SPONTANEOUSLY OCCURRING FIBROID TUMORS OF THE

LAYING HEN OVIDUCT

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# SPONTANEOUSLY OCCURRING FIBROID TUMORS OF THE LAYING HEN OVIDUCT

Amy Lynn Doernte

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SPONTANEOUSLY OCCURRING FIBROID TUMORS OF THE

LAYING HEN OVIDUCT

Amy Lynn Doernte



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# VITA

Amy Lynn Doernte, daughter of Harry Robert and Deborah Lynn Doernte, was born January 15th, 1982, in Newport News, Virginia. She graduated from Poquoson High School in 2000. She attended Virginia Polytechnic Institute and State University in Blacksburg, Virginia, and graduated in July, 2004, with a Bachelor of Science degree in Animal and Poultry Sciences. In August, 2004, she entered Graduate School at Auburn University under the direction of Wallace Berry, Ph.D.

THESIS ABSTRACT

SPONTANEOUSLY OCCURRING FIBROID TUMORS OF THE

LAYING HEN OVIDUCT

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Spontaneously occurring benign uterine leiomyomas (fibroids) are the most common reproductive tract tumor of women in the United States. It is estimated that more than 70% of women will develop uterine fibroids and the presence of these tumors is a primary reason for hysterectomies. Research into the causes and treatment of uterine fibroids is hampered by a lack of reliable animal models for the disease. Leiomyomas that appear to be outwardly similar to human uterine fibroid tumors are known to occur on the oviducts of laying hens over two years of age. However, the cellular and molecular similarities of those tumors to human leiomyomas have not been determined. The objective of this study was to characterize the avian tumors and compare them to human uterine fibroids for the purpose of determining the suitability of the aging hen as a model system for the study of the disease. Hens at 5 years of age were examined for the

presence of oviduct-associated fibroid tumors. Tumors were found attached to the internal surface of the oviduct, embedded in the oviduct wall, or attached to the exterior of the magnum and isthmus. Tumor and normal oviduct samples were frozen or fixed in formalin for histological and immunohistochemical analyses of biomarkers characteristic of human fibroids including estrogen receptor-α (ER-α), progesterone receptor (PR), proliferating cell nuclear antigen (PCNA), B-cell leukemia/lymphoma 2 (Bcl-2), transforming growth factor-β3 (TGF-β3), insulin-like growth factor-2 (IGF-2), and insulin-like growth factor binding protein-5 (IGFBP-5). Human uterine fibroid samples were acquired and evaluated in comparison to hen oviduct fibroids. The results indicate that laying hen oviduct associated fibroids are similar to human uterine leiomyomas with respect to the defined biomarkers; therefore, it appears the hen may provide a useful model for the study of the disease in humans.

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# I. INTRODUCTION

Uterine leiomyomas, otherwise known as fibroids, are the most common type of reproductive tract tumor of women in the United States. Modern imaging techniques and pathological examination of specimens indicate that the incidence of uterine fibroids is as high as 77%, suggesting that these tumors are far more prevalent than previously estimated by clinical cases (20-50%) (Stewart, 2001). Regardless of their generally benign neoplastic characteristics, uterine fibroids are responsible for significant morbidity in a large segment of the female population. These tumors are the single most common indication for a hysterectomy, accounting for approximately one-third (200,000) of all hysterectomies per year (Wilcox et al., 1994, Gambone et al., 1990). The clinical effects of these tumors are related to their local mass effect, resulting in pressure upon adjacent organs, fatigue, prolonged or heavy menstrual bleeding, pelvic pressure or pain, and, in rare cases, reproductive dysfunction (Haney, 2000).

In spite of their high prevalence, little is known concerning the etiology and molecular basis for the development and growth of uterine fibroids. It is well established that human uterine leiomyomas possess receptors for estrogen and progesterone and grow under the influence of ovarian steroid hormones (Newbold et al., 2000). Acting as intermediate elements, cytokines and growth factors regulated by ovarian steroids are thought to promote the growth of leiomyomas. Abnormal production of cytokines and growth factors may lead to an increase in cell proliferation, cellular hypertrophy,

accumulation of extracellular matrix (ECM), or a combination of these phenomena to cause fibroid initiation and growth (Sozen and Arici, 2002).

Recently, Matsuo *et al.* (1999) proposed another potential mechanism, decreased apoptosis, which may contribute to the growth and maturation of leiomyomas. Bcl-2, a proto-oncogene product, is an integral membrane protein located in the endoplasmic reticulum (ER), nuclear envelope, and in the outer membranes of the mitochondria (Kimball, 2005). Matsuo *et al.* (1999) found that the Bcl-2 protein that functions to inhibit apoptosis was abundantly expressed in leiomyoma relative to that in normal myometrium. Reduced apoptosis leading to reduced cell turnover in the myometrium results in neoplastic accumulation of cells that eventually become fibrotic due to cellular senescence.

In the course of studies using the aged laying hen as a model for development of ovarian cancer, the authors noticed that fibroid tumors of the oviduct were common in hens over two years of age. It was decided to characterize these tumors and compare them to human uterine fibroids for the purpose of determining the suitability of the hen as a model system for the study of the disease. In addition to the high rate of spontaneously occurring fibroid tumors on the oviduct, hens have proven to be excellent research subjects for modeling human reproductive tract disorders. Laying hens, like humans, are in a persistent reproductive state, whereas common laboratory animals are estrous cycle breeders. In addition, they have an ovulatory hormonal cycle similar to that of humans, but compressed in time. Validation of the hen as an experimental model for reproductive tract fibroids will allow the study of disease origin, early detection, development of treatments, and ultimately, prevention. The specific objective of the present study was to

evaluate hen reproductive tract fibroids for the presence of biomarkers normally associated with human fibroids: estrogen receptor (ER), progesterone receptor (PR), proliferating cell nuclear antigen (PCNA), B-cell leukemia/lymphoma 2 (Bcl-2), transforming growth factor-β3 (TGF-β3), insulin-like growth factor-2 (IGF-2), and insulin-like growth factor binding protein-5 (IGFBP-5). The results of the current study demonstrate that hen fibroids possess the defined biomarkers typical of human fibroids; therefore, it appears the hen may provide a useful model for the study of the disease in humans.

# II. LITERATURE REVIEW

## Uterine Leiomyomas: An Overview

Uterine leiomyomas, otherwise known as fibroids, are the most common pelvic smooth muscle tumor of women in the United States. These benign tumors are the single most common indication for a hysterectomy, accounting for approximately one-third or about 200,000 of all hysterectomies per year (Wilcox et al., 1994, Gambone et al., 1990). Due to newer imaging techniques and careful pathological examination of surgical specimens, true prevalence is thought to be as high as 77%, suggesting that these tumors are far more prevalent than estimated by clinical cases (20-30%) (Stewart, 2001). The initiator or initiators of uterine fibroids remain unknown, however, several predisposing factors have been identified including age (late reproductive years), African-American ethnicity, nulliparity, and obesity (Flake et al., 2003). The majority of patients have multiple leiomyomas, and each tumor is thought to be clonal, arising independently from a single smooth muscle cell (Buttram et al., 1981). Leimyomas vary in size and are classified based on location (cervical, isthmic, or corporal) and uterine layer affected (subserosal, submucosal, or intramural). Uterine leiomyomas are most commonly located in the intramural layer of the body of the uterus frequently causing no symptoms (Wexler and Pernoll, 1994).

The risk of malignant transformation of uterine fibroids to leiomyosarcoma has been reported to range from 0.29% to 1% (Banu and Manyonda, 2004). Regardless of their generally benign neoplastic characteristics, uterine fibroids are responsible for significant morbidity in a large segment of the female population. The clinical effects of these tumors are related to their local mass effect, resulting in pressure upon adjacent organs, prolonged or heavy menstrual bleeding, pelvic pressure or pain, as well as problems related to pregnancy, including infertility and repetitive pregnancy loss (Haney, 2000).

In spite of their high prevalence, little is known concerning the etiology and molecular basis of the development and growth of uterine fibroids. It is well established that leiomyomas grow under the influence of the ovarian steroid hormones estrogen and progesterone (Newbold et al., 2000). An up-regulation of expression of proliferating cell nuclear antigen (PCNA) in leiomyomas by progesterone and estradiol has been reported in the literature (Matsuo et al., 1999). This suggests that ovarian steroid hormones are exerting growth-stimulatory effects on leiomyomas through the presence of intermediate elements such as cytokines and growth factors. Estrogen and progesterone may regulate gene expression of these cytokines and growth factors, which in turn modify other genes' transcription. The result of this abnormal production of cytokines and growth factors may be an increase in cell proliferation, cellular hypertrophy, accumulation of extracellular matrix (ECM), or a combination of these phenomena (Sozen and Arici, 2002).

Recently, Matsuo *et al*. proposed another potential mechanism, decreased apoptosis, which is thought to contribute to the growth and maturation of leiomyomas. Matsuo and his colleagues found that the Bcl-2 protein, an apoptosis-inhibiting gene

product, was abundantly expressed in leiomyoma relative to that in normal myometrium. In this study, Bcl-2 expression in leiomyoma cells was up-regulated by progesterone, but down-regulated by estradiol (Matsuo et al., 1997).

Leiomyomas are characterized by tissue fibrosis. One distinctive feature of leiomyoma is the presence of abundant fibrous connective tissue elements and extracellular matrix (ECM), hence the name “fibroids”. Overexpression of collagen, fibronectin, and glycosaminoglycans contributes to the formation and growth of the tumor, but it is also thought that this overproduced ECM may itself play a dynamic role in the development of fibroids by influencing cellular proliferation and differentiation in addition to providing a repository for biologically active growth factors and cytokines (Stewart et al., 1994, Arici and Sozen, 2000, Wolanska et al., 1998, Hulboy et al., 1997).

## Medical Treatment

Uterine fibroids, as benign tumors, can generally be managed expectantly unless they become symptomatic (50-75% of affected patients) (Guarnaccia and Rein, 2001, Towbin et al., 1996). Several factors determine treatment, including the size and location of the fibroids, the presenting symptoms, and the age and reproductive desires of the patient. Available treatment options include hormonal, surgical, and radiological modalities (Aubuchon et al., 2002).

### Hormonal Therapies for Leiomyomas

To prevent surgical treatment, new hormonal therapies are being discovered to regress leiomyomas. Hormonal therapy is based on the observation that leiomyoma

growth is influenced by estrogen and progesterone and prevented by the presence of androgens (Aubuchon et al., 2002). One of the most promising therapies includes the use of gonadotropin-releasing hormone (GnRH) treatments. GnRH is a decapeptide synthesized in the hypothalamus and secreted in a pulsatile manner into the anterior pituitary. This pulsing secretion of GnRH promotes secretion of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the anterior pituitary. When GnRH is administered in a continuous fashion, there is a brief increase in gonadotropin release followed by receptor down-regulation resulting in a hypogonadal state within 1-3 weeks. During this time, both serum estrogen and progesterone levels are suppressed. This down-regulated phase can be maintained with continuous treatments for many months, preventing the enlargement of leiomyomas (Nowak, 1999).

Other hormonal therapies include androgen therapy, anti-steroidal therapy, and manipulation of growth factors (Aubuchon et al., 2002). These all have potentially positive effects when used for treatment of uterine leiomyomas.

**Surgical Treatment of Leiomyomas**

Uterine fibroid tumors have conventionally been treated with hysterectomy accounting for 30% of the >590,000 hysterectomies performed in the United States per year (Guarnaccia and Rein, 2001). Hysterectomy, however, is associated with substantial morbidity and recovery times ranging from 2 to 8 weeks (Carlson et al., 1994). The majority of hysterectomies are performed via the abdominal route, but can now be done using the vaginal approach with the advantages that the patient will have no abdominal scar, decreased post-operative discomfort, decreased hospital stay, and decreased risk of

infection and overall cost (Aubuchon et al., 2002, Dicker et al., 1982, Stovall, 1992). Vaginal hysterectomies are not recommended for the patient who has a uterus larger than 10 to 14 weeks gestational size (Stovall, 1992).

Although hysterectomy has been the traditional treatment or cure for leiomyomas for many years, myomectomies have become more frequent as surgical techniques have improved for women who desire uterine preservation. It can be performed via the vaginal route or by laparotomy, laparoscopy, or hysteroscopy (Aubuchon et al., 2002). After myomectomy, 80% of patients have resolution of menorrhagia and anemia (Hurst et al., 2000).

**Radiological Treatment**

Uterine fibroid embolization (UFE) is one of the newest treatments of uterine fibroids as an alternative to surgery. The main purpose is to reduce the size of the fibroids and to treat excessive uterine bleeding (Al-Fadhli et al., 2004). UFE is a procedure in which an interventional radiologist inserts a catheter in the femoral artery and, with imaging guidance, deposits embolic material to block blood flow to the fibroid, causing it to regress (Smith, 2000). McLucas and Adler (2000) reported that in a review of 119 cases of UFE, about 70% of the patients had an immediate cessation of menorrhagia and improvement of pain and pressure symptoms after the procedure. At 6 months follow-up, the total uterine volume decreased by 56% and the average diameter of the largest myoma decreased by 36%.

UFE is not typically offered to women who desire to preserve their fertility. The long-term effects mainly relate to the possible concerns for ovarian failure, as well as the

unknown effects on the endometrium that may follow the procedure (Hurst et al., 2000). A few investigators, however, recently reported that ovarian function, especially in younger women, is not affected by UFE and as well as several reports of pregnancy following UFE (Tulandi 2003, Ravina et al., 2000). In an older woman (>45 years), the ovary might be less tolerant to embolization of the utero-ovarian collateral circulation resulting in compromised blood flow to the ovaries and possible permanent ovarian failure with severe menopausal symptoms. Based on these observations, it appears that myomectomy is a better alternative than UFE in women of reproductive age who wish to preserve their fertility (Al-Fadhli et al., 2004).

## Biology of Uterine Leiomyoma

### Initiators of Leiomyoma

The most important aspect of the etiology of human uterine fibroids, the initiator(s), remains unknown, although many theories have been advanced (Flake et al., 2003). In 1930, Mayer suggested that leiomyoma cells originate from myoblasts of uterine musculature. This theory has generally been accepted regarding the histogenesis of uterine leiomyoma. The progenitors of leiomyomas, however, have not clearly been identified as well as why neoplastic transformation of smooth muscle cells into leiomyoma predominantly occurs in the uterus despite the wide distribution of smooth muscle cells within the body (Fujii et al., 2004).

One hypothesis investigated the expression of apoptotic-positive cells in the myometrium during the menstrual cycle (Fujii et al., 2004). The apoptotic-positive cells were only observed in the follicular phase of the menstrual cycle, suggesting that smooth

muscle cells that are injured during menstruation appear to be eliminated in the follicular phase of the menstrual cycle. The majority of injured cells would be eliminated as apoptotic cells, but some may survive, acquiring a protective mechanism against oxidative stress and apoptosis indicating logical candidates for the progenitor cell of uterine leiomyoma. Uterine leiomyoma cells do in fact have a protective mechanism against oxidative stress expressing manganese superoxide dismutase (MnSOD) and against apoptosis expressing Bcl-2, PEP-19, and sFRP-1 (Kanamori et al., 2003, Fukuhara et al., 2002, Gao et al., 2001).

Another hypothesis states that increased levels of estrogen and progesterone result in an increased mitotic rate that may contribute to the likelihood of somatic mutations leading to leiomyoma formation (Rein, 2000). Others have suggested a predisposing genetic factor based on ethnic and familial predilections (Marshall et al., 1997, Schwartz and Marshall et al., 2000). Another theory postulates that the pathogenesis of uterine leiomyomas might be similar to the response to injury. Stewart and Nowak (1998) suggested that smooth muscle cells could respond to injury or ischemia with increased cellular proliferation and production of extracellular matrix (ECM), which are critical components for the pathogenesis of uterine leiomyomas.

Based on the literature, the possibility of hereditary genetic predisposition to fibroids cannot be excluded. On the other hand, evidence has been presented that karyotypic changes may occur secondarily during the evolution or aging of some fibroids (Mashal et al., 1994). It is assumed that preceding stimuli, conditions, or injuries are responsible for the induction of genetic changes, and in this sense, acquired genetic changes may be regarded as secondary (Flake et al., 2003).

**Differential Gene Expression in Leiomyoma**

Leiomyomas are true neoplasms, as detected by X-linked glucose-6-phosphate dehydrogenase enzyme analysis and by androgen receptor gene analysis (Fujii et al., 2004). Cytogenic studies have revealed that leiomyomas are monoclonal and specific chromosomal regions may be abnormal in up to 40% of tumors, specifically chromosomes 6, 7, 12, and 14 (Ligon and Morton, 2001). Interestingly, 60% of leiomyomas do not exhibit a cytogenic abnormality (Tsibris et al., 2002). This finding suggests that cytogenic abnormalities are secondary changes in tumorigenesis, and that primary genetic change may be responsible for tumor growth in genetically susceptible cells (Gross, 2001).

Microarray analysis revealed genes such as Purkinje cell protein 4 (PEP-19), secreted frizzled related protein 1 (sFRP1), stromelysin 3, IGF-2, TGF-β3, and IGFBP-5 are upregulated in leiomyoma when compared to matched normal myometrium (Kanamori et al., 2003). Tsibris et al. (2002) found similar results with upregulated expression of PEP-19, stromelysin 3, IGF-2, IGFBP-5, versican, and TGF-β3 in leiomyoma relative to normal matched myometrium.

Among these genes, PEP-19, a calmodulin regulatory protein found within neurons, exhibited the most striking difference in expression between leiomyoma and normal myometrium. The function of this protein in the pathogenesis of uterine leiomyoma is unknown; however, it might be involved in the control of apoptosis of leiomyoma cells (Kanamori et al., 2003).

Fujii *et al*. (2004) reported an over-expression of secreted frizzled related protein 1 (sFRP1), a modulator of Wnt signaling in uterine leiomyoma with the strongest expression in the late follicular phase of the menstrual cycle when estrogen levels are the highest. The authors suggest that strong sFRP1 expression under high estrogenic conditions is thought to contribute to the development of uterine leiomyomas through the anti-apoptotic effect of sFRP1, which appears to be independent of cell proliferation (Fukuhara et al., 2002).

**Promoters: Estrogen and Progesterone**

Although the pathogenesis of leimomyomas is not clearly understood, there is considerable evidence that estrogen and progesterone are involved in promoting tumor growth. In humans, leiomyomas only occur after menarche, develop during the reproductive years, and may increase in size during pregnancy or after administration of oral contraceptives (Stewart, 2001). Cessation of fibroid growth and, often, regression occur following menopause (Nowak, 1999). Furthermore, treatment with gonadotropin releasing hormone (GnRH) analogs, which reduces the steroid hormone concentrations, leads to a reduction in the size of leiomyomas; however, enlargement of leiomyomas recurs after therapy with GnRH analogs is discontinued (Maruo et al., 2004). This indicates ovarian steroid-dependent growth potential.

Because leiomyoma growth is closely associated to reproductive years, and the vital role of estrogen in uterine growth has been established (Murphy and Ghahary, 1990), estrogen has received much attention as the major factor responsible for leiomyoma development. The mechanism underlying the stimulatory effects of

progesterone on leiomyoma growth has not been as fully defined. The clinical observations that support the estrogen hypothesis also support the involvement of progesterone in the development of uterine fibroids. Progesterone levels, in a manner similar to that of estrogen, are also clinically elevated during the reproductive years, are significantly elevated during pregnancy, and are suppressed after menopause rendering it difficult to distinguish the relative importance between estrogen versus progesterone involvement in the development of uterine fibroids (Rein et al., 1995).

A great deal of biochemical and molecular evidence has been uncovered supporting a role for estrogen and progesterone in tumor growth. Elevated expression of estrogen-regulated genes has been seen in leiomyomas compared with autologous myometrium, including connexin 43 gap junction protein, type 1 and 3 collagen, insulin-like growth factor-1 (IGF-1) and its receptor, parathyroid hormone-related peptide, progesterone receptor (PR), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (Huet-Hudson et al., 1990; Murphy and Ghahary, 1990; Nelson et al., 1992; Andersen et al., 1995; Barbarisi et al., 2001; Murphy et al. 1994). In addition, it has been reported that aromatase, an estrogen synthetase that catalyzes androgen to estrogens, is expressed at higher levels in leiomyomas when compared to normal myometrium (Yamamoto et al., 1984).

It has been suggested that progesterone may stimulate the mitotic activity and proliferation of leiomyoma cells. In both myometrium and leiomyomas, it appears that peak cell proliferation occurs during the secretory phase of the menstrual cycle when progesterone levels are high (Nowak, 1999). Brandon *et al*. (1993) demonstrated increased PR mRNA and protein levels in human leiomyoma together with elevated

proliferation-associated antigen Ki-67 in comparison to adjacent myometrium, suggesting the association of progesterone-mediated signaling with leiomyoma growth. These findings support the view that estrogen and progesterone play a vital role in promoting the growth of uterine leiomyomas.

**Estrogen and Progesterone Receptors**

From review of the literature, it can be concluded that leiomyomas have significantly elevated levels of estrogen and progesterone receptors when compared to the normal myometrium (Flake et al., 2003). Furthermore, Sadan *et al.* (1987) found ER and PR levels to be elevated in fibroids during all phases of the menstrual cycle when compared with matched myometrium. In addition, this study reported that receptor concentrations were independent of the size of the tumor (Sadan et al., 1987). Interestingly, one study reported that ER and PR levels were significantly higher in submucosal than subserosal leiomyomas, leading the authors to speculate about different etiologies and types of leiomyomas (Marugo et al., 1989).

Throughout the entire myometrium, nuclear expression of both ER-α and ER-β has been demonstrated immunohistochemically (Taylor and Al-Azzawi, 2000). The significance of ER-β relative to the classic ER-α has not been fully determined due to its fairly recent discovery in 1996 (Kuiper et al., 1996; Mosselman et al., 1996).

Progesterone receptor-A (PR-A) and progesterone receptor-B (PR-B) are both found to be expressed in leiomyomas and myometrium, with the concentrations of PR-A reported higher than that of PR-B in both tissues (Viville et al., 1997).

The interaction between estrogen and progesterone with their respective receptors has been reviewed extensively with regard to the role of the steroid hormones in promoting fibroid growth. It is well established that estrogen increases the levels of both ER and PR in the myometrium, whereas, the effect of progesterone is to decrease the levels of ER (Hsueh et al., 1975, Katzenellenbogen, 1980, Thi et al., 1975). These observations are consistent with the sequential presentation of these two hormones during the menstrual cycle and the observations that in the myometrium, both ER and PR levels rise during the follicular (proliferative) phase when estrogen levels are highest and subsequently fall during the luteal (secretory) phase when progesterone is the dominant steroid hormone (Flake et al., 2003).

**Role of Bcl-2 Protein in Leiomyoma Development**

Leiomyomas show an increase in expression of the Bcl-2 (B-cell leukemia/lymphoma 2) protein, which is an apoptosis-inhibiting gene product that prevents the normal course of apoptotic cell death in a variety of cells (Bodner et al., 2004). Additionally, Bcl-2 can promote cell replication by reducing the requirement for growth factors (Reed et al., 1991, Nunez et al., 1990). The Bcl-2 protein and its possible prognostic significance have been demonstrated in a variety of human cancers (Barreton et al., 1996, Nakanishi et al., 1997, Nakopoulou et al., 1998). The greater abundance of Bcl-2 in leiomyomas relative to myometrium suggests that the protein is likely to play an important role in the growth of fibroid tumors.

Matsuo et al. (2000) reported that Bcl-2 protein expression in leiomyoma cells predominated in the secretory, progesterone-dominated, phase of the menstrual cycle

compared to that in the proliferative phase. In addition, production of Bcl-2 protein in leiomyoma cells cultured *in vitro* was significantly increased by progesterone (Matsuo et al., 2000). Consistent with these findings, Nowak (1999) suggested that peak mitotic activity in both myometrium and leiomyomas occurred during the secretory phase of the cycle when progesterone levels are high. Since Bcl-2 production has been shown to prolong cell survival by preventing apoptotic cell death, progesterone may act as a growth-promoting factor in regulating leiomyoma growth through the enhanced inhibition of cell death in leiomyomas.

**Effectors: Growth Factors Identified in Fibroids**

There is increasing evidence to suggest that the growth-promoting effects of estrogen and progesterone on uterine leiomyomas are mediated by local growth factors. Those that have received the most attention in the literature include epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor-β (TGFβ), heparin-binding epidermal growth factor (HBEGF), and platelet derived growth factor (PDGF) (Sozen and Arici, 2002). A brief review of each growth factor will be presented.

**Epidermal growth factor**

One of the earliest investigated and characterized growth factors in the uterus is epidermal growth factor (EGF). Epidermal growth factor is a 53 amino acid polypeptide with well-documented mitogenic and differentiating effects in various reproductive tissues. Studies on EGF and its receptor in leiomyoma date back to 1984 when the presence of EGF binding sites in human myometrium and leiomyoma were reported

(Hoffman et al., 1984). Myometrial and leiomyoma cells both express EGF mRNA and EGF protein throughout the cycle, but a clear differential level of expression in these tissues is not defined. One study showed that there were significantly decreased levels of EGF in leiomyoma tissue compared to matched myometrium (Dixon et al., 2000). In contrast, an earlier study reported that leiomyomas produce greater amounts of EGF mRNA than normal myometrium during the luteal phase of the cycle (Harrison-Woolrych et al., 1994). In line with this study, an *in vitro* study demonstrated an up-regulation of EGF in leiomyoma tissue compared to matched myometrium (Shimomura et al., 1998).

It has been shown that progesterone up-regulates EGF-like protein expression in cultured leiomyoma cells, whereas EGF-R expression in those cells was up-regulated by estrogen. As EGF is known to play a crucial role as a local factor in the autocrine/paracrine regulation of leiomyoma growth, it is conceivable that progesterone and estrogen act in combination to stimulate the proliferative potential of leiomyoma cells through the induction of EGF-like proteins and EGF-R expression in human uterine leiomyoma (Matsuo et al., 2000).

EGF receptors have been quantified in leiomyomas and myometrium using radioligand-binding techniques and polymerase chain reaction. The number of EGF receptors in the two tissues is fairly similar. EGF has also been shown to be mitogenic for both myometrial and leiomyoma smooth muscle cells in culture (Fayed et al., 1989).

The accumulated evidence supports the hypothesis that EGF is an important growth factor in the development of leiomyomas and further suggests that progesterone is an important factor influencing EGF mRNA production.

**Insulin-like growth factor**

Insulin-like growth factors (IGFs) are small polypeptides that are structurally related to proinsulin and promote cellular proliferation, differentiation, and cell survival (Strawn et al., 1995, Yu and Berkel, 1999). There are two well-characterized IGFs noted as the 70 amino acid IGF-1 and the 67 amino acid IGF-2. Production of these growth factors has been observed in a wide variety of tissues (Han et al., 1987). The actions of IGFs are mediated through the IGF receptors, primarily IGF-1 receptor, and regulated by the IGF-binding proteins. The sources of IGFs include endocrine, paracrine, or autocrine products. Autocrine production is a primary mode of IGF action on some tumor cells (Toretsky and Helman, 1996).

Human myometrium and leiomyoma both express the mRNAs for IGF-1 and IGF-2. The relative levels of expression of these two growth factors have been analyzed. Results showed that the levels of IGF-1 mRNA were similar in both tissues. On the other hand, the levels of IGF-2 mRNA were consistently higher in leiomyomas than in normal myometrial tissue (Hoopner et al., 1988, Boehm et al., 1990, Vollenhoven et al., 1993) and are further increased in leiomyosarcomas (Daughaday et al., 1988, Gloudemans et al., 1990).

Both IGF 1 and 2 can bind to the IGF-1 receptor with equal binding affinity, whereas the IGF-2 receptor preferentially binds to IGF-2. The IGF-1 receptor mediates most of the biological actions of IGFs including mitogenic, metabolic, and cell-survival properties through tyrosine kinase signaling (Flake et al., 2003). Leiomyomas contained higher number of IGF-1 receptors than myometrium when measured by radioligand-binding assays (Nowak, 1999). Localization of IGF-1 receptors by autoradiography

showed that the majority of IGF-1 binding to uterine secretions occurred in the smooth muscle cells of the myometrium (Murphy and Ghahary, 1990). Thus, both IGF-1 and its receptors are most abundant in the myometrial layer of the uterus. *In vitro* studies using myometrial cells and leiomyoma cells in culture have shown that IGF-1 is a mitogen for these cells, particularly in combination with EGF or platelet-derived growth factor (Nowak, 1999).

In most situations, the IGF binding proteins inhibit the actions of IGFs by blocking their binding to the receptor; in certain circumstances, however, these binding proteins may be able to enhance the action of IGF-1 by increasing its bioavailability in target tissues by binding to it and preventing its degradation (Yu and Berkel, 1999). Of the IGF-binding proteins, IGFBP-5, was over-expressed in leiomyoma, although at the protein level, IGFBP-5 may be regulated by pregnancy-associated plasma protein A, a protease that was consistently over-expressed in leiomyomata (Tsibris et al., 2002). One function of IGFBP-5 is to protect IGF from proteolytic cleavage and clearance, however, when bound to a target cell, the binding protein may change conformation and release IGF locally. In summary, these findings suggest that differences in IGF production, levels of IGF receptors, or expression of binding proteins may be partially responsible for the enhanced growth of leiomyomas.

**Transforming growth factor-β**

Transforming growth factor-β (TGF-β), a dimeric polypeptide composed of 112 amino acid subunits, belongs to a family comprised of polypeptide growth factors that exist in three isoforms (TGF-β1, 2, 3) encoded by distinct but closely related genes

(Sozen and Arici, 2002). Transforming growth factor-βs are multifunctional growth factors that regulate many aspects of cell function including proliferation, differentiation, migration and adhesiveness (Massague, 1990). A function of TGF-β of particular interest is its ability to up-regulate the synthesis of many components of the extracellular matrix, leading to fibrosis, a characteristic of uterine fibroids (Lyons and Moses, 1990).

The presence of TGF-βs and their receptors in human myometrium and leiomyoma was first demonstrated by Chegini *et al*. and Arici *et al*. in 1994. One study (Lee and Nowak, 2001) found TGF-β3 expression to be elevated in leiomyomas relative to the adjacent myometrium. In support of these findings, Arici and Sozen (2000) found that the TGF-β3 mRNA levels were 3.5-fold higher than in the myometrium. In contrast, no significant difference in the levels of TGF-β1 mRNA abundance was observed between the myometrium and leiomyomas (Vollenhoven et al., 1995). On the other hand, TGF-β1 inhibited the proliferation of normal myometrial cells, although leiomyoma smooth muscle cells in culture did not show similar growth inhibition by TGF-β1 (Sozen et al., 2000).

One study reported a significant increase in the expression of TGF-β3 in luteal phase leiomyoma samples. This observation suggests a potential stimulatory role for progesterone (possibly in combination with estradiol, which up-regulated progesterone receptors) in the expression of TGF-β3 (Arici and Sozen, 2000). Interestingly, the levels of TGF-β1 and TGF-β3 mRNAs were reduced in patients receiving GnRH therapy (Dou et al., 1996). This further suggests that ovarian steroid hormones may regulate the production of these growth factors.

The conclusive data suggests that TGF-β3 has a potentially important role in uterine leiomyoma growth by stimulating cellular proliferation and the production of ECM, however, the effects of TGF-β3 depend upon multiple factors, including the concentration of TGF-β3, the specific target cell, the levels of ovarian steroid hormones, and the presence of other growth-regulatory molecules. Investigators have found that low concentrations of TGF-β3 lead to a significant increase in cell proliferation in both the myometrium and leiomyomas (Arici and Sozen, 2000, Lee and Nowak, 2001). On the other hand, TGF-β3 does not appear to have this effect at high concentrations. In a similar fashion, TGF-β1 appears to stimulate leiomyoma cellular proliferation at low levels, but this mitogenic effect disappears at higher concentrations (Sozen et al., 2000).

In evaluation of the potential role of TGF-β in the pathophysiology of fibroids, it is of particular interest that the gene encoding for TGF-β3 is located near the 14q23-24 break-points, one of the most common translocation sites identified in cytogenic studies of fibroids (Flake et al., 2003).

**Heparin-binding growth factor**

Heparin-binding growth factors are a family of mitogenic proteins that have varying affinities for heparin and heparin-like molecules. They include platelet derived growth factor (PDGF), basic and acidic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and heparin-binding epidermal growth factor (HBEGF). The fact that these growth factors bind to heparin allows them to be sequestered in the extra-cellular matrix (ECM), typically abundant in fibroids, making the

ECM an important reservoir of biologically active growth factors. These heparin binding growth factors may not only act as mitogens for leiomyoma cells but may also stimulate angiogenesis, blood vessel formation (Nowak, 1999).

Levels of PDGF, a potent mitogen for mesenchymal tissues, were not found to differ in leiomyomas relative to normal myometrium (Boehm et al., 1990). On the other hand, PDGF receptor sites in leiomyoma outnumbered those in myometrium, although the binding affinity for PDGF of those sites in leiomyoma tissue was lower than those in myometrium (Fayed et al., 1989). Basic FGF has also been examined in uterine leiomyoma. Both the levels of bFGF mRNA and protein were elevated in leiomyoma when compared to the normal myometrium. Immunohistochemical staining for bFGF in fibroids demonstrated much stronger staining in fibroids than in the myometrium because of the large amount of extracellular matrix in uterine leiomyoma (Mangrulkar et al., 1995). This finding suggests large quantities of bFGF are stored in the extracellular matrix of these tumors, further demonstrating the importance of ECM as a reservoir of growth factors that could promote leiomyoma growth (Nowak, 1999). The presence of VEGF and HBEGF expression has also been examined in uterine leiomyomas and normal myometrium. VEGF levels appear to be similar in the two tissues (Harrison-Woolrych et al., 1995), whereas, HBEGF was found to have a decreased expression in leiomyoma when compared to normal myometrium (Mangrulkar et al., 1995), a finding that essentially argues against a possible role for this factor.

## Summary of Growth Factors

A number of growth factors have been investigated in leiomyoma to determine which factors may be responsible for mediating the growth-promoting effects of ovarian hormones. Many of these growth factors interact, sometimes resulting in a synergistic effect, demonstrated by the two angiogenic mitogens VEGF and bFGF (Flake et al., 2003). Other situations indicate one growth factor's dependency upon the presence of another as illustrated by IGF-1 acting as a progression factor in the cell cycle when competence factors such as PDGF and FGF are also present (Cohick and Clemmons 1993). Based on this review of the literature, one can conclude that these major growth factors are potentially important in the pathogenesis of leiomyomas.

## Extracellular Matrix in Leiomyomas

Uterine leiomyomas contain large amounts of ECM, hence the name "fibroids". This ECM consists of an overexpression of collagen, fibronectin, and glycosaminoglycans contributing to the formation and growth of the bulk of the tumor (Stewart et al., 1994, Arici and Sozen, 2000, Wolanska et al., 1998). It is suggested that the overproduced ECM itself may play a dynamic role in the metabolic processes leading to tumor growth by influencing cellular proliferation and differentiation as well as a repository for biologically active growth factors and cytokines. Extracellular matrix components such as collagen, fibronectin, and proteoglycans, serve to confine cytokines and growth factors in the vicinity of tumor cells by binding tightly to them and preventing them from moving away to distant sites, thus, contributing to the development of leiomyomas (Arici and Sozen, 2002). In addition, authors suggest that ovarian steroid

hormones or other growth factors that vary throughout the menstrual cycle may be important in modulating the production of certain ECM proteins in leiomyomas accounting for the rapid and significant change in size of these tumors (Fujita, 1985, Puistola et al., 1990, Stewart et al., 1994, Nowak, 1999).

## Experimental Model Systems

Progress toward a better understanding of the biology of uterine leiomyomas and how their growth can be controlled is limited by a lack of suitable *in vivo* models. In contrast to the many well-characterized models of epithelial oncogenesis, relatively few animal models of soft tissue tumorigenesis have been developed.

### Mammalian

There is a rare occurrence of spontaneous leiomyomas in rodents. A low incidence of reproductive tract leiomyomas has been reported as an aging lesion in an inbred strain of the Brown Norway rat. There had been no reports of a high incidence of spontaneously occurring reproductive tract leiomyomas in any outbred stocks or inbred strains of laboratory rodents until fairly recent studies using Eker rats. Eker rats are genetically predisposed to tumor development by a germline mutation in the Tuberous Sclerosis-2 (Tsc-2) tumor suppressor gene. Leiomyomas develop in Eker rats in the uterine horns and body and resemble their human counterparts histologically, thus, providing a potential valuable new rodent model of smooth-muscle leiomyomas (Walker et al., 1996). Development of rodent models of uterine leiomyoma has allowed use of an *in vivo/in vitro* approach that combines studies in tumor-derived cell lines with

experimental manipulations in live animals. The combination of *in vivo* and *in vitro* tools has contributed to the understanding of leiomyoma cell signaling pathways and the response of tumors to hormonal modulation (Newbold et al., 2000).

**Avian: Potential Use for a Diagnostics Model of Uterine Leiomyoma**

Smooth muscle tumors (leiomyomas and leiomyosarcomas) are among the most common neoplasms in the avian species (Feldman and Olson, 1959). In the early research on muscle tumors in chickens, Feldman and Olson (1959) noted that leiomyomas and leiomyosarcomas may arise from any smooth muscle tissue but are more common in the ligament of the oviduct or the oviduct itself. In 1972, Valsala and Sivadas reported that out of 80 chickens that had tumors, 11.2% had leiomyomas. In addition, Ramakrishnan *et al.* (1980) reported the occurrence of a leiomyoma in an 11-mo-old White Leghorn hen and Anderson *et al.* (1985) reported a leiomyosarcoma in a 7-wk-old female broiler chicken.

In other avian species, Foster *et al.* (1989) observed the frequency of these tumors in adult laying Japanese quail (*Coturnix coturnix japonica*), with a high incidence of leiomyomas and leiomyosarcomas in the dorsal and ventral ligaments of the oviduct in growth-selected lines. Helmboldt and Wyand (1972) reported a leiomyoma found in the cecal wall of three golden pheasants (*Chrysolophus pictus*) believed to be caused by *Heterakis sp*. Furthermore, Sasipreeyajan *et al.* (1988) and Steinberg (1988) reported the occurrence of leiomyosarcomas in the Budgerigar (*Melopsittacus undulatus*).

It was decided to characterize the oviduct smooth muscle tumors in the laying hen and compare them to human uterine fibroids for the purpose of determining their

suitability as a model system for the study of the disease. In addition to the high rate of spontaneously occurring fibroid tumors on the oviduct, hens have proven to be excellent research subjects for modeling human reproductive tract disorders. Laying hens, like humans, are in a persistent reproductive state, whereas common laboratory animals, such as rodents, are estrous cycle breeders. In addition, they have an ovulatory hormonal cycle similar to that of humans, but compressed in time. Validation of the hen as an experimental model for reproductive tract fibroids will allow the study of disease origin, early detection, development of treatments, and ultimately, prevention.

## III. RESEARCH OBJECTIVES

To evaluate hen reproductive tract fibroids for the presence of biomarkers characteristic of human uterine fibroids: ER-α, PR, PCNA, Bcl-2, TGF-β3, IGF-2, and IGFBP-5.

To quantify the relative abundance of biomarkers normally associated with human fibroids in hen reproductive tract fibroids: ER-α, PR, PCNA, Bcl-2, TGF-β3, IGF-2, and IGFBP-5.

## IV. MATERIALS AND METHODS

Fifty White Leghorn hens, 5 year of age, were examined for the presence of oviduct associated fibroid tumors. A portion of fibroid and unaffected oviduct tissue was collected and immediately fixed in formalin for immunohistochemical analyses. The remaining tissue was homogenized in phosphate-buffered saline (PBS), centrifuged, and the supernatant was aliquoted and stored at $-20^0$ C for future analyses. This experiment and all animal procedures were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee.

### Immunohistochemistry

Fibroid and normal oviduct tissues were fixed in 10% formalin, dehydrated in a graded ethanol series, embedded in paraffin blocks, and sliced into 5-μm sections. Formalin fixed, paraffin embedded human fibroid samples were obtained for comparison. Samples were first deparaffinized in Hemo-D followed by a transfer to ethanol and then PBS. Deparaffinized sections were incubated with PBS containing 1% BSA as a blocking step for 60 min at room temperature. Sections were then incubated with mouse monoclonal antibodies for ER-α Ab-14 (Clone 1D5+6F11), PR Ab-2 (Clone hPRa 2), PCNA Ab-1 (Clone PC10), goat polyclonal TGF-β3, and monoclonal anti-human Bcl-2, IGF-2, and IGFBP-5 for 12 hours at $4^0$ C at a concentration of 4 μg/mL, 4 μg/mL, 2 μg/mL, 1 μg/mL, 0.4 μg/mL, 5 μg/mL , and 1 μg/mL , respectively. The primary

monoclonal antibodies against ER-α, PR, and PCNA were obtained from Lab Vision Corporation (Fremont, CA). The monoclonal anti-human Bcl-2, IGF-2, and IGFBP-5 antibodies were obtained from R&D Systems, Inc. (Minneapolis, MN) and the goat polyclonal TGF-β3 antibody was obtained from Abcam (Cambridge, MA). For comparison, normal hen oviduct and human uterine fibroid tissue received the same concentration of monoclonal antibody for all biomarkers. Slides subjected to the same staining procedure without primary antibody were used as controls for non-specific staining. Antibody binding was visualized using biotinylated goat anti-mouse secondary antibody followed by streptavidin peroxidase binding with diaminobenzidine (0.1%) as the color reagent (Lab Vision Corporation, Fremont, CA).

**Electrophoresis and Western Blot Analysis**

Fibroid and normal oviduct pooled samples (0.33 g/mL) were homogenized in ice-cold PBS. Total lysate was centrifuged at 15,000 g for 30 min at 4$^{o}$C. Supernatant aliquots were used for total protein determination and polyacrylamide gel electrophoresis (Laemmli, 1970). The protein concentration of supernatant aliquots was determined using the Bio-Rad protein assay kit. Standard curves were obtained from duplicate samples of bovine serum albumin solution (Sigma) ranging from 0.2, to 0.3, 0.6, and 0.9 mg/mL. For electrophoresis, sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% (w/v) SDS, 0.05% (w/v) bromophenol blue, and 400 µL β-mercaptoethanol) was added to the fibroid supernatants at a dilution of 1:7, whereas the normal oviduct samples were prepared by the addition of sample buffer for a 1:9 dilution. A total of 25 µg of total protein for

fibroid and normal oviduct tissues were loaded and separated on 7.5% polyacrylamide gels.

Proteins were transferred from the SDS-polyacrylamide gels to nitrocellulose membranes using the Western blotting technique (Burnette, 1981). Membranes were then incubated for 2 h at room temperature under constant agitation with ER, PR, PCNA, Bcl-2, TGF-β3, IGF-2, and IGFBP-5 primary antibodies diluted with BSA at 2 μg/mL, 4 μg/mL, 1 μg/mL, 2 μg/mL, 1 μg/mL , 4 μg/mL , and 4μg/mL , respectively. After three washings over 15 min in BSA, membranes were then incubated with biotinylated secondary antibody for 30 min at room temperature with gentle agitation. The membranes were then washed with BSA under the same conditions as described above and incubated in Vectastain Elite ABC Reagent at room temperature for 30 min. The color reaction was developed in the presence of the chromogenic substrate, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) until bands became visible (approximately 30 min). The Vectastain Elite ABC Reagent kit as well as the ABTS Substrate Kit for Peroxidase was obtained from Vector Laboratories (Burlingame, CA). Membrane blots were digitally scanned on a flatbed scanner. Molecular weight (MW) values were determined by calculating Rf ratios as the distance from the bottom of the well to the protein band of interest divided by the distance from the bottom of the well to the dye front. Standard curves were developed using log MW of protein standards versus Rf ratio of those standards. Apparent molecular weights of proteins identified by western blot analysis were determined by extrapolating MW from the standard curve using measured Rf ratios. The optical density of the bands corresponding to specific proteins was determined by densitometry using ImageJ software and biomarker levels in hen

fibroid tissue were quantified relative to normal oviduct tissue.

**Statistical Analysis**

Analysis of differences in the abundance of ER, PR, PCNA, Bcl-2, TGF-β3, IGF-2, and IGFBP-5 between normal hen oviduct and fibroid tissue was carried out using analysis of variance (ANOVA) with the general linear model procedure of SAS 9.1 software. $P < 0.05$ was considered statistically significant.

# V. RESULTS

Fibroids were identified in 100% of the examined hens. These tumors were attached to the internal surface of the oviduct, embedded in the oviduct wall, or attached to the exterior of the oviduct, specific to the magnum and isthmus. No fibroids were found associated with the eggshell gland.

Hemotoxylin and Eosin staining for overall morphology revealed a distinct similar appearance between the connective portion of normal hen oviduct tissue and fibroid tissue, suggesting hen fibroids are primarily composed of dense connective tissue (Figure 2).

Estrogen and progesterone receptors were identified in connective tissue and secretory epithelial cells in normal and fibroid tissue of the hen (Figure 3 and Figure 4). Diffuse cytoplasmic staining along with distinct nuclear staining was visualized in normal hen oviduct glandular epithelium cells (Figure 3A and Figure 4A), whereas, connective tissue of the hen fibroid demonstrated prominent nuclear staining of presumably active cells of the fibroid (Figure 3B and Figure 4B). The human fibroid exhibited similar results, with estrogen and progesterone receptor positive nuclei demonstrating discrete nuclear staining throughout the connective tissue (Figure 5). Comparison of estrogen and progesterone receptor abundance by western blot in matched pairs of normal oviduct tissue and fibroid specimens in the hen showed that fibroids had a

significantly higher level of expression of both receptors than did the corresponding normal oviduct tissue ($P < 0.05$; Table 1).

Immunohistochemical examinations of hen fibroid and adjacent normal oviduct tissue demonstrated nuclear PCNA labeling in fibroid tissue with greater staining intensity of cells at the interface of the normal oviduct and fibroid tissue (Figure 6A). Western blot analysis revealed an up-regulation of PCNA in the hen fibroid relative to the normal oviduct tissue ($P < 0.05$; Table 1). Different levels of staining intensity for PCNA in the human fibroid was observed, indicating areas of presumptive active growth in the fibroid (Figure 6B).

Bcl-2 protein in hen fibroid and normal oviduct tissues as well as human fibroids was evaluated. Immunohistochemical analysis illustrated that both the hen and the human fibroid displayed relatively diffuse cytoplasmic staining with some discrete staining of nuclei indicating regions of putatively higher growth (Figure 7). In comparison, the normal hen oviduct tissue exhibited dense staining in the glandular epithelium indicating areas of higher Bcl-2 abundance in actively secreting cells (Figure 7A). In the hen, western blot analysis revealed significantly elevated levels of the Bcl-2 protein in the fibroid relative to the normal oviduct tissue ($P < 0.05$, Table 1).

Immunohistochemical analysis revealed cytoplasmic localization of TGF-β3 in normal hen oviduct and fibroid tissue as well as human fibroid tissue (Figure 8). Unlike ER, PR, PCNA, and Bcl-2, there was no significant difference in the relative abundance of TGF-β3 in hen fibroids when compared to normal oviduct tissue ($P > 0.05$, Table 1).

The immunohistochemical comparison of IGF-2 in the hen and human fibroid demonstrated distinct similarities in the localization of IGF-2 between the respective

tissues. Both the hen and human fibroid connective tissue contained patches of intense staining that presumably indicate active areas of the fibroid (Figure 9). Similar to TGF-β3, there was no significant difference in the levels of IGF-2 between the hen fibroid and normal oviduct tissue ($P > 0.05$, Table 1).

Normal oviduct tissue in the hen demonstrated intense cytoplasmic staining for IGFBP-5 throughout the glandular epithelium, whereas, the hen and human fibroid displayed moderate cytoplasmic staining in the connective tissue (Figure 10). Similar to IGF-2, concentrated regions of higher IGFBP-5 abundance in the human fibroid were revealed (Figure 10B). Western blot analysis indicated no significant difference in the relative abundance of IGFBP-5 in the hen fibroid compared to normal oviduct tissue ($P > 0.05$, Table 1).

Table 1. Western blot analysis of ER, PR, PCNA, Bcl-2, TGF-β3, IGF-2, and IGFBP-5 protein expression in normal hen oviduct and fibroid tissues

| | Band Density[1] | | | |
|---|---|---|---|---|
| | Normal Oviduct Tissue | Fibroid Tissue | SEM[2] | *P*-Value[3] |
| ER | 6391[a] | 10203[b] | 877.2 | 0.0048 |
| PR | 81870[a] | 1220874[b] | 13983.2 | 0.0012 |
| PCNA | 112473[a] | 162555[b] | 7704.7 | < 0.0001 |
| Bcl-2 | 572227[a] | 730118[b] | 30031.8 | 0.0343 |
| TGF-β3 | 137727[a] | 131981[a] | 9386.8 | 0.4199 |
| IGF-2 | 314992[a] | 320999[a] | 5921.6 | 0.4171 |
| IGFBP-5 | 305741[a] | 310699[a] | 2724.9 | 0.2105 |

[a-b] Means within a row with different superscripts are significantly different at $P < 0.05$
[1] Averages obtained from both normal oviduct and fibroid tissue relative to the biomarker of interest (Arbitrary Unit)
[2] Pooled standard error of the mean
[3] $P < 0.05$ considered statistically significant

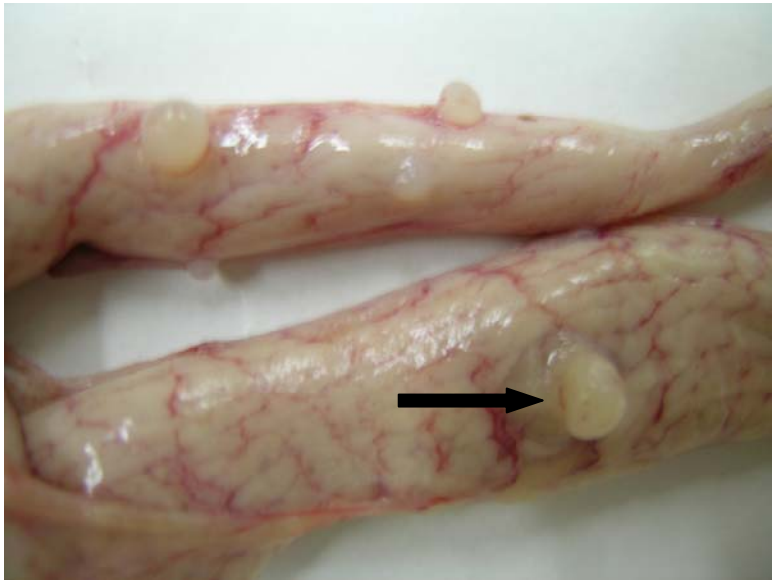

Figure 1. White Leghorn hen oviduct invested with fibroids along the magnum and isthmus. Arrow depicts a fibroid located on the exterior of the magnum.

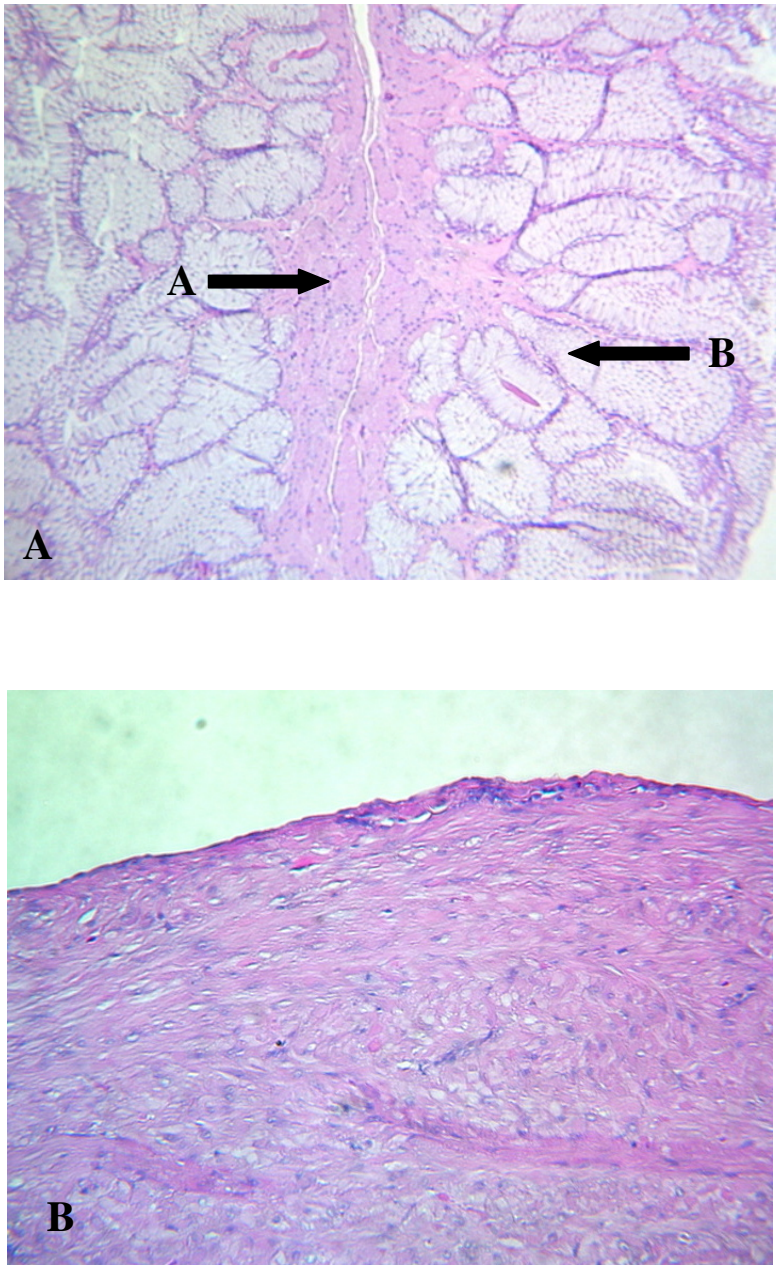


Figure 2. Hemotoxylin and Eosin staining of normal hen oviduct (A) and fibroid (B) tissues. Arrow A depicts connective portion and arrow B depicts glandular portion of normal hen oviduct tissue. 100X.

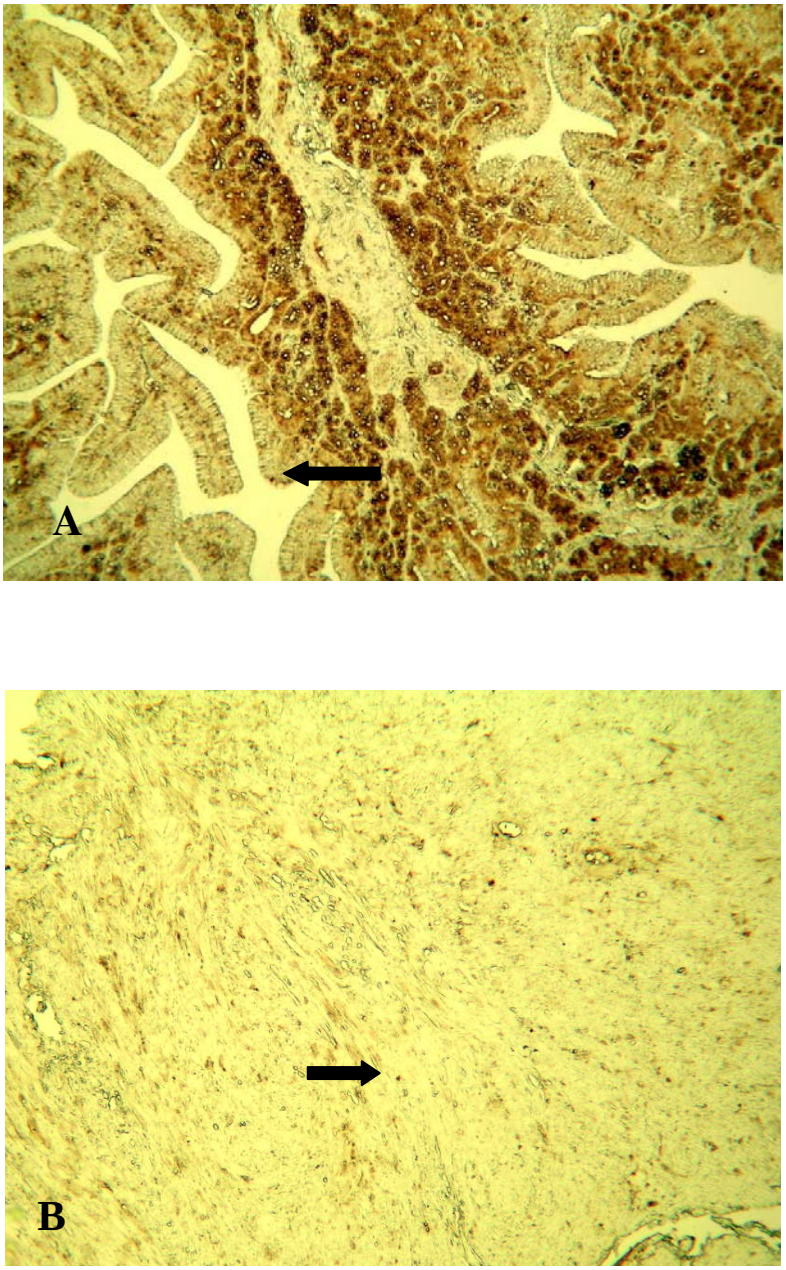


Figure 3. Immunohistochemical localization of estrogen receptor (ER) in normal hen oviduct tissue (A) and fibroid tissue (B). Arrows depict specific positive nuclei staining in the respective tissues. 100 X.

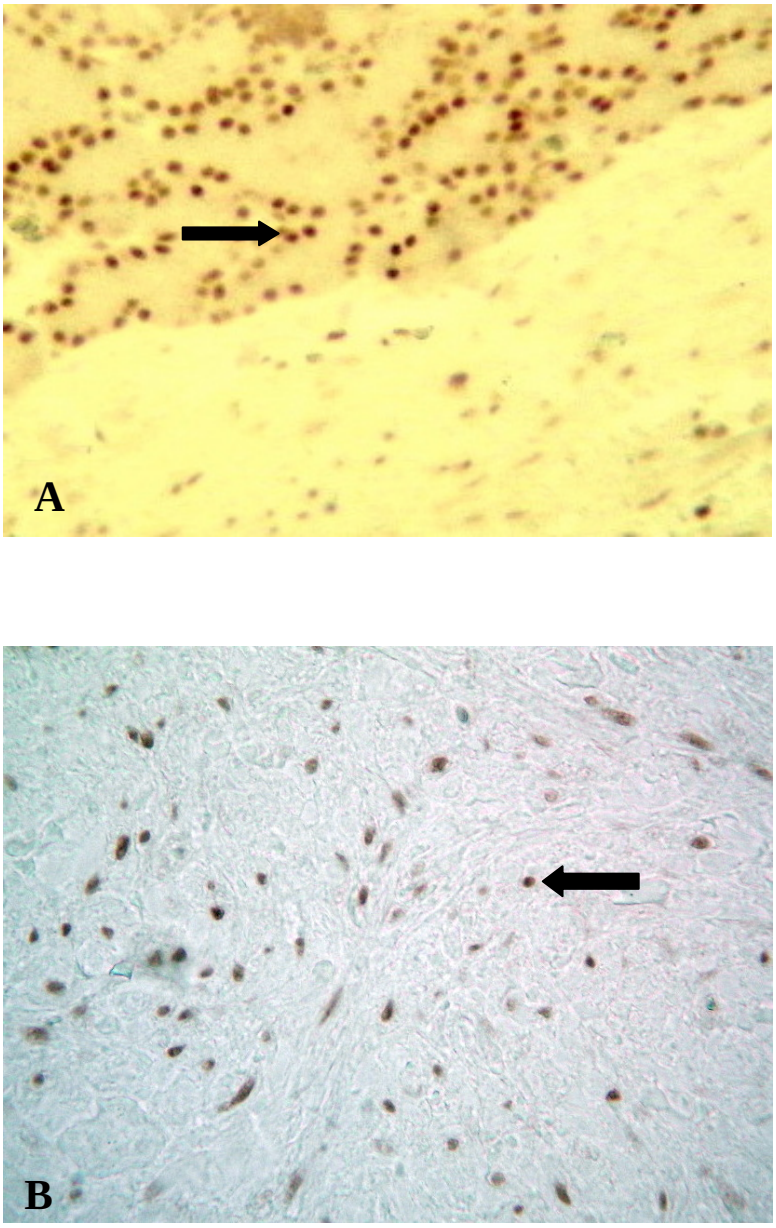


Figure 4. Immunohistochemical localization of progesterone receptor (PR) in normal hen oviduct tissue (A) and fibroid tissue (B). Arrows depict specific positive nuclei staining in the respective tissues. 400X.

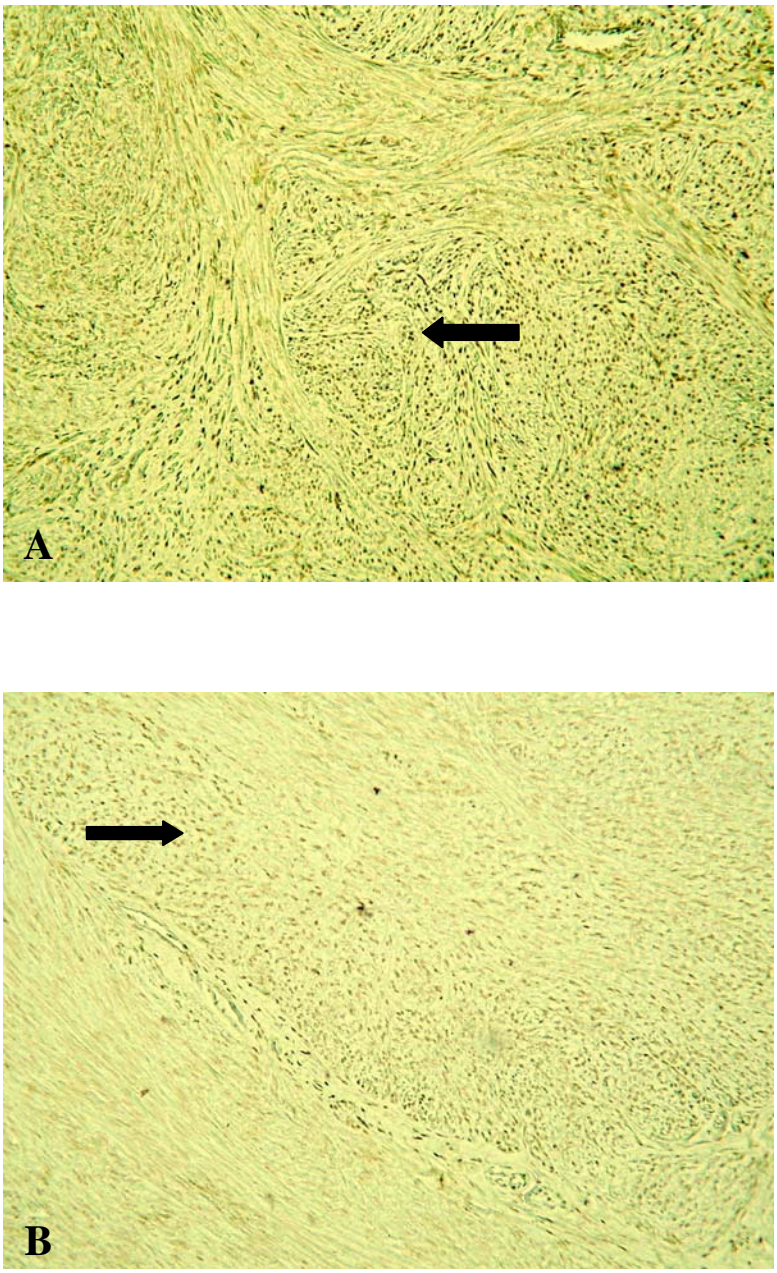


Figure 5. Immunohistochemical localization of estrogen receptor (A) and progesterone receptor (B) in human fibroid tissue. Arrows depict specific positive nuclei staining in the respective tissues. 100X.

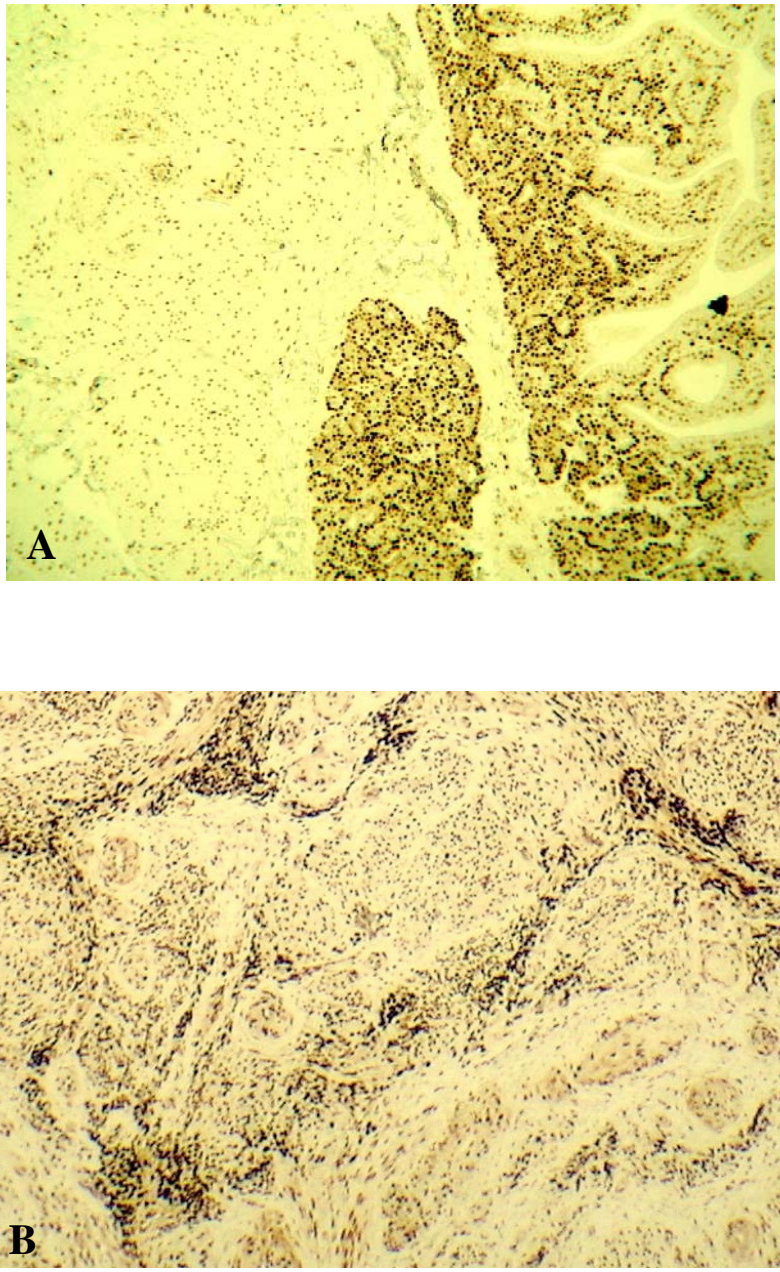


Figure 6. Immunohistochemical labeling of PCNA in normal hen oviduct and fibroid tissue (A) and human fibroid tissue (B). 100X.

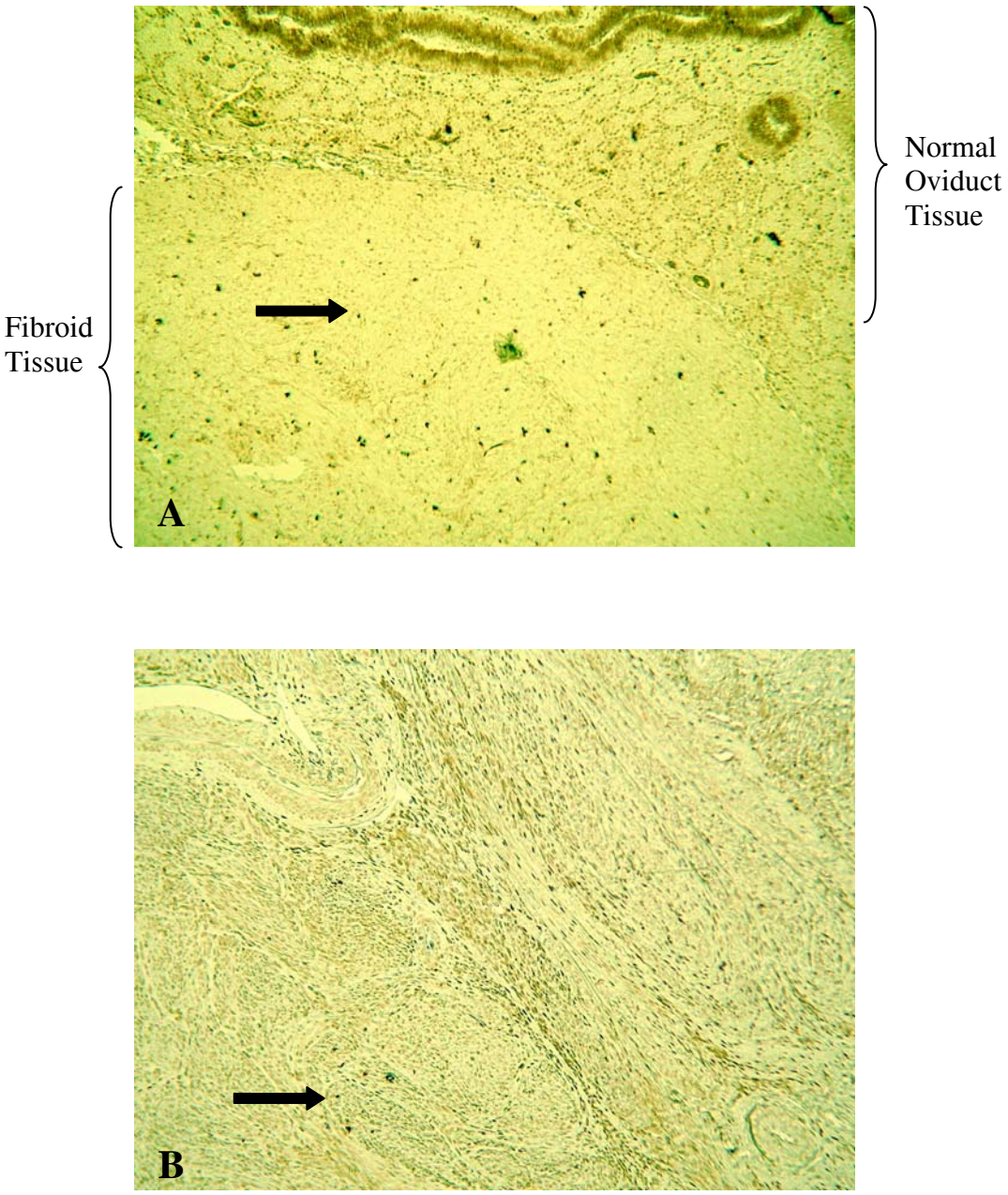


Figure 7. Immunohistochemical localization of Bcl-2 protein in normal hen oviduct and fibroid tissue (A) and human fibroid tissue (B). The arrows depict positive staining for Bcl-2 protein in a fibroid cell. 100X.

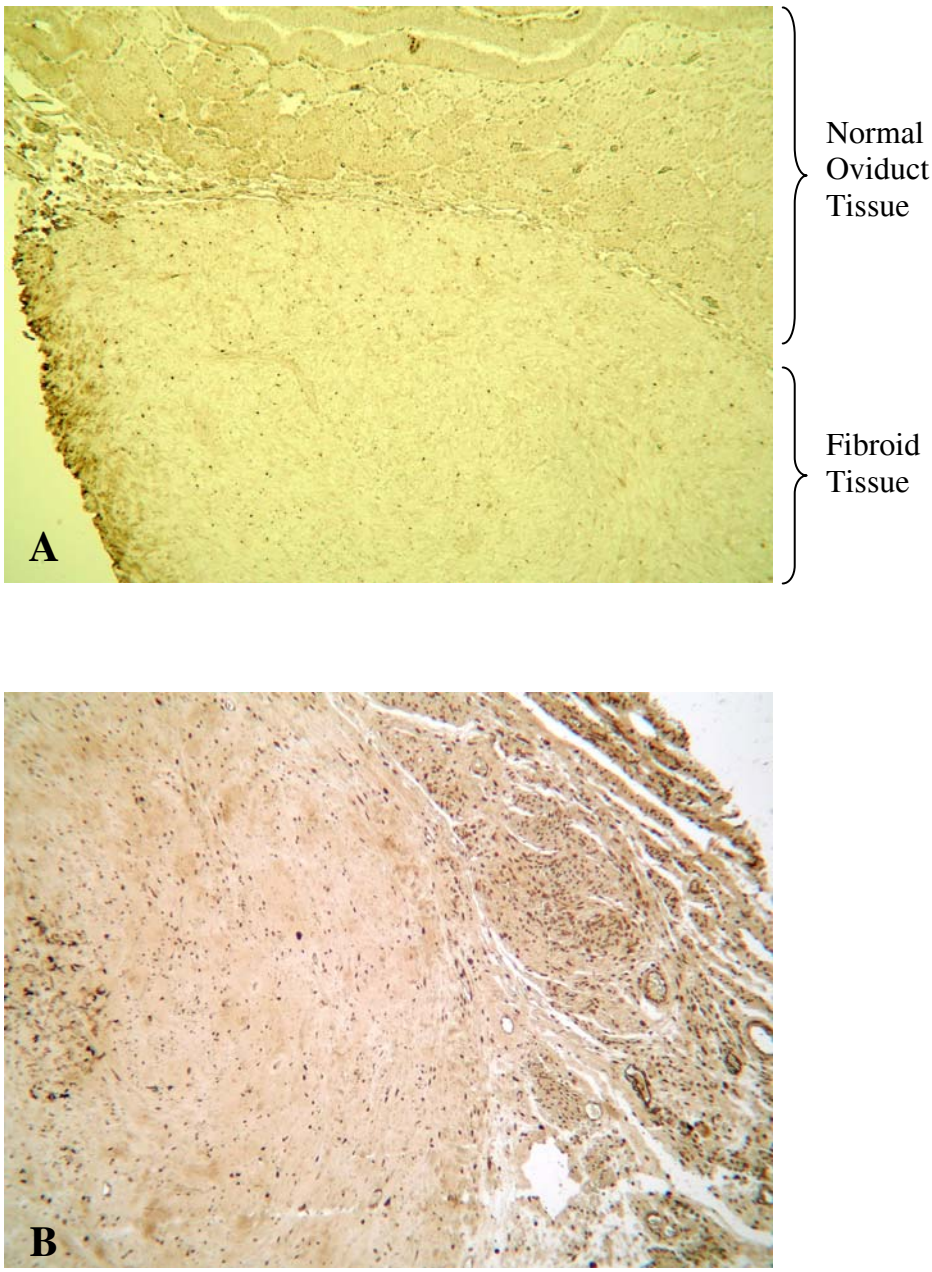


Figure 8. Immunohistochemical localization of TGF-β3 in normal hen oviduct and fibroid tissue (A) and human fibroid tissue (B). 100X.

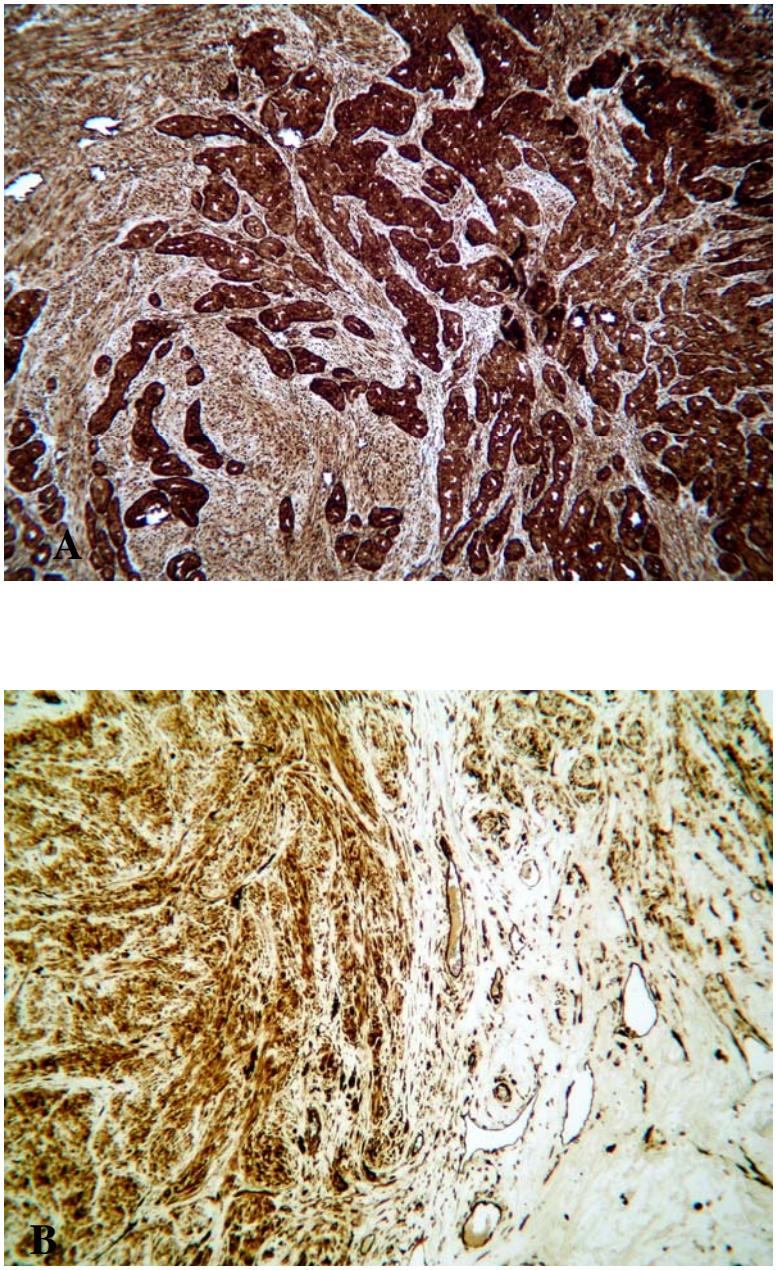


Figure 9. Immunohistochemical localization of IGF-2 in hen (A) and human (B) fibroid tissue. 100X.

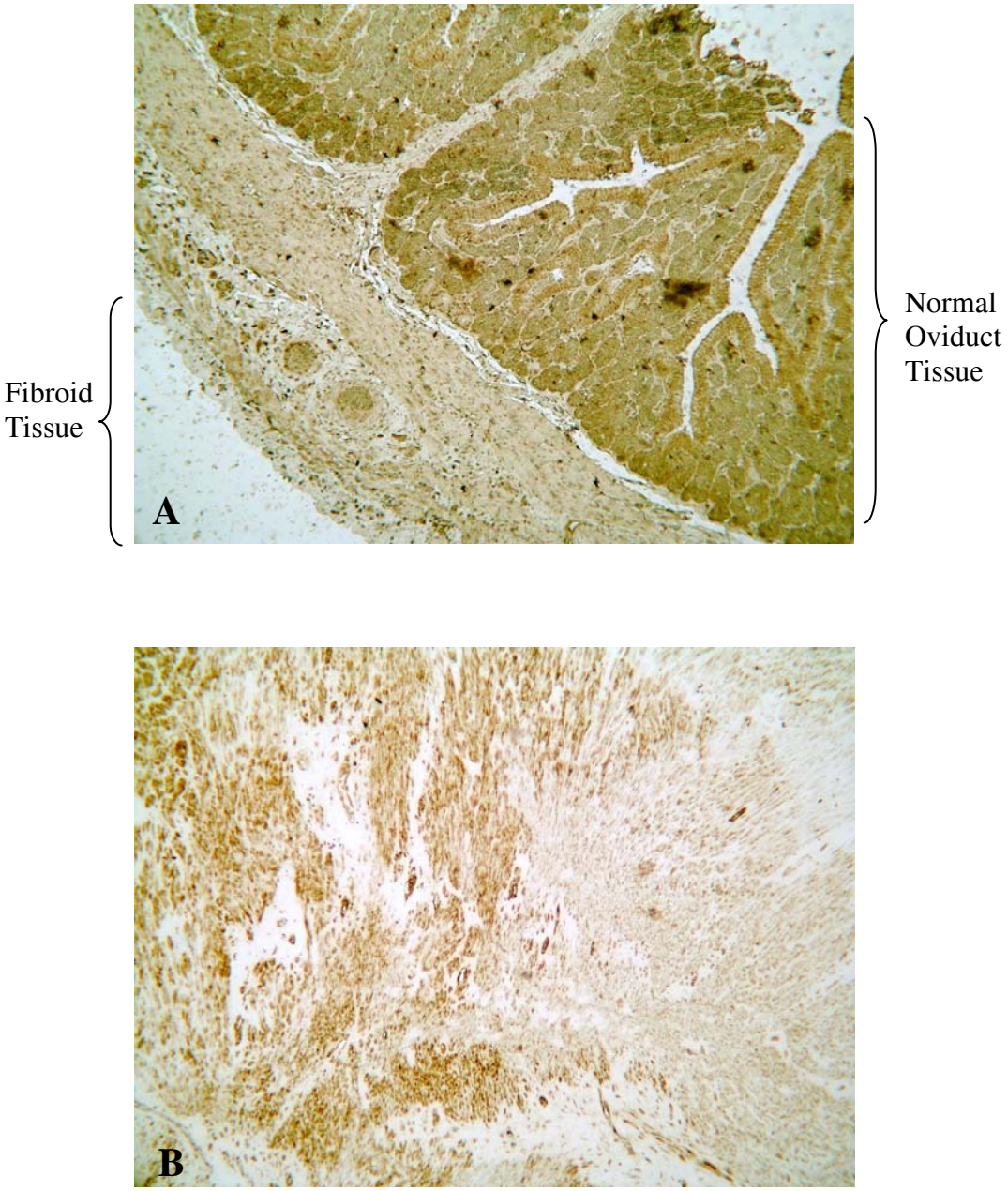


Figure 10. Immunohistochemical localization of IGFBP-5 in normal hen oviduct and fibroid tissue (A) and human fibroid tissue (B). 100X.

## VI. DISCUSSION

In humans, there is considerable evidence that estrogen and progesterone are involved in uterine fibroid growth. Leiomyomas develop only during the reproductive years and may increase in size during pregnancy or after administration of oral contraceptives (Stewart, 2001). Cessation of fibroid growth and regression occur following menopause (Nowak, 1999). Furthermore, long-term treatment with gonadotropin releasing hormone (GnRH) analogs reduces plasma sex steroid hormone concentrations and leads to a reduction in leiomyoma size. Leiomyoma enlargement, however, recurs after therapy with GnRH analogs is discontinued (Maruo et al., 2004). In addition, it has been reported that human leiomyomas contain elevated levels of estrogen and progesterone receptors when compared to normal myometrium (Nowak, 1999), suggesting ovarian steroid-dependent growth of the tumors.

Accumulated evidence supports the concept that estrogen and estrogen receptivity is closely related to the tumorigenesis and growth of leiomyomas (Maruo et al., 2004). Elevated expression of estrogen-regulated genes has been seen in leiomyomas compared with autologous myometrium, including connexin 43 gap junction protein, type 1 and 3 collagen, insulin-like growth factor-1 (IGF-1) and its receptor, parathyroid hormone-related peptide, progesterone receptor (PR), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (Huet-Hudson et al., 1990; Murphy and Ghahary, 1990; Nelson et al., 1992; Andersen et al., 1995; Barbarisi et

al., 2001; Murphy et al., 1994). In addition, it has been reported that aromatase, an estrogen synthetase that catalyzes androgen to estrogens, is expressed at higher levels in leiomyomas when compared to normal myometrium (Yamamoto et al., 1984).

Clinical observations that support the estrogen hypothesis also support a role for progesterone in the pathogenesis of uterine fibroids. Similar to estrogen, progesterone levels are also elevated during reproductive years, pregnancy, and further suppressed after menopause, making it difficult to distinguish the relative importance of estrogen versus progesterone (Rein, 2000). It has been suggested that progesterone may stimulate the mitotic activity and proliferation of leiomyoma cells, however, the mechanism underlying the stimulatory effects of progesterone on leiomyoma growth has not been fully defined. In both myometrium and leiomyomas, it appears that peak cell proliferation occurs during the secretory phase of the menstrual cycle when progesterone levels are high (Nowak, 1999). Brandon *et al*. (1993) demonstrated increased PR mRNA and protein levels in human leiomyoma together with elevated proliferation-associated antigen Ki-67 in comparison to adjacent myometrium, suggesting the association of progesterone-mediated signaling with leiomyoma growth. These findings support the view that progesterone may play a vital role in promoting the growth of uterine leiomyomas.

The observations in the present study demonstrate that hen fibroid tumors contain significantly elevated levels of estrogen and progesterone receptors compared to normal oviduct tissue. These findings are consistent with a role for estrogen and progesterone in the initiation and growth of hen oviduct fibroids as well as human uterine fibroids.

Human leiomyomas show an increase in expression of the Bcl-2 protein, which has been shown to prevent programmed cell death. The analysis of the nucleotide sequence of an isolated chicken homologue to the human bcl-2 gene showed that the organization of the chicken bcl-2 gene is very similar to that of the human bcl-2 gene (Eguchi et al., 1992). Production of Bcl-2 is significantly increased by progesterone (Matsuo et al., 1997). Since Bcl-2 production has been shown to prolong cell survival by preventing apoptotic cell death, progesterone may act as a growth-promoting factor in regulating leiomyoma growth through the inhibition of cell death in leiomyomas. By contrast, estrogen inhibited the induction of Bcl-2 protein in leiomyoma cells (Matsuo et al., 1999). The present observation that Bcl-2 protein is over-expressed in hen fibroid cells is consistent with the results from human studies, which suggests a role for the protein in the growth of the tumor by preventing apoptotic cell death.

PCNA is a cell cycle-related nuclear protein, and labeling of PCNA provides a useful evaluation of the proportions of proliferating cells in normal and neoplastic cell populations (Robbins et al., 1987). The current study demonstrates an abundance of PCNA in normal hen oviduct and fibroid tissue as well as human fibroids. Western blot analysis validates an elevated proliferation index in the hen fibroid relative to normal oviduct tissue. Scattered patches of intense staining for PCNA throughout the connective tissue in the hen and human fibroid tumors suggests increased cellular proliferation in specific regions of the fibroid.

Transforming growth factor-βs are multifunctional growth factors that regulate many aspects of cell function including proliferation, differentiation, migration and adhesiveness (Massague, 1990). A function of TGF-β of particular interest is its ability to

up-regulate the synthesis of many components of the extracellular matrix, leading to fibrosis, a characteristic of uterine fibroids (Lyons and Moses, 1990). One study (Lee and Nowak, 2001) found TGF-β3 expression to be elevated in leiomyomas relative to the adjacent myometrium. In support of these findings, Arici and Sozen (2000) found that the TGF-β3 mRNA levels were 3.5-fold higher than in the myometrium. One study reported a significant increase in the expression of TGF-β3 in luteal phase leiomyoma samples, suggesting a potential stimulatory role for progesterone in the expression of TGF-β3 (Arici and Sozen, 2000). In evaluation of hen fibroids for expression of the TGF-β3 protein, it appears that the laying hen does express the growth factor, however, unlike human leiomyoma studies, there was no significant difference in the abundance of TGF-β3 between the hen fibroid and normal oviduct tissue.

The biological actions of IGF-2 on tumor cells are thought to include mitogenic and metabolic effects as well as cell-survival properties. Human myometrium and leiomyoma both express the mRNAs for IGF-1 and IGF-2, demonstrating consistently higher levels of IGF-2 mRNA in leiomyomas than in normal myometrial tissue (Hoopner et al., 1988, Boehm et al., 1990, Vollenhoven et al., 1993). In the current study, IGF-2 was not significantly over-expressed in the hen fibroid relative to the normal oviduct tissue despite highly concentrated areas of IGF-2 abundance in the fibroid revealed by immunohistochemistry. The main actions of IGF-2 on leiomyoma cells in the human have not fully been determined, however, it is of particular interest that both the hen and human fibroid demonstrated a comparable staining pattern in the connective tissue suggesting that IGF-2 involvement during hen fibroid growth may be similar to its role in the growth of human uterine fibroids.

The role of IGFBP-5 on fibroid growth is uncertain, however, this binding protein may be able to enhance the actions of IGF-1 on tumor cells by increasing its bioavailability in target tissues by binding to it and preventing its degradation (Yu and Berkel, 1999). In human studies, IGFBP-5 was over-expressed in leiomyoma when compared to normal myometrium (Tsibris et al., 2002). Contrary to those findings, the present study indicates no significant difference in the relative abundance of IGFBP-5 in the hen fibroid compared to the normal oviduct tissue. Like IGF-2, there was a similar pattern of staining intensity of IGFBP-5 abundance between the hen and human fibroid suggesting a similar role of this binding protein in the development of these tumors.

In conclusion, the results of this study demonstrate that hen fibroids possess biomarkers typical of human uterine fibroids including ER, PR, PCNA, Bcl-2, TGF-β3, IGF-2, and IGFBP-5. Furthermore, the biomarkers ER, PR, PCNA, and Bcl-2 appear to be significantly elevated in the hen fibroid tumor, proving similar results to that of human leiomyoma studies. The accumulated evidence supports the idea that the hen may provide a useful model to gain a better understanding of the biology of human uterine leiomyomas.

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## VIII. APPENDIX

LIST OF ABBREVIATIONS USED IN THIS THESIS

| | |
|---|---|
| ABTS | 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) |
| Bcl-2 | B-cell leukemia/lymphoma 2 |
| bFGF | basic fibroblast growth factor |
| BSA | bovine serum albumin |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| ER | estrogen receptor |
| FSH | follicle-stimulating hormone |
| GnRH | gonadotropin-releasing hormone |
| HBEGF | heparin-binding epidermal growth factor |
| HCl | hydrochloric acid |
| IGF | insulin-like growth factor |
| IGFBP | insulin-like growth factor binding protein |
| LH | luteinizing hormone |
| MW | molecular weight |
| PBS | phosphate-buffered saline |

| | |
|---|---|
| PCNA | proliferating cell nuclear antigen |
| PDGF | platelet-derived growth factor |
| PEP-19 | purkinje cell protein 4 |
| PR | progesterone receptor |
| Rf | retention factor |
| SDS | sodium dodecyl sulfate |
| sFRP1 | secreted frizzled related protein 1 |
| TGF | transforming growth factor |
| UFE | uterine fibroid embolization |
| VEGF | vascular endothelial growth factor |

# Rf RATIO DATA

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.992047306 |
| R Square | 0.984157858 |
| Adjusted R Square | 0.978877144 |
| Standard Error | 0.04655976 |
| Observations | 5 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 0.404011366 | 0.404011366 | 186.3683338 | 0.000850328 |
| Residual | 3 | 0.006503434 | 0.002167811 | | |
| Total | 4 | 0.4105148 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* |
|---|---|---|---|---|---|---|
| Intercept | 7.506550888 | 0.511010018 | 14.68963548 | 0.000684289 | 5.880288946 | 9.132812831 |
| X Variable 1 | -1.522767275 | 0.111544323 | -13.65167879 | 0.000850328 | -1.877751095 | -1.167783455 |

PROBABILITY OUTPUT

| *Percentile* | *Y* |
|---|---|
| 10 | 0.157 |
| 30 | 0.32 |
| 50 | 0.547 |
| 70 | 0.672 |
| 90 | 0.985 |

y = mx + b

x = (7-b)/m

x = (y-7.506551)/-1.52277

| Rf Standards | MW Standards | LOG MW |
|---|---|---|
| 0.15700 | 70000 | 4.84509804 |
| 0.32000 | 52400 | 4.719331287 |
| 0.54700 | 34900 | 4.542825427 |
| 0.67200 | 29100 | 4.463892989 |
| 0.98500 | 20700 | 4.315970345 |

| Biomarker | Rf unknown | Log MW unk | MW unk | Expected MW |
|---|---|---|---|---|
| PCNA | 0.92 | 4.32537481 | 21153 | 30000 |
| Bcl-2 | 0.903 | 4.346737243 | 22219 | 25000 |
| ER | 0.624 | 4.533085092 | 34125 | 67000 |
| PR | 0.333 | 4.727447903 | 53388 | 99000 |
| TGF-B3 | 0.442 | 4.654645338 | 45148 | 47000 |
| IGF-2 | 0.989 | 4.289296687 | 19466 | 7500 |
| IGFBP-5 | 0.806 | 4.411524846 | 25794 | 28200 |