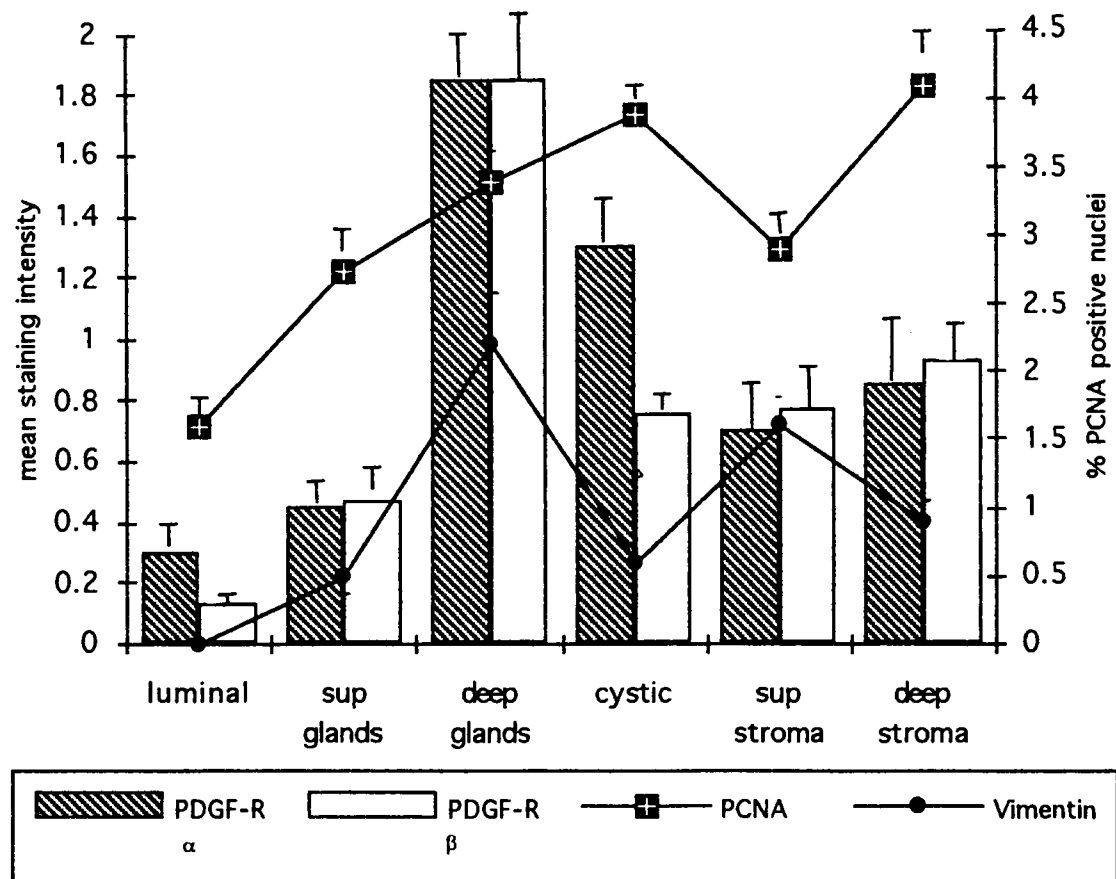


Figure 3.2: Association between PCNA, PDGF-R α , PDGF-R β and vimentin immunoreactivity in endometrial cystic hyperplasia of women.

Association of PCNA, PDGF-R α , PDGF-R β and vimentin expression is illustrated in this overlay graph of the endometrium from women with cystic endometrial hyperplasia. Results are represented as mean values for a minimum of 3 samples in each morphologic group.

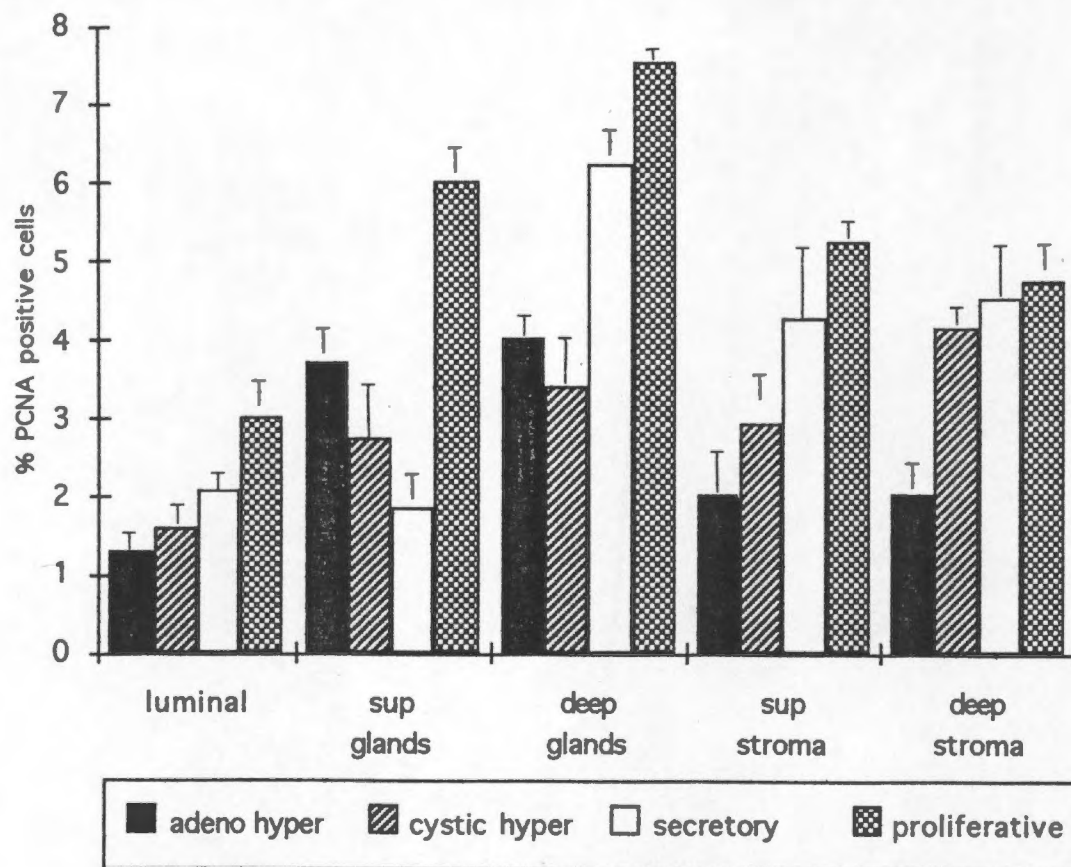


Figure 3.3: Proportion of PCNA immunoreactive nuclei in hyperplastic, proliferative and secretory human endometrium

Values represent the mean of the combined percentages of PCNA positive cells from a minimum of three cases for each endometrial stage examined: proliferative, secretory and inactive. Two slides were immunostained for each case. Percentages were derived from the number of positive staining nuclei out of one hundred cells in each tissue compartment of each slide examined..

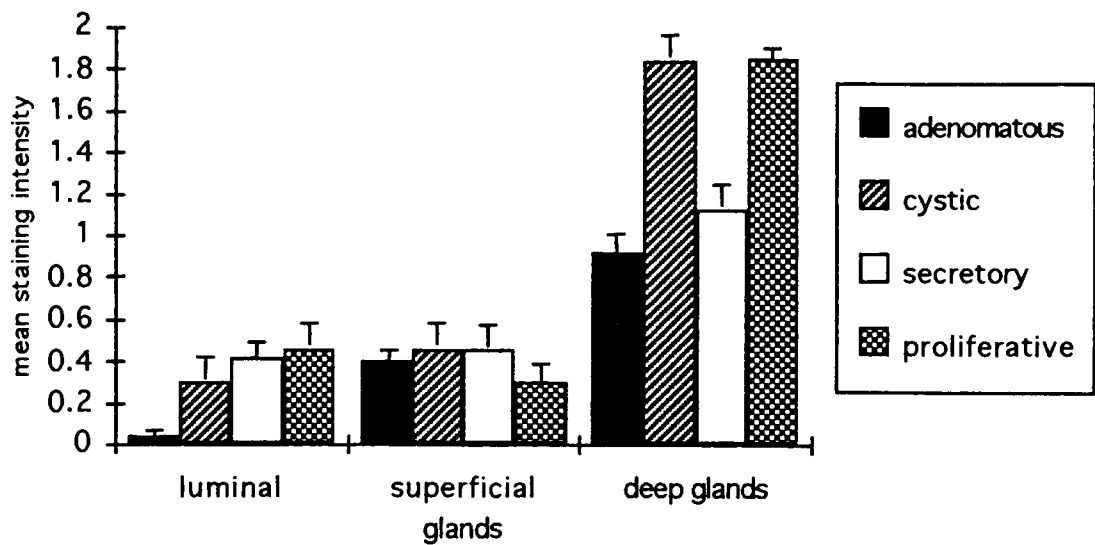


Figure 3.4: Immunoreactivity for PDGF-R α in hyperplastic, proliferative and secretory human endometrium

Values represent the mean staining intensity from a minimum of three cases of proliferative and secretory stage endometrium and adenomatous and cystic endometrial hyperplasia.. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1= in each mild staining, 2 = moderate staining, 3 = strong staining.

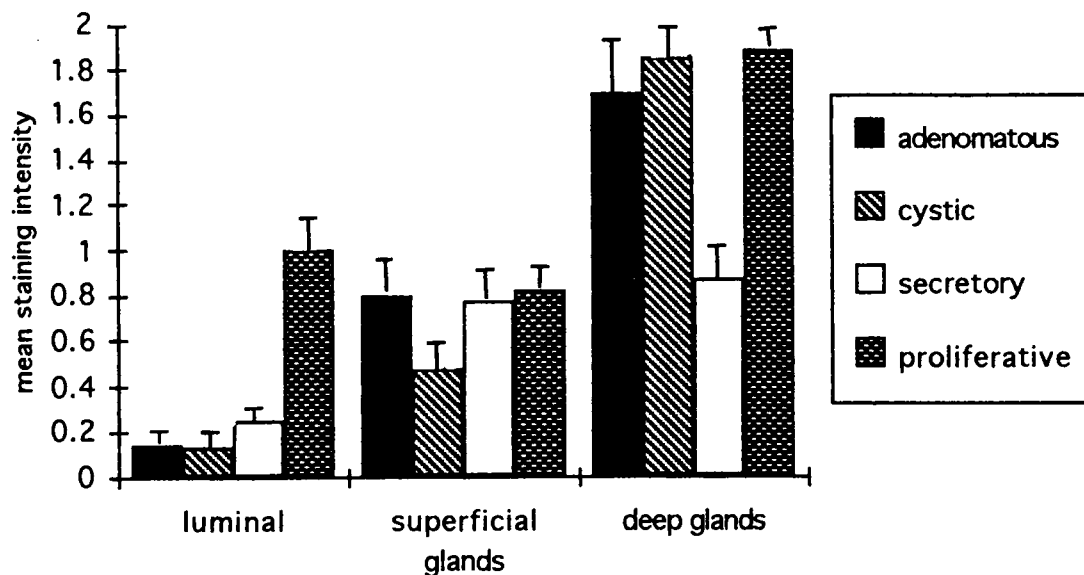


Figure 3.5: Immunoreactivity for PDGF-R β in hyperplastic, proliferative and secretory human endometrium

Values represent the mean staining intensity from a minimum of three cases of proliferative and secretory stage endometrium and adenomatous and cystic endometrial hyperplasia. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

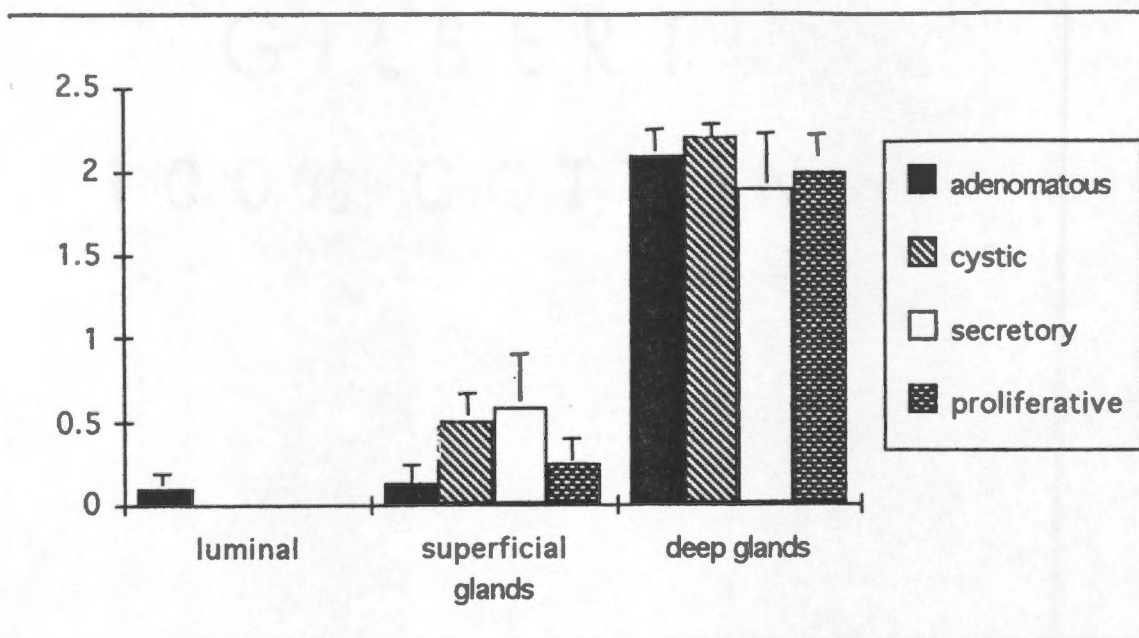


Figure 3.6: Immunoreactivity for vimentin in hyperplastic, proliferative and secretory human endometrium

Values represent the mean staining intensity from a minimum of three cases of proliferative and secretory stage endometrium and adenomatous and cystic endometrial hyperplasia. Two slides were immunostained for each case. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

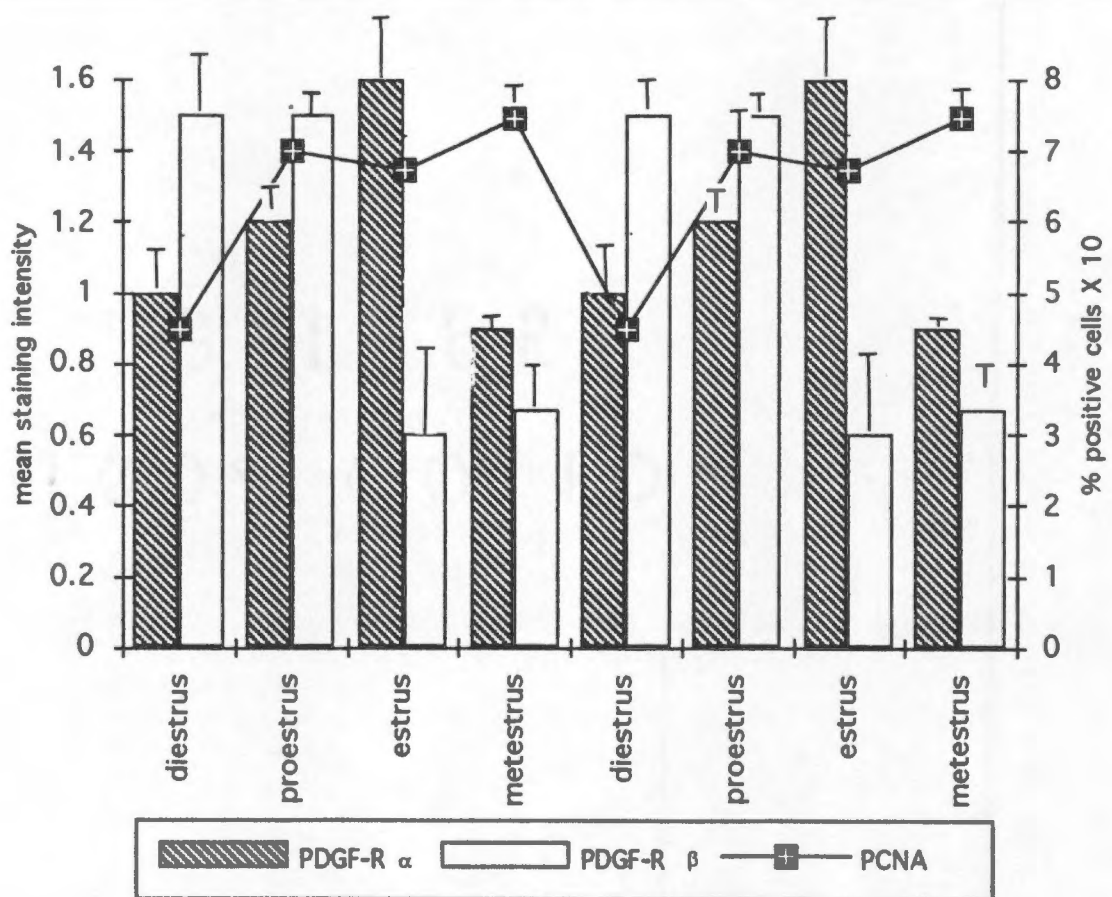


Figure 4.1: Association between PCNA, PDGF-R α and PDGF-R β immunoreactivity in the luminal epithelium of the endometrium of mice

Association of PCNA, PDGF-R α and PDGF-R β expression is illustrated in this overlay graph of the luminal epithelium in the diestrus, proestrus, estrus and metestrus stages of the murine endometrium. Results are represented as mean values for a minimum of 3 samples in each morphologic group. Results have been duplicated in this graph to emphasize the cyclic expression of the immunoreactivity. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

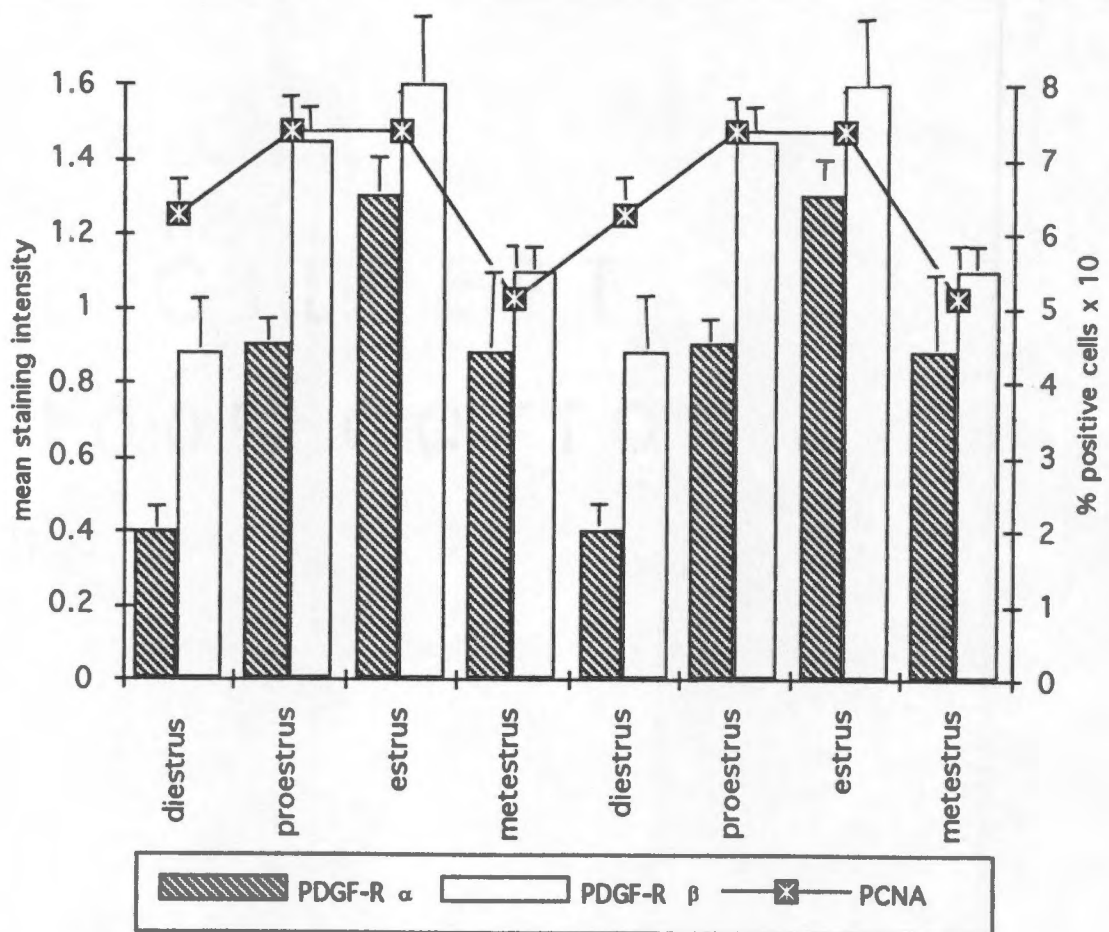


Figure 4.2: Association between PCNA, PDGF-R α and PDGF-R β immunoreactivity in the glandular epithelium of the endometrium of mice

Association of PCNA, PDGF-R α and PDGF-R β expression is illustrated in this overlay graph of the glandular epithelium in the diestrus, proestrus, estrus and metestrus stages of the murine endometrium. Results are represented as mean values for a minimum of 3 samples in each morphologic group. Results have been duplicated in this graph to emphasize the cyclic expression of the immunoreactivity. Immunoreactivity was graded on a 0-3 scale: 0 = no staining, 1 = in each mild staining, 2 = moderate staining, 3 = strong staining.

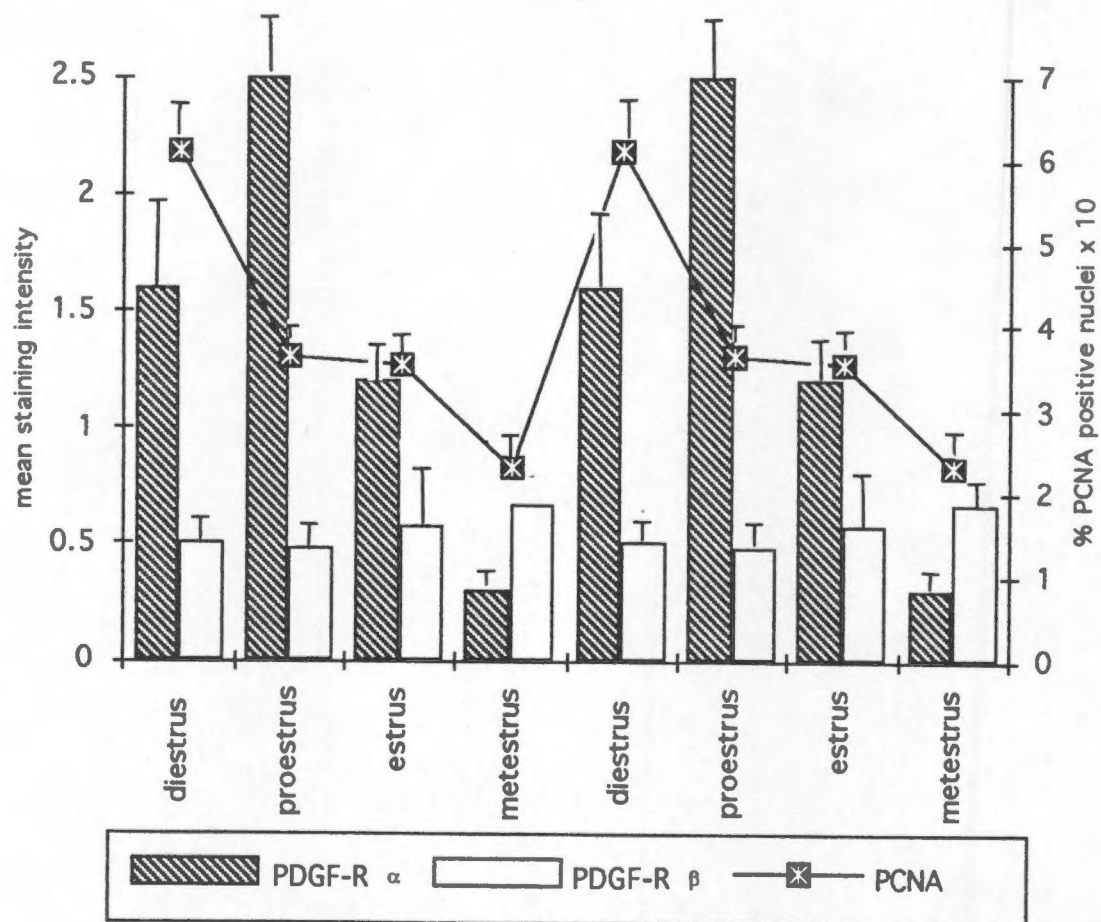


Figure 4.3: Association between PCNA, PDGF-R α and PDGF-R β immunoreactivity in the stroma of the endometrium of mice

Association of PCNA, PDGF-R α and PDGF-R β expression is illustrated in this overlay graph of the stroma in the diestrus, proestrus, estrus and metestrus stages of the murine endometrium. Results are represented as mean values for a minimum of 3 samples in each morphologic group. Results have been duplicated in this graph to emphasize the cyclic expression of the immunoreactivity.

Appendix C

Color Plates

Color Plate 1

Photomicrograph 1.1: Three day outgrowth of epithelial cells from isolated human endometrial glandular explant.

Photomicrograph 1.2: Confluent six month monolayer of human endometrial epithelial cells (hEEC). Cells exhibited polygonal morphology and contact inhibition growth behavior.

Photomicrograph 1.3: Immunoreactivity of monolayer hEEC to pancytokeratin intermediate filament antibodies. Positive perinuclear staining was present in all cells.

Photomicrograph 1.4: Immunoreactivity of monolayer hEEC to monoclonal antibody for cytokeratin 8, 18, 19 intermediate filaments which are associated with glandular epithelia. Immunoreactivity varied from strong to weak. NBT-BCIP chromagen. no counterstain

Photomicrograph 1.5: Immunoreactivity of monolayer hEEC to polyclonal PDGF-R β antibody. Positive granular cytoplasmic and membrane staining was present in cells. NBT-BCIP chromagen. no counterstain

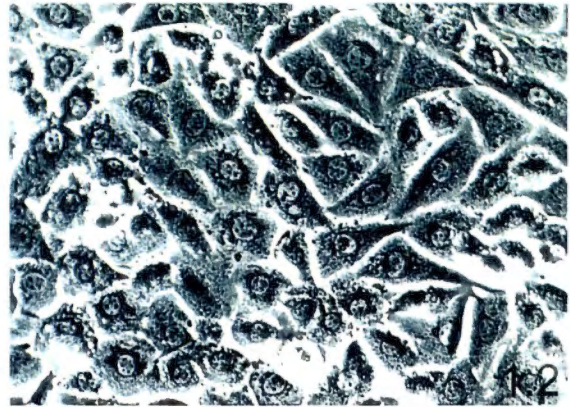
Photomicrograph 1.6: Immunoreactivity of monolayer hEEC to polyclonal PDGF-R α antibody. Positive granular cytoplasmic and membrane staining was present in cells. NBT-BCIP chromagen. no counterstain

Photomicrograph 1.7: Immunoreactivity of monolayer hEEC to vimentin intermediate filament antibody. Positive perinuclear staining was present in all cells. Fast red chromagen with hematoxylin counterstain.

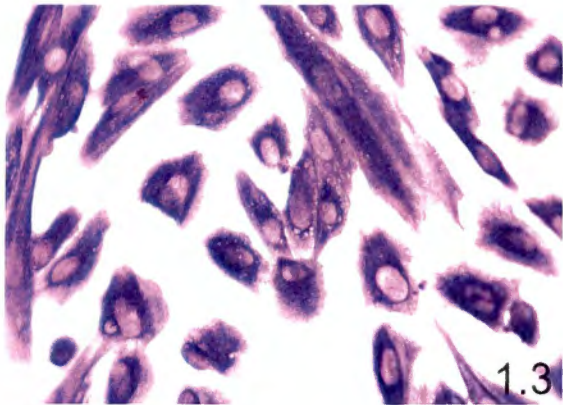
Photomicrograph 1.8: Dual staining of monolayer hEEC for pancytokeratin (brown, DAB chromagen) and PDGF-R β (blue, NBT-BCIP chromagen)



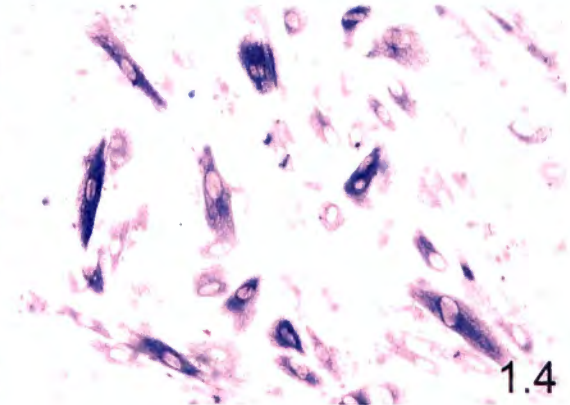
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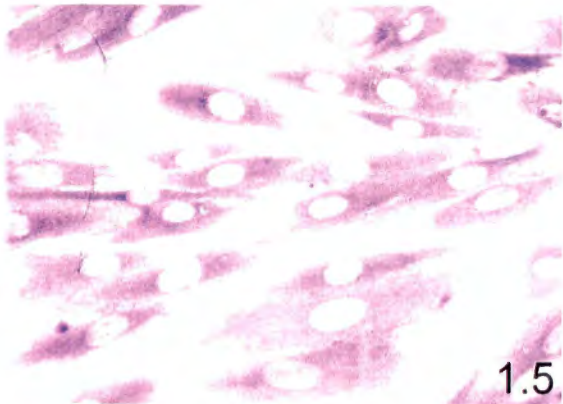
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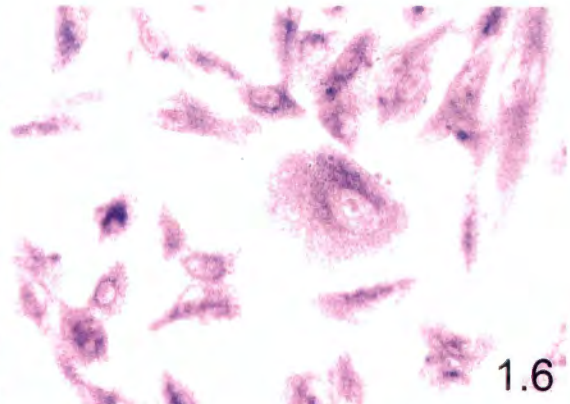
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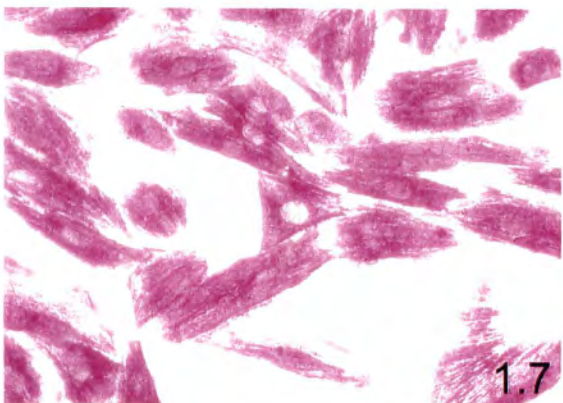
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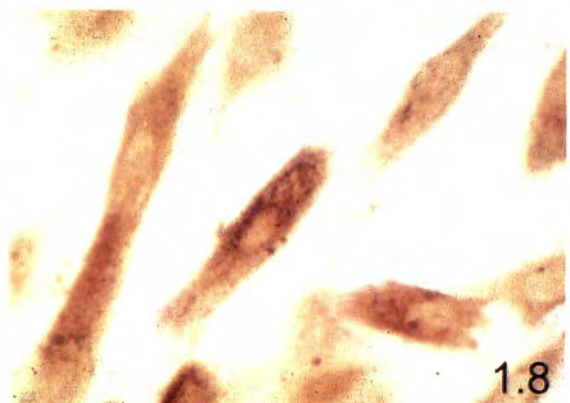
1.5



1.6



1.7



1.8

Color Plate 2

Photomicrograph 2.1: Positive immunoreactivity for PDGF-R α in malignant epithelium of human prostatic carcinoma served as the positive control. Fast red chromagen with light green counterstain

Photomicrograph 2.2: Negative control for PDGF-R α in human prostatic carcinoma. Non immune sera was substituted for PDGF-R α antibody.

Photomicrograph 2.3: Proliferative endometrium negative control for PDGF-R β . Fast red chromagen with hematoxylin counterstain. (compare to 2.5 & 2.7)

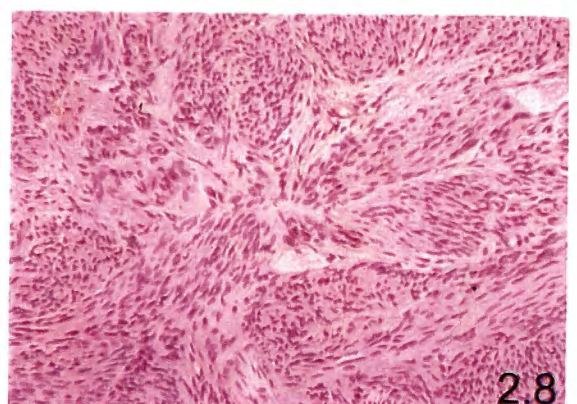
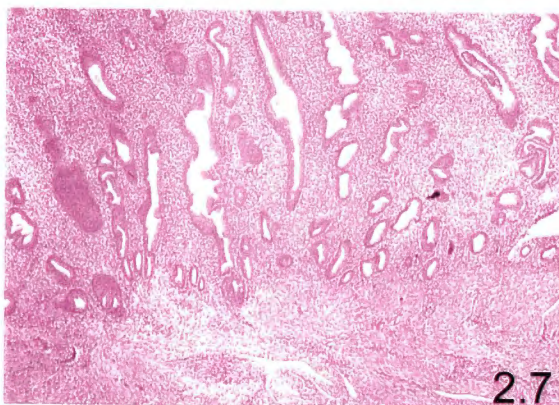
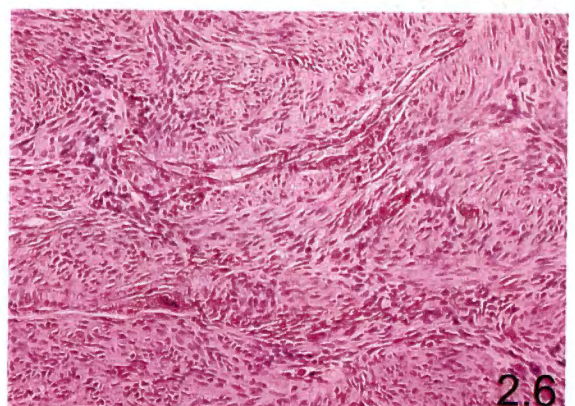
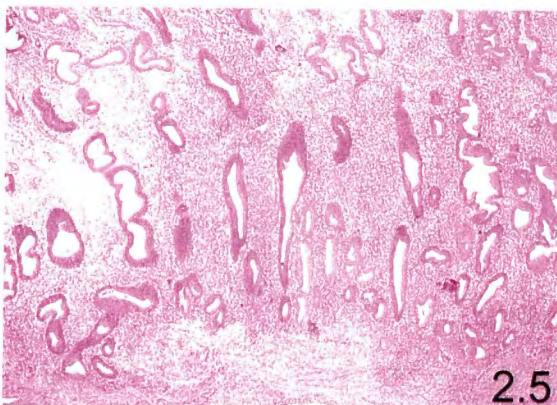
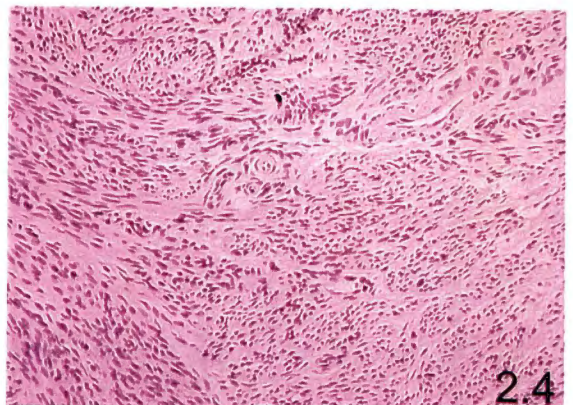
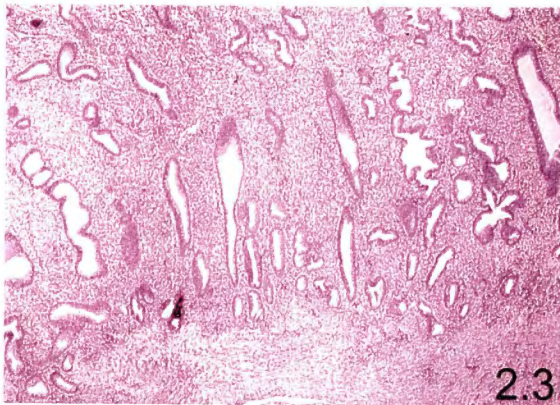
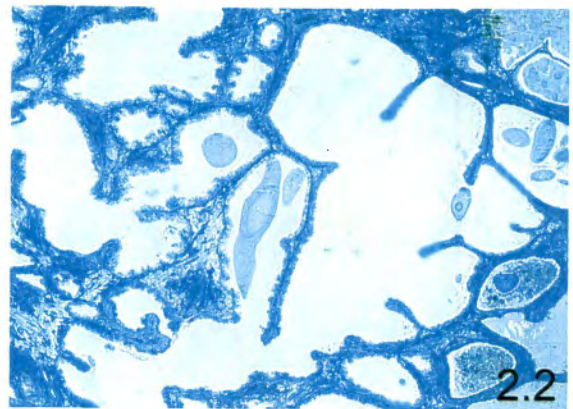
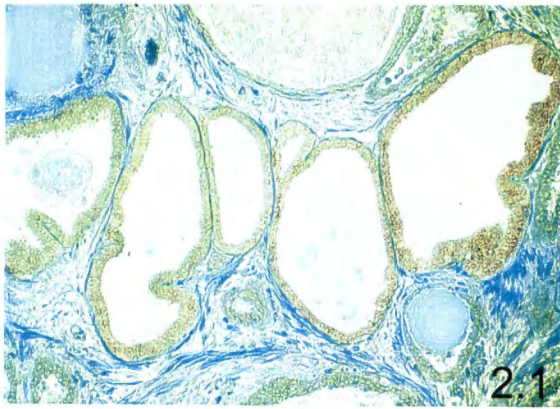
Photomicrograph 2.4: Myometrium and arterioles of proliferative stage endometrium. Negative control for PDGF-R β . (compare to 2.6 & 2.8)

Photomicrograph 2.5: Immunoreactivity of glands to PDGF-R β (1:1000) can be seen in this proliferative stage endometrium. Fast red chromagen with hematoxylin counterstain. (compare to 2.3 & 2.7)

Photomicrograph 2.6: Immunoreactivity of vessels to PDGF-R β (1:1000) can be seen in the myometrium of this proliferative stage endometrium. Fast red chromagen with hematoxylin counterstain. (compare to 2.4 & 2.8)

Photomicrograph 2.7: Immunoneutralization of PDGF-R β (1:1000) can be seen in the glands of this proliferative stage endometrium. Prior to immunostaining the PDGF-R β antibody was incubated with 10 μ m immunizing peptide for 1 hr at RT. Fast red chromagen with hematoxylin counterstain. (compare to 2.3 & 2.5)

Photomicrograph 2.8: Immunoneutralization of PDGF-R β (1:1000) can be seen in the myometrial arterioles of this proliferative stage endometrium. Prior to immunostaining the PDGF-R β antibody was incubated with 10 μ m immunizing peptide 1 hr at RT. Fast red chromagen with hematoxylin counterstain. (compare to 2.4 & 2.6)



Color Plate 2 (continued)

Photomicrograph 2.9: Human colon for PCNA immunoreactivity. No staining is apparent in this negative control. DAB chromagen (brown) with hematoxylin counterstain.

Photomicrograph 2.10: Human colon served as positive control for PCNA immunoreactivity. Positive nuclear immunoreactivity for PCNA can be seen in the colonic crypts. DAB chromagen with hematoxylin counterstain.

Photomicrograph 2.11: Proliferate stage human endometrium. H & E stain. (20X)

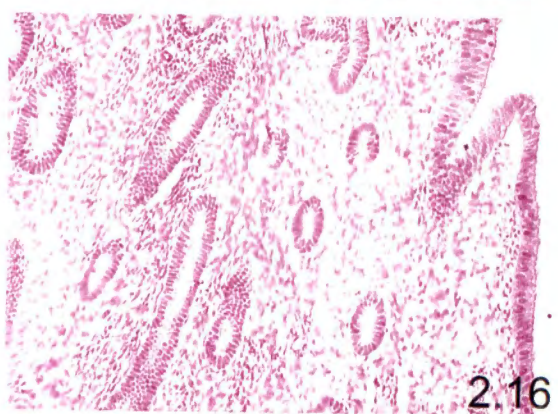
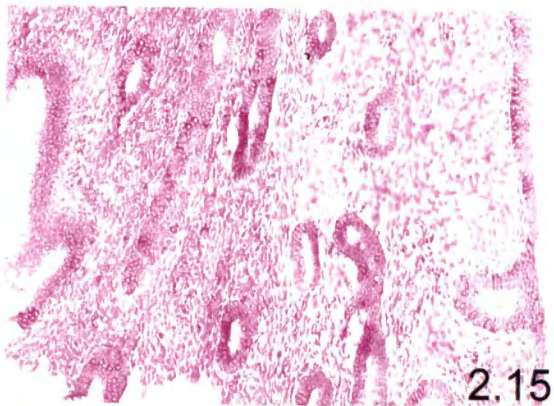
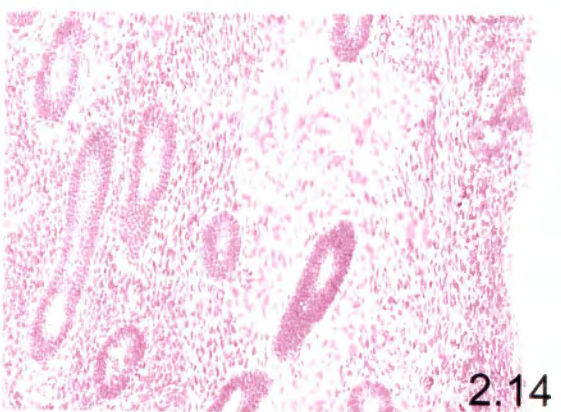
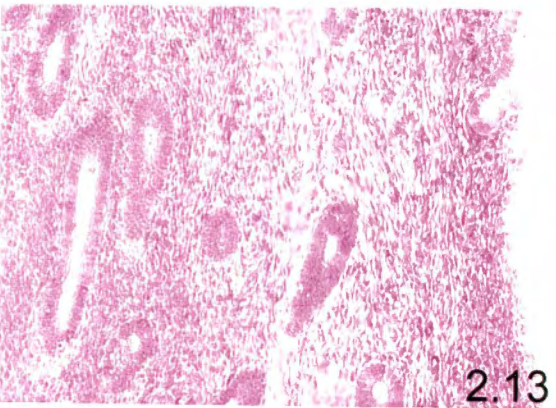
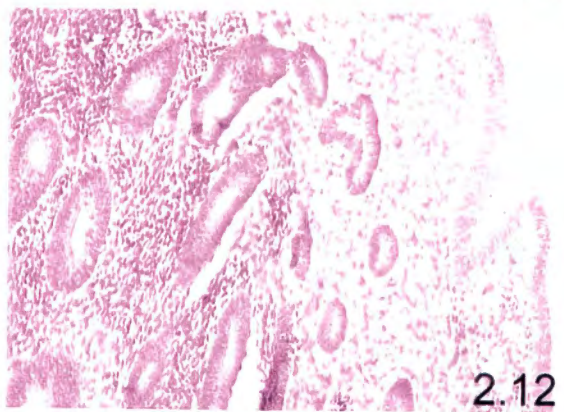
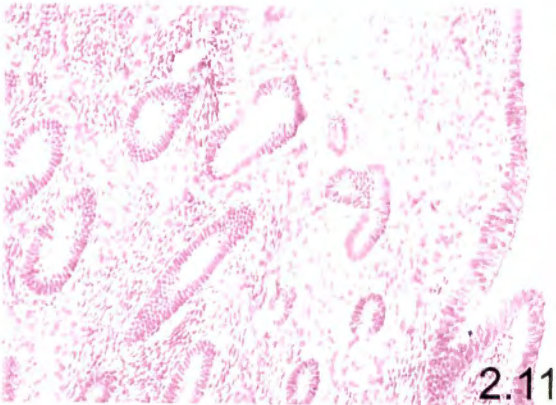
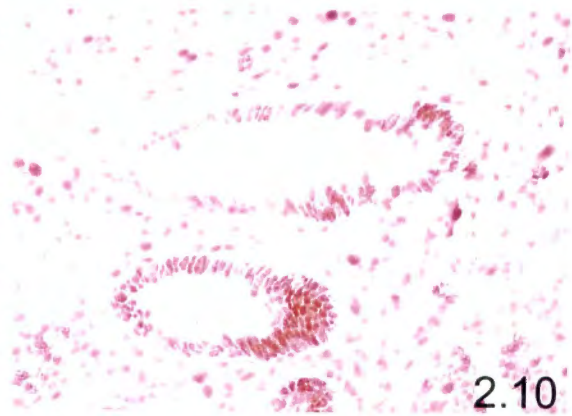
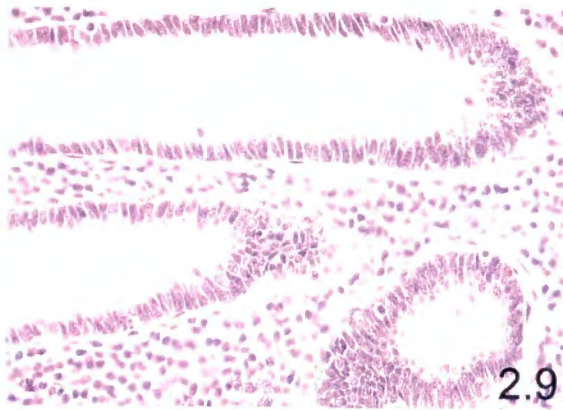
Photomicrograph 2.12: Proliferate stage human endometrium. Negative control with Vector red chromagen and hematoxylin counterstain. (20X)

Photomicrograph 2.13: Immunoreactivity for PDGF-R β is strongest in the deep glands of this proliferative stage human endometrium. Vector red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 2.14: Immunoreactivity for PDGF-R α is strongest in the deep glands of this proliferative stage human endometrium. Vector red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 2.15: Immunoreactivity for vimentin is strongest in the deep glands of this proliferative stage human endometrium. Vector red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 2.16: Positive nuclear immunoreactivity for PCNA is apparent in the epithelium and stroma of this proliferative stage human endometrium. Chromagen DAB with hematoxylin counterstain. (20X)



Color Plate 2 (continued)

Photomicrograph 2.17: Secretory stage human endometrium. H & E stain.

Photomicrograph 2.18: Secretory stage human endometrium. Negative control for the PDGF-R antibodies with Vector red chromagen and hematoxylin counterstain.

Photomicrograph 2.19: Immunoreactivity for PDGF-R β is strongest the stroma of this secretory stage human endometrium. (compare to 2.13) Vector red chromagen with hematoxylin counterstain. (10X)

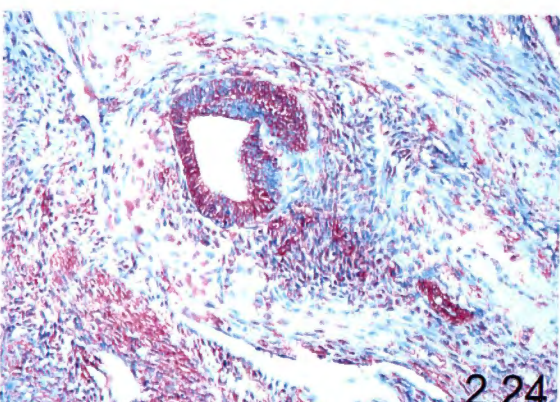
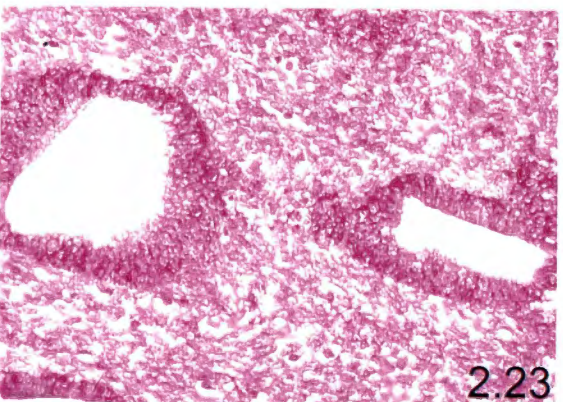
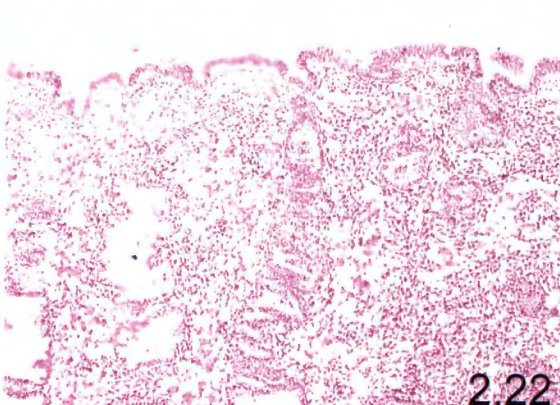
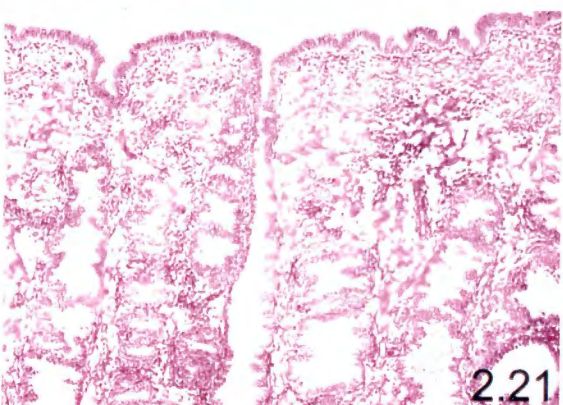
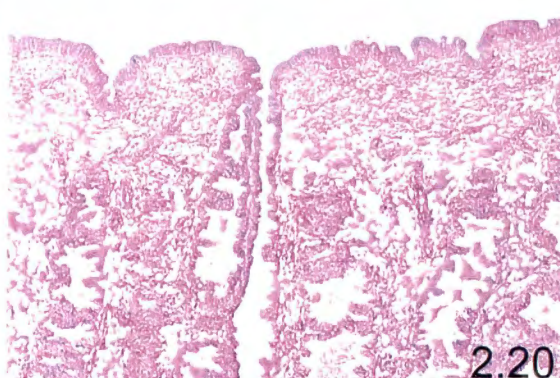
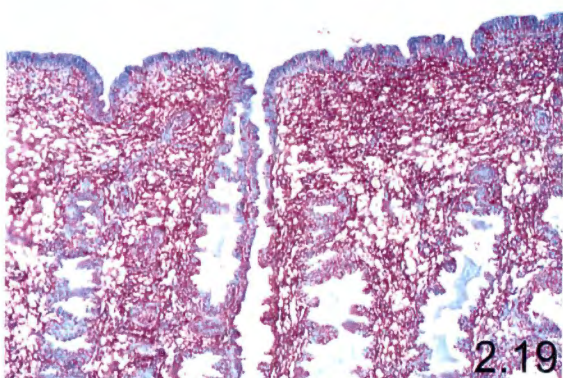
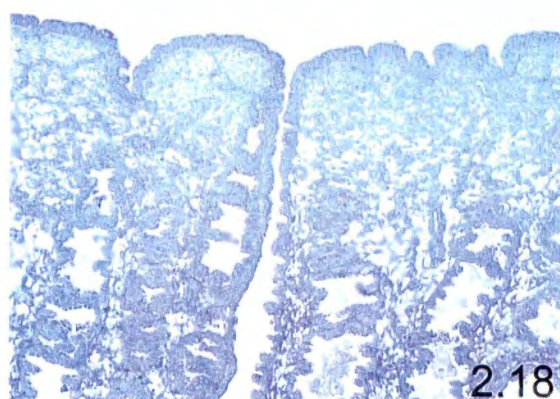
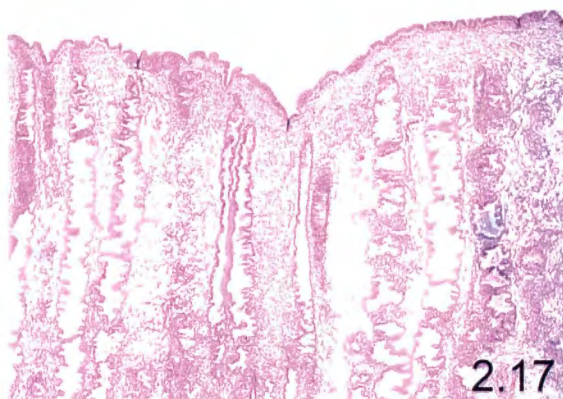
Photomicrograph 2.20: Immunoreactivity for PDGF-R α is strongest in the deep glands and arterioles of this secretory stage human endometrium. Vector red chromagen with hematoxylin counterstain. (10X)

Photomicrograph 2.21: Immunoreactivity for vimentin is strongest in the superficial stroma and deep glandular epithelium this secretory stage human endometrium than in the epithelium. Vector red chromagen with hematoxylin counterstain. (10X)

Photomicrograph 2.22: Positive nuclear immunoreactivity for PCNA is diminished in the epithelium (particularly the superficial glands) and stroma of this secretory stage human endometrium when compared to the proliferative stage endometrium (2.16). Chromagen DAB with hematoxylin counterstain. (10X)

Photomicrograph 2.23: Immunoreactivity for vimentin was strongest in the deep glands of this secretory stage human endometrium. Vector red chromagen with hematoxylin counterstain. (40X)

Photomicrograph 2.24: Immunoreactivity for PDGF-R β in the epithelium was strongest in the deep glands of this secretory stage human endometrium. Vector red chromagen with hematoxylin counterstain. (20X)



Color Plate 2 (continued)

Photomicrograph 2.25: Inactive human endometrium. H & E stain. (10X)

Photomicrograph 2.26: Inactive human endometrium. Negative control with Vector red chromagen and hematoxylin counterstain. (10X)

Photomicrograph 2.27: Immunoreactivity for PDGF-R α is limited to the superficial stroma in this inactive human endometrium. Vector red chromagen with hematoxylin counterstain. (20X)

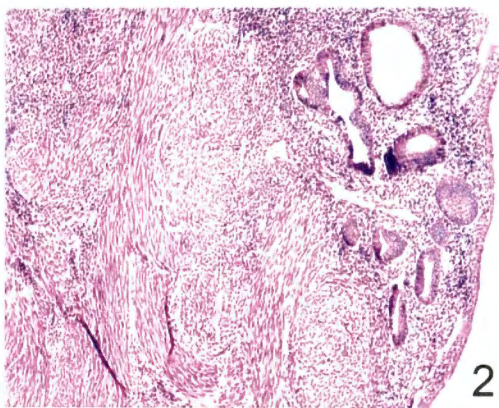
Photomicrograph 2.28: Immunoreactivity for PDGF-R β is strongest in the stroma and luminal epithelium in this inactive human endometrium. Vector red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 2.29: Immunoreactivity for vimentin is strongest in the deep glandular epithelium and stroma in this inactive human endometrium. Vector red chromagen with hematoxylin counterstain. (20X)

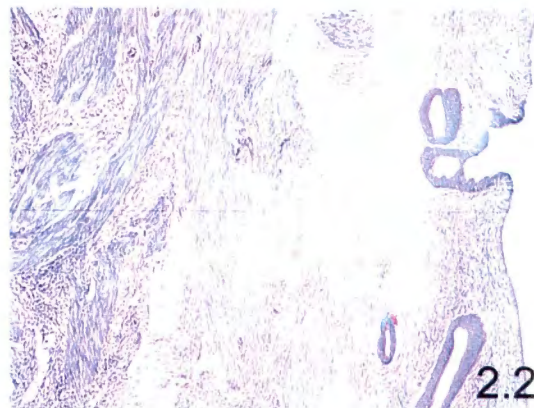
Photomicrograph 2.30: PCNA positive immunoreactivity is most pronounced in the luminal epithelium and stroma of this inactive human endometrium. DAB chromagen with hematoxylin counterstain. (20X)

Photomicrograph 2.31: Immunoreactivity for both PDGF-R β (red, Vector red chromagen) and vimentin (bluish-black, metal enhanced DAB chromagen) can be seen in the epithelial cells in this dual stain of human proliferative stage endometrial deep glands. Hematoxylin counterstain. (40X)

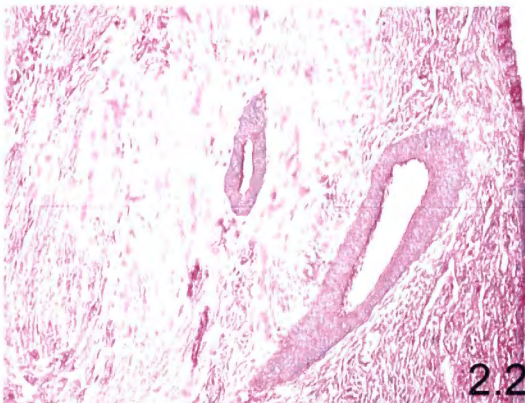
Photomicrograph 2.32: Immunoreactivity for PDGF-R β is (green, Vector red chromagen) and vimentin (bluish-black, metal enhanced DAB chromagen) can be seen in the epithelial cells with this fluorescent stain of human proliferative stage endometrial deep glands. (40x)



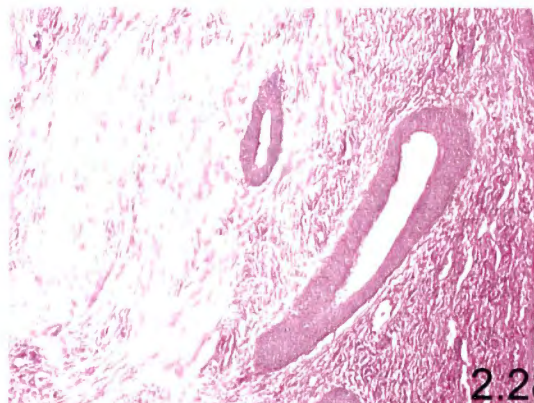
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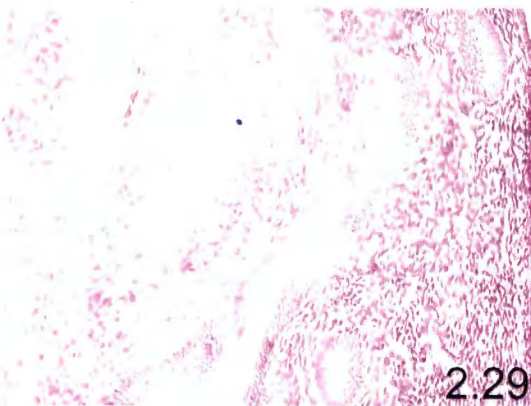
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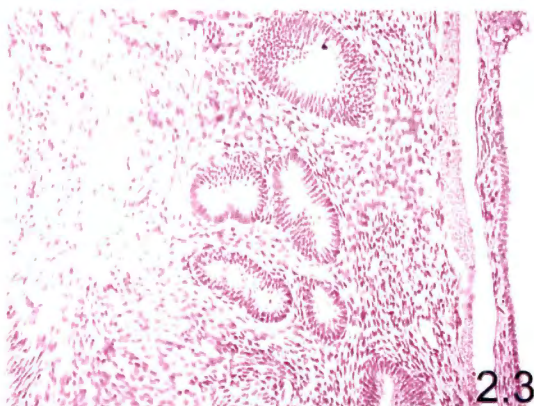
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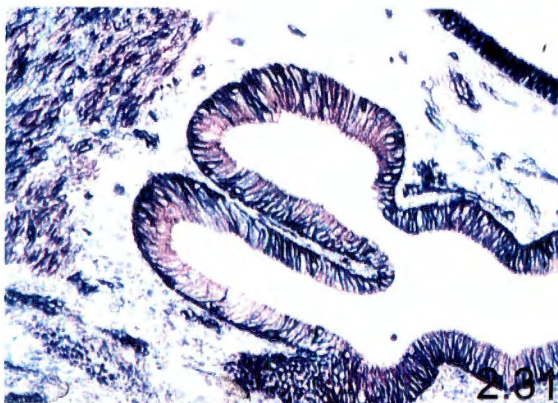
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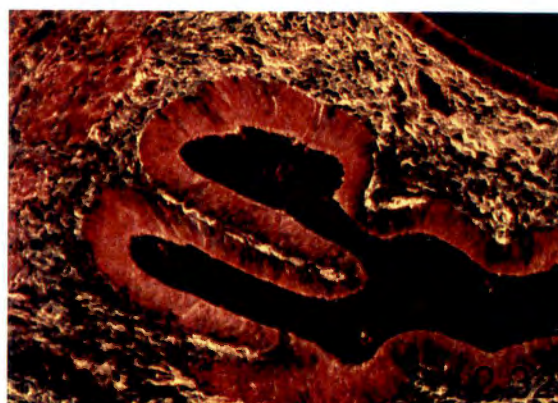
2.29



2.30



2.31



2.32

Color Plate 3

Photomicrograph 3.1: Human endometrial cystic hyperplasia. H & E stain (10X)

Photomicrograph 3.2: Human endometrial cystic hyperplasia. Negative control with Vector red chromagen and hematoxylin counterstain. (4X)

Photomicrograph 3.3: Immunoreactivity for PDGF-R β is strongest in the deep glands and stroma of human cystic endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (4X)

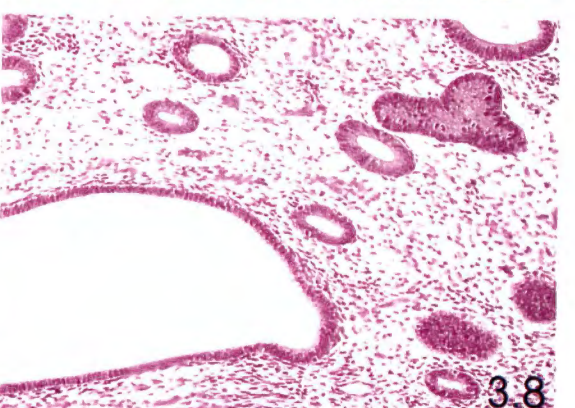
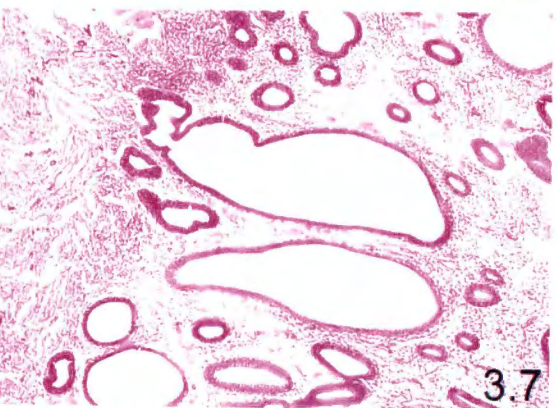
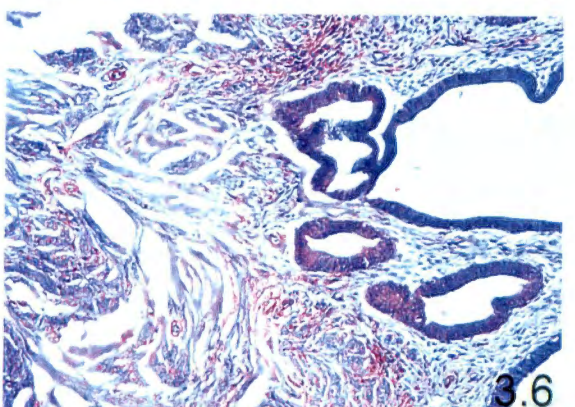
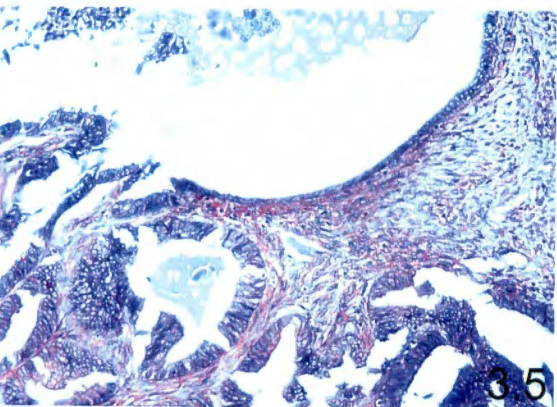
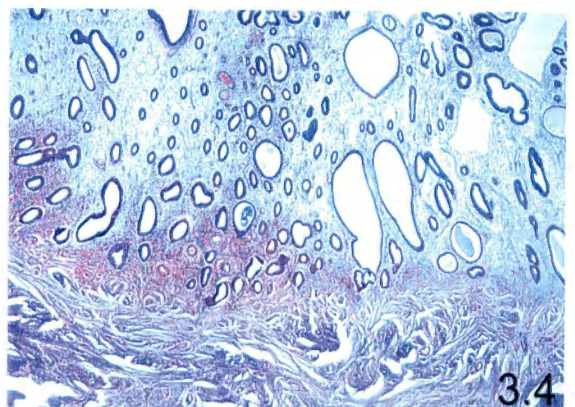
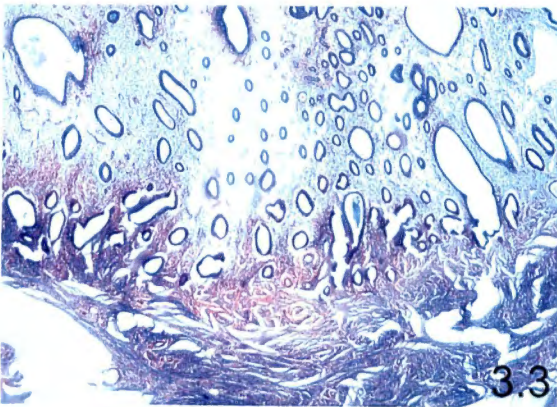
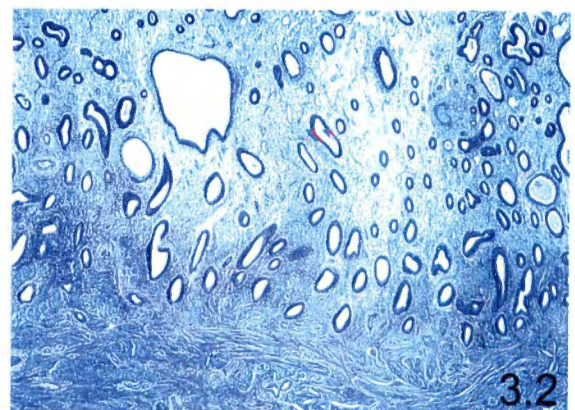
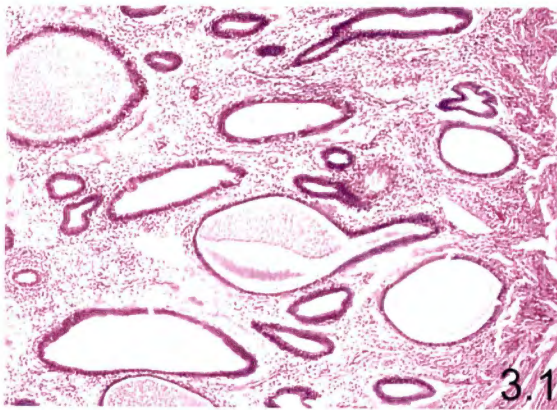
Photomicrograph 3.4: Immunoreactivity for PDGF-R α is strongest in the deep glands and stroma of human cystic endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (4X)

Photomicrograph 3.5: Higher magnification view of immunoreactivity for PDGF-R β in the deep glandular epithelium and stroma of human cystic endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 3.6: Higher magnification view of immunoreactivity for PDGF-R β in the deep glandular epithelium and stroma of human cystic endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 3.7: Immunoreactivity for vimentin intermediate filament is predominantly seen in the deep glands and superficial stroma of human cystic endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (10X)

Photomicrograph 3.8: Positive nuclear immunoreactivity for PCNA was strongest in the deep glandular epithelium and deep stroma of in endometrial cystic hyperplasia. DAB chromagen with hematoxylin counterstain. (20X)



Color Plate 3 (continued)

Photomicrograph 3.9: Human endometrial adenomatous hyperplasia. H & E stain (10X)

Photomicrograph 3.10: Human endometrial adenomatous hyperplasia. Negative control with Vector red chromagen and hematoxylin counterstain. (20X)

Photomicrograph 3.11: Immunoreactivity for PDGF-R β is strongest in the deep glands and stroma of human adenomatous endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (4X)

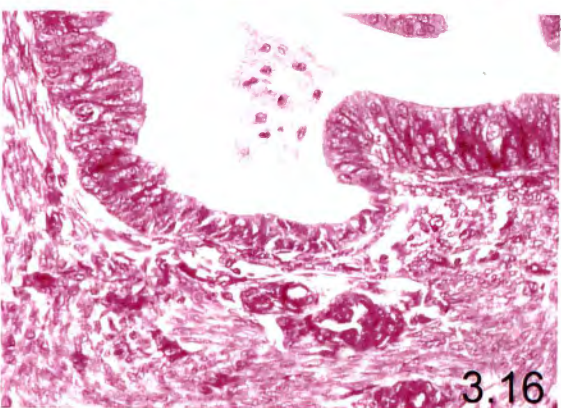
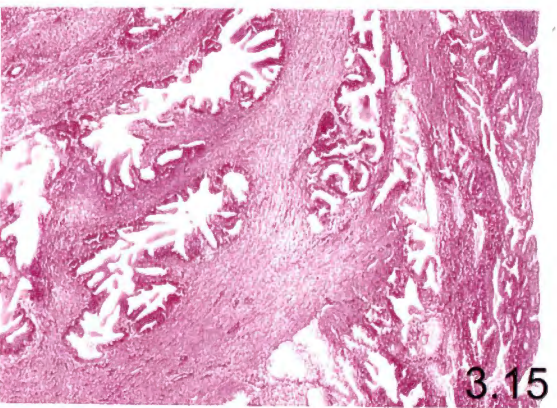
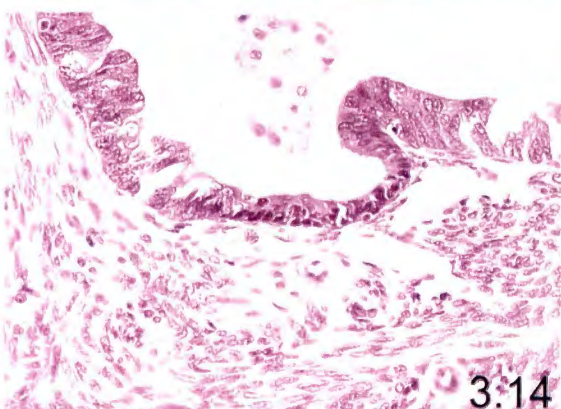
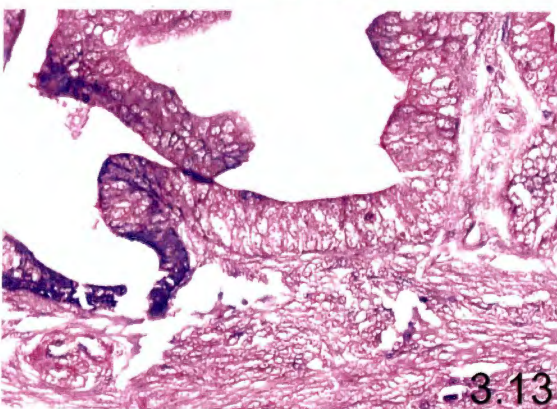
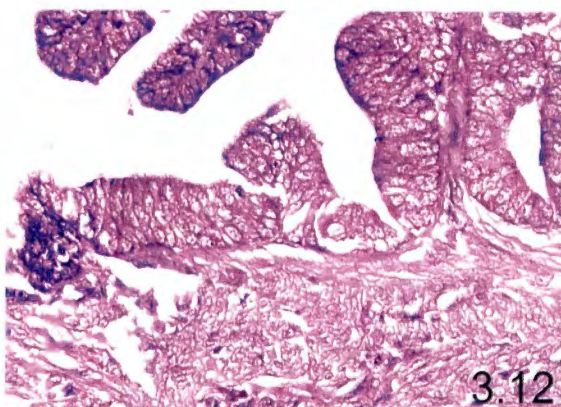
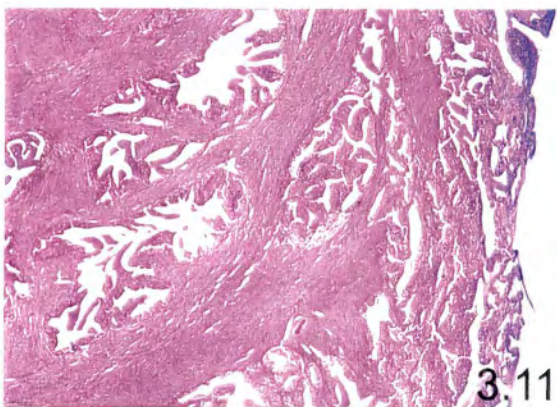
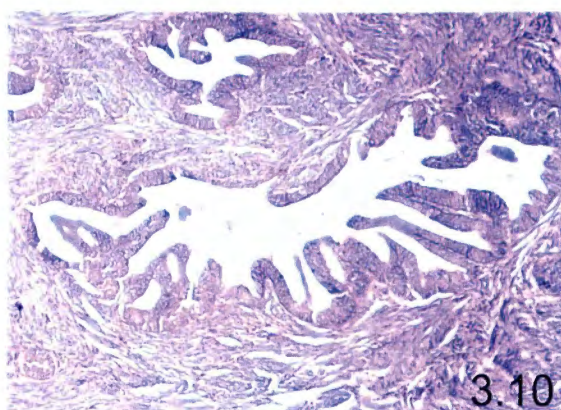
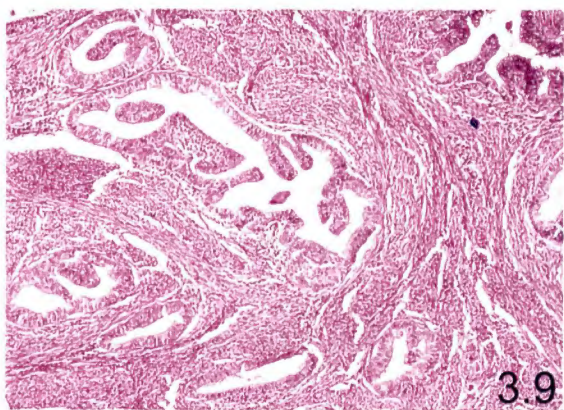
Photomicrograph 3.12: Higher magnification view of immunoreactivity for PDGF-R β in the deep glandular epithelium and stroma of human adenomatous endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (40X)

Photomicrograph 3.13: Immunoreactivity for PDGF-R α is strongest in the deep glands and stroma of human adenomatous endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (40X)

Photomicrograph 3.14: Positive nuclear immunoreactivity for PCNA was strongest in the deep glandular epithelium of endometrial adenomatous hyperplasia. DAB chromagen with hematoxylin counterstain. (40X)

Photomicrograph 3.15: Immunoreactivity for vimentin intermediate filament is predominantly seen in the deep glands and deep stroma of human adenomatous endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (4X)

Photomicrograph 3.16: Higher magnification view of immunoreactivity for vimentin in human adenomatous endometrial hyperplasia. Vector red chromagen with hematoxylin counterstain. (40X)



Color Plate 3 (continued)

Photomicrograph 3.17: Human endometrial adenocarcinoma. H & E stain. (10X)

Photomicrograph 3.18: Human endometrial adenocarcinoma. Negative control with Vector red chromagen and toluidine blue counterstain. (10X)

Photomicrograph 3.19: Immunoreactivity for PDGF-R β was strong in the malignant glandular epithelial cells of this human endometrial adenocarcinoma. Stromal and fibrous connective tissue immunoreactivity was minimal. Vector red chromagen with toluidine blue counterstain. (10X)

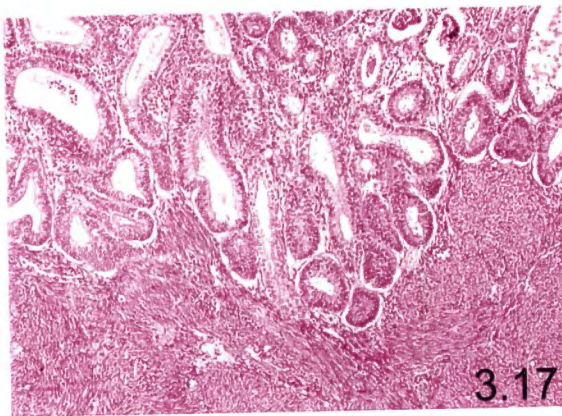
Photomicrograph 3.20: Immunoreactivity for PDGF-R α was weak in the malignant glandular epithelial cells, stroma and fibrous connective tissue of this human endometrial adenocarcinoma. Vector red chromagen with toluidine blue counterstain. (10X)

Photomicrograph 3.21: Higher magnification of photomicrograph 3.19. (40X). The strong epithelial immunoreactivity for PDGF-R β can be seen in the basal aspect of the neoplastic glandular cells. Vector red chromagen with toluidine blue counterstain. (4X)

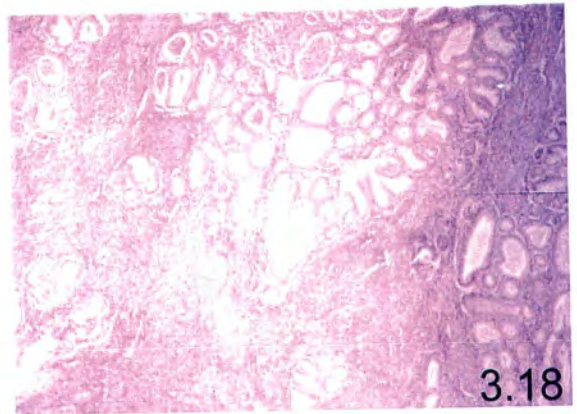
Photomicrograph 3.22: Higher magnification of 3.20. (40X).

Photomicrograph 3.23: Immunoreactivity for vimentin was moderate in the malignant glandular epithelial cells of this endometrial adenocarcinoma and predominantly localized to the basal aspect of the cells. Vector red chromagen with toluidine blue counterstain. (4X)

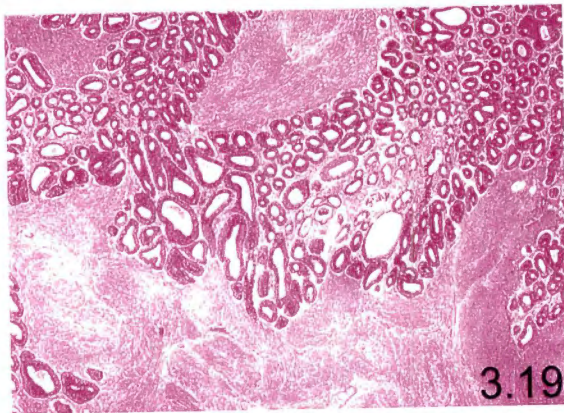
Photomicrograph 3.24: The greatest positive nuclear immunoreactivity for PCNA was present at the leading edge of the neoplastic glandular epithelium in this endometrial adenocarcinoma. DAB chromagen with hematoxylin counterstain. (40X)



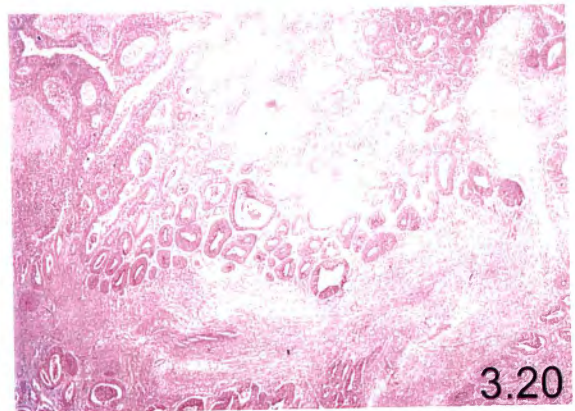
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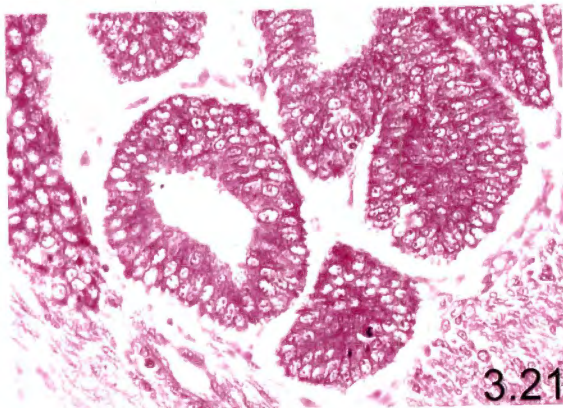
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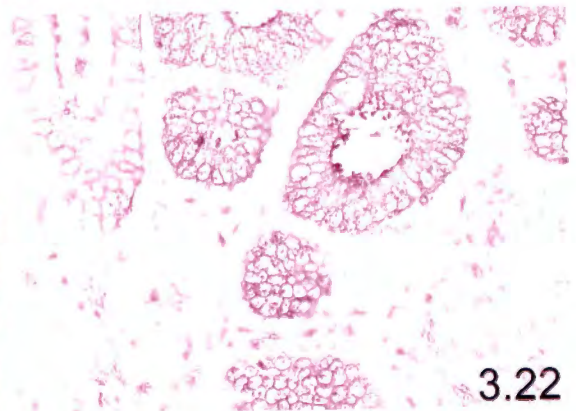
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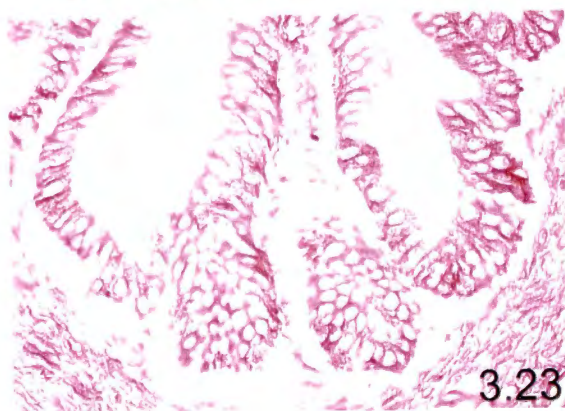
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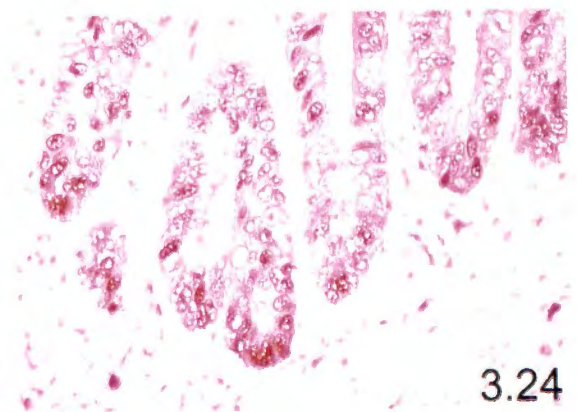
3.21



3.22



3.23



3.24

Color Plate 3 (continued)

Photomicrograph 3.25: Human endometrial adenocarcinoma. H & E stain. (10X)

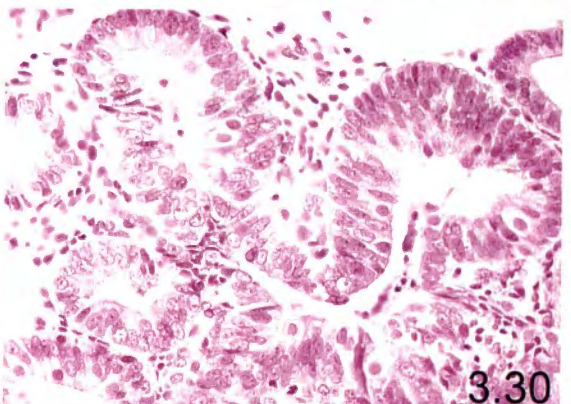
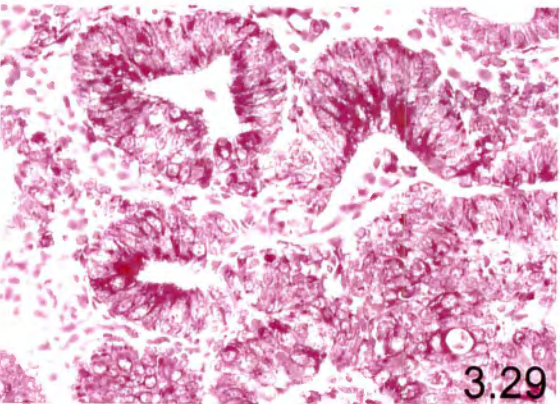
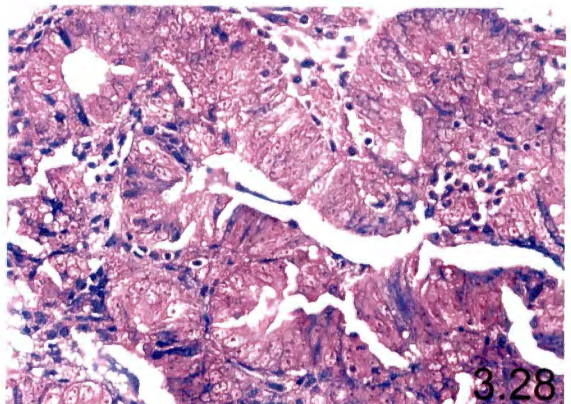
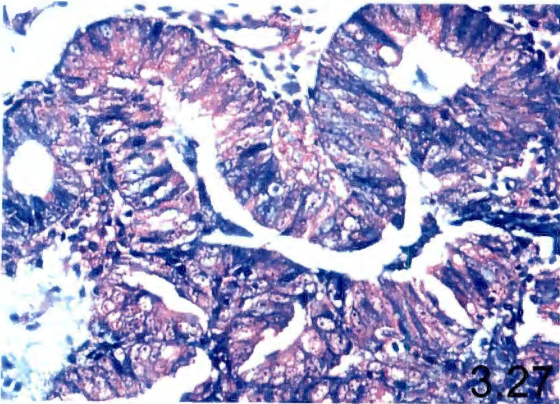
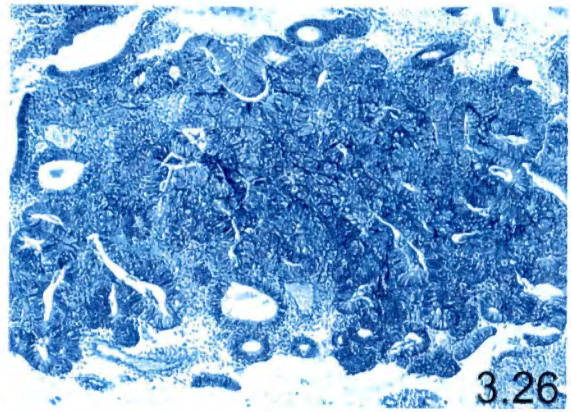
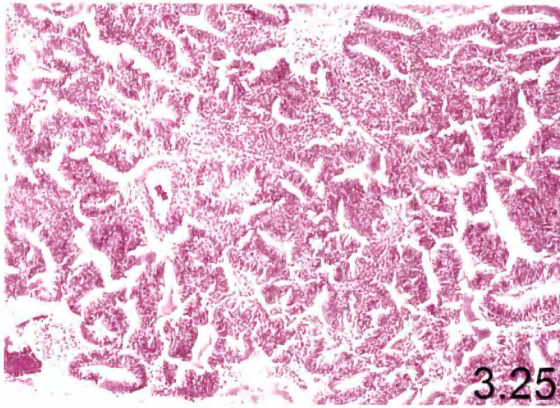
Photomicrograph 3.26: Human endometrial adenocarcinoma. Negative control with Vector red chromagen and toluidine blue counterstain. (10X)

Photomicrograph 3.27: Immunoreactivity for PDGF-R β was strong in the malignant glandular epithelial cells of this human endometrial adenocarcinoma.(compare to photomicrograph 3.20) Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 3.28: Immunoreactivity for PDGF-R α was strong in the malignant glandular epithelial cells of this human endometrial adenocarcinoma.(compare to photomicrograph 3.21) Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 3.29: Immunoreactivity for vimentin was moderate to strong in the malignant epithelial cells of this endometrial adenocarcinoma. Immunoreactivity for vimentin was present in both the apical and basal aspects of the epithelial cells in this carcinoma. Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 3.30: The positive nuclear immunoreactivity for PCNA was present throughout the neoplastic glandular epithelium in this endometrial adenocarcinoma. Numerous mitotic figures were also present throughout the neoplastic focus. DAB chromagen with hematoxylin counterstain. (40X)



Color Plate 3 (continued)

Photomicrograph 3.31: Ovarian endometriosis. Endometriotic foci of ectopic glandular and stromal endometrial tissue can be seen above hilar corpora albicans in this ovary. H &E stain. (4x)

Photomicrograph 3.32: Ovarian endometriosis. Flattened to cuboidal endometriotic epithelium line a cystic gland. Moderately decidualized stroma can be seen subjacent to the endometriotic epithelium. H &E stain. (20x)

Photomicrograph 3.33: Immunoreactivity for PDGF-R β was weak in the majority of the endometriotic stroma and epithelia. Vector red chromagen with toluidine blue counterstain. (4X)

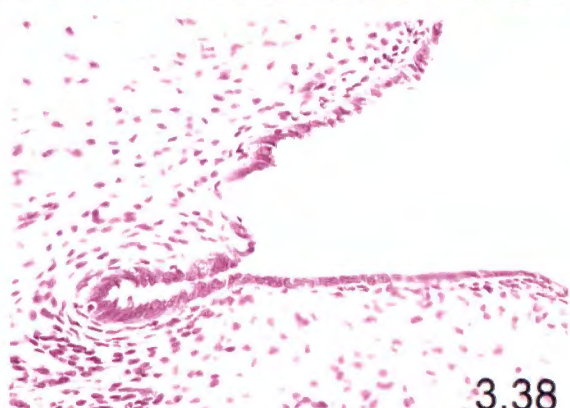
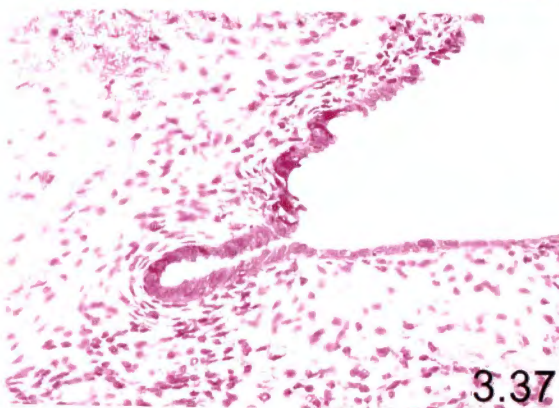
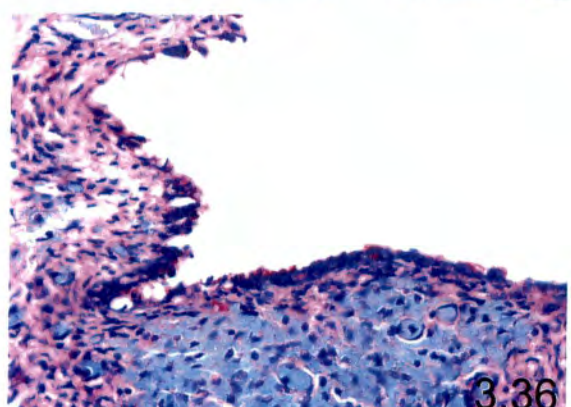
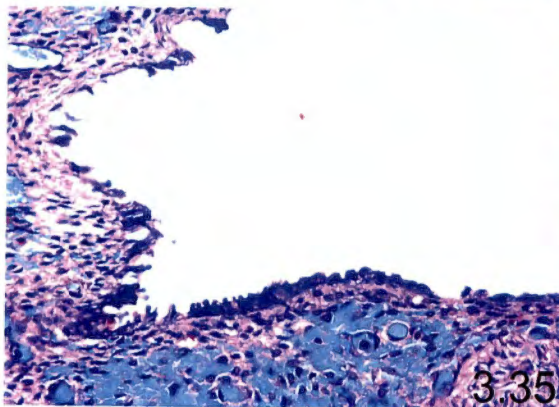
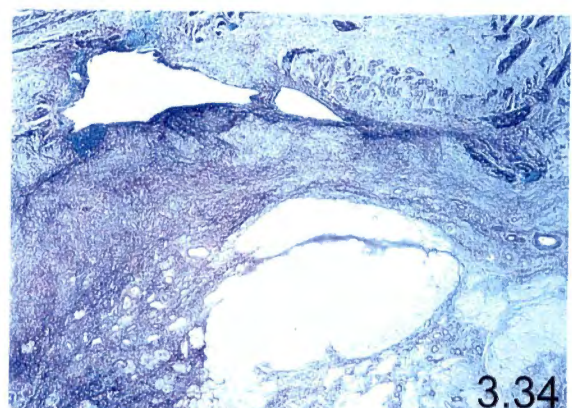
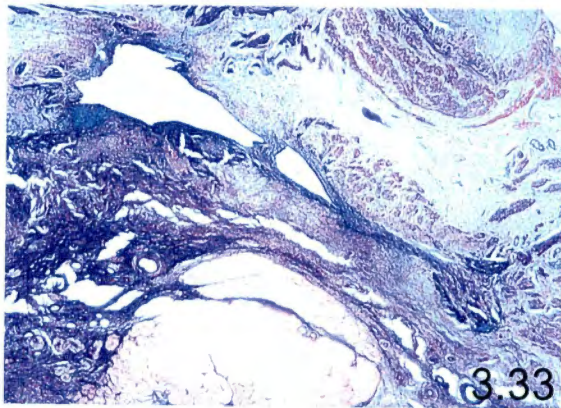
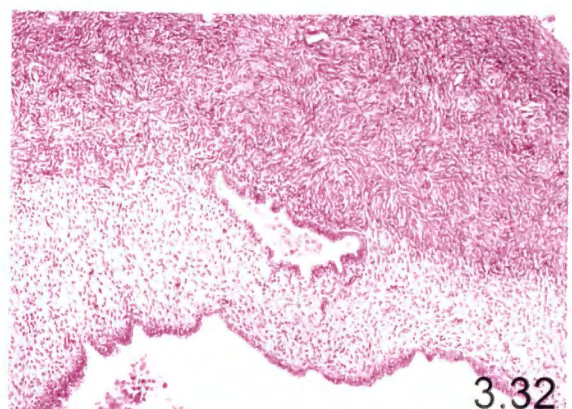
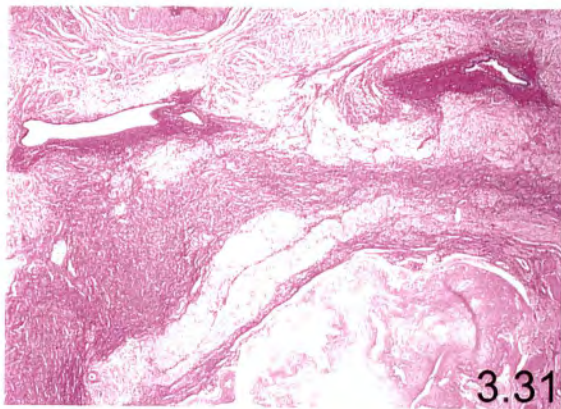
Photomicrograph 3.34: Immunoreactivity for PDGF-R α was weak in the majority of the endometriotic stroma and epithelia. Vector red chromagen with toluidine blue counterstain. (4X)

Photomicrograph 3.35: Higher magnification of photomicrograph 3.33. (40X). Focal areas of moderate immunoreactivity for PDGF-R β in the endometriotic epithelium corresponded to immunoreactivity for PDGF-R α , vimentin and PCNA. (compare with 3.36, 3.37, 3.38) Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 3.36: Higher magnification of photomicrograph 3.34. (40X). There were focal areas of moderate immunoreactivity for PDGF-R α in the endometriotic epithelium which corresponded to sections exhibiting staining for PDGF-R β , vimentin and PCNA. (compare with 3.35, 3.37, 3.38) Vector red chromagen with toluidine blue counterstain. (40X) (over)

Photomicrograph 3.37: The positive nuclear immunoreactivity for PCNA was weak to moderate in the majority of endometriotic epithelium and stroma. There were focal areas of moderate immunoreactivity for PCNA in the endometriotic epithelium which corresponded to sections exhibiting staining for PDGF-R β , PDGF-R α and vimentin. (compare with 3.35, 3.36, 3.38) DAB chromagen with hematoxylin counterstain. (40X)

Photomicrograph 3.38: Immunoreactivity for vimentin was weak in the majority of the endometriotic stroma and epithelia. There were focal areas of moderate immunoreactivity for PDGF-R α in the endometriotic epithelium which corresponded to sections exhibiting staining for PDGF-R β , (over)



Color Plate 3 (continued)

PDGF-R α and PCNA. (compare with 3.35, 3.37, 3.37) Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 3.39: Mesenteric endometriosis. Focal areas of moderate immunoreactivity for PDGF-R α in the endometriotic epithelium corresponded immunoreactivity for PDGF-R β , vimentin and PCNA. (compare with 3.40, 3.41, 3.42) Vector red chromagen with toluidine blue counterstain. (20X)

Photomicrograph 3.40: Mesenteric endometriosis. Focal areas of moderate immunoreactivity for PDGF-R β in the endometriotic epithelium corresponded immunoreactivity for PDGF-R α , vimentin and PCNA. (compare with 3.39, 3.41, 3.42) Vector red chromagen with toluidine blue counterstain. (20X)

Photomicrograph 3.41: Mesenteric endometriosis. Focal areas of moderate immunoreactivity for vimentin in the endometriotic epithelium corresponded immunoreactivity for PDGF-R α , PDGF-R β and PCNA. (compare with 3.39, 3.40, 3.42) Vector red chromagen with toluidine blue counterstain. (20X)

Photomicrograph 3.42: The positive nuclear immunoreactivity for PCNA was weak to moderate in the majority of endometriotic epithelium and stroma. There were focal areas of moderate immunoreactivity for PCNA in the (over) endometriotic epithelium which corresponded to sections exhibiting staining for PDGF-R β , PDGF-R α and vimentin. (compare with 3.39, 3.40, 3.42) DAB chromagen with hematoxylin counterstain. (20X)

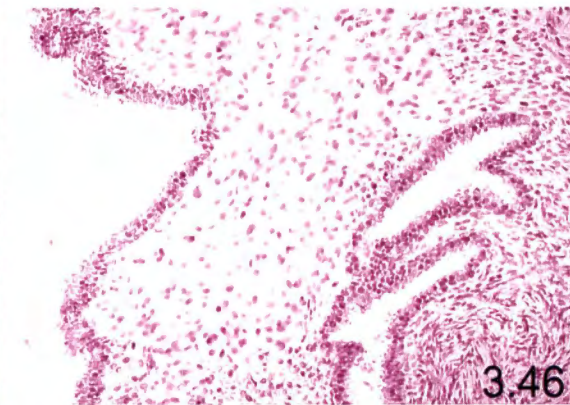
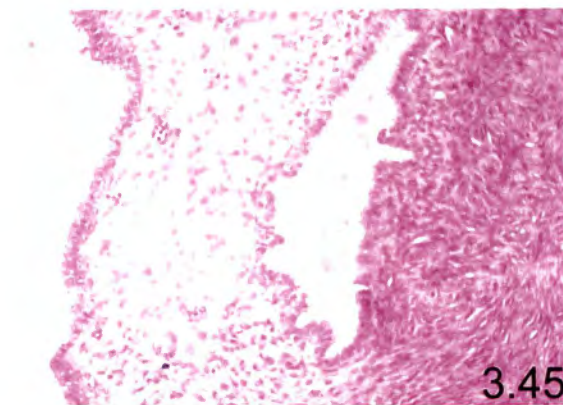
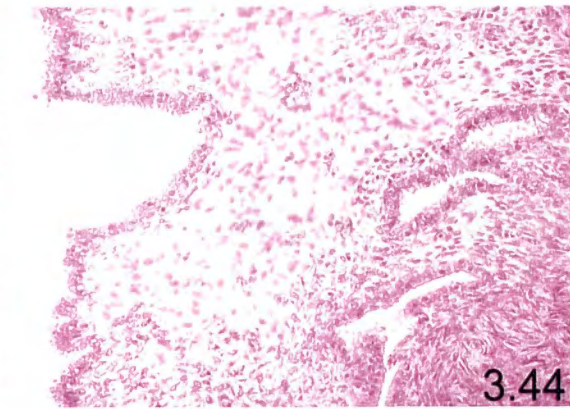
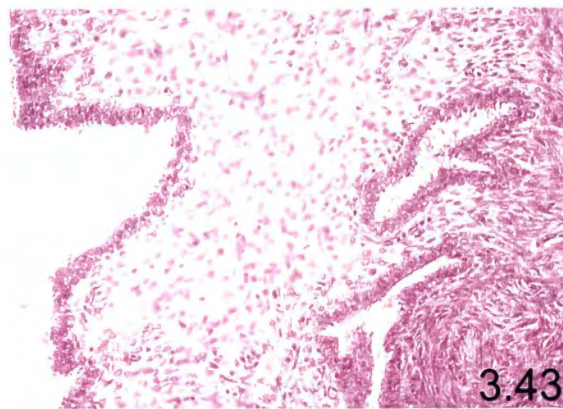
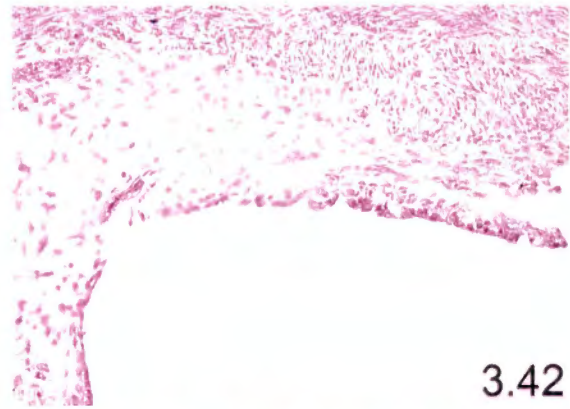
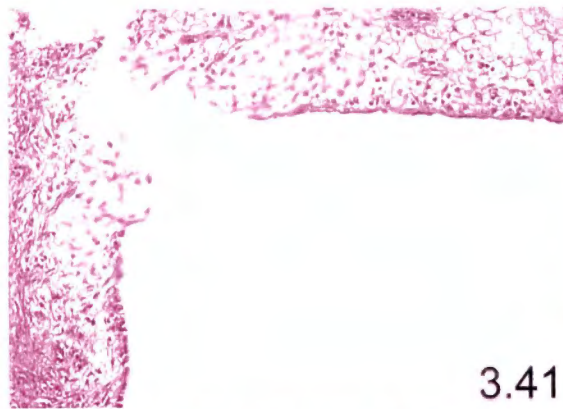
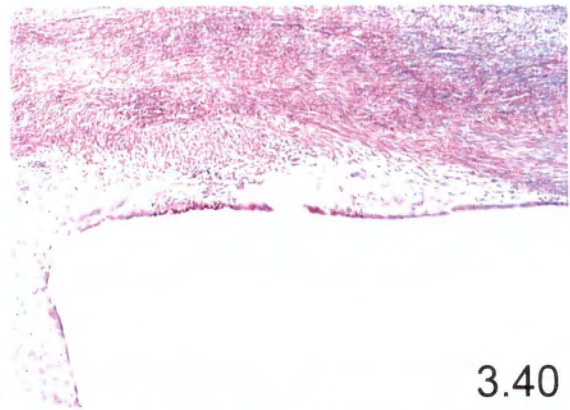
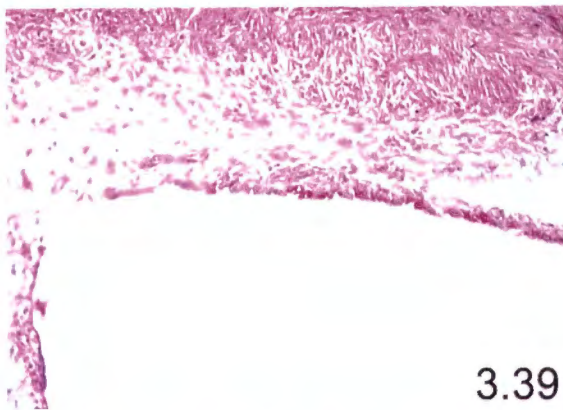
Photomicrograph 3.43: Mesenteric endometriosis. Focal areas of moderate immunoreactivity for PDGF-R α in the endometriotic epithelium corresponded immunoreactivity for PDGF-R β , vimentin and PCNA. (compare with 3.44, 3.45, 3.46) Vector red chromagen with toluidine blue counterstain. (20X)

Photomicrograph 3.44: Mesenteric endometriosis. Focal areas of moderate immunoreactivity for PDGF-R β in the endometriotic epithelium corresponded immunoreactivity for PDGF-R α , vimentin and PCNA. (compare with 3.43, 3.45, 3.46) Vector red chromagen with toluidine blue counterstain. (20X)

Photomicrograph 3.45: Mesenteric endometriosis. Focal areas of moderate immunoreactivity for vimentin in the endometriotic epithelium corresponded immunoreactivity for PDGF-R α , PDGF-R β and PCNA. (compare with 3.43, 3.44, 3.46) Vector red chromagen with toluidine blue counterstain. (20X)
(over)

Color Plate 3 (continued)

Photomicrograph 3.46: The positive nuclear immunoreactivity for PCNA was weak to moderate in the majority of endometriotic epithelium and stroma. There were focal areas of moderate immunoreactivity for PCNA in the endometriotic epithelium which corresponded to sections exhibiting staining for PDGF-R β , PDGF-R α and vimentin. (compare with 3.43, 3.44, 3.345) DAB chromagen with hematoxylin counterstain. (20X)



Color Plate 4

Photomicrograph 4.1: Diestrus stage mouse vaginal vault. H & E stain (10x)

Photomicrograph 4.2: Diestrus stage mouse uterus. Negative control with Vector red chromagen and toluidine blue counterstain. (10X)

Photomicrograph 4.3: Immunoreactivity for PDGF-R β was strongest on the luminal epithelium of the diestrus stage mouse endometrium. Vector red chromagen with toluidine blue counterstain. (10X)

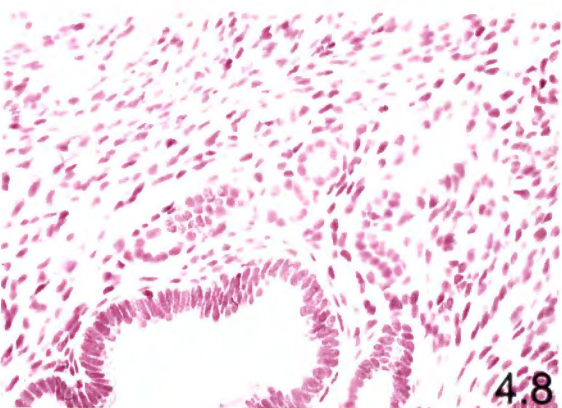
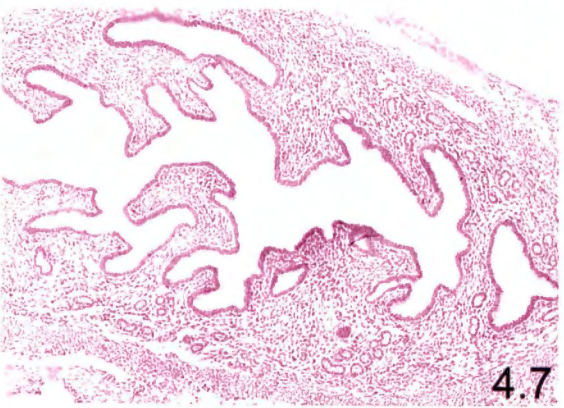
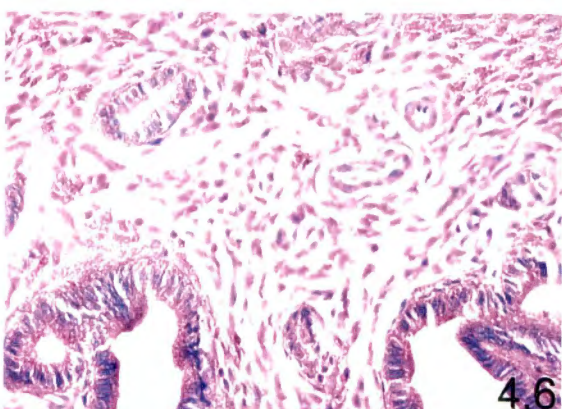
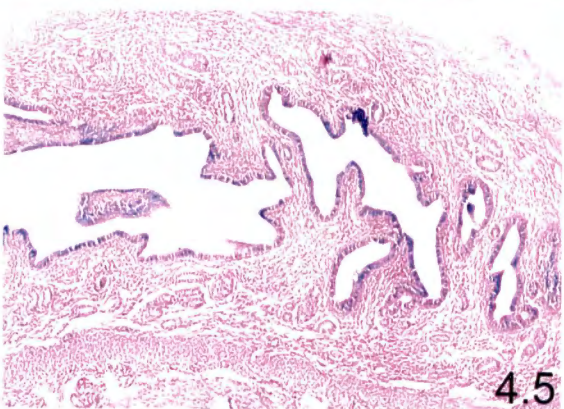
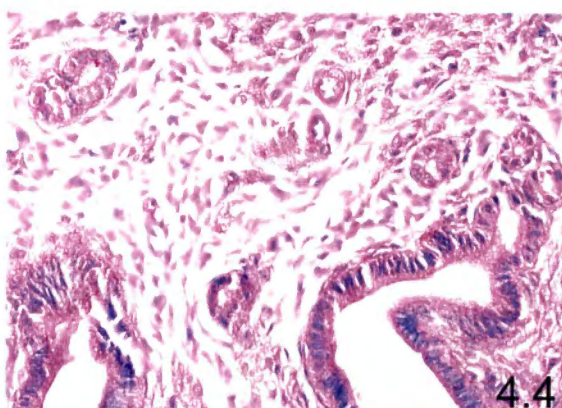
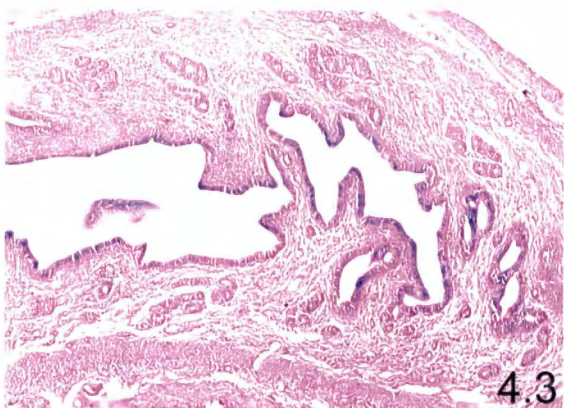
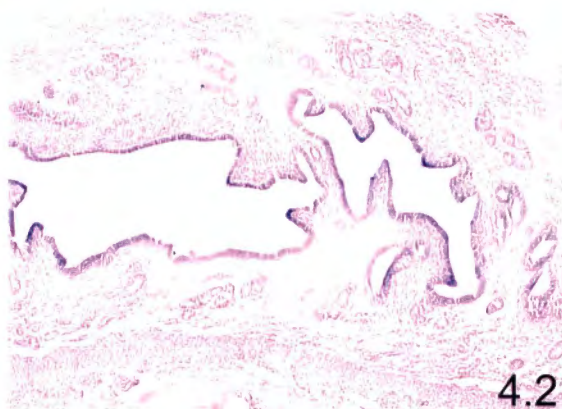
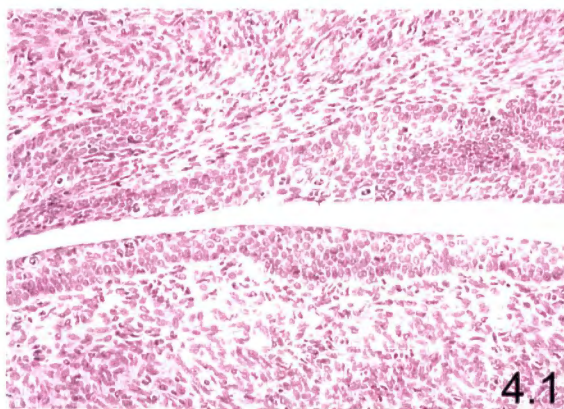
Photomicrograph 4.4: Higher magnification of 4.3. Diffuse, moderate, cytoplasmic, immunoreactivity can be seen in the luminal epithelium. Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 4.5: Immunoreactivity for PDGF-R α was strongest in the stroma of the diestrus stage mouse endometrium. Immunoreactivity in the luminal epithelium was stronger than in the glands. Vector red chromagen with toluidine blue counterstain. (10X)

Photomicrograph 4.6: Higher magnification of 4.5. Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 4.7: Positive nuclear immunoreactivity for PCNA was strongest in the glandular epithelium and stroma of the diestrus stage mouse endometrium. DAB chromagen with hematoxylin counterstain. (10X)

Photomicrograph 4.8: Higher magnification of 4.7. DAB chromagen with hematoxylin counterstain. (40X)



Color Plate 4 (continued)

Photomicrograph 4.9: Proestrus stage murine vaginal vault. H & E stain (20x)

Photomicrograph 4.10: Proestrus stage mouse uterus. Negative control with Vector red chromagen and toluidine blue counterstain. (10X)

Photomicrograph 4.11: Immunoreactivity for PDGF-R β was strongest in the luminal and glandular epithelium of the proestrus stage mouse endometrium. Vector red chromagen with toluidine blue counterstain. (10X)

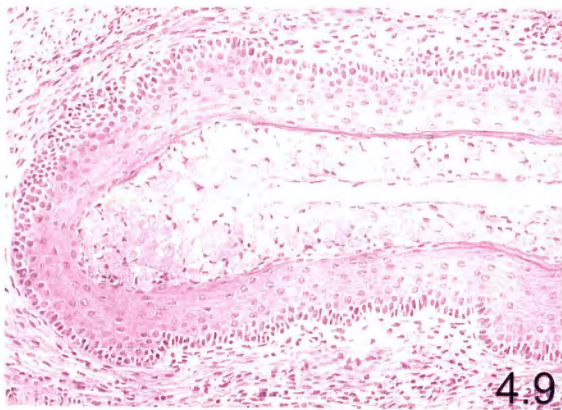
Photomicrograph 4.12: Higher magnification of 4.11. Diffuse, moderate, cytoplasmic, immunoreactivity can be seen in the luminal and glandular epithelium. Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 4.13: Immunoreactivity for PDGF-R α was strongest in the stroma and luminal epithelium of the proestrus stage mouse endometrium. Vector red chromagen with toluidine blue counterstain. (10X)

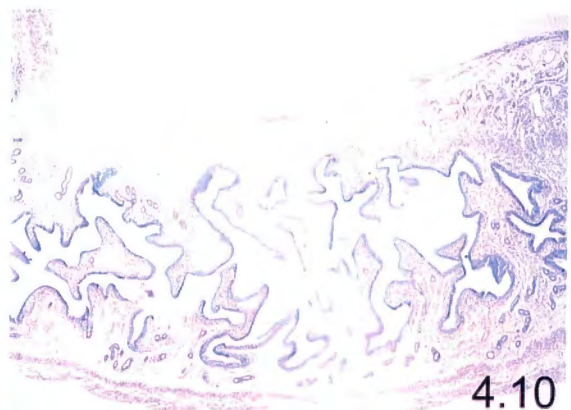
Photomicrograph 4.14: Higher magnification of 4.13. Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 4.15: Positive nuclear immunoreactivity for PCNA was strong in both the glandular and luminal epithelium of the proestrus stage mouse endometrium. DAB chromagen with hematoxylin counterstain. (10X)

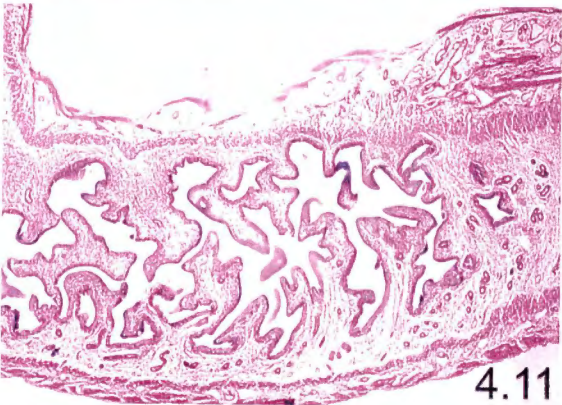
Photomicrograph 4.16: Higher magnification of 4.15. DAB chromagen with hematoxylin counterstain. (40X)



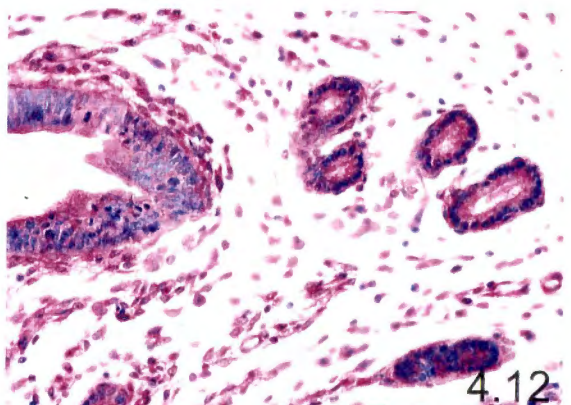
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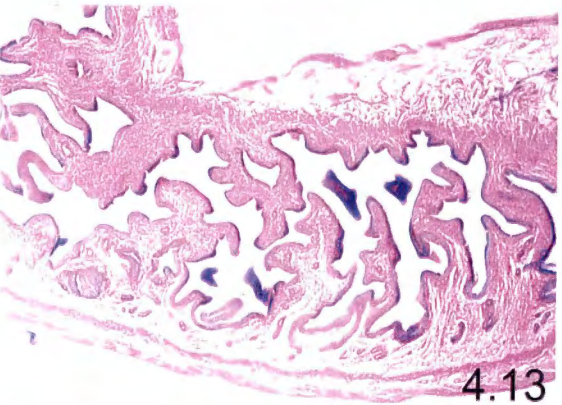
4.10



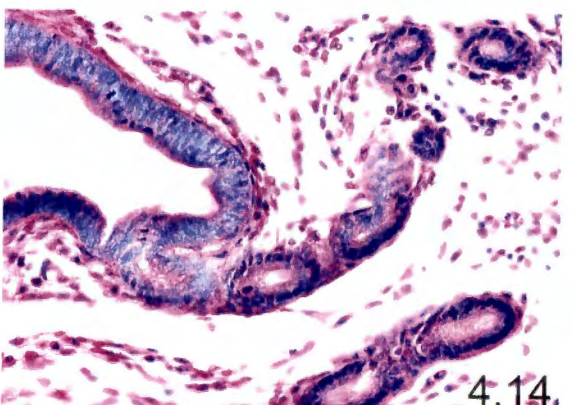
4.11



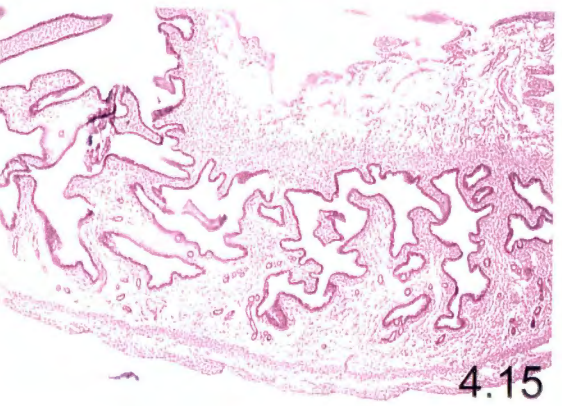
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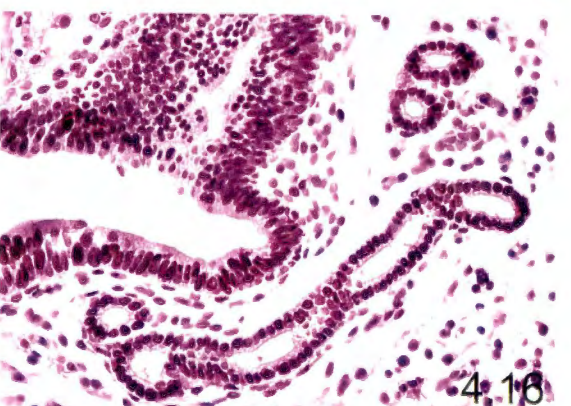
4.13



4.14



4.15



4.16

Color Plate 4 (continued)

Photomicrograph 4.17: Estrus stage murine vaginal vault. H & E stain (10x)

Photomicrograph 4.18: Estrus stage mouse uterus. Negative control with Vector red chromagen and toluidine blue counterstain. (10X)

Photomicrograph 4.19: Immunoreactivity for PDGF-R β was strongest in the luminal and glandular epithelium of the estrus stage mouse endometrium. Vector red chromagen with toluidine blue counterstain. (10X)

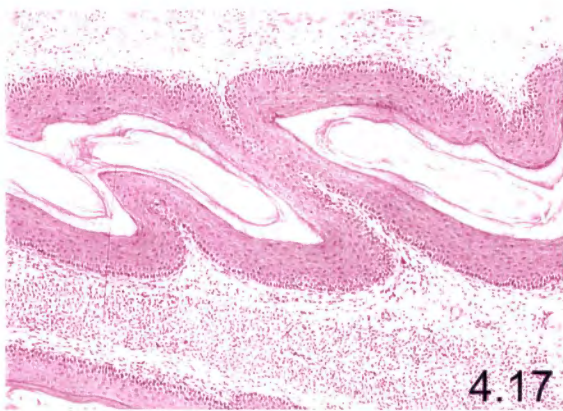
Photomicrograph 4.20: Higher magnification of 4.19. Diffuse, moderate, cytoplasmic, immunoreactivity can be seen in the luminal and glandular epithelium. Vector red chromagen with toluidine blue counterstain.(40X)

Photomicrograph 4.21: Immunoreactivity for PDGF-R α was moderately present in both the epithelial and stromal compartments of the estrus stage mouse endometrium. Vector red chromagen with toluidine blue counterstain. (10X)

Photomicrograph 4.22: Higher magnification of 4.21. The stromal and epithelial immunoreactivity can be seen. (compare to PDGF-R β : 4.20) Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 4.23: Positive nuclear immunoreactivity for PCNA was strong in both the glandular and luminal epithelium of the estrus stage mouse endometrium. DAB chromagen with hematoxylin counterstain. (10X)

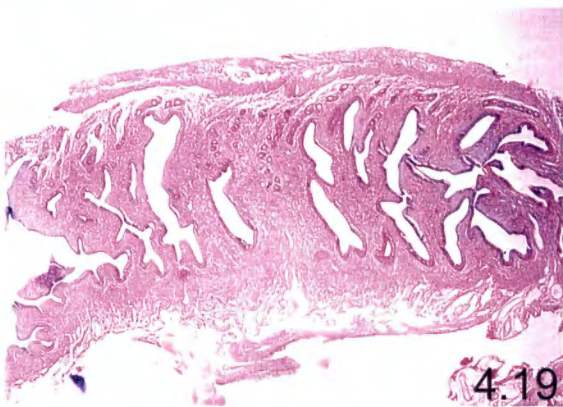
Photomicrograph 4.24: Higher magnification of 4.15. DAB chromagen with hematoxylin counterstain. (40X)



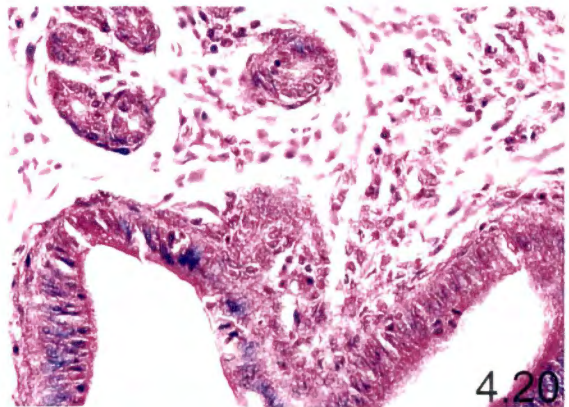
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4.18



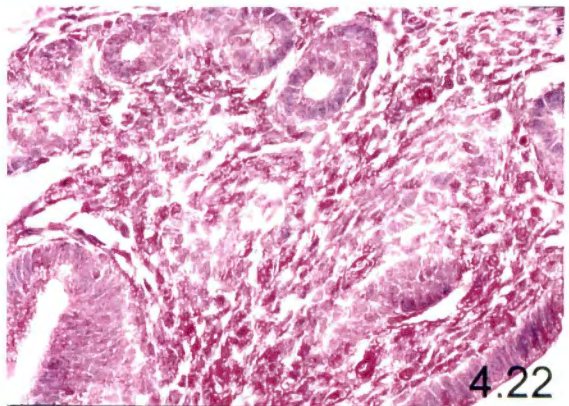
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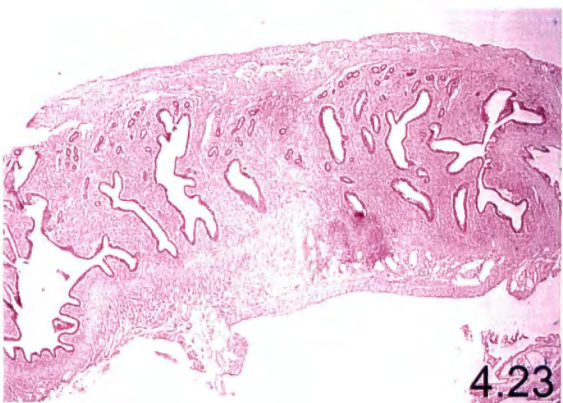
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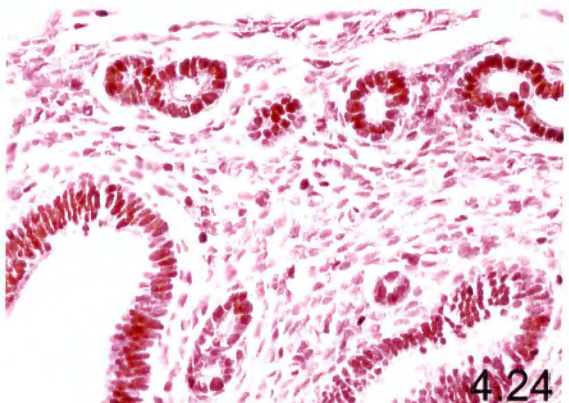
4.21



4.22



4.23



4.24

Color Plate 4 (continued)

Photomicrograph 4.25: Metestrus stage murine vaginal vault. H & E stain (10x)

Photomicrograph 4.26: Metestrus stage mouse uterus. Negative control with Vector red chromagen and toluidine blue counterstain. (10X)

Photomicrograph 4.27: Immunoreactivity for PDGF-R β was strongest in the luminal and glandular epithelium of the metestrus stage mouse endometrium. Vector red chromagen with toluidine blue counterstain. (10X)

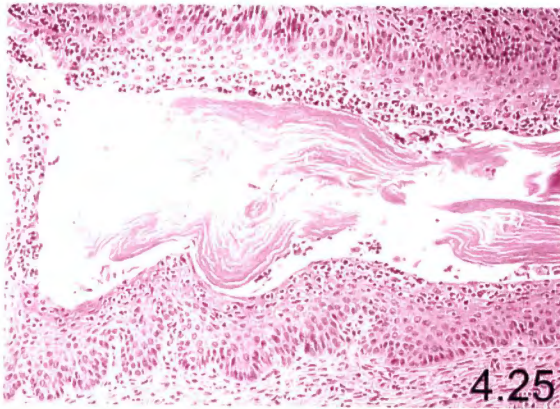
Photomicrograph 4.28: Higher magnification of 4.27. Diffuse, moderate, cytoplasmic, immunoreactivity can be seen in the luminal and glandular epithelium. Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 4.29: Immunoreactivity for PDGF-R α was moderately present in both the luminal and glandular epithelial compartments of the metestrus stage mouse endometrium. Vector red chromagen with toluidine blue counterstain. (10X)

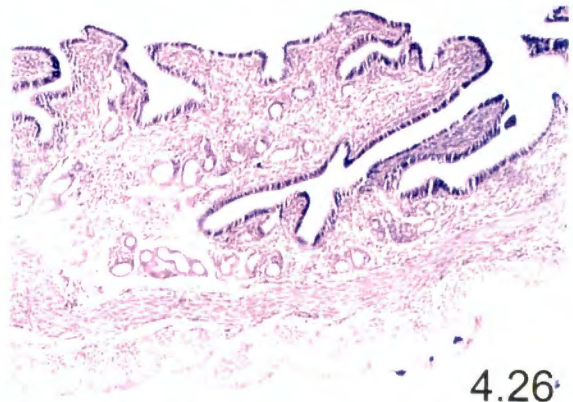
Photomicrograph 4.30: Higher magnification of 4.29. Vector red chromagen with toluidine blue counterstain. (40X)

Photomicrograph 4.31: Positive nuclear immunoreactivity for PCNA was strongest in the luminal epithelium of the metestrus stage mouse endometrium. DAB chromagen with hematoxylin counterstain. (10X)

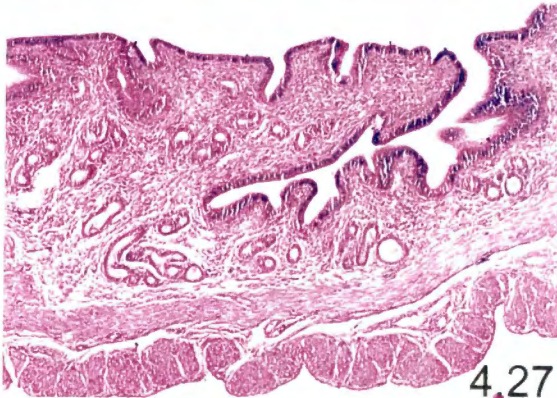
Photomicrograph 4.32: Higher magnification of 4.31. DAB chromagen with hematoxylin counterstain. (40X)



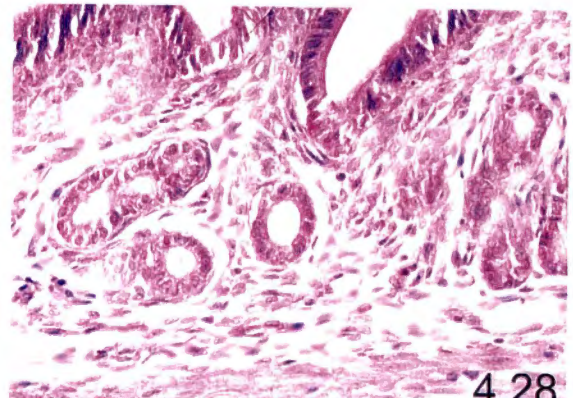
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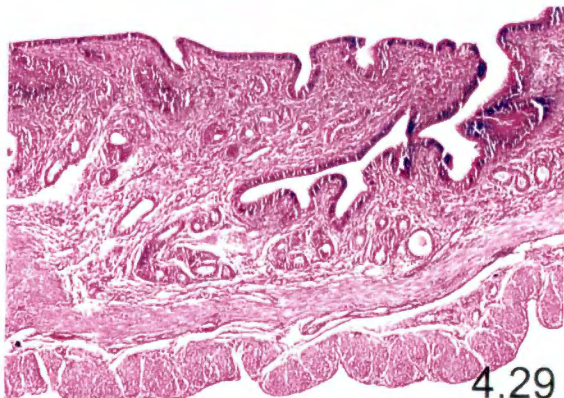
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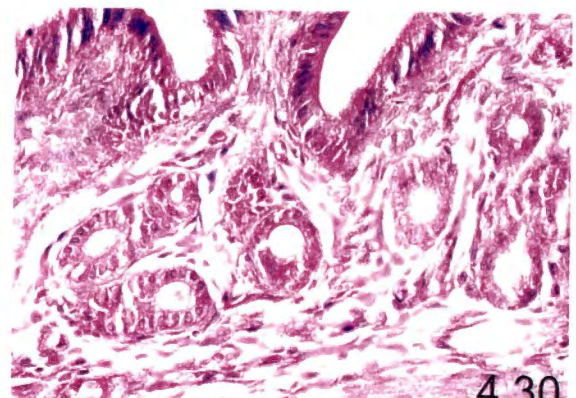
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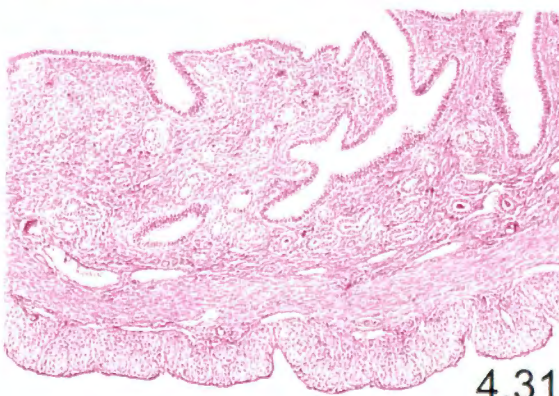
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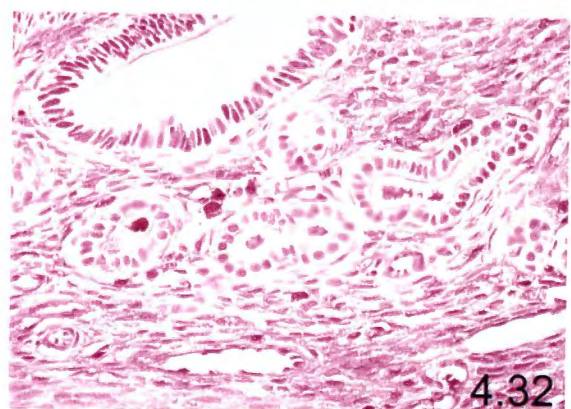
4.29



4.30



4.31



4.32

Color Plate 5

Photomicrograph 5.1: Human endometrial epithelial cells (hEEC) cultured on submerged, unattached human amniotic membrane for 3 days. (phase: 20X)

Photomicrograph 5.2: hEEC cultured on submerged, unattached human amniotic membrane. Note fracturing of amniotic membrane. H & E stain. (20X)

Photomicrograph 5.3: Positive immunoreactivity for vimentin was present in hEEC cultured on submerged, unattached human amniotic membrane. DAB chromagen with hematoxylin counterstain. (40X)

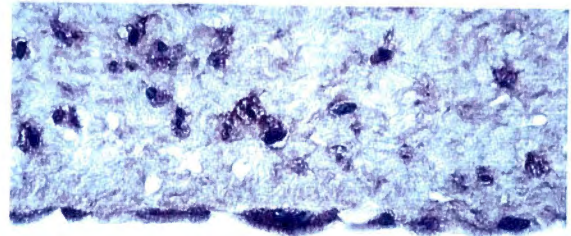
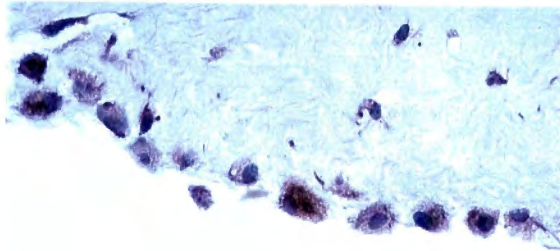
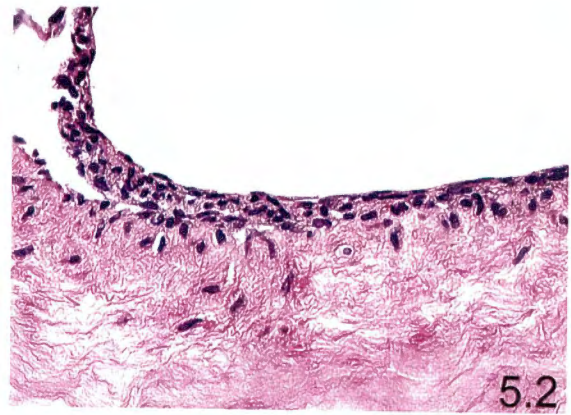
Photomicrograph 5.4: Positive immunoreactivity for PDGF-R β was present in hEEC cultured on submerged, unattached human amniotic membrane. DAB chromagen with hematoxylin counterstain. (40X)

Photomicrograph 5.5: Positive immunoreactivity for PDGF-R α was present in hEEC cultured on submerged, unattached human amniotic membrane. DAB chromagen with hematoxylin counterstain. (40X)

Photomicrograph 5.6: Negative control of hEEC cultured on submerged, unattached human amniotic membrane. DAB chromagen with hematoxylin counterstain. (40X)

Photomicrograph 5.7: Gelfoam sponge. Note the interconnecting trabecular network.

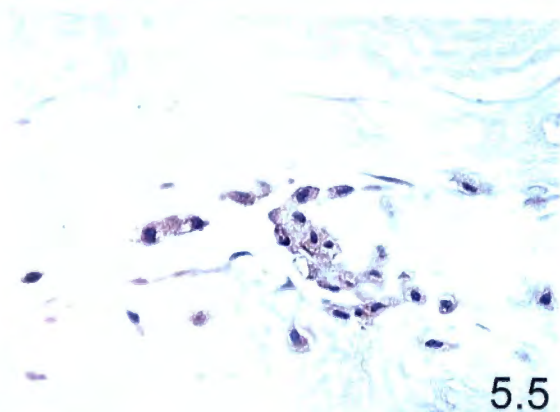
Photomicrograph 5.8: Human endometrial epithelial cells (hEEC) cultured on submerged, unattached Matrigel / Gelfoam matrix for 3 days. (phase: 20X)



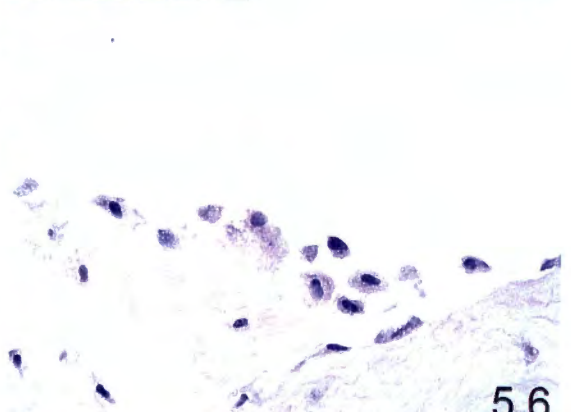
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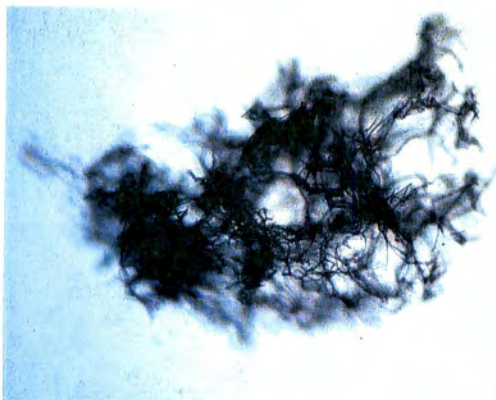
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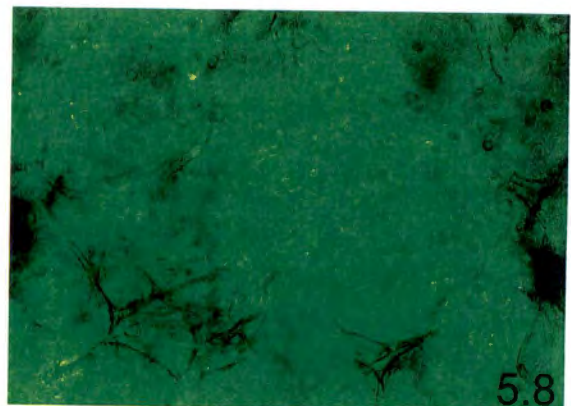
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5.6



5.7



5.8

Color Plate 5 (continued)

Photomicrograph 5.9: hEEC on Matrigel / Gelfoam matix. H & E stain. (20X)

Photomicrograph 5.10: hEEC on Matrigel / Gelfoam matix. Negative control.

Fast red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 5.11: Immunoreactivity for pancytokeratin in hEEC on Matrigel / Gelfoam matix. Note predominance of growth of cells on the matrix surface. Fast red chromagen with hematoxylin counterstain. (20X)

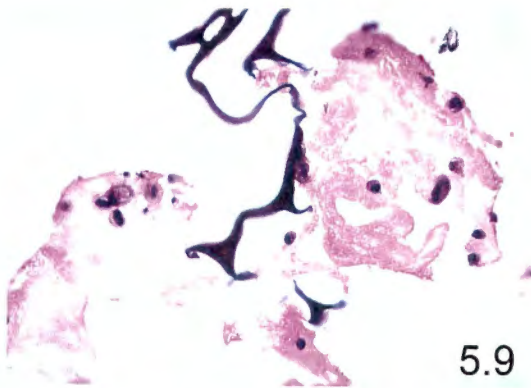
Photomicrograph 5.12: Immunoreactivity for PDGF-R β in hEEC on Matrigel / Gelfoam matix. Fast red chromagen with hematoxylin counterstain. (20X)

Photomicrograph 5.13: Immunoreactivity for PDGF-R α in hEEC on Matrigel / Gelfoam matix. Fast red chromagen with hematoxylin counterstain. (20X)

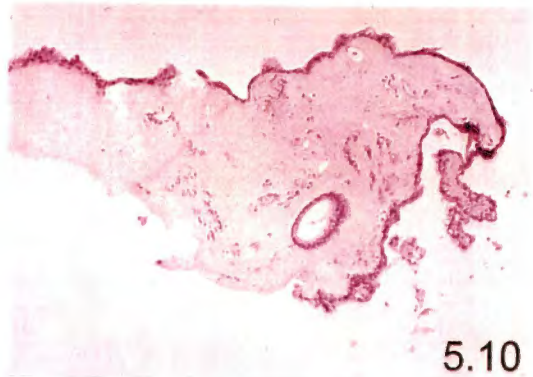
Photomicrograph 5.14: Example of attachment distribution of free floating implants (arrows), Millicell membrane (cranial) in the peritoneal cavity of scid mouse two weeks post transplantation.

Photomicrograph 5.15: Millicell membrane attached to the right caudal liver lobe two weeks post implantation. Note neovascularization extending from the hepatic capsule onto the implant.

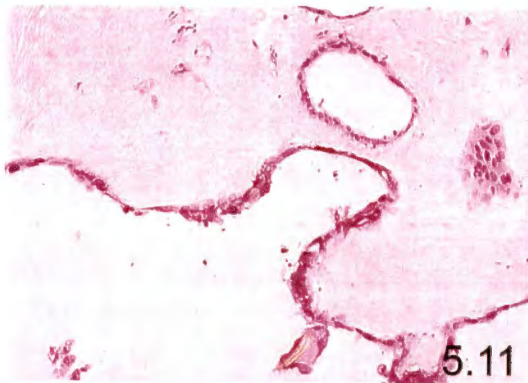
Photomicrograph 5.16: Matrigel / Gelfoam implant adhesion to the intestinal and lateral body wall at two weeks post transplantation. Neovascularization can be seen extending onto the implant.



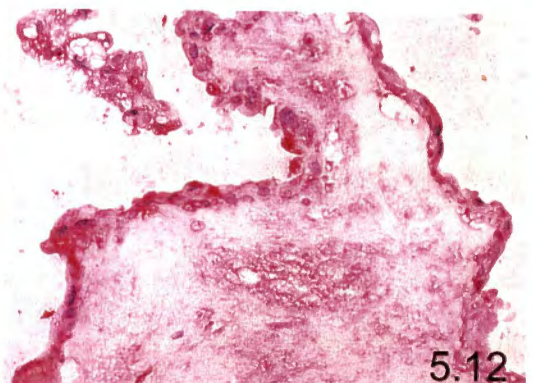
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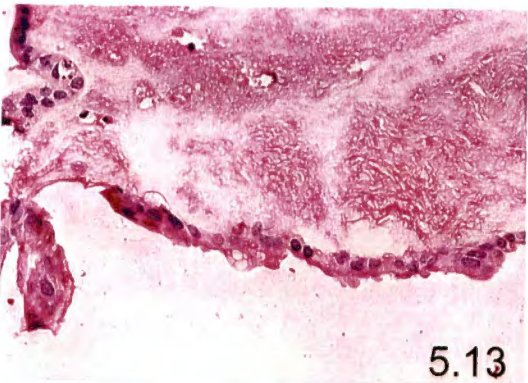
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5.11



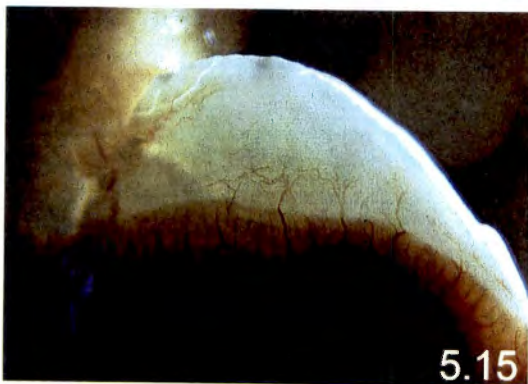
5.12



5.13



5.14



5.15



5.16

Color Plate 5 (continued)

Photomicrograph 5.17: Clumping and attachment of hEEC cell in mesothelial cleft at 2 hours post peritoneal infusion. H & E stain. (40X)

Photomicrograph 5.18: Attachment of hEEC-seeded Matrigel / Gelfoam implant to the intestinal wall of a scid mouse at two weeks post-transplantation. Note the paucity of inflammatory cellular infiltrates in the implant. H & E stain. (10X)

Photomicrograph 5.19: hEEC seeded Matrigel / Gelfoam implant two weeks post transplantation in to the peritoneal cavity of a scid mouse. A glandular type structure of HEEC (arrow) is present in the Matrigel (asterisk) / Gelfoam (arrowheads) matrix. H & E stain. (40X)

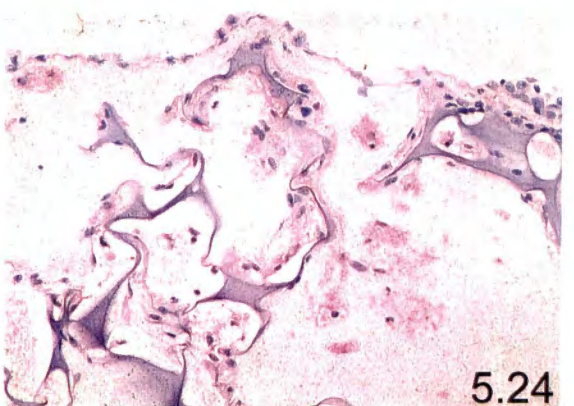
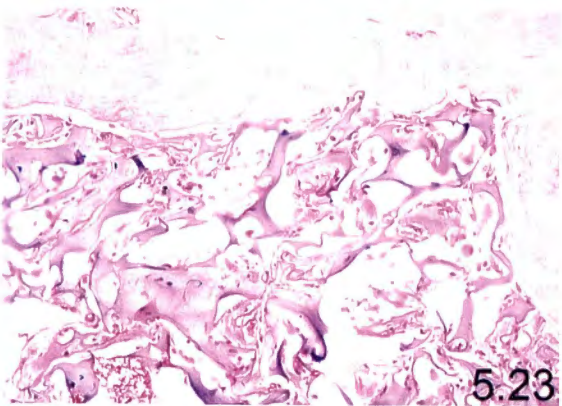
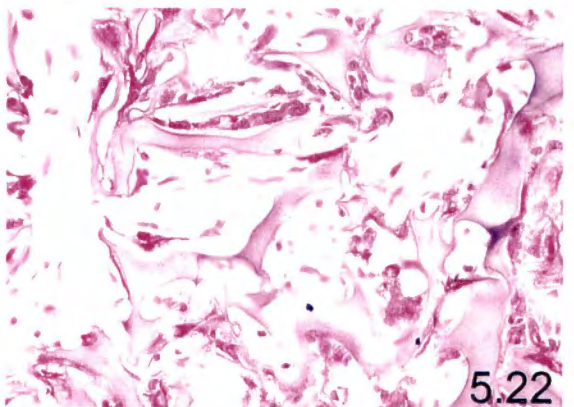
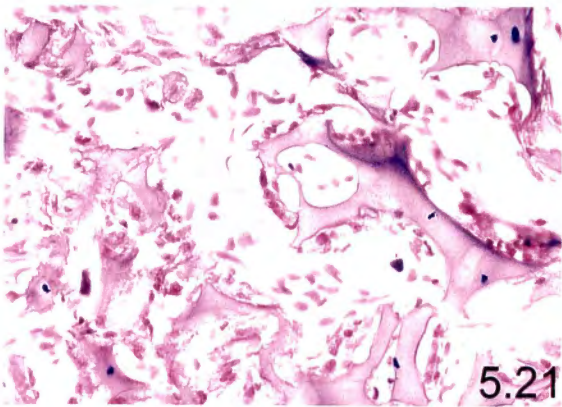
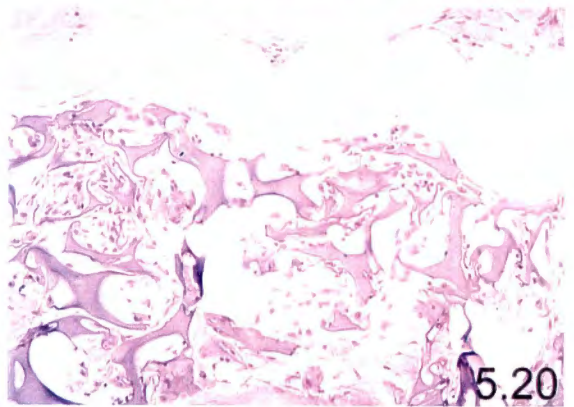
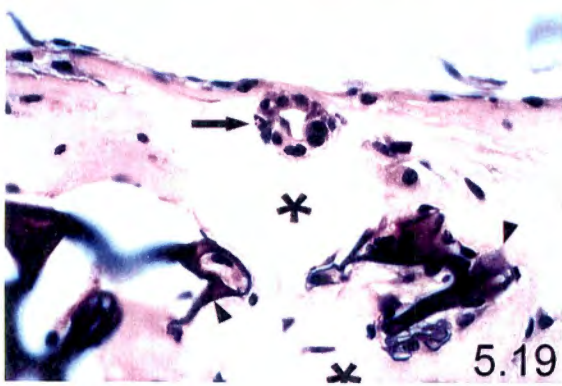
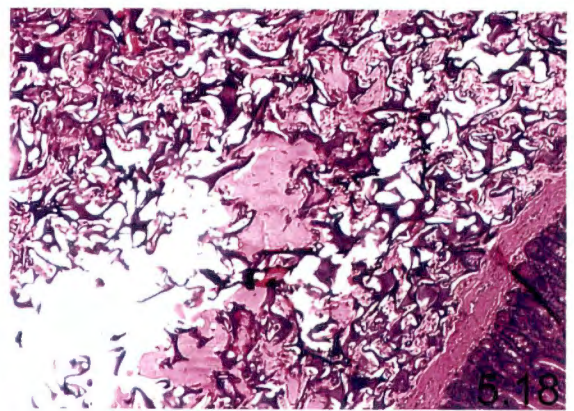
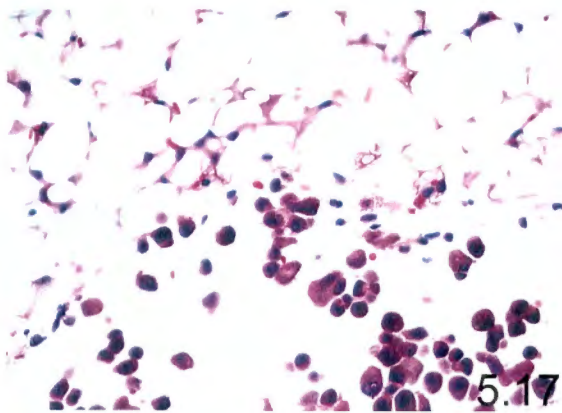
Photomicrograph 5.20: Positive immunoreactivity to vimentin IF of the hEEC in a Matrigel / Gelfoam implant six weeks post transplantation into the peritoneal cavity of a scid mouse can be seen in this photomicrograph. Because the monoclonal (V9) antibody to the vimentin IF does not cross react with the mouse vimentin IF and hEEC contain vimentin IF the vimentin antibody was used to positively identify hEEC. Chromagen Vector red with hematoxylin counterstain. (10X)

Photomicrograph 5.21: Higher magnification of 5.20. Vimentin positive immunoreactive cells can be seen to line the Matrigel / Gelfoam implant trabeculae. Vector red chromagen with hematoxylin counterstain. (40X)

Photomicrograph 5.22: Immunoreactivity for PDGF-R β is present in the transplanted hEEC within the Matrigel / Gelfoam implant six weeks post transplantation into the peritoneal cavity of a scid mouse. Vector red chromagen with hematoxylin counterstain. (40X)

Photomicrograph 5.23: Immunoreactivity for PDGF-R α is present in the transplanted hEEC within the Matrigel / Gelfoam implant six weeks post transplantation into the peritoneal cavity of a scid mouse. Vector red chromagen with hematoxylin counterstain. (10X)

Photomicrograph 5.24: Immunoreactivity for pancytokeratin is present in the transplanted hEEC within the Matrigel / Gelfoam implant six weeks post transplantation into the peritoneal cavity of a scid mouse. Vector red chromagen with hematoxylin counterstain. (40X)



Vita

Robert Lindsey Donnell was born in Lumberton, North Carolina on April 9, 1954. He attended public schools in the DeKalb County, Georgia, and after attending the Georgia Institute of Technology in lieu of his senior year of high school, graduated from Clarkston High School in June 1972. He entered Southwestern at Memphis in 1972, from which he received a Bachelor of Arts with distinction in Art in 1976. He returned to academia in 1982, at the University of Tennessee, in order to fulfill prerequisite coursework for pursuit of a career in veterinary medicine. He entered the University of Tennessee College of Veterinary Medicine in the fall of 1985 and received his Doctor of Veterinary Medicine degree in the spring of 1988. After working in private practice for two years he returned to the University of Tennessee, College of Veterinary Medicine as a medical resident in the Department of Pathology and as a graduate student in the Comparative and Experimental Medicine program. In 1994, he successfully completed his residency requirements, passed the anatomic specialty exam of the American College of Veterinary Pathologists boards and was recognized as a Diplomate of the American College of Pathology. During the period of November 1994 until October 1997, he worked to complete his doctoral research while participating as chief pathologist investigating "Phenotypic Changes Associated with Murine Chromosomal Translocations" for a Cooperative Research and Development Award project administered by the University of Tennessee Department of Pathology, Martin-Marietta Corporation (ORNL- Oak Ridge, TN) and Darwin Corporation (Seattle, WA). The doctoral degree was received in December 1997.