

EXTRACELLULAR-MATRIX MEDIATED REGULATION OF HUMAN UTERINE
LEIOMYOMA SMOOTH MUSCLE CELLS AND POTENTIAL TARGETING BY THE
ANTI-FIBROTIC DRUG HALOFUGINONE

BY

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DISSERTATION

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ABSTRACT

Uterine leiomyomas are known as the most commonly occurring neoplasms in women during their reproductive years. The overall incidence of these benign tumors can be as high as 70-80%, which represents a significant health problem to women. Patients suffer from abnormal uterine bleeding, pelvic pain, and reproductive dysfunctions. Available treatments for symptomatic patients are limited and this is in large part due to the fact that the mechanisms regulating the development and growth of these tumors are still not well understood.

Two essential features of leiomyoma tumors, which arise from the uterine smooth muscle cells, are an increase in smooth muscle cell (SMC) proliferation and excessive, aberrant deposition of extracellular matrix (ECM) collagen. The objective of this dissertation was to first understand the mechanisms regulating the development and growth of leiomyoma tumors in the context of ECM collagen and second, to test the inhibitory effects of the anti-fibrotic drug, halofuginone (HF), on the growth of leiomyoma tumors in both *in vitro* and *in vivo* model systems.

Our research in understanding the pathogenesis of leiomyomas showed that (1) monomeric and fibrillar forms of ECM collagen can differentially regulate the morphology and proliferation of leiomyoma smooth muscle cells (LSMCs); (2) physical properties of fibrillar collagen such as density and thickness can modulate the morphology and growth of LSMCs; (3) expression and localization of actin stress fibers as well as focal adhesion proteins such as vinculin and focal adhesion kinase are altered on different collagen matrices; (4) a synergistic effect between monomeric collagen and PDGF can further enhance the proliferation of LSMCs; and (5) the MAPK

signaling pathway is involved in the interaction of LSMCs with collagen matrices. These findings demonstrate how the excess, aberrant deposition of ECM collagen in fibrotic uterine tissue can contribute to the the formation and growth of leiomyoma tumors through modulation of major cell signaling pathways.

Our *in vitro* studies with the anti-fibrotic drug HF demonstrated that (1) this drug can inhibit LSMC proliferation under both basal and PDGF-stimulated conditions; (2) HF can significantly reduce transcript levels of *COL11A1*, *COL3A1*, *TGF β 1*, *TGF β 3*, *DMT* and *LOX3* in a time-depenedent manner; (3) expression of collagen I and III proteins were reduced by HF in a time-dependent manner; (4) PDGF-stimulated activation of the MAPK and SRC signaling pathways is not significantly inhibited by HF; and (5) HF itself could also activate the MAPK and SRC signaling pathways. These findings suggest that the antifibrotic drug HF may be an effective therapeutic treatment for patients with ULs.

Our complementary *in vivo* studies on the effects of HF in a mouse model xenografted with human LSMCs showed that (1) treatment with HF at 0.25 mg/kg body weight/day and 0.5 mg/kg body weight/ every-other-day (EOD) were safe for host mice while 0.50 mg/kg body weight treatment on a daily basis was not well tolerated by mouse hosts; (2) all HF treatment regiments significantly reduced the volume of xenografted tumors; (3) tumor reduction in ULs xenografted on mice treated with 0.25 mg/kg body weight/day or 0.50 mg/kg body weight/EOD HF occurred through decreased cellular proliferation and increased apoptosis; (4) 0.25 mg/kg body weight/day or 0.50 mg/kg body weight/EOD HF did not change the expression of collagens I and III at the protein or transcript level; (5) transcripts of *COL13A1* and

TGFβ1, but not *COL1A1*, *TGFβ3*, *DMT* and *LOX3*, were altered by EOD HF treatment at 0.5 mg/kg body weight. These findings not only further validate the use of the UL-xenografted mouse model as an excellent model system to explore the mechanisms involved in the regulation of leiomyoma tumor growth, but also as a tool to fully assess the effectiveness of novel therapeutic drugs such as HF as well as their potential side effects for future clinical trials.

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Chapter One

Introduction

Uterine leiomyomas (UL) or fibroids are benign neoplasms of the reproductive tract that occur in 70-80% of reproductive-aged women. Among women with ULs, 25-60% show symptoms such as pelvic pain and pressure, menorrhagia, hypermenorrhea, and in some cases infertility (Kroon *et al.*, 2011; Lee, *et al.* 2009). The high prevalence of these tumors is estimated to cost the United States health care system between \$5.9-34.4 billion annually (Cardozo *et al.*, 2011). Available treatments for leiomyomas are limited and this is due to the fact that the mechanisms regulating the development and growth of these tumors are still not well understood. The goal of this project was to better understand these mechanisms in order to identify new therapeutic treatments. Two essential features of leiomyoma tumors are an increase in smooth muscle cell (SMC) proliferation and excessive, aberrant deposition of extracellular matrix (ECM). The correct formation and deposition of ECM has been shown to influence major cell functions such as growth, survival, development and differentiation. This critical role of ECM is known to be mediated through the formation of focal adhesion kinases and augmented through interplay with growth factors such as PDGF. Fibrotic diseases such as liver fibrosis, keloids, and atherosclerosis are well-established examples of how an imbalance in ECM can lead to major symptoms and in some cases death. The use of chemicals, called anti-fibrotic drugs, that target molecules involved in the initiation and progression of fibrosis, has been one of the major approaches for re-establishing the correct balance of ECM in fibrotic tissue. In recent years, the drug halofuginone (HF) has been identified as one of these candidate therapeutic treatments.

The fact that leiomyoma cells are surrounded by an excess of disorganized collagen and are exposed to high levels of growth factors such as PDGF prompted us to investigate whether different forms of ECM collagen can differentially alter the morphology and proliferation of leiomyoma smooth muscle cells (LSMCs) through focal adhesions. We also investigated the modulatory role of ECM collagen on PDGF signaling through effects on PDGF receptor phosphorylation and activation of the MAPK signaling pathway.

The anti-fibrotic drug HF has been considered a promising therapeutic option for the treatment of fibrotic diseases such as hepatic fibrosis and scleroderma. The inhibitory effect of this drug appears to occur through its suppressive effect on the production of collagens I and III. Furthermore, research in our lab has shown that HF inhibits cell proliferation in a dose-dependent manner and is also able to inhibit PDGF and EGF-induced DNA synthesis. Due to the similar nature of ULs to other fibrotic diseases, we decided to investigate similar inhibitory effects of HF on LSMCs. We hypothesized that inhibition of collagen type I and III production through treatment with HF would suppress the proliferation of LSMCs through the MAPK, SRC, and PI3K/AKT signaling pathways.

The above-mentioned studies in an *in vitro* culture system with HF provided us with unique opportunities to explore the detailed mechanisms involved in the regulation of LSMC growth. However, it is only through the use of a live animal model that one can fully assess the effectiveness of a drug as well as its potential side effects. Immunocompromised mice xenografted with human leiomyoma tissue have been successfully used as an *in vivo* model for human leiomyomas (Ishikawa, *et al.* 2010).

We, therefore, tested the efficacy of HF as a potential non-surgical treatment in such an *in vivo* mouse model xenografted with UL tissue. In doing so, we also assessed any potential systemic effects of the drug on the host animals.

The overall objective of this dissertation was to understand how ECM collagen may regulate the proliferation of ULs and whether it can be targeted by HF. In chapter 2, an overview of ULs, ECM and its role in pathogenesis, the properties of HF and its mode of action are presented. Chapter 3 discusses our findings on how ECM collagens in either the fibrillar (cross-linked) or monomeric (non-crosslinked) forms regulate proliferation and collagen expression in LSMCs. The anti-fibrotic effects of HF on LSMCs *in vitro* are discussed in chapter 4. Our findings on the effects of HF in an *in vivo* UL-xenografted animal model are presented in chapter 5. Chapter 6 contains a summary of our findings and directions for future studies. Lastly, chapter 7 includes detailed information on the materials and techniques used in all experimental studies.

Chapter Two

Literature Review

A. Uterine Leiomyoma

A.1. Classification and Epidemiology

Uterine leiomyomas, also called myomas, leiomyomata, fibromyomas, leiomyofibromas, fibroleiomyoma or fibroids, are benign tumors arising from smooth muscle cells (SMC) of the human myometrium. The main characteristics of leiomyomas are an increase in the proliferation of SMCs as well as an excessive deposition of extracellular matrix proteins mainly collagens type I and type III (Kawaguchi *et al.*, 1989; Leppert *et al.*, 2004; Stewart *et al.*, 1994; Stewart, 2001). Based on their position in the uterine cavity, these tumors can be classified as intramural (within the myometrium), submucosal (lying just beneath or within the endometrium), or subserosal (located just beneath the outer serosa) (Fig. 2.1) (Benda, 2001). These tumors vary in size from seedlings to large tumors and can be solitary or multiple (Fig. 2.2). The location as well as the size of these tumors can determine the symptomatic state of the disease as well as its severity (Mavrellos *et al.*, 2010). Although most patients with leiomyomas are symptom free, the prognostics in 25% of symptomatic women include pelvic pressure, abnormal uterine bleeding, anemia, and reproductive dysfunction. Infertility is not commonly caused by leiomyomas, and if this occurs, it is due to the submucosal location of the tumor (Kroon *et al.*, 2011; Lee *et al.*, 2009; Wallach and Vlahos, 2004).

Epidemiological studies have shown that the prevalence of symptomatic leiomyomas in African-American women is higher than in Caucasian women (Day Baird *et al.*, 2003). African-American women are also more likely to develop these benign

tumors at an earlier age and with greater severity (Catherino *et al.*, 2011; D'Aloisio *et al.*, 2011; Faerstein *et al.*, 2001; Huyck *et al.*, 2008). Other factors such as early menarche (<10 years old), high body mass index (BMI), diabetes, hypertension, chlamydial infection, current smoking, and environmental toxicants such as Fenvalerate are also positively correlated with higher risk of symptomatic uterine leiomyomas (ULs), whereas use of oral contraceptives, pregnancy, and dietary intake of fruit and preformed vitamin A have been shown to have a negative effect on leiomyoma growth (Catherino *et al.*, 2011; Faerstein *et al.*, 2001; Flake *et al.*, 2003; Gao *et al.*, 2010; Huyck *et al.*, 2008).

A.2. Etiology of Leiomyoma

Despite the prevalence of ULs, the important aspects regarding the initiating factors for their development remain unknown. There are, however, several suggested theories (Fig. 2.3). One hypothesis refers to the role of genetic or epigenetic changes in uterine SMCs. Leiomyoma tumors are monoclonal in origin, meaning that a single SMC carrying a mutation that confers a growth advantage will give rise to the entire tumor cell population (Townsend *et al.*, 1970). Common chromosomal abnormalities identified in leiomyoma tumors include a translocation between chromosomes 12 and 14, deletion of chromosome 7, trisomy of chromosome 12, and rearrangements of 6p10q and 13q. Translocations between chromosomes 12 and 14 also occur in other mesenchymal solid tumors and involve HMGA2, a gene encoding a DNA binding protein that induces conformational changes in DNA and may regulate transcription (Hodge *et al.*, 2009; Meloni *et al.*, 1992; Rein *et al.*, 1991). Genetic markers such as cytokine gene polymorphisms of the IL4 and TNFA α genes are also known to be positively associated

with an increased risk of these tumors (Sosna *et al.*, 2010). Recently, Makinen *et al.* identified tumor-specific mutations in the mediator complex subunit 12 (MED12) gene in 70% of UL tumors analyzed (Makinen *et al.*, 2011). MED12 is a 26-subunit transcriptional regulator that bridges DNA regulatory sequences to the RNA polymerase II initiation complex. Apparently all found mutations in MED12 reside in exon 2, suggesting that aberrant function of this region of MED12 contributes to tumorigenesis (Makinen *et al.*, 2011; McGuire *et al.*, 2012).

The presence of various genetic mechanisms potentially affecting leiomyoma development suggests that these benign tumors are composed of different, distinct subtypes of neoplasms (Stewart and Morton, 2006). Polymorphisms in genes such as CYP17, catechol-O-methyltransferase, and estrogen receptor have all been associated with a higher risk of developing leiomyoma (Amant *et al.*, 2004). Tuberin, a tumor suppressor encoded by the tuberous sclerosis complex 2 (*TSC2*) gene, is similarly found to have elevated levels in samples of human ULs (Cui *et al.*, 2011) whereas in the Eker rat model where deletion of the *TSC2* gene results in the spontaneous formation of leiomyomas, tuberin levels were found to be reduced (Walker *et al.*, 2003). Despite this discrepancy between the human and rat model of ULs, upregulation of components of the mammalian target of rapamycin (mTOR) pathway, which is negatively regulated by tuberin, has been confirmed in both species leading to tumorigenesis.

Recent studies have also indicated a role for miRNAs in that many miRNAs including miR-363, miR-21, miR-490, miR-137, miR-34a, miR-125b, miR-139, miR-29b, miR-323, miR-217 and miR-4792 are shown to be differentially expressed in ULs

(Georgieva *et al.*, 2012). The overexpressed levels of miR-21 in human ULs were found to be elevated during the secretory phase of the menstrual cycle, but reduced in patients receiving gonadotropin releasing hormone analogue (GnRHa) therapy (Pan *et al.*, 2009). Modulation of cellular apoptosis and translation by miR-21 seems to be involved in the tumorigenic role of this miRNA in ULs (Fitzgerald *et al.*, 2012).

Epigenetic regulators such as acetyltransferases, deacetylases, methyltransferases and miRNAs have recently been recognized as factors involved in fibrosis and tumorigenesis (Ghosh and Vaughan, 2012; Sandoval and Esteller, 2012). A genome-wide DNA methylation study also identified silencing of known tumor suppressor genes such as *KLF11*, *DLEC1*, and *KRT19* in ULs as compared to adjacent myometrial tissue (Navarro *et al.*, 2012). Major components of the miRNA-processing machinery, Drosha and Dicer, were also found to be elevated in ULs compared to adjacent myometrium implicating a role in the formation of these tumors (Papachristou *et al.*, 2012).

Another theory argues for the role of sex steroid hormones in the pathogenesis of leiomyoma. Significantly increased levels of estrogen receptors (ESR1 and ESR2) have been found in ULs (Richards and Tiltman, 1996). Increased levels of progesterone receptor along with a higher proliferative rate during the secretory phase of the cycle have also been observed (Brandon *et al.*, 1993; Maruo *et al.*, 2000). In addition, treatment of leiomyoma cells with estrogen or progesterone increases cell proliferation (Maruo *et al.*, 2000) whereas use of respective antagonists blocks this effect (Barbarisi *et al.*, 2001; Chegini *et al.*, 2002). GnRH treatment causes suppression of the hypophyseal-gonadal axis, decreasing ovarian steroid levels and leading to tumor

regression (Felberbaum *et al.*, 1998; Mizutani *et al.*, 1998). Leiomyomas have also been found to express the aromatase P450 enzyme suggesting the presence of an autocrine/paracrine system within ULs by which the SMCs produce their own estradiol that stimulates growth independently of systemic sex steroid hormones (Bulun *et al.*, 1994; Sumitani *et al.*, 2000). Genetic polymorphism for another enzyme, catechol-O-methyltransferase, involved in the conversion of catechol estrogens into the active derivatives, is also thought to be a risk factor for ULs (Al-Hendy and Salama, 2006a). Sex steroid hormones also exert mitogenic effects through stimulation of growth factor production by their target cells leading to autocrine/paracrine modulation of cell growth (Barbarisi *et al.*, 2001; Di Lieto *et al.*, 2002; Flake *et al.*, 2003).

A third interesting theory postulates that the initiating factor in the formation of leiomyomas is some type of injury to normal myometrial cells followed by disrupted wound healing (Stewart and Nowak, 1998). In this scenario, myometrial cells might be injured through ischemia associated with the release of increased vasoconstrictive substances at the time of menses. Following the release of prostaglandins and vasopressin, myometrial cells could undergo transformation from a contractile phenotype to a proliferative-synthetic one. This transformation is one that has been observed in several tissues, including skin and vasculature, in response to injury. In normal tissues, proper wound healing requires the successive stages of inflammation and proliferation followed by differentiation of cells to a myofibroblast phenotype which after the completion of healing, return back to their normal phenotype. As part of this tissue remodeling, collagens and other ECM components are secreted while matrix metalloproteinases degrade the existing ECM. However, if for any reason, cells do not

progress through these stages or return back to their normal state, conditions such as fibrosis may occur (Leppert *et al.*, 2006). Several clinical and laboratory observations are in support of this theory. In women with dysmenorrhea, the incidence of ULs has been recorded to be higher due to an increased expression of prostaglandins and vasopressin (Emans *et al.*, 2005), which can trigger an ischemic injury to uterine tissue (Stewart and Nowak, 1998). Furthermore, overexpression of basic fibroblast growth factor (bFGF), a known mitogen for SMCs in response to injury, has also been established in leiomyoma (Lindner and Reidy, 1991). Frequent mucosal injury with stromal repair reactions has been suggested to induce the release of growth factors initiating ULs (Cramer *et al.*, 2009). In addition, histopathologic examination of paired myometrial and leiomyoma tissues have also provided evidence of altered healing in UL tissue (Roeder *et al.*, 2012). As part of this evidence, myofiber disarray, elastosis, tissue edema, and inflammation in UL were documented. Therefore, the abnormal response to tissue injury can lead to a disrupted wound healing process and development of a fibrous tissue abundant with ECM collagens (Leppert *et al.*, 2006).

A.3. Sex steroid Hormones in Leiomyoma

While the initial factors leading to the development of ULs are not fully known, the development and growth of these tumors are clearly shown to be dependent on sex steroid hormones. Development of ULs in women during their reproductive years and their shrinkage after menopause strongly supports a role for ovarian steroid hormones in the pathogenesis of uterine fibroids. Analysis of circulating hormone levels in white and African-American cycling women have indicated higher estradiol levels in African-Americans across the menstrual cycle which might contribute to racial disparities in ULs

(Marsh *et al.*, 2011). In addition, the pattern of the menstrual cycle has been shown to be associated with developing ULs in that women with shorter cycles are at a higher risk for developing ULs (Amanti *et al.*, 2011). Leiomyoma cells have increased cellular proliferation and express estrogen and progesterone receptors at higher mRNA and protein levels compared to adjacent normal myometrium (Benassayag *et al.*, 1999; Brandon *et al.*, 1993; Kovacs *et al.*, 2001; Viville *et al.*, 1997). Several *in vitro* studies have indicated that treatment of LSMCs with estrogen or progesterone increases cell proliferation, while treatment with respective antagonists blocks this effect (Barbarisi *et al.*, 2001; Chegini *et al.*, 2002). Furthermore, tumor size in women with ULs who receive GnRH agonists treatment have been shown to be reduced confirming the involvement of ovarian hormones in UL growth. Decreased cell proliferation in ULs in peri-menopausal women compared to tumors originating in women of reproductive age is another indication of the role of ovarian hormones in the development of ULs (Plewka *et al.*, 2011).

The specific and key role of estrogen in the tumorigenesis of ULs has been validated by numerous studies. In addition to the presence of both estrogen receptors ESR1 and 2, estrogen-regulated genes such as connexin 43 gap junction protein, collagens I and III, IGF-1 and progesterone receptor have also been shown to be upregulated in ULs (Andersen *et al.*, 1995). The expression of aromatase P450 in ULs, which converts local androgen to estrogens, provides an additional autocrine/paracrine system within ULs for production of estradiol which stimulates tumor growth independent of systemic sex steroid hormones (Bulun *et al.*, 1994; Sumitani *et al.*, 2000).

In recent years, growing evidence has highlighted the role of the other ovarian steroid hormone, progesterone, in the pathogenesis of ULs. The increased mitotic activity in LSMCs during the secretory phase of the menstrual cycle, increased cellularity of LSMCs in women undergoing progesterone treatment, increase in the size of ULs during the first 10 weeks of pregnancy, reduced viability and increased apoptosis induced by the levonorgestrel intrauterine system device all suggest that leiomyoma growth is affected by progesterone levels (Kawaguchi *et al.*, 1989). Furthermore, data from numerous clinical studies on the use of anti-progestins or selective progesterone receptor modulators showing significant reductions in both tumor volume and UL-associated symptoms support of a role for progesterone (Kim and Sefton, 2011; Socolov *et al.*, 2011)

The stimulatory effects of estrogen and progesterone on the growth of ULs appear to be mediated through their classical receptors and also via non-genomic mechanisms. The mitogen-activated protein kinase (MAPK), protein kinase C α , and AKT/PI3K have all been demonstrated to be rapidly activated in ULs in response to sex steroid hormone treatment (Barbarisi *et al.*, 2001; Hoekstra *et al.*, 2009; Nierth-Simpson *et al.*, 2009). A few studies have also demonstrated that such activation in ULs is further modulated by the crosstalk of ovarian hormones with growth factors such as platelet-derived growth factor (PDGF) and neurotensin (Maruo *et al.*, 2004; Rodriguez *et al.*, 2011).

A.4. Growth Factors in Leiomyoma

Several growth factors and their receptors have been identified in myometrial cells and LSMCs. The most important ones include epidermal growth factor (EGF), platelet-

derived growth factor (PDGF), insulin-like growth factor I and III (IGF-I/III), transforming growth factor beta 3 (TGF- β 3), (bFGF), vascular endothelial growth factor (VEGF), and prolactin (Flake *et al.*, 2003; Mangrulkar *et al.*, 1995). There is numerous direct and indirect evidence of the regulation of these growth factors by estrogen and progesterone in uterine fibroids (Flake *et al.*, 2003). For example, progesterone upregulates EGF during the luteal phase of the menstrual cycle in leiomyoma while EGFR is upregulated by estrogen (Maruo *et al.*, 2000). Treatment of women with GnRH agonists reduces the binding of EGF to LSMCs when compared to leiomyomas from untreated patients (Lumsden *et al.*, 1988; Maruo *et al.*, 2000; Maruo *et al.*, 2004). Despite the aberrant expression of these growth factors in LSMCs compared to normal myometrium, it is not clear whether these factors are involved in the development or initiation of the disease or are secondary effectors in fibroid tumorigenesis.

The TGF β superfamily includes more than 30 polypeptides with related structures that can act either as inhibitors or stimulators of cell proliferation (Arici and Sozen, 2000; Miyazono, 2000). The three isoforms of the TGF β subfamily play significant roles in the development of fibrotic diseases due to their effects on mitogenesis and synthesis of extracellular matrix proteins, which are the hallmarks of fibrosis (Lyons and Moses, 1990; Arici and Sozen, 2000). Expression of all three isoforms of TGF- β and their receptors has been detected in leiomyoma and myometrial SMCs (Arici and Sozen, 2003; Chegini *et al.*, 1999; Lee and Nowak, 2001). TGF- β 3 mRNA levels were increased during the luteal phase of the menstrual cycle, and treatment with the GnRH agonist leuprolide acetate caused a decrease in expression of TGF- β family members (Arici and Sozen, 2003; Dou *et al.*, 1996). In contrast, no

significant difference was observed in the TGF- β 1 mRNA levels between leiomyoma and myometrical SMCs (Vollenhoven *et al.*, 1995). Lee and Nowak also found that inhibition of TGF- β 3 caused a decrease in collagen I and III mRNAs in both myometrial and leiomyoma SMCs whereas its presence stimulated DNA synthesis only in leiomyoma cells (Lee and Nowak, 2001). The effect of TGF- β 3 on the growth of leiomyoma is not always stimulatory as high concentrations of this growth factor show an inhibitory effect on SMCs (Arici and Sozen, 2000; Battegay *et al.*, 1990). Thus, it appears that the effect of TGF- β on leiomyomas is dependent on factors such as concentration, cell type, and the presence of other growth factors (Flake *et al.*, 2003).

IGF-I is another growth factor implicated in the pathogenesis of ULs. Both the expression of IGF-I and its binding are increased by exposure to estrogen, and IGF-I appears to preferentially induce mitogenic activity in leiomyoma SMCs through up-regulation of PCNA and Bcl-2 expression, which inhibits apoptosis (Gao *et al.*, 2001; Strawn *et al.*, 1995).

EGF has been found to have mitogenic effects on both myometrial and leiomyoma SMCs (Fayed *et al.*, 1989). The mRNA levels of EGF are similar in both tissues during the follicular phase, but significantly elevated in leiomyomas in the luteal phase (Harrison-Woolrych *et al.*, 1994). Treatment with GnRH agonists resulted in a sharp decrease in EGF receptor levels (Lumsden *et al.*, 1988) as well as shrinkage of the tumor (Rein and Nowak, 1992). In addition, progesterone treatment induces an up-regulation of EGF in fibroid SMCs while not affecting myometrial SMCs (Maruo *et al.*, 2000). These data indicate that the mitogenic effects of ovarian steroids on ULs may be due, in part, to their effect on EGF synthesis (Flake *et al.*, 2003). EGF itself can then

lead to the transient activation of EGFR-MAPK pathway to induce increased proliferation and collagen synthesis (Ren *et al.*, 2011).

Another important growth factor present in UL is PDGF. This known mitogen of smooth muscle cells and fibroblasts exists predominantly as a heterodimer of A and B chains or homodimers of AA or BB although newer members of this family, C and D, were recently discovered (Hwu *et al.*, 2008). Both myometrial and leiomyoma SMCs contain PDGF intracellularly, but not in the ECM (Mangrulkar *et al.*, 1995; Rossi *et al.*, 1992), suggesting that PDGF action is local and occurs through a paracrine or autocrine fashion (Mangrulkar *et al.*, 1995). Transcripts for this potent mitogen of SMCs have been reported to be expressed in equal or varying amounts in both myometrial and leiomyoma tissue (Boehm *et al.*, 1990; Hwu *et al.*, 2008; Liang *et al.*, 2006; Mangrulkar *et al.*, 1995; Palman *et al.*, 1992), indicating the local production of this growth factor in uterine tissue (Boehm *et al.*, 1990). In clinical studies with GnRH analogs, decreased production of PDGF was associated with a greater shrinkage in uterine volume suggesting a mitogenic action in leiomyomas (Di Lieto *et al.*, 2002; Di Lieto *et al.*, 2005;). In study by Liang *et al* (2006), it was reported that the PDGF-BB levels were higher during the secretory phase of the menstrual cycle in leiomyoma and treatment with PDGF increased the expression of PCNA and collagen α 1(I) in both leiomyoma and myometrial cells (Liang *et al.*, 2006).

There is, however, a difference in the number of PDGF receptor sites per cell between leiomyoma and myometrial SMCs in that LSMCs contain more PDGF receptors but with a lower binding affinity (Fayed *et al.*, 1989). These increased concentrations of PDGF receptor may lead to a greater mitogenic response to PDGF in

leiomyoma tissue (Barbarisi *et al.*, 2001; Boehm *et al.*, 1990). In addition, growth factors such as EGF and IGF have been shown to have synergistic effects with PDGF to increase the proliferation LSMC (Boehm *et al.*, 1990; Fayed *et al.*, 1989). The mitogenic effects of PDGF in LSMCs appear to be mediated through induction of NADPH oxidase-dependent ROS production and AMPK signaling pathways (Mesquita *et al.*, 2009). In addition, PDGF alone or in association with other growth factors has been shown to be involved in the proliferative response of LSMCs to estradiol and in mediating the early signaling responses to estradiol. Increased production of PDGF upon stimulation with estradiol may propagate an autocrine loop involving PDGF that continuously stimulates proliferation of E2-treated LSMCs (Barbarisi *et al.*, 2001).

A.5. Therapeutic Approaches for Leiomyoma

Management of ULs can be categorized into three groups: surgical, drug-based, and non-invasive interventions. Historically, surgical management of uterine fibroids has been the only available treatment option for women. This form of treatment involves removal of the entire uterus (hysterectomy) or removal of only the fibroids (myomectomy) (Bouchard *et al.*, 2011). Either procedure can be performed via laparotomy, laparoscopy, or vaginally (Plotti *et al.*, 2008). In general, myomectomy is usually attempted in women who desire to maintain their fertility, but the most common treatment is still hysterectomy (Al-Hendy and Salama, 2006b). Recent surveillance on the rate of hysterectomies in the United States shows that about 40% of the 600,000 hysterectomies annually performed were due to ULs (Whiteman *et al.*, 2008). While these types of surgery are the only definitive treatment for uterine fibroids, they can be costly and confer subsequent complications (Lee *et al.*, 2009; Stewart, 2001). The

existing risk of 15-38% for major complications includes post-operative hemorrhage, fever, or injury to adjacent organs (Al-Hendy and Salama, 2006b).

Current trends in reproductive medicine are moving toward less invasive treatments for uterine fibroids. These treatment options, which are still in relatively early stages, include uterine artery embolization (UAE), high frequency ultrasonography, cryotherapy and thermoablation (Stein and Ascher-Walsh, 2009; Wallach and Vlahos, 2004). In UAE, the goal is to block the blood supply to the fibroid. So far patients undergoing this procedure have been satisfied with the outcome, but there are concerns with the possibility of missing some fibroids or uterine malignancies (Al-Hendy and Salama, 2006b). The other methods show promise, but more randomized studies are needed to prove their efficacy and document associated complications (Stein and Ascher-Walsh, 2009).

The other major non-surgical treatment for leiomyomas involves the use of gonadotropin-releasing hormone agonists (GnRHa) that suppress the production of ovarian estradiol and progesterone (Chavez and Stewart, 2001; Pinkerton, 2011; Stewart, 2002). The use of these drugs was first tested in the late 1980s and showed a significant decrease in the size of leiomyomas within 3-6 months. These drugs also reduce uterine vascularity (Duhan, 2011). However, due to their effect on bone density and the relatively rapid resumption of tumor growth once treatment is terminated, they are not considered suitable as long-term therapies. Selective estrogen receptor modulators (SERMs) are another class of drug-based treatments for leiomyoma, which show antagonist activity only in the breast and uterus and not in other tissues (Nowak, 2004). However, as demonstrated in studies with raloxifene, these types of drugs did

not show a significant efficacy in cycling women at lower doses (Palomba *et al.*, 2002). Selective progesterone receptor modulators (SPRMs), on the other hand, have shown great potential in treatment of leiomyomas (Stewart, 2012). These compounds have a high binding affinity for progesterone receptor and can also modulate the effects of estrogen in specific tissues (Engman *et al.*, 2009). Asoprisnil is one of these drugs and has been tested as a treatment for leiomyomas. This drug reduced the volume of the largest fibroids by 36.1% after 12 weeks of treatment and induced amenorrhea in a dose-dependent manner (Chwalisz *et al.*, 2007). Similarly, in a randomized study, mifepristone was shown to reduce the pain and bleeding associated with uterine fibroids and also decreased the size of tumors without inducing endometrial hyperplasia (Engman *et al.*, 2009).

Non-hormone based drugs designed to either remedy or reduce altered expression of various factors in ULs are another category of compounds that have gained traction in recent years. For example, Ro 41-0960, a synthetic catechol-O-methyl transferase inhibitor (COMTI) has been successfully tested in the Eker rat model of ULs and shown to shrink fibroid tumors (Hassan *et al.*, 2011). Natural and synthetic derivatives of vitamins such as all-trans-retinoic acid and 1,25-dihydroxyvitamin D3 have also been proven to be safe and effective non-surgical therapies for ULs (Halder *et al.*, 2011; Halder *et al.*, 2012).

The fibrotic nature of ULs has directed research towards the use of anti-fibrotic drugs as potential non-invasive treatments for uterine fibroids. These candidate drugs include anti-TGF β compounds, halofuginone, and interferons. Pirfenidone, an anti-TGF β drug, was found to decrease the proliferation of LSMCs as well as the production

of collagen type I (Lasky, 2004; Lee *et al.*, 1998). Similarly, halofuginone has been shown to reduce basal and growth factor-stimulated proliferation of leiomyoma SMCs in a dose dependent manner (Grudzien *et al.*, 2009). Interferons are cytokines that antagonize the actions of several growth factors such as bFGF, TGF β , and TNF α . Clinical studies have reported a size reduction in fibroids by up to 50% upon treatment with these drugs (Ali *et al.*, 2000; Minakuchi *et al.*, 1999).

Gene therapy has also been the focus of research for the treatment of leiomyomas. Due to the localized nature of these tumors and the availability of imaging and endoscopic techniques to visualize and access uterine fibroids, the delivery of gene-based vectors seems like an attractive target. Candidate genes in these attempts have been the dominant negative form of estrogen receptor and a thymidine kinase-gancyclovir-expressing adenovirus (Hassan *et al.*, 2009; Hassan *et al.*, 2010). *In vivo* results using this approach in the Eker rat reported a decrease in Bcl-2, cyclin D1 and PCNA along with an inhibition of proliferation. None of these potential promising treatments has yet moved into full clinical trials due to the preliminary nature of *in vitro* and studies and limited success in recruiting subjects (Catherino *et al.*, 2011).

Overall, both the direct and indirect costs for the management of ULs in the United States alone are estimated to be \$5.9-9.34 billion annually (Cardozo *et al.*, 2011). The key to successful use of new treatment options for women with ULs and reduced costs is understanding the needs of each patient and the nature of the associated symptoms in a multidisciplinary approach in order to find the most effective treatment program (Stovall, 2011).

A.6. Animal Models for Leiomyoma

Several animal models for studying ULs exist. The best characterized and most widely used is the Eker rat model (Walker *et al.*, 2003). These rats carry a germline mutation in the rat homolog of the Tsc2 gene and develop spontaneous renal cell carcinomas and ULs with a high frequency. Eker rat leiomyomas share phenotypic, biochemical, and genetic characteristics similar to human ones. They express ER and PR and are responsive to steroid hormones, have aberrant HMGA2 expression, overexpress IGF-1, and show reduced growth during pregnancy. Cell lines derived from these tumors have been successfully established and have been shown to retain their hormone responsiveness in culture (Walker *et al.*, 2003). This system has been widely used to demonstrate the potential efficacy of selective estrogen modulators (SERMs), progesterone modulators, and therapeutic agents for fibroids (Arslan *et al.*, 2005; Buchanan *et al.*, 2002; Cook and Walker, 2004; Crabtree *et al.*, 2009; Halder *et al.*, 2012; Hassan *et al.*, 2011; Miyake *et al.*, 2009).

Another animal model for ULs in the guinea pig came about after the spontaneous development of leiomyomas was observed in 8% of aged animals (Porter *et al.*, 1995). Ovariectomy of young animals combined with high-doses of estrogen can also cause the development of uterine and abdominal leiomyomas with high frequency. This model has also been used to study SERMs (Porter *et al.*, 1998).

Other studies have shown the induction of leiomyomas in mice treated with the β -adrenoceptor antagonist levobunolo (Romagnolo *et al.*, 1996; Rothwell *et al.*, 1992) as well as in transgenic mice expressing SV40 T antigen driven by the estrogen-responsive calbindin promoter (Romagnolo *et al.*, 1996). There are also reports of

chicken (Berry *et al.*, 2006; Machado *et al.*, 2012), guinea pig (Tsibris, 2004) and dog models for this disease. Rats injected with estradiol benzoate have been shown to develop nodules similar to human ULs (Hai-Gang *et al.*, 2011). Finally, a recent model has utilized xenografting of human leiomyoma tissue under the kidney capsule of ovariectomized mice (Ishikawa *et al.*, 2010). This model, which has been successfully used to investigate the role of sex steroid hormones on the growth of leiomyomas, has a success rate of 100% and maintains the histological characteristics of the original tissue.

B. Extracellular Matrix Collagen

B.1. Classification and Functions of Extracellular Matrix

Most cells in multi-cellular organisms are surrounded by a complex mixture of non-living material that is called the extracellular matrix (ECM). In animal cells, ECM is the major component of connective tissue and is comprised of the interstitial space and basement membrane. This complex in vertebrates is composed of proteins, either alone or in combination with carbohydrates and minerals. Based on the molecular components of ECM, it can be categorized into proteoglycans (heparan sulfate, chondroitin sulfate, keratan sulfate), non-proteoglycan polysaccharides (hyaluronic acid), fibers (collagen and elastin) and other types such as laminin and fibronectin.

The diverse nature of ECM allows it to serve various functions within the body. ECM can provide structural support and anchorage for cells, serve as a barrier between different cell types, regulate intercellular communications through its structural characteristics, and mediate the effects of growth factors leading to cellular growth and differentiation. In addition, tissue-remodeling-dependent processes such as embryonic

development and wound healing rely on the formation of ECM and the correct interaction between ECM and cells. Aberrant formation and regulation of ECM can lead to tumor invasion and metastasis in cancer (Hynes and Yamada, 2011).

B.2. Molecular Structure and Types of Collagens

Collagens are the most abundant proteins in the human body (Prockop and Kivirikko, 1995). Tropocollagen, the basic unit of collagen, is rigid and rod-shaped with an approximate length of 3000 Å and 15 Å in diameter (Beard *et al.*, 1977).

Depending on their structural domains collagens can be globular or triple-helical (Burgeson and Nimni, 1992). Triple-helical collagens are unique amongst other proteins in that their structure is composed of three left-handed twists of α -chains with an average number of 100 amino acids per chain. In each α -chain, the third residue needs to be a Gly in order to allow for the three helices to wind into a helical structure (Patino *et al.*, 2002). This well-packed structure contributes to the stress-bearing characteristics of connective tissue in bone, skeletal muscle and fibrous tissues such as skin and blood vessels (Voet and Voet, 2010).

Based on their structure and supramolecular organization, collagens can be grouped into fibril-forming collagens, fibril-associated collagens, network-forming collagens, anchoring fibrils, transmembrane collagens, basement membrane collagens and a few others with distinctive functions (Gelse *et al.*, 2003). Twenty-eight different types of collagens have been identified in vertebrates, of which collagen type IV and fibrillar collagens are found in species ranging from sponges to humans suggesting a common ancestral gene for collagen alpha chain in metazoans (Exposito *et al.*, 2010). Fibril-forming collagens, such as collagens I and III, are

approximately 300 nm in length and 1.5 nm in diameter and their formation is dependent on the presence of short extrahelical telopeptides flanking the molecule (Achilli *et al.*, 2009; Kadler *et al.*, 1996). In a typical fibrillar procollagen, the α chain consists of an uninterrupted major triple helix (collagenous domain) made up of approximately 338 Gly-Xaa-Yaa triplets. This region is flanked by the extra-helical telopeptides and by two non-collagenous domains, the N- and the C-propeptides (Exposito *et al.*, 2010) (Fig. 2.4).

B.3. Collagen Biosynthesis

The production of collagen involves a complex biosynthetic pathway. In the first step, the precursor form of collagen, procollagen, is produced from the selection and association of three procollagen chains in the cytoplasm. Following extensive post-translational modification, the soluble procollagen molecules are assembled into the triple helix, packed into secretory vesicles and released from the surface of the cell by exocytosis. Once in the extracellular environment, procollagen molecules are processed by different enzymes for fibril formation and cross-linking. The enzyme procollagen N-proteinase removes the N-propeptides and procollagen while the C-proteinase removes C-propeptides. The resultant collagen molecules spontaneously self-assemble into cross-striated fibrils present in the ECM of connective tissue. Formation of covalent links by lysyl oxidase further stabilizes the cross-links (Kadler *et al.*, 1996; Patino *et al.*, 2002) (Fig. 2.5).

Collagen may be extracted from tissues in neutral salt buffers or in dilute acidic solutions. Typically the collagen in mouse skin and tendon is solubilized by mechanical disruption in an acetic acid solution. The yield of such preparations is usually milligram

quantities of type I collagen, mainly in the form of monomers but can also include variable amounts of cross-linked components (dimers, trimers and some higher components) (Kadler *et al.*, 1996). The collagen obtained in this manner loses its structural organization and mechanical features while maintaining good biological properties. By changing the temperature and pH of the solution, the solubilized collagen can be manipulated to polymerize and become a gel. The change in pH to the isoelectric point of 6.5-7 influences the formation of electric charges in the amino acids of collagen causing electrostatic attractions or repulsions between different fibrils (Achilli *et al.*, 2009).

B.4. Collagen Receptors

The interaction of cells with ECM occurs through several families of receptors (Heino, 2000). These collagen receptors, which can be divided into four groups based on the motifs in collagens that they recognize are integrins, receptor tyrosine kinases (DDR), immunoglobulin like receptors, and macrophage mannose receptors (Heino *et al.*, 2009). Among these, integrins and DDRs are considered the most important receptors for collagens.

Integrins, cell-surface glycoprotein receptors, exist as heterodimers of α and β transmembrane subunits in a variety of combinations (Aplin *et al.*, 1998). Collagens bind to integrins containing a $\beta 1$ subunit combined with any of the α subunits (Fig. 2.6). The extracellular domains recognize short peptide sequences (e.g., Arg-Gly-Asp [RGD]) found on some ECM proteins (e.g., fibronectin, vitronectin and collagens, while the intracellular domains are involved in the formation of focal adhesion complexes and in signaling events with implications for cell adhesion, migration, differentiation, and cell

survival (Freyer *et al.*, 2001). Different combinations of α and β receptors can provide tissue specificity for the integrin receptors. For example, $\alpha1\beta1$ is mainly expressed in mesenchymal cells, whereas $\alpha2\beta1$ is most abundant in epithelial cells and platelets (Heino, 2000). Fibril forming collagens such as collagens I and III have high avidity for the $\alpha2\beta1$ integrin. Whereas the avidity of $\alpha2\beta1$ to fibrillar or cross-linked collagens is less compared to monomeric collagen, cell adhesion and migration can still be mediated by $\alpha2\beta1$ integrin on fibrillar collagen matrix compared to monomeric collagen (Jokinen *et al.*, 2004). It is, therefore, conceivable to think of integrins as mechanosensors that undergo complex conformational changes to interact with ECM proteins and intracellular cytoskeletal proteins (Schwartz, 2010).

Collagens type I and III (fibril-forming collagens) are also ligands of dimeric discoidin receptors DDR1 and DDR2, which have tyrosine kinase activity (Ricard-Blum, 2011). An important aspect of their phosphorylation is that, contrary to receptor tyrosine kinases (RTKs), they can remain phosphorylated for several hours. DDR1 is considered an epithelial cell receptor while DDR2 is found on mesenchymal cells. The extracellular domains of these receptors can regulate the deposition of ECM by inhibiting fibrillogenesis (Flynn *et al.*, 2010). DDR1 has similar affinities for fibril-forming and non-fibril forming collagens, whereas DDR2 can be activated by fibril-forming collagens only. The expression of these collagen receptors is dependent on the specific chemical and physical structures of their ligands. Early studies by Klein *et al.* showed that culture of fibroblasts on three dimensional collagen lattices can induce the synthesis and transcription of $\alpha2\beta1$ integrin (but not of $\alpha1\beta1$ or $\alpha3\beta1$) in parallel with gel contraction (Klein *et al.*, 1991). This activation was later found to be able to activate the PKC

pathway (Xu and Clark, 1997). Subsequently Xu and Clark (1996) showed that culture of fibroblasts on polymerized collagen matrices could stimulate the transcription of integrins $\alpha 2\beta 1$, while suppressing integrins $\alpha 3\beta 1$ and $\alpha 2\beta 1$. These alterations proved to be responsible for the differential response of cells to PDGF (Xu and Clark, 1996). In addition, $\alpha 2\beta 1$ integrin has been shown to mediate the antiproliferative effects of polymerized collagen on VSMCs (Koyama *et al.*, 1996).

Collagen types I and III can exist as non-fibrillar forms that do not contribute to cell structure, but retain their cell signaling characteristics (Davis *et al.*, 2000; Emsley *et al.*, 2004; Knight *et al.*, 2000). The non-fibril forming (non-crosslinked) collagen or monomeric collagen have been found to stimulate SMC proliferation (Fassett *et al.*, 2003; Fassett *et al.*, 2006; Nguyen *et al.*, 2005; Schuliga *et al.*, 2010), cell survival (Freyer *et al.*, 2001; Peng *et al.*, 2005), and cytokine release (Peng *et al.*, 2005). Similarly, thin films of heat-denatured collagen promote cellular proliferation (Carragher *et al.*, 1999; Elliott *et al.*, 2003; Jones *et al.*, 1997). On the other hand, thick gels of heat-denatured collagen induce a cellular morphology and proliferation that resembles native fibrillar collagen gels. Thus, the interaction between cells and surrounding matrix is not only dependent on the bulk material properties of collagen gels, but also on the micron-scale interactions of cells and ECM (Elliott *et al.*, 2003).

B.5. Signaling Pathways Activated by Collagens

Collagens can activate three main signaling pathways leading to cytoskeletal organization, cell proliferation and cell survival (Fig. 2.7). The binding of ligands to integrins leads to cross-linking or clustering of receptors and the formation of structures called focal adhesions. These protein assemblies link integrin receptors to a variety of

structural and signaling proteins, such as the actin cytoskeleton and specific kinases (Kumar, 1998). In this process, proteins such as α -actinin and talin bind to the cytoplasmic tails of integrins and the actin-binding protein, vinculin (Martin *et al.*, 2002). Integrin aggregation by ligand binding results in the oligomerization of focal adhesion kinase (FAK), which is mediated by talin. Autophosphorylation of FAK at residue Tyr397 results in the binding of the SH2 domain of SRC and Fyn, which phosphorylates a number of FAK-associated proteins including paxilin, tensin and the docking protein p130CAS (Crk-associated substrate). Phosphorylation of Tyr397 also leads to the recruitment of other SH2-containing proteins, such as phosphatidylinositol-3 kinase (PI3K) (Parsons *et al.*, 2000).

Collagen signaling also involves activation of SRC kinases. SRC phosphorylates FAK at Tyr 925, creating a binding site for the growth-factor-receptor-bound protein complex, the GRB2-SOS complex, and activates another small G-protein, Ras. Once activated by FAK, Ras activates PI3K and Raf at the cytoplasmic membrane. Activated Raf can then activate the kinase activity of MEK followed by phosphorylation of ERK. ERK in turn activates transcription factors such as serum response factor (SRF) and c-Myc that are involved in regulating growth and differentiation. Engagement of integrins linked to SHC activates transcription from the SRF and promotes progression through the G1-phase of the cell cycle in response to growth factors. Since growth factors also stimulate the Ras-MAPK pathway, the integrins and growth factor receptors synergize to enhance Ras-MAPK activation and also promote cell migration on the ECM in a transcription-independent manner. PI3K is also associated with integrin-associated focal adhesion complexes and provides protective signals acting through AKT/ PKB (protein

kinase-B), which blocks entry into apoptosis (Kumar, 1998). In addition, linkage of the exogenous force through matrix stiffness and endogenous force through cytoskeletal tension to the MAPK and ROCK pathways have been proven to be involved in the way focal adhesions and growth factor signaling are modulated for tissue function (Paszek *et al.*, 2005; Provenzano and Keely, 2011).

B.6. Extracellular Matrix in Leiomyoma

Leiomyomas are made up of excessive ECM relative to the surrounding myometrial SMCs. Immunohistochemical staining of myometrial and leiomyoma tissues by Stewart *et al.* showed that while in normal myometrium SMCs are well organized and have small areas of ECM between the bundles of cells, the ECM in leiomyoma tissues are significantly greater than myometrial tissue (Stewart *et al.*, 1994). The ECM in leiomyoma tissue stains more strongly for collagens I and III in leiomyoma tissue than corresponding myometrium (Stewart *et al.*, 1994; Wolanska *et al.*, 1998) (Fig. 2.8). Further electron microscopy analysis showed that collagen fibers in leiomyoma were loosely packed and organized in a non-parallel manner compared with myometrium (Fig. 2.9). Ordered and barbed appearance of collagen fibrils was also absent in leiomyomas and showed an altered orientation (Catherino *et al.*, 2004; Leppert *et al.*, 2004).

Consistent with these changes at the protein level, altered mRNA levels for ECM proteins have also been reported in LSMCs. Stewart *et al.* reported increased mRNA levels of collagens I and III in the proliferative phase of the menstrual cycle (Stewart *et al.*, 1994). Iwahashi and Masaaki also found an increase in the levels of collagens I and V, but this was consistent across the two phases of the menstrual cycle (Iwahashi and

Muragaki, 2011). Subsequently, a microarray study reported differential expression of several genes involved in the formation of ECM in LSMCs. Among these genes, dermatopontin and versican coding transcripts showed the greatest differences between the leiomyoma and myometrial tissues (Tsibris *et al.*, 2002, Catherino *et al.*, 2004). Due to the role of these proteins in the proper organization of ECM collagen, such alterations probably contribute to the disordered growth and ECM organization of ULs through various molecular mechanisms (Catherino *et al.*, 2004).

There are also reports of differences in other types of ECM proteins in leiomyoma and myometrial tissues. For example, fibronectin and hyaluronic acid contents were found to be similar in both leiomyoma and myometrial tissue (Stewart *et al.*, 1994; Wolanska *et al.*, 1998), whereas the levels of sulphated glycosaminoglycans (GAGs) were found to be distinctly higher in leiomyomas. The increase in GAGs, accompanied by the simultaneous increase in the amount of interstitial collagen, is thought to be induced by the persistent stimulation of growth factors stored in GAGs (Wolanska *et al.*, 1998). The ECM protein versican has also been reported to have elevated levels in ULs. Since this protein interacts with other ECM proteins such as collagens, any change in its expression is thought to modulate the organization of the ECM and promote tumor growth and cellular proliferation (Carrino *et al.*, 2012; Norian *et al.*, 2009).

The viscoelastic properties of leiomyoma tissue also seem to differ from those of myometrium causing them to show reduced sensitivity to mechanical cues (Norian *et al.*, 2011). Since mechanical stress has recently been shown to play a role in tumorigenesis (Butcher *et al.*, 2009; Ingber, 2008), it is conceivable that the tissue

stiffness of leiomyomas can alter the signaling pathways linking the ECM to the intracellular machinery leading to altered growth and pathogenesis of fibroids.

C. Halofuginone

C.1. Structure and Effects

Halofuginone (HF, Stenorol, Tempostatin) (7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone) is a plant alkaloid originally isolated from the plant *Dichroa febrifuga* (Fig. 2.10). For many years, extracts from either the roots or the leaves of this plant have been used against malarial fever and the protozoan parasite, *Plasmodium gallinaceum* that infects chickens (Pines *et al.*, 2000). In the late 1980s, halofuginone, which was widely used as a coccidiostat in poultry industry, was found to specifically suppress collagen synthesis in chicken skin (Granot *et al.*, 1991; Weinberg *et al.*, 1985). Further *in vitro* studies on mouse skin fibroblasts, avian growth plate chondrocytes, vascular smooth muscle cells, bovine aortic endothelial cells as well as rat liver stellate cells showed that this inhibition occurs at the transcriptional level for collagen type I (McGaha *et al.*, 2002b) and without affecting cell proliferation and collagen degradation (Granot *et al.*, 1993; McGaha *et al.*, 2002b; Pines *et al.*, 2003).

Halofuginone, at non-cytotoxic concentrations, was also found to diminish the ability of fibroblasts to contract collagen lattices through p42/44 MAPK signaling pathway (Tacheau *et al.*, 2007). In LSMCs, HF inhibited DNA synthesis within 24 hours and lead to cellular apoptosis by 48-72 hours. Transcripts of collagen I and III as well as TGF β -1 were also found to be reduced in both leiomyoma and myometrial SMCs treated with HF (Grudzien *et al.*, 2009).

C.2. Halofuginone as an Anti-Fibrotic Drug

Systemic administration of HF has been shown to be well tolerated in a variety of animal models with different types of fibrotic diseases. In both a mouse model for Duchenne muscle dystrophy (DMD) as well as the tight skin mice model for skin fibrosis, HF was shown reduce muscle fibrosis and hyperplasia and improve muscle function and regeneration (Huebner *et al.*, 2008; McGaha *et al.*, 2002a; Nevo *et al.*, 2010; Pines and Halevy, 2011; Pines *et al.*, 2003; Turgeman *et al.*, 2008). In a db/db mice model of diabetic nephropathy, HF suppressed mesangial expansion and fibronectin overexpression in the kidney, while reducing oxidative stress in the glomerulus (Sato *et al.*, 2009). Similarly, the efficacy of HF as a potential therapeutic agent for liver cirrhosis, corneal fibrosis, esophageal and hypopharyngeal fibrosis has been established (Bruck *et al.*, 2001; Dabak *et al.*, 2010; Nelson *et al.*, 2012; Spira *et al.*, 2002). HF has also been successfully tested for healing skin wounds in a rat model (Yoon *et al.*, 2011) as well as reduction of burn scar formation in a mouse model (Zeplin, 2012). Pre-treatment of a rat model for ischemia/reperfusion renal injury with HF at 100ug/kg body weight was found to reduce oxidative injury and improve renal function by suppressing the generation of reactive oxygen species, depressing lipid peroxidation, and increasing glutathione levels (Karadeniz Cerit *et al.*, 2012).

Pharmacokinetic studies on HF in a rat model have shown that intravenous administration of HF leads to its distribution in all tissues except the brain (Steckclair *et al.*, 2001). Most interestingly, HF was found to remain present more consistently in lung and skeletal muscle than in plasma, which is of importance in fibrotic diseases such as pulmonary fibrosis and DMD. This study also showed that no metabolites of the drug

can be measured in the plasma or tissue and that cellular toxicity was observed at only very high doses.

In recent years, the anti-tumor activities of HF have been the focus of several studies. HF has been shown to reduce tumor growth in models of brain tumors, pheochromocytoma of adrenal glands, hepatocellular carcinoma, and AIDS-related Kaposi-sarcoma (Koon *et al.*, 2011; Taras *et al.*, 2006). An in-depth study on acute promyelocytic leukemia showed inhibition of cell growth accompanied by the up-regulation of TGF β target genes involved in cell cycle regulation (TGF β , TGF β RI, SMAD3, p15, and p21) and down-regulation of MYC (de Figueiredo-Pontes *et al.*, 2011). In the same study, treatment of immunodeficient mice bearing leukemia cells with 150 mg/kg/day HF for 21 days induced partial hematological remission in the peripheral blood, bone marrow, and spleen. More recently, the potential of HF as an anti-angiogenic factor against tumor metastasis has been explored in mice (Jordan and Zeplin, 2012). Mice implanted with HF-coated silicon tubes for three months showed a significant reduction in local angiogenesis mediated by a decrease in the level of angiogenic factors such as TGF- β -1, bFGF, COL1A1, MMP-2, MMP-9, VEGF and PDGF.

Based on the good tolerance of HF in animal studies, clinical trials for the treatment of some fibrotic diseases and cancers have been underway. In 1999, after a successful phase I trial, the first application of HF on a human patient with cutaneous chronic graft versus host disease in the neck area was reported (Nagler and Pines, 1999). Although effects of HF treatment were reversed after the cessation of treatment, for the duration of treatment, HF inhibited collagen synthesis leading to improved neck

rotation. A phase II clinical trial for systemic sclerosis was also reported to be successful (Ong and Denton, 2010; Silman *et al.*, 1995). Last year, the AIDS Malignancy Consortium carried out a phase II study of topical HF treatment in patients with AIDS-related Kaposi-sarcoma (Koon *et al.*, 2011). More recently, in an *in vivo* using a myeloma xenograft mouse model, HF was shown to not only have an anti-tumour activity against a panel of human multiple-myeloma (MM) cell lines and primary patient-derived MM cells, but also a synergistic cytotoxicity with traditional anti-tumor drugs (Leiba *et al.*, 2012). In all these cases, HF treatment proved to be safe and with few side effects (de Jonge *et al.*, 2006).

C.3. Mode of Action

Numerous *in vitro* cell culture systems as well as human and animal models of liver cirrhosis, scleroderma, dermal fibrosis, and solid tumors have been used to determine the mechanisms involved in the inhibitory effects of HF on fibrosis. These effects seem to be mediated primarily through the MAPK and TGF β signaling pathways to reduce the expression of collagens and fibronectin synthesis (Genin *et al.*, 2008; Gnainsky *et al.*, 2007; McGaha *et al.*, 2002b; Nagler *et al.*, 2000; Pines and Nagler, 1998; Pines *et al.*, 2003; Pines *et al.*, 2003; Sato *et al.*, 2009; Yee *et al.*, 2006).

The efficacy of HF in improving radiation-induced fibrosis in a mouse model was demonstrated to occur through the down-regulation of TGF β -RII as well as induction of the inhibitory Smad 7 leading to inhibition of Smad 2/3 phosphorylation (Xavier *et al.*, 2004). Similarly, in mesangial cells, HF decreased the expression of TGF β -RII and suppressed phosphorylation of Smad 2 as well as expression of collagen I and fibronectin induced by TGF β (Sato *et al.*, 2009).

HF also inhibited caerulein-induced collagen synthesis, collagen cross-linking enzyme prolyl-4-hydroxylase, along with MMP2 (Zion *et al.*, 2009). Furthermore, HF was shown to inhibit the proliferation of hepatic stellate cells in culture by decreasing Smad 3 phosphorylation and increasing c-Jun N-terminal kinase activation (Popov *et al.*, 2006). Treatment of human fibroblasts with 50 nM HF was shown to induce a rapid and prolonged phosphorylation of p38, ERK1/2 and JNK1/2 as early as 15 minutes and lasted for two hours (Tacheau *et al.*, 2007). Additionally, the activation of ERK1/2 was specifically correlated to decreased collagen contraction induced by HF.

Another study using a C2 muscle cell line and primary myoblasts derived from wild type and mdx mice diaphragm showed that 10 nM HF promoted the activation of PI3K/AKT and MAPK pathways in muscle cells, while reducing the phosphorylation of Smad3 through direct molecular associations (Roffe *et al.*, 2010). The PI3K/AKT and MAPK signaling pathways both play a crucial role in myoblast differentiation and proliferation. Therefore, the effect of HF on these pathways resulted in an enhancement of myotube formation in muscle cells of a mouse model for muscular dystrophy (Roffe *et al.*, 2010).

More recently, activation of other signaling pathways, such as the amino acid starvation response by HF have been shown to inhibit inflammatory T-cell differentiation *in vitro* (Sundrud *et al.*, 2009) and to confer stress resistance (Peng *et al.*, 2012) by directly inhibiting the prolyl-tRNA synthetase activity of glutamyl-prolyl-tRNA synthetase (Keller *et al.*, 2012). In another study, up-regulation of MMP13 in mammary epithelial cells treated with HF was observed to be mediated by p38 and NF κ B, and not through Smad7 activation and TGF β signaling inhibition (Kamberov *et al.*, 2011; Popov *et al.*,

2006). A subsequent microarray study on these cells correlated this observation with transcriptional responses characteristic of the Integrated Stress Response (ISR), suggesting that this pathway may be the underlying mechanism for the biological activities of HF (Kamberov *et al.*, 2011).

Figures

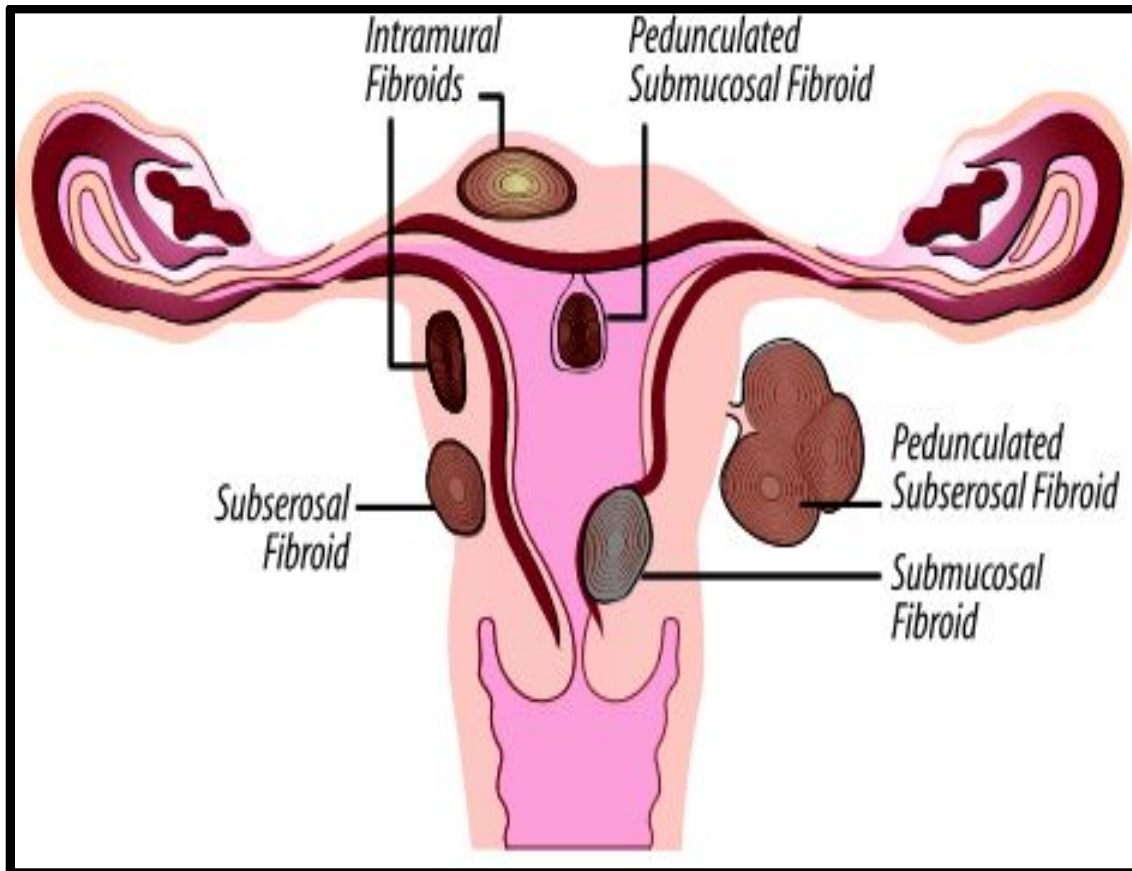


Figure 2.1. Formation of leiomyoma tumors in different locations within the uterus. (National Uterine Fibroid Foundation)

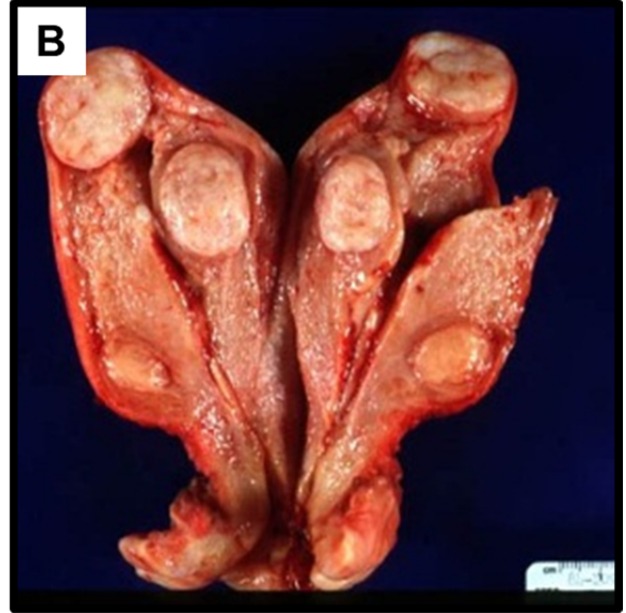
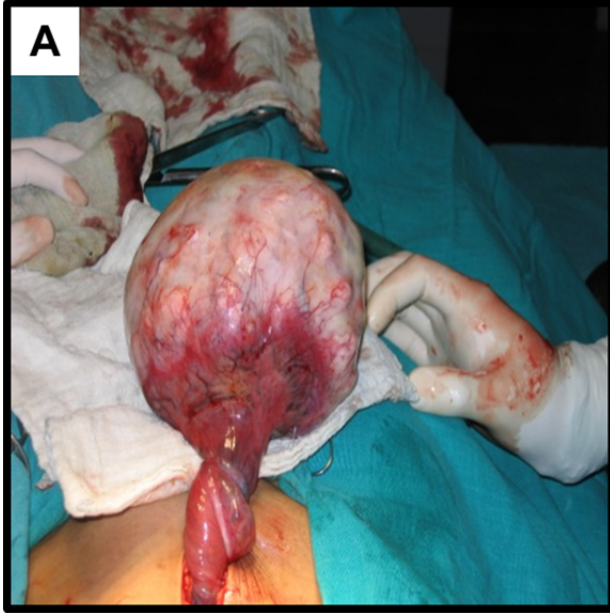


Figure 2.2. Uterine leiomyoma tumors. (A) A large uterine fibroid being removed from a 58 year old patient with excruciating abdominal pain; (B) Presence of multiple ULs in different locations within the uterine cavity. Image courtesy of Lady Willingdon Hospital in India and PathosWeb.

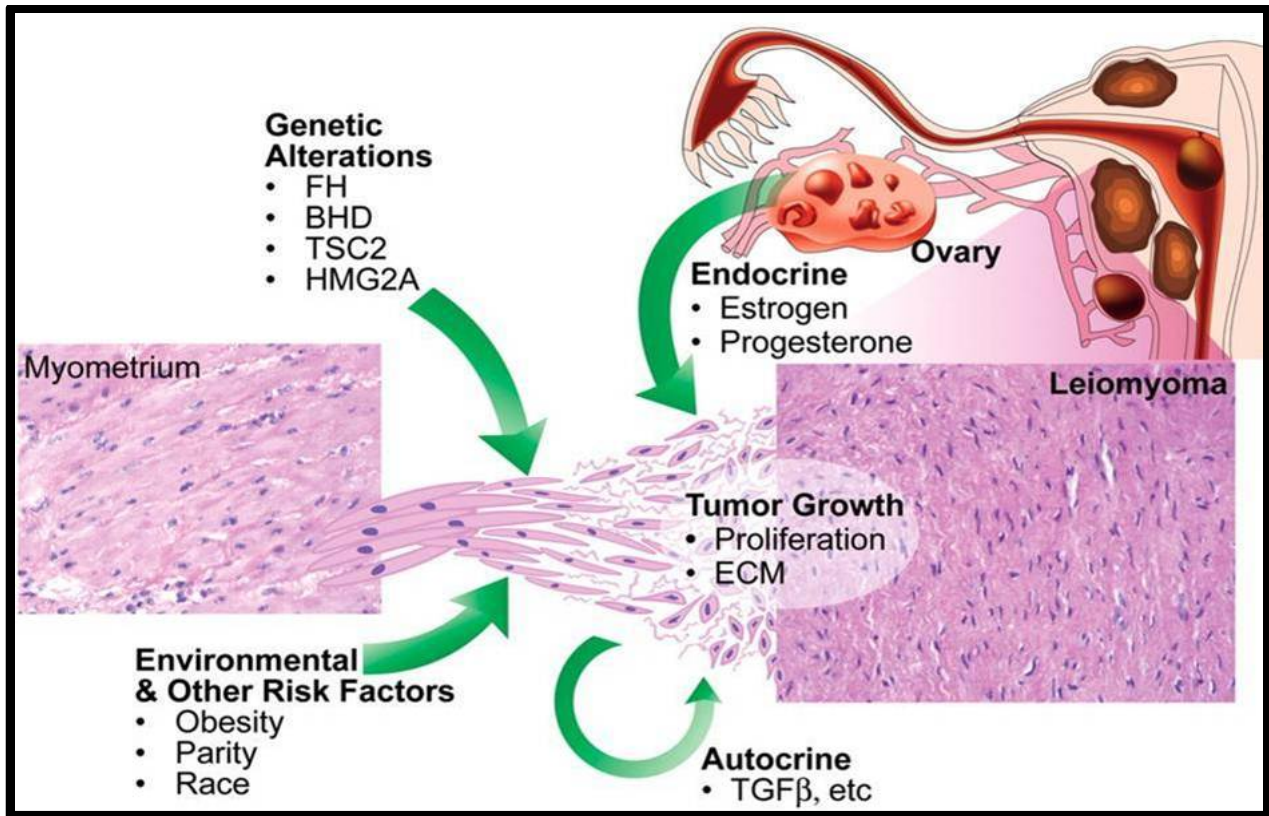


Figure 2.3. Etiology of ULs. Initiation of ULs can be linked to several heterogeneous factors. (Walker and Stewart. 2005)

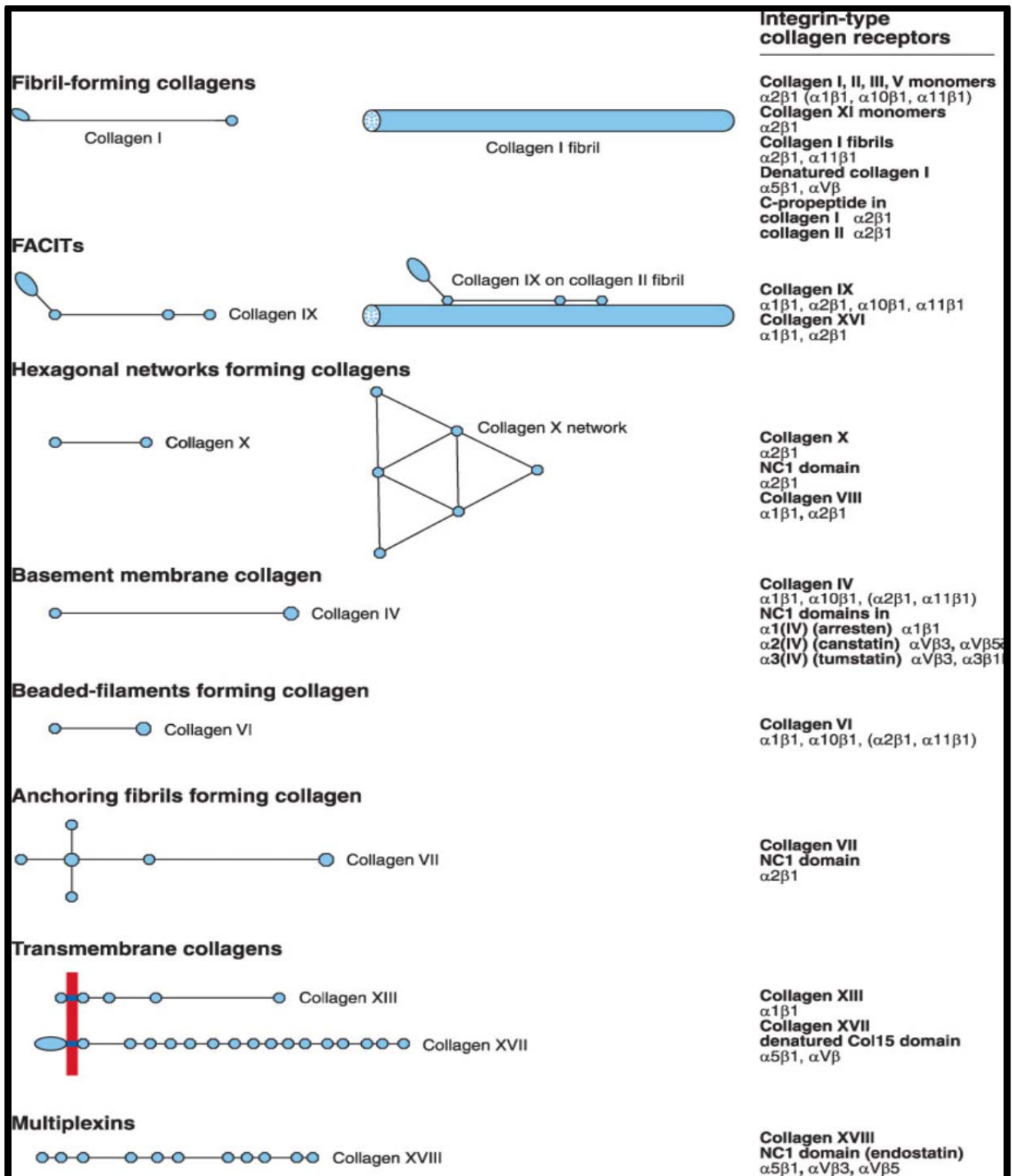


Figure 2.4. Collagen subfamilies and their associated integrin receptors (Heino et al. 2007).

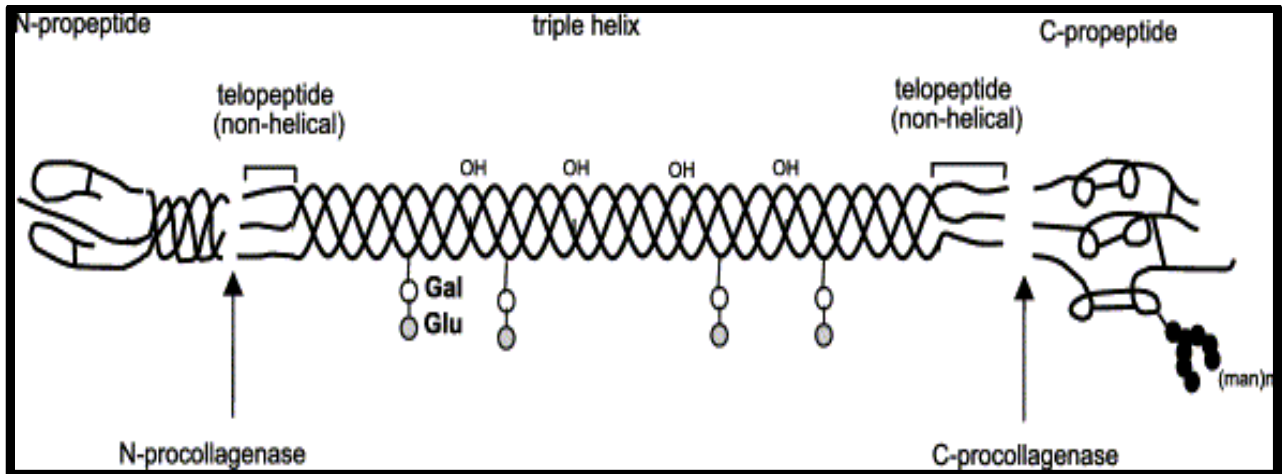


Figure 2.5. Molecular structure of fibrillar collagens (Gelse *et al.* 2003).

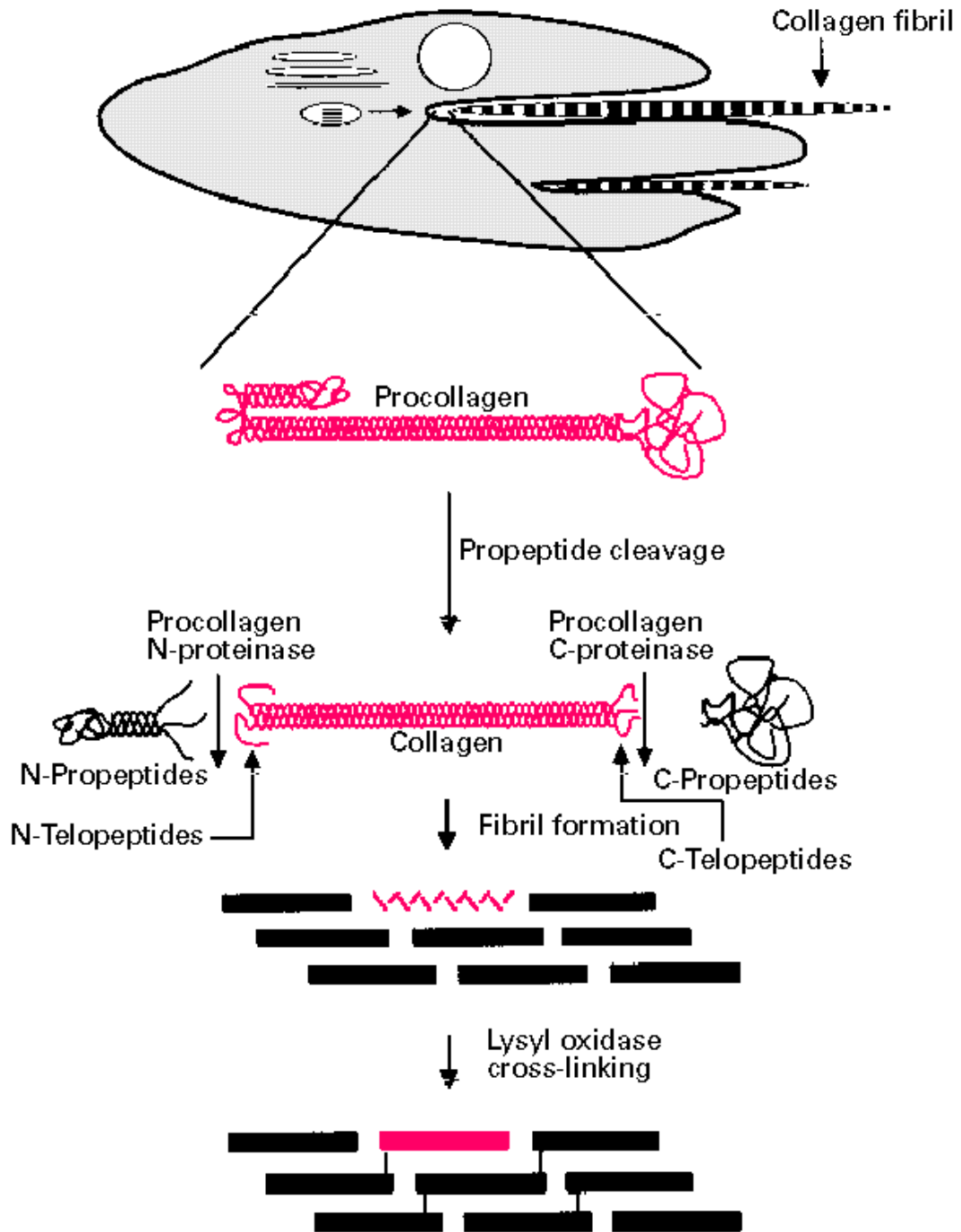


Figure 2.6. Biosynthesis of collagens (Kadler *et al.* 1996).

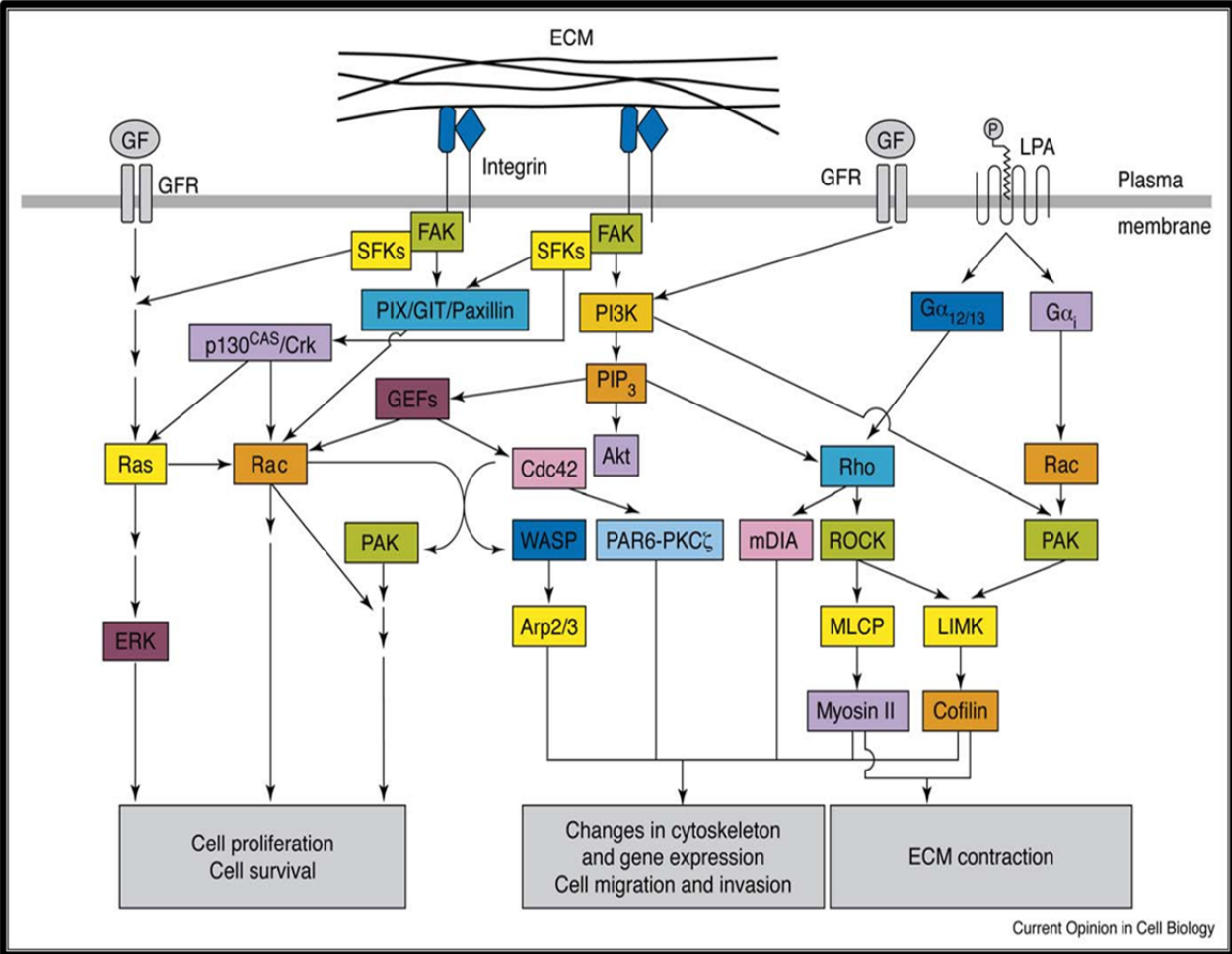


Figure 2.7. Signaling pathways activated by collagens (Larsen *et al.* 2006).

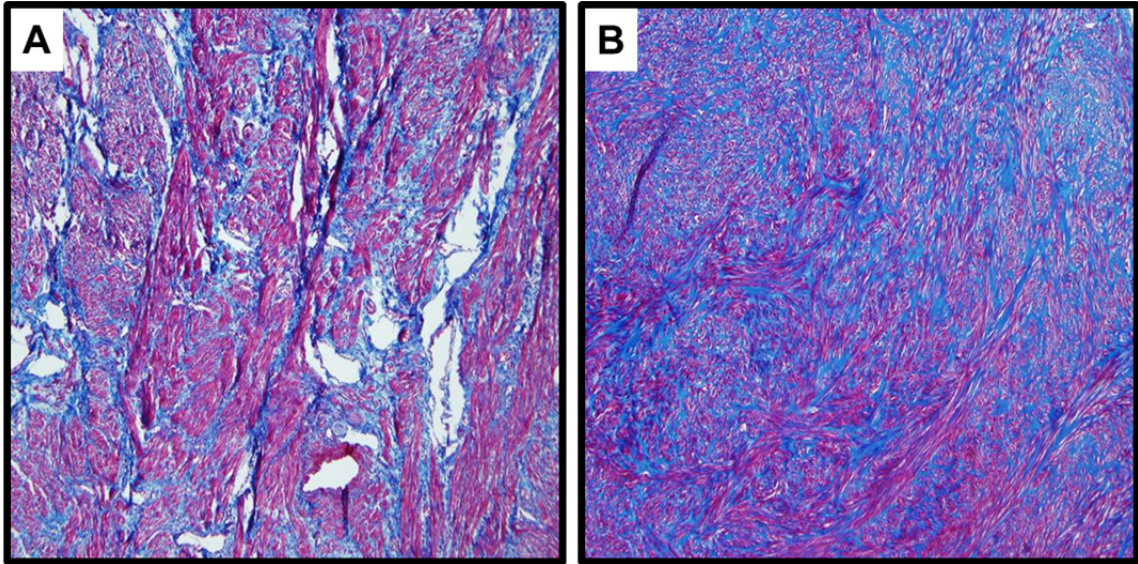


Figure 2.8. Excessive deposition of ECM in ULs. Masson's trichrome staining in matched myometrial (A) and leiomyoma (B) tissue shows increased deposition of ECM stained in blue in ULs.

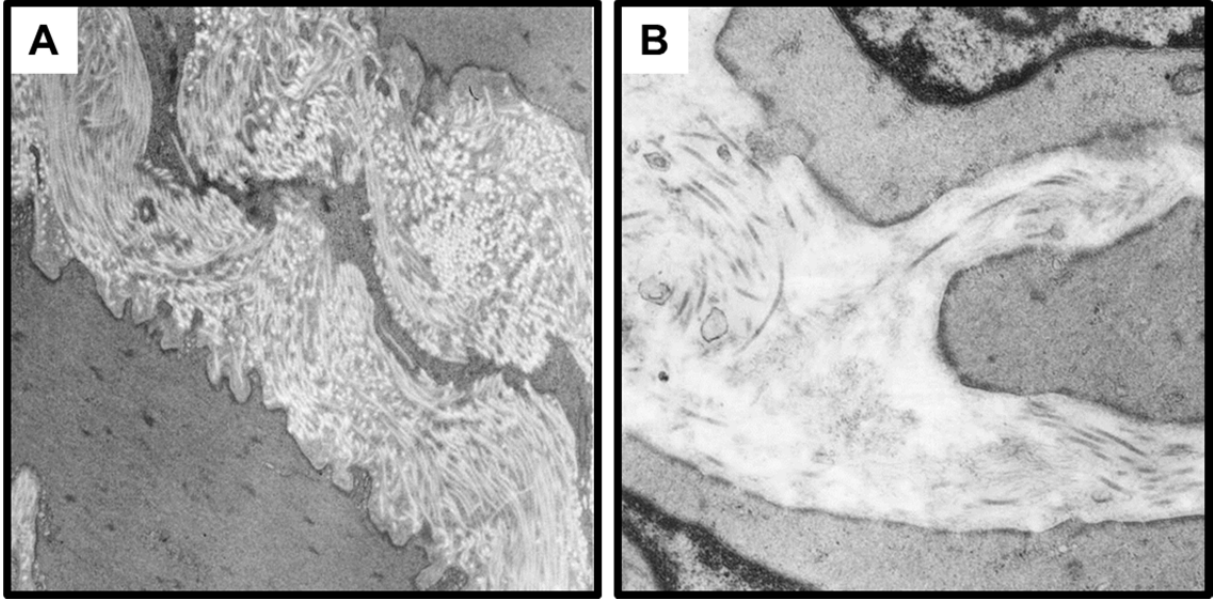


Figure 2.9. Electron microscopy of ECM in myometrium (A) and leiomyoma (B).

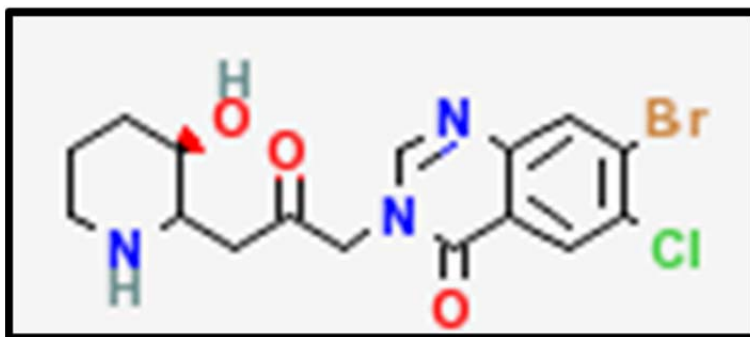


Figure 2.10. Molecular structure of halofuginone and the plant *Dichroa febrifuga* from which it is derived.

Reference List

- Achilli, M., Lagueux, J., and Mantovani, D. (2009). On the effects of UV-C and pH on the mechanical behavior, molecular conformation and cell viability of collagen-based scaffold for vascular tissue engineering. *Macromol. Biosci.* *10*, 307-416.
- Al-Hendy, A., and Salama, S.A. (2006a). Catechol-O-methyltransferase polymorphism is associated with increased uterine leiomyoma risk in different ethnic groups. *J. Soc. Gynecol. Investig.* *13*, 136-144.
- Al-Hendy, A., and Salama, S. (2006b). Gene therapy and uterine leiomyoma: a review. *Hum. Reprod. Update* *12*, 385-400.
- Ali, A.F.M., Fateen, B., Ezzet, A., Badawy, H., Ramadan, A., and El-tobge, A. (2000). Elevated level of basic fibroblast growth factor in leiomyoma-related abnormal uterine bleeding and its reversal by interferon- α . *Obstet. Gynecol.* *95*, S6-S6.
- Amant, F., Dorfling, C.M., de Brabanter, J., Vandewalle, J., Vergote, I., Lindeque, B.G., and van Rensburg, E.J. (2004). A possible role of the cytochrome P450c17alpha gene (CYP17) polymorphism in the pathobiology of uterine leiomyomas from black South African women: a pilot study. *Acta Obstet. Gynecol. Scand.* *83*, 234-239.
- Amanti, L., Sadeghi-Bazargani, H., Abdollahi, H., and Ehdaevand, F. (2011). Uterine leiomyoma and its association with menstrual pattern and history of depo-medroxyprogesterone acetate injections. *Int. J. Gen. Med.* *4*, 535-538.
- Andersen, J., DyReyes, V.M., Barbieri, R.L., Coachman, D.M., and Miksicek, R.J. (1995). Leiomyoma primary cultures have elevated transcriptional response to estrogen compared with autologous myometrial cultures. *J. Soc. Gynecol. Investig.* *2*, 542-551.
- Aplin, A.E., Howe, A., Alahari, S.K., and Juliano, R.L. (1998). Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol. Rev.* *50*, 197-263.
- Arici, A., and Sozen, I. (2003). Expression, menstrual cycle-dependent activation, and bimodal mitogenic effect of transforming growth factor-beta1 in human myometrium and leiomyoma. *Am. J. Obstet. Gynecol.* *188*, 76-83.
- Arici, A., and Sozen, I. (2000). Transforming growth factor-beta3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. *Fertil. Steril.* *73*, 1006-1011.
- Arslan, A.A., Gold, L.I., Mittal, K., Suen, T., Belitskaya-Levy, I., Tang, M., and Toniolo, P. (2005). Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: New evidence and a systematic review. *Hum. Reprod.* *20*, 852-863.

Barbarisi, A., Petillo, O., Di Lieto, A., Melone, M.A., Margarucci, S., Cannas, M., and Peluso, G. (2001). 17-beta estradiol elicits an autocrine leiomyoma cell proliferation: evidence for a stimulation of protein kinase-dependent pathway. *J Cell. Physiol.* *186*, 414-424.

Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen-Pope, D.F., and Ross, R. (1990). TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. *Cell.* *63*, 515-524.

Beard, H.K., Faulk, W.P., Conochie, L.B., and Glynn, L.E. (1977). Some immunological aspects of collagen. *Prog. Allergy.* *22*, 45-106.

Benassayag, C., Leroy, M.J., Rigourd, V., Robert, B., Honore, J.C., Mignot, T.M., Vacher-Lavenu, M.C., Chapron, C., and Ferre, F. (1999). Estrogen receptors (ERalpha/ERbeta) in normal and pathological growth of the human myometrium: pregnancy and leiomyoma. *Am. J. Physiol.* *276*, E1112-1118.

Benda, J.A. (2001). Pathology of smooth muscle tumors of the uterine corpus. *Clin. Obstet. Gynecol.* *44*, 350-363.

Berry, W., Doernte, A., Conner, M., Barnes, M., and Oates, S. (2006). Spontaneously occurring fibroid tumors of the laying hen oviduct. *Poult. Sci.* *85*, 1969-1974.

Boehm, K.D., Daimon, M., Gorodeski, I.G., Sheean, L.A., Utian, W.H., and Ilan, J. (1990). Expression of the insulin-like and platelet-derived growth factor genes in human uterine tissues. *Mol. Reprod. Dev.* *27*, 93-101.

Bouchard, P. (2011). Current and future medical treatments for menometrorrhagia during the premenopause. *Gynecol. Endocrinol.* *27 Suppl 1*, 1120-1125.

Bouchard, P., Chabbert-Buffet, N., and Fauser, B.C. (2011). Selective progesterone receptor modulators in reproductive medicine: pharmacology, clinical efficacy and safety. *Fertil. Steril.* *96*, 1175-1189.

Brandon, D.D., Bethea, C.L., Strawn, E.Y., Novy, M.J., Burry, K.A., Harrington, M.S., Erickson, T.E., Warner, C., Keenan, E.J., and Clinton, G.M. (1993). Progesterone receptor messenger ribonucleic acid and protein are overexpressed in human uterine leiomyomas. *Am. J. Obstet. Gynecol.* *169*, 78-85.

Bruck, R., Genina, O., Aeed, H., Alexiev, R., Nagler, A., Avni, Y., and Pines, M. (2001). Halofuginone to prevent and treat thioacetamide-induced liver fibrosis in rats. *Hepatology.* *33*, 379-386.

Buchanan, D.L., Ohsako, S., Tohyama, C., Cooke, P.S., and Iguchi, T. (2002). Dioxin inhibition of estrogen-induced mouse uterine epithelial mitogenesis involves changes in cyclin and transforming growth factor-beta expression. *Toxicol. Sci.* *66*, 62-68.

Bulun, S.E., Simpson, E.R., and Word, R.A. (1994). Expression of the CYP19 gene and its product aromatase cytochrome P450 in human uterine leiomyoma tissues and cells in culture. *J. Clin. Endocrinol. Metab.* *78*, 736-743.

Burgeson, R.E., and Nimni, M.E. (1992). Collagen types. Molecular structure and tissue distribution. *Clin. Orthop. Relat. Res.* *282*, 250-272.

Butcher, D.T., Alliston, T., and Weaver, V.M. (2009). A tense situation: forcing tumour progression. *Nat. Rev. Cancer.* *9*, 108-122.

Cardozo, E.R., Clark, A.D., Banks, N.K., Henne, M.B., Stegmann, B.J., and Segars, J.H. (2011). The estimated annual cost of uterine leiomyomata in the United States. *Am. J. Obstet. Gynecol.* *206*, 211.

Carragher, N.O., Levkau, B., Ross, R., and Raines, E.W. (1999). Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J. Cell Biol.* *147*, 619-630.

Carrino, D.A., Mesiano, S., Barker, N.M., Hurd, W.W., and Caplan, A.I. (2012). Proteoglycans of uterine fibroids and keloid scars: similarity in their proteoglycan composition. *Biochem. J.* *443*, 361-368.

Catherino, W.H., Leppert, P.C., Stenmark, M.H., Payson, M., Potlog-Nahari, C., Nieman, L.K., and Segars, J.H. (2004). Reduced dermatopontin expression is a molecular link between uterine leiomyomas and keloids. *Genes Chrom. Cancer* *40*, 204-217.

Catherino, W.H., Parrott, E., and Segars, J. (2011). Proceedings from the national institute of child health and human development conference on the uterine fibroid research update workshop. *Fertil. Steril.* *95*, 9-12.

Chavez, N.F., and Stewart, E.A. (2001). Medical treatment of uterine fibroids. *Clin. Obstet. Gynecol.* *44*, 372-384.

Chegini, N., Ma, C., Tang, X.M., and Williams, R.S. (2002). Effects of GnRH analogues, 'add-back' steroid therapy, antiestrogen and antiprogestins on leiomyoma and myometrial smooth muscle cell growth and transforming growth factor-beta expression. *Mo.I Hum. Reprod.* *8*, 1071-1078.

Chegini, N., Tang, X.M., and Ma, C. (1999). Regulation of transforming growth factor-beta1 expression by granulocyte macrophage-colony-stimulating factor in leiomyoma and myometrial smooth muscle cells. *J. Clin. Endocrinol. Metab.* *84*, 4138-4143.

Chwalisz, K., Larsen, L., Mattia-Goldberg, C., Edmonds, A., Elger, W., and Winkel, C.A. (2007). A randomized, controlled trial of asoprisnil, a novel selective progesterone receptor modulator, in women with uterine leiomyomata. *Fertil. Steril.* *87*, 1399-1412.

Cook, J.D., and Walker, C.L. (2004). The Eker rat: establishing a genetic paradigm linking renal cell carcinoma and uterine leiomyoma. *Curr. Mol. Med.* 4, 813-824.

Crabtree, J.S., Jelinsky, S.A., Harris, H.A., Choe, S.E., Cotreau, M.M., Kimberland, M.L., Wilson, E., Saraf, K.A., Liu, W., McCampbell, A.S., Dave, B., Broaddus, R.R., Brown, E.L., Skotnicki, J.S., Abou-Gharbia, M., Winneker, R.C., Walker, C.L. (2009). Comparison of human and rat uterine leiomyomata: identification of a dysregulated mammalian target of rapamycin pathway. *Cancer Res.* 69, 6171-6178.

Cramer, S.F., Mann, L., Calianese, E., Daley, J., and Williamson, K. (2009). Association of seedling myomas with myometrial hyperplasia. *Hum. Pathol.* 40, 218-225.

Cui, L., Ren, Y., Yin, H., Wang, Y., Li, D., Liu, M., Zhu, Y., Lin, W., Tang, X.D., Gui, Y., and Zheng, X.L. (2011). Increased expression of tuberin in human uterine leiomyoma. *Fertil. Steril.* 95, 1805-1808.

Dabak, H., Karlidag, T., Akpolat, N., Keles, E., Alpay, H.C., Serin, M., Kaygusuz, I., Yalcin, S., and Isik, O. (2010). The effects of methylprednisolone and halofuginone on preventing esophageal and hypopharyngeal fibrosis in delivered radiotherapy. *Eur. Arch. Otorhinolaryngol.* 267, 1429-1435.

D'Aloisio, A.A., Baird, D.D., Deroo, L.A., and Sandler, D.P. (2011). Early-life exposures and early onset uterine leiomyomata in black women in the sister study. *Environ. Health Perspect.* 120, 406-412.

Davis, G.E., Bayless, K.J., Davis, M.J., and Meininger, G.A. (2000). Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules. *Am. J. Pathol.* 156, 1489-1498.

Day Baird, D., Dunson, D.B., Hill, M.C., Cousins, D., and Schectman, J.M. (2003). High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. *Am. J. Obstet. Gynecol.* 188, 100-107.

de Figueiredo-Pontes, L.L., Assis, P.A., Santana-Lemos, B.A., Jacomo, R.H., Lima, A.S., Garcia, A.B., Thome, C.H., Araujo, A.G., Panepucci, R.A., Zago, M.A., Nagler A, Falcão RP, Rego EM. (2011). Halofuginone has anti-proliferative effects in acute promyelocytic leukemia by modulating the transforming growth factor beta signaling pathway. *PLoS One.* 6, e26713.

de Jonge, M.J.A., Dumez, H., Verweij, J., Yarkoni, S., Snyder, D., Lacombe, D., Marréaud, S., Yamaguchi, T., Punt, C.J.A., and van Oosterom, A. (2006). Phase I and pharmacokinetic study of halofuginone, an oral quinazolinone derivative in patients with advanced solid tumours. *Eur. J. Cancer.* 42, 1768-1774.

Di Lieto, A., De Falco, M., Mansueto, G., De Rosa, G., Pollio, F., and Staibano, S. (2005). Preoperative administration of GnRH-a plus tibolone to premenopausal women

with uterine fibroids: evaluation of the clinical response, the immunohistochemical expression of PDGF, bFGF and VEGF and the vascular pattern. *Steroids* 70, 95-102.

Di Lieto, A., De Rosa, G., De Falco, M., Iannotti, F., Staibano, S., Pollio, F., Scaramellino, M., and Salvatore, G. (2002). Relationship between platelet-derived growth factor expression in leiomyomas and uterine volume changes after gonadotropin-releasing hormone agonist treatment. *Hum. Pathol.* 33, 220-224.

Dou, Q., Zhao, Y., Tarnuzzer, R.W., Rong, H., Williams, R.S., Schultz, G.S., and Chegini, N. (1996). Suppression of transforming growth factor-beta (TGF beta) and TGF beta receptor messenger ribonucleic acid and protein expression in leiomyomata in women receiving gonadotropin-releasing hormone agonist therapy. *J. Clin. Endocrinol. Metab.* 81, 3222-3230.

Duhan, N. (2011). Current and emerging treatments for uterine myoma - an update. *Int. J. Womens Health.* 3, 231-241.

Elliott, J.T., Tona, A., Woodward, J.T., Jones, P.L., and Plant, A.L. (2003). Thin films of collagen affect smooth muscle cell morphology. *Langmuir.* 19, 1506-1514.

Emans, S.J., Laufer, M.R., and Goldstein, D.P. (2005). *Pediatric and Adolescent Gynecology* (Philadelphia: Lippincott Williams Wilkins Publishing Company).

Emsley, J., Knight, C.G., Farndale, R.W., and Barnes, M.J. (2004). Structure of the Integrin $\alpha 2\beta 1$ -binding Collagen Peptide. *J. Mol. Biol.* 335, 1019-1028.

Engman, M., Granberg, S., Williams, A.R., Meng, C.X., Lalitkumar, P.G., and Gemzell-Danielsson, K. (2009). Mifepristone for treatment of uterine leiomyoma. A prospective randomized placebo controlled trial. *Hum. Reprod.* 24, 1870-1879.

Exposito, J.Y., Valcourt, U., Cluzel, C., and Lethias, C. (2010). The fibrillar collagen family. *Int. J. Mol. Sci.* 11, 407-426.

Faerstein, E., Szklo, M., and Rosenshein, N. (2001). Risk factors for uterine leiomyoma: a practice-based case-control study. I. African-American heritage, reproductive history, body size, and smoking. *Am. J. Epidemiol.* 153, 1-10.

Fassett, J.T., Tobolt, D., Nelsen, C.J., Albrecht, J.H., and Hansen, L.K. (2003). The role of collagen structure in mitogen stimulation of ERK, cyclin D1 expression, and G1-S progression in rat hepatocytes. *J. Biol. Chem.* 278, 31691-31700.

Fassett, J., Tobolt, D., and Hansen, L.K. (2006). Type I Collagen Structure Regulates Cell Morphology and EGF Signaling in Primary Rat Hepatocytes through cAMP-dependent Protein Kinase A. *Mol. Bio. Cell* 17, 345-356.

- Fayed, Y.M., Tsibris, J.C., Langenberg, P.W., and Robertson, A.L., Jr. (1989). Human uterine leiomyoma cells: binding and growth responses to epidermal growth factor, platelet-derived growth factor, and insulin. *Lab. Invest.* *60*, 30-37.
- Felberbaum, R.E., Germer, U., Ludwig, M., Riethmuller-Winzen, H., Heise, S., Buttge, I., Bauer, O., Reissmann, T., Engel, J., and Diedrich, K. (1998). Treatment of uterine fibroids with a slow-release formulation of the gonadotrophin releasing hormone antagonist Cetrorelix. *Hum. Reprod.* *13*, 1660-1668.
- Fitzgerald, J.B., Chennathukuzhi, V., Koohestani, F., Nowak, R.A., and Christenson, L.K. (2012). Role of microRNA-21 and programmed cell death 4 in the pathogenesis of human uterine leiomyomas. *Fertil. Steril.* ePub.
- Flake, G.P., Andersen, J., and Dixon, D. (2003). Etiology and pathogenesis of uterine leiomyomas: a review. *Environ. Health. Perspect.* *111*, 1037-1054.
- Flynn, L.A., Blissett, A.R., Calomeni, E.P., and Agarwal, G. (2010). Inhibition of collagen fibrillogenesis by cells expressing soluble extracellular domains of DDR1 and DDR2. *J. Mol. Biol.* *395*, 533-543.
- Freyer, A.M., Johnson, S.R., and Hall, I.P. (2001). Effects of growth factors and extracellular matrix on survival of human airway smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* *25*, 569-576.
- Gao, X., Yu, L., Castro, L., Moore, A.B., Hermon, T., Bortner, C., Sifre, M., and Dixon, D. (2010). An endocrine-disrupting chemical, fenvalerate, induces cell cycle progression and collagen type I expression in human uterine leiomyoma and myometrial cells. *Toxicol. Lett.* *196*, 133-141.
- Gao, Z., Matsuo, H., Wang, Y., Nakago, S., and Maruo, T. (2001). Up-regulation by IGF-I of proliferating cell nuclear antigen and Bcl-2 protein expression in human uterine leiomyoma cells. *J. Clin. Endocrinol. Metab.* *86*, 5593-5599.
- Gelse, K., Poschl, E., and Aigner, T. (2003). Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* *55*, 1531-1546.
- Genin, O., Rechavi, G., Nagler, A., Ben-Itzhak, O., Nazemi, K.J., and Pines, M. (2008). Myofibroblasts in pulmonary and brain metastases of alveolar soft-part sarcoma: a novel target for treatment? *Neoplasia* *10*, 940-948.
- Georgieva, B., Milev, I., Minkov, I., Dimitrova, I., Bradford, A.P., and Baev, V. (2012). Characterization of the uterine leiomyoma microRNAome by deep sequencing. *Genomics.* *99*, 275-281.
- Ghosh, A.K., and Vaughan, D.E. (2012). Fibrosis: is it a coactivator disease? *Front. Biosci. (Elite Ed.)* *4*, 1556-1570.

Gnainsky, Y., Kushnirsky, Z., Bilu, G., Hagai, Y., Genina, O., Volpin, H., Bruck, R., Spira, G., Nagler, A., Kawada, N., Yoshizato K, Reinhardt, D. P., Libermann, T. A., Pines, M. (2007). Gene expression during chemically induced liver fibrosis: effect of halofuginone on TGF-beta signaling. *Cell Tissue Res.* 328, 153-166.

Granot, I., Bartov, I., Plavnik, I., Wax, E., Hurwitz, S., and Pines, M. (1991). Increased skin tearing in broilers and reduced collagen synthesis in skin *in vivo* and *in vitro* in response to the coccidiostat halofuginone. *Poult. Sci.* 70, 1559-1563.

Granot, I., Halevy, O., Hurwitz, S., and Pines, M. (1993). Halofuginone: an inhibitor of collagen type I synthesis. *Biochim. Biophys. Acta* 1156, 107-112.

Grudzien, M.M., Low, P.S., Manning, P.C., Arredondo, M., Belton, R.J., Jr, and Nowak, R.A. (2009). The antifibrotic drug halofuginone inhibits proliferation and collagen production by human leiomyoma and myometrial smooth muscle cells. *Fertil. Steril.* 93:1290-1298.

Hai-Gang, C., Lan, Z., Quan-Cai, C., Jing-He, L., and Bin, L. (2011). Estrogen induced rat model of uterine leiomyoma. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 33, 408-411.

Halder, S.K., Goodwin, J.S., and Al-Hendy, A. (2011). 1,25-Dihydroxyvitamin D3 reduces TGF- β 3-induced fibrosis-related gene expression in human uterine leiomyoma cells. *J. Clin. Endocrinol. Metab.* 96:E754-62.

Halder, S.K., Sharan, C., and Al-Hendy, A. (2012). 1,25-Dihydroxyvitamin D3 treatment shrinks uterine leiomyoma tumors in the eker rat model. *Biol. Reprod.* 86,116.

Harrison-Woolrych, M.L., Charnock-Jones, D.S., and Smith, S.K. (1994). Quantification of messenger ribonucleic acid for epidermal growth factor in human myometrium and leiomyomata using reverse transcriptase polymerase chain reaction. *J. Clin. Endocrinol. Metab.* 78, 1179-1184.

Hassan, M., Zhang, D., Salama, S., Hamada, F., Arafa, H., Fouad, H., Walker, C., and Al-Hendy, A. (2009). Towards fibroid gene therapy: adenovirus-mediated delivery of herpes simplex virus 1 thymidine kinase gene/ganciclovir shrinks uterine leiomyoma in the Eker rat model. *Gynecol. Obstet. Invest.* 68, 19-32.

Hassan, M.H., Fouad, H., Bahashwan, S., and Al-Hendy, A. (2011). Towards non-surgical therapy for uterine fibroids: catechol-O-methyl transferase inhibitor shrinks uterine fibroid lesions in the Eker rat model. *Hum. Reprod.* 26, 3008-3018.

Hassan, M.H., Salama, S.A., Zhang, D., Arafa, H.M., Hamada, F.M., Fouad, H., Walker, C.C., and Al-Hendy, A. (2010). Gene therapy targeting leiomyoma: adenovirus-mediated delivery of dominant-negative estrogen receptor gene shrinks uterine tumors in Eker rat model. *Fertil. Steril.* 93, 239-250.

Heino, J. (2000). The collagen receptor integrins have distinct ligand recognition and signaling functions. *Matrix Biology* 19, 319-323.

Heino, J., Huhtala, M., Käpylä, J., and Johnson, M.S. (2009). Evolution of collagen-based adhesion systems. *Int. J. Biochem. Cell Biol.* 41, 341-348.

Hodge, J.C., T Cuenco, K., Huyck, K.L., Somasundaram, P., Panhuysen, C.I., Stewart, E.A., and Morton, C.C. (2009). Uterine leiomyomata and decreased height: a common HMGA2 predisposition allele. *Hum. Genet.* 125, 257-263.

Hoekstra, A.V., Sefton, E.C., Berry, E., Lu, Z., Hardt, J., Marsh, E., Yin, P., Clardy, J., Chakravarti, D., Bulun, S., and Kim, J.J. (2009). Progestins activate the AKT pathway in leiomyoma cells and promote survival. *J. Clin. Endocrinol. Metab.* 94, 1768-1774.

Huebner, K.D., Jassal, D.S., Halevy, O., Pines, M., and Anderson, J.E. (2008). Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone. *Am. J. Physiol. Heart Circ. Physiol.* 294, H1550-1561.

Huyck, K.L., Panhuysen, C.I.M., Cuenco, K.T., Zhang, J., Goldhammer, H., Jones, E.S., Somasundaram, P., Lynch, A.M., Harlow, B.L., Lee, H., Stewart, E.A., and Morton, C.C. (2008). The impact of race as a risk factor for symptom severity and age at diagnosis of uterine leiomyomata among affected sisters. *Obstet. Gynecol.* 198, 168.e1-168.e9.

Hwu, Y.M., Li, S.H., Lee, R.K., Tsai, Y.H., Yeh, T.S., and Lin, S.Y. (2008). Increased expression of platelet-derived growth factor C messenger ribonucleic acid in uterine leiomyomata. *Fertil. Steril.* 89, 468-471.

Hynes, R.O. and Yamada, K.M. (2011). *Extracellular Matrix Biology*. Cold Spring Harbor.

Ingber, D.E. (2008). Can cancer be reversed by engineering the tumor microenvironment? *Semin. Cancer Biol.* 18, 356-364.

Ishikawa, H., Ishi, K., Serna, V.A., Kakazu, R., Bulun, S.E., and Kurita, T. (2010). Progesterone is essential for maintenance and growth of uterine leiomyoma. *Endocrinology* 151, 2433-2442.

Iwahashi, M., and Muragaki, Y. (2011). Increased type I and V collagen expression in uterine leiomyomas during the menstrual cycle. *Fertil. Steril.* 95, 2137-2139.

Jokinen, J., Dadu, E., Nykvist, P., Kapyla, J., White, D.J., Ivaska, J., Vehvilainen, P., Reunanen, H., Larjava, H., Hakkinen, L., and Heino, J. (2004). Integrin-mediated cell adhesion to type I collagen fibrils. *J. Biol. Chem.* 279, 31956-31963.

Jones, P.L., Crack, J., and Rabinovitch, M. (1997). Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to

promote epidermal growth factor receptor phosphorylation and growth. *J. Cell Biol.* **139**, 279-293.

Jordan, M.C., and Zeplin, P.H. (2012). Local inhibition of angiogenesis by halofuginone coated silicone materials. *J. Mater. Sci. Mater. Med.* **23**, 1203-1210.

Kadler, K.E., Holmes, D.F., Trotter, J.A., and Chapman, J.A. (1996). Collagen fibril formation. *Biochem. J.* **316**, 1-11.

Kamberov, Y.G., Kim, J., Mazitschek, R., Kuo, W.P., and Whitman, M. (2011). Microarray profiling reveals the integrated stress response is activated by halofuginone in mammary epithelial cells. *BMC Res. Notes* **4**, 381.

Karadeniz Cerit, K., Karakoyun, B., Yuksel, M., Ozkan, N., Cetinel, S., Tolga Dagli, E., Yegen, B.C., and Tugtepe, H. (2012). The antifibrotic drug halofuginone reduces ischemia/reperfusion-induced oxidative renal damage in rats. *J. Pediatr. Urol.* ePub.

Kawaguchi, K., Fujii, S., Konishi, I., Nanbu, Y., Nonogaki, H., and Mori, T. (1989). Mitotic activity in uterine leiomyomas during the menstrual cycle. *Am. J. Obstet. Gynecol.* **160**, 637-641.

Keller, T.L., Zocco, D., Sundrud, M.S., Hendrick, M., Edenius, M., Yum, J., Kim, Y.J., Lee, H.K., Cortese, J.F., Wirth, D.F., Dignam, J.D., Rao, A., Yeo, C.Y., Mazitschek, R., Whitman, M. (2012). Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase. *Nat. Chem. Biol.* **8**, 311-317.

Kim, J.J., and Sefton, E.C. (2011). The role of progesterone signaling in the pathogenesis of uterine leiomyoma. *Mol. Cell. Endocrinol.* **358**, 223-231.

Klein, C.E., Dressel, D., Steinmayer, T., Mauch, C., Eckes, B., Krieg, T., Bankert, R.B., and Weber, L. (1991). Integrin alpha 2 beta 1 is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils. *J. Cell Biol.* **115**, 1427-1436.

Knight, C.G., Morton, L.F., Peachey, A.R., Tuckwell, D.S., Farndale, R.W., and Barnes, M.J. (2000). The collagen-binding A-domains of Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ recognize the same specific amino acid sequence, GFOGER, in Native (Triple-helical) Collagens. *JBC.* **275**, 35-40.

Koon, H.B., Fingleton, B., Lee, J.Y., Geyer, J.T., Cesarman, E., Parise, R.A., Egorin, M.J., Dezube, B.J., Aboulafia, D., and Krown, S.E. (2011). Phase II AIDS malignancy consortium trial of topical halofuginone in AIDS-related Kaposi sarcoma. *J. Acquir. Immune Defic. Syndr.* **56**, 64-68.

Kovacs, K.A., Oszter, A., Gocze, P.M., Kornyei, J.L., and Szabo, I. (2001). Comparative analysis of cyclin D1 and oestrogen receptor (alpha and beta) levels in human leiomyoma and adjacent myometrium. *Mol. Hum. Reprod.* 7, 1085-1091.

Koyama, H., Raines, E.W., Bornfeldt, K.E., Roberts, J.M., and Ross, R. (1996). Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell.* 87, 1069-1078.

Kroon, B., Johnson, N., Chapman, M., Yazdani, A., Hart, R., and on behalf of the Australasian CREI Consensus Expert Panel on Trial evidence (ACCEPT) group. (2011). Fibroids in infertility - consensus statement from ACCEPT (Australasian CREI Consensus Expert Panel on Trial evidence). *Aust. N. Z. J. Obstet. Gynaecol.* 51, 289-295.

Kumar, C.C. (1998). Signaling by integrin receptors. *Oncogene* 17, 1365-1373.

Lasky, J. (2004). Pirfenidone. *IDrugs* 7, 166-172.

Lee, B.S., Margolin, S.B., and Nowak, R.A. (1998). Pirfenidone: a novel pharmacological agent that inhibits leiomyoma cell proliferation and collagen production. *J. Clin. Endocrinol. Metab.* 83, 219-223.

Lee, B.S., and Nowak, R.A. (2001). Human leiomyoma smooth muscle cells show increased expression of transforming growth factor-beta 3 (TGF beta 3) and altered responses to the antiproliferative effects of TGF beta. *J. Clin. Endocrinol. Metab.* 86, 913-920.

Lee, D.W., Gibson, T.B., Carls, G.S., Ozminkowski, R.J., Wang, S., and Stewart, E.A. (2009). Uterine fibroid treatment patterns in a population of insured women. *Fertil. Steril.* 91, 566-574.

Leiba, M., Jakubikova, J., Klippel, S., Mitsiades, C.S., Hideshima, T., Tai, Y.T., Leiba, A., Pines, M., Richardson, P.G., Nagler, A., and Anderson, K.C. (2012). Halofuginone inhibits multiple myeloma growth *in vitro* and *in vivo* and enhances cytotoxicity of conventional and novel agents. *Br. J. Haematol.* 157, 718-731.

Leppert, P.C., Baginski, T., Prupas, C., Catherino, W.H., Pletcher, S., and Segars, J.H. (2004). Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertil. Steril.* 82 *Suppl.* 3, 1182-1187.

Leppert, P.C., Catherino, W.H., and Segars, J.H. (2006). A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. *Obstet. Gynecol.* 195, 415-420.

Liang, M., Wang, H., Zhang, Y., Lu, S., and Wang, Z. (2006). Expression and functional analysis of platelet-derived growth factor in uterine leiomyomata. *Cancer. Biol. Ther.* 5, 28-33.

Lindner, V., and Reidy, M.A. (1991). Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 88, 3739-3743.

Lumsden, M.A., West, C.P., Bramley, T., Rungay, L., and Baird, D.T. (1988). The binding of epidermal growth factor to the human uterus and leiomyomata in women rendered hypo-oestrogenic by continuous administration of an LHRH agonist. *Br. J. Obstet. Gynaecol.* 95, 1299-1304.

Lyons, R.M., and Moses, H.L. (1990). Transforming growth factors and the regulation of cell proliferation. *Eur. J. Biochem.* 187, 467-473.

Machado, S.A., Bahr, J.M., Hales, D.B., Braundmeier, A.G., Quade, B.J., and Nowak, R.A. (2012). Validation of the Aging Hen (*Gallus gallus domesticus*) As an Animal Model for Uterine Leiomyomas. *Biol. Reprod.* 87, 86.

Makinen, N., Mehine, M., Tolvanen, J., Kaasinen, E., Li, Y., Lehtonen, H.J., Gentile, M., Yan, J., Enge, M., Taipale, M., Aavikko, M., Katainen, R., Virolainen, E., Böhling, T., Koski, T.A., Launonen, V., Sjöberg, J., Taipale, J., Vahteristo, P., Aaltonen, L.A. (2011). MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. *Science.* 334, 252-255.

Mangrulkar, R.S., Ono, M., Ishikawa, M., Takashima, S., Klagsbrun, M., and Nowak, R.A. (1995). Isolation and characterization of heparin-binding growth factors in human leiomyomas and normal myometrium. *Biol. Reprod.* 53, 636-646.

Marsh, E.E., Shaw, N.D., Klingman, K.M., Tiamfook-Morgan, T.O., Yialamas, M.A., Sluss, P.M., and Hall, J.E. (2011). Estrogen levels are higher across the menstrual cycle in African-American women compared with caucasian women. *J. Clin. Endocrinol. Metab.* 96, 3199-3206.

Martin, K.H., Slack, J.K., Boerner, S.A., Martin, C.C., and Parsons, J.T. (2002). Integrin connections map: to infinity and beyond. *Science.* 296, 1652-1653.

Maruo, T., Matsuo, H., Samoto, T., Shimomura, Y., Kurachi, O., Gao, Z., Wang, Y., Spitz, I.M., and Johansson, E. (2000). Effects of progesterone on uterine leiomyoma growth and apoptosis. *Steroids.* 65, 585-592.

Maruo, T., Ohara, N., Wang, J., and Matsuo, H. (2004). Sex steroidal regulation of uterine leiomyoma growth and apoptosis. *Hum. Reprod. Update* 10, 207-220.

- Mavrelou, D., Ben-Nagi, J., Holland, T., Hoo, W., Naftalin, J., and Jurkovic, D. (2010). The natural history of fibroids. *Ultrasound Obstet. Gynecol.* 35, 238-242.
- McGaha, T., Koder, T., Phelps, R., Spiera, H., Pines, M., and Bona, C. (2002a). Effect of halofuginone on the development of tight skin (TSK) syndrome. *Autoimmunity* 35, 277-282.
- McGaha, T.L., Phelps, R.G., Spiera, H., and Bona, C. (2002b). Halofuginone, an inhibitor of type-I collagen synthesis and skin Sclerosis, blocks transforming-growth-factor-[bgr]-mediated Smad3 activation in fibroblasts. *118*, 461-470.
- McGuire, M.M., Yatsenko, A., Hoffner, L., Jones, M., Surti, U., and Rajkovic, A. (2012). Whole exome sequencing in a random sample of North American women with leiomyomas identifies MED12 mutations in majority of uterine leiomyomas. *PLoS One* 7, e33251.
- Meloni, A.M., Surti, U., Contento, A.M., Davare, J., and Sandberg, A.A. (1992). Uterine leiomyomas: cytogenetic and histologic profile. *Obstet. Gynecol.* 80, 209-217.
- Mesquita, F.S., Dyer, S.N., Heinrich, D.A., Bulun, S.E., Marsh, E.E., and Nowak, R.A. (2009). Reactive oxygen species mediate mitogenic growth factor signaling pathways in human leiomyoma smooth muscle cells. *Bio. Repro.* 82, 341-351.
- Minakuchi, K., Kawamura, N., Tsujimura, A., and Ogita, S. (1999). Remarkable and persistent shrinkage of uterine leiomyoma associated with interferon alfa treatment for hepatitis. *Lancet* 353, 2127-2128.
- Miyake, A., Takeda, T., Isobe, A., Wakabayashi, A., Nishimoto, F., Morishige, K., Sakata, M., and Kimura, T. (2009). Repressive effect of the phytoestrogen genistein on estradiol-induced uterine leiomyoma cell proliferation. *Gynecol. Endocrinol.* 25, 403-409.
- Miyazono, K. (2000). Positive and negative regulation of TGF-beta signaling. *J. Cell. Sci.* 113, 1101-1109.
- Mizutani, T., Sugihara, A., Nakamuro, K., and Terada, N. (1998). Suppression of cell proliferation and induction of apoptosis in uterine leiomyoma by gonadotropin-releasing hormone agonist (leuprolide acetate). *J. Clin. Endocrinol. Metab.* 83, 1253-1255.
- Nagler, A., Gofrit, O., Ohana, M., Pode, D., Genina, O., and Pines, M. (2000). The effect of halofuginone, an inhibitor of collagen type i synthesis, on urethral stricture formation: and *in vitro* study in a rat model. *J. Urol.* 164, 1776-1780.
- Nagler, A., and Pines, M. (1999). Topical treatment of cutaneous chronic graft versus host disease with halofuginone: a novel inhibitor of collagen type I synthesis. *Transplantation* 68, 1806-1809.

- Navarro, A., Yin, P., Monsivais, D., Lin, S.M., Du, P., Wei, J.J., and Bulun, S.E. (2012). Genome-wide DNA methylation indicates silencing of tumor suppressor genes in uterine leiomyoma. *PLoS One*. 7, e33284.
- Nelson, E.F., Huang, C.W., Ewel, J.M., Chang, A.A., and Yuan, C. (2012). Halofuginone down-regulates Smad3 expression and inhibits the TGFbeta-induced expression of fibrotic markers in human corneal fibroblasts. *Mol. Vis.* 18, 479-487.
- Nevo, Y., Halevy, O., Genin, O., Moshe, I., Turgeman, T., Harel, M., Biton, E., Reif, S., and Pines, M. (2010). Fibrosis inhibition and muscle histopathology improvement in laminin-alpha2-deficient mice. *Muscle Nerve*. 42, 218-229.
- Nguyen, T.T., Ward, J.P.T., and Hirst, S.J. (2005). β 1-Integrins mediate enhancement of airway smooth muscle proliferation by collagen and fibronectin. *Am. J. Respir. Crit. Care Med.* 171, 217-223.
- Nierth-Simpson, E.N., Martin, M.M., Chiang, T.C., Melnik, L.I., Rhodes, L.V., Muir, S.E., Burow, M.E., and McLachlan, J.A. (2009). Human uterine smooth muscle and leiomyoma cells differ in their rapid β -estradiol signaling: implications for proliferation. *Endocrinology* 150, 2436-2445.
- Norian, J.M., Malik, M., Parker, C.Y., Joseph, D., Leppert, P.C., Segars, J.H., and Catherino, W.H. (2009). Transforming growth factor B3 regulates the versican variants in the extracellular matrix-rich uterine leiomyomas. *Reprod. Sci.* 16, 1153-1164.
- Norian, J.M., Owen, C.M., Taboas, J., Korecki, C., Tuan, R., Malik, M., Catherino, W.H., and Segars, J.H. (2011). Characterization of tissue biomechanics and mechanical signaling in uterine leiomyoma. *Matrix Biol.* 31, 57-65.
- Nowak, R.A. (2004). Drug therapies for uterine fibroids: a new approach to an old problem. *Drug Discovery Today: Therapeutic Strategies* 1, 237-242.
- Ong, V.H., and Denton, C.P. (2010). Innovative therapies for systemic sclerosis. *Curr. Opin. Rheumatol.* 22, 264-272.
- Palman, C., Bowen-Pope, D.F., and Brooks, J.J. (1992). Platelet-derived growth factor receptor (beta-subunit) immunoreactivity in soft tissue tumors. *Lab. Invest.* 66, 108-115.
- Palomba, S., Orio, F., Jr, Morelli, M., Russo, T., Pellicano, M., Zupi, E., Lombardi, G., Nappi, C., Panici, P.L., and Zullo, F. (2002). Raloxifene administration in premenopausal women with uterine leiomyomas: a pilot study. *J. Clin. Endocrinol. Metab.* 87, 3603-3608.
- Pan, Q., Luo, X., and Chegini, N. (2009). microRNA 21 (miR-21): Response to hormonal therapies and regulatory function in leiomyoma, transformed leiomyoma and leiomyosarcoma cells. *Mol. Hum. Reprod.* 16, 215-227.

Papachristou, D.J., Sklirou, E., Corradi, D., Grassani, C., Kontogeorgakos, V., and Rao, U.N. (2012). Immunohistochemical analysis of the endoribonucleases Drosha, Dicer and Ago2 in smooth muscle tumours of soft tissues. *Histopathology*. *60*, E28-36.

Parsons, J.T., Martin, K.H., Slack, J.K., Taylor, J.M., and Weed, S.A. (2000). Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* *19*, 5606-5613.

Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., Hammer, D.A., and Weaver, V.M. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell*. *8*, 241-254.

Patino, M.G., Neiders, M.E., Andreana, S., Noble, B., and Cohen, R.E. (2002). Collagen: An overview. *Implant Dent*. *11*, 280-285.

Peng, Q., Lai, D., Nguyen, T.T., Chan, V., Matsuda, T., and Hirst, S.J. (2005). Multiple beta 1 integrins mediate enhancement of human airway smooth muscle cytokine secretion by fibronectin and type I collagen. *J. Immunol*. *174*, 2258-2264.

Peng, W., Robertson, L., Gallinetti, J., Mejia, P., Vose, S., Charlip, A., Chu, T., and Mitchell, J.R. (2012). Surgical stress resistance induced by single amino acid deprivation requires gcn2 in mice. *Sci. Transl. Med*. *4*, 118ra11.

Pines, M., and Halevy, O. (2011). Halofuginone and muscular dystrophy. *Histol. Histopathol*. *26*, 135-146.

Pines, M., and Nagler, A. (1998). Halofuginone: A novel antifibrotic therapy. *Gen. Pharmacol*. *30*, 445-450.

Pines, M., Snyder, D., Yarkoni, S., and Nagler, A. (2003). Halofuginone to treat fibrosis in chronic graft-versus-host disease and scleroderma. *Biol. Blood Marrow Transplant*. *9*, 417-425.

Pines, M., Vlodavsky, I., and Nagler, A. (2000). Halofuginone: From veterinary use to human therapy. *Drug Dev. Res*. *50*, 371-378.

Pinkerton, J.V. (2011). Pharmacological therapy for abnormal uterine bleeding. *Menopause* *18*, 453-461.

Plewka, A., Plewka, D., Madej, P., Nowaczyk, G., Sieron-Stoltny, K., and Jakubiec-Bartnik, B. (2011). Processes of apoptosis and cell proliferation in uterine myomas originating from reproductive and perimenopausal women. *Folia Histochem. Cytobiol*. *49*, 398-404.

- Plotti, G., Plotti, F., Di Giovanni, A., Battaglia, F., and Nagar, G. (2008). Feasibility and safety of vaginal myomectomy: a prospective pilot study. *J. Minim. Invasive. Gynecol.* *15*, 166-171.
- Popov, Y., Patsenker, E., Bauer, M., Niedobitek, E., Schulze-Krebs, A., and Schuppan, D. (2006). Halofuginone induces matrix metalloproteinases in rat hepatic stellate cells via activation of p38 and NFkappaB. *J. Biol. Chem.* *281*, 15090-15098.
- Porter, K.B., Tsibris, J.C., Porter, G.W., Fuchs-Young, R., Nicosia, S.V., O'Brien, W.F., and Spellacy, W.N. (1998). Effects of raloxifene in a guinea pig model for leiomyomas. *Am. J. Obstet. Gynecol.* *179*, 1283-1287.
- Porter, K., Tsibris, J., Nicosia, S., Murphy, J., O'Brien, W., Rao, P., and Spellacy, W. (1995). Estrogen-induced guinea pig model for uterine leiomyomas: do the ovaries protect? *Biol. Reprod.* *52*, 824-832.
- Prockop, D.J., and Kivirikko, K.I. (1995). Collagens: molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* *64*, 403-434.
- Provenzano, P.P., and Keely, P.J. (2011). Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. *J. Cell. Sci.* *124*, 1195-1205.
- Rein, M.S., Friedman, A.J., Barbieri, R.L., Pavelka, K., Fletcher, J.A., and Morton, C.C. (1991). Cytogenetic abnormalities in uterine leiomyomata. *Obstet. Gynecol.* *77*, 923-926.
- Rein, M.S., and Nowak, R.A. (1992). Biology of uterine myomas and myometrium *in vitro*. *Semin. Reprod. Endocrinol.* *10*, 310-319.
- Ren, Y., Yin, H., Tian, R., Cui, L., Zhu, Y., Lin, W., Tang, X.D., Gui, Y., and Zheng, X.L. (2011). Different effects of epidermal growth factor on smooth muscle cells derived from human myometrium and from leiomyoma. *Fertil. Steril.* *96*, 1015-1020.
- Ricard-Blum, S. (2011). The collagen family. *Cold Spring Harb Perspect. Biol.* *3*, a004978.
- Richards, P.A., and Tiltman, A.J. (1996). Anatomical variation of the oestrogen receptor in the non-neoplastic myometrium of fibromyomatous uteri. *Virchows Arch.* *428*, 347-351.
- Rodriguez, Y., Baez, D., de Oca, F.M., Garcia, C., Dorta, I., Reyes, R., Valladares, F., Almeida, T.A., and Bello, A.R. (2011). Comparative analysis of the ERalpha/ERbeta ratio and neurotensin and its high-affinity receptor in myometrium, uterine leiomyoma, atypical leiomyoma, and leiomyosarcoma. *Int. J. Gynecol. Pathol.* *30*, 354-363.

Roeder, H.A., Cramer, S.F., and Leppert, P.C. (2012). A look at uterine wound healing through a histopathological study of uterine scars. *Reprod. Sci.* 19, 463-473.

Roffe, S., Hagai, Y., Pines, M., and Halevy, O. (2010). Halofuginone inhibits Smad3 phosphorylation via the PI3K/Akt and MAPK/ERK pathways in muscle cells: effect on myotube fusion. *Exp. Cell Res.* 316,1061-1069.

Romagnolo, B., Molina, T., Leroy, G., Blin, C., Porteux, A., Thomasset, M., Vandewalle, A., Kahn, A., and Perret, C. (1996). Estradiol-dependent uterine leiomyomas in transgenic mice. *J. Clin. Invest.* 98, 777-784.

Rossi, M.J., Chegini, N., and Masterson, B.J. (1992). Presence of epidermal growth factor, platelet-derived growth factor, and their receptors in human myometrial tissue and smooth muscle cells: their action in smooth muscle cells *in vitro*. *Endocrinology* 130, 1716-1727.

Rothwell, C.E., McGuire, E.J., Martin, R.A., and De la Iglesia, F.A. (1992). Chronic toxicity and carcinogenicity studies with the beta-adrenoceptor antagonist levobunolol. *Fundam. Appl. Toxicol.* 18, 353-359.

Sandoval, J., and Esteller, M. (2012). Cancer epigenomics: beyond genomics. *Curr. Opin. Genet. Dev.* 22, 50-55.

Sato, S., Kawamura, H., Takemoto, M., Maezawa, Y., Fujimoto, M., Shimoyama, T., Koshizaka, M., Tsurutani, Y., Watanabe, A., Ueda, S., Halevi, K., Saito, Y., Yokote, K. (2009). Halofuginone prevents extracellular matrix deposition in diabetic nephropathy. *Biochem. Biophys. Res. Commun.* 379, 411-416.

Schuliga, M., Ong, S.C., Soon, L., Zal, F., Harris, T., and Stewart, A.G. (2010). Airway smooth muscle remodels pericellular collagen fibrils: implications for proliferation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 298, 584-592.

Schwartz, M.A. (2010). Integrins and extracellular matrix in mechanotransduction. *Cold Spring Harb. Perspect. Biol.* 2, a005066.

Silman, A., Harrison, M., and Brennan, P. (1995). Is it possible to reduce observer variability in skin score assessment of scleroderma? The ad hoc International Group on the Assessment of Disease Outcome in Scleroderma. *J. Rheumatol.* 22, 1277-1280.

Socolov, D., Blidaru, I., Tamba, B., Miron, N., Boiculese, L., and Socolov, R. (2011). Levonorgestrel releasing-intrauterine system for the treatment of menorrhagia and/or frequent irregular uterine bleeding associated with uterine leiomyoma. *Eur. J. Contracept. Reprod. Health Care.* 16, 480-487.

- Sosna, O., Kolesar, L., Slavcev, A., Skibova, J., Fait, T., Mara, M., Striz, I., and Kuzel, D. (2010). TH1/TH2 cytokine gene polymorphisms in patients with uterine fibroid. *Folia Biol. (Praha)* 56, 206-210.
- Spira, G., Mawasi, N., Paizi, M., Anbinder, N., Genina, O., Alexiev, R., and Pines, M. (2002). Halofuginone, a collagen type I inhibitor improves liver regeneration in cirrhotic rats. *J. Hepatol.* 37, 331-339.
- Steckclair, K.P., Hamburger, D.R., Egorin, M.J., Parise, R.A., Covey, J.M., and Eiseman, J.L. (2001). Pharmacokinetics and tissue distribution of halofuginone (NSC 713205) in CD2F1 mice and Fischer 344 rats. *Cancer Chemother. Pharmacol.* 48, 375-382.
- Stein, K., and Ascher-Walsh, C. (2009). A comprehensive approach to the treatment of uterine leiomyomata. *Mt. Sinai J. Med.* 76, 546-556.
- Stewart, E.A. (2012). Uterine fibroids and evidence-based medicine-not an oxymoron. *N. Engl. J. Med.* 366, 471-473.
- Stewart, E.A. (2002). Nonsurgical treatment of uterine fibroids. *Infertility and Reproductive Medicine Clinics of North America* 13, 393-405.
- Stewart, E.A. (2001). Uterine fibroids. *Lancet* 357, 293-298.
- Stewart, E.A., Friedman, A.J., Peck, K., and Nowak, R.A. (1994). Relative overexpression of collagen type I and collagen type III messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. *J. Clin. Endocrinol. Metab.* 79, 900-906.
- Stewart, E.A., and Morton, C.C. (2006). The genetics of uterine leiomyomata: what clinicians need to know. *Obstet. Gynecol.* 107, 917-921.
- Stewart, E.A., and Nowak, R.A. (1998). New concepts in the treatment of uterine leiomyomas. *Obstet. Gynecol.* 92, 624-627.
- Stovall, D.W. (2011). Alternatives to hysterectomy: focus on global endometrial ablation, uterine fibroid embolization, and magnetic resonance-guided focused ultrasound. *Menopause* 18, 437-444.
- Strawn, E.Y., Jr, Novy, M.J., Burry, K.A., and Bethea, C.L. (1995). Insulin-like growth factor I promotes leiomyoma cell growth *in vitro*. *Am. J. Obstet. Gynecol.* 172, 1837-1843.
- Sumitani, H., Shozu, M., Segawa, T., Murakami, K., Yang, H.J., Shimada, K., and Inoue, M. (2000). In situ estrogen synthesized by aromatase P450 in uterine leiomyoma cells promotes cell growth probably via an autocrine/intracrine mechanism. *Endocrinology* 141, 3852-3861.

Sundrud, M.S., Koralov, S.B., Feuerer, M., Calado, D.P., Kozhaya, A.E., Rhule-Smith, A., Lefebvre, R.E., Unutmaz, D., Mazitschek, R., Waldner, H., Whitman, M., Keller, T., Rao, A. (2009). Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. *Science*. 324, 1334-1338.

Tacheau, C., Michel, L., Farge, D., Mauviel, A., and Verrecchia, F. (2007). Involvement of ERK signaling in halofuginone-driven inhibition of fibroblast ability to contract collagen lattices. *Eur. J. Pharmacol.* 573, 65-69.

Taras, D., Blanc, J.F., Rullier, A., Dugot-Senant, N., Laurendeau, I., Bieche, I., Pines, M., and Rosenbaum, J. (2006). Halofuginone suppresses the lung metastasis of chemically induced hepatocellular carcinoma in rats through MMP inhibition. *Neoplasia* 8, 312-318.

Townsend, D.E., Sparkes, R.S., Baluda, M.C., and McClelland, G. (1970). Unicellular histogenesis of uterine leiomyomas as determined by electrophoresis by glucose-6-phosphate dehydrogenase. *Am. J. Obstet. Gynecol.* 107, 1168-1173.

Tsibris, J. (2004). The guinea pig model for uterine leiomyomata: gene-hormone interaction? *Fertil. Steril.* 82, 988-989.

Tsibris, J.C., Segars, J., Coppola, D., Mane, S., Wilbanks, G.D., O'Brien, W.F., and Spellacy, W.N. (2002). Insights from gene arrays on the development and growth regulation of uterine leiomyomata. *Fertil. Steril.* 78, 114-121.

Turgeman, T., Hagai, Y., Huebner, K., Jassal, D.S., Anderson, J.E., Genin, O., Nagler, A., Halevy, O., and Pines, M. (2008). Prevention of muscle fibrosis and improvement in muscle performance in the mdx mouse by halofuginone. *Neuromuscul. Disord.* 18, 857-868.

Viville, B., Charnock-Jones, D.S., Sharkey, A.M., Wetzka, B., and Smith, S.K. (1997). Distribution of the A and B forms of the progesterone receptor messenger ribonucleic acid and protein in uterine leiomyomata and adjacent myometrium. *Hum. Reprod.* 12, 815-822.

Voet, D., and Voet, J.G. (2010). *Biochemistry* (New York: J. Wiley & Sons).

Vollenhoven, B.J., Herington, A.C., and Healy, D.L. (1995). Epidermal growth factor and transforming growth factor-beta in uterine fibroids and myometrium. *Gynecol. Obstet. Invest.* 40, 120-124.

Walker, C.L., Hunter, D., and Everitt, J.I. (2003). Uterine leiomyoma in the Eker rat: a unique model for important diseases of women. *Genes Chromosomes Cancer* 38, 349-356.

Wallach, E.E., and Vlahos, N.F. (2004). Uterine myomas: an overview of development, clinical features, and management. *Obstet. Gynecol.* 104, 393-406.

Weinberg, A., Z.G., Polishuk, O., Heit, M., and Plavnik, I. and Bartov, I. (1985). A connection between a dietary coccidiostat and skin tears of female broiler chickens. *Poult. Sci.* 64, 294-296.

Whiteman, M.K., Hillis, S.D., Jamieson, D.J., Morrow, B., Podgornik, M.N., Brett, K.M., and Marchbanks, P.A. (2008). Inpatient hysterectomy surveillance in the United States, 2000-2004. *Am. J. Obstet. Gynecol.* 198, 34.e1-34.e7.

Wolanska, M., Sobolewski, K., Drozdewicz, M., and Bankowski, E. (1998). Extracellular matrix components in uterine leiomyoma and their alteration during the tumour growth. *Mol. Cell. Biochem.* 189, 145-152.

Xavier, S., Piek, E., Fujii, M., Javelaud, D., Mauviel, A., Flanders, K.C., Samuni, A.M., Felici, A., Reiss, M., Yarkoni, S., Sowers, A., Mitchell, J.B., Roberts, A.B., Russo, A. (2004). Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor-beta signaling by halofuginone. *J. Biol. Chem.* 279, 15167-15176.

Xu, J., and Clark, R.A. (1997). A three-dimensional collagen lattice induces protein kinase C-zeta activity: role in alpha2 integrin and collagenase mRNA expression. *J. Cell Biol.* 136, 473-483.

Xu, J., and Clark, R.A. (1996). Extracellular matrix alters PDGF regulation of fibroblast integrins. *J. Cell Biol.* 132, 239-249.

Yee, K.O., Connolly, C.M., Pines, M., and Lawler, J. (2006). Halofuginone inhibits tumor growth in the polyoma middle T antigen mouse via a thrombospondin-1 independent mechanism. *Cancer. Biol. Ther.* 5, 218-224.

Yoon, Y.H., Rha, K.S., Kim, D.H., Kim, E.H., Kim, J.M., and Koo, B.S. (2011). Is there any synergic effect for coadministration of mitomycin C and halofuginone on the skin wound healing? *Am. J. Otolaryngol.* 32, 130-134.

Zeplin, P.H. (2012). Reduction of burn scar formation by halofuginone-eluting silicone gel sheets: a controlled study on nude mice. *Ann. Plast. Surg.* 68, 271-275.

Zion, O., Genin, O., Kawada, N., Yoshizato, K., Roffe, S., Nagler, A., Iovanna, J.L., Halevy, O., and Pines, M. (2009). Inhibition of transforming growth factor beta signaling by halofuginone as a modality for pancreas fibrosis prevention. *Pancreas* 38, 427-435.

Chapter Three

Effect of Extracellular Matrix Collagen on Cell Morphology, Proliferation, Focal Adhesions, and MAPK Signaling in Human Leiomyoma Smooth Muscle Cells

Abstract

Uterine leiomyomas (ULs) are benign tumors occurring in the majority of women during their reproductive years. Despite the prevalence of these tumors, little is known about their etiology. A hallmark of leiomyomas is the excessive deposition of extracellular matrix (ECM), primarily collagens. Collagens are known to modulate cell behavior and function singularly or through interactions with integrins and growth factor mediated mitogenic pathways. To better understand the pathogenesis of ULs and the role of ECM collagens in their growth, we investigated the interaction of leiomyoma smooth muscle cells (LSMCs) with two different forms of collagen, namely monomeric, non-polymerized collagen or fibrillar, polymerized collagen, in the absence or presence of platelet-derived growth factor (PDGF); an abundant growth factor in ULs. Primary cultures of human LSMCS from symptomatic patients were grown on these two different collagen matrices and their morphology, cytoskeletal organization, cell proliferation, and signaling pathways were evaluated. Our results showed that LSMCs had distinct morphologies on the different collagen matrices and their basal as well as PDGF-stimulated proliferation varied on these matrices. These differences in proliferation were accompanied by changes in the phosphorylation of focal adhesion kinase, cytoskeletal reorganization, and activation of mitogen activated protein kinase (MAPK) signaling pathway. In conclusion, our results demonstrate a direct effect of ECM collagen on the proliferation of LSMCs through interplay between the collagen matrix and the PDGF-stimulated

MAPK pathway. In addition, these findings pave the way for identifying alternative therapeutic approaches for ULs that target ECM proteins and their signaling pathways in ULs.

Introduction

Uterine leiomyomas (ULs) are one of the most common pelvic neoplasms in reproductive aged women, with a reported incidence of 25-70% depending on age (Bowden *et al.*, 2009; Day Baird *et al.*, 2003; Houston *et al.*, 2001). These benign tumors originate from uterine smooth muscle cells (SMCs) and can cause severe symptoms such as abnormal uterine bleeding, pelvic pain, and infertility (Bowden *et al.*, 2009). Despite the prevalence of these tumors, not many treatments are available for patients and this is due to the limited understanding of their nature.

The most distinct feature of ULs is the excess synthesis and deposition of ECM proteins, mainly collagen type I and III (Iwahashi and Muragaki, 2011; Kawaguchi *et al.*, 1989; Leppert *et al.*, 2004; Malik *et al.*, 2010; Stewart *et al.*, 1994; Stewart, 2001; Wolanska *et al.*, 1998). Early studies by Stewart and colleagues (Stewart *et al.*, 1994) showed that collagen types I and III are both upregulated in ULs compared to normal myometrium. Recent global gene-profiling experiments have also shown that ECM genes encoding collagen proteins are differentially expressed in ULs compared to normal myometrial SMCs (Catherino *et al.*, 2004; Leppert *et al.*, 2006; Malik *et al.*, 2010; Tsibris *et al.*, 2002). In addition, the structure and composition of collagen fibrils in ULs were found to be altered such that collagens are loosely packed and arranged in a nonparallel manner (Leppert *et al.*, 2004). These changes are thought to be factors

contributing to the altered mechanical homeostasis in ULs leading to changes in cell signaling (Norian *et al.*, 2011; Rogers *et al.*, 2008).

ECM collagens are known to both maintain cellular morphology and act as conduits between extracellular stimuli and cells regulating proliferation, migration, differentiation, and survival (Pickering, 2001). The ultrastructure of fibril-forming collagens I and III has been shown to have distinct effects on cell morphology and proliferation mediated by focal adhesions and signaling pathways such as mitogen activated protein kinase (Henriet *et al.*, 2000; Koyama *et al.*, 1996; Sato *et al.*, 2003). Normal and malignant cells, such as fibroblasts (Grinnell, 2000; Sato *et al.*, 2003), endothelial cells (Kuzuya *et al.*, 1999), hepatic stellate cells (Kojima *et al.*, 1998), vascular SMCs (Koyama *et al.*, 1996), bladder SMCs (Herz *et al.*, 2003) and melanoma cells (Henriet *et al.*, 2000) all show an expanded morphology on monomeric collagen in contrast to a more dendritic morphology on fibrillar collagen. Moreover, vascular SMCs and hepatic stellate cells cultured on polymerized collagen show reduced cell proliferation in contrast to cells grown on monomeric, unpolymerized collagen (Fassett *et al.*, 2006; Koyama *et al.*, 1996). These effects are likely modulated through growth factors such as PDGF because ECM can act as a repository for growth factors changing their bioavailability and function (Hollenbeck *et al.*, 2004; Nakagawa *et al.*, 1989; Raines *et al.*, 2000; Wren *et al.*, 1986).

Recently, some mechanisms have been proposed to explain the growth modulatory effects of different forms of ECM collagen. One mode of action is through integrins, which are the main collagen receptors. Clustering and activation of integrin receptors induces cytoskeletal reorganization and formation of focal adhesions followed

by activation of specific focal adhesion kinases leading to the activation of signaling pathways such as mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. These pathways in turn can alter the expression of cell cycle regulatory proteins and promote proliferation (Klein *et al.*, 2009; Mettouchi *et al.*, 2001; Provenzano *et al.*, 2009; Provenzano and Keely, 2011; Wall *et al.*, 2005; Zhao *et al.*, 2001). The direct effect of collagen matrices on cell growth can also be explained through interactions with discoidin domain receptors (DDR) independent of cell spreading and cytoskeletal organization (Hou *et al.*, 2011; Lu *et al.*, 2010; Wall *et al.*, 2005).

The fact that ULs are fibrotic tumors containing an abundance of disorganized ECM collagen (Leppert *et al.*, 2004; Rogers *et al.*, 2008) led us to investigate the pathogenesis of these tumors in the context of how these ECM collagens may interact with PDGF; a growth factor that is abundantly expressed in ULs. Using an *in vitro* model system of ECM collagen, we examined the interaction of cultured LSMCs with monomeric, unpolymerized collagen films and fibrillar, polymerized collagen gels in modulating cellular morphology, cell proliferation, and the associated signaling pathways.

Results

Morphology of LSMCs on Different Collagen Matrices

As the first step in understanding the interaction of LSMCs with different collagen matrices, we cultured primary LSMCs on plastic, monomeric collagen, or fibrillar collagen-coated dishes and examined cell morphology 48 hours later. LSMCs on monomeric collagen-coated dishes displayed a spindle-like morphology typical of SMCs

that was similar to LSMCs on control, plastic dishes (Fig. 3.1A & B). In contrast, LSMCs on fibrillar collagen-coated dishes had a star-like morphology, with reduced cell size and numerous cellular projections (Fig. 3.1C). The star-like morphology was found to be reversible as once the fibrillar coating was removed, LSMCs returned to their spindle-like morphology and grew similarly to cells on plastic and monomeric collagen matrices (Fig. 3.1D). Growth of LSMCs on fibrillar collagen was also slower than cells on plastic or monomeric collagen-coated dishes, as observed by the confluence of dishes 48 hours after initial seeding.

To test whether the physical properties of fibrillar collagen such as density and thickness would affect the behavior of LSMCs, cells were cultured on fibrillar collagen-coated dishes with densities of 0.05%, 0.1%, and 0.2% and thicknesses of 0.4×10^{-3} or 1.2×10^{-3} mm². Results indicated that LSMCs on a fibrillar collagen matrix with a reduced thickness appeared more spindle-like (Fig. 3.2A-C) compared to cells on a thicker collagen gel (Fig 3.2D-E). In addition, within each gel thickness, growth of LSMCs was reduced with decreasing rigidity of the fibrillar collagen matrix (Fig 3.2A-D). Taken together, these results show that the form and physical properties of collagen matrices affect the morphology and growth of LSMCs.

Proliferation of LSMCs on Collagen Matrices

To quantify the growth of LSMCs on different collagen matrices, cell proliferation overtime was measured using both cell counts and triated thymidine incorporation assays. Growth curves for LSMCs cultured on collagen matrices in the presence of 10% serum showed that both monomeric and fibrillar collagen are permissive to cell growth. However, the rate of LSMC proliferation on monomeric collagen was significantly

greater than for cells on fibrillar collagen (Fig. 3.3). LSMCs on monomeric collagen grew at a rate of 76.6×10^3 cells per day whereas cells on fibrillar collagen had a growth rate of 17×10^3 cells per day. To confirm that these differences in growth rates were not due to different adhesive properties of the cells on monomeric and fibrillar collagen, LSMCs were seeded on these matrices and allowed to attach for three hours before washing the unattached cells and counting the remaining attached cells. Results indicated that although there was a slight difference between the two collagen matrices in adhesion properties, the difference was not significant enough to cause a difference in rate of cell proliferation (Fig. 3.4). Therefore, the two forms of collagen can differentially modulate the growth of LSMCs independent of their adhesion properties.

LSMCs within leiomyoma tissues are exposed to many growth factors including PDGF which can exist bound to the ECM. Thus, we next examined whether the interaction of LSMCs with ECM collagen alters the way these cells respond to the stimulatory effects of PDGF. Basal and PDGF-stimulated proliferation of LSMCs cultured on either monomeric or fibrillar collagen matrices for 24 hours was measured. Results showed that, similar to our growth curve analysis, both matrices were permissive to proliferation of LSMCs in the presence or absence of PDGF with monomeric collagen, having a significant potentiating effect over fibrillar collagen (Fig. 3.5). Interestingly, the increase in LSMC proliferation on monomeric collagen in the presence of PDGF was greater than the effects of monomeric collagen matrix or PDGF alone, suggesting a synergistic effect between the monomeric, unpolymerized collagen and PDGF. LSMCs on fibrillar collagen were also responsive to the stimulatory effects

of PDGF on fibrillar collagen (40% increase), although to a lesser extent than cells on monomeric collagen (60% increase).

Localization of Cytoskeletal F-actin and Focal Adhesion Components in LSMCs Cultured on Collagen Matrices

ECM exerts its effects on cells through the activation of several mediatory proteins including focal adhesion kinase (FAK) and vinculin leading to the assembly of cytoskeletal components. To investigate whether the differential proliferative responses of LSMCs cultured on monomeric and fibrillar collagen matrices occur through such mediatory proteins, we immunostained LSMCs cultured on collagen matrices for phospho-FAK (pY397), total FAK, vinculin, and F-actin stress. Our results showed that LSMCs on monomeric collagen had abundant activation (phosphorylation) of FAK, specifically at the sites of matrix adhesions (Fig. 3.6). In contrast, LSMCs cultured on fibrillar collagen on the other hand, showed low, diffuse expression of FAK pY397 throughout the cell. Localization of vinculin in LSMCs on monomeric collagen showed a punctate pattern which differed from the uniform localization observed in cells on fibrillar collagen (Fig. 3.6). Formation of F-actin stress fibers in LSMCs on monomeric collagen was significantly pronounced with distinct lines of central and peripheral fibers compared to the presence of thin, lightly stained peripheral fibers in cells on fibrillar collagen (Fig. 3.6).

Activation of MAPK Signaling Pathway

To understand the signaling mechanisms involved in the interaction of collagen matrices with LSMCs, we focused on the MAPK signaling pathway, which has been shown to be associated with the effects of ECM collagen in a variety of cell types. LSMCs cultured

on plastic were stimulated with 10 ng/ml PDGF for 10 minutes to determine if the major signaling molecule in the MAPK pathway, ERK1/2, is activated. Results showed that ERK1/2 is robustly activated in LSMCs upon PDGF treatment (Fig. 3.7). Pre-treatment of LSMCs for two hours with inhibitors specific to PDGFR and ERK1/2, AG1296 and PD98059 respectively, completely inhibited the phosphorylation of ERK1/2 (Fig. 3.7), demonstrating the specificity of the MAPK signaling pathway in the stimulatory effects of PDGF in LSMCs.

To see whether the activation of MAPK is different on collagen matrices, LSMCs were cultured on plastic, monomeric collagen or fibrillar collagen-coated dishes and stimulated with 10 ng/ml PDGF for 0, 10, 30, and 60 minutes. Results showed that as early as 10 minutes after PDGF stimulation, ERK1/2 was strongly activated in LSMCs cultured on all matrices (Fig. 3.8 A). Activation of ERK1/2 on plastic and monomeric collagen matrices returned to basal levels in an hour, whereas it persisted in LSMCs cultured on fibrillar collagen matrix. We next evaluated at the activation of PDGFR on matrices. Basal phosphorylation level of PDGFR on plastic and monomeric collagen was found to be higher than fibrillar collagen (Fig. 3.8 B). Upon PDGF treatment, a robust activation of PDGFR in 10 minutes was observed on all matrices. This activation was stronger on monomeric and fibrillar collagen compared to plastic matrix. In contrast, the duration of this phosphorylation was shorter on fibrillar collagen than on the other two matrices. These findings may explain the involvement of different signaling mechanisms on matrices leading to the differential rate of cell proliferation upon stimulation with PDGF in LSMCs cultured on monomeric and fibrillar collagen matrices.

Discussion

In the present study, we investigated interactions of primary, human uterine LSMCs on different collagen matrices representing normal and fibrotic uterine SMCs. In our model, we used non-polymerized, monomeric collagen matrix to mimic the ECM environment in ULs where SMCs continuously synthesize nascent collagen leading to a higher proportion of non-cross linked collagen that is laid down in a disorganized manner (Leppert *et al.*, 2004; Rogers *et al.*, 2008). Uterine LSMCs cultured on monomeric collagen showed typical cell spreading with an increased rate of cell proliferation compared to cells cultured on fibrillar collagen matrix, which was used to replicate the highly ordered, polymerized ECM in normal myometrial tissue. Our findings are in agreement with previous observations in other cell types where distinct morphologies and biological functions were observed for cells grown on monomeric versus fibrillar forms (ultra-structures) of collagens (Elliott *et al.*, 2003; Henriot *et al.*, 2000; Sato *et al.*, 2003).

Cell shape and extracellular environment (in addition to genotype) have been recognized as major determinants of cell behavior and function (Chen *et al.*, 1997; Cretel *et al.*, 2008; Hintze *et al.*, 2012; Le Beyec *et al.*, 2007; Raines, 2000; Tee *et al.*, 2011). Cells can sense the degree of extension or compression in their surroundings to modulate specific cell processes that lead to cell survival, differentiation, or apoptosis (Chen *et al.*, 1997; Engler *et al.*, 2004; Engler *et al.*, 2006; Georges *et al.*, 2006; Goffin *et al.*, 2006; Lo *et al.*, 2000; Yeung *et al.*, 2005). Mammary epithelial cells lose their epithelial morphology once matrix rigidity decreases below a specific threshold (Wozniak *et al.*, 2004). Moreover, chromosomal abnormalities in these cells are not

sufficient to lead to tumor formation and metastasis unless the cells are surrounded by a rigid stromal matrix (Paszek *et al.*, 2005). In the case of ULs, the existence of altered mechanical homeostasis along with increased sensitivity to mechanical cues has been linked to the excess production of ECM and increased proliferation (Norian *et al.*, 2011; Rogers *et al.*, 2008). In our *in vitro* system, LSMCs showed the greatest cell spreading and proliferation when grown on the monomeric collagen matrix compared to a gelatinous, fibrillar collagen matrix. In addition, altering the rigidity and pliability of fibrillar collagen matrix altered the way LSMCs expanded on the matrix and grew. It is therefore, possible to think of uterine LSMCs having a higher rate of proliferation because they are not only being exposed to high amounts of monomeric collagen continuously laid down within the ECM, but also disorganized fibrillar collagens which accumulate over time and perhaps become more rigid. Both of these factors are known to change the degree of mechanical stress exerted on cells (Provenzano and Keely, 2011). Thus, this alteration can in turn feed back to the intracellular machinery to increase the rate of cell proliferation.

Our knowledge of molecular mechanisms involved in the transduction of signals from the ECM leading to changes in biochemical and intracellular processes has significantly increased in recent decades. In both normal and diseased tissues, form and ultrastructure of the ECM can impose varying levels of mechanical stress upon cells, which is transmitted through the intracellular signaling machinery to alter cellular functions. The main molecular players in this interaction are integrin receptors, focal adhesions, cytoskeletal proteins, and signaling pathways such as MAPK, PI3K/AKT, and Rho-GTPase (Bhadriraju *et al.*, 2009; Mierke *et al.*, 2010; Tsai *et al.*, 2010) . In this

study, we noted an increased phosphorylation of FAK at Y397 as well as its specific localization at adhesion sites in LSMCs cultured on monomeric collagen compared to fibrillar collagen. The fact that phosphorylation of FAK at Y397 is known to be regulated by the physical properties of the matrix (Cukierman *et al.*, 2002) is suggestive of the active formation of focal adhesion sites in LSMCs cultured on monomeric collagen matrix. Based on these facts, we could speculate that such activation along with the pronounced expression and clustered placement of vinculin, and F-actin stress fibers observed on the monomeric collagen could then lead to that the hyper-activation of the MAPK signaling pathway on the monomeric collagen matrix which, in turn could cause an increase in the rate of cell proliferation on this matrix. Although we did examine alterations in integrins and major components of the MAPK, PI3K/AKT or Rho-GTPase in LSMCs using immunofluorescent staining 24 hours after seeding in our cell signaling studies with PDGF, higher (but not statistically significant) levels of ERK1/2 and PDGFR activation were observed on non-stimulated cells cultured on plastic and monomeric collagen matrices compared to fibrillar collagen.

The decreased level of activation or localization of vinculin, FAK, and F-actin stress fibers in LSMCs cultured on fibrillar collagen observed in this study could explain the state of quiescence or decreased cell proliferation in LSMCs grown on this matrix. In fact, potent suppression of focal adhesion formation as indicated by vinculin has been linked to changes in gene expression and cell function in VSMCs cultured on polymerized, fibrillar collagen (Ichii *et al.*, 2001). Cell cycle arrest at the G0/G1-S and G2-M transition in cells cultured on fibrillar collagen has also been linked to alterations in cell proliferation and tumor progression (Henriet *et al.*, 2000; Koyama *et al.*, 1996;

Wall *et al.*, 2005). It was also interesting to see how changes in the physical properties of the polymerized collagen, such as density and pliability, could affect cell morphology and function. Although we did not quantify changes in LSMC proliferation under these conditions, gross analysis of cell shape and confluence were indications of the effects of the collagen ultra-structure on the biology of LSMCs.

In ULs, along with alteration in ECM collagens, an increased presence of and response to growth factors such as PDGF have also been documented (Hwu *et al.*, 2008; Liang *et al.*, 2006; Mesquita *et al.*, 2009). While our findings from our proliferation studies are in support of such studies, the observed cross-talk between collagens and PDGF in regulating cell proliferation in LSMCs was novel. Interactions between integrin receptors and growth factors have been the subject of numerous studies in recent decades. The pioneering study of Sundberg and Rubin (Sundberg and Rubin, 1996), clearly demonstrated the cross-talk between $\beta 1$ integrin-mediated adhesion reactions and tyrosine phosphorylation of PDGFR independent of PDGF stimulation. Similar cross-talk was observed by the synergism between integrin and growth factor receptors in several other cell lines (DeMali *et al.*, 1999; Hollenbeck *et al.*, 2004; Mahabeleshwar *et al.*, 2007; Plopper *et al.*, 1995; Ross, 2004; Schneller *et al.*, 1997; Soung *et al.*, 2010; Woodard *et al.*, 1998; Yamada and Even-Ram, 2002). The synergistic effect of monomeric collagen in augmenting the stimulatory effects of PDGF in LSMC proliferation, which was not present in cells cultured on fibrillar collagen, is suggestive of a similar interaction in LSMCs.

Moreover, stimulation of LSMCs with PDGF resulted in the activation of the MAPK signaling pathway on both collagen matrices at the level of both PDGFR and

ERK1/2. However, this activation was different in LSMCs cultured on monomeric and fibrillar collagen matrices. While the dynamics of PDGFR activation was similar on both monomeric and fibrillar collagen matrices upon PDGF treatment, the activation pattern differed for ERK1/2. Rapid activation of ERK1/2 on monomeric collagen nicely followed the activation of PDGF-R in response to PDGF treatment. In contrast, we observed persistent ERK1/2 activation in LSMCs cultured on fibrillar collagen in response to PDGF stimulation. Similar sustained activation of ERK1/2 has been observed in other cell types, indicating the involvement of a different cellular process leading to cell survival and quiescence (Andreadi *et al.*, 2012; Ebisuya *et al.*, 2005; Kolch, 2005; Roovers and Assoian, 2003; Yamamoto *et al.*, 2006). Mitogenic stimulation in fibroblasts is also shown to contribute to sustained ERK1/2 activation which in turn maintains decreased expression levels of anti-proliferative genes (Yamamoto *et al.*, 2006).

Although our study was focused on the molecular processes downstream of collagen receptors, the lack of any significant differences between the intensity of ERK1/2 activation in LSMCs stimulated with PDGF and cultured on monomeric collagen matrix and other matrices as well as differential phosphorylation of PDGFR on this matrix in the absence of PDGF implies the involvement of other signaling pathways. Indeed, activation of signaling pathways like PI3K/AKT or JAK/STAT in cells grown on different matrices has been reported. In hepatocytes, matrix stiffness enhanced ERK1/2, protein kinase B (PKB/AKT) and signal transducer and activator of transcription-3 (STAT3) phosphorylation and resulted in enhanced mitogenic signaling in response to hepatocyte growth factor (Schrader *et al.*, 2011). Similar activation of

MAPK and AKT pathway was observed in mesenchymal stem cells cultured on different matrices (Tsai *et al.*, 2010). It will be of interest to further validate our *in vitro* model of ULs by investigating the dynamics of expression and distribution of collagen and PDGF receptors in activation of other major signaling pathways involved in cell proliferation.

In conclusion, our findings on the characterization of LSMCs cultured on different forms of collagen as a simple model to replicate the ECM environment in normal myometrium and fibrotic leiomyoma tissue were two fold. One aspect of the current findings demonstrated the importance of proper culture systems for studying specific cell functions. This study along with many more in other cell lines, including LSMCs (Hinz, 2010; Malik and Catherino, 2012), show that growth of cells on conventional plastic or monomeric-coated dishes induces a different cell growth and morphology than cellular characteristics induced by fibrillar collagen matrix. The second, and more important, aspect of our findings is on the critical role of the ECM collagen in the pathogenesis of ULs as a fibrotic disease. The interactions between matrix stiffness, increased mechanical stress and the pro-fibrotic factor, TGF β 1, has been proven to be involved in the differentiation of fibroblasts to myofibroblasts leading to the development of fibrosis (Hinz, 2010). In this context, our findings are in support of the theory of injury and disrupted wound healing process as the initiating factors for the formation of UL tumors.

Figures

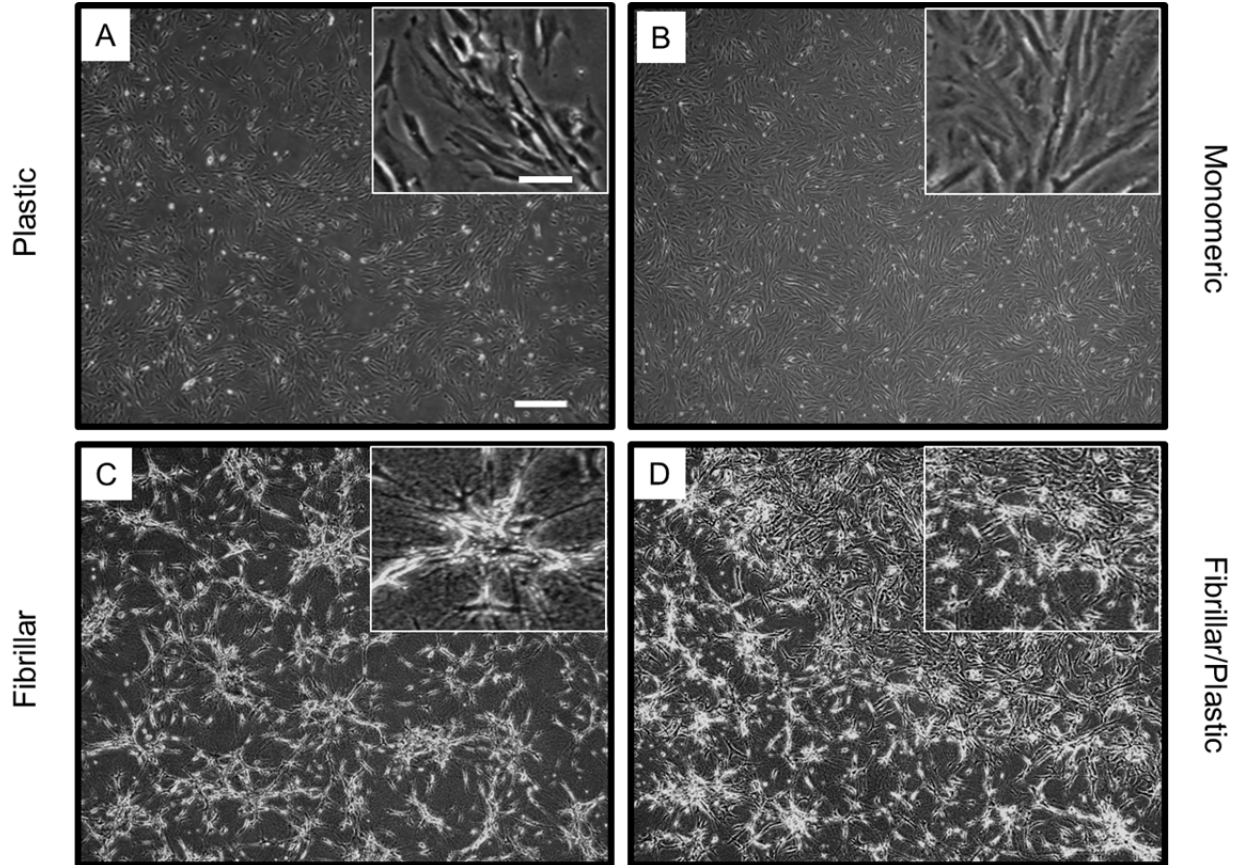


Figure 3.1. Morphology of LSMCs is altered on different collagen matrices.

LSMCs were cultured on plastic (A), monomeric collagen (B), or fibrillar collagen (C) coated dishes in serum containing medium for two days and then imaged. The fibrillar collagen coating on some dishes was partially removed to expose LSMCs to the plastic matrix (D). (Bars 200 μm in main figures, 50 μm in insets).

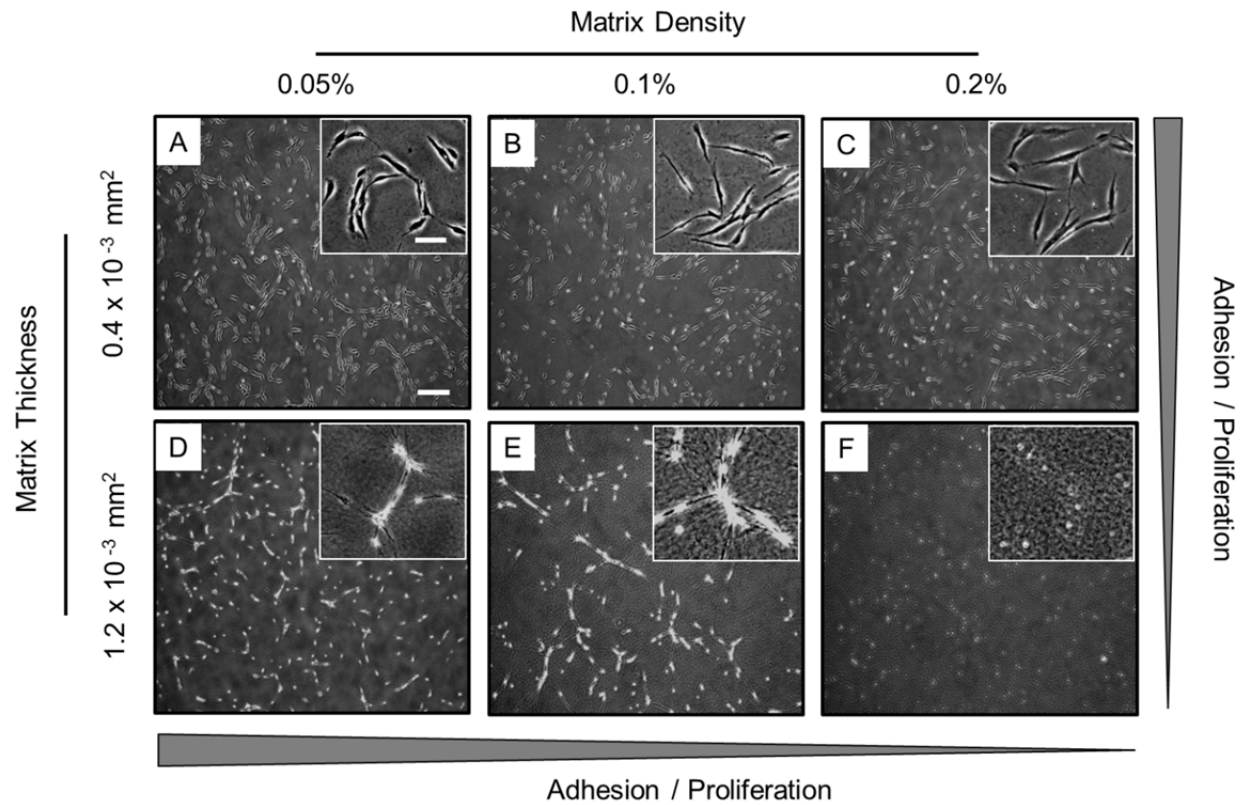


Figure 3.2. Physical properties of the ECM affect cell growth. Plastic dishes were coated with different volumes of fibrillar collagen at different concentrations. Cells were cultured in serum-containing medium for two days and then imaged (Bars 200 μm in main figures; 50 μm in inserts).

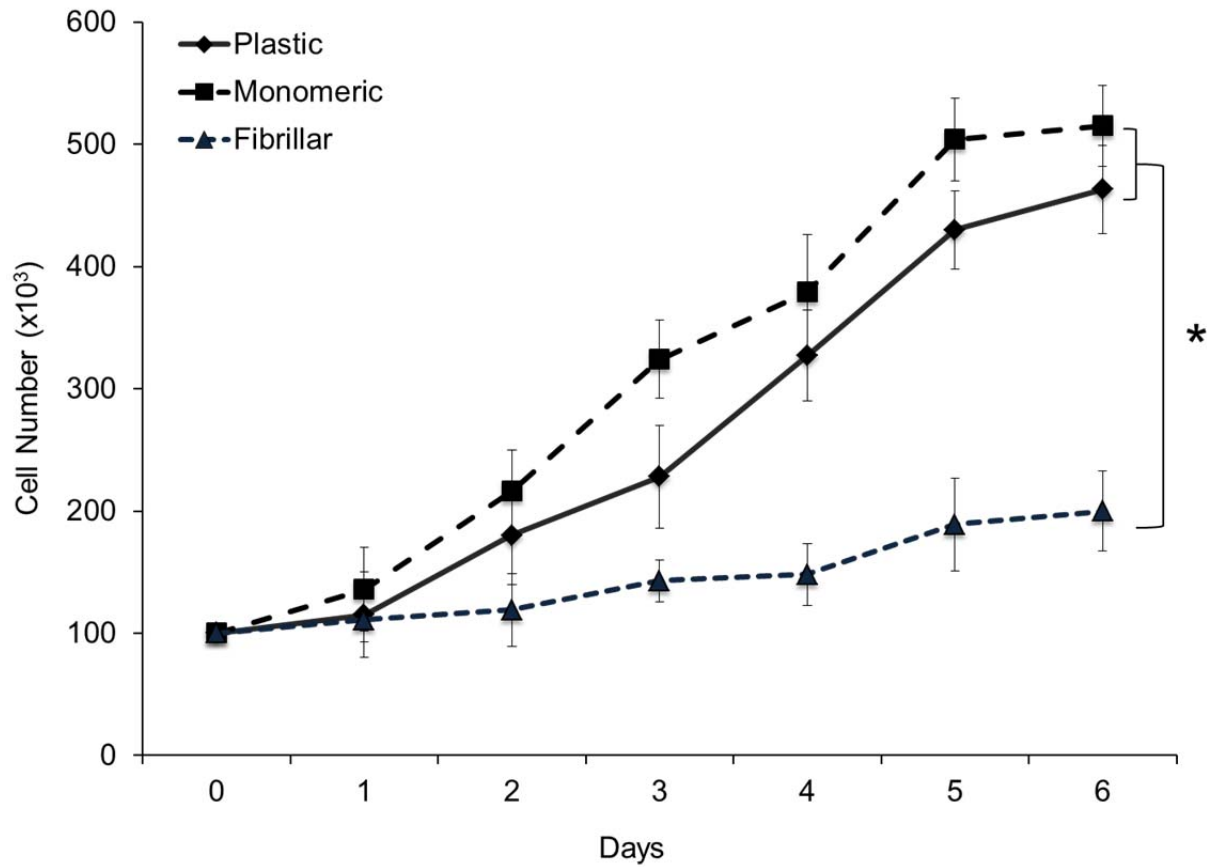


Figure 3.3. Proliferation of LSMCs is altered on different collagen matrices.

LSMCs were cultured on plastic, monomeric, or fibrillar collagen coated dishes in serum-containing medium. Cells were trypsinized and counted using a hemacytometer every 24 hours. Statistical significance between matrices is indicated by asterisk.(n=3, P<0.05)

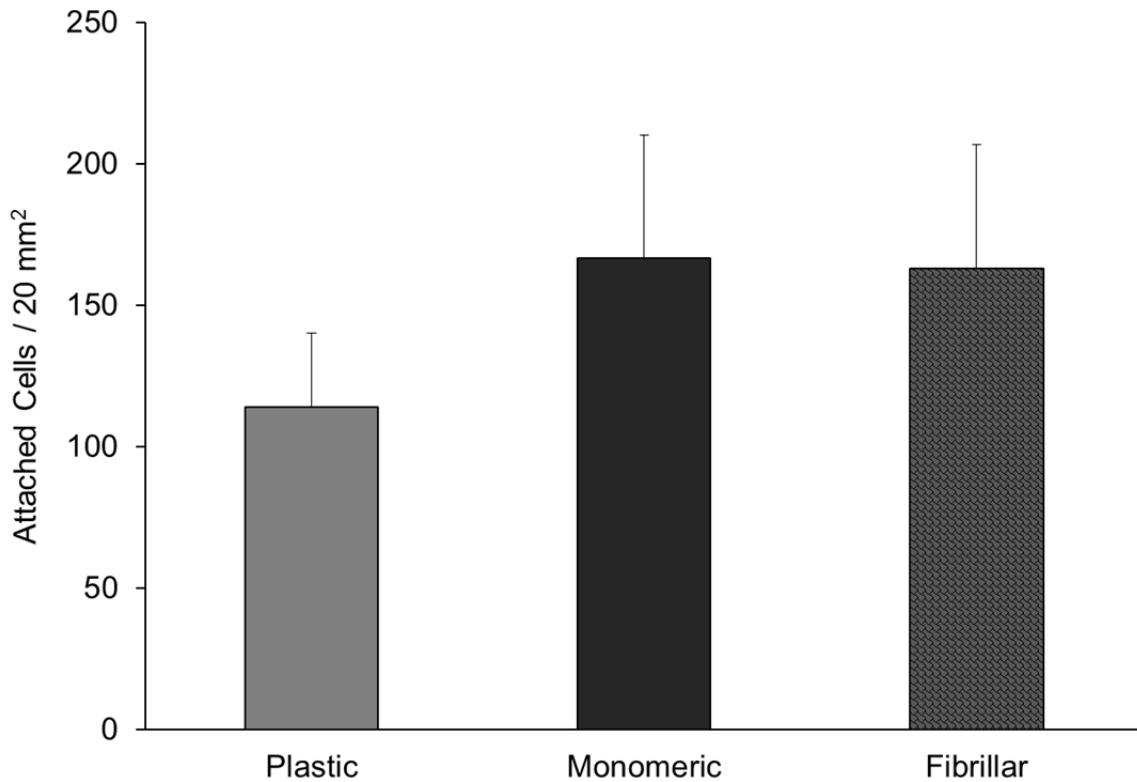


Figure 3.4. Adhesion of LSMCs to different matrices is similar. LSMCs were seeded at equal numbers on plastic, monomeric or fibrillar collagen coated dishes. Three hours after culture, medium was removed and the number of attached cells to each matrix surface was counted. (n=3).

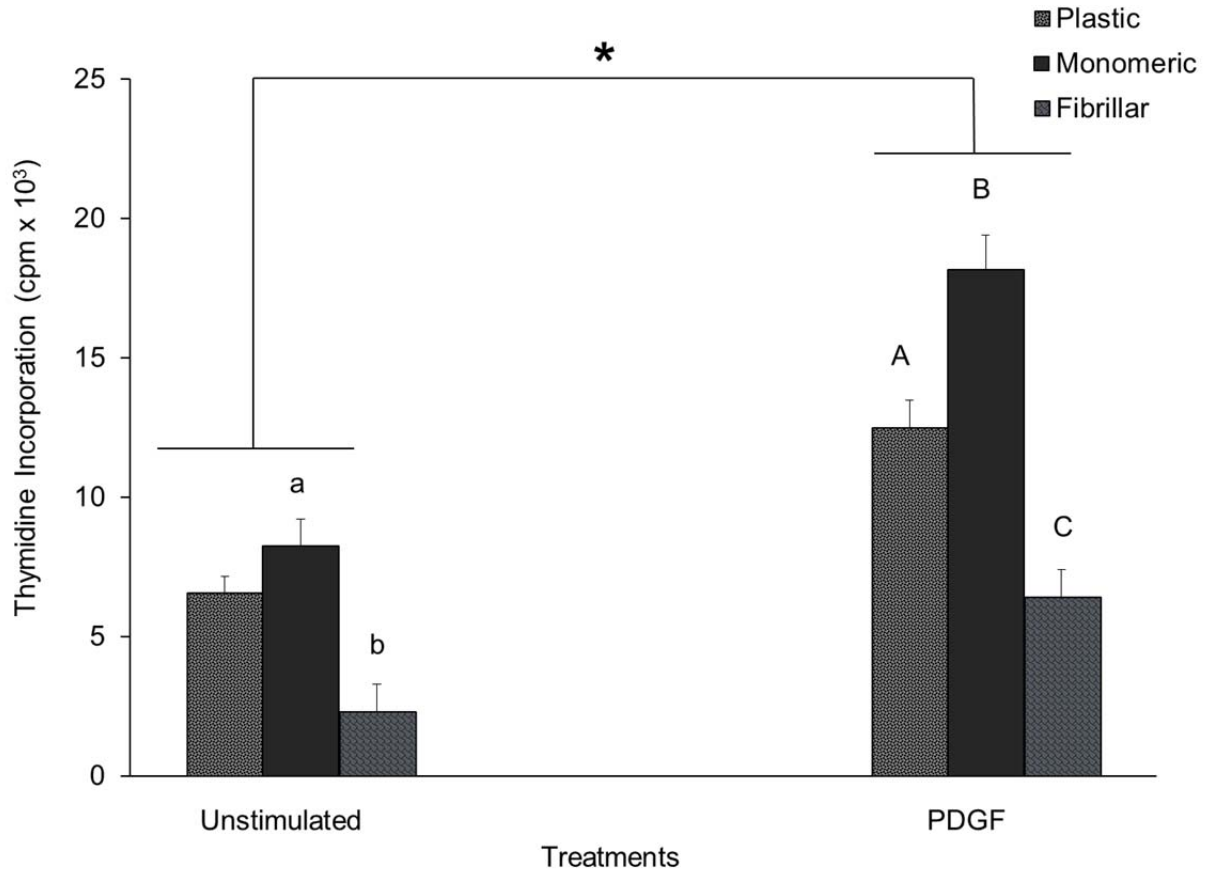


Figure 3.5. Basal and PDGF-stimulated proliferation of LSMCs on different collagen matrices varies. LSMCs were cultured in 96-well plates coated with different forms of collagen in medium with or without 10 ng/ml PDGF for 24 hours. At the end of the treatment, cell proliferation was measured using thymidine incorporation assays. Statistical significance between matrices is indicated by different letters. Significant differences from unstimulated cells within each matrix are indicated by asterisks. (n=3, P<0.05).

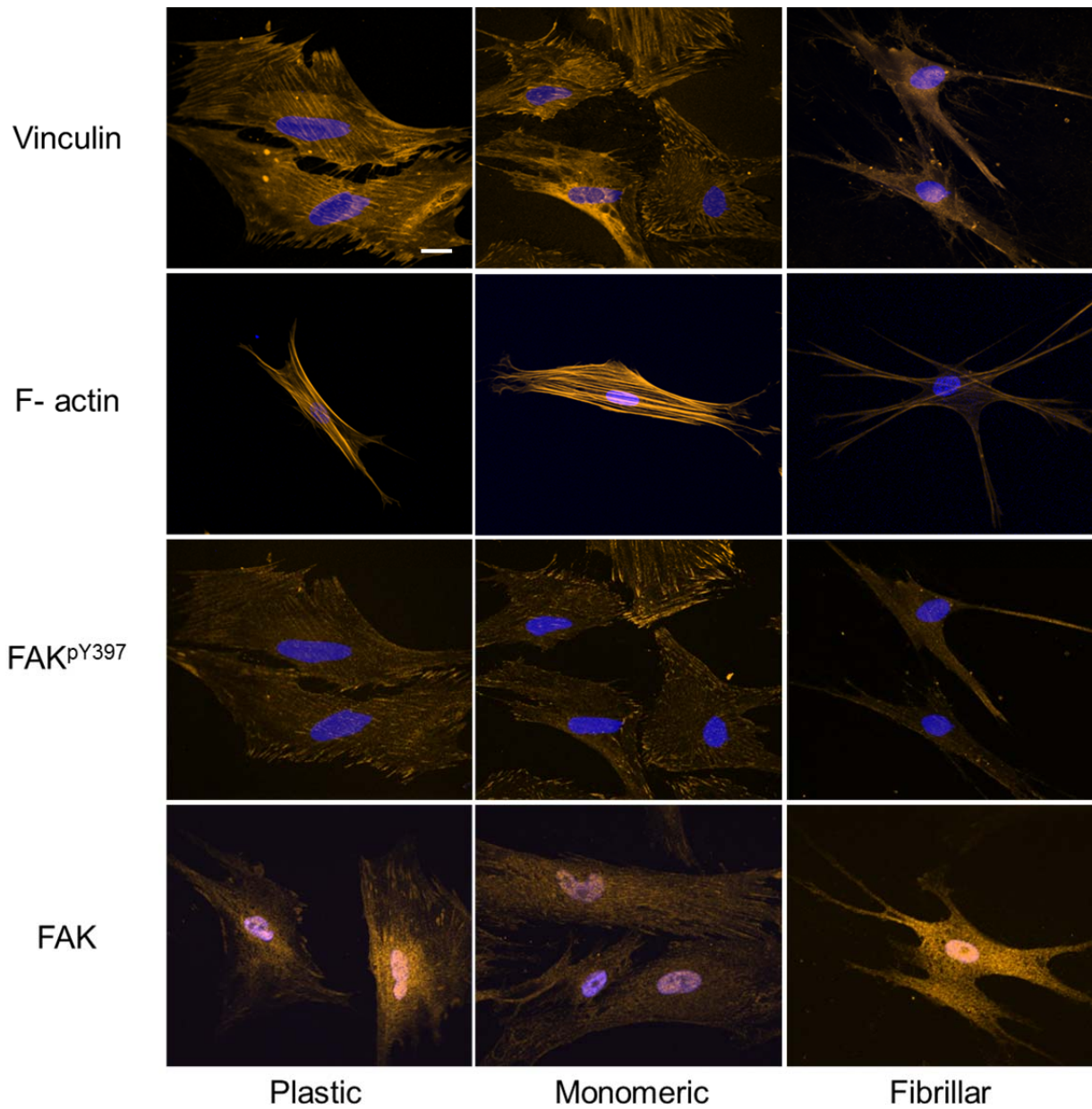


Figure 3.6. Activation of focal adhesion complexes and F-actin stress fibers in LSMCs is altered on different collagen matrices. LSMCs were cultured on different matrices for 24 hours before being fixed and stained with fluorescent-labeled antibodies against vinculin, F-actin, FAK (pY397), and FAK. (Bar 20 μ m)

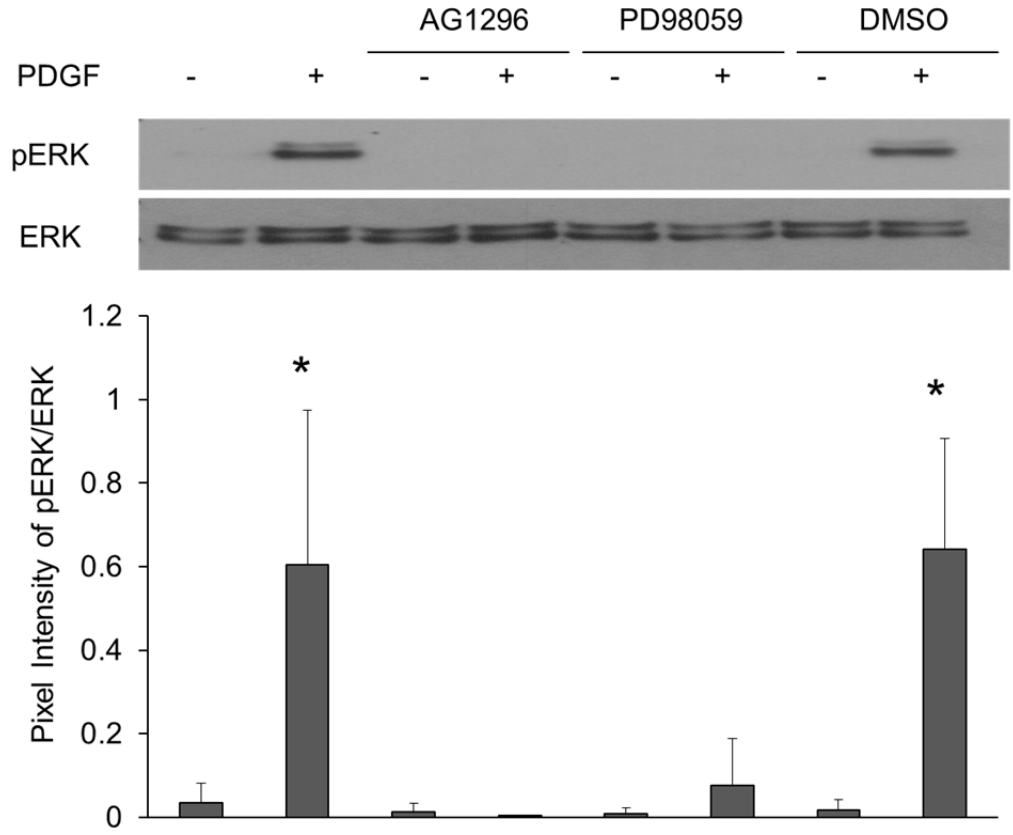


Figure 3.7. PDGF activates MAPK signaling pathway in LSMCs. LSMCs were cultured on plastic matrix in the presence or absence of inhibitors specific to PDGF-R and MEK or DMSO control for 2 hours before treatment with 10 ng/ml PDGF for 10 minutes. Cell lysates were probed for pERK1/2. Asterisks indicate significant difference compared to control, non-PDGF and non-inhibitor treated sample. (n=3, P<0.05)

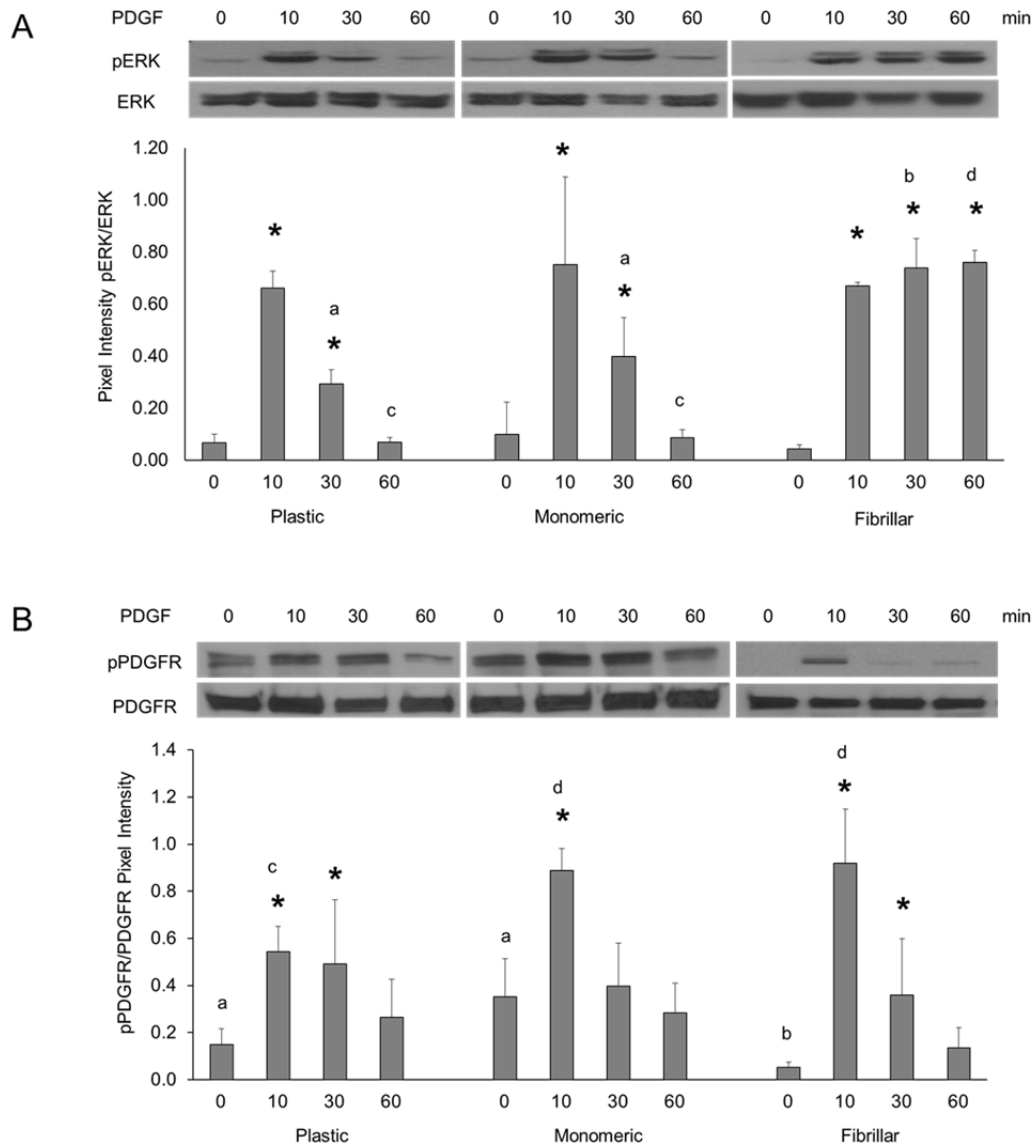


Figure 3.8. MAPK signaling pathway is differentially activated in LSMCs cultured on different matrices. LSMCs were cultured on plastic, monomeric or fibrillar collagen matrices and then stimulated with 10 ng/ml PDGF for different time points. Cell lysates were probed for pERK1/2 (A) and pPDGFR (B). Asterisks indicate significant difference compared to time zero within each matrix. Letters indicate significant difference across matrices at specific time points. (n=3, P<0.05)

Reference List

- Andreadi, C., Noble, C., Patel, B., Jin, H., Aguilar Hernandez, M.M., Balmanno, K., Cook, S.J., and Pritchard, C. (2012). Regulation of MEK/ERK pathway output by subcellular localization of B-Raf. *Biochem. Soc. Trans.* *40*, 67-72.
- Bhadriraju, K., Chung, K.H., Spurlin, T.A., Haynes, R.J., Elliott, J.T., and Plant, A.L. (2009). The relative roles of collagen adhesive receptor DDR2 activation and matrix stiffness on the downregulation of focal adhesion kinase in vascular smooth muscle cells. *Biomaterials* *30*(35):6687-6694.
- Bowden, W., Skorupski, J., Kovanci, E., and Rajkovic, A. (2009). Detection of novel copy number variants in uterine leiomyomas using high-resolution SNP arrays. *Mol. Hum. Reprod.* *15*, 563-568.
- Catherino, W.H., Leppert, P.C., Stenmark, M.H., Payson, M., Potlog-Nahari, C., Nieman, L.K., and Segars, J.H. (2004). Reduced dermatopontin expression is a molecular link between uterine leiomyomas and keloids. *Genes Chromosomes Cancer* *40*, 204-217.
- Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., and Ingber, D.E. (1997). Geometric control of cell life and death. *Science* *276*, 1425-1428.
- Cretel, E., Pierres, A., Benoliel, A.M., and Bongrand, P. (2008). How cells feel their environment: a focus on early dynamic events. *Cell. Mol. Bioeng.* *1*, 5-14.
- Cukierman, E., Pankov, R., and Yamada, K.M. (2002). Cell interactions with three-dimensional matrices. *Curr. Opin. Cell Biol.* *14*, 633-639.
- Day Baird, D., Dunson, D.B., Hill, M.C., Cousins, D., and Schectman, J.M. (2003). High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. *Am. J. Obstet. Gynecol.* *188*, 100-107.
- DeMali, K.A., Balciunaite, E., and Kazlauskas, A. (1999). Integrins enhance platelet-derived growth factor (PDGF)-dependent responses by altering the signal relay enzymes that are recruited to the PDGF beta receptor. *J. Biol. Chem.* *274*, 19551-19558.
- Ebisuya, M., Kondoh, K., and Nishida, E. (2005). The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. *J. Cell Sci.* *118*, 2997-3002.
- Elliott, J.T., Tona, A., Woodward, J.T., Jones, P.L., and Plant, A.L. (2003). Thin films of collagen affect smooth muscle cell morphology. *Langmuir.* *19*, 1506-1514.

Engler, A.J., Griffin, M.A., Sen, S., Bonnemann, C.G., Sweeney, H.L., and Discher, D.E. (2004). Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *J. Cell Biol.* *166*, 877-887.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell.* *126*, 677-689.

Fassett, J., Tobolt, D., and Hansen, L.K. (2006). Type I collagen structure regulates cell morphology and EGF signaling in primary rat hepatocytes through cAMP-dependent protein kinase A. *Mol. Biol. Cell.* *17*, 345-356.

Georges, P.C., Miller, W.J., Meaney, D.F., Sawyer, E.S., and Janmey, P.A. (2006). Matrices with compliance comparable to that of brain tissue select neuronal over glial growth in mixed cortical cultures. *Biophys. J.* *90*, 3012-3018.

Goffin, J.M., Pittet, P., Csucs, G., Lussi, J.W., Meister, J.J., and Hinz, B. (2006). Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J. Cell Biol.* *172*, 259-268.

Grinnell, F. (2000). Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading. *Trends Cell Biol.* *10*, 362-365.

Henriet, P., Zhong, Z.D., Brooks, P.C., Weinberg, K.I., and DeClerck, Y.A. (2000). Contact with fibrillar collagen inhibits melanoma cell proliferation by up-regulating p27KIP1. *Proc. Natl. Acad. Sci.* *97*, 10026-10031.

Herz, D.B., Aitken, K., and Bagli, D.J. (2003). Collagen directly stimulates bladder smooth muscle cell growth in vitro: regulation by extracellular regulated mitogen activated protein kinase. *J. Urol.* *170*, 2072-2076.

Hintze, V., Miron, A., Moller, S., Schnabelrauch, M., Heinemann, S., Worch, H., and Scharnweber, D. (2012). Artificial extracellular matrices of collagen and sulphated hyaluronan enhance the differentiation of human mesenchymal stem cells in the presence of dexamethasone. *J. Tissue Eng. Regen. Med.* ePub

Hinz, B. (2010). The myofibroblast: Paradigm for a mechanically active cell. *J. Biomech.* *43*, 146-155.

Hollenbeck, S.T., Itoh, H., Louie, O., Faries, P.L., Liu, B., and Kent, K.C. (2004). Type I collagen synergistically enhances PDGF-induced smooth muscle cell proliferation through pp60src-dependent crosstalk between the $\alpha 2\beta 1$ integrin and PDGF β receptor. *Biochem. Biophys. Res. Commun.* *325*, 328-337.

Hou, G., Wang, D., and Bendeck, M.P. (2011). Deletion of discoidin domain receptor 2 does not affect smooth muscle cell adhesion, migration, or proliferation in response to type I collagen. *Cardiovasc. Pathol.* *21*, 214-218.

- Houston, K.D., Hunter, D.S., Hodges, L.C., and Walker, C.L. (2001). Uterine leiomyomas: mechanisms of tumorigenesis. *Toxicol. Pathol.* **29**, 100-104.
- Hwu, Y.M., Li, S.H., Lee, R.K., Tsai, Y.H., Yeh, T.S., and Lin, S.Y. (2008). Increased expression of platelet-derived growth factor C messenger ribonucleic acid in uterine leiomyomata. *Fertil. Steril.* **89**, 468-471.
- Ichii, T., Koyama, H., Tanaka, S., Kim, S., Shioi, A., Okuno, Y., Raines, E.W., Iwao, H., Otani, S., and Nishizawa, Y. (2001). Fibrillar collagen specifically regulates human vascular smooth muscle cell genes involved in cellular responses and the pericellular matrix environment. *Circ. Res.* **88**, 460-467.
- Iwahashi, M., and Muragaki, Y. (2011). Increased type I and V collagen expression in uterine leiomyomas during the menstrual cycle. *Fertil. Steril.* **95**, 2137-2139.
- Kawaguchi, K., Fujii, S., Konishi, I., Nanbu, Y., Nonogaki, H., and Mori, T. (1989). Mitotic activity in uterine leiomyomas during the menstrual cycle. *Am. J. Obstet. Gynecol.* **160**, 637-641.
- Klein, E.A., Yin, L., Kothapalli, D., Castagnino, P., Byfield, F.J., Xu, T., Levental, I., Hawthorne, E., Janmey, P.A., and Assoian, R.K. (2009). Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. *Curr. Biol.* **19**, 1511-1518.
- Kojima, N., Sato, M., Imai, K., Miura, M., Matano, Y., and Senoo, H. (1998). Hepatic stellate cells (vitamin A-storing cells) change their cytoskeleton structure by extracellular matrix components through a signal transduction system. *Histochem. Cell Biol.* **110**, 121-128.
- Kolch, W. (2005). Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat. Rev. Mol. Cell Biol.* **6**, 827-837.
- Koyama, H., Raines, E.W., Bornfeldt, K.E., Roberts, J.M., and Ross, R. (1996). Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell* **87**, 1069-1078.
- Kuzuya, M., Satake, S., Ramos, M.A., Kanda, S., Koike, T., Yoshino, K., Ikeda, S., and Iguchi, A. (1999). Induction of apoptotic cell death in vascular endothelial cells cultured in three-dimensional collagen lattice. *Exp. Cell Res.* **248**, 498-508.
- Le Beyec, J., Xu, R., Lee, S.Y., Nelson, C.M., Rizki, A., Alcaraz, J., and Bissell, M.J. (2007). Cell shape regulates global histone acetylation in human mammary epithelial cells. *Exp. Cell Res.* **313**, 3066-3075.
- Leppert, P.C., Baginski, T., Prupas, C., Catherino, W.H., Pletcher, S., and Segars, J.H. (2004). Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertil. Steril.* **82 Suppl. 3**, 1182-1187.

- Leppert, P.C., Catherino, W.H., and Segars, J.H. (2006). A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. *Obstet. Gynecol.* *195*, 415-420.
- Liang, M., Wang, H., Zhang, Y., Lu, S., and Wang, Z. (2006). Expression and functional analysis of platelet-derived growth factor in uterine leiomyomata. *Cancer. Biol. Ther.* *5*, 28-33.
- Lo, C.M., Wang, H.B., Dembo, M., and Wang, Y.L. (2000). Cell movement is guided by the rigidity of the substrate. *Biophys. J.* *79*, 144-152.
- Lu, K.K., Trcka, D., and Bendeck, M.P. (2010). Collagen stimulates discoidin domain receptor 1-mediated migration of smooth muscle cells through Src. *Cardiovasc. Pathol.* *20*,71-76.
- Mahabeleshwar, G.H., Feng, W., Reddy, K., Plow, E.F., and Byzova, T.V. (2007). Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis. *Circ. Res.* *101*, 570-580.
- Malik, M., and Catherino, W.H. (2012). Development and validation of a three-dimensional in vitro model for uterine leiomyoma and patient-matched myometrium. *Fertil. Steril.* *97*,1287-1293.
- Malik, M., Norian, J., McCarthy-Keith, D., Britten, J., and Catherino, W.H. (2010). Why leiomyomas are called fibroids: the central role of extracellular matrix in symptomatic women. *Semin. Reprod. Med.* *28*, 169-179.
- Mesquita, F.S., Dyer, S.N., Heinrich, D.A., Bulun, S.E., Marsh, E.E., and Nowak, R.A. (2009). Reactive oxygen species mediate mitogenic growth factor signaling pathways in human leiomyoma smooth muscle cells. *Biol. Reprod.* *82*, 341-351.
- Mettouchi, A., Klein, S., Guo, W., Lopez-Lago, M., Lemichez, E., Westwick, J.K., and Giancotti, F.G. (2001). Integrin-specific activation of Rac controls progression through the G(1) phase of the cell cycle. *Mol. Cell.* *8*, 115-127.
- Mierke, C.T., Kollmannsberger, P., Zitterbart, D.P., Diez, G., Koch, T.M., Marg, S., Ziegler, W.H., Goldmann, W.H., and Fabry, B. (2010). Vinculin facilitates cell invasion into three-dimensional collagen matrices. *J. Biol. Chem.* *285*, 13121-13130.
- Nakagawa, S., Pawelek, P., and Grinnell, F. (1989). Extracellular matrix organization modulates fibroblast growth and growth factor responsiveness. *Exp. Cell Res.* *182*, 572-582.
- Norian, J.M., Owen, C.M., Taboas, J., Korecki, C., Tuan, R., Malik, M., Catherino, W.H., and Segars, J.H. (2011). Characterization of tissue biomechanics and mechanical signaling in uterine leiomyoma. *Matrix Biol.* *31*, 57-65.

Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., Hammer, D.A., and Weaver, V.M. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell*. **8**, 241-254.

Pickering, J.G. (2001). Regulation of vascular cell behavior by collagen : form is function. *Circ. Res.* **88**, 458-459.

Plopper, G.E., McNamee, H.P., Dike, L.E., Bojanowski, K., and Ingber, D.E. (1995). Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Molecular Biology Cell*. **6**, 1349-1365.

Provenzano, P.P., Inman, D.R., Eliceiri, K.W., and Keely, P.J. (2009). Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. *Oncogene*. **28**, 4326-4343.

Provenzano, P.P., and Keely, P.J. (2011). Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. *J. Cell. Sci.* **124**, 1195-1205.

Raines, E.W. (2000). The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationships to vascular disease. *Int. J. Exp. Pathol.* **81**, 173-182.

Raines, E.W., Koyama, H., and Carragher, N.O. (2000). The extracellular matrix dynamically regulates smooth muscle cell responsiveness to PDGF. *Ann. N. Y. Acad. Sci.* **902**, 39-51.

Rogers, R., Norian, J., Malik, M., Christman, G., Abu-Asab, M., Chen, F., Korecki, C., Iatridis, J., Catherino, W.H., Tuan, R.S., Dhillon, N., Leppert, P., Segars, J.H. (2008). Mechanical homeostasis is altered in uterine leiomyoma. *Am. J. Obstet. Gynecol.* **198**, 474.e1-474.11.

Roovers, K., and Assoian, R.K. (2003). Effects of rho kinase and actin stress fibers on sustained extracellular signal-regulated kinase activity and activation of G(1) phase cyclin-dependent kinases. *Mol. Cell. Biol.* **23**, 4283-4294.

Ross, R.S. (2004). Molecular and mechanical synergy: cross-talk between integrins and growth factor receptors. *Cardiovasc. Res.* **63**, 381-390.

Sato, K., Hattori, S., Irie, S., and Kawashima, S. (2003). Spike formation by fibroblasts adhering to fibrillar collagen I gel. *Cell Struct. Funct.* **28**, 229-241.

Schneller, M., Vuori, K., and Ruoslahti, E. (1997). α v β 3 integrin associates with activated insulin and PDGF β receptors and potentiates the biological activity of PDGF. *EMBO J.* **16**, 5600-5607.

Schrader, J., Gordon-Walker, T.T., Aucott, R.L., van Deemter, M., Quaas, A., Walsh, S., Benten, D., Forbes, S.J., Wells, R.G., and Iredale, J.P. (2011). Matrix stiffness modulates proliferation, chemotherapeutic response, and dormancy in hepatocellular carcinoma cells. *Hepatology* 53, 1192-1205.

Soung, Y.H., Clifford, J.L., and Chung, J. (2010). Crosstalk between integrin and receptor tyrosine kinase signaling in breast carcinoma progression. *BMB Rep.* 43, 311-318.

Stewart, E.A. (2001). Uterine fibroids. *Lancet* 357, 293-298.

Stewart, E.A., Friedman, A.J., Peck, K., and Nowak, R.A. (1994). Relative overexpression of collagen type I and collagen type III messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. *J. Clin. Endocrinol. Metab.* 79, 900-906.

Sundberg, C., and Rubin, K. (1996). Stimulation of beta1 integrins on fibroblasts induces PDGF independent tyrosine phosphorylation of PDGF beta-receptors. *J. Cell Biol.* 132, 741-752.

Tee, S.Y., Fu, J., Chen, C.S., and Janmey, P.A. (2011). Cell shape and substrate rigidity both regulate cell stiffness. *Biophys. J.* 100, L25-27.

Tsai, K.S., Kao, S.Y., Wang, C.Y., Wang, Y.J., Wang, J.P., and Hung, S.C. (2010). Type I collagen promotes proliferation and osteogenesis of human mesenchymal stem cells via activation of ERK and Akt pathways. *J. Biomed. Mater. Res. A.* 94, 673-682.

Tsibris, J.C., Segars, J., Coppola, D., Mane, S., Wilbanks, G.D., O'Brien, W.F., and Spellacy, W.N. (2002). Insights from gene arrays on the development and growth regulation of uterine leiomyomata. *Fertil. Steril.* 78, 114-121.

Wall, S.J., Werner, E., Werb, Z., and DeClerck, Y.A. (2005). Discoidin domain receptor 2 mediates tumor cell cycle arrest induced by fibrillar collagen. *J. Biol. Chem.* 280, 40187-40194.

Wolanska, M., Sobolewski, K., Drozdewicz, M., and Bankowski, E. (1998). Extracellular matrix components in uterine leiomyoma and their alteration during the tumour growth. *Mol. Cell. Biochem.* 189, 145-152.

Woodard, A.S., Garcia-Cardena, G., Leong, M., Madri, J.A., Sessa, W.C., and Languino, L.R. (1998). The synergistic activity of alphavbeta3 integrin and PDGF receptor increases cell migration. *J. Cell. Sci.* 111, 469-478.

Wozniak, M.A., Modzelewska, K., Kwong, L., and Keely, P.J. (2004). Focal adhesion regulation of cell behavior. *Biochimica Et Biophysica Acta (BBA) - Mol. Cell Res.* 1692, 103-119.

Wren, F.E., Schor, A.M., Schor, S.L., and Grant, M.E. (1986). Modulation of smooth muscle cell behaviour by platelet-derived factors and the extracellular matrix. *J. Cell. Physiol.* *127*, 297-302.

Yamada, K.M., and Even-Ram, S. (2002). Integrin regulation of growth factor receptors. *Nat. Cell Biol.* *4*, E75-E76.

Yamamoto, T., Ebisuya, M., Ashida, F., Okamoto, K., Yonehara, S., and Nishida, E. (2006). Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. *Curr. Biol.* *16*, 1171-1182.

Yeung, T., Georges, P.C., Flanagan, L.A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P.A. (2005). Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil. Cytoskeleton.* *60*, 24-34.

Zhao, J., Pestell, R., and Guan, J.L. (2001). Transcriptional activation of cyclin D1 promoter by FAK contributes to cell cycle progression. *Mol. Biol. Cell.* *12*, 4066-4077.

Chapter Four

Anti-fibrotic Effects of Halofuginone on Human Uterine Leiomyoma Smooth Muscle Cells *in vitro*

Abstract

Uterine leiomyomas (UL) are the most prevalent gynecological complications occurring in reproductive-aged women. These hormone-dependent, benign tumors are characterized by increased cell proliferation and excess deposition of extracellular matrix, primarily collagens I and III. Available treatment options for patients with ULs consist mainly of hormonal therapies or surgical interventions. However, due to the diverse nature and heterogeneity of these tumors and the non-fertility preserving characteristics of currently available treatments, alternative therapies are needed. We tested the anti-fibrotic drug halofuginone (HF) as an alternative therapeutic treatment in primary human uterine leiomyoma smooth muscle cells (LSMCs) cultured on plastic or monomeric collagen matrices. Results showed that 50 nM HF significantly reduced the rate of basal and PDGF-stimulated cell proliferation of LSMCs on both matrices without causing any toxic effects. Results also showed no compensatory effect of monomeric collagen on the inhibitory effects of HF on LSMC proliferation. HF reduced the expression of collagens I and III at the level of both RNA and protein in a time-dependent manner. In addition, HF altered transcription of *TGFβ1*, *TGFβ3*, lysyl oxidase and dermatopontin. These inhibitory effects of HF on LSMCs appeared to occur through alterations in the MAPK and SRC signaling pathways. These findings suggest that HF can effectively target fibrosis in LSMCs and be considered as an alternative therapeutic option for ULs.

Introduction

Uterine fibroids or leiomyomas (ULs) are benign tumors arising from myometrial smooth muscle cells. These tumors develop in the majority of women during their reproductive years and can cause symptoms such as pelvic pain and pressure, abnormal uterine bleeding, frequent miscarriages and fertility problems (Kroon *et al.*, 2011; Lee *et al.*, 2009; Wallach and Vlahos, 2004). At the cellular level, ULs are characterized by an enhanced level of cell proliferation and increased synthesis and expression of ECM proteins such as collagens I and III (Kawaguchi *et al.*, 1989; Leppert *et al.*, 2004; Stewart *et al.*, 1994; Stewart, 2001).

The high prevalence of this reproductive disease has not led to a great understanding of its etiology. Numerous *in vitro* and *in vivo* studies have highlighted the role of ovarian steroid hormones in the pathogenesis of ULs. These tumors have also been found to have diverse genetic and epigenetic alterations compared to normal adjacent myometrial cells (Ghosh and Vaughan, 2012; Makinen *et al.*, 2011; Meloni *et al.*, 1992; Stewart and Morton, 2006). The fibrotic nature of ULs, which is due to the excess deposition of ECM collagen, has linked their formation to injury occurring in the uterine myometrium (Leppert *et al.*, 2006; Roeder *et al.*, 2012; Stewart and Nowak, 1998).

Based on the evidence that ovarian steroid hormones play a role in the pathogenesis of ULs, treatment options for women with ULs have been limited to either surgical intervention through myomectomy or hysterectomy or the use of various hormone therapies (Al-Hendy and Salama, 2006; Pinkerton, 2011; Stewart, 2002). While these treatment options alleviate the symptoms associated with UL, they are

accompanied by high costs, significant side effects, and also not suitable for women who would like to maintain their fertility. There is, therefore, a need for developing alternative therapeutic treatments which not only help with alleviating symptoms but also target the underlying cause of these tumors.

In recent years, the anti-fibrotic drug halofuginone (HF) has been tested in a variety of fibrotic diseases as well as cancers as a possible therapeutic treatment. This drug is a low molecular weight plant alkaloid derived from *Dichora febrifuga* and has been used as an anti-fever and anti-malaria herb in Chinese medicine as well as a coccidiostat for poultry (Pines *et al.*, 2000). More recently, HF was found to target collagen synthesis in numerous *in vivo* and *in vitro* cell models. Stellate cells, mesangial cells, mammary epithelial cells, and fibroblasts are among the cell types that show sensitivity to the inhibitory effects of HF (Gnainsky *et al.*, 2004b; McGaha *et al.*, 2002; Nagler *et al.*, 1997; Xavier *et al.*, 2004). In addition, HF has been found to significantly reduce the formation of microvessels in bladder carcinoma and silicone implants containing HF lead to limited angiogenesis in *in vivo* models (Elkin *et al.*, 2000; Jordan and Zeplin, 2012).

The inhibitory effects of HF appear to occur through several signaling pathways such as MAPK, TGF β , SRC, AKT and amino acid starvation. As the major fibrosis-inducing pathway, TGF β -SMAD signaling has been identified as a key pathway modulated by HF in a variety of cell types (Genin *et al.*, 2008; Gnainsky *et al.*, 2007; Grudzien *et al.*, 2009; McGaha *et al.*, 2002; Nagler *et al.*, 2000; Sato *et al.*, 2009; Xavier *et al.*, 2004; Yee *et al.*, 2006). Indeed, up-regulation of the anti-fibrotic Smad7 and down-regulation of the pro-fibrotic Smad3 by HF was observed in cultured fibroblasts and mesangial

cells (Sato *et al.*, 2009; Xavier *et al.*, 2004). Similarly, the MAPK and AKT signaling pathways involved in cell proliferation, myofibroblast differentiation and matrix contraction have been shown to be up-regulated by HF treatment (Leiba *et al.*, 2012; Roffe *et al.*, 2010).

In our goal to find an alternative therapeutic treatment for ULs, we investigated the effects of HF on primary human LSMCs in an *in vitro* culture system. We assessed cell proliferation and expression of ECM proteins including collagens I, III, and dermatopontin as well as major pro-fibrotic factors such as TGF β 1, TGF β 3, and lysyl oxidase of cultured primary LSMCs treated with HF. We also tested the effects of HF on LSMCs cultured on monomeric collagen matrix to more closely model *in vivo* leiomyoma tissue surrounded by abundant collagen matrix.

Results

Dose Response Study for HF and Cytotoxicity Assay

A dose response study was performed to determine the inhibitory dose of HF that would cause approximately 50% reduction in cell proliferation. LSMCs cultured on plastic or monomeric collagen coated dishes were treated with 0, 10, 25, 50, 100, 200 and 400 nM HF in serum-containing medium for 24 hours before assessing their rate of cell proliferation using the thymidine incorporation assay. Results showed that 50 nM HF reduced the rate of proliferation by 50-70% depending on the patient/sample on both matrices (Fig. 4.1-A). To test whether the observed reduction in cell proliferation was due to cytotoxic effects of HF on LSMCs, trypan blue exclusion assays were performed.

**Please insert sentence stating what the percentage of dead cells was for each treatment and that they were not significantly different. Treatment of LSMCs with 50 nM

HF showed no cytotoxic effects during the 24 hour treatment period (Fig. 4.1-B). We, therefore, used the 50 nM concentration of HF for the subsequent studies on the effects of HF on LSMCs.

Effects of HF on LSMC Cell Proliferation

Increased rate of cell proliferation is one of the characteristics of ULs. To test the inhibitory effects of HF on LSMC proliferation, several treatment schemes with HF and/or PDGF, an abundant growth factor in ULs, were carried out with cells cultured on both plastic and monomeric collagen coated matrices. Treatment schemes were co-treatment of HF with PDGF for 24 hours, treatment with HF or PDGF for 24 hours followed by a 24-hour treatment with PDGF or HF respectively, and finally treatment with HF or PDGF and their removal at the end of 24 hours followed by treatment with PDGF or HF, respectively, for 24 hours.

In all three treatment schemes, treatment of LSMCs with 50 nM HF alone for 24 hours significantly reduced the rate of cell proliferation by 60-90% (Figs. 4.2-5). The degree of reduction was found to be similar in LSMCs cultured on either of the matrices indicating that HF was a potent inhibitor of cell proliferation of LSMCs even in the presence of monomeric collagen matrix, which is a known stimulator of cell proliferation.

Co-treatment of LSMCs with 50 nM HF and 10 ng/ml PDGF for 24 hours reduced the rate of proliferation on both matrices by 86% in comparison to PDGF-treated cells (Fig. 4.2), confirming that even in the presence of growth-stimulating factors such as PDGF, HF could still inhibit the cellular machinery involved in cell proliferation.

In the second treatment scheme, LSMCs cultured on both matrices were pre-treated with either HF or PDGF for 24 hours and then stimulated or inhibited with PDGF

or HF, respectively, for an additional 24 hours (Fig. 4.3). Results showed that pre-treatment of LSMCs with 50nM HF for 24 hours followed by stimulation with 10 ng/ml PDGF could still significantly reduce the rate of cell proliferation by at least 80% compared to cells treated with PDGF alone (Fig. 4.3). In addition, compared to cells which were treated with just PDGF, proliferation of LSMCs pre-treated with PDGF and then treated with HF was also found to be significantly reduced. This reduction was 30% less compared to cells which were first pre-treated with HF and then stimulated with PDGF (Fig. 4.4), indicating that the inhibitory effects of HF on LSMC proliferation can effectively overcome the stimulatory effects of PDGF.

Finally, in the last treatment scheme, LSMCs were initially treated with 50 nM HF or 10 ng/ml PDGF before these initial treatments were removed at the end of 24 hours. Next, cells which were treated with HF were stimulated with PDGF for 24 hours and the ones which had received the initial treatment of PDGF, were treated with HF. Thymidine incorporation assays performed on these cells showed that PDGF stimulation following the removal of the pre-treatment with HF increased the rate of proliferation by about 155% compared to cells which were treated with just HF and had not been stimulated with PDGF (Fig. 4.4). In addition, HF treatment on cells pre-treated with PDGF significantly reduced the rate of cell proliferation by 89% compared to LSMCs treated with just PDGF for the same time period (Fig. 4.4). These results support the strong inhibitory effects of HF on LSMCs observed in other treatment schemes, and show the temporary inhibitory effects of this drug on the proliferation of LSMCs.

Analysis of cell proliferation in LSMCs cultured on monomeric collagen coated dishes in all HF treatments schemes did not show statistically significant differences

from cells cultured on plastic matrix at the 0.05 P value (Fig. 4.2-4). Statistical differences were only observed at the 0.1 Pvalue.

To understand whether the inhibitory effect of HF on LSMC proliferation was as a result of its inhibitory effect on the expression of *COL1A1* and *COL3A1*, we silenced expression of each or both of these ECM proteins using 60 nM siRNAs specific for each protein for 24 hours and then assessed the rate of cell proliferation in the presence or absence of 10ng/ml PDGF on both plastic or monomeric collagen matrices. Our results showed that compared with either mock or negative control siRNA-transfected LSMCs, silencing of either collagen alone or of both together did not alter cell proliferation (Appendix C). There were also no significant differences between cells cultured on the two matrices. These results suggest that the inhibitory effect of HF on LSMCs proliferation probably do not occur through its inhibitory effect on collagen production.

Overall, findings from proliferation assays with HF in the present or absence of PDGF indicate that the inhibitory effects of HF at the 50 nM concentration on LSMCs, though potent, are not permanent and cells exposed to HF are still able to respond to the stimulatory signals of PDGF for cell proliferation. Moreover, pre-treatment of LSMCs with HF was found to be more effective in reducing cell proliferation as compared to post-treatment after PDGF stimulation.

Effects of HF on the Expression of COL1A1 and COL3A1

A hallmark of ULs is the excess presence of ECM collagens mainly collagen I and collagen III. One of the well-known properties of HF is its inhibitory effects on collagen production. To test whether a similar inhibitory effect would be observed in ULs, LSMCs were treated with 50 nM HF for 0, 2, 6, 12, 24 and 48 hours and then assessed for the

expression of collagen I and collagen III proteins by immunoblotting. Results from these experiments showed that the inhibitory effects of HF on the expression of these two major collagens in LSMCs were detected as early as 2 hours after treatment with significant reduction in levels of both proteins by 6 hours (Fig. 4.5). Thus, the inhibitory effect of HF on ECM collagens I and III was validated in LSMCs as well.

Effects of HF on the Expression of Genes Involved in Fibrosis

In addition to collagens I and III, a number of other proteins such as TGF β 1, TGF β 3, lysyl oxidase and dermatopontin are associated with fibrotic diseases and have been shown to be dysregulated in ULs. To determine whether HF had any effect on the transcription of these genes in ULs, LSMCs were treated with 50 nM HF for 0, 6, 12, 24 and 48 hours and analyzed by qRT-PCR. Results showed that HF could reduce transcript levels of all genes 24 or 48 hours after treatment (Fig 4.6. A-F). These findings suggest that transcriptional inhibition of the major genes involved in fibrosis may be one of the mechanisms through which HF exerts its effects on LSMCs.

Signaling Pathways Altered by HF in LSMCs

Following our observation of the effects of HF on cell proliferation and expression of genes involved in fibrosis in LSMCs, we next looked into the mechanism through which such effects might be mediated. LSMCs were first treated with 50 nM HF for 0, 15, 30 and 60 minutes and then assessed for alterations in the expression of key molecules involved in the MAPK and SRC pathways. Immunoblot analysis of these cells showed that HF phosphorylated ERK1/2 rapidly in 15 minutes and this activation lasted for an hour (Fig. 4.7A). A similar pattern of activation, but with a delayed phosphorylation at 30 minutes was observed for SRC (Fig. 4.7B).

Based on the inhibitory effects of HF on cell proliferation in the presence of PDGF, we next pre-treated LSMCs with 50 nM HF for 6 hours and then stimulated them with 10 ng/ml PDGF for 10 minutes. Results showed PDGFR, ERK1/2 SRC, and AKT were robustly activated by PDGF (Fig. 4.8,A-D). Pre-treatment of these cells with HF for 6 hours followed by stimulation by PDGF, however, did not alter the activation of ERK1/2, SRC, AKT or PDGFR at significant levels (Fig. 4.8, A-D). These results indicate that HF itself can activate major signaling pathways such as SRC and MAPK without interfering with the major signaling pathways activated by PDGF at the dose and pre-treatment period used in this study.

Discussion

HF has been shown to significantly inhibit collagen synthesis and cell proliferation in variety of cell types, including LSMCs (Grudzien *et al.*, 2009; Nelson *et al.*, 2012; Pines and Halevy, 2011). To further advance our understanding of how HF mediates these inhibitory effects on LSMCs, we designed the present study with two objectives: (i) to assess the inhibitory effect of HF on LSMC proliferation in the presence of PDGF, an abundant growth factor in ULs, and how these effects might be modulated in cells grown on monomeric collagen, an abundant form of ECM collagen in ULs; and (ii) to investigate the signaling mechanisms responsible for the inhibitory effects of HF on LSMCs.

Cell proliferation assays showed that a 24-hour treatment with 50 nM HF could significantly reduced the proliferation of LSMCs in the absence or presence of PDGF, regardless of the sequence of treatment with PDGF. In addition, the inhibitory effect of HF on LSMC proliferation seemed to be non-toxic and reversible because cells were

able to respond to the growth stimulatory signals of PDGF both when HF was concomitantly present with PDGF or once HF treatment was removed and PDGF was then added. These results are in agreement with other studies on HF and growth factors. Treatment of vascular SMCs with 100-300 nM HF in the presence of serum or bFGF caused an abrogation of DNA synthesis and cell proliferation, with minimal effects on vascular endothelial cells (Nagler *et al.*, 1997). HF at concentrations above 10^{-8} M was found to affect basal proliferation of fibroblasts (McGaha *et al.*, 2002) and IGF-I-induced proliferation in human myeloma cells and rat stellate cells (Gnainsky *et al.*, 2004a; Leiba *et al.*, 2012). Earlier results in our lab also showed the inhibitory effects of HF on LSMCs proliferation in the presence of serum in a dose-dependent manner (Grudzien *et al.*, 2009). The reversibility of HF's effect on LSMC proliferation observed in our study is also in support of previous investigations in LSMCs and other cell types (Grudzien *et al.*, 2009; Leiba *et al.*, 2012; Nagler *et al.*, 1997). Together, these data suggest that HF can effectively target one of the main features of ULs, which is an increased rate of cell proliferation, even in the presence of a potent stimulator of growth such as PDGF.

The suppressive effect of HF on collagen synthesis in dermal fibroblast cells was the first indication of its inhibitory effect on fibrosis (Granot *et al.*, 1991). Follow-up studies in other cell types proved this to be the main feature of HF as an anti-fibrotic drug (Dabak *et al.*, 2010; Genin *et al.*, 2008; Gnainsky *et al.*, 2007; Grudzien *et al.*, 2009; McGaha *et al.*, 2002; Nagler *et al.*, 2000; Nelson *et al.*, 2012; Sato *et al.*, 2009; Xavier *et al.*, 2004; Yee *et al.*, 2006). Similarly, in our study, HF showed a time-dependent inhibition of the expression of both collagens I and III. Protein levels of both

collagens were found to be greatly reduced as early as 6 hours after treatment with 50 nM HF and eventually returned to normal by 48 hours. We hypothesized that the decrease in collagen I and III proteins was mediated through inhibition of procollagen transcripts. However, treatment of LSMCs with HF at the same time points, failed to decrease transcripts of both genes in early hours corresponding to the time points of protein down-regulation. Indeed, mRNA levels were not reduced until 24 or 48 hours after HF treatment.

This time difference between the transcriptional and translational inhibition of collagens I and III in LSMCs is suggestive of several possible mechanisms. One possibility may be the extended half-life of procollagen mRNA in LSMCs. Another mechanism might be through activation of the ubiquitination pathway by HF, which can rapidly lead to the degradation of existing collagen proteins in LSMCs. Although this mode of action for HF has not been reported in other cell types previously, there are reports of other anti-fibrotic agents such as S-adenosyl methionine (SAM-e) reducing collagen protein expression through polyubiquitination of intracellular type I collagen (Thompson *et al.*, 2011). It is also possible that the inhibitory effect of HF on collagen expression in LSMCs can occur through inhibition of gene transcription, but as a late, secondary molecular response to HF. Furthermore, due to the multi-step process of collagen synthesis and various routes of collagen degradation, HF could disrupt these processes at several points at earlier times of exposure. In fact, in hepatic stellate cells, it was found that HF could target collagen synthesis and degradation through the crosslinking enzyme prolyl 4-hydroxylase β (P4H β) and tissue inhibitor of matrix metalloproteinase II (TIMP-II) as well as MMPs 2, 3, 13 and 9 (Popov *et al.*, 2006; Zion

et al., 2009). Endocytic collagen degradation through uPARAP/Endo180 in the same cell type has also been shown to act as a protective mechanism against fibrosis (Madsen *et al.*, 2012). Further investigation into these possible mechanisms is needed. Overall, these data provide strong evidence for the potent effect of HF on this major feature of ULs, which is the abundant production of collagens I and III, leading to fibrotic tissue.

We hypothesized that the inhibitory effects of HF on collagen expression may be one of the underlying causes for the reduced rate of cell proliferation observed in LSMCs treated with HF in the absence or presence of PDGF. However, our results with siRNAs that silenced *COL1A1*, *COL3A1*, or both in LSMCs showed no difference in cell proliferation compared to negative control siRNA-transfected cells. These results indicate that the inhibitory effects of HF on cell proliferation in LSMCs are definitely not due to its inhibitory effect on collagen expression, that these two effects are separate from each other. Instead, the inhibitory effect of HF on cell proliferation may be due to alterations in cell cycle progression. In concordance with another study by deFigueiredo-Pontes *et al.* (de Figueiredo-Pontes *et al.*, 2011), unpublished results in our lab have indeed shown that HF increases the expression of p21, an inhibitor of cell cycle progression. Moreover, modulatory effects of HF on other molecules in LSMCs such as cell signaling proteins or other cell cycle regulatory molecules may also contribute to decreased rates of cell proliferation in LSMCs.

Molecular mechanisms involved in the inhibitory effects of HF on fibrosis have been partially elucidated in several different cell types (Genin *et al.*, 2008; Gnainsky *et al.*, 2007; McGaha *et al.*, 2002; Xavier *et al.*, 2004; Yee *et al.*, 2006). Signaling

pathways such as the amino acid starvation pathway, p38 and NF κ B, as well as integrated stress response pathways, have all been found to be modulated by HF (Kamberov *et al.*, 2011; Keller *et al.*, 2012; Peng *et al.*, 2012; Popov *et al.*, 2006). However, inhibition of the Smad3 signaling pathway downstream of TGF β receptors and effects on phosphorylation of the AKT and MAPK pathways seem to be major mechanisms involved (Roffe *et al.*, 2010; Tacheau *et al.*, 2007). In the present study, short treatment with HF alone was able to activate the MAPK and SRC signaling pathways in LSMCs. In a study by Zion *et al.*, treatment of pancreatic stellate cells, which are the major collagen producing cells in pancreas, with HF resulted in increased activation of ERK1/2, but not AKT or p38MAPK (Zion *et al.*, 2009). Similarly, 50nM HF robustly activated ERK1/2, JNK1/2 and p38MAPK in as early as 15 minutes in fibroblasts (Tacheau *et al.*, 2007). Furthermore, while Roffe *et al.* reported a similar activation for ERK1/2 in myofibroblasts upon treatment with 10nM HF, they showed phosphorylation of AKT as well (Roffe *et al.*, 2010).

In this study, phosphorylation of ERK1/2 in response to stimulation by 10 ng/ml PDGF for 10 minutes was slightly, but not significantly, reduced in LSMCs following a 6-hour pre-treatment with HF. Likewise no changes in the activation of PDGFR, SRC or AKT were observed. A similar study in hepatic stellate cells using the same treatment scheme as ours but with 200nM HF confirms our observations (Popov *et al.*, 2006). These apparently opposite effects of HF on signaling pathways upon short or long treatments in the absence or presence of PDGF are suggestive of several possible processes. Signaling pathways from the starting point of ligand-receptor binding to the transmission of signal to the site of action involve a complicated and intricate system of

molecular interaction whose spatial and temporal positioning along the signaling network can critically affect the final outcome. In addition, strength and duration of these interactions can greatly affect the dynamics of signaling cascades. In this context, it is possible to assume that the short, minutes-long stimulation of LSMCs with HF could transiently activate signaling pathways such as MAPK and SRC to either initiate specific cellular responses or prime other molecular pathways for later, secondary responses. Pre-treatment of LSMCs with HF for longer duration, hence, could have similar effects on the cell whereby stable changes in the signaling cascade occur that can override the stimulatory effects induced by PDGF. In contrast, it is possible that pre-treatment of LSMCs with HF for a long time would de-sensitize PDGF receptor and associated signaling molecules such that the stimulatory signals of PDGF are mitigated. Lastly, modulatory effects of HF on signaling pathways may have not lasted long enough to interfere with the molecular mechanisms modulated by PDGF.

The process of fibrosis, which is a pathological manifestation of tissue injury and loss of tissue homeostasis, is accompanied by disrupted ECM protein synthesis and turnover (Chen and Raghunath, 2009; Ghosh, 2002; Ghosh and Vaughan, 2012). During this process, pro-fibrotic factors such as TGF β , as well as enzymes involved in matrix synthesis and degradation such as lysyl oxidase and MMPs are altered. In addition to targeting collagens I and III, HF has been shown to target other factors involved in tissue fibrosis such as TGF β 1, MMP-2 and 9, as well as the crosslinking enzyme P4H β (Elkin *et al.*, 1999; Jordan and Zeplin, 2012; Kamberov *et al.*, 2011; Popov *et al.*, 2006; Taras *et al.*, 2006; Zion *et al.*, 2009). Our analysis of gene transcription on a few of these factors upon treatment with HF in LSMCs was in

agreement with previous studies. HF inhibited transcription of *COL1A1*, *COL3A1*, *TGFβ1*, *TGFβ3*, *LOX3*, and *DMT* in a time-dependent manner, suggesting a multi-modal form of intervention in UL fibrosis.

In conclusion, we have demonstrated that HF is a potent inhibitor of both proliferation and collagen expression in LSMCs. In addition, HF targets transcription of major factors involved in tissue fibrosis. Alteration of MAPK and SRC signaling pathways, which seemed to be differentially altered by HF in a time-dependent manner of exposure, provides a framework for finding target molecules to control UL fibrosis. The results of this study also provide a strong basis for future *in vivo* and pre-clinical investigations in pursuit of alternative therapeutic options for women with ULs.

Figures

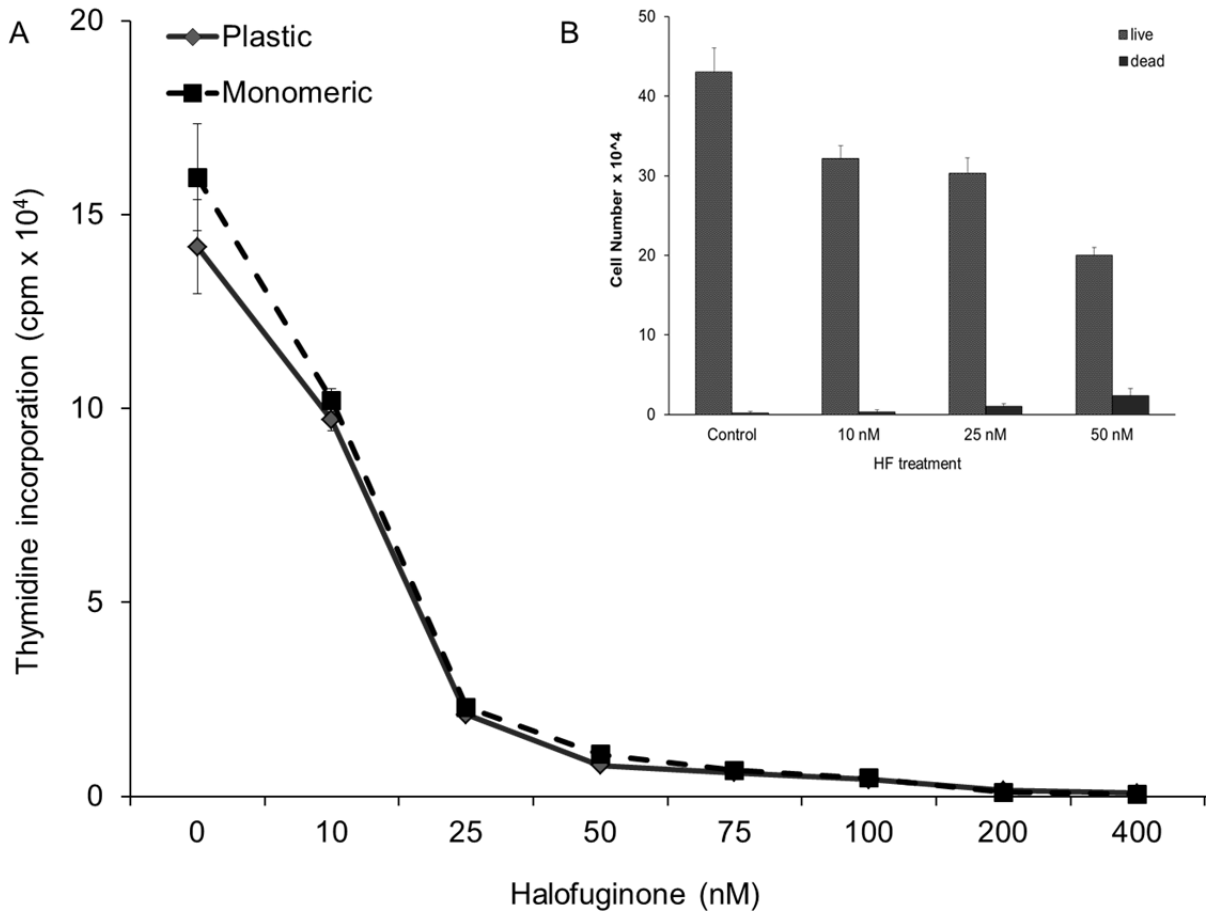


Figure 4.1. HF significantly reduced LSMC proliferation without significantly affecting cell viability. (A) LSMCs were cultured on plastic or monomeric collagen coated matrices and once they reached 90% confluence were treated with different concentrations of HF in serum containing medium for 24 hours before being assessed for cell proliferation. (B) LSMCs cultured on plastic matrix and treated with different concentrations of HF were stained with trypan blue to detect non-viable cells. (n=8 for dose response, n=4 for viability assay, P<0.05)

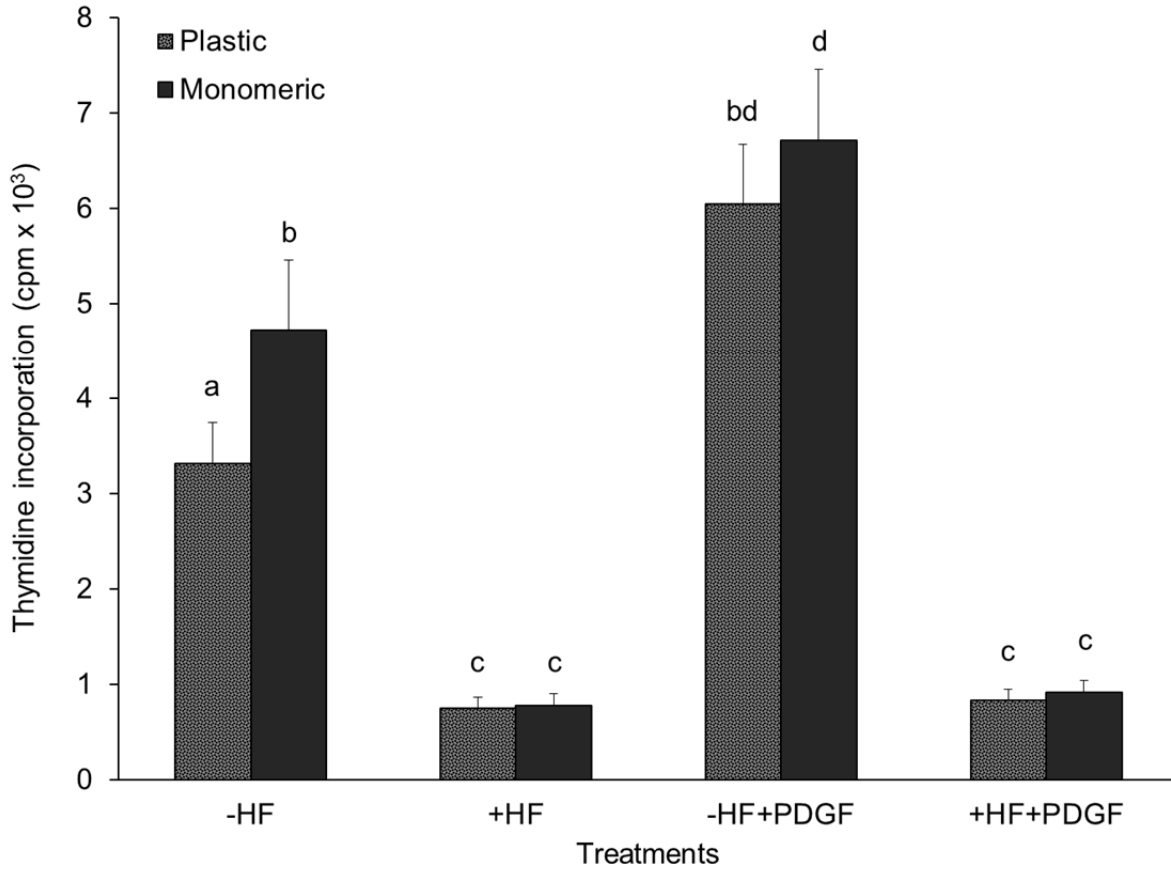
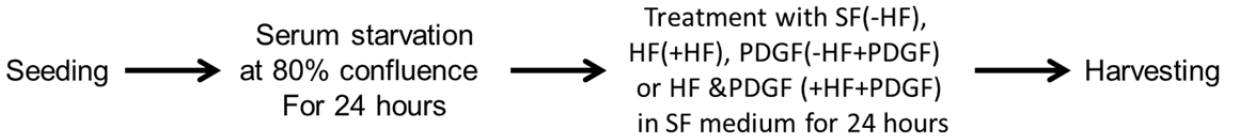


Figure 4.2. HF reduced proliferation of LSMCs cultured on either plastic or monomeric collagen matrices in the presence of PDGF. Cell-cycle synchronized LSMCs were treated with 50 nM HF, 10 ng/ml PDGF or both for 24 hours before cell proliferation assessment with thymidine incorporation assay. Letters indicate statistical significance between groups. (n=7, P<0.05, SF: serum-free).

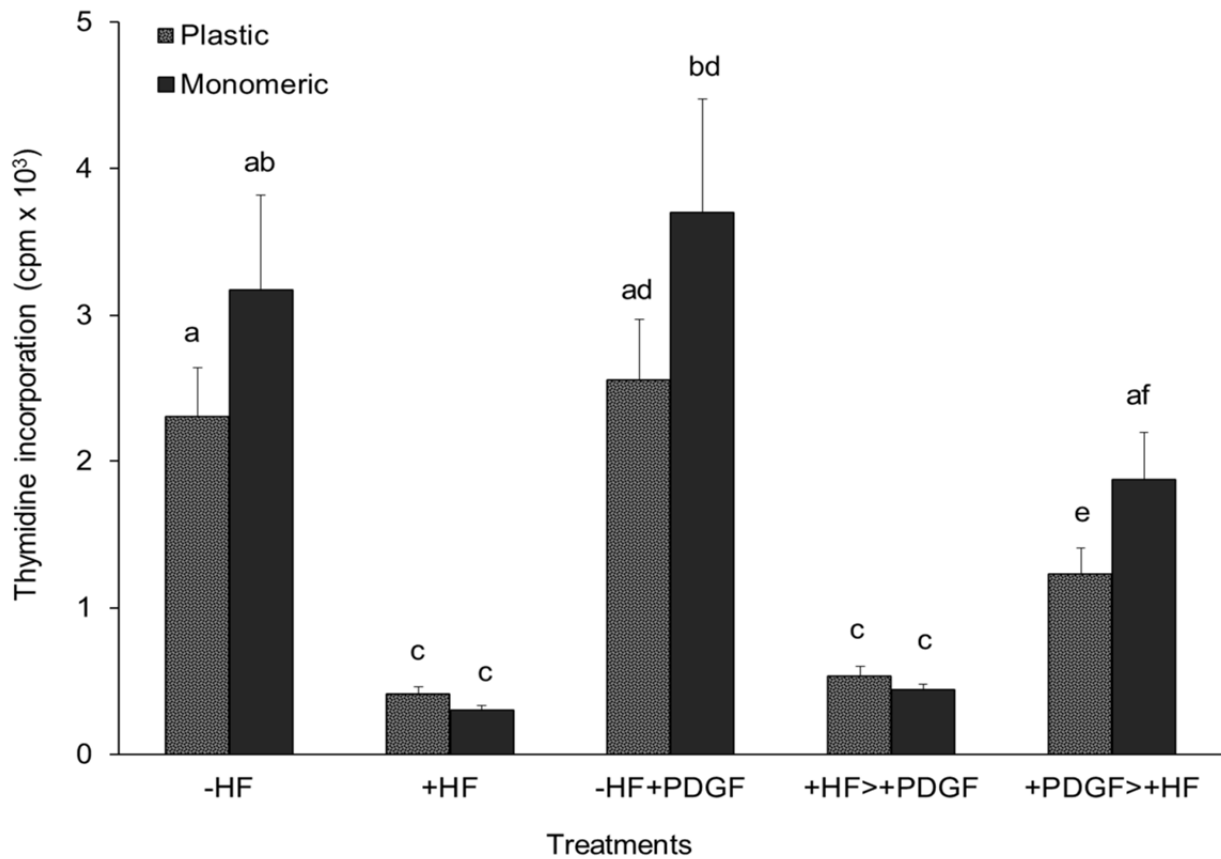
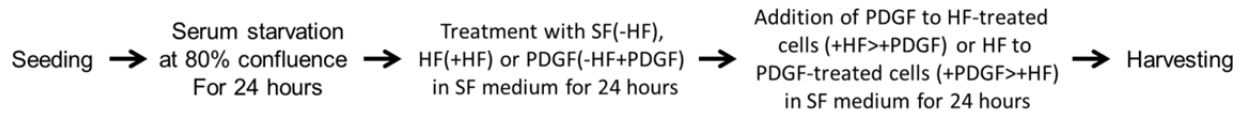


Figure 4.3. Pre-treatment of LSMCs with HF reduced proliferation of LSMCs cultured on either plastic or monomeric collagen matrices. LSMCs were cell-cycle synchronized and then subjected to initial and secondary treatments for 24 hours each before cell proliferation assessment with thymidine incorporation assay. Letters indicate statistical significance between groups. (n=8, P<0.05, SF: serum-free).

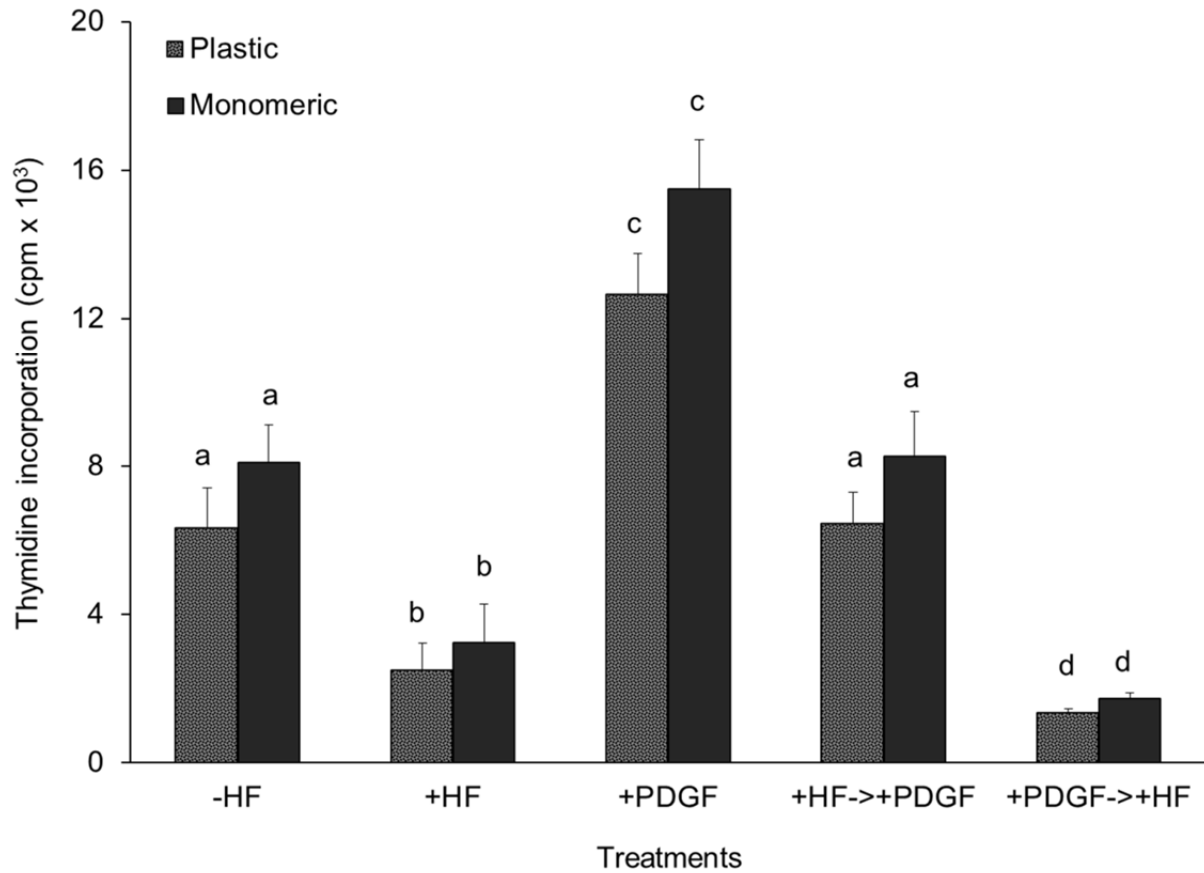
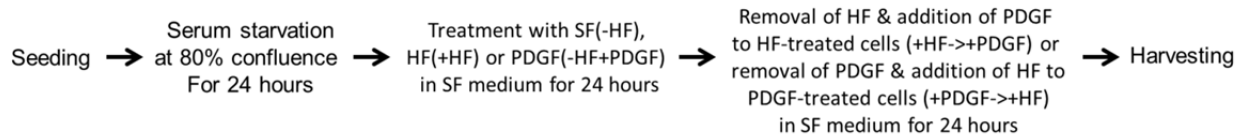


Figure 4.4. Inhibitory effect of HF on LSMC proliferation is reversible. Cell-cycle synchronized LSMCs were cell-cycle synchronized and then subjected to initial and secondary treatments for 24 hours each before cell proliferation assessment with thymidine incorporation assay. Letters indicate statistical significance between groups. (n=8, P<0.05).

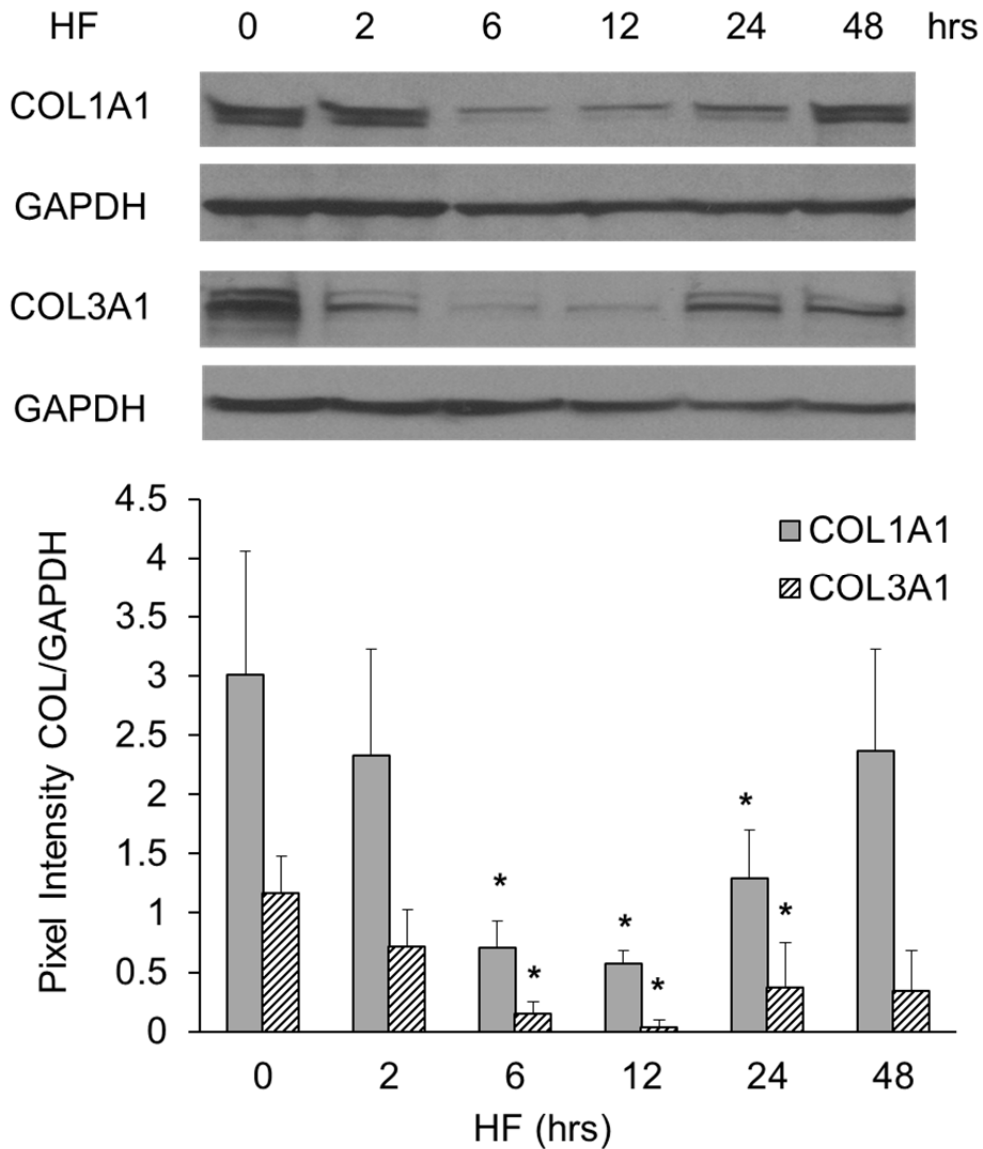


Figure 4.5. HF reduced the expression of COL1A1 and COL3A1 proteins in a time-dependent manner. LSMCs cultured on plastic matrix were treated with 50 nM HF for 2, 6, 12, 24, and 48 hours in serum containing medium. Changes in the expression of COL1A1 and COL3A1 were detected by immunoblotting using specific antibodies against each protein. Asterisks indicate statistical significance compared to non-treated samples. (n=3, P<0.05).

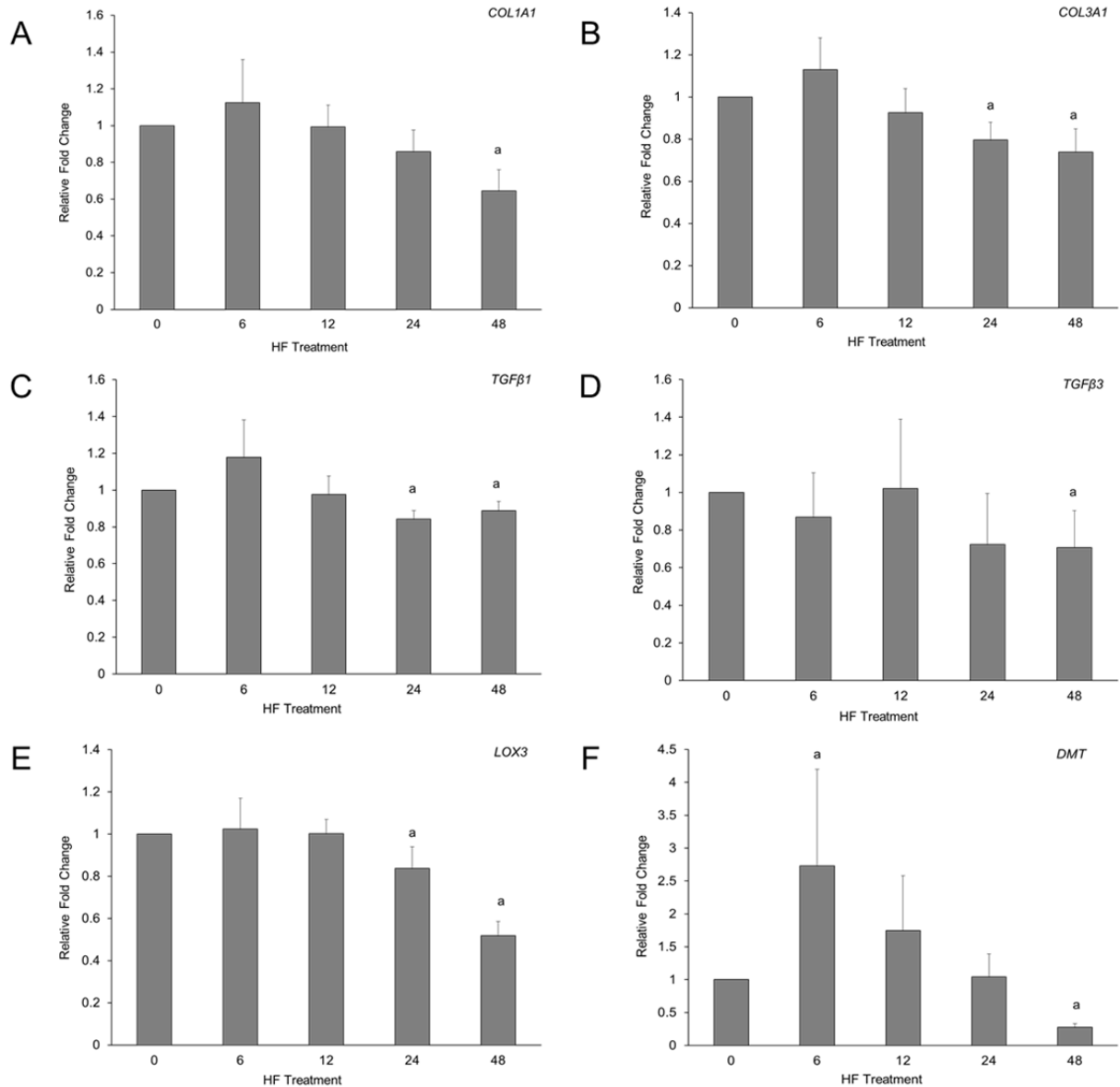


Figure 4.6. HF alters transcription of major fibrotic factors in a time-dependent manner. LSMCs cultured on plastic matrix were treated with 50 nM HF for 6, 12, 24 and 48 hours. Transcript levels for (A) *COL1A1*, (B) *COL3A1*, (C) *TGFβ1*, (D) *TGFβ3*, (E) *LOX3*, and (F) *DMT* were quantified by qRT-PCR. Letters indicate significance compared to time zero. (n=3, P<0.05).

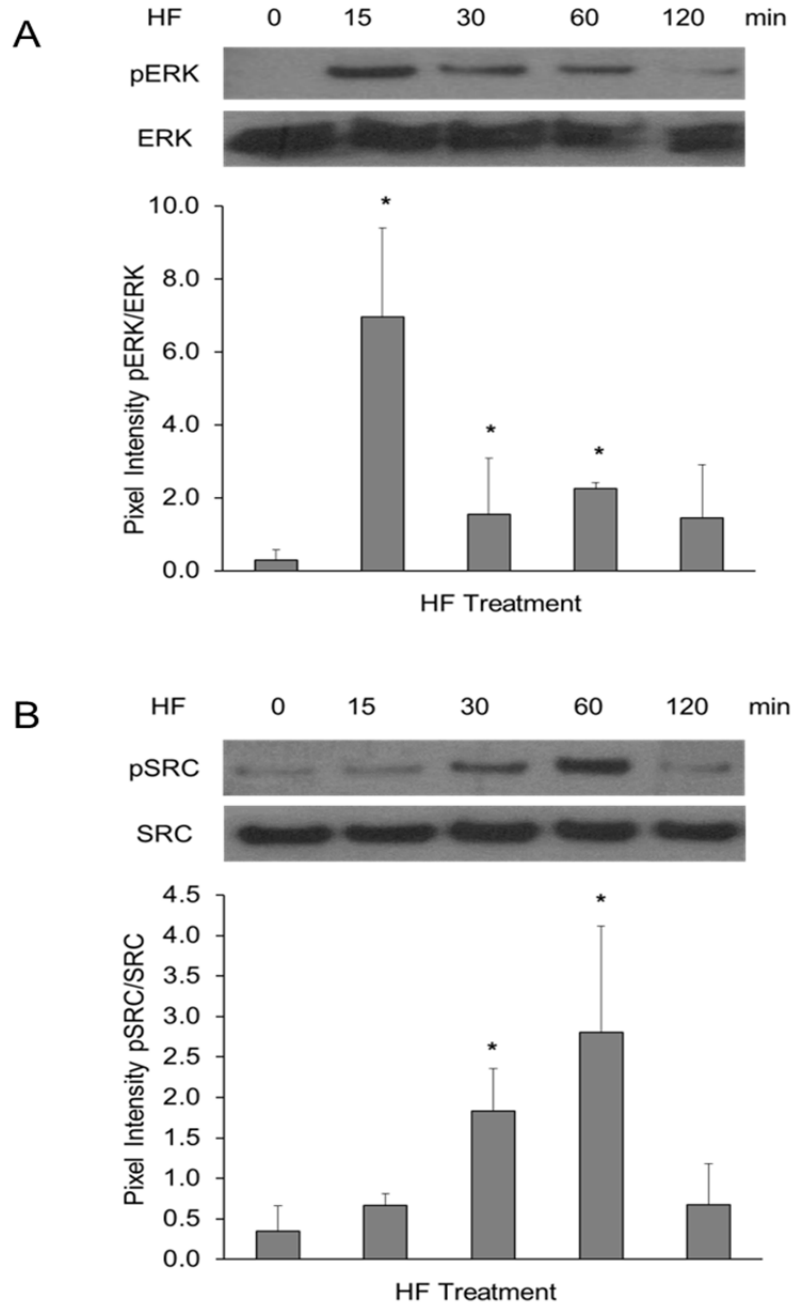


Figure 4.7. HF activates the MAPK and SRC signaling pathways. LSMCs cultured on plastic matrix were stimulated with 50 nM HF for 15, 30, 60 and 120 minutes. Cell lysates were blotted with antibodies specific to (A) pERK1/2 & ERK and (B) pSRC & SRC. Asterisks represent significance compared to non-HF treated samples. (n=2, P<0.05).

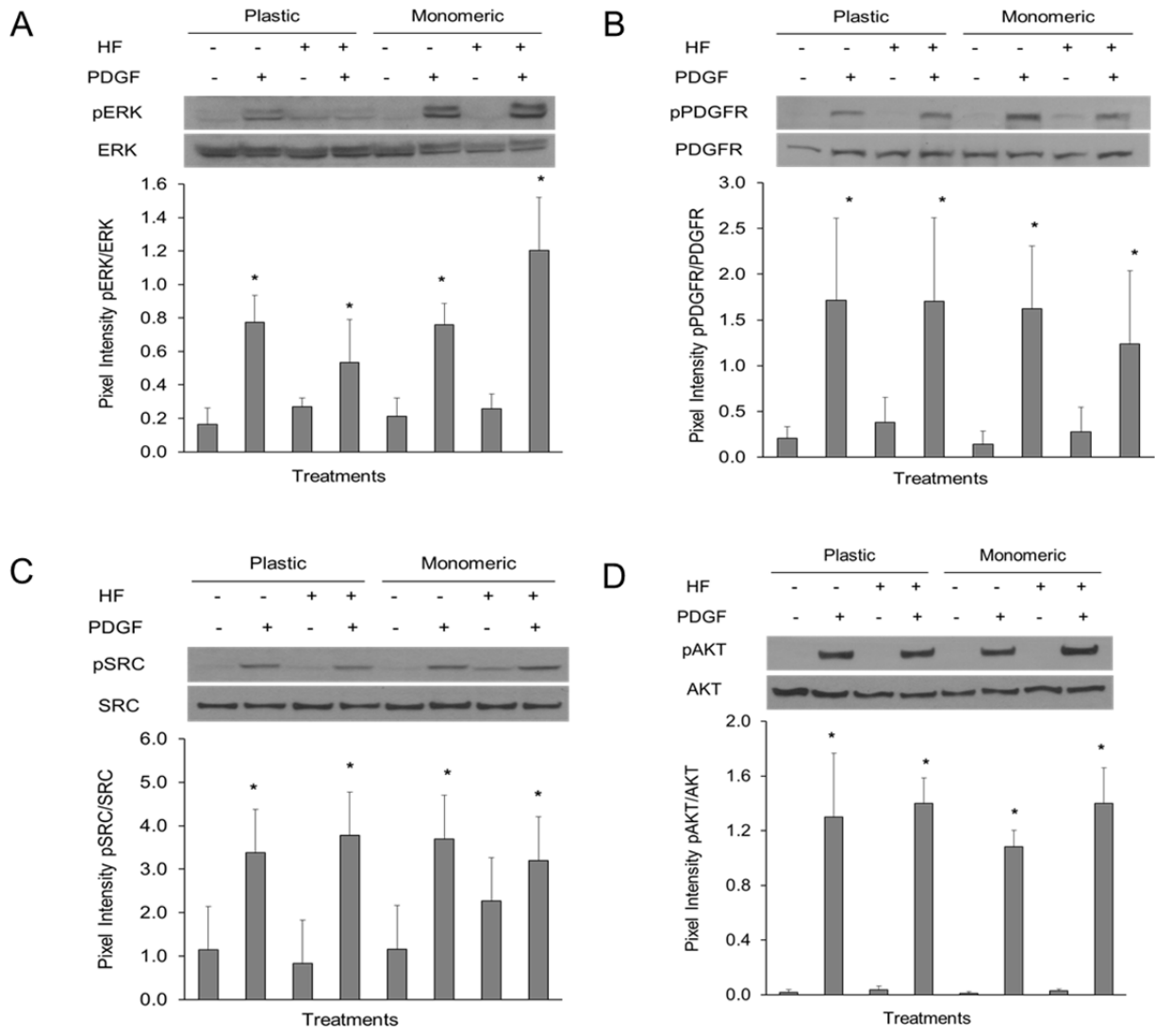


Figure 4.8. HF modulates signaling pathways activated by PDGF. LSMCs cultured on plastic matrix were pre-treated with 50 nM HF for 6 hours prior to stimulation with 10 ng/ml PDGF for 10 minutes. Cell lysates were blotted with antibodies specific to (A) pPDGFR and PDGFR, (B) pERK1/2 and ERK1/2, (C) pSRC and SRC, and (D) pAKT and AKT. Asterisks represent significance compared to non-treated control samples within each matrix. (n=3, P<0.05).

Reference List

Al-Hendy, A., and Salama, S. (2006). Gene therapy and uterine leiomyoma: a review. *Hum. Reprod. Update* 12, 385-400.

Chen, C.Z., and Raghunath, M. (2009). Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis -- state of the art. *Fibrogenesis Tissue Repair*. 2, 7.

Dabak, H., Karlidag, T., Akpolat, N., Keles, E., Alpay, H.C., Serin, M., Kaygusuz, I., Yalcin, S., and Isik, O. (2010). The effects of methylprednisolone and halofuginone on preventing esophageal and hypopharyngeal fibrosis in delivered radiotherapy. *Eur. Arch. Otorhinolaryngol.* 267, 1429-1435.

de Figueiredo-Pontes, L.L., Assis, P.A., Santana-Lemos, B.A., Jacomo, R.H., Lima, A.S., Garcia, A.B., Thome, C.H., Araujo, A.G., Panepucci, R.A., Zago, M.A., Nagler, A., Falcão, R.P., Rego, E.M. (2011). Halofuginone has anti-proliferative effects in acute promyelocytic leukemia by modulating the transforming growth factor beta signaling pathway. *PLoS One*. 6, e26713.

Elkin, M., Miao, H.Q., Nagler, A., Aingorn, E., Reich, R., Hemo, I., Dou, H.L., Pines, M., and Vlodavsky, I. (2000). Halofuginone: a potent inhibitor of critical steps in angiogenesis progression. *FASEB J.* 14, 2477-2485.

Elkin, M., Reich, R., Nagler, A., Aingorn, E., Pines, M., de-Groot, N., Hochberg, A., and Vlodavsky, I. (1999). Inhibition of matrix metalloproteinase-2 expression and bladder carcinoma metastasis by halofuginone. *Clin. Cancer Res.* 5, 1982-1988.

Genin, O., Rechavi, G., Nagler, A., Ben-Itzhak, O., Nazemi, K.J., and Pines, M. (2008). Myofibroblasts in pulmonary and brain metastases of alveolar soft-part sarcoma: a novel target for treatment? *Neoplasia* 10, 940-948.

Ghosh, A.K. (2002). Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. *Exp. Biol. Med.* 227, 301-314.

Ghosh, A.K., and Vaughan, D.E. (2012). Fibrosis: is it a coactivator disease? *Front. Biosci.* 4, 1556-1570.

Gnainsky, Y., Kushnirsky, Z., Bilu, G., Hagai, Y., Genina, O., Volpin, H., Bruck, R., Spira, G., Nagler, A., Kawada, N., Yoshizato, K., Reinhardt, D.P., Libermann, T.A., Pines, M. (2007). Gene expression during chemically induced liver fibrosis: effect of halofuginone on TGF-beta signaling. *Cell Tissue Res.* 328, 153-166.

Gnainsky, Y., Spira, G., Paizi, M., Bruck, R., Nagler, A., Abu-Amara, S.N., Geiger, B., Genina, O., Monsonogo-Ornan, E., and Pines, M. (2004a). Halofuginone, an inhibitor of collagen synthesis by rat stellate cells, stimulates insulin-like growth factor binding protein-1 synthesis by hepatocytes. *J. Hepatol.* 40, 269-277.

Gnainsky, Y., Spira, G., Paizi, M., Bruck, R., Nagler, A., Abu-Amara, S.N., Geiger, B., Genina, O., Monsonogo-Ornan, E., and Pines, M. (2004b). Halofuginone, an inhibitor of collagen synthesis by rat stellate cells, stimulates insulin-like growth factor binding protein-1 synthesis by hepatocytes. *J. Hepatol.* *40*, 269-277.

Granot, I., Bartov, I., Plavnik, I., Wax, E., Hurwitz, S., and Pines, M. (1991). Increased skin tearing in broilers and reduced collagen synthesis in skin in vivo and in vitro in response to the coccidiostat halofuginone. *Poult. Sci.* *70*, 1559-1563.

Grudzien, M.M., Low, P.S., Manning, P.C., Arredondo, M., Belton, R.J., Jr, and Nowak, R.A. (2009). The antifibrotic drug halofuginone inhibits proliferation and collagen production by human leiomyoma and myometrial smooth muscle cells. *Fertil. Steril.* *93*, 1290-1298.

Jordan, M.C., and Zeplin, P.H. (2012). Local inhibition of angiogenesis by halofuginone coated silicone materials. *J. Mater. Sci. Mater. Med.* *44*, 22-26.

Kamberov, Y.G., Kim, J., Mazitschek, R., Kuo, W.P., and Whitman, M. (2011). Microarray profiling reveals the integrated stress response is activated by halofuginone in mammary epithelial cells. *BMC Res. Notes* *4*, 381.

Kawaguchi, K., Fujii, S., Konishi, I., Nanbu, Y., Nonogaki, H., and Mori, T. (1989). Mitotic activity in uterine leiomyomas during the menstrual cycle. *Am. J. Obstet. Gynecol.* *160*, 637-641.

Keller, T.L., Zocco, D., Sundrud, M.S., Hendrick, M., Edenius, M., Yum, J., Kim, Y.J., Lee, H.K., Cortese, J.F., Wirth, D.F., Dignam, J.D., Rao, A., Yeo, C.Y., Mazitschek, R., Whitman, M. (2012). Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase. *Nat. Chem. Biol.* *8*, 311-317.

Kroon, B., Johnson, N., Chapman, M., Yazdani, A., Hart, R., and on behalf of the Australasian CREI Consensus Expert Panel on Trial evidence (ACCEPT) group. (2011). Fibroids in infertility - consensus statement from ACCEPT (Australasian CREI Consensus Expert Panel on Trial evidence). *Aust. N. Z. J. Obstet. Gynaecol.* *51*, 289-295.

Lee, D.W., Gibson, T.B., Carls, G.S., Ozminkowski, R.J., Wang, S., and Stewart, E.A. (2009). Uterine fibroid treatment patterns in a population of insured women. *Fertil. Steril.* *91*, 566-574.

Leiba, M., Jakubikova, J., Klippel, S., Mitsiades, C.S., Hideshima, T., Tai, Y.T., Leiba, A., Pines, M., Richardson, P.G., Nagler, A., and Anderson, K.C. (2012). Halofuginone inhibits multiple myeloma growth in vitro and in vivo and enhances cytotoxicity of conventional and novel agents. *Br. J. Haematol.* *157*, 718-731.

Leppert, P.C., Baginski, T., Prupas, C., Catherino, W.H., Pletcher, S., and Segars, J.H. (2004). Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertil. Steril.* 82 Suppl 3, 1182-1187.

Leppert, P.C., Catherino, W.H., and Segars, J.H. (2006). A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. *Obstet. Gynecol.* 195, 415-420.

Madsen, D.H., Jurgensen, H.J., Ingvarsen, S., Melander, M.C., Vainer, B., Egerod, K.L., Hald, A., Rono, B., Madsen, C.A., Bugge, T.H., Engelholm, L.H., and Behrendt, N. (2012). Endocytic collagen degradation: a novel mechanism involved in protection against liver fibrosis. *J. Pathol.* 227, 94-105.

Makinen, N., Mehine, M., Tolvanen, J., Kaasinen, E., Li, Y., Lehtonen, H.J., Gentile, M., Yan, J., Enge, M., Taipale, M., Aavikko, M., Katainen, R., Virolainen, E., Böhling, T., Koski, T.A., Launonen, V., Sjöberg, J., Taipale, J., Vahteristo, P., Aaltonen, L.A. (2011). MED12, the Mediator Complex Subunit 12 Gene, Is Mutated at High Frequency in Uterine Leiomyomas. *Science.* 76, 30-33.

McGaha, T.L., Phelps, R.G., Spiera, H., and Bona, C. (2002). Halofuginone, an Inhibitor of Type-I Collagen Synthesis and Skin Sclerosis, Blocks Transforming-Growth-Factor-[bgr]-Mediated Smad3 Activation in Fibroblasts. *J. Invest. Dermatol.* 118, 461-470.

Meloni, A.M., Surti, U., Contento, A.M., Davare, J., and Sandberg, A.A. (1992). Uterine leiomyomas: cytogenetic and histologic profile. *Obstet. Gynecol.* 80, 209-217.

Nagler, A., Gofrit, O., Ohana, M., Pode, D., Genina, O., and Pines, M. (2000). The effect of halofuginone, an inhibitor of collagen type i synthesis, on urethral stricture formation: in vivo and in vitro study in a rat model. *J. Urol.* 164, 1776-1780.

Nagler, A., Katz, A., Aingorn, H., Miao, H.Q., Condiotti, R., Genina, O., Pines, M., and Vlodavsky, I. (1997). Inhibition of glomerular mesangial cell proliferation and extracellular matrix deposition by halofuginone. *Kidney Int.* 52, 1561-1569.

Nelson, E.F., Huang, C.W., Ewel, J.M., Chang, A.A., and Yuan, C. (2012). Halofuginone down-regulates Smad3 expression and inhibits the TGFbeta-induced expression of fibrotic markers in human corneal fibroblasts. *Mol. Vis.* 18, 479-487.

Peng, W., Robertson, L., Gallinetti, J., Mejia, P., Vose, S., Charlip, A., Chu, T., and Mitchell, J.R. (2012). Surgical stress resistance induced by single amino Acid deprivation requires gcn2 in mice. *Sci. Transl. Med.* 4, 118ra11.

Pines, M., and Halevy, O. (2011). Halofuginone and muscular dystrophy. *Histol. Histopathol.* 26, 135-146.

Pines, M., Vlodaysky, I., and Nagler, A. (2000). Halofuginone: From veterinary use to human therapy. *Drug Dev. Res.* *50*, 371-378.

Pinkerton, J.V. (2011). Pharmacological therapy for abnormal uterine bleeding. *Menopause* *18*, 453-461.

Popov, Y., Patsenker, E., Bauer, M., Niedobitek, E., Schulze-Krebs, A., and Schuppan, D. (2006). Halofuginone induces matrix metalloproteinases in rat hepatic stellate cells via activation of p38 and NFkappaB. *J. Biol. Chem.* *281*, 15090-15098.

Roeder, H.A., Cramer, S.F., and Leppert, P.C. (2012). A look at uterine wound healing through a histopathological study of uterine scars. *Reprod. Sci.* *19*, 463-473.

Roffe, S., Hagai, Y., Pines, M., and Halevy, O. (2010). Halofuginone inhibits Smad3 phosphorylation via the PI3K/Akt and MAPK/ERK pathways in muscle cells: effect on myotube fusion. *Exp. Cell Res.* *316*, 1061-1069

Sato, S., Kawamura, H., Takemoto, M., Maezawa, Y., Fujimoto, M., Shimoyama, T., Koshizaka, M., Tsurutani, Y., Watanabe, A., Ueda, S., Halevi, K., Saito, Y., Yokote, K. (2009). Halofuginone prevents extracellular matrix deposition in diabetic nephropathy. *Biochem. Biophys. Res. Commun.* *379*, 411-416.

Stewart, E.A. (2002). Nonsurgical treatment of uterine fibroids. *Infertility and Reproductive Medicine Clinics of North America* *13*, 393-405.

Stewart, E.A. (2001). Uterine fibroids. *Lancet.* *357*, 293-298.

Stewart, E.A., Friedman, A.J., Peck, K., and Nowak, R.A. (1994). Relative overexpression of collagen type I and collagen type III messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. *J. Clin. Endocrinol. Metab.* *79*, 900-906.

Stewart, E.A., and Morton, C.C. (2006). The genetics of uterine leiomyomata: what clinicians need to know. *Obstet Gynecol* *107*, 917-921.

Stewart, E.A., and Nowak, R.A. (1998). New concepts in the treatment of uterine leiomyomas. *Obstet. Gynecol.* *92*, 624-627.

Tacheau, C., Michel, L., Farge, D., Mauviel, A., and Verrecchia, F. (2007). Involvement of ERK signaling in halofuginone-driven inhibition of fibroblast ability to contract collagen lattices. *Eur. J. Pharmacol.* *573*, 65-69.

Taras, D., Blanc, J.F., Rullier, A., Dugot-Senant, N., Laurendeau, I., Bieche, I., Pines, M., and Rosenbaum, J. (2006). Halofuginone suppresses the lung metastasis of chemically induced hepatocellular carcinoma in rats through MMP inhibition. *Neoplasia* *8*, 312-318.

Thompson, K.J., Lakner, A.M., Cross, B.W., Tsukada, S., Rippe, R.A., McKillop, I.H., and Schrum, L.W. (2011). S-adenosyl-L-methionine inhibits collagen secretion in hepatic stellate cells via increased ubiquitination. *Liver Int.* *31*, 891-901.

Wallach, E.E., and Vlahos, N.F. (2004). Uterine myomas: an overview of development, clinical features, and management. *Obstet. Gynecol.* *104*, 393-406.

Xavier, S., Piek, E., Fujii, M., Javelaud, D., Mauviel, A., Flanders, K.C., Samuni, A.M., Felici, A., Reiss, M., Yarkoni, S., Sowers, A., Mitchell, J.B., Roberts, A.B., Russo, A. (2004). Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor-beta signaling by halofuginone. *J. Biol. Chem.* *279*, 15167-15176.

Yee, K.O., Connolly, C.M., Pines, M., and Lawler, J. (2006). Halofuginone inhibits tumor growth in the polyoma middle T antigen mouse via a thrombospondin-1 independent mechanism. *Cancer. Biol. Ther.* *5*, 218-224.

Zion, O., Genin, O., Kawada, N., Yoshizato, K., Roffe, S., Nagler, A., Iovanna, J.L., Halevy, O., and Pines, M. (2009). Inhibition of transforming growth factor beta signaling by halofuginone as a modality for pancreas fibrosis prevention. *Pancreas* *38*, 427-435.

Chapter Five

Anti-fibrotic Effects of Halofuginone on Human Uterine Leiomyoma Smooth Muscle Cells in a Mouse Xenograft Model

Abstract

Uterine leiomyomas (UL) are the most common pelvic tumor in reproductive-aged women arising from localized fibrosis in the myometrium. Hormone therapy and hysterectomy are the leading management options for these benign tumors that are associated with abnormal uterine bleeding, anemia, pelvic pain, recurrent pregnancy loss and infertility. In an attempt to find an alternative therapeutic treatment for ULs with more specificity and fewer side effects, we evaluated the potential of the anti-fibrotic drug halofuginone (HF) in reducing fibrosis and growth of UL xenograft tumors. Treatment of mice carrying xenografts of primary human leiomyoma smooth muscle cells (LSMCs) underneath the kidney capsule with either 0.25 or 0.50 mg/kg body weight/day HF for four weeks resulted in a 35-40% ($P < 0.05$) reduction in tumor volume. At the cellular level, this decrease was accompanied by increased apoptosis and decreased cell proliferation. Masson's trichrome staining as well as specific staining for collagens I and III did not show any changes in collagen content for either dose of HF during the treatment period. Quantitative RT-PCR on xenografts from mice treated with HF did not show reductions in the transcript levels of major genes involved in fibrosis, except for TGF β 1. While neither dose of HF caused any harmful side effects in major body organs of the mouse hosts, the higher dose of 0.5 mg/kg body weight/day was not well tolerated by mice as indicated by decreased appetite, weight loss and some deaths. In conclusion, HF was found to be effective in reducing UL tumor growth by

interfering with major cellular processes regulating cell proliferation and death. The use of this mouse xenograft for human UL appears to be a valid model for pre-clinical drug trials for ULs.

Introduction

Tissue fibrosis in major body organs such as heart, kidney and liver has been the underlying cause of numerous diseases associated with high morbidity and mortality (Ghosh, 2002; 1985; Last, 1985; Schuppan and Pinzani, 2012). A similar fibrotic process occurs in uterine myometrium of reproductive-aged women, which, despite its high prevalence of 70-80%, is not well understood. These benign tumors arising from increased proliferation and collagen deposition of uterine smooth muscle cells are called uterine leiomyomas (UL) and can lead to symptoms such as abnormal uterine bleeding, anemia, pelvic pain and pressure, recurrent miscarriages and infertility (Levy. et al, 2012; Medikare *et al.*, 2011).

While the etiology and pathogenesis of fibroids are still unknown, development and growth of ULs have been linked to age, ethnicity, parity, environmental factors, and specifically to ovarian steroid hormones (Arslan *et al.*, 2005; Catherino *et al.*, 2011; Flake *et al.*, 2003; Gao *et al.*, 2010; Stewart and Morton, 2006). Individual and combination of these factors are known to manifest their effects as increased rate of cell proliferation and excess deposition of extracellular matrix (ECM) collagens in the fibrotic leiomyoma tissue (Dixon *et al.*, 2002; Kawaguchi *et al.*, 1989; Leppert *et al.*, 2004; Stewart *et al.*, 1994).

Major management options for UL tumors are currently limited to either hormone therapy or surgical intervention in the form of myomectomy or hysterectomy. Although

these treatments provide temporary relief of symptoms or may permanently alleviate the problem all together, they are associated with serious side effects and high costs.

Hormone therapy causes menopause-associated symptoms such as hot flashes and osteoporosis (Chavez and Stewart, 2001; Pinkerton, 2011; Stewart, 2002). Surgical interventions can lead to reduced fecundity, hemorrhage or in rare instances, death (Al-Hendy and Salama, 2006). It has been estimated that the average indirect and direct costs of ULs in the USA alone are as high as \$5.9-9.34 billion annually (Cardozo *et al.*, 2011). It is, therefore, important to develop alternative cost-effective and more efficient treatments for ULs.

Among currently studied anti-fibrotic drugs, halofuginone (HF) has shown high efficiency in reducing fibrosis and disease burden in a variety of fibrotic diseases (Dabak *et al.*, 2010; Huebner *et al.*, 2008; Karatas *et al.*, 2008; Nelson *et al.*, 2012; Pines and Halevy, 2011; Pines *et al.*, 2003; Zeplin *et al.*, 2010; Zeplin, 2012). HF, which is a halogenated derivative of ferbrifugine, has been used as an antimalarial agent in China and an antiprotozoal agent in the poultry and cattle industries (Derbyshire *et al.*, 2012; Dorne *et al.*, 2011; Pines *et al.*, 2000; Trotz-Williams *et al.*, 2011). Extensive *in vitro* studies in a variety of fibrotic cells as well as tumor cells indicate that HF specifically targets collagens and MMPs, cell proliferation and angiogenesis through signaling pathways such as TGF β -SMADs, MAPK and the amino acid starvation response system (Genin *et al.*, 2008; Gnainsky *et al.*, 2007; McGaha *et al.*, 2002; Nelson *et al.*, 2012; Sato *et al.*, 2009; Tacheau *et al.*, 2007; Yee *et al.*, 2006). Animal models for fibrotic disease such as Duchenne muscle dystrophy (DMD), scleroderma, diabetic nephropathy, liver cirrhosis, and pulmonary fibrosis have all been successfully

used to show the *in vivo* anti-fibrotic effects of HF in both the establishment and development of tissue fibrosis (Huebner *et al.*, 2008; Pines *et al.*, 2001; Sheffer *et al.*, 2007; Spector *et al.*, 2010; Spira *et al.*, 2002; Turgeman *et al.*, 2008). More recently, HF has been shown to induce anti-proliferative, anti-angiogenic, and anti-metastatic processes in tumor models of sarcoma, brain, bladder, breast, hepatocellular carcinoma, multiple myeloma and prostate cancer (Cook *et al.*, 2010; de Figueiredo-Pontes *et al.*, 2011; de Jonge *et al.*, 2006; Jordan and Zeplin, 2012; Koon *et al.*, 2011; Leiba *et al.*, 2012; Taras *et al.*, 2006).

We have previously shown that HF induces apoptosis and inhibits basal and serum-stimulated cell proliferation in primary human LSMCs *in vitro* (Grudzien *et al.*, 2009). Furthermore, HF targeted the expression of collagens I and III, which are the two major ECM proteins upregulated in ULs. Studies in Chapter 4 of this dissertation have shown that these inhibitory effects of HF are mediated through effects on the TGF β , SRC and MAKP signaling pathways. Taken together, our data suggest the potential of HF as an alternative therapeutic treatment for human ULs.

The aim of the present study was to test the inhibitory effects of HF on human uterine LSMCs xenografted into immunocompromised mouse hosts. Effects of HF on tumor growth were assessed by measuring changes in tumor volume and examining changes in cell proliferation and apoptosis. Daily body weight measurements and pathological analysis of several key organs were used to assess potential side effects of the drug on mouse hosts. Our results showed a strong inhibitory effect of HF on the growth of ULs through direct effects on cell proliferation and apoptosis, which can serve as a preclinical framework for future clinical studies on HF in ULs.

Results

Effect of Different Doses of Halofuginone on Mouse Host Body Weights

To assess the effect of HF treatment on the overall health of mice hosts, two doses of HF at 0.25 and 0.50 mg/kg/body weight along with vehicle controls were used for treatment of UL-xenografted mice. Intraperitoneal (i.p.) administration of HF was scheduled on a daily basis for 5 days a week for 4 weeks (Fig. 5.1). Daily weight measurement of mice indicated that treatment of mice with 0.25 mg/kg/body weight on a daily basis was well tolerated and compared to vehicle-treated animals no change in body weight was observed in this group (Fig. 5.2A). In contrast, the higher dose (0.5 mg/kg/body weight) of HF caused significant loss of appetite and some deaths in the animals (Fig. 5.2B). We therefore, split animals on the higher dose of treatment into two groups. One group continued to receive daily treatments of 0.5mg/kg while the other received this higher dose treatment of HF every-other-day (EOD). This change in the treatment plan seemed to help animals better tolerate the higher dose as observed by recovered appetite and more stable body weights (Fig. 5.2C). These observations showed that 0.25 mg/kg body weight HF treatment administered daily or 0.50 mg body weight administered EOD, respectively; were better treatment schemes compared to daily treatment with the higher dose.

Effect of Halofuginone on Growth of UL-Xenografted Tumors

To determine whether treatment of UL-xenografted tumors with HF could reduce the size of tumors, mice hosts were treated for 4 weeks with either vehicle, 0.25 mg/kg/body weight of HF daily for 5 days a week, or 0.5 mg/kg/body weight of HF daily or EOD for five days a week. Measurement of xenografted tumor volumes at the end of 4 weeks for

each treatment group showed that, in comparison to tumors from matched vehicle-treated mice, all HF treatment schemes had significantly reduced tumor volumes (Fig 5.3). Interestingly, the percent reduction achieved by the lower dose (0.25) was similar (40%) to that achieved with the higher dose (0.5) given on a daily basis. The EOD treatment with 0.50 mg/kg/body weight reduced tumor volume by 35% which was not significantly different from the other two treatment schemes. These data demonstrate the potent inhibitory effect of HF on the growth of ULs within a relatively short treatment period.

Effect of Halofuginone on Cell Proliferation and Apoptosis in UL-Xenografted Tumors

To investigate the underlying mechanisms for reduction in tumor volumes in response to HF treatment, cell proliferation and apoptotic programmed cell death were assessed in UL-xenografts. Specimens from both vehicle and HF treatments were analyzed by immunohistochemistry for Ki67, a marker for cell proliferation, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect apoptosis. Results showed that four weeks of treatment with 0.25 mg/kg/body weight HF daily or 0.50 mg/kg/body weight HF EOD reduced LSMC proliferation and increased apoptotic programmed cell death compared to vehicle-treated xenografts (Fig. 5.4 & 5.5). Overall, the results support that HF targets cell proliferation along with apoptosis in LSMCs resulting in reduced tumor volume.

Effect of Halofuginone on Collagens I and III Expression in UL-Xenografted

Tumors

The anti-fibrotic drug HF has been shown to target collagens in both *in vitro* and *in vivo* systems. In UL-xenografts, treatment with 0.25 mg/kg/body weight HF daily or 0.50 mg/kg/body weight HF EOD for four weeks did not alter the expression of collagen I and III proteins as observed by Masson's trichrome staining or IHC (Fig. 5.6-8). Quantitative RT-PCR with primer probes specific to *COL1A1* and *COL3A1* on a limited number of vehicle (n=1 from one patient and 4 from the second patient) and HF-treated (n=3 from one patient and 1 from the second patient) xenografts from two patient specimens at the 0.5 mg/kg body weight EOD treatment showed non-significant decreases for *COL1A1* mRNA, but with a significant increase for *COL3A1* mRNA (Fig. 5.9A&B). These results are contrary to our hypothesis of an inhibitory effect of HF on collagen I and III expression.

Effect of Halofuginone on Other Pro-Fibrotic Factors in UL-Xenografted Tumors

To investigate whether HF could alter the expression of fibrotic factors other than collagens I and III, we also examined the expression of TGF β 1, TGF β 3, lysyl oxidase III, dermatopontin and TGF β RII at either the protein or transcript levels. Quantitative RT-PCR on a limited number of xenografts treated with 0.5 mg/kg body weight of HF EOD for 4 weeks, showed no changes at the transcript level of any of these genes except for a significant decrease in TGF β 1 (Fig. 5.9A-F). Immunohistochemical analysis of xenografts treated with 0.25 mg/kg body weight HF or vehicle on a daily basis did not show any significant differences in dermatopontin or TGF β RII protein levels in xenografts (Fig. 5.10A-D). In spite of the limited number of xenografts analyzed for

these studies, our current results support targeting of the TGF β pathway as a possible underlying mechanism behind the inhibitory effect of HF on UL-xenografted tumors.

Effect of Halofuginone on Specific Organs/Tissues of Mouse Hosts

To evaluate potential side effects of HF treatment on mice hosts, major body organs including lung, liver, heart, kidney, female reproductive tract, spleen, bladder, pancreas and skin were collected from vehicle and HF-treated animals and subjected to blind examination by a veterinary pathologist. Gross analysis of tissues stained with hematoxylin and eosin for both vehicle and HF-treated animals in all treatment schemes showed no sign of abnormal pathology resulting from any of the treatments (Fig. 5.11). These results indicate the relative safety of HF treatment at either 0.25 or 0.50 mg/kg body weight/day for mouse hosts.

Discussion

HF has been shown to have inhibitory effects on cell proliferation, collagen expression, angiogenesis, and tumor progression in numerous animal models for tissue fibrosis and cancer. These well-known characteristics of HF along with our own *in vitro* studies with HF led us to investigate whether similar inhibitory effects would occur in an *in vivo* model of ULs. The results of the present study, using three different treatment plans with HF, demonstrated the potent inhibitory effects of HF on the growth of UL-xenografts in a four week treatment period.

Growth of pre-existing or newly formed tumors and fibrotic tissues of liver, bladder, skin, breast, prostate, and adrenal gland in animal models has been shown to be inhibited by microgram treatments of HF over a 4-8 week treatment period (Huebner *et al.*, 2008; Pines *et al.*, 2001; Sheffer *et al.*, 2007; Spector *et al.*, 2010; Spira *et al.*,

2002; Turgeman *et al.*, 2008; Cook *et al.*, 2010; de Figueiredo-Pontes *et al.*, 2011; de Jonge *et al.*, 2006; Jordan and Zeplin, 2012; Koon *et al.*, 2011; Leiba *et al.*, 2012; Taras *et al.*, 2006;). In UL-xenografts, both the low and high doses of HF at 0.25 and 0.50 mg/kg/day, respectively, reduced the volume of tumors by similar levels of 35-40%. In a similar study, administration of 10 µg HF three times a week after implantation of pancreatic tumor cells into nude mice resulted in 50% reduction in tumor development in a month (Spector *et al.*, 2010). Xenografted Wilms tumor as well as prostate cancer treated with lesser doses of HF showed significant decreases in tumor volume (Gavish *et al.*, 2002; Pinthus *et al.*, 2005).

Moreover, the inhibitory effect of HF in reported *in vivo* studies was found to be specific to the diseased tissue, leaving healthy tissues unaffected. HF treatment of normal mice, which served as control for mice induced for cirrhosis, did not interfere with normal collagen synthesis and liver regeneration (Spira *et al.*, 2002). In a mouse model of muscular dystrophy, HF treatment at 5 µg for 10 weeks caused a significant decrease in fibrosis only in organs like diaphragm where the highest collagen content was present (Turgeman *et al.*, 2008). Although we did not measure the rate of collagen synthesis in mouse tissues, morphological characteristics of host tissues treated with either doses of HF seemed to be normal and intact. The observed loss of appetite and weight loss in mice treated daily with the higher dose of HF seems to be a side effect of this drug at higher doses, which has also been reported in other studies. In a clinical study on patients with advanced solid tumors, a similar side effect on the gastrointestinal system was documented for patients taking a high dose of HF (de Jonge *et al.*, 2006). The same study also showed that treatment of mice with 6

mg/kg/day HF leads to mortality. A very limited number of patients under clinical testing of HF for AIDS-related Kaposi Sarcoma showed skin irritations after the topical application of the drug (Koon *et al.*, 2011). Taken together, our findings suggest that there is a threshold dose for HF above which no further inhibitory effect can be achieved without affecting the overall health of the host animal.

To understand the underlying cellular processes targeted by HF, which could contribute to tumor reduction, we looked into two major regulators of cell growth: cell proliferation and death. Our results showed that both doses of HF reduced the rate of cell proliferation along with an induction of apoptosis in xenografted-LSMCs. In addition to several *in vitro* studies confirming such observations, *in vivo* studies on HF validate alterations of cell proliferation and apoptosis as mechanisms involved in the inhibitory effects of HF on tumor development (Elkin *et al.*, 1999; Gavish *et al.*, 2002; Huebner *et al.*, 2008; Pines *et al.*, 2001; Sheffer *et al.*, 2007; Spira *et al.*, 2002). Interestingly, in all these studies, the effect of HF on these two major cellular pathways were found to be specific to cells with dysregulated rate of proliferation belonging to the TGF β -dependent fibrotic organs (Levi-Schaffer *et al.*, 1996; Pines *et al.*, 2001; Spector *et al.*, 2010)

In addition to cell proliferation and death, tumor growth is dependent on other factors such as individual cell size, abundance of ECM proteins and the water content within tissues (Jackson and Byrne, 2002). The first well characterized property of HF is its specific targeting of collagen α 1 in fibroblast-like cells (Pines *et al.*, 2000). Indeed, numerous *in vivo* and *in vitro* studies on fibrosis and cancers as well as evidence from clinical studies on HF are in support of this inhibitory effect (Huebner *et al.*, 2008; Leiba *et al.*, 2012; Pines and Halevy, 2011; Pines *et al.*, 2003; Turgeman *et al.*, 2008). The

lack of a significant change in ECM collagen I and III proteins or mRNAs in UL-xenografts treated with either doses of HF does not support our hypothesis on the effect of HF on ECM production or turnover in xenografted-ULs. Besides the availability of very limited number of samples for mRNA analysis in our study, it is possible that the lack of such effect is due to the four-week treatment regimen for HF. This time frame may not have provided enough time for HF to cause measurable changes in production or degradation of the stable, highly cross linked collagens in UL tissue. It will be very important to repeat this analysis with a greater number of samples to confirm the observed targeting of collagen genes by HF that we observed in our *in vitro* study with LSMCs. Furthermore, extending the treatment period to longer times will help in understanding the effect of HF on collagen content in xenografted-ULs.

Tissue fibrosis is thought to occur following a dysregulated healing response to tissue injury (Ghosh, 2002). During this process, cells and tissues are subjected to ongoing synthesis and turnover of ECM proteins through activation of enzymes such as prolyl hydroxylase and matrix metalloproteinases (MMPs). A major growth factor involved in fibrosis and tumorigenesis is TGF β , which induces the synthesis of many ECM proteins including collagens and also inhibits the expression of anti-fibrotic MMPs for ECM degradation (Flanders, 2004; Horbelt *et al.*, 2012). The inhibitory effects of HF on tissue fibrosis and angiogenesis have been shown to be strongly mediated through modulation of the TGF β pathway (Barrientos *et al.*, 2008; Nelson *et al.*, 2012; Wynn, 2008; Zion *et al.*, 2009; Turgeman *et al.*, 2008). In an animal model of diabetic nephropathy, 1mg treatment with HF on a daily basis for 15 weeks decreased the mRNA expression of TGF β R1 and R2 with no effect on the TGF β 1 mRNA (Sato *et al.*,

2009). Down-regulation of TGF β RII by HF has also been observed in mammary gland epithelial cells (Xavier *et al.*, 2004). On the other hand, McGaha *et al.* found no change on either receptors at 10⁻⁸ M in fibroblasts (McGaha *et al.*, 2002; Zion *et al.*, 2009) and in leukemia cell lines HF, in a dose-dependent manner, upregulated TGF β 1 and TGF β R1 (de Figueiredo-Pontes *et al.*, 2011). In pancreatic stellate cells, HF acted downstream of TGF β receptors (Popov *et al.*, 2006; Zion *et al.*, 2009).

In LSMCs, different doses of HF were shown to reduce levels of TGF β 1 mRNA, but not TGF β 3 (Grudzien *et al.*, 2009). Our results with TGF β signaling further confirm the differential, cell-type dependent effect of HF on this signaling pathway. In UL-xenografted tumors treated with 0.50 mg/kg/body weight of HF we observed no changes at TGF β RII protein level or TGF β 3 mRNA. However, transcript levels of TGF β 1 were significantly reduced in UL-xenografts treated with 0.5 mg/kg body weight HF. In addition, although the inhibitory effect of HF on the expression of the cross-linking enzyme lysyl oxidase has been reported in hepatocyte after 8 weeks of treatment (Gnainsky *et al.*, 2007), we did not observe any changes in the expression of this gene in UL-xenografts treated with the higher dose of HF. Due to the limited number of xenografts available for analysis, we can hypothesize that with a greater sample size we might see statistical differences or if we used an alternative method such as in situ hybridization to assess differences. Moreover, it is possible that extending the HF treatment period to longer than 4 weeks would result in significant changes in the expression of fibrotic factors.

In conclusion, findings of our current study demonstrate an inhibitory effect of HF on human UL-xenografts in a mouse model and support this model as a pre-clinical

framework for further work on this anti-fibrotic drug. Both 0.25 and 0.50 mg/kg body weight/day HF used in our study significantly reduced tumor volume through an increase in apoptosis and a reduction in cell proliferation after 4 weeks of treatment without any pathological side effects on host animals. However, the specific pathways and molecular regulators of these processes remain unknown. Based on mechanistic studies of HF in other fibrotic and cancerous cells, one would speculate that pathways such as TGF β -SMADs, MAPK, and immune regulatory systems could be altered in UL-xenografted tumors by HF. Further understanding of molecular mechanisms underlying the effects of HF in ULs can lead the way for designing derivatives of HF, which would have maximum target efficiency for better management treatment of UL tumors in diseased patients with minimum side effects.

Figures

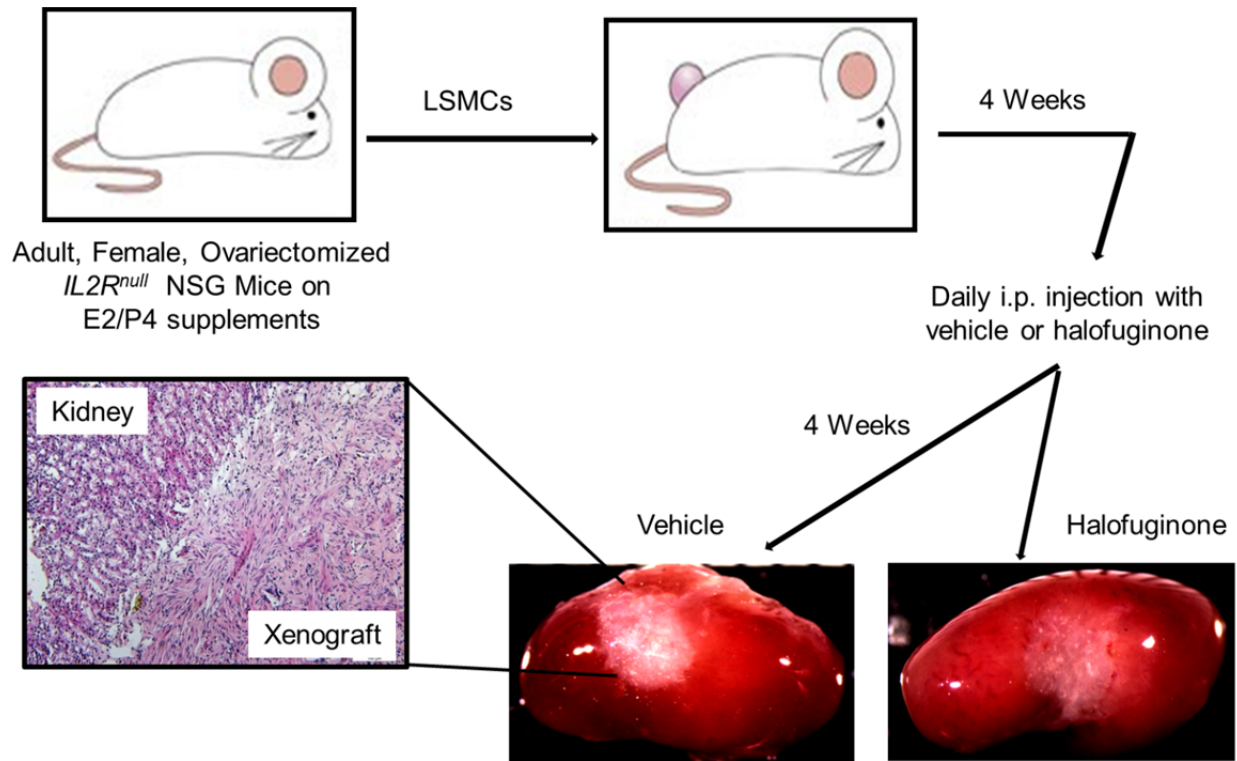


Figure 5.1. Treatment schemes for UL-xenografted mice. LSMCs embedded in a collagen gel were transplanted under the kidney capsule of 8-week-old, immunocompromised, nude female mice. Four weeks after the establishment of the tumors, mice were divided into two groups of either vehicle or HF treatment. The three HF treatment schemes used were 0.25 mg/kg body weight daily or 0.5 mg/kg body weight daily or every-other-day via *i.p.* injection. Staining of mouse kidney shows UL-xenografted tissue adjacent to mouse nephrons.

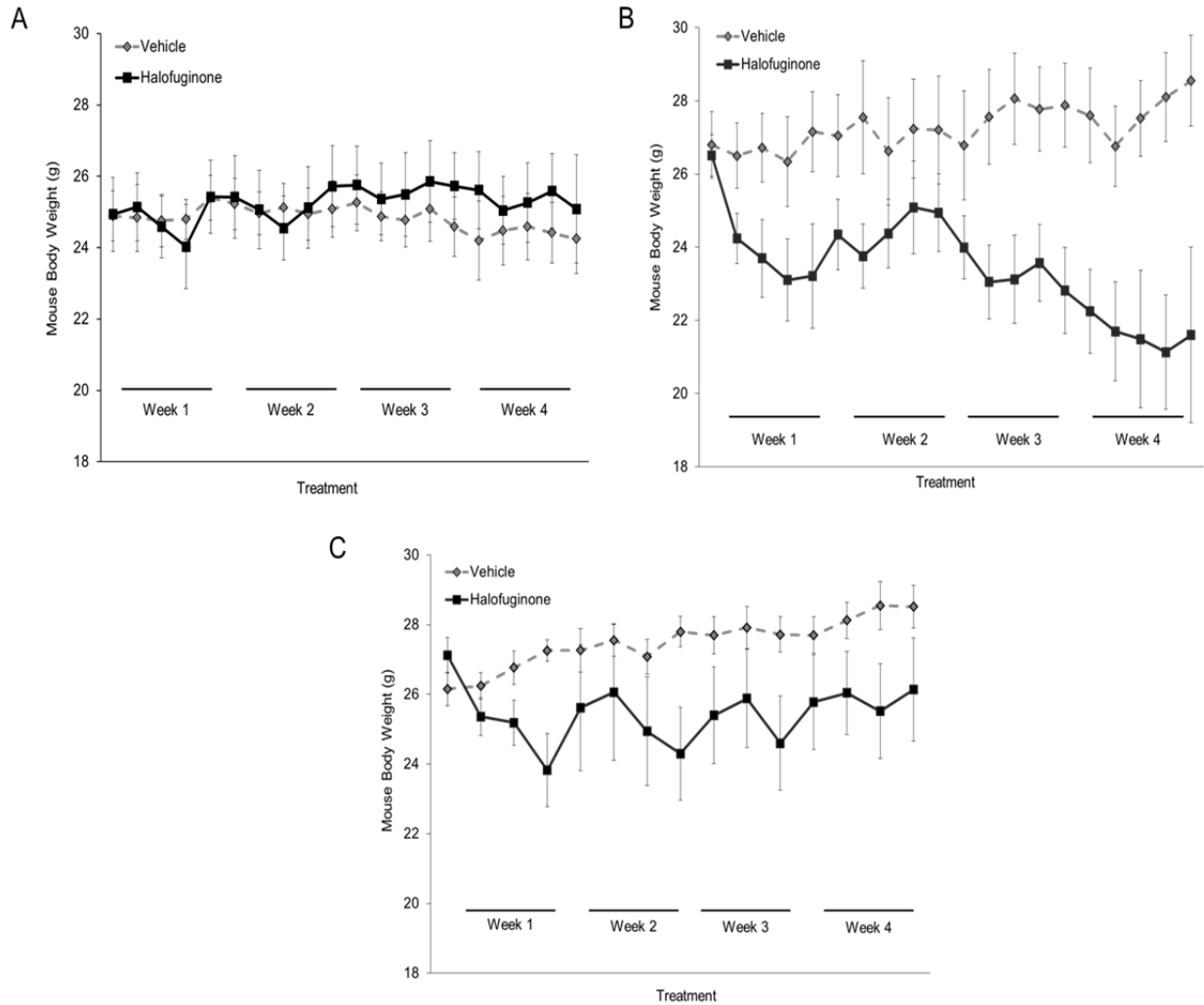


Figure 5.2. Effect of HF treatment on the body weight of mouse hosts. Beginning from the first day of treatment, body weight measurements of mice treated with vehicle or HF were recorded every day for the 4-week duration of treatments. (A) Body weight of mice treated with vehicle (n=6) or 0.25 mg/kg body weight HF (n=8) on a daily basis; (B) Body weight of mice treated with vehicle (n=7) or 0.50 mg/kg body weight HF (n=8) on a daily basis; (C) Body weight of mice treated with vehicle (n=6) or 0.50 mg/kg body weight HF (n=6) every-other-day.

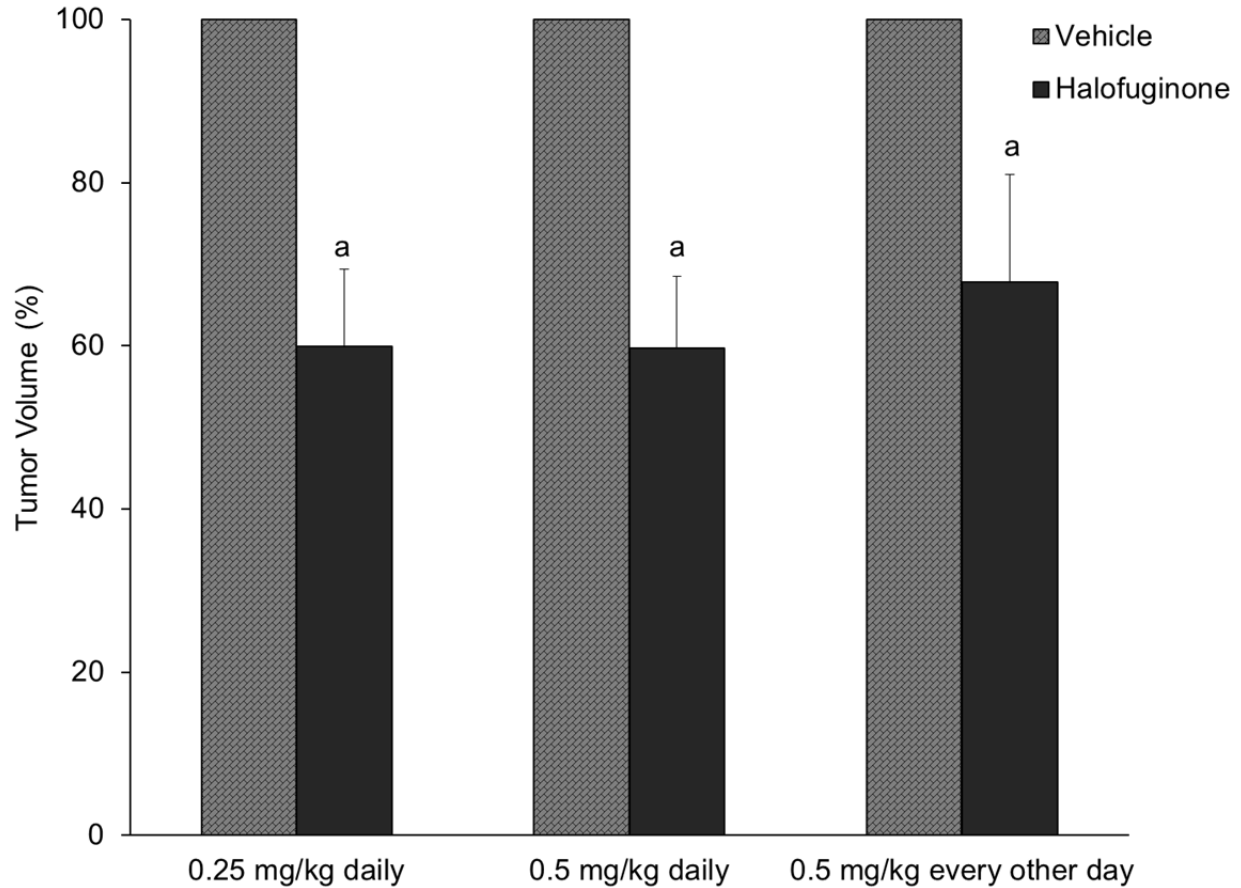


Figure 5.3. Effect of HF treatment on volume of UL-xenografts. Primary LSMCs embedded in collagen gels were grown as subrenal grafts in mouse hosts and treated with either vehicle or HF for 4 weeks. At the end of the treatment period, grafts were harvested and the volumes of the xenografts were measured and normalized to the volumes of matched vehicle-treated xenografts from the same patient specimen. Statistical significance relative to vehicle-treated xenografts within each treatment scheme is indicated by letters. LSMCs from two patients are xenografted into 6-8 animals per treatment group. ($P < 0.05$).

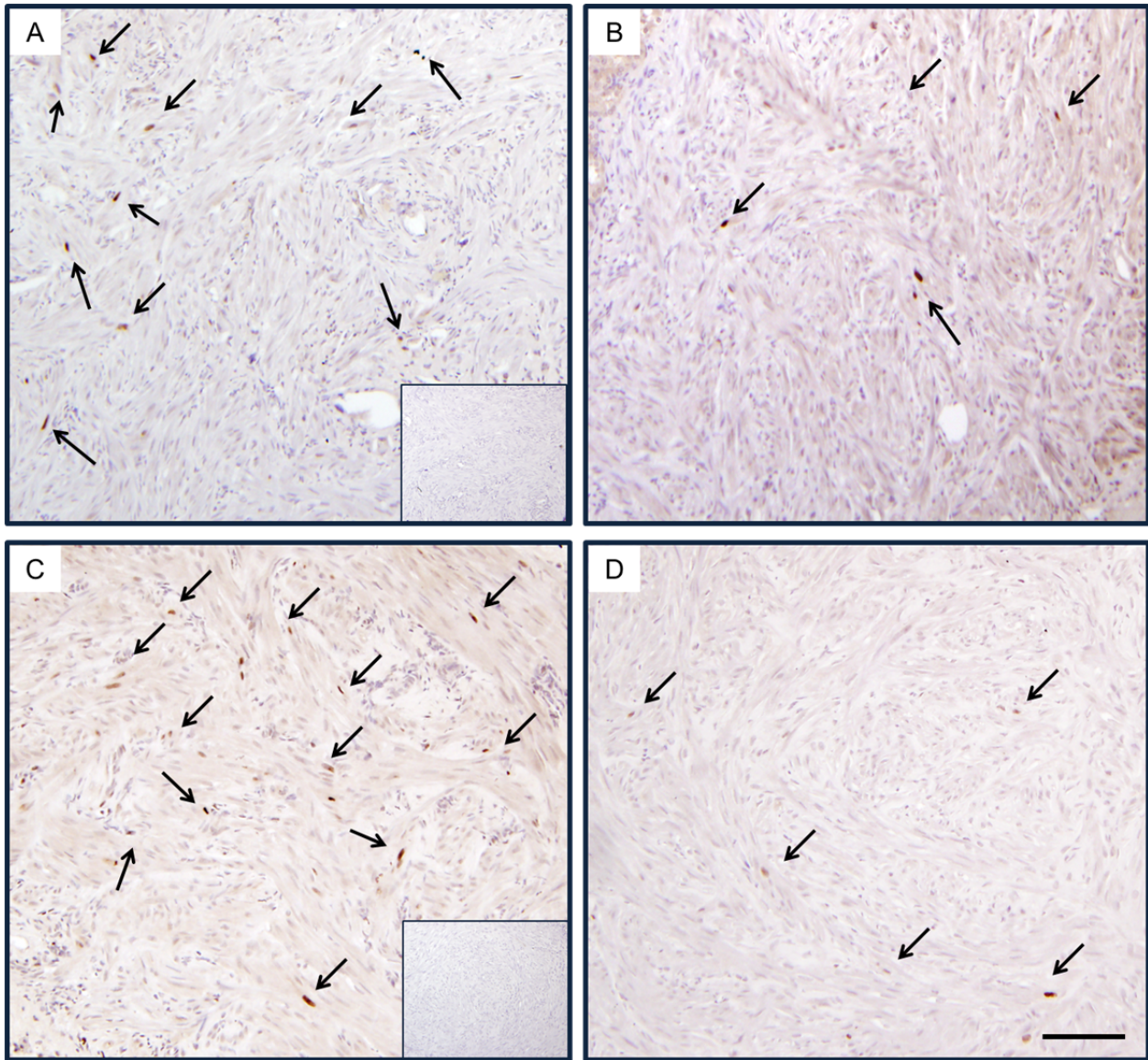


Figure 5.4. Effect of HF treatment on proliferation of ULSMCS in UL-xenografts.

Sections of embedded xenografts treated with vehicle (A & C), 0.25 mg/kg HF daily (B) or 0.50 mg/kg HF EOD (D) were immunostained with an antibody specific for the proliferation marker, Ki67. Images are representative of tumors from either 4 (for 0.25 mg/kg/body weight HF) or 2 (for 0.50 mg/kg/body weight HF) different patients xenografted into 4-6 animals for each treatment (vehicle vs HF). (Bar=100 μ m, insets: negative control).

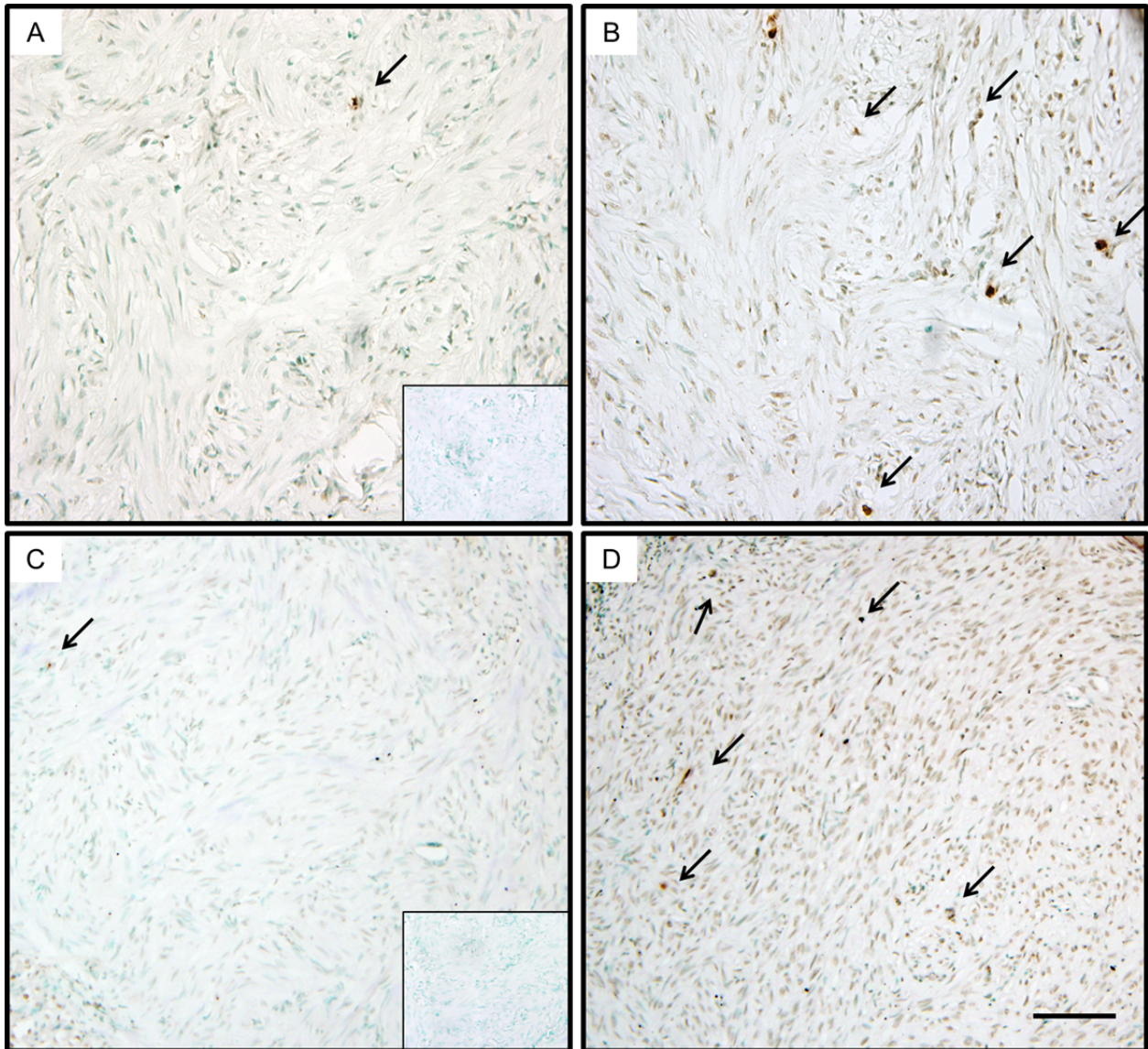


Figure 5.5. Effect of HF treatment on programmed cell death in UL-xenografts.

Sections of embedded xenografts treated with vehicle (A & C), 0.25 mg/kg HF daily (B) or 0.50 mg/kg HF EOD (D) were evaluated for apoptosis using TUNEL assay. Images are representative of either 4 (for low dose HF) or 2 (for high dose HF) patient tumors xenografted on 4-6 animals per vehicle or HF-treated mice. (Bar=100 μ m, insets: negative control)

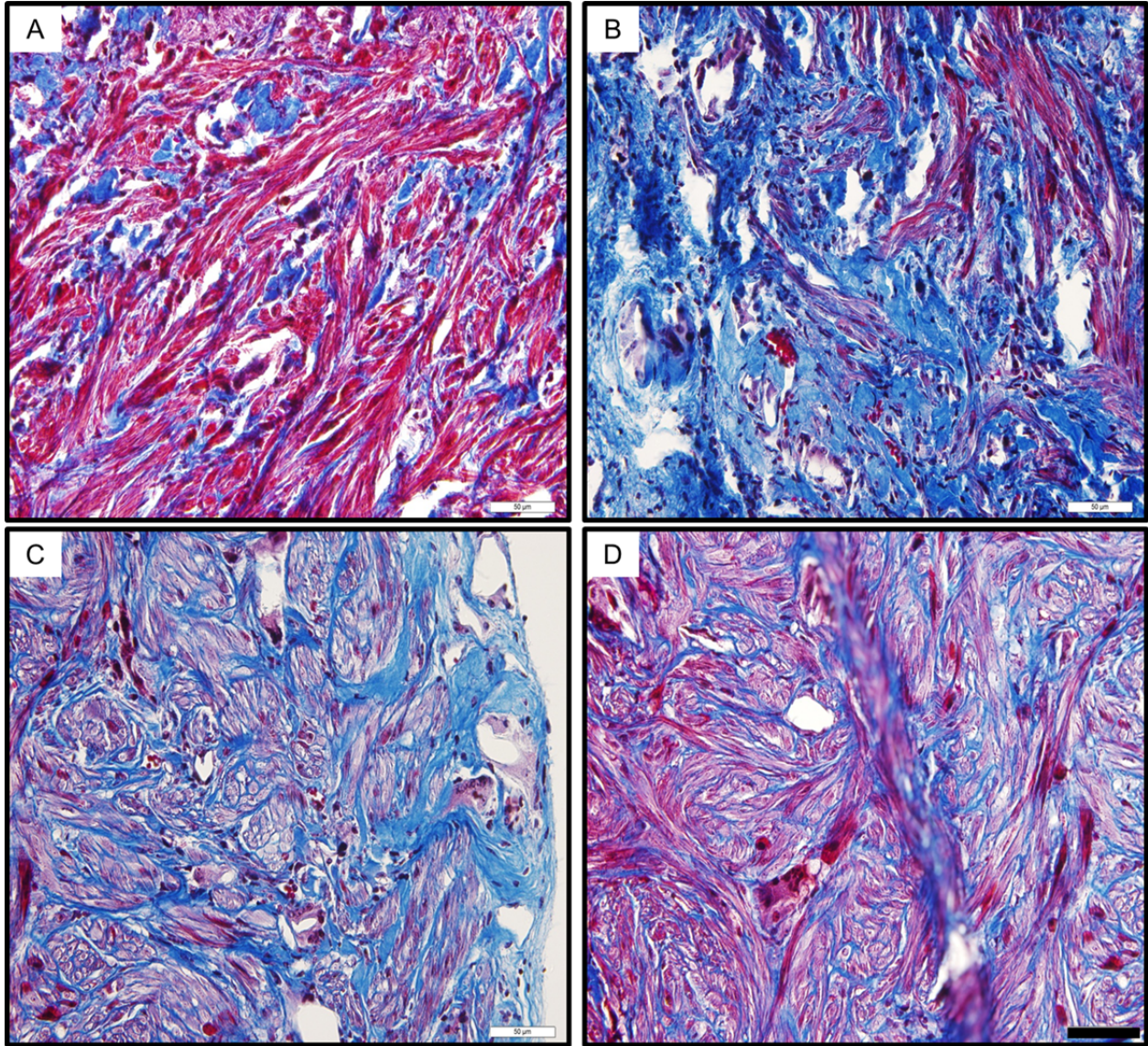


Figure 5.6. Effect of HF treatment on ECM collagens in UL-xenografts. Different sections from embedded xenografts treated with vehicle (A & B) or 0.25 mg/kg HF daily (C& D) were stained with Masson's trichrome to assess the presence of ECM collagens. Images are representative of either 4 patient tumors xenografted on 4-6 animals per vehicle or HF-treated mice. (Bar=100μm)

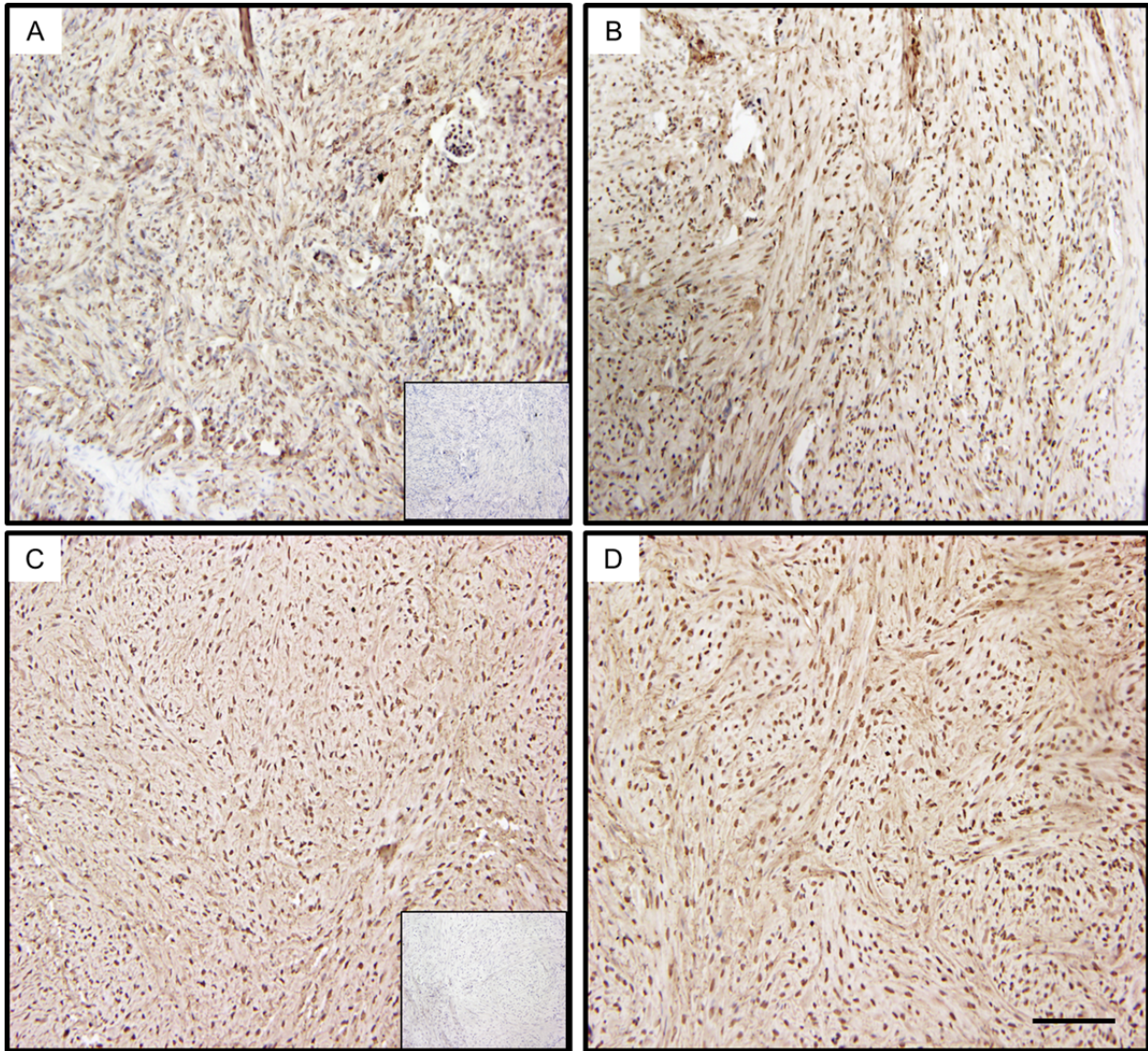


Figure 5.7. Effect of HF treatment on the expression of collagen I in UL-xenografts. Sections of xenografts from animals treated with either vehicle (A & C), 0.25 mg/kg HF daily (B) or 0.50 mg/kg HF EOD (D) were immunostained with a primary antibody specific for collagen I. Images are representative examples of tumors from either 4 (for low dose HF) or 2 (for high dose HF) different patients xenografted into 4-6 animals per treatment. (Bar=100 μ m, insets: negative control).

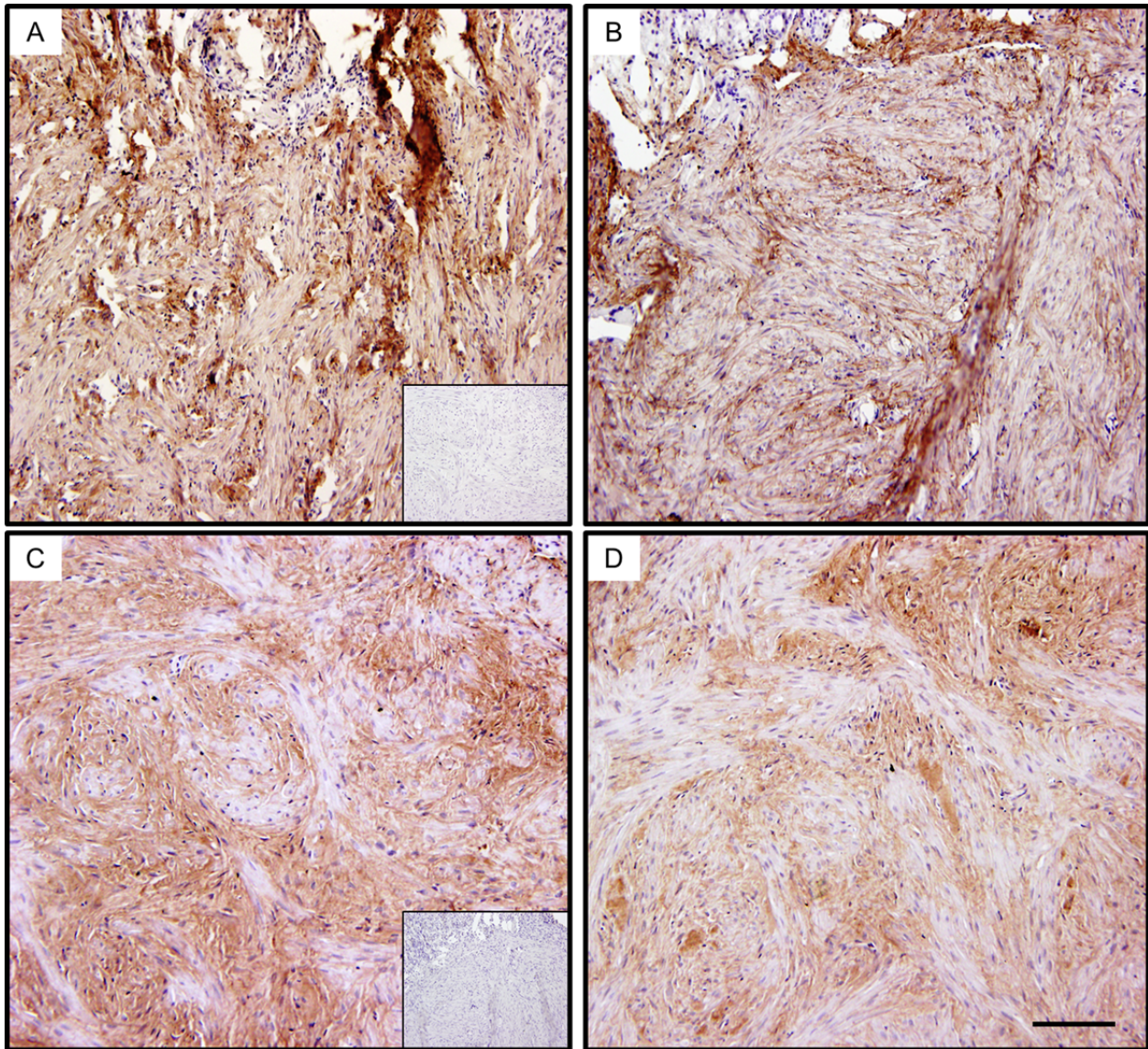


Figure 5.8. Effect of HF treatment on the expression of collagen III in UL-xenografts. Sections of xenografts treated with either vehicle (A & C), 0.25 mg/kg HF daily (B) or 0.50 mg/kg HF EOD (D) were immunostained with a primary antibody specific for collagen III. Images are representative examples of tumors from either 4 (for low dose HF) or 2 (for high dose HF) different patients xenografted into 4-6 animals per treatment. (Bar=100 μ m, insets: negative control)

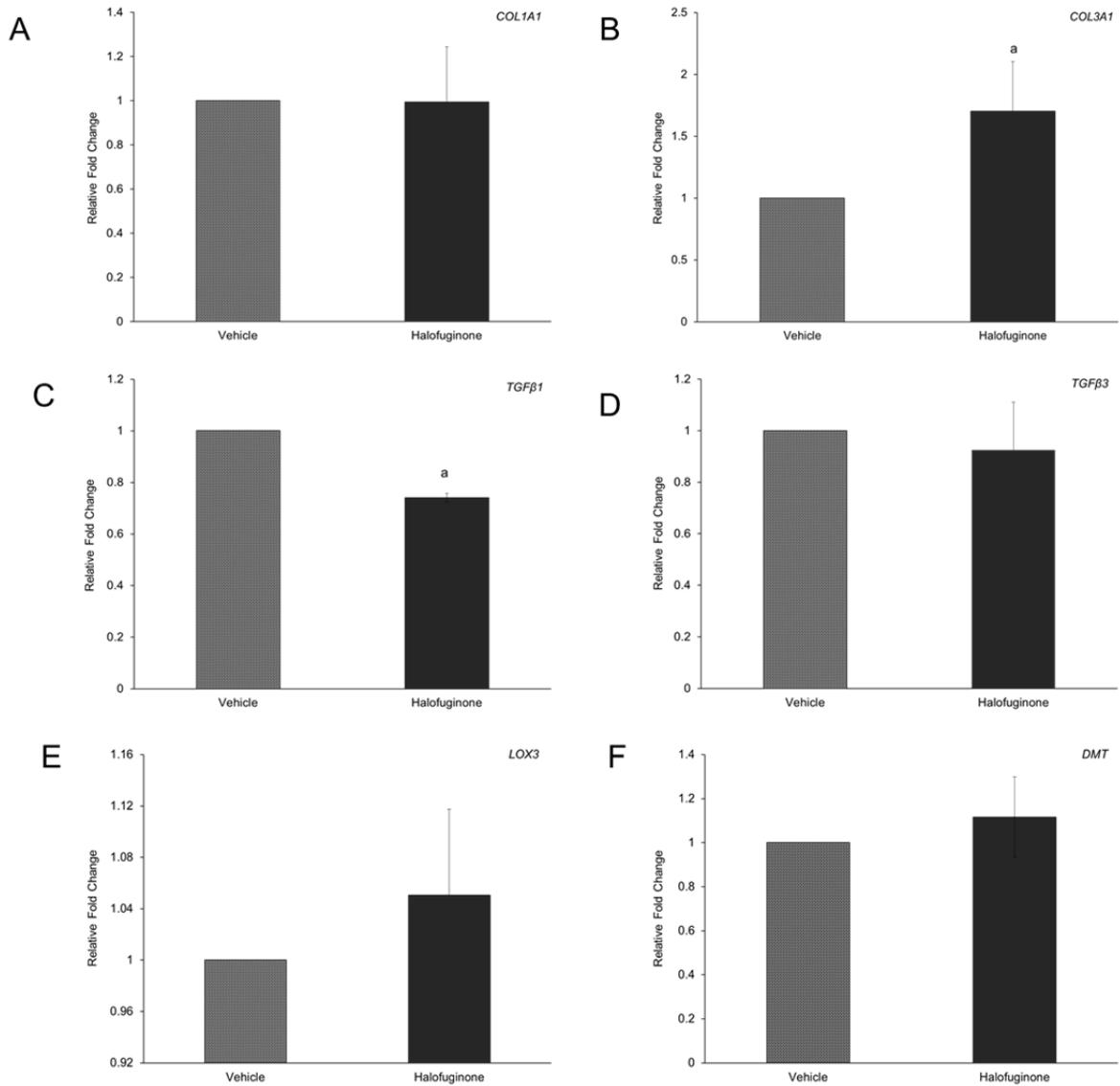


Figure 5.9. Effect of HF on mRNA levels of genes involved in tissue fibrosis. RNA samples isolated from xenografts from animals treated with either vehicle or 0.50 mg/kg HF EOD for 4 weeks were subjected to qRT-PCR using primers specific for *COL1A1* (A), *COL3A1* (B), *TGFβ1* (C), *TGFβ3* (D), *LOX3* (E), and *DMT* (F). Xenografts established from two different patients transplanted into 1-4 mice per treatment group were used for analysis. ($P < 0.05$)

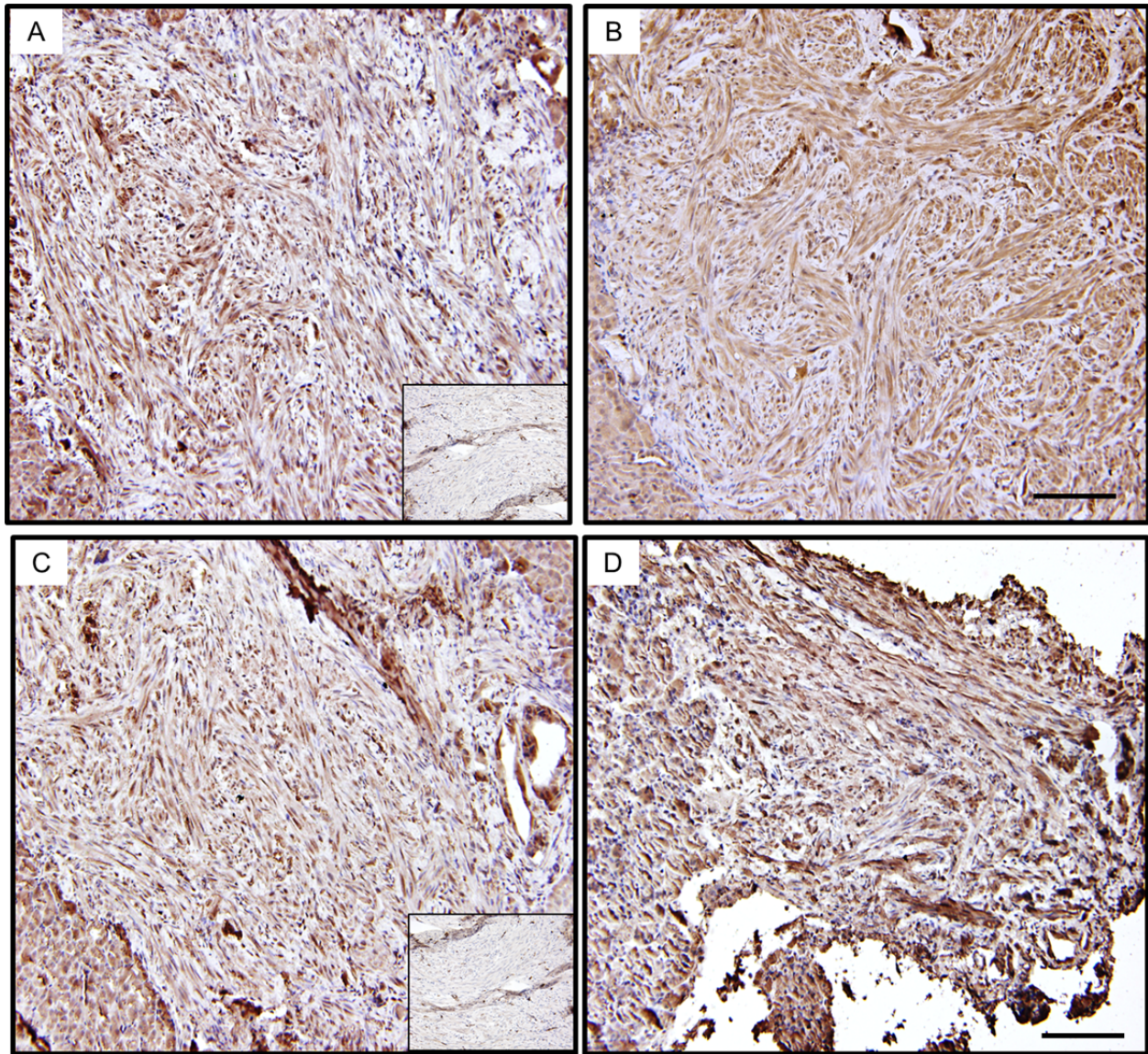


Figure 5.10. Effect of HF treatment on the expression of Bcl2, dermatopontin and TGFβRII and in UL-xenografts. Sections of xenografts from mice treated with either vehicle (A, C & E) or 0.25 mg/kg HF daily (B, D & F) were immunostained with primary antibodies specific for dermatopontin (A & B) or TGFβRII (C & D). Images are representative examples of tumors from 4 different patients xenografted into 4-6 animals per treatment group. (Bar=100µm, insets: negative control).

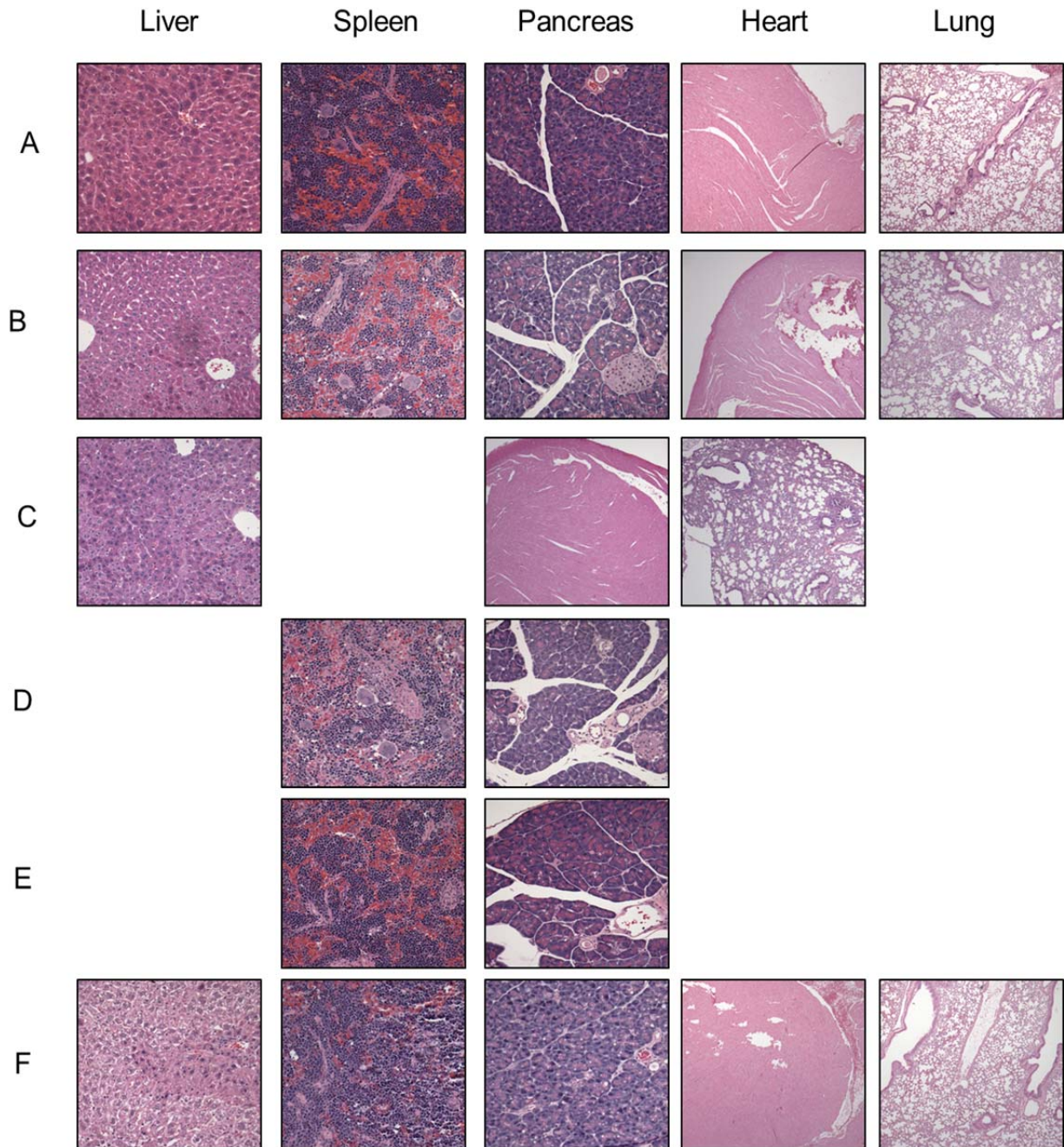


Figure 5.11. Effect of HF on specific organs and tissues of mouse hosts.

Histopathological characteristics of paraffin-embedded body organs from mice xenografted with UL-tumors under different treatments were assessed by hematoxylin and eosin staining. Treatments were: (A) control, (B) vehicle for 0.25 mg/kg HF daily, (C) 0.25 mg/kg HF daily (continued on next page)

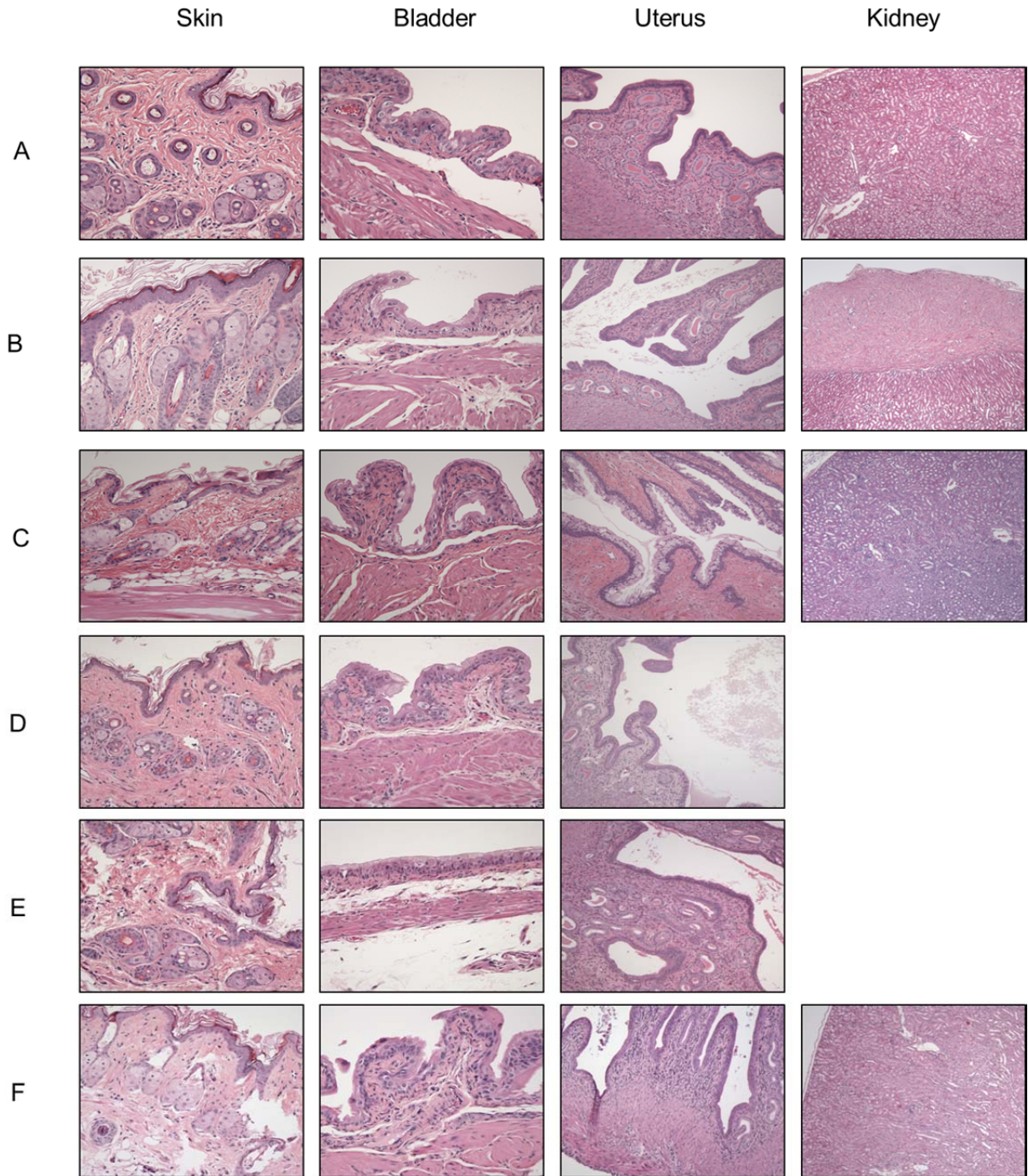


Figure 5.11 (cont'd) (D) vehicle for 0.5 mg/kg HF daily or EOD, (E) 0.5 mg/kg HF daily, and (F) 0.5 mg/kg HF EOD. Images are representative examples of sections from 5-12 xenografts for each treatment group. (Bar=100µm)

Reference List

Al-Hendy, A., and Salama, S. (2006). Gene therapy and uterine leiomyoma: a review. *Hum. Reprod. Update.* 12, 385-400.

Arslan, A.A., Gold, L.I., Mittal, K., Suen, T., Belitskaya-Levy, I., Tang, M., and Toniolo, P. (2005). Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: New evidence and a systematic review. *Hum. Reprod.* 20, 852-863.

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., and Tomic-Canic, M. (2008). Growth factors and cytokines in wound healing. *Wound Repair Regen.* 16, 585-601.

Cardozo, E.R., Clark, A.D., Banks, N.K., Henne, M.B., Stegmann, B.J., and Segars, J.H. (2011). The estimated annual cost of uterine leiomyomata in the United States. *Am. J. Obstet. Gynecol.* 206, 211.e1-9.

Catherino, W.H., Parrott, E., and Segars, J. (2011). Proceedings from the national institute of child health and human development conference on the uterine fibroid research update workshop. *Fertil. Steril.* 95, 9-12.

Chavez, N.F., and Stewart, E.A. (2001). Medical treatment of uterine fibroids. *Clin. Obstet. Gynecol.* 44, 372-384.

Cook, J.A., Choudhuri, R., Degraff, W., Gamson, J., and Mitchell, J.B. (2010). Halofuginone enhances the radiation sensitivity of human tumor cell lines. *Cancer Lett.* 289, 119-126.

Dabak, H., Karlidag, T., Akpolat, N., Keles, E., Alpay, H.C., Serin, M., Kaygusuz, I., Yalcin, S., and Isik, O. (2010). The effects of methylprednisolone and halofuginone on preventing esophageal and hypopharyngeal fibrosis in delivered radiotherapy. *Eur. Arch. Otorhinolaryngol.* 267, 1429-1435.

de Figueiredo-Pontes, L.L., Assis, P.A., Santana-Lemos, B.A., Jacomo, R.H., Lima, A.S., Garcia, A.B., Thome, C.H., Araujo, A.G., Panepucci, R.A., Zago, M.A., *et al.* (2011). Halofuginone has anti-proliferative effects in acute promyelocytic leukemia by modulating the transforming growth factor beta signaling pathway. *PLoS One.* 6, e26713.

de Jonge, M.J.A., Dumez, H., Verweij, J., Yarkoni, S., Snyder, D., Lacombe, D., Marréaud, S., Yamaguchi, T., Punt, C.J.A., and van Oosterom, A. (2006). Phase I and pharmacokinetic study of halofuginone, an oral quinazolinone derivative in patients with advanced solid tumours. *Eur. J. Cancer* 42, 1768-1774.

Derbyshire, E.R., Mazitschek, R., and Clardy, J. (2012). Characterization of plasmodium liver stage inhibition by halofuginone. *Chem. Med. Chem.* 7, 844-849.

Dixon, D., Flake, G.P., Moore, A.B., He, H., Haseman, J.K., Risinger, J.I., Lancaster, J.M., Berchuck, A., Barrett, J.C., and Robboy, S.J. (2002). Cell proliferation and apoptosis in human uterine leiomyomas and myometria. *Virchows Arch.* 441, 53-62.

Dorne, J.L., Fernandez-Cruz, M.L., Bertelsen, U., Renshaw, D.W., Peltonen, K., Anadon, A., Feil, A., Sanders, P., Wester, P., and Fink-Gremmels, J. (2011). Risk assessment of coccidiostats during feed cross-contamination: Animal and human health aspects. *Toxicol. Appl. Pharmacol.* ePub

Elkin, M., Ariel, I., Miao, H., Nagler, A., Pines, M., de-Groot, N., Hochberg, A., and Vlodavsky, I. (1999). Inhibition of bladder carcinoma angiogenesis, stromal support, and tumor growth by halofuginone. *Cancer Res.* 59, 4111-4118.

Flake, G.P., Andersen, J., and Dixon, D. (2003). Etiology and pathogenesis of uterine leiomyomas: a review. *Environ. Health Perspect.* 111, 1037-54.

Flanders, K.C. (2004). Smad3 as a mediator of the fibrotic response. *Int. J. Exp. Pathol.* 85, 47-64.

Gao, X., Yu, L., Castro, L., Moore, A.B., Hermon, T., Bortner, C., Sifre, M., and Dixon, D. (2010). An endocrine-disrupting chemical, fenvalerate, induces cell cycle progression and collagen type I expression in human uterine leiomyoma and myometrial cells. *Toxicol. Lett.* 196,133-141.

Gavish, Z., Pinthus, J.H., Barak, V., Ramon, J., Nagler, A., Eshhar, Z., and Pines, M. (2002). Growth inhibition of prostate cancer xenografts by halofuginone. *Prostate.* 51, 73-83.

Genin, O., Rechavi, G., Nagler, A., Ben-Itzhak, O., Nazemi, K.J., and Pines, M. (2008). Myofibroblasts in pulmonary and brain metastases of alveolar soft-part sarcoma: a novel target for treatment? *Neoplasia* 10, 940-948.

Ghosh, A.K. (2002). Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. *Exp. Biol. Med.* 227, 301-314.

Gnainsky, Y., Kushnirsky, Z., Bilu, G., Hagai, Y., Genina, O., Volpin, H., Bruck, R., Spira, G., Nagler, A., Kawada, N., *et al.* (2007). Gene expression during chemically induced liver fibrosis: effect of halofuginone on TGF- β signaling. *Cell Tissue Res.* 328, 153-166.

Grudzien, M.M., Low, P.S., Manning, P.C., Arredondo, M., Belton, R.J., Jr, and Nowak, R.A. (2009). The antifibrotic drug halofuginone inhibits proliferation and collagen production by human leiomyoma and myometrial smooth muscle cells. *Fertil. Steril.* 93,1290-1298.

Horbelt, D., Denkis, A., and Knaus, P. (2012). A portrait of Transforming Growth Factor beta superfamily signalling: Background matters. *Int. J. Biochem. Cell Biol.* *44*, 469-474.

Huebner, K.D., Jassal, D.S., Halevy, O., Pines, M., and Anderson, J.E. (2008). Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone. *Am. J. Physiol. Heart Circ. Physiol.* *294*, H1550-1561.

Jackson, T.L., and Byrne, H.M. (2002). A mechanical model of tumor encapsulation and transcapsular spread. *Math. Biosci.* *180*, 307-328.

Jordan, M.C., and Zeplin, P.H. (2012). Local inhibition of angiogenesis by halofuginone coated silicone materials. *J. Mater. Sci. Mater. Med.* *23*, 1203-1210.

Karatas, A., Paksoy, M., Erzin, Y., Carkman, S., Gonenc, M., Ayan, F., Aydogan, F., Uzun, H., and Durak, H. (2008). The effect of halofuginone, a specific inhibitor of collagen type 1 synthesis, in the prevention of pancreatic fibrosis in an experimental model of severe hyperstimulation and obstruction pancreatitis. *J. Surg. Res.* *148*, 7-12.

Kawaguchi, K., Fujii, S., Konishi, I., Nanbu, Y., Nonogaki, H., and Mori, T. (1989). Mitotic activity in uterine leiomyomas during the menstrual cycle. *Am. J. Obstet. Gynecol.* *160*, 637-641.

Koon, H.B., Fingleton, B., Lee, J.Y., Geyer, J.T., Cesarman, E., Parise, R.A., Egorin, M.J., Dezube, B.J., Aboulafia, D., and Krown, S.E. (2011). Phase II AIDS malignancy consortium trial of topical halofuginone in AIDS-related kaposi sarcoma. *J. Acquir. Immune Defic. Syndr.* *56*, 64-68.

Last, J.A. (1985). Changes in the collagen pathway in fibrosis. *Toxicol. Sci.* *5*, 210-218.

Leiba, M., Jakubikova, J., Klippel, S., Mitsiades, C.S., Hideshima, T., Tai, Y.T., Leiba, A., Pines, M., Richardson, P.G., Nagler, A., and Anderson, K.C. (2012). Halofuginone inhibits multiple myeloma growth in vitro and in vivo and enhances cytotoxicity of conventional and novel agents. *Br. J. Haematol.* *157*, 718-731.

Leppert, P.C., Baginski, T., Prupas, C., Catherino, W.H., Pletcher, S., and Segars, J.H. (2004). Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertil. Steril.* *82 Suppl. 3*, 1182-1187.

Levi-Schaffer, F., Nagler, A., Slavin, S., Knopov, V., and Pines, M. (1996). Inhibition of collagen synthesis and changes in skin morphology in murine graft-versus-host disease and tight skin mice: effect of halofuginone. *J. Invest. Dermatol.* *106*, 84-88.

Levy, G., Hill, M.J., Beall, S., Zarek, S.M., Segars, J.H., and Catherino, W.H. (2012). Leiomyoma: genetics, assisted reproduction, pregnancy and therapeutic advances. *J. Assist. Reprod. Genet.* *29*, 703-712.

McGaha, T.L., Phelps, R.G., Spiera, H., and Bona, C. (2002). Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-[bgr]-mediated Smad3 activation in fibroblasts. *J. Invest. Dermatol.* *118*, 461-470.

Medikare, V., Rao Kandukuri, L., Ananthapur, V., Deenadayal, M., and Nallari, P. (2011). The genetic bases of uterine fibroids; a review. *J.Repro.Infert.* *12*, 181-192.

Nelson, E.F., Huang, C.W., Ewel, J.M., Chang, A.A., and Yuan, C. (2012). Halofuginone down-regulates Smad3 expression and inhibits the TGFbeta-induced expression of fibrotic markers in human corneal fibroblasts. *Mol. Vis.* *18*, 479-487.

Pines, M., and Halevy, O. (2011). Halofuginone and muscular dystrophy. *Histol. Histopathol.* *26*, 135-146.

Pines, M., Domb, A., Ohana, M., Inbar, J., Genina, O., Alexiev, R., and Nagler, A. (2001). Reduction in dermal fibrosis in the tight-skin (Tsk) mouse after local application of halofuginone. *Biochem. Pharmacol.* *62*, 1221-1227.

Pines, M., Snyder, D., Yarkoni, S., and Nagler, A. (2003). Halofuginone to treat fibrosis in chronic graft-versus-host disease and scleroderma. *Biol. Blood Marrow Transplantn* *9*, 417-425.

Pines, M., Vlodayky, I., and Nagler, A. (2000). Halofuginone: From veterinary use to human therapy. *Drug Dev. Res.* *50*, 371-378.

Pinkerton, J.V. (2011). Pharmacological therapy for abnormal uterine bleeding. *Menopause* *18*, 453-461.

Pinthus, J.H., Sheffer, Y., Nagler, A., Fridman, E., Mor, Y., Genina, O., and Pines, M. (2005). Inhibition of Wilms tumor xenograft progression by halofuginone is accompanied by activation of WT-1 gene expression. *J. Urol.* *174*, 1527-1531.

Popov, Y., Patsenker, E., Bauer, M., Niedobitek, E., Schulze-Krebs, A., and Schuppan, D. (2006). Halofuginone induces matrix metalloproteinases in rat hepatic stellate cells via activation of p38 and NFkappaB. *J. Biol. Chem.* *281*, 15090-15098.

Sato, S., Kawamura, H., Takemoto, M., Maezawa, Y., Fujimoto, M., Shimoyama, T., Koshizaka, M., Tsurutani, Y., Watanabe, A., Ueda, S., *et al.* (2009). Halofuginone prevents extracellular matrix deposition in diabetic nephropathy. *Biochem. Biophys. Res. Commun.* *379*, 411-416.

Schuppan, D., and Pinzani, M. (2012). Anti-fibrotic therapy: lost in translation? *J. Hepatol.* *56 Suppl 1*, S66-74.

Sheffer, Y., Leon, O., Pinthus, J.H., Nagler, A., Mor, Y., Genin, O., Iluz, M., Kawada, N., Yoshizato, K., and Pines, M. (2007). Inhibition of fibroblast to myofibroblast transition by

halofuginone contributes to the chemotherapy-mediated antitumoral effect. *Mol. Cancer Ther.* **6**, 570-577.

Spector, I., Honig, H., Kawada, N., Nagler, A., Genin, O., and Pines, M. (2010). Inhibition of pancreatic stellate cell activation by halofuginone prevents pancreatic xenograft tumor development. *Pancreas*. **39**,1008-1015.

Spira, G., Mawasi, N., Paizi, M., Anbinder, N., Genina, O., Alexiev, R., and Pines, M. (2002). Halofuginone, a collagen type I inhibitor improves liver regeneration in cirrhotic rats. *J. Hepatol.* **37**, 331-339.

Stewart, E.A. (2002). Nonsurgical treatment of uterine fibroids. *Infertility and Reproductive Medicine Clinics of North America*. **13**, 393-405.

Stewart, E.A., Friedman, A.J., Peck, K., and Nowak, R.A. (1994). Relative overexpression of collagen type I and collagen type III messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. *J. Clin. Endocrinol. Metab.* **79**, 900-906.

Stewart, E.A., and Morton, C.C. (2006). The genetics of uterine leiomyomata: what clinicians need to know. *Obstet. Gynecol.* **107**, 917-921.

Tacheau, C., Michel, L., Farge, D., Mauviel, A., and Verrecchia, F. (2007). Involvement of ERK signaling in halofuginone-driven inhibition of fibroblast ability to contract collagen lattices. *Eur. J. Pharmacol.* **573**, 65-69.

Taras, D., Blanc, J.F., Rullier, A., Dugot-Senant, N., Laurendeau, I., Bieche, I., Pines, M., and Rosenbaum, J. (2006). Halofuginone suppresses the lung metastasis of chemically induced hepatocellular carcinoma in rats through MMP inhibition. *Neoplasia* **8**, 312-318.

Trotz-Williams, L.A., Jarvie, B.D., Peregrine, A.S., Duffield, T.F., and Leslie, K.E. (2011). Efficacy of halofuginone lactate in the prevention of cryptosporidiosis in dairy calves. *Vet. Rec.* **168**, 509.

Turgeman, T., Hagai, Y., Huebner, K., Jassal, D.S., Anderson, J.E., Genin, O., Nagler, A., Halevy, O., and Pines, M. (2008). Prevention of muscle fibrosis and improvement in muscle performance in the mdx mouse by halofuginone. *Neuromuscul. Disord.* **18**, 857-868.

Wynn, T.A. (2008). Cellular and molecular mechanisms of fibrosis. *J. Pathol.* **214**, 199-210.

Xavier, S., Piek, E., Fujii, M., Javelaud, D., Mauviel, A., Flanders, K.C., Samuni, A.M., Felici, A., Reiss, M., Yarkoni, S., Sowers, A., Mitchell, J.B., Roberts, A.B., Russo, A.

(2004). Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor-beta signaling by halofuginone. *J. Biol. Chem.* 279, 15167-15176.

Yee, K.O., Connolly, C.M., Pines, M., and Lawler, J. (2006). Halofuginone inhibits tumor growth in the polyoma middle T antigen mouse via a thrombospondin-1 independent mechanism. *Cancer. Biol. Ther.* 5, 218-224.

Zeplin, P.H. (2012). Reduction of burn scar formation by halofuginone-eluting silicone gel sheets: a controlled study on nude mice. *Ann. Plast. Surg.* 68, 271-275.

Zeplin, P.H., Larena-Avellaneda, A., and Schmidt, K. (2010). Surface modification of silicone breast implants by binding the antifibrotic drug halofuginone reduces capsular fibrosis. *Plast. Reconstr. Surg.* 126, 266-274.

Zion, O., Genin, O., Kawada, N., Yoshizato, K., Roffe, S., Nagler, A., Iovanna, J.L., Halevy, O., and Pines, M. (2009). Inhibition of transforming growth factor beta signaling by halofuginone as a modality for pancreas fibrosis prevention. *Pancreas* 38, 427-435.

Chapter Six

Conclusions and Future Directions

The overall goal of my dissertation work was to understand the pathogenesis of human UL tumors in the context of the abundant extracellular matrix collagen and to determine the potential of the anti-fibrotic drug HF as a therapeutic for ULs by testing its inhibitory effects on the growth of these tumors. In doing so, I hypothesized that: (i) type and ultra-structure of the ECM collagens, namely whether it is monomeric or fibrillar, would differentially affect the growth and morphology of LSMCs, (ii) the anti-fibrotic drug HF targets cell proliferation and collagen expression in LSMCs *in vitro*, and (iii) immunocompromised mice xenografted with human LSMCs serve as a good pre-clinical model to test the use of HF as an alternative therapeutic treatment for ULs and treatment with HF would negatively affect tumor growth.

Using *in vitro* and *in vivo* model systems of primary human LSMCs cultured on different collagen matrices or xenografted under the kidney capsule of immunocompromised mouse hosts, I was able to confirm all three hypotheses. In summary, the main findings of my research studies are:

1. LSMCs cultured on monomeric collagen matrix maintained their smooth muscle cell morphology along with increased activation of FAK, showed increased accumulation of vinculin, and formation of central and peripheral actin stress fibers. In contrast, cells cultured on fibrillar collagen matrix developed star-like morphologies with reduced phosphorylation of FAK, dispersed vinculin distribution, and minimal assembly of actin stress fibers;

2. Monomeric collagen matrix increased basal and PDGF-stimulated cell proliferation, whereas fibrillar collagen, although permissive to proliferation, was not as potent in the induction of proliferation;
3. Stimulation of LSMCs with PDGF caused rapid and transient phosphorylation of PDGFR on both monomeric and fibrillar collagen;
4. PDGF treatment led to transient and robust activation of ERK1/2 in LSMCs cultured on monomeric collagen in contrast to the sustained activation of ERK1/2 on fibrillar collagen matrices;
5. HF at 50 nM concentration significantly reduced both basal and PDGF-stimulated cell proliferation in LSMCs cultured on either plastic or monomeric collagen matrices without affecting cell viability;
6. Protein and transcript levels of both collagens I and III in LSMCs were reduced by HF treatment of LSMCs *in vitro* in a time-dependent manner;
7. HF also altered gene transcription of several major fibrotic genes including *TGFβ1*, *TGFβ3*, *LOX3* and *DMT*, which are involved in the pathogenesis of ULs;
8. The SRC and MAPK signaling pathways were activated by HF over a short-term treatment whereas the activation of MAPK, SRC, and AKT were not significantly reduced by a 6-hour pre-treatment with 50 nM HF;
9. Treatment of mouse hosts xenografted with LSMCs with either 0.25 or 0.50 mg/kg body weight/day HF over four weeks significantly reduced the size of xenografted tumors through decreased proliferation and increased apoptosis;

10. Treatment of mice hosts with UL-xenografted tumors with either doses of HF did not alter the expression of collagen I, collagen III, TGF β RII and dermatopontin proteins.

11. HF treatment, at both doses, of mice hosts altered the transcription of only *COL3A1* and *TGF β 1*, but not *COL1A1*, *TGF β 3*, *DMT* and *LOX* in UL-xenografts.

12. In contrast to the 0.50 mg/kg body weight/day treatment with HF, the 0.25 mg/kg body weight/day dose was well tolerated by mouse hosts. Histopathological analysis on major body organs of mice treated with either doses of HF, however, showed no adverse side effects of HF on mouse hosts.

A model summarizing these findings on the role of ECM collagen in regulating the proliferation of ULs and the modulatory effects of HF on growth of these tumors is depicted in Fig. 6.1. Our results demonstrating that interaction of ECM collagens with LSMCs leads to alterations in their morphology and growth add to the growing body of evidence in a variety of cell types and tissues demonstrating how the extracellular environment of a cell, particularly the ECM, along with its genetic makeup can regulate cell function. The *in vitro* model system of monomeric and fibrillar collagen matrices that I used in these studies is a simple replication of the ECM environment that LSMCs are exposed to *in vivo*. While this model provided us with valuable insights into the manner by which the specific type and ultra-structure of ECM collagens regulate the growth of LSMCs, it could not provide us with a complete view of how cells in an *in vivo* environment interact with the matrix. Interaction of LSMCs with ECM collagens would best be studied in a three dimensional collagen matrix where cells are surrounded by the matrix from all sides and exposed to both biochemical and mechanical changes

occurring in their extracellular environment. In addition, since the ECM *in vivo* is composed of proteins other than collagens, our simplified collagen-only matrix could not recapitulate the combinatorial effect of all ECM proteins on LSMCs. Future work with more complex *in vitro* systems based on our initial studies would better determine the exact role of ECM collagen on the morphology and proliferation of LSMCs.

The findings of our studies, however, have opened up new avenues for future research on ULs. The following is a list of questions and topics worth pursuing in future research investigations:

1. How is the expression and localization of major collagen receptors, namely DDRs and integrins, altered on different collagen matrices? If the expression and localization of these receptors change differentially on each matrix, how would the down-stream signaling pathways be altered to affect LSMCs growth? Given the presence of a feedback mechanism on collagen synthesis from integrin receptors, it would be interesting to know whether the expression of collagen receptors is different between normal myometrial SMCs and LSMCs and if so, how such differences could affect the process of collagen synthesis in uterine SMCs.
2. Is there a feedback mechanism that controls the process of collagen synthesis in LSMCs when they are exposed to different types and ultra-structures of collagens? In other words, does the excess presence of malformed or disorganized collagen in the ECM provide a positive feedback to the process of collagen synthesis such that more collagen will be synthesized to keep the tumor

growing? If so, what are the major steps in this process that can be identified as points of intervention for therapeutic purposes?

3. Is there an interplay between PDGFR and collagen receptors in regulating the proliferation of LSMCs? If there is an interaction between the two, how is this interaction altered in the leiomyoma vs. normal myometrial tissue? It would be also important to examine the dynamics of interaction between PDGFR and collagen receptors in LSMCs cultured on various collagen matrices. Co-immunoprecipitation and localization studies as well as development of dominant negative mutants of these receptors in myometrial and leiomyoma tissues could demonstrate the presence of such interaction on LSMCs proliferation and collagen synthesis.

4. Does the transformation of fibroblasts to myofibroblasts change when cells are cultured on different collagen matrices? This process is known to be the key event in the formation of fibrotic tissue in response to an initial injury in a normal tissue. In light of the proposed theory of injury to the uterus being the initiating event that leads to ULs, it is worth investigating the process of fibroblast to myofibroblast conversion on different collagen matrices. Due to the anti-fibrotic effects of HF and its role in regulating fibroblast conversion, it would also be interesting to see if HF can modulate major markers of the transition of fibroblasts to myofibroblasts, such as α SMC, transgelin, cygb/stap, and cytoglobin in ULs.

5. Does proliferation of LSMCs treated with TGF β , a known pro-fibrotic factor in ULs, change depending on whether these cells are cultured on monomeric or fibrillar collagen matrices? Will HF have modulatory effects on the signaling

pathways activated by TGF β in LSMCs? Can it reduce both the expression of collagens and proliferation induced by TGF β in LSMCs similar to findings in other cell types?

6. Increased deposition of ECM collagens is considered a major characteristic of LSMCs. The multi-step, complex process of collagen synthesis and deposition makes it susceptible to many changes at the gene and protein levels. Studies in ULs and similar fibrotic diseases have indicated mutations in collagen genes, alterations in post-translational modifications of collagen proteins as well as enzymes involved in collagen synthesis. While such alterations represent the observed heterogeneity in ULs, it is worthwhile to identify major abnormal steps which are commonly shared between patients. Identification of such tissue-specific targets will serve as biomarkers for early detection of the disease as well as help with the design of therapeutic or preventive treatments for uterine fibroids.

7. Vitamin C has been shown to have both a protective effect in tumor formation and therapeutic effect in tumor development. These tumors include prostate, cervix, breast, lung, colon, and esophagus. In addition, this vitamin is involved in the proper synthesis and deposition of ECM collagen. Moreover, a correlation between the consumption of fruits and vegetables, which mostly contain considerable amounts of vitamin C and the reduced risk of ULs has been established. Based on these facts, it is plausible to ask whether: (a) levels of vitamin C are different in women with ULs compared to healthy ones, (b) these differences are due to biological abnormalities or lifestyle; (c) alterations in the

level of vitamin C can alter the ultra-structure of collagen deposited in either *in vivo* or *in vitro* models of ULs, and (c) HF can ameliorate the possible alteration in the synthesis and function of vitamin C in UL tissue. Similar questions can be asked about other anti-oxidative agents.

8. ULs are sex steroid hormone-responsive tumors. Numerous investigations looking at the efficacy of anti-estrogen and progesterone therapies in the treatment of leiomyomas have implied the role of these hormones in the abundance of collagens in UL tissue. Moreover, limited *in vitro* studies in collagen-synthesizing cells have shown the stimulatory effect of female hormones in collagen production. It, therefore, seems necessary to fully delineate the exact role of these two hormones on the expression and deposition of collagen in LSMCs. Identification of biomarkers and development of effective therapeutics will emerge from such studies. On the other hand, it would be interesting to know if the anti-fibrotic agent HF could play a role in the hormonal regulation of collagen synthesis. Research on the effects of HF on sex steroid hormones is lacking. Investigating such effects on a hormone-dependent disease like ULs in proper animal models would be a good starting point in that direction.

9. Epigenetic regulators such as acetyltransferases and miRNAs have recently been recognized to be involved in fibrosis and tumorigenesis. Alterations in similar epigenetic factors have also been identified in ULs. It will be intriguing to know whether HF can alter known epigenetic factors in ULs such that the rate of fibrosis/tumor development be reduced in diseased women.

10. Mechanistic studies on HF have identified the amino acid starvation pathway in immune responses to be a target for this drug. In light of injury and the aberrant wound healing process followed the injury one could stipulate that the inhibitory effects of HF on ULs, documented in this study, could be mediated through a similar pathway. After all, ovulation is considered an inflammatory process and women with early age of menarche experiencing more ovulation are considered at higher risk of developing ULs. In addition, menstruation has been considered a form of injury to the myometrium.

In conclusion, the findings of my dissertation have made a substantial contribution to understanding some of the underlying causes of ULs as well as key molecular targets for therapeutic treatments of this disease. The fact that the type and ultra-structure of ECM collagens can affect the morphology and growth of LSMCs will enable us to further delineate cellular and molecular processes through which these factors can be involved in the pathogenesis of ULs. Alterations in these factors can be considered as either the initiating elements in the formation of ULs or manifestations of other primary effectors. Thus, it will be of importance to not only identify the effect of risk factors such as race and the use of oral contraceptives associated with the etiology of ULs on these processes, but also discover how aberrations in these processes would lead to increased risk of developing ULs.

In addition, the promising results obtained in comprehensive *in vitro* and *in vivo* studies on HF as a candidate drug for the treatment of HF, which showed efficient targeting of the major characteristics of ULs, have paved the way for testing HF in a clinical setting. Considering the non-toxic and reversible effects of HF on cell

proliferation and collagen synthesis in LSMCs, one would be hopeful that in near future, this drug would be of help to women with ULs who are not benefitting from other treatments due to the desire to maintain their fertility or avoid side effects.

Figures

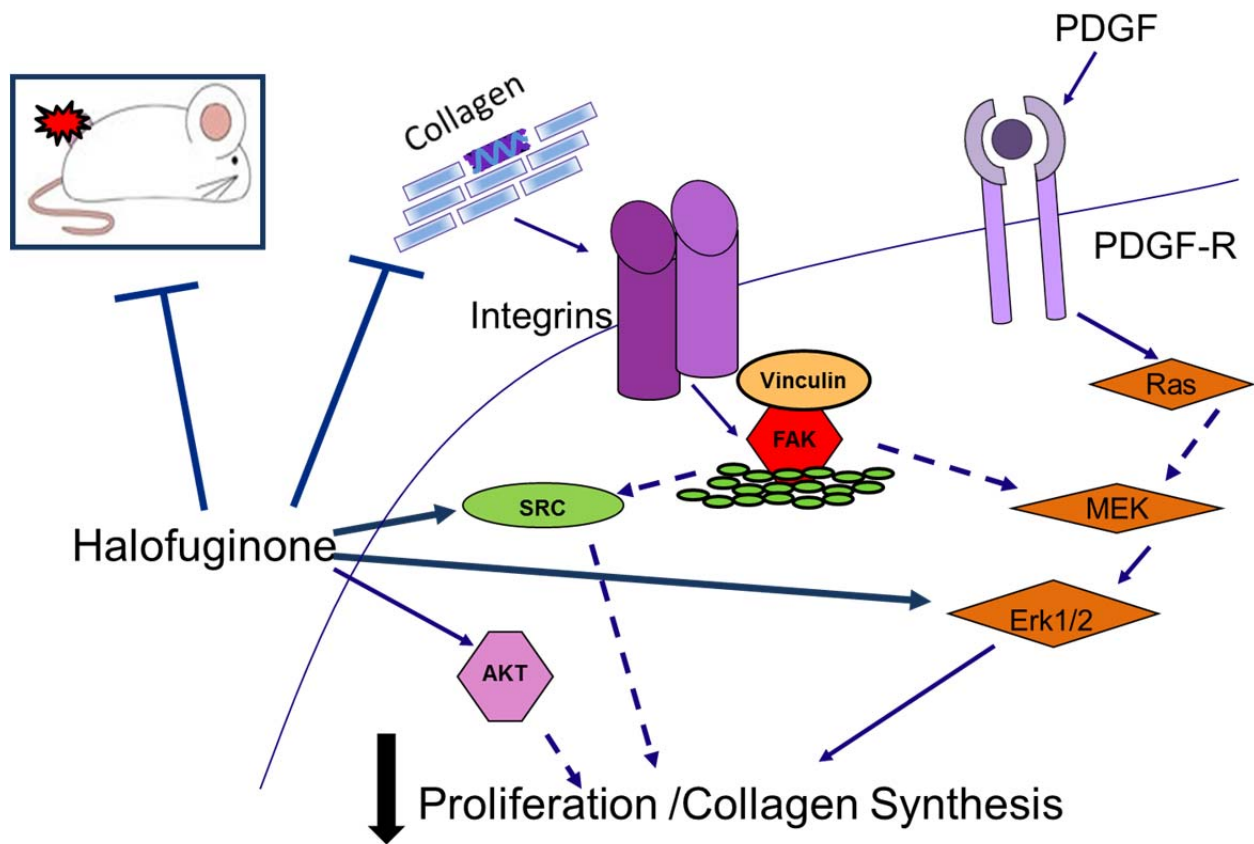


Figure 6.1. Suggested model for how ECM collagen regulates LSMCs proliferation and how the anti-fibrotic drug HF targets increased cell proliferation and excess deposition of ECM collagen in ULs.

Chapter Seven

Materials and Methods

Chemicals and Reagents

Dulbecco's Modified Eagle's medium (DMEM), penicillin-streptomycin, and L-glutamine were purchased from Biowhittaker (Walkersville, MD). Dulbecco's PBS/Modified medium, fetal bovine serum (FBS) and bovine calf serum (BCS) were purchased from Hyclone (Logan, UT). PureCol Collagen was purchased from Advanced Biomatrix (San Diego, CA.). Recombinant human PDGF-AB was purchased from R&D Systems (Minneapolis, MN). Tritiated thymidine ($[^3\text{H}]$ thymidine) was purchased from PerkinElmer (Waltham, MA). Precise Protein Gels, Restore Western Blot Stripping Buffer, Pierce® BCA protein assay kit, Nitrocellulose transfer membranes, Scintiverse scintillation liquid, nuclease-free water, hydrogen peroxide, xylenes, DAB EASY tablets, methyl green, eosin, paraffin, microscope slides, Sodium Dodecyl Sulfate (SDS), Restrore™ western blot stripping buffer, and SuperSignal West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Rockford, IL). All antibodies, except for FAK, were purchased from Cell Signaling (Danvers, MA). FAK antibody was purchased from BD Transduction Laboratories (Lexington, KY). All signaling inhibitors, propidium iodide, Mayer's Hematoxilin solution, sodium orthovanadate, trichostatin-A, Crude collagenase, Diethyl pyrocarbonate (DEPC), Triton X-100, Ponceau S solution, calcium chloride, Trichostatin A, and hemacytometer were purchased from Sigma (St. Louis, MO). Fluorophore antibodies, alamarBlue® Cell Viability Reagent, Collagenase type II, Image-iT Signal FX Enhancer, HRP-linked secondary antibodies, OPTI-MEM I reduced-serum medium, Platinum® Taq DNA Polymerase, Platinum® PCR SuperMix, TRIzol®

Reagent, BLOCK-iT™ Fluorescent Oigo, Lipofectamine™ 2000 SuperScript III™ Reverse Transcriptase, DAPI, vitamins, essential and non-essential amino acids, High Capacity cDNA Reverse Transcription kit, TaqMan Universal Master Mix, Assays-on-Demand™ Gene Expression Assay, RNAlater and ProLong Gold were purchased from Life Technologies (Carlsbad, CA). RNase A and Dithiothreitol (DTT) were from Roche (Indianapolis, IN). DAKo Target Retrieval Solution was from DAKO, Denmark. IHC SELECT Proteinase K and ApopTag® Plus Peroxidase In Situ Apoptosis Kit were from Millipore (Temecula, CA). Fetal bovine serum (FBS) and bovine calf serum (BCS) were purchased from Atlanta Biologicals (Norcross, GA). Ibidi dishes were purchased from ibidi GmbH (Munich, Germany), paraformaldehyde from Electron Microscopy Sciences (Hatfield, PA), Precision Plus Protein All Blue Standards from BioRad (Hercules, CA), HyBlot CL autoradiography films from Denville Scientific (Metuchen, NJ), ABI PRISM® 7900 Sequence Detection System from ABI (ABI; Branchburg, NJ), G 418 Sulfate from Calbiochem (Calbiochem), and Protein A/G Plus-Agarose Immunoprecipitation Reagent from Santa Cruz Biotechnology (Santa Cruz, CA). The list of antibodies used in different assays throughout the study along with their specifications is in appendix B.

Tissue Collection and Cell Culture

Leiomyoma samples were obtained from premenopausal women undergoing hysterectomy at either Carle Foundation Hospital (Urbana, IL) or the Feinberg School of Medicine, Northwestern University (Chicago, IL). Permission to use these samples was approved by the Institutional Review Boards at the University of Illinois at Urbana-Champaign and Northwestern University.

Tissue samples were manually minced into different sized pieces depending on their intended use. For cell culture, tissue samples were minced into 2-3 mm pieces to increase the surface area for efficient enzymatic digestion. These pieces were placed in 10% medium (DMEM medium containing 5% FBS, 5% BCS, and 10,000 U pen/ml penicillin, 10,000 µg strep/ml streptomycin, and 200 mM L-glutamine), 200 U/ml collagenase type II and 3mM CaCl₂ for 4-6 hours at 37°C in a shaking water bath. Total volume of the digesting solution was 8 times the volume of minced tissue pieces in the tube. Once digestion was finished, tissue samples were further dissociated by gentle, repeated pipetting 3-5 times. Digested tissues were next centrifuged at 65 x g for 3-5 minutes, supernatant was removed and the pellet was resuspended in 10% medium at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Immortalized LSMCs, DD-HLMs, were cultured similarly to primary LSMCs, but in minimum essential medium (MEM) containing 300 µg/ml G418, 16% FBS, and 1% vitamins (100X), L-Glutamine, and essential and non-essential amino acids.

Collagen Matrix Preparation

PureCol collagen solution was diluted to 1.0 mg/ml with 0.1 M acetic acid at room temperature. For monomeric collagen coating, plastic dishes were incubated with 0.5 M acetic acid for 20 minutes at room temperature, rinsed once with distilled water and coated with diluted collagen (2 mls for 60 mm and 4 mls for 100 mm dishes) under sterile conditions for 4-6 hours. For fibrillar collagen coating, diluted collagen was neutralized with 10x PBS at half the volume of concentrated collagen used to reach to the 1x concentration along with addition of 0.1 N NaOH and 0.1 N HCl, if necessary, on ice. Plastic 60 mm dishes were coated with 3 mls and 100 mm dishes were coated with

7 mls of fibrillar collagen. Plastic dishes coated with fibrillar collagen were incubated at 37 degrees for 4 hours or overnight also at 37°C for the gel to polymerize. Coated dishes were then rinsed with PBS several times before seeding cells.

Cell Proliferation Assay: Cell Count and Growth Curve

LSMCs were cultured in standard 10% medium until reaching the desired confluence or the end of the treatment period. Cultured cells were then washed once with 1x PBS to remove dead, floating cells before dissociation by 0.05% Trypsin in 0.53 mM EDTA for 5-8 minutes at 37°C. Once cells were lifted off from the dishes, 10% serum-containing medium was added to the dishes for enzyme inactivation. Cell suspensions were next centrifuged at 65 X g for 5 minutes, supernatant was removed, and cell pellets were resuspended in 10% serum-containing medium. A 10 ul aliquot of this cell suspension was placed on two separate chambers of a hemocytometer for counting of cells under the microscope.

To obtain the growth curves for LSMCs, equal numbers of LSMCs were cultured in DMEM/10% serum-containing medium on three different matrices (plastic, monomeric collagen, and fibrillar collagen). Cells were trypsinized and counted every other day using a hemacytometer.

Cell Proliferation Assay: Thymidine Incorporation Assay

Thymidine incorporation assays were also used to quantify proliferation of LSMCs on different matrices in response to different treatments. LSMCs were plated at equal numbers in 96-well plates coated with either monomeric or fibrillar collagen. Cell cycle synchronization was achieved by growing LSMCs to 90% confluence followed by serum starvation for 24 hours. Cells were then treated with either 200µl of 10% serum-

containing medium, serum-free medium or medium containing 10 ng/ml PDGF +/- HF at different concentrations. Cells were labeled with 0.01 $\mu\text{Ci}/\mu\text{l}$ triated thymidine in 20 μl 18 hours after treatments were initiated. Finally, cells were trypsinized in 100 μl of 0.05% trypsin-EDTA and harvested on filter papers for quantification in a Wallace Microbeta liquid scintillation counter (Oy, Finland). Three to six experimental replicates were carried out per treatment. Primary cells from at least three different patients were used as biological replicates.

Cell Adhesion Assay

Adhesion of LSMCs to different matrices was compared in cells seeded onto either plastic, monomeric collagen or fibrillar collagen coated dishes. Three hours after seeding, dishes were gently rinsed with PBS to remove unattached cells and imaged to count the number of attached cells to each matrix. Adhesion assays were performed on cells from three different patients with two experimental replicates for each patient. The number of attached cells in 10 fields of 20 mm^2 surface area was averaged and represented as mean \pm SEM.

Viability Assay

To determine the cytotoxicity of 50 nM HF on LSMCs, cells treated with HF for 24 hours were diluted to approximately 10^6 cells/ml. Next, a 1:1 dilution of the cell suspension was prepared using a 0.4% trypan blue solution. Chambers of the hemocytometer were then loaded with 10 μl of the diluted cell suspension in trypan blue. After letting cells sit for 1-2 minutes, the number of stained cells was counted. The calculated percentage of unstained cells over the total number of cells represented the percent viable cells.

Gene Silencing Using siRNA

For specific gene silencing using siRNAs, LSMCs were seeded in antibiotic-free medium and grown to 50% confluence for transfection. The following Gene specific siRNAs from Life Technologies Inc. were used for transfection: Silencer® Select Negative Control #1 siRNA, Silencer® Select siRNA *COL11A1* (4392422, GCGAUGACGUGAUCUGUGAtt), Silencer® Select siRNA *COL3A1* (4392421, CUUUGAAUGUCCACGGAAtt). LSMCs were cultured in 96-well plates in a total volume of 200 ul for transfection. Once the cells reached 50% confluence and were ready for transfection, 0.2 ul of each siRNA at 60 nM final concentration was mixed with 25 ul Opti-MEM for each well and incubated at room temperature for 5 minutes. A mixture of 0.5 ul lipofectamine and 25 ul Opti-MEM was then also mixed and incubated at room temperature for 5 minutes. Next, this diluted lipofectamine was gently mixed with the diluted siRNA and incubated at room temperature for 20 minutes. For mock transfection, siRNAs were left out of the transfection mix. After washing cells with 2X Opti-MEM, 50.7 ul of the transfection mix and 150 ul Opti-MEM were added to each well and cells were placed in the incubator at 37 Cr for 4 hours. Thereafter, medium was changed to the normal growth medium without antibiotics before using cells for subsequent experiments.

Immunofluorescence Microscopy

Immunofluorescence staining of cells was carried out by first fixing cells in 4% paraformaldehyde for 30 minutes at room temperature. Following three washes in PBS, cells were permeabilized in 0.1% triton X-100 for 15 minutes. Dishes were next incubated in ultra-cold methanol for 15 minutes at -20 degrees and washed for half an

hour before blocking with Image-iT FX Signal FX Enhancer for 30 minutes. Following a PBS wash, cells were incubated with 1:100 dilutions of primary antibodies against vinculin, pFAK, and FAK for 2 hours at room temperature. Cells were then incubated in a 1:200 dilution of secondary antibodies conjugated with different fluorophores for 2 hours. For F-actin staining, fixed cells were washed in PBS three times and then permeabilized in 0.1% Triton X-100 in PBS for 3-5 minutes. After 3 washes in PBS, cells were incubated in Image-iT FX Signal Enhancer for 30 minutes. Next, cells were stained with 6.6 μ M fluorescent phalloidin for 20 minutes at room temperature. Finally, all stained cells received three washes of PBS and were stained with 10 μ g/ml DAPI for 15 minutes. Dishes were cured in Prolong Gold for 24 hours in the dark before imaging with a Zeiss LSM710 microscope.

Collecting Cell Lysate for Immunoblotting

Total cell lysate from LSMCs that had received various treatments in specific experiments were harvested by scraping cells into hot (95 °C) 1x Laemmli sample buffer (LSB) (10% glycerol, 62.5 mM Tris pH 6.8, and 2% Sodium Dodecyl Sulfate, and 5 mM sodium orthovanadate). Harvested cell lysates were then collected in eppendorf tubes, sonicated for 10 seconds, and then spun at 14,000xg for 10 min at 4 °C to remove nuclear and cell debris. The supernatant was then transferred to fresh eppendorf tubes for immediate use or storage at -20°C for future use.

Immunoblotting

BCA protein assays were performed on cell lysates to determine protein concentrations. Proteins were loaded onto gradient SDS-PAGE gels before being transferred to nitrocellulose membranes. Membranes were stained for 5-10 minutes with panceau to

check for proper transfer of proteins to the membrane. Next, membranes were blocked in 5% non-fat dry milk in TBST (20 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20) for 90 minutes and then incubated at 4°C overnight in primary antibody according to conditions specific for each primary antibody listed in appendix B. After 3 washes in TBST, membranes were incubated in a 1:10000 dilution of HRP-conjugated secondary antibody for 60 min at room temperature. Finally, after three 10-minute washes in TBST, the SuperSignal West Pico Chemiluminescent Substrate was applied to membranes for 5 minutes before exposure to autoradiography films. Membranes were first probed with phosphorylated protein antibody and then stripped in Restore western blotting stripping buffer for 15 minutes at room temperature and re-probed with antibody against the total protein as a loading control. For collagen I and III antibodies, GAPDH was used as loading control. (Conditions for these specific antibodies are described in the appendix B)

Densitometric Analysis of Western Blots

To quantify changes in protein expression in response to various treatments, films developed after exposure to chemiluminescence were scanned. Images were saved at high resolution in a TIFF format and analyzed using the ImageJ software from the National Institutes of Health (available at <http://rsbweb.nih.gov/ij/download.html>). Software was calibrated for optical density measurements using a Kodak no. 3 calibrated step tablet available at <http://rsb.info.nih.gov/ij/docs/examples/calibration>. Bands of interest were measured by drawing the smallest rectangle capable of measuring the optical density of each of the bands. The same rectangle was used to measure all the bands on a given film. Measurements of bands for the activated or

phosphorylated form of a specific protein) were normalized to measurements for total proteins as a loading control. Immunoblots for collagens I and III were normalized to GAPDH.

RNA Isolation

TriZol™ reagent was used to isolate RNA from both cultured LSMCs and leiomyoma tissues. Once the cell culture medium was removed, cells were washed with 1x PBS and then 1ml TriZol™ was added to each 60 mm dish. Dishes were placed on a shaker at medium speed for 5 minutes before collecting the lysate into an eppendorf tube for storage at -80°C or immediate isolation. Frozen sample tubes were thawed at room temperature for 5 minutes before continuing the isolation procedure. Two hundred µl of pure chloroform per 1 ml of TRIZol volume were added to each tube in a fume hood. Tubes were tightly closed and shaken vigorously by hand for at least 15 sec and then left at room temperature for 3 min with a quick shake every minute. Tubes were then spun at 12000 X g for 15 min at 4°C. Following centrifugation the mixture separated into a lower phenol-chloroform phase, an interphase, and a colorless aqueous phase on top. Since RNA is present in the aqueous phase, this phase was transferred into a fresh 1.5 ml eppendorf tube. Next RNA was precipitated by adding 0.5 ml ice-cold isopropyl alcohol per 1 ml of original TRIZol volume. Tubes were inverted a few times to mix and left at room temperature for 10 minutes before being spun at 12000 X g for 10 minutes at 4°C. Supernatant was removed and the RNA pellet was washed with 1ml of 75% ice-cold EtOH. Samples were next vortexed and spun at 7,500 X g for 5 minutes at 4°C. The supernatant was discarded and the pellet was air-dried for 5-10 minutes at room temperature. Finally, the RNA pellet was resuspended in 10-20 µl of DEPC-

treated water and incubated for 10 min at 55°C to help improve re-suspension before storage at -80°C.

To obtain samples for RNA isolation from UL specimen, pieces of tissue were sliced thinly and soaked in twice the volume of the tissue in RNAlater[®] solution overnight before freezing at -80°C for future use. UL-xeongrafted tissue was homogenized in TRIZol and RNA was isolated as mentioned above.

Reverse Transcription

Reverse transcription was carried out on 1 ug of RNA from each sample using a High-Capacity cDNA Reverse Transcription Kit. Ten µl of RNA were mixed with 10 µl 2X RT master mix (2 µl 10X RT buffer, 0.8 µl of 25X of 100mM dNTP mix, 2 µl of 10X RT random primers, 1 µl of MultiScribe[™] reverse transcriptase, 4.2 µl of nuclease-free water). After a brief spin, RNA was transcribed under the following thermal cycle conditions: 25°C for 10 minutes, 37°C for 120 minutes and 85 °C for 5 minutes. Samples were then stored at -20°C for future assays.

Quantitative Real Time PCR

Synthesized cDNA samples from different experimental treatments were analyzed for changes in gene expression by real time PCR using TaqMan[®] Universal PCR Master Mix. The following 20x Assays-on-Demand[™] Gene Expression Assay primer-probe sets from Applied Biosystems were used for our studies: *COL1A1* (Hs00164004_m1), *COL3A1* (Hs00943809_m1), *TGFβ1* (Hs00998133_m1), *TGFβ3* (Hs01086000_m1), *lysyl oxidase III (LOX III)* (Hs01046945_m1), *dermatopontin (DMT)* (Hs00355056_m1), and POP4 (HS00198357_m1) as an endogenous control to amplify the corresponding genes. Briefly, 4.5 µl of a 1:10 diluted cDNA sample was mixed with 5.5 µl of master mix

(5µl of TaqMan Universal PCR Mix and 0.5 µl of 20X Assay on Demand) for a total volume of 10 µl per well in a MicroAmp optical 384-well reaction plate. Three experimental replicates were performed for each sample.

Real time PCR amplification and quantitation was performed using the ABI 7900 sequence detection system. We first validated the use of *POP4* as an appropriate endogenous control in ULs. Next, the comparative CT method ($\Delta\Delta Ct$) was used for quantification of gene expression. Relative fold differences in gene expression for all tested genes were normalized to the *POP4* endogenous control and then calibrated to the no treatment sample at the corresponding time.

Immunohistochemistry

To assess the effects of halofuginone (HF) on UL-xenografted tissues and mouse body organs, immunohistochemical analysis was performed. Following harvest of the xenograft specimens, tissues were placed in cold, 4% paraformaldehyde (a 1:10 ratio of tissue to PFA) and left at 4°C overnight. The next day, tissues were rinsed with ethanol and paraformaldehyde was replaced with 70% ethanol. Specimens were then kept at 4°C for future processing, embedding and sectioning.

Slides were stained with Hematoxylin and eosin to assess the effect of treatment on the overall morphology of tissues and also to detect the presence of xenografted ULs in the kidney capsule specimens. Slides were deparaffinized in a series of xylene incubations for 5 minutes, rehydrated in serial (100%, 100%, 90% and 70%) 1-minute washes of EtOH followed by a quick wash in tap water. Nuclei were then stained with Mayer's hematoxylin for 10 minutes before being washed in running tap water for at least 15 minutes. Slides were next stained in 1% eosin for one minute followed by tissue

dehydration in a series of EtOH washes (70%, 80%, 90%, 100%, and 100%), each for one minute. Clearing was done last with three 1-minute washes in xylene and slides were covered in Permount.

After tissue deparaffinization and rehydration, an antigen retrieval method using DAKO solution was used to detect the presence and abundance of specific proteins in fixed tissues. Slides were placed in a 1:10 dilution of DAKO solution in PBS at 90°C in a waterbath for 30 minutes and brought to room temperature with incubation in pre-warmed (37°C)-PBS at room temperature. Endogenous peroxidase activity in specimens was inactivated by incubating slides in 0.3% H₂O₂ in methanol for 15 minutes. After three 5-minute washes in PBS, sections were blocked for half an hour in 5% normal serum of the animal species in which the secondary antibody was raised in. Sections were then washed in PBS three times and incubated in a 1:100 dilution of biotin-secondary antibody in 1% BSA/PBS for an hour. After a short wash in PBS, sections were incubated in ABC solution for 30 minutes at room temperature before 3 washes in PBS. Incubation with DAB was next performed using various incubation times depending on the particular antibody. Finally, counterstaining in Mayer's hematoxylin was carried out for 1 minute before washing sections in tap water, dehydration, clearing and mounting.

TUNEL Assay

The ApopTag® Plus Peroxidase in Situ Apoptosis Kit or the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to assess the rate of apoptosis in UL-xenografted tissues in HF and vehicle-treated animals. Following deparaffinization, tissue was pretreated with freshly diluted proteinase K (20 µl/ml) for

15 min at room temperature in a coplin jar or directly on the slide. Endogenous peroxidase (remove this big space) was next quenched in 3% hydrogen peroxide in PBS for 5 min at RT either directly on the slide or in a coplin jar. Slides were rinsed 2X with PBS for 5 minutes each and then incubated with the equilibration buffer for 60 minutes at room temperature. Working strength TdT Enzyme was next applied to sections for an hour at room temperature. Slides were incubated for 15 seconds in the stop/wash buffer before a 10-minute incubation at room temperature. After three washes with PBS, anti-digoxigenin conjugate was applied to slides in a humid chamber for 30 min at room temperature. Following a PBS wash, color was developed using the peroxidase substrate (3,3'-Diaminobenzidine Tetrahydrochloride hydrate or DAB), slides were washed in 3X dH₂O for 1 min each and the last wash was followed by an incubation in dH₂O for 5 min at room temperature. Slides were incubated in 0.5% (w:v) methyl green in a coplin jar for 10 min at room temperature to counterstain specimens, and then washed a few times in dH₂O before a final wash in 3X 100% n-butanol followed by mounting with coverslips.

Statistical Analysis

Experimental variability between treatments/matrices was determined by first conducting the Kolmogorov test for normality on obtained data. Based on the results, parametric (ANOVA) or non-parametric (Kruskal-Wallis) hypothesis tests were conducted using STATA (6.0). To compare groups pair-wised, *post-hoc* comparisons were performed. Values are expressed as mean \pm SEM and $P < 0.05$ was considered statistically significant.

Xenografting of Human Leiomyomas Underneath Mouse Kidney Capsule

The detailed procedure for transplanting ULs under the kidney capsule of mice host can be found at Kurita *et al.* 2010.

Treatment of UL-Xenografted Mouse with Halofuginone

Based on previously published studies on HF treatment in animal models, we chose two doses of HF at 0.25 and 0.50 mg/kg/body weight along with vehicle controls for treatment of UL-xenografted mice. Intraperitoneal (i.p.) administration of HF was scheduled on a daily basis for 5 days a week for 4 weeks. Since the daily treatment of mice with 0.50 mg/kg/body weight was found to be not well tolerated by mouse host, animals on the higher dose of treatment were split into two groups. One group continued to receive daily treatments of 0.5mg/kg while the other received this higher dose treatment of HF every-other-day (EOD).

Appendix A

List of Abbreviations

ABC: avidin biotinylated enzyme complex

ANOVA: analysis of variance

BCA: bicinchoninic acid

BCS: bovine calf serum

bFGF: basic fibroblast growth factor

BMI: body mass index

BSA: bovine serum albumin

β -ME: beta mercaptoethanol

CPM: counts per minute

DAB: 3,3-Diaminobenzidine, liquid chromogen substrate kit

DMEM: dulbecco's modified eagle medium

DMSO: dimethylsulphoxide

DPBS: dulbecco's PBS

DTT: dithiothreitol, reducing agent

DMT: dermatopontin

EGF-R: EGF receptor

Eker rat: animal model for leiomyoma tumors

ELT-3: leiomyom smooth muscle cell line from Eker rat

ER/ESR: estrogen receptor

ERK: extracellular signal regulated kinase

FBS: fetal bovine serum

FGF: fibroblast growth factor
GAGs: glycosaminoglycans
GDP: guanosine diphosphate
GnRH: gonadotropin releasing hormone
HGF-R: hepatocyte growth factor receptor
HRP: horseradish peroxidase
IGF-I: insulin-like growth factor-I
JNK: c-jun N-terminal kinase
LSB: Laemmli sample buffer
LSMCS: leiomyoma smooth muscle cells
LOX 3: Lysyl oxidase 3
MAPK: mitogen-activated protein kinase
MAPK1/2: ERK1/2
MMP: matrix metalloproteinase
PBS: phosphate buffer saline
PCNA: proliferating cell nuclear antigen
PDGF: platelet-derived growth factor
PDGF-R: PDGF receptor
PGF2 α : prostaglandin F2 alpha
PI3K: phosphatidyl inositol 3 phosphate
PKC: protein kinase C
PR: progesterone receptor
PTEN: phosphatase and tensin homolog

qRT-PCR: real-time quantitative reverse transcription PCR

ROS: reactive oxygen species

RU-486: progesterone receptor antagonist

SEM: standard error of the mean

SRF: serum response factor

SMC: smooth muscle cells

TBST: tris buffer saline with Tween

TGF β 1: transforming growth factor beta 1

TGF β 3: transforming growth factor beta 3

TIMP: tissue inhibitor of metalloproteinase

TNF α : tumor necrosis factor alpha

VEGF: vascular endothelial growth factor

VSMCs: vascular SMCs

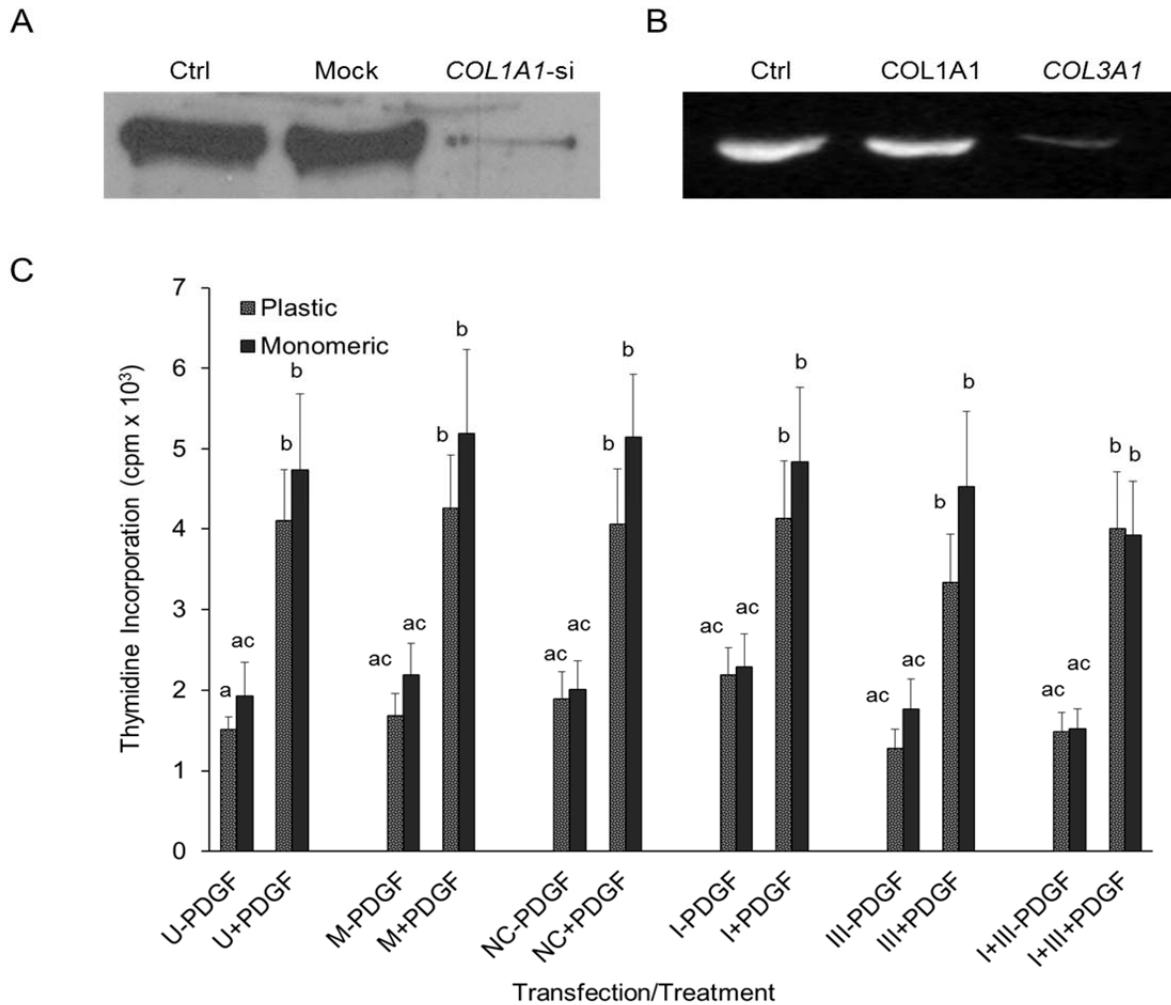
Appendix B

List of Antibodies

Antibody	Band Size(kDa)	Species	Clone	Dilution	Diluent	Company	Cat #
pERK (Thr202/Tyr204)	42-44	Rabbit	Mono	1:1000 (WB)	5% BSA	Cell Signaling	4370
ERK	42-44	Rabbit	Poly	1:1000 (WB)	5% BSA	Cell Signaling	9102
pSRC (Tyr416)	60	Rabbit	Poly	1:1000 (WB)	5% BSA	Cell Signaling	2101
SRC	60	Mouse	Mono	1:1000 (WB)	5% milk	Cell Signaling	2110
pAkt (Thr308)	60	Rabbit	Mono	1:500	5% BSA	Cell Signaling	4056
Akt	60	Rabbit	Poly	1:1000	5% BSA	Cell Signaling	9272
pPDGF-R(Y751)	190	Rabbit	Mono	1:500 (WB)	5% BSA	Cell Signaling	4549
PDGF-R	190	Rabbit	Mono	1:1000 (WB)	5% BSA	Cell Signaling	3169
pFAK (Tyr397)	125	Rabbit	Poly	1:100 (IF)		Cell Signaling	3283
FAK	116-125	Mouse		1:100 (IF)		BD Transduction Laboratories	610087
Vinculin	116	Mouse	Mono	1:100 (IF)		Sigma	V 9131
Collagen III	100-120	Rabbit	Poly	1:1000 (WB), 1:100 (IHC)	2.5% milk	Millipore	MAB3392
Collagen I	80-120	Rabbit	Poly	1:2000 (WB), 1:100 (IHC)	2.5% milk	Antibodies-online	ABIN97498
GAPDH	36-40	Mouse	Mono	1:3000 (WB)	5% BSA	IMGENEX	IMG-5019A-1
TGFBR1		Rabbit	Poly	1:100 (IHC)		Santa Cruz Biotechnology	sc-400
Ki67		Mouse	Mono	1:100 (IHC)		BD Pharmingen	550609
Bcl2		Rabbit	Mono	1:100 (IHC)		Millipore	04-436
Dermatopontin		Goat	Poly	1:100 (IHC)		Santa Cruz Biotechnology	sc-16080

Appendix C

siRNA-Mediated Silencing of *COL1A1* and *COL3A1*



Silencing collagen expression did not affect LSMC proliferation. (A) Immunoblot for collagen I in LSMCs silenced for *COL1A1*, (B) RT-PCR for *COL1A1* and *COL3A1* in LSMCs silenced for *COL3A1*, (C) proliferation of LSMCs silenced with negative control (NC) siRNA, or siRNAs specific for *COL1A1* and *COL3A1* or both in the absence of presence of 10 ng/ml PDGF for another 24 hours. Letters indicate statistical significance between groups. (n=5, P<0.05.)