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NOVEL EFFECTS OF SIMVASTATIN ON UTERINE FIBROIDS

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NOVEL EFFECTS OF SIMVASTATIN ON UTERINE FIBROIDS

by

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Dedication

To my parents, Abdel-Aziz and Fatima, whose inspiration was my principal motivator,

To my wife, Rania, and kids, Jana, Kareem and Bassem, whose support at difficult times was crucial for this work to come to light,

To my mentor, Darren Boehning, who has been an outstanding mentor, not only in scientific research, but also in life,

To my brothers, sisters, friends, and colleagues,

Thank you!

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NOVEL EFFECTS OF SIMVASTATIN ON UTERINE FIBROIDS

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Abstract: Uterine fibroids (also known as leiomyomas) represent a common gynecologic problem with a significant medical and economic burden. Unfortunately, no satisfactory long-term medical treatment is currently available. Statins are drugs commonly used for the treatment of high plasma cholesterol levels. Beyond these well-known lipid-lowering properties, they possess broad reaching effects in vivo which include anti-tumor effects. Statins inhibit the growth of multiple tumors; however the mechanisms remain incompletely understood. The purpose of this study was to examine the effects of simvastatin on uterine fibroid, both in vitro and using an animal model. For the in vitro studies, we used primary and immortalized human fibroid cells. For the in vivo studies, we used immunodeficient mice supplemented with estrogen/progesterone pellets xenografted with human fibroid tissue explant. We found that simvastatin inhibits the proliferation of human fibroid cells. This was associated with decreased mitogen-activated protein kinase signaling and multiple changes in cell cycle progression. Simvastatin potently induced fibroid cell apoptosis in a manner mechanistically dependent upon apoptotic calcium release from L-type voltage-gated calcium channels. Thus, simvastatin possesses anti-tumor effects which are dependent upon the apoptotic calcium release machinery. For the in vivo studies, animals were treated with simvastatin

vs vehicle control. The treatment inhibited tumor growth as measured weekly using calipers and/ or ultrasound. Finally, simvastatin decreased the expression of the proliferation marker Ki67 in xenograft tumor tissue as examined by immunohistochemistry. In conclusion**,** simvastatin can be a promising treatment for uterine fibroid. Further studies, including pharmacokinetic and drug delivery studies, are required.

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List of Abbreviations

DMSO	Dimethyl Sulfoxide
ERE	Estrogen Response Elements
ERK	Extracellular Signal-Regulated Kinases
ERs	Estrogen Receptors
GnRHa	Gonadotropin Releasing Hormone Agonist
GPR30	G Protein-Coupled Receptor 30
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HuLM	Immortalized Human Leiomyoma Cells
IGF	Insulin-Like Growth Factor
IP_3R	with inositol 1,4,5-trisphosphate receptor
MAPK	Mitogen-activated protein kinases
MTD	Maximum Tolerated Dose
PCNA	Proliferating Cell Nuclear Antigen
PCOS	Polycystic Ovarian Syndrome
PLC	Phospholipase C
PRs	Progesterone Receptors
RTK	Receptor Tyrosine Kinase
SEM	Standard Error of the Mean
SMA	Smooth Muscle Actin
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated 2'-Deoxyuridine 5'-Triphosphate Nick End Labeling

CHAPTER 1: INTRODUCTION

Signaling Pathways in Leiomyoma

Modified from Signaling Pathways in Leiomyoma: Understanding Pathobiology and Implications for Therapy

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INTRODUCTION

Uterine leiomyomas are the most common tumors of the female genital tract. They were first described in 1793 by Matthew Baillie, a Scottish physician and pathologist at St George's Hospital in London (1). Since then, they were found to cause several gynecologic problems with significant medical and economic burden. In fact, the lifetime incidence of leiomyomas ranges from 50-80% (2) while their total annual cost (both direct and indirect) in the US is estimated to be $34.4 billion (3).

Despite this enormous impact, the exact underlying pathobiology of uterine leiomyomas is not clear. This leads to inability to develop a satisfactory medical treatment. Although the initial event in leiomyoma development is considered proliferation of a single smooth muscle cell (4, 5), additional complex signaling alterations are thought as necessary (6).

Understanding aberrations of these signaling pathways and their interconnections is critical for directing research aimed at discovering potential therapeutic targets. The aim of this review is to discuss what is known about these pathways, their interconnections and their role in leiomyoma pathobiology and to identify potential therapeutic targets.

STEROID SIGNALING

Estrogen

Uterine leiomyomas have classically been considered estrogen-dependent tumors as they tend to grow during reproductive years and shrink after menopause (7) In addition, no cases have been described before puberty (8). All these observation have led to the early recognition of the critical role of estrogen in leiomyoma tumorigenesis. This role was later supported by the finding that continuous gonadotropin releasing hormone

agonist (GnRHa) treatment, which significantly decreases ovarian estrogen production, is associated with reduction in tumor size (9).

ESTROGEN RECEPTORS

Estrogens exert their effects on target cells through activation of estrogen receptors. First described in mouse uterus and vagina by Jensen and colleagues in 1973 (10), estrogen receptors are now known to be present in many tissues and organs. They are currently classified into nuclear and plasma membrane-bound.

Nuclear estrogen receptors (also called classical and simply referred to as ERs) are modular proteins composed of five domains. They are further subdivided into ERα (which describes initially discovered ERs), and ERβ (recently identified in 1996) (11). ERα and ERβ exhibit DNA- and ligand-binding domain sequence conservation and are encoded by two distinct genes (*ESR1* and *ESR2*, respectively) on different chromosomes (12, 13). Furthermore, they have different transcriptional activation domains and diverse tissue distribution (14, 15). While ERα is mainly expressed in uterus and breast, ERβ is more broadly distributed in ovary, brain, bone, and other organs. However, both ERα and ERβ are co-expressed in several organs (16).

Membrane-bound estrogen receptors include the same nuclear estrogen receptors localized to the plasma membrane (mERs) and unique ones such as the more recently identified G protein-coupled receptor 30 (GPR30) (17-19). In fact, about 5-10% of total ERs of the cells are localized at plasma membrane where palmitoylation (attachment of palmitate residue) is important for localization at this membrane (19). Both α and β subtypes are localized at the plasma membrane (known as mERα and mERß) (19, 20). On the other hand, the transmembrane G protein-coupled receptor 30 (GPR30), first described in late 1990s, is structurally and functionally different (17, 18). Detailed description of the structure and function of these receptors is beyond the scope of this review and can be found elsewhere (18, 19).

ESTRADIOL SIGNALING

Cellular estrogen effects are mediated through two main pathways: modulation of transcriptional activities and rapid signal transduction.

In the classic transcriptional modulation, 17β-estradiol (E2) binds to ERs and then E2-ER complexes regulate transcriptional activities. Although unbound ERs were initially thought to be mostly cytosolic and translocate to nucleus only after ligand binding (21), they were later found to be mostly nuclear (22). These unbound receptors are chaperoned by heat shock protein 90 (HSP90), which also helps in their nuclear trafficking (23, 24). Binding of E2 to ER leads to dissociation of HSP90, receptor dimerization and conformational changes that allow ER to bind estrogen response elements (EREs) of DNA at target genes promoters (23). Transcription of these target genes is also regulated by a number of coactivators such as Steroid Receptor Coactivator 1 (SRC-1) and corepressors such as Nuclear Receptor Co-Repressor (NCoR) (25). Another mode of action of classic pathway is interaction of ligand-ER complexes with certain transcription factors such as Nuclear Factor –Kappa B (NF-κB) and specificity protein-1 (SP-1) (26).

On the other hand, the rapid signaling pathway works in a similar way to growth factor receptors. Upon ligand binding, mERs form homodimers, then activate several kinases including src followed by activation of PI3K and ERK pathways (19). In addition, activated mERs may transactivate growth factor receptors e.g. epidermal growth factor receptors (19). As a G protein-coupled receptor, GPR30 is involved in rapid signaling events including cAMP generation, calcium release and protein kinase activation as well as regulation of transcriptional activation of certain genes such as c-fos (18).

While nuclear receptors are generally involved in transcriptional activity, membrane receptors are more commonly involved in rapid signaling. However, there is

evidence that both receptor categories are capable of rapid signaling as well as transcriptional activity modulation (18). A simplified illustration of estrogen signaling in presented in figure 1.

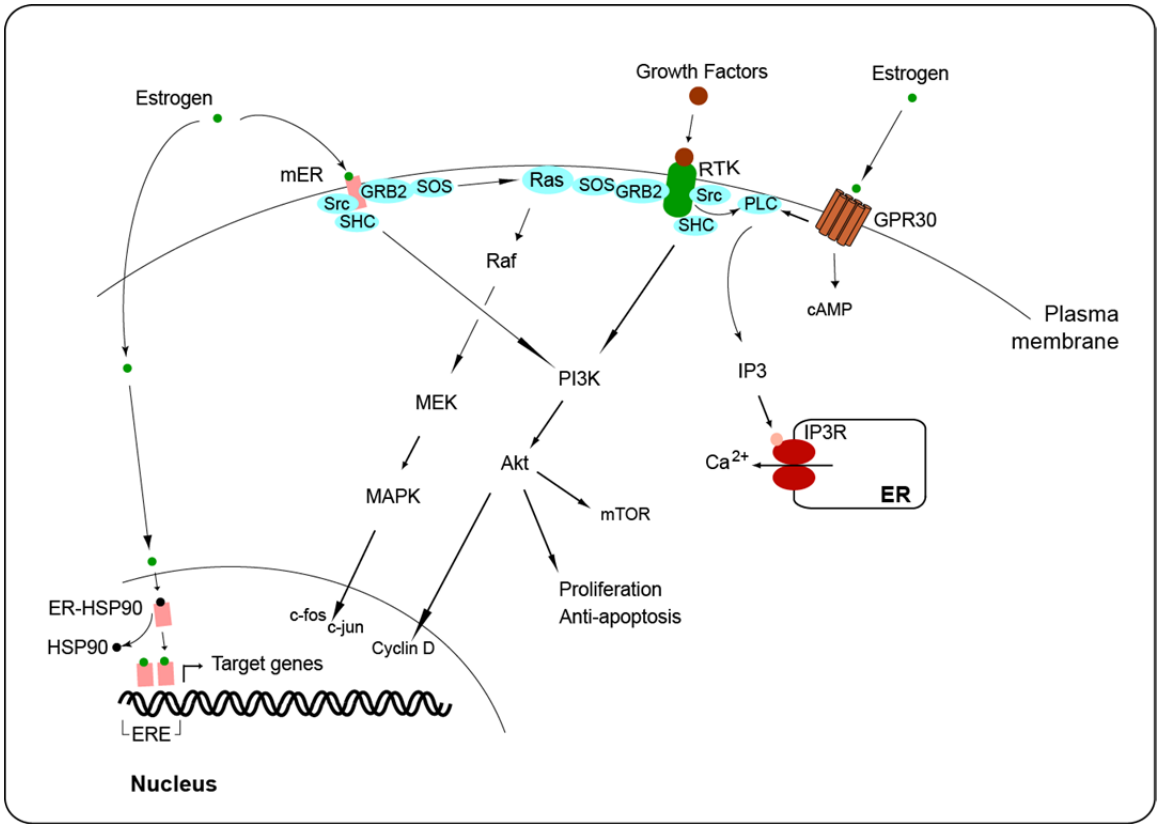

Figure 1: Schematic presentation of estrogen and growth factor signaling pathways in uterine leiomyoma cell.

ER: estrogen receptor; mER: membrane-bound estrogen receptor; HSP90: heat shock protein 90; ERE: estrogen response element; RTK: receptor tyrosine kinase; GPR30: G protein coupled receptor 30; PLC: phospholipase C; IP3: inositol triphosphate; IP3R: inositol triphosphate receptor; ER: endoplasmic reticulum

ROLE OF ESTRADIOL SIGNALING IN LEIOMYOMA PATHOBIOLOGY

There is clear evidence that aberrant estrogen receptor signaling contributes to leiomyoma development and growth. For example, leiomyomas overexpress ERα and ERβ mRNA compared to surrounding myometrium (27, 28). In addition, Maekawa and colleagues (29) demonstrated that epigenetic regulation of ERα through DNA methylation plays a role in leiomyoma. More recently, uterine leiomyomas were found to overexpress GPR30 in comparison to surrounding myometrial tissue (30).

In addition to ER expression, receptor phosphorylation can be a contributing factor to leiomyoma development. ERα is phosphorylated at a higher rate on serine in leiomyoma compared to surrounding myometrium and colocalizes with phospho-p44/42 MAPK. Therefore, it is reasonable to assume that phosphorylated ERα, possibly regulated by p44/42 MAPK, may play a role in leiomyoma development (31).

There is evidence that rapid E_2 signaling plays a role in leiomyoma pathobiology. For example, Barbarisi and colleagues demonstrated that E_2 treatment for 1, 5, and 30 minutes triggers rapid activation of phosphatidylinositide 3-kinases (PI3K), mitogen-activated protein kinase (MAP Kinase) and phosphoinositide phospholipase Cγ (PLCγ) (32). In addition, Nierth-Simpson and colleagues (33) showed that E_2 treatment for 5 minutes activates protein kinase C alpha (PKCα) in both human leiomyoma cells and human smooth muscle cells. Furthermore, they noted that it increases phosphorylated MAP Kinase in human leiomyoma cells but not human smooth muscle cells. Therefore, they concluded that aberrant and rapid MAP kinase signaling responses to estradiol may play a role in leiomyoma proliferation.

Finally, it is evident that uterine leiomyomas have aberrations in E_2 biosynthesis. Ishikawa and colleagues (34), Bulun and colleagues (35, 36) and Shozu and colleagues (37) demonstrated that aromatase enzyme is significantly overexpressed in leiomyoma compared to normal myometrium. In addition, Kasai and colleagues (38) demonstrated

that leiomyoma tissue overexpresses type I 17β-hydroxysteroid dehydrogenase (17β-HSD) compared to myometrial tissue. Together with aromatase overexpression, this means that leiomyoma tissue converts circulating androstenedione into estrone (via aromatase), then into estradiol (via 17β-HSD type I) in situ (37). This represents the molecular basis of potentially using aromatase inhibitors as therapeutic agents in uterine leiomyomas.

TARGETING ESTROGEN SIGNALING IN LEIOMYOMA TREATMENT

Modulating estrogen signaling represents an attractive therapeutic opportunity. First, lowering estrogen levels through inducing a menopause-like state by continuously administering GnRHa can lead to shrinkage of tumors (39). However, due to significant side effects such as loss of bone mineral density, it can only be used for a short period. As such, it is almost conclusively used only pre-operatively. Second, administering aromatase inhibitors lowers local estrogen levels by suppressing conversion of androgens to estrogen (40). Similar to GnRHa, aromatase inhibitors are associated with significant estrogen-deprivation side effects.

Selective estrogen receptor modulators (SERMs) are chemicals that exert a mixture of agonistic and antagonistic effects on estrogen receptors. Reports of tamoxifen (one of the SERMs) effects on uterine leiomyomas are conflicting (41, 42). Similarly, a Cochrane review (43) summarized three randomized controlled trials using raloxifene (another SERM) in uterine leiomyomas with two describing a significant benefit while the third found no benefit. However the quality of evidence of the three trials was described as low or very low (43).

In addition, the natural estrogen metabolite 2-methoxyestradiol represents another potential therapeutic agent. Salama and colleagues found that it induces apoptosis and inhibits proliferation of leiomyoma cells through several mechanisms including modulating estrogen signaling pathways (44, 45).

Finally, gene therapy targeting ERs represents another potential leiomyoma therapy. Al-Hendy and colleagues (46) demonstrated that adenovirus used to express dominant negative estrogen receptors can lead to tumor growth inhibition in nude mice (46).

Progesterone

While early research has mostly focused on the role of estrogen in leiomyomas (47, 48), more recent evidence points to a significant role of progesterone. Initially dubbed as "the progesterone hypothesis" (6), the role of progesterone in leiomyoma pathobiology is becoming more established (8, 49). Kawaguchi and colleagues (50) demonstrated that mitotic activity in uterine leiomyomas is significantly higher in secretory (progesterone-dominant) phase compared to proliferative (estrogen-dominant) phase of the menstrual cycle. Furthermore, the necessity of progesterone for growth of uterine leiomyoma was supported by leiomyoma xenograft animal models. For example, Ishikawa and colleagues (51) implanted human uterine leiomyoma tissue underneath the kidney capsule of immunodeficient mice. They found that treatment of mice with estrogen alone was not associated with tumor growth while treatment with estrogen and progesterone was associated with tumor growth. Interestingly, progesterone withdrawal (or antagonism with RU486) was associated with decreased tumor size, emphasizing the critical role of progesterone. Finally, they found that estradiol induces expression of progesterone receptors in leiomyoma cells (51) giving a potential explanation for the interaction between estrogen and progesterone. For all this, there is current near consensus that progesterone is at least as important as estrogen for leiomyoma development.

PROGESTERONE RECEPTORS

Similar to estrogen receptors, progesterone receptors exist in two main categories, nuclear and membrane-bound. Nuclear progesterone receptors (PRs) work as ligand-activated transcription factors in the same way as nuclear estrogen receptors. There are two main isoforms of PR in humans, PR-A and PR-B. Both are transcribed from the same gene with PRB being larger by 164 amino acids (52). Genes for membrane progesterone receptors (mPR) were initially identified in fish and later in human and are present in three isoforms; mPRα, mPRβ and mPRγ (53, 54). Although these membrane receptors are expressed in uterus (54), there are no published studies to address their expression or role in uterine leiomyoma.

PROGESTERONE SIGNALING PATHWAYS

Ligand-bound PR binds to DNA at progesterone response elements (PRE) and in the presence of other transcription factors such as SP-1 regulates transcription of several target genes (8). In addition to this transcriptional pathway, progesterone can activate rapid signaling pathways. Ligand-bound PRs can activate several protein kinases involved in growth factor signaling such as MEK MAPK (55). Furthermore, there is evidence that PRs contain a proline-rich motif that can directly interact and active c-src tyrosine kinases and thereby activate ERK signaling pathway (56).

INTERACTION WITH OTHER SIGNALING PATHWAYS

The role of progesterone in uterine leiomyoma development is complex. First, there is evidence to support estrogen-progesterone "cross-talk" in leiomyoma cells. For example, as previously described, Ishikawa and colleagues found that estradiol induces expression of progesterone receptors in leiomyoma cells (51).

Second, there is evidence to support interaction between progesterone and growth factors signaling. Maruo group in Japan found that progesterone down-regulates

expression of insulin-like growth factor-I (IGF-I) in human leiomyoma cells (57). They also demonstrated that progesterone up-regulates the expression of proliferating cell nuclear antigen (PCNA) and epidermal growth factor (EGF), both are known regulators of leiomyoma cellular proliferation (58, 59). Furthermore, Hoekstra and colleagues (60) demonstrated that R5020 (a synthetic progestin that acts as an agonist of progesterone receptors) induces proliferation of leiomyoma cells in vitro. In addition, they found that R5020 activates (through phosphorylation) AKT and glycogen synthase kinase-3B (GSK3B). They also found that API-59 (an AKT inhibitor) abrogates R5020-induced cellular proliferation. Therefore, they concluded that progesterone can induce proliferation of leiomyoma cells through activation of AKT pathway.

Finally, Yin and colleagues (61) found that the transcription factor KLF11 integrates progesterone receptor signaling pathway with proliferation in uterine leiomyoma cells. From all this, It seems that progesterone signaling is an integrated part of a complex signaling network in leiomyoma.

TARGETING PROGESTERONE SIGNALING IN LEIOMYOMA TREATMENT

Fiscella and colleagues (62) randomized 42 women with symptomatic uterine leiomyomas into treatment with progesterone antagonist mifepristone or placebo for 26 weeks. They found that mifepristone treatment was associated with a significant tumor size reduction, improvement of anemia, and improved subjective assessment of quality of life. The same group followed patients for 12 months and confirmed tumor shrinkage. However, they found modest increase in endometrial hyperplasia (63).

Donnez and colleagues (64) randomized patients with symptomatic uterine leiomyomas to receiving 10 mg orally daily of the selective progesterone receptor modulator (SPRM) ulipristal acetate (96 patients), 5 mg orally daily of ulipristal acetate (96 women) or placebo (48 women). After 13 weeks of treatment, there was significant improvement of uterine bleeding and reduction in total leiomyoma volume in the treated

groups. This study confirms that modulating progesterone signaling pathway presents a potential therapeutic target in leiomyoma treatment. Side effects included headache and breast pain and discomfort. However, long term data is needed before widespread use of SPRMs in uterine leiomyoma treatment.

To further study the underlying leiomyoma signaling pathways involved in SPRM treatment, Ohara and colleagues (65) used primarily cultured leiomyoma and myometrial cells. They demonstrated that SPRM asoprisnil (J867) decreased expression of proliferating cell nuclear antigen (PCNA) while increasing terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) positive cells. In addition, asoprisnil decreased expression of anti-apoptotic Bcl-2 protein while increasing active caspase-3. All these effects are consistent with induction of apoptotic signaling pathways and inhibition of proliferative signaling pathways. Importantly, these changes were noted in leiomyoma but not myometrial cells. Furthermore, they demonstrated that asinoprisnil decreases expression of certain growth factors and growth factors receptors. These findings are significant as they represent another example of interaction between steroid and growth factor signaling pathways in leiomyoma (65).

GROWTH FACTORS SIGNALING

Growth factors are relatively small proteins secreted into extracellular space to bind to cell membrane receptors of target cells. This results in activation of intracellular signal transduction pathways which modulate several processes including cell proliferation and growth (66). Since the first discovery of nerve growth factor (NGF) by Rita Levi-Montalcini in the 1950s (67), many growth factors have been characterized. Perturbation of growth factor signaling plays a significant role in the development and growth of many tumors (66).

Substantial evidence exists to suggest that aberrations of certain growth factors and/or their receptors or signaling pathways play a significant role in growth and

development of uterine leiomyomas (68-71). These include insulin-like growth factor-I (IGF-I) (72, 73), platelet-derived growth factor (PDGF) (71), vascular endothelial growth factor (VEGF)(74), epidermal growth factor (EGF) (75) and fibroblast growth factor (FGF) (76). A simplified illustration of growth factor signaling in presented in figure 1.

Receptor tyrosine kinases (RTKs)

Receptor tyrosine kinases (RTKs) are cell-surface growth factor receptors with 58 known members in humans categorized into 20 subfamilies (77). They share a similar structure composed of 3 parts: an extracellular ligand binding domain, a transmembrane helix and an intracellular domain that contains the tyrosine kinase (TK). Generally, growth factor binding to the RTK leads to receptor dimerization and autophosphorylation. This leads to downstream activation of several pathways including Grb2-Sos-Ras-Raf-MEK-ERK and PI3K-PIP3-Akt. Therefore, RTKs are important regulators of important cellular processes including proliferation, differentiation, survival and metabolism. Aberrations in RTKs are linked to several disease processes including cancer, diabetes and inflammations (77).

There is growing body of evidence for role of RTKs in growth and development of uterine leiomyomas. Yu and colleagues (78) demonstrated that several RTKs are overexpressed in leiomyoma. Using an RTK array technique, they found that 39 out of 42 RTKs are differentially over phosphorylated in leiomyomas compared to myometrial tissues. In addition, Swartz and colleagues (79) found that 17β estradiol treatment leads an increase in insulin-like growth factor (IGF)-1 mRNA in leiomyoma cells. Therefore, they concluded a “cross talk” where estradiol leads to up regulation of growth factors and RTKs in uterine leiomyomas and therefore, growth factors and RTKs represent intermediate effectors for sex steroids effects on leiomyomas (78).

Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK signaling pathway regulates several critical processes including cellular proliferation and survival (77, 80, 81). Mitogen-activated protein kinases (MAP kinases) are serine/threonine-specific protein kinases that regulate many cellular functions including proliferation, survival and apoptosis. They include several subfamilies, e.g. extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK (82).

The binding of growth factors to their receptors (RTKs) leads to a cascade of molecular changes. These include the recruitment and anchoring of Son of sevenless protein (Sos) to RTK with Grb2 (either directly or through Shc protein linker) working as adapter or bridge proteins. The Sos works as a guanine nucleotide exchange factor (GEF) to stimulate the exchange of a GDP for a GTP molecule to (and therefore activation) the small guanine nucleotide-binding protein (G protein) Ras. Thereafter, activated Ras recruits and activates the Raf kinases which subsequently phosphorylates (and therefore activates) MEK protein which phosphorylates and activates the extracellular signal-regulated kinase (ERK) (66). In turn, ERK activates several of the activating protein-1 (AP-1) family of transcription factors including c-Fos and c-Jun which leads to a complex series of nuclear events modulating transcription of several target genes (83).

It is critical to understand the complex and bidirectional interaction between steroid signaling and the Ras/Raf/MEK/ERK signaling pathway. On one hand, estrogen can induce activation of ERK pathway through GPCR as well as transactivation of EGFR (84). In addition, estrogen can activate ERK through pathway involving the non-receptor tyrosine kinase c-src (85-87). On the other hand, growth factors can modulate the response to steroids through effects of ERK on transcriptional activity of steroid receptors (88).

There is evidence that Ras/Raf/MEK/ERK pathway plays a significant role in leiomyoma pathobiology. Yu and colleagues (78) demonstrated that several of the signaling molecules involved in this pathway are overexpressed in leiomyoma compared

to myometrium including Shc, Grb2, and ERK. They also found that 15 out of 17 RTKs were more expressed in leiomyoma. Therefore, it seems that RTKs and their downstream signaling through ERK pathway may play a role in leiomyoma. In addition, Nierth-Simpson and colleagues (33) demonstrated aberrant ERK signaling in leiomyoma. They found that rapid 17β-estradiol (E_2) signaling is associated with ERK activation in leiomyoma cells where in normal myometrium it decreases phosphorylated ERK.

PI3K/Akt/mTOR pathway

The phosphatidylinositide 3-kinase (PI3K)/Akt pathway represents another important RTK-ligand activated signaling pathway (66). In addition to RTKs-ligand binding, PI3K can be activated by G protein-coupled receptors (GPCRs) and membrane-bound steroid receptors. PI3K activation phosphorylates the plasma membrane lipid molecule phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) or PIP_2 to phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) or PIP_3. PIP_3 leads to recruitment of several pleckstrin-homology (PH) domain-containing signaling proteins including Akt CITE <EndNote><Cite -dependent kinase-1(PDK1). This in turn regulates mammalian target of rapamycin (mTOR), Bcl-2 family proteins and glycogen synthase kinase 3 (GSK3), several transcription factors and many other molecules. PIP_3 is inactivated by dephosphorylation at carbon 3 by the tumor suppressor PTEN. These pathways control important processes including survival, proliferation, cell cycle and apoptosis.. Further details of the Akt signaling pathway are beyond the scope of this review but can be found elsewhere (89).

There is recent evidence of aberrant PI3K/Akt/mTOR signaling in uterine leiomyoma. Karra and colleagues (90) found increased expression of glycogen synthase kinase 3 (GSK3) and cyclin D_2 in leiomyoma compared to myometrium, with interaction between phosphorylated GSK3 and Akt. In addition, Jeong and colleagues (91) demonstrated that leiomyomas express lower levels of phospho-Akt and

phosphatidylinositol-3,4,5-triphosphate (PIP_3) but higher PTEN levels. In addition, they found that E_2 treatment increases PTEN in leiomyoma, but not myometrial cells. Finally, Crabtree and colleagues (92) found upregulated mTOR pathway in leiomyoma both in human and in the Eker rat animal model. These findings suggest that aberrant PI3K/Akt/mTOR signaling plays a role in leiomyoma pathobiology.

Role of individual growth factors in leiomyoma pathobiology

Several studies point to a role for insulin-like growth factor I in leiomyoma pathobiology. For example, Peng and colleagues (73) found that one third of uterine leiomyomas demonstrate dysregulation in IGFs. They obtained uterine leiomyomas samples from hysterectomy cases where they performed microarray analysis, immunohistochemistry, RT-PCR, methylation analysis and Western blotting. While IGF-2 protein and mRNA transcript levels were increased, IGF-1 protein levels were increased, but with no change in mRNA transcript level. In addition, they noted that IGF-1 level correlates with activation of AKT. Interestingly, they noted that overexpression of IGF-1 and p-AKT correlate with leiomyoma size.

In addition, Burroughs and colleagues (72) used Eker rat leiomyoma animal model to demonstrate that insulin-like growth factor 1 (IGF-I) is expressed in leiomyoma tissues 7.5 times normal tissues. In addition, they demonstrated that tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), the signal transduction protein downstream of IGF-1 signaling, in leiomyoma is 4 folds higher compared to myometrium (72). Importantly, IGF-1 signaling seems to be regulated by estrogen. Swartz and colleagues (79) found that in vitro E_2 treatment of uterine leiomyoma cells leads to upregulation of IGF-I and Myb (a transcription factor promoting cell cycle progression) genes.

Fayed and colleagues (93) studied specific binding of EGF, PDGF and insulin in leiomyoma and myometrium. They found that protein synthesis was stimulated by EGF,

PDGF and insulin in both leiomyoma and myometrial cells. This denotes that these signaling pathways are operational in leiomyoma and myometrium.

More recently, Liang and colleagues (94) demonstrated using human leiomyoma and myometrial tissues that leiomyoma expresses PDGF more than the surrounding myometrium. In addition, they found that PDGF increases expression of PCNA and collagen α1 in leiomyoma compared to myometrial cells. Therefore, it seems reasonable to conclude that PDGF signaling is aberrant in leiomyoma and that it may contribute not only to proliferation but excessive deposition of extracellular matrix (94).

Rossi and colleagues (75) demonstrated using immunohistochemical studies and cultures of human primary cells that myometrial cells express EGF, PDGF, EGFR, and PDGFR-β. More recently, Ren and colleagues (95) demonstrated that EGF stimulates DNA synthesis in leiomyoma but not myometrial smooth muscle cells. In addition, they found that EGF-induced effects can be blocked by AG1478, an EGFR inhibitor as well as by PD98059, a MAPK kinase inhibitor. However, they noted that both leiomyoma and myometrial cells equally express EGFR. Importantly, they found that EGF-induced stimulation pattern in leiomyoma is different from myometrial cells suggesting fundamental alterations in EGF signaling in leiomyomas.

Modulating growth factor signaling as a potential target in leiomyoma treatment

Growth factors signaling pathways represent a potential target for leiomyoma treatment (96). For example, Di Lieto and colleagues (97) demonstrated that reduction of uterine volume after gonadotropin releasing hormone agonists (GnRHa) treatment was associated with a reduction in expression of fibroblast growth factor (FGF) and vascularity of leiomyomas. In addition, Ohara and colleagues (65) found that the selective progesterone receptor modulator (SPRM) asoprisnil (J867), known to inhibit proliferation and induces apoptosis of leiomyoma cells, also decreases expression of EGF, IGF-I, TGF-β mRNA and protein. In addition, it decreases expression of EGFR,

IGF-IR alpha and TGFRII protein in leiomyoma but not myometrial cells. Therefore, they suggested that the potential therapeutic effects of asoprisnil are mediated through downregulating the expression of certain growth factors and growth factor receptors in leiomyoma cells. This further supports the notion that growth factors signaling pathways present a potential therapeutic target in leiomyoma therapy.

Modulating PI3K/Akt/mTOR pathway seems to be an intriguing target in leiomyoma therapy. For example, Sefton and colleagues (98) demonstrated that Akt inhibition by MK-2206, an investigational drug currently in phase II trials, inhibits leiomyoma growth and induces cellular death. In addition, there is a published patent to use an mTOR inhibitor as a treatment of leiomyoma (99).

OTHER PATHWAYS

Transforming growth factor-beta (TGF-β3)/Smads signaling pathway

Transforming growth factor-β (TGF-β) is a ubiquitous small polypeptide involved in regulation of cellular proliferation, differentiation, survival and other processes. Three isoforms of TGF-β are present in humans; TGF-β1, TGF-β2 and TGF-β3. These three isoforms are secreted from cells into extracellular matrix as latent proteins. Once activated by tissue proteases, TGF-β ligands bind to and activate its receptors (TGFBR1, TGFBR2 and TGFBR3). Ligand binding to receptors lead to receptor heterotetrameric complex formation and phosphorylation, which in turn phosphorylates a series of transcription factors called Smads. These, in turn, translocate into nucleus and regulate transcription of several target genes. In addition, intracellular signaling of TGF-β involves non-Smad pathways. Ikushima and Miyazono (100) as well as Elliott and Blobe (101) provide a detailed review of TGF-β signaling pathways in human tumors. Aberrations of TGF-β signaling are considered central in tumorigenesis and tumor progression(102). The role of TGF-β signaling in tumors is complex and context and type-specific (100).

There is a growing body of evidence for a role of aberrations of TGF-β signaling in growth and development of uterine leiomyoma. Lee and Nowak (103) found that TGF-β3 mRNA was 5-fold higher in leiomyoma compared to normal myometrium. In addition, they demonstrated that leiomyomas are refractory to anti-proliferative effects of TGF-β1 and TGF-β3 observed in normal myometrium. Finally, they found that a TGF-β neutralizing antibody decreases levels of type I and III collagen mRNA in leiomyoma and myometrial cells. Therefore, they concluded that leiomyoma cells demonstrate abnormal TGF-β signaling pathways rendering cells resistant to anti-proliferative effects of TGF-β (103). Arici and Sozen (104) found that leiomyoma tissue overexpress TGF-β3 compared to myometrial tissue. In addition, they found that TGF-β3 induces fibronectin expression by leiomyoma cells. However, in contrast to Lee and Nowak (103), they found that TGF-β3 stimulates proliferation in both leiomyoma and myometrial cells. Again, this underlines the complex and context-dependent nature of outcome of TGF-β3 signaling. Furthermore, Salama and colleagues (105) demonstrated that TGF-β3 induces profibrotic effects (expression of type I and III collagen and others) on leiomyoma cells through Smad and non-Smad pathways. Finally, leiomyoma was found to overexpress TGFBR1, TGFBR2, Smad3, Smad4 and phosphorylated Smad3 compared to myometrium (106). All these studies point to a potential rule for aberrant TGF-β-Smad signaling in leiomyoma.

TGFβ-SMADs signaling pathway represent a potential target for therapeutics development. Chegini and colleagues(106) demonstrated that gonadotropin-releasing hormone-analog (GnRHa) treatment decreases expression of TGF-βRs, Smad4 and phosphorylated Smad3. Furthermore they found that GnRHa treatment reduces expression of TGF-β3 and CTGF in leiomyoma, but not myometrial tissues. Therefore, they concluded that TGF-β3 and CTGF may play a role in leiomyoma tumorigenesis and may represent potential therapeutic targets. Finally, Salama and colleagues (105) found that 2-methoxyestradiol (an estrogen metabolite with anti-tumor properties) inhibits TGF-

β3 profibrotic effects in leiomyoma cells through Smad and non-Smad mediated pathways. Furthermore, De Falco and colleagues (107) used tissues obtained from hysterectomy patients and demonstrated that TGF-β3 and connective tissue growth factor are overexpressed in leiomyoma compared to myometrium.

Wnt/β-Catenin

The wingless-type (Wnt) signaling pathways represent a group of signal transduction pathways (canonical and noncanonical) where the binding of Wnt protein ligands to a cell surface Frizzled family receptors leads to receptor activation and phosphorylation of the disheveled (Dsh) cytoplasmic protein. The canonical Wnt pathway leads to accumulation of beta catenin in the cytoplasm and its subsequent translocation into the nucleus and subsequent activation of transcription factors (108).

There are several recent reports of role of Wnt/beta catenin in leiomyoma growth and development. For example, Mangioni and colleagues (109) demonstrated that Wnt5b gene is overexpressed in uterine leiomyomas, suggesting a possible role in its pathogenesis.

The mediator complex subunit 12 (MED12) is a large protein (1.2 MDa) involved in initiation of transcription. Aberrations of MED12 were found in 70% uterine leiomyomas (110). In addition, Markowski and colleagues found that leiomyomas with MED12 mutations overexpress Wnt protein family member Wnt4 (111). They further hypothesized that estrogen along with MED12 mutations lead to Wnt/beta catenin activation with leiomyoma-like lesions in murine models. In addition, Tanwar and colleagues demonstrated that constitutively expressing activated beta-catenin in Cre-recombinase mouse model leads to myometrial hyperplasia and development of leiomyoma-like uterine tumors (112).

More recently, Ono and colleagues (113) demonstrated that Wnt/beta catenin signaling mediates a novel interaction between leiomyoma stem cells (representing 1% of

tumor cells; also called leiomyoma side-population, LMSP) and mature leiomyoma cells that promotes tumor growth. They also found that estrogen and progesterone induce expression of Wnt11 and Wnt16 in mature leiomyoma cells which, through paracrine effects, leads to nuclear translocation of beta catenin in LMSP with subsequent transcription of their target genes including AXIN2 and proliferation of LMSP cells. Therefore, they concluded that estrogen and progesterone-induced proliferation is leiomyomas is modulated, at least in part, through Wnt expression by mature leiomyoma cells and its paracrine response on beta catenin signaling in LMSP.

Retinoic acid (RA) signaling

Retinoic acid (RA), the active metabolite of vitamin A (retinol), is involved in several functions especially those related to growth and development (114). It acts as the ligand for the nuclear retinoic acid receptors (RARs: alpha, beta and gamma) which binds DNA at retinoic acid response elements (RAREs) regulating transcription of target genes. Of note, RARs act as heterodimers with retinoid X receptors (RXRs: alpha, beta and gamma) (115). Interestingly, RXRs acts as heterodimers for several nuclear receptors including vitamin D receptor (VDR), Peroxisome proliferator-activated receptor (PPAR) and thyroid hormone receptor (TR). In addition, RXRs can directly bind 9-*cis* retinoic acid (9-*cis*-RA) and other retinoids (115).

There is strong evidence that aberrations in retinoic acid signaling pathway play a role in leiomyoma development and growth. First, Boettger-Tong and colleagues (116) demonstrated that leiomyoma cells express receptors involved in RA signaling (RARs and RXRs). In addition, they demonstrated that all-trans retinoic acid inhibits proliferation of leiomyoma cells. Therefore, they concluded that leiomyoma cells are retinoid responsive (116).

More recently, Zaitseva and colleagues (117) used microarray analysis and found differential gene expression of several proteins, enzymes and receptors in the retinoic

acid signaling pathway affecting leiomyoma compared to myometrium. The same group of investigators later described an additional group of genes differentially expressed in leiomyomas and also found that the expression of these genes itself is differentially regulated by retinoid in leiomyoma versus myometrium (118). Catherino and Malik found lower expression of several genes in RA pathway including alcohol dehydrogenase-1, aldehyde dehydrogenase-1, cellular retinol binding protein-1 (CRBP-1) and retinoic acid binding protein-1 in leiomyoma compared to adjacent myometrium (119). Furthermore, they found that cytochrome P450, which catabolizes retinoic acid, is up-regulated in leiomyoma. Therefore, they concluded that leiomyoma display a molecular pattern associated with lower exposure to RA. Importantly, there is evidence that leiomyoma displays different levels of RXR alpha than myometrium (120) and that this difference can be due to alteration in its degradation and transcriptional activity (121).

In addition to the in vitro evidence (116), Gamage and colleagues (122) demonstrated that treatment with retinoid X receptor ligand LGD1069 reduces the size of leiomyoma tumors in the Eker rat animal model. Therefore, it seems that modulating RA signaling pathway can be a potential target in leiomyoma treatment.

Vitamin D signaling

Although the classical role of vitamin D has been regulating calcium metabolism including bone mineralization (123), there has been recent strong evidence for an association with several other biologic processes including tumorigenesis (124).

Similar to other steroids discussed in this review, 1α, $25(OH)_2D_3$ (the active metabolite of vitamin D) activates 2 main cellular signaling pathways: classic/nuclear and rapid/non-genomic. In the classic nuclear pathway, $1\alpha,25(OH)_2D_3$ forms a complex with vitamin D receptor (VDR) and retinoic X receptor (RXR) that binds vitamin D response elements (VDREs) to modulate expression of target genes. In the rapid/non-genomic

pathway, 1α,25(OH)$_2$D$_3$ binds to membrane vitamin D receptors (memVDR) including GPCRs to activate several signaling pathways including Ras-Raf-MEK-ERK, PLCγ-PKC and AC-cAMP-PKA pathways (124). Vitamin D signaling pathways seem to modulate several processes including G0/G1 progression, apoptosis, differentiation and angiogenesis (124). A simplified illustration of vitamin D signaling in leiomyoma is presented in figure 2.

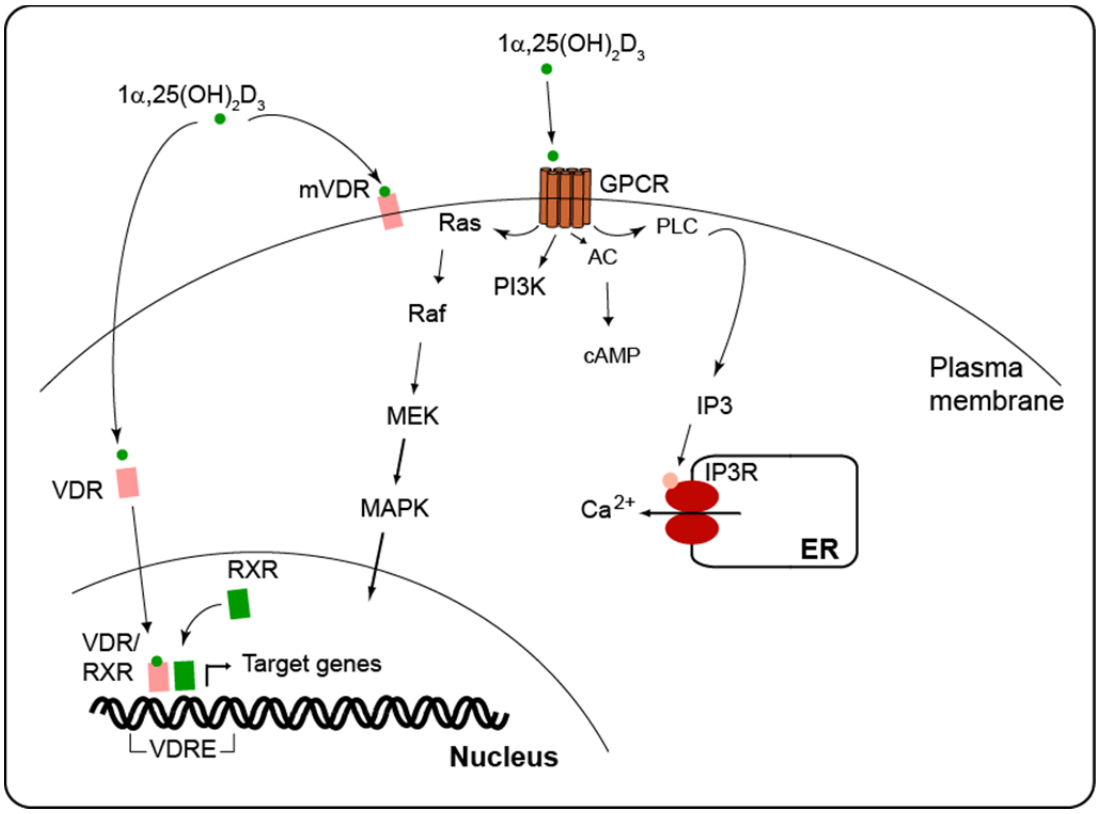

Figure 2: Schematic presentation of vitamin D signaling pathways in uterine leiomyoma cell.

VDR: vitamin D receptor; mVDR: membrane-bound vitamin D receptor; RXR: retinoid X receptor; VDRE: vitamin D response element; GPCR: G protein coupled receptor; PLC: phospholipase C; AC: adenylate cyclase; IP3: inositol triphosphate; IP3R: inositol triphosphate receptor; ER: endoplasmic reticulum

Recent evidence from three independent research groups in populations in North Africa, East US and central Europe clearly demonstrates an association between serum vitamin D deficiency and increased risk of uterine leiomyomas. Al-Hendy group first reported on the association between lower serum vitamin D levels and increased susceptibility to uterine leiomyomas in 2012 in a cohort of black and white women in North Africa (125, 126). This was followed by two other major studies including a cohort of women from eastern United States in 2013 by Baird and colleagues (127) and another cohort of Italian women in 2013 by Paffoni and colleagues (128). The high prevalence of vitamin D deficiency in African Americans may explain, at least in part, the high prevalence of uterine leiomyomas in this patient population (129).

In addition to the epidemiologic evidence, treatment with vitamin D was demonstrated to inhibit leiomyoma cells proliferation in vitro (130) and shrink leiomyoma tumors in Eker rat animal model (131). Furthermore, Sharan and colleagues (132) demonstrated that the anti-proliferative effect of vitamin D on leiomyoma cells noted in vitro is mediated through modulating expression and activity of catechol-O-methyltransferase enzyme. In addition to the anti-proliferative effects, Halder and colleagues (133) demonstrated that vitamin D reduces expression of certain TGF-β3-induced proteins in leiomyoma cells including fibronectin, type I collagen and plasminogen activator inhibitor-1. These proteins together are involved in development of fibrotic tissue in leiomyomas, and therefore, vitamin D can play anti-fibrotic role in leiomyoma through modulating TGF-β3 signaling. There is additional evidence that vitamin D modulates extracellular matrix in uterine leiomyomas (134, 135) which is an important aspect of the pathobiology with a therapeutic potential. Other vitamin D analogues and VDR agonists are currently under evaluation as potential therapeutic options for women with symptomatic uterine leiomyoma.

Peroxisome proliferator-activated receptor gamma (PPARγ)

Peroxisome proliferator-activated receptors (PPARs) are members of the ligand-activated nuclear transcription factors superfamily (136). Three PPARs have been described in humans: PPARα, PPARβ/δ and PPARγ with PPARγ present in three isoforms PPARγ1, PPARγ2 and PPARγ3. PPARs form heterodimers with retinoid X receptor (RXR) to regulate transcription of several genes involved in lipid metabolism, adipogenesis, proliferation and other important cellular processes (137). Thiazolidinediones (TZDz), insulin sensitizers used in treatment of type 2 diabetes, are among the well-known PPARγ ligands (137).

There is growing evidence for a role of PPARγ signaling in leiomyoma development and growth. For example, Jeong and colleagues (91) found higher PPARγ levels in leiomyoma compared to myometrium. In addition, they found that 17β-E_2 treatment increases levels of PPARγ in leiomyoma, but not myometrial cells. These findings points to an underlying aberrant PPARγ signaling in leiomyoma. In addition, Houston and colleagues (138) demonstrated that leiomyoma cells express PPARα, PPARβ/δ and PPARγ. In addition, they found that one pan-PPAR ligand and several PPARγ ligands inhibit 17βE_2–induced leiomyoma cell proliferation. Furthermore, they noted that stimulation of PPARγ leads to inhibition of E_2-mediated gene expression. Therefore, they concluded that PPARγ signaling may lead to inhibition of leiomyoma growth through modulating estrogen signaling. In addition, Nam and colleagues (139) found that leiomyoma cells are more sensitive than myometrium cells to the proliferation-inhibiting effect of ciglitizone, a member of the thiazolidinedione family of PPARγ ligands. This implies that PPARγ signaling may modulate leiomyoma growth. Finally, modulating this signaling pathway through PPARγ ligands may present a potential therapeutic target (139, 140).

CONCLUSIONS

It is clear that leiomyoma growth and development is regulated by a complex network of signaling pathways including steroids, growth factors and other pathways. It is important to understand that these pathways don't work separately and interact with each other at several points. It seems that aberrations in these pathways contribute to the development and growth of leiomyomas. Finally, there is evidence that several of the current treatments attempt to target these pathways. More importantly, these pathways present several important potential therapeutic targets for future research.

Statins and cellular proliferation

Statins are 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors that block an early step of the mevalonate pathway of cholesterol synthesis (figure 3). They have been used for many years for treatment of hypercholesterolemia. In addition, statins were demonstrated to have profound and broad-reaching effects on certain types of tissues beyond their well-known properties as lipid lowering drugs (141, 142). These include effects on endothelial cell function, oxidative stress, angiogenesis, anti-inflammatory and anti-allergic actions, and effects on cellular proliferation (143-145). The effects of statins on proliferation are cell-type specific. While they induce proliferation in certain cell types, e.g. endothelial progenitor cells (146), they inhibit proliferation of vascular smooth muscles, hepatocytes, and other cell types (147-150).

Statins have been shown to have general anti-tumor properties (147, 151, 152). Tumors whose growth have been shown to be inhibited by statins include breast (149, 153), ovarian (154, 155), prostate (156), colon (157) leukemia (158), and more recently certain variants of lung cancer (159). The mechanisms by which statins inhibit tumor growth are incompletely understood. Multiple mechanisms have been proposed, including blocking protein geranylgeranylation (160), activation of the mitochondrial pathway of apoptosis (158, 161), arrest of cell cycle progression and inhibition of invasion through modulating RhoA-dependent signaling pathways (162). While the effects of statins on certain tumors were demonstrated to depend on HMG-CoA reductase expression (155), it was found to be independent of HMG-CoA reductase in other cell types (163). In addition to anti-tumor properties, recent studies showed that that certain statins, specifically simvastatin and atorvastatin, have beneficial effects on other gynecologic conditions, such as endometriosis (144, 164-166) and polycystic ovarian syndrome (PCOS) (167-169).

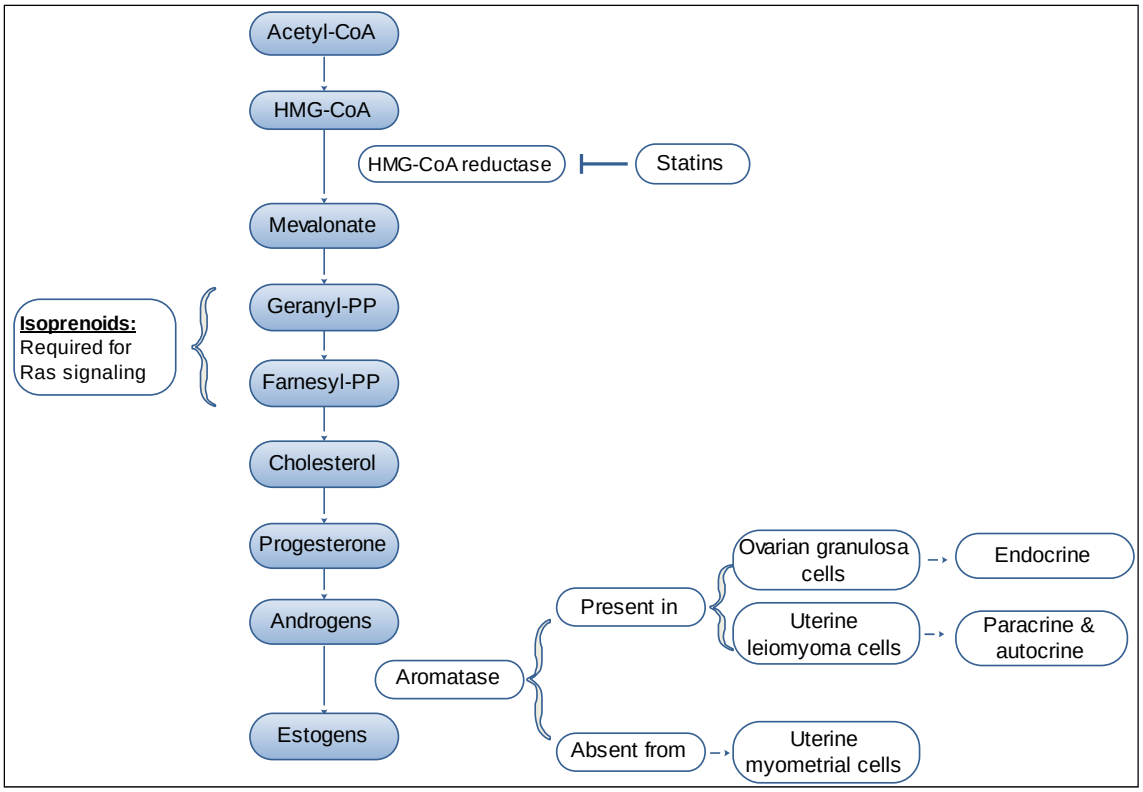

Figure 3: Mevalonate and steroidogenesis pathways.

Acetyl-CoA substrate is converted to cholesterol through mevalonate pathway. Statins inhibit HMG-CoA reductase enzyme, the rate-limiting enzyme in cholesterol biosynthesis. Farnesyl pyrophosphate and geranylgeranyl pyrophosphate are required for prenylation of Ras. Sex steroids (progesterone, androgens and estrogens) are synthesized from cholesterol through steroidogenesis pathway.

STATINS AND GYNECOLOGIC CONDITIONS

There are several studies that addressed the effects of statins on gynecologic malignancies. For example, Jacobs and colleagues reported that statin use was associated with a decrease in the risk of endometrial cancer (170). In addition, Blais and colleagues reported a 70% reduction of gynecologic cancers (endometrial, cervical, and ovarian) in statin users compared to bile acid-sequestrant users in patients older than 65 years (171).

However, in some other studies, long term use of statins (more than 5 years) for the treatment of hypercholesterolemia was not associated with a decreased risk of gynecologic malignancy (172-174). Also, some randomized controlled studies did not demonstrate any clinically significant reduction in the incidence of gynecologic malignancies on statin therapy after the conclusion of the follow up period (79, 175, 176).

Cervical cancer

Crescencio and colleagues studied the effects of statins on cervical cancer cell lines (177). They evaluated the effect of atorvastatin, fluvastatin and simvastatin on the cell cycle, proliferation, oxidative stress and cell death in CaSki, HeLa (HPV^+) and ViBo (HPV^-) cervical cancer cell lines. All three statins were able to induce cell death and inhibit proliferation in a dose-dependent manner. Simvastatin was the most effective inhibitor (177).

Ovarian cancer

Lui and colleagues demonstrated that lovastatin and atorvastatin induce apoptosis in ovarian cancer cell lines via JNK activation and increasing the expression of Bim (178). In another study, Martirosyan and colleagues used a panel of ovarian cancer cell lines and tested the effect of lovastatin on cellular apoptosis alone or in combination with doxorubicin. They found that lovastatin induces apoptosis in ovarian cancer cells through inhibition of mevalonate pathway. Furthermore, they found that lovastatin effects are synergetic with those of doxorubicin via mevalonate-independent pathway (179).

In addition, Taylor-Harding and colleagues measured the effect of fluvastatin and its synergistic effect with cisplatin (a chemotherapeutic drug commonly used in the treatment of ovarian cancer) on epithelial ovarian cell cancer. They found that fluvastatin inhibits cellular proliferation by blocking HMG-CoA reductase activity. They also found that fluvastatin effects are synergistic with cisplatin (180).

Osteopontin (OPN) is a secreted calcium-binding gylcophosphoprotein known to be a mediator of bone remodeling. Its expression in ovarian cancer cells has been shown to be related to tumor progression and metastasis (181). Matsuura and colleagues evaluated the effects of simvastatin on ovarian cell cancer cells including the expression of OPN. Results showed that simvastatin treatment inhibits ovarian cell cancer cells proliferation by 40-50%. In addition, simvastatin induces apoptosis and decreases the expression of osteopontin protein at the level of mRNA (181).

Endometrial cancer

Some studies investigated the effect of statins on endometrial cell cancer. The first study found that the lipophilic statins (e.g. simvastatin and lovastatin), but not the hydrophilic statins (e.g. pravastatin) induce apoptosis in Ishikawa endometrial cell cancer cell line (182). The second study, recently published in 2014, investigated the effects of simvastatin on cell proliferation, apoptosis, and invasion in endometrial cancer cell lines and primary cultures of endometrial cancer cells. They found that simvastatin was a potent inhibitor of endometrial cancer cellular growth that possesses anti-metastatic properties. Mechanism of action was mediated through inhibition of HMG-CoA reductase activity, alteration of the MAPK and AKT/mTOR pathways, G1 cell cycle arrest, induction of apoptosis as well as a rise on reactive oxygen species leading to DNA damage and cellular stress (183).

Endometriosis

Endometriosis is a common gynecologic condition where endometrium (tissue normally lining the cavity of the uterus) is present in other locations. This happens commonly in the ovaries and pelvic peritoneum. Endometriosis causes several symptoms including pelvic pain, pain during sexual intercourse, painful periods, and infertility.

Although endometriosis has several risks factors and theories of pathogenesis, the exact mechanisms of its development are not completely understood (184).

Some studies addressed the effects of statins on endometriosis. One study used an endometriosis rat model and found that atorvastatin causes regression of the endometriosis implants (185). Similarly, another group found that simvastatin treatment protects against the development of endometriosis in a murine model (144). In addition, atorvastatin was found to induce inhibition of the expression of Ki-67 and LPS-induced expression of COX-2, VEGF, RAGE, and EN-RAGE (inflammatory and angiogenic genes) in endometriotic stromal cells (186).

Lipophilic versus hydrophilic statins

The study of statins was further dissected by its relative water or fat solubility. In general, statins are either hydrophilic (pravastatin and rosuvastatin) or lipophilic (cerivastatin, simvastatin, lovastatin, fluvastatin and atorvastatin). Kato and colleagues studied the difference in effect of lipophilic (lovastatin and simvastatin) and hydrophilic (pravastatin) statins on cellular death of ovarian, cervical and endometrial cell lines. Lipophilicity allows easier entry to non-hepatic cells while hydrophilicity renders the statin to be more hepatoselective. They found that cellular death of ovarian, endometrial and cervical cell lines took place in the presence of lipophilic and not hydrophilic statins (182). Their findings could provide explanation to the differences in effects of statins on same cell lines found in other studies.

CHAPTER 2: SIMVASTATIN POTENTLY INDUCES CALCIUM-DEPENDENT APOPTOSIS OF HUMAN LEIOMYOMA CELLS

Modified from: Simvastatin Potently Induces Calcium-Dependent Apoptosis of Human Leiomyoma Cells

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INTRODUCTION

As previously discussed in the introduction chapter, statins were found to have profound and broad-reaching effects on certain types of tissues beyond their lipid lowering properties (141, 142). These include effects on cellular proliferation (143-145). The effects of statins on proliferation are cell-type specific. While they induce proliferation in certain cell types, e.g. endothelial progenitor cells (146), they inhibit proliferation of certain cell types including certain tumors (147-150), including breast (149, 153), ovarian (154, 155), prostate (156), colon (157) leukemia (158), and more recently certain variants of lung cancer (159).

In this study, we examined the effects of simvastatin, one of the lipophilic statins, on uterine fibroids. We report for the first time that simvastatin (a semi-synthetic lipophilic HMG-CoA reductase inhibitor) inhibits the proliferation of human leiomyoma cells. In addition, we demonstrate that this anti-proliferative effect is associated with modulation of ERK1/2 signaling and alterations in cell cycle progression. Moreover, we demonstrate that simvastatin induces apoptosis in human leiomyoma cells. Intracellular calcium chelation completely inhibited apoptosis induced by simvastatin. Mechanistically, activation of L-type voltage-gated calcium channels likely mediates calcium-dependent apoptosis induced by simvastatin. Thus, we have identified a novel calcium-dependent pathway by which simvastatin induces apoptosis in tumor cells.

EXPERIMENTAL PROCEDURES

MATERIALS

Simvastatin was purchased from Cayman Chemical (Ann Arbor, MI). It was dissolved in dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich (St. Louis, MO). Stock solution (10 mM) was prepared and kept at -20ºC till use. Final concentration of

DMSO in culture medium was ≤0.1% vol/vol. Complete protease inhibitor cocktail without EGTA was purchased from Roche Applied Science (Indianapolis, IN). Z-DEVD-R110 used for the caspase-3 assay was purchased from American Peptide Company (Sunnyvale, CA). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was purchased from Calbiochem (EMD Millipore, Merck KGaA, Darmstadt, Germany). Collagenase and deoxyribonuclease I (used for primary cell isolation), propidium iodide, ribonuclease A (used for cell cycle analysis), non-selective voltage-gated calcium channel blockers mibefradil and SKF96365 and specific T-type voltage-gated calcium channel blocker NNC 55-0396 were purchased from Sigma-Aldrich (St. Louis, MO). The specific L-type voltage-gated calcium channel blocker nimodipine was purchased from Cayman Chemical. Fura-2 AM and BAPTA-AM were purchased from Molecular Probes®, Invitrogen (Grand Island, NY). U-73122, cell permeable, isotype non-selective phospholipase C (PLC) inhibitor, was purchased from MP Biomedicals (Solon, OH). Xestospongin C, cell permeable, potent blocker of inositol 1,4,5-trisphosphate (IP_3)-mediated calcium release, was purchased from EMD Millipore Corporation (Billerica, MA). Cells were treated with U-73122, Xestospongin C, mibefradil, SKF96365, nimodipine and NNC 55-0396 for 30 minutes before simvastatin treatment

ANTIBODIES

Monoclonal anti-human smooth muscle actin (SMA) was purchased from Dako (Glostrup, Denmark). Alexa Fluor labeled secondary antibody was purchased from Invitrogen (Grand Island, NY). Monoclonal anti-ERK1/2, anti-phospho ERK1/2, anti-Proliferating Cell Nuclear Antigen (PCNA), and anti-Bim, antibodies were purchased from Cell Signaling (Danvers, MA). Anti-alpha tubulin antibody and anti-β actin used as a loading control was purchased from Abcam (Cambridge, MA) and ThermoFisher Scientific (Rockford, IL), respectively. Rabbit polyclonal anti-pan α1 voltage-gated

calcium channels (L-type) was purchased from Millipore Corporation (Billerica, MA). Rabbit polyclonal anti-voltage-gated calcium channels (T-type) CACNA1G antibody was purchased from Novus Biologicals (Littleton, CO).

IMMORTALIZED AND PRIMARY CELLS

Immortalized human leiomyoma cell line (HuLM) was a gift from Dr. Salama A. Salama, Baylor College of Medicine, Houston, TX. In this cell line, human leiomyoma cells obtained from a patient after surgery were immortalized using a retroviral vector carrying human telomerase reverse transcriptase hTERT (187). Cells were confirmed to maintain expression of molecular markers of human leiomyoma cells including estrogen and progesterone receptors and smooth muscle actin (187). HuLM cells were cultured and maintained in Smooth Muscle Basal Medium containing 5% fetal bovine serum (FBS), 0.1% insulin, 0.2% basic human fibroblast growth factor, 0.1% gentamicin/amphotericin B, and 0.1% human epidermal growth factor; all purchased from Lonza (Walkersville, MD). Cells were incubated in 5% CO_2 atmosphere under 37 ºC and split once 70-80% confluent. Primary non-pregnant human leiomyoma and myometrial cells were obtained from patients undergoing hysterectomy for symptomatic uterine leiomyomas at the University of Texas Medical Branch (UTMB) John Sealy Hospital at Galveston, TX. The study protocol was reviewed and approved by the Institutional Review Board (IRB) at the University of Texas Medical Branch. Primary leiomyoma cells and primary myometrial cells were isolated using a modification of a previously described protocol (75). In brief, tissues were obtained immediately at surgical excision. After washing in cold sterile Dulbecco's Phosphate Buffered Saline (DPBS) solution without calcium or magnesium (Sigma-Aldrich, St. Louis, MO), tissues were mechanically minced into around 1 mm pieces. Next, they were agitated at 37ºC for 4 hours at sterile Hanks Buffered Salt (HBSS) solution without phenol, calcium or magnesium (Sigma-Aldrich, St. Louis, MO) to which collagenase and deoxyribonuclease

I were added. Medium was subsequently filtered through a 70 μm filter and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 Ham 1:1 mixture (Sigma-Aldrich, St. Louis, MO) supplemented with HEPES, pyridoxine, L-glutamine, 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin vol/vol. Medium was changed on daily basis. Cells were split once at 70-80% confluent and were used without further passaging to maintain the differentiated phenotype.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed using a modification of a previously described method (188). In brief, cells were seeded on poly-L-lysine-coated coverslips. After reaching 70-90% confluence, medium was aspirated and cells were washed with 1X PBS solution. Slides were then fixed using a 4% paraformaldehyde solution followed by quenching with 30 mM glycine and permeabilization with 0.25% Triton X-100 in 1% BSA solution. The slides were blocked with 2% BSA and stained with monoclonal anti-human smooth muscle actin (SMA) followed by Alexa Fluor 594-labeled secondary antibody. Finally, the slides were exposed to DAPI nuclear stain and mounted. Images were acquired on a Nikon Eclipse TE2000-U epifluorescent inverted light microscope using a 40× oil immersion objective (SuperFluor; Nikon). QED Capture software, part of QED Imaging Solutions (Media Cybernetics, Inc., Rockville, MD) was used for image acquisition.

MTT ASSAY

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to monitor cellular proliferation. HuLM cells were seeded into 96-well plates. Cells were treated with medium containing 0, 0.1, 0.5, 1, 5, and 10 μM simvastatin and MTT assay was performed as previously described (189). We chose to investigate doses of simvastatin ranging from 0.1 to 10 μM. This decision was based on dosages currently

used to treat hypercholesterolemia (up to 1 mg/kg body weight/day) which is associated with peak serum levels of ~0.3 μM (190, 191). The maximum tolerated dose (MTD) of simvastatin in humans was found to be 15 mg/kg body weight/day (192), thus providing the rationale of simvastatin concentrations of 0.1-10 μM.

CELL CYCLE ANALYSIS

Cells were plated in medium containing serum and growth factors. The next day, the medium was changed and the cells were kept overnight in serum-free, growth factor-free medium for cell cycle synchronization. Thereafter, the cells were treated for 3 hours with simvastatin in serum-free, growth factor-free medium before replacing medium with various simvastatin concentrations in serum and growth factor replete medium. After 48 hours, cells were collected and cell cycle analysis was performed as previously described (45) with some modifications. In brief, the cells were fixed and permeabilized by adding cold absolute acetone-free methanol while vortexing. Cells were saved in cold methanol until flow cytometric analysis (less than 7 days). Prior to flow cytometry, the cells were centrifuged and resuspended in solution containing propidium iodide and ribonuclease A and incubated in the dark for 30 min. Flow cytometry was performed using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) using the FL2 parameter with excitation wavelength at 488 nm and emission at 670 nm. Data was collected using FACSDiva software (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree star Inc., Ashland, OR). Gating was used to exclude cell aggregates. Thereafter, cell cycle analysis was performed and percentage of cells in each cell cycle phase was calculated. In addition, pre G0/G1 (apoptotic) cell population was calculated.

CELL DEATH ANALYSIS

Propidium iodide staining for the quantification of cell death was performed as previously described (193). The percentage of propidium iodide-positive cells was

counted in a blinded manner. Caspase-3 activity was measured using a quantitative fluorometric assay as previously described (194).

CYTOSOLIC CALCIUM IMAGING

HuLM cells were seeded onto coverslips in 6-well plates. Thereafter, cells were treated with 0.1 μM simvastatin as well as DMSO (control). After 24 hours, resting cytosolic calcium levels were quantified using Fura-2 AM. In brief, the medium was replaced with imaging solution (107 mM NaCl, 7.25 mM KCl, 20 mM HEPES, 2.5 mM $MgCl_2$, 11.5 mM glucose pH 7.2) containing 2.5 μM Fura-2 AM for 20 min at room temperature while protected from light. Next, the solution was aspirated and replaced with imaging solution without Fura-2 AM and incubated for another 20 min at room temperature protected from light. Cover slips were then examined under 40X oil immersion using a Nikon Eclipse TE2000 epifluorescence inverted microscope. Images were captured (340nm and 380nm excitation, 510nm emission) and transferred to personal computer using MetaFluor® Imaging Software (Molecular Devices, LLC; Sunnyvale, CA). Five fields on each cover slip were randomly chosen and cytoplasmic regions of interest in all cells in the field were quantified. This was repeated two more times for a total of 3 separate experiments comprising hundreds of cells. The ratiometric data was converted to calcium concentrations by utilizing a calcium calibration buffer kit (Molecular Probes, Eugene OR). The data in Figure 4A is represented as a histogram to better appreciate the heterogeneity in cytoplasmic calcium. To measure calcium release kinetics continuously during the first five hours of simvastatin exposure, we used the genetically encoded calcium indicator protein GCaMP6s (195). The expression plasmid driving expression of GCaMP6s off of a CMV promoter was kindly provided by Dr. Douglas Kim (HHMI Janelia Farm) and obtained through Addgene (plasmid 40753). The huLM cells were transfected with Lipofectamine 3000 and imaged after 48 to 72 hours. Fluorescence was monitored by excitation at 480nm and emission at 510nm. Cells were

imaged at 37°C in growth medium. Images were taken every 30 seconds for 5 hours. For each experiment, approximately 10-20 cells could be imaged simultaneously. After acquiring baseline calcium measurements, cells were treated with vehicle, 0.1μM, 1μM or 10μM simvastatin. Each experiment was repeated 3 times with essentially identical results. The data in Figure 4D-G is a representative experiment showing the calcium responses in all cells on a single coverslip.

MITOCHONDRIAL CALCIUM IMAGING

Mitochondrial calcium was measured by loading the cells with 1μM Rhod-2 AM for 30 minutes at 37°C in imaging solution. Cells were imaged by excitation at 565nm and monitoring emission at 610nm. At least five fields on each cover slip were randomly chosen and mitochondrial regions of interest in all cells in the field were quantified. This was repeated two more times for a total of 3 separate experiments from which the data were pooled.

MITOCHONDRIAL MEMBRANE POTENTIAL

Mitochondrial membrane potential was examined using cationic dye JC-1 (Invitrogen/Molecular Probes). This dye is red in polarized mitochondria, and green in depolarized mitochondria. Cells were loaded with 10μg/ml JC-1 for 10 minutes at 37°C. Red and green emission were monitored simultaneously by excitation at 480nm and emission at 620nm and 525nm. The ratio of red/green was used as a measure of mitochondrial membrane potential, with a drop in this ratio indicative of depolarization. As in Rhod-2 imaging, five fields on each cover slip were randomly chosen and mitochondrial regions of interest in all cells in the field were quantified. This was repeated two more times for a total of 3 separate experiments from which the data were pooled.

INTRACELLULAR CALCIUM CHELATION

Cells were seeded into 6-well plates. Thereafter, 3 wells were loaded with BAPTA-AM at 1 μM, 10 μM and 20 μM concentrations. After 30 minutes, medium was changed to BAPTA-AM-free medium and treated with simvastatin (10 μM). In addition, three more wells were treated with DMSO (control), BAPTA-AM (10 μM) without simvastatin and simvastatin (10 μM) without BAPTA-AM. Forty eight hours later, morphologic appearance of cells was observed and recorded. Thereafter, cells were harvested, cell lysates obtained and caspase-3 activity assay was performed as described (194).

STATISTICAL ANALYSIS

Experiments were performed in triplicates and independently repeated at least three times. Whenever applicable, data were presented as mean±SEM and student's t-test was used for statistical analysis. Results were considered statistically significant when p value was < 0.05. We used SigmaPlot software (Systat Software Inc, San Jose, CA) for statistical analysis.

RESULTS

SIMVASTATIN INHIBITS THE PROLIFERATION OF LEIOMYOMA CELLS

Treatment of a human leiomyoma cell line (HuLM); (187) with simvastatin induced dose-dependent morphological changes consistent with the inhibition of cellular proliferation and induction of cell death (figure 4A). Almost identical results were found with primary leiomyoma cells isolated from a patient (figure 4B). We confirmed that isolated primary cells are smooth muscle-derived by performing immunocytochemistry for smooth muscle actin (196) (data not shown). To quantify the effects of simvastatin on cellular proliferation, we performed a MTT assay. Simvastatin induced dose-dependent and time-dependent inhibition of cellular proliferation (figure 4C-D). These findings are

evident at concentrations as low as 100 nM, while doses of 1 μM or more were associated with cell death. These effects were evident at 48 hours, and were more pronounced at 72 hours of treatment (figure 4D). To confirm that simvastatin was inhibiting cellular proliferation, we investigated protein levels of proliferating cell nuclear antigen (PCNA). We found that simvastatin induced a dose-dependent decrease in PCNA expression in HuLM cells (figure 4E), confirming the results obtained by the MTT assay.

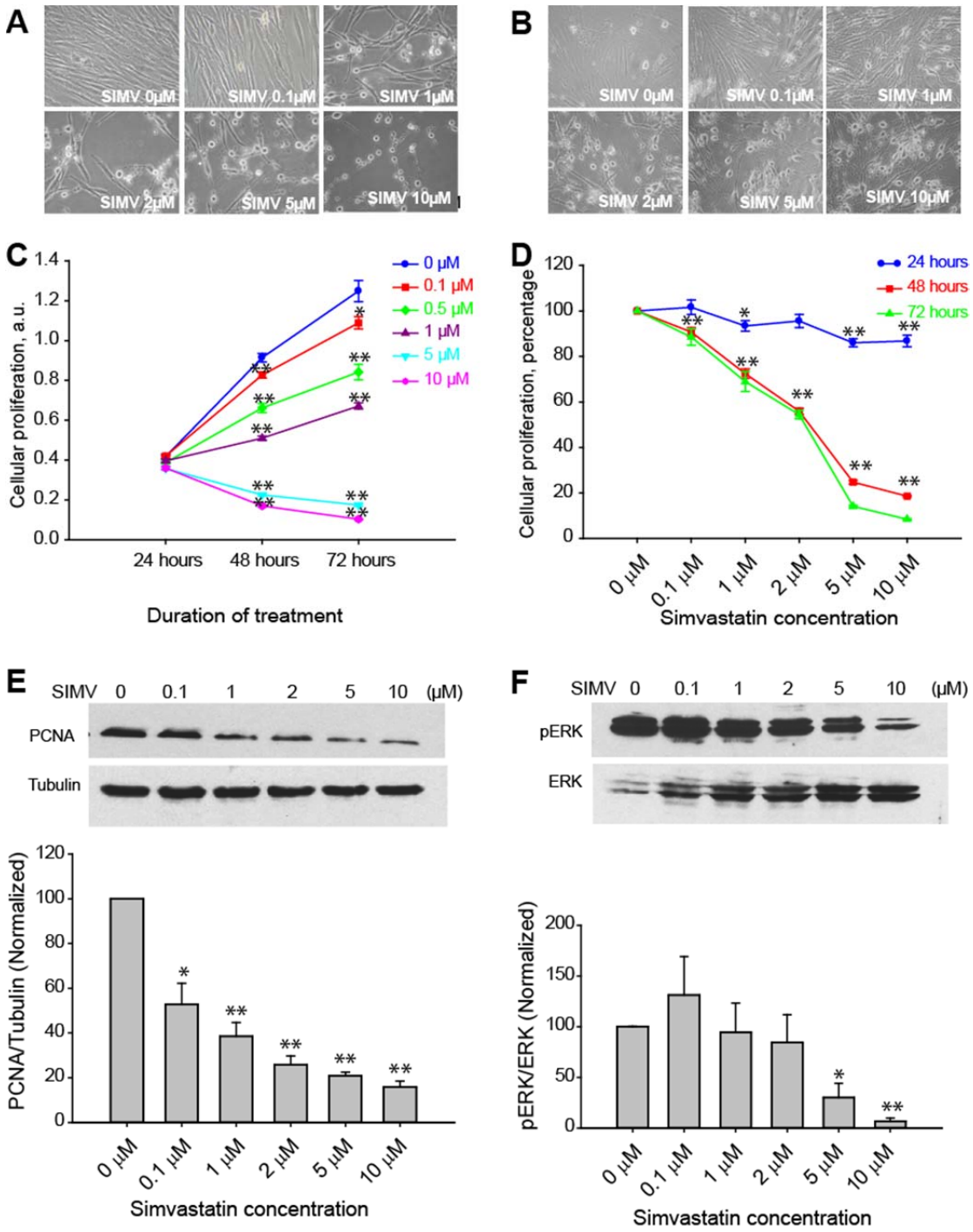

Figure 4: Anti-proliferative effects of simvastatin on human leiomyoma cells.

A and B, Morphologic effects of simvastatin (SIMV) treatment for 48 hours on HuLM cells (A) and primary human leiomyoma cells (B). C and D, MTT proliferation assay results for cells treated with 0 μM to 10 μM SIMV with treatment end points at 24, 48,

and 72 hours showing a dose-response curve (C) and a time-response curve (D). E and F, Western blotting showing effects of SIMV treatment for 48 hours on Proliferating Cell Nuclear Antigen (PCNA) (E) and phosphorylated and total ERK expression (F) along with quantification. Alpha-tubulin was used as a loading control. *$p<0.05$; **$p<.0.01$ versus 0 μM. For panel D, significance signs at lower part of graph apply to both 48 and 72 hours.

We next determined whether these effects were mediated through modulating growth factor signaling. Growth factors such as EGF, VEGF and PDGF are known to be significant contributors to development and growth of uterine leiomyomas (69, 71-73, 75). To investigate effects of simvastatin on the Ras/Raf/MEK/ERK pathway, we treated HuLM cells with different simvastatin concentrations for 48 hours and monitored ERK phosphorylation. Simvastatin treatment was associated with significantly decreased ERK1/2 phosphorylation at concentrations 1μM and higher (figure 4F). Interestingly, there was a moderate increase in ERK phosphorylation at the lowest dose tested (0.1μM), however the significance of this effect is unclear as this dose was associated with reduced proliferation (figure 4C). Simvastatin increased the total amount of ERK1/2 at all concentrations, suggesting compensatory upregulation of the kinase by the tumor cells. Notably, as shown in figure 4F, baseline phosphorylation of ERK is relatively high. This can be due to one of the growth factor signaling aberration associated with leiomyoma development. Alternatively, this can be caused by the growth factors present in the cell culture medium used (see experimental procedures section).

SIMVASTATIN INDUCES CHANGES IN CELL CYCLE PROGRESSION

To determine the effects of simvastatin on cell cycle progression, we performed flow cytometry. Synchronized cells were treated with different concentrations of simvastatin in addition to vehicle. After 48 hours, the cells were collected and cell cycle

analysis was performed. Simvastatin induced a dose-dependent decrease in the number of cells in S phase and a concomitant increase in the sub-G0/G1 (apoptotic) population (figure 5A-H).

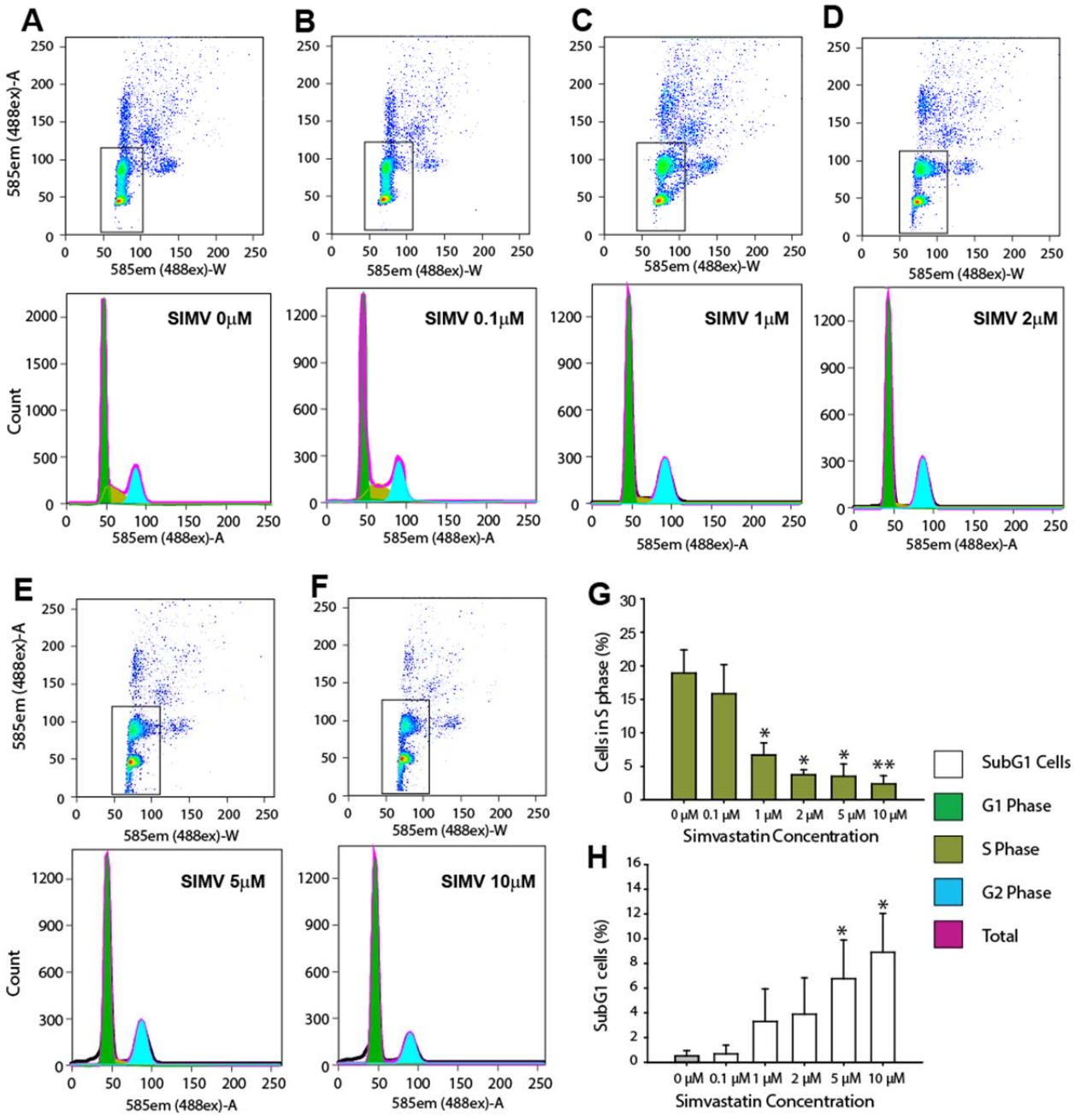

Figure 5: Effects of simvastatin on human leiomyoma cell cycle progression.

Cells were synchronized by overnight serum-starvation then treated with 0 μM to 10 μM simvastatin (SIMV) for 48 hours. Cells were then stained with propidium iodide and flow cytometry. A-F, Cell cycle analysis results at 0 to 10 μM simvastatin concentrations. Top part of each panel represents two-dimensional frequency pseudocolor plot. Rectangles represent gating of events to exclude cellular aggregates. Lower panel represents

analyzed data using FlowJo software where each cell cycle phase population is presented in the colors shown in the legend. G and H, Histogram showing percentage of cells at the S phase of cell cycle (G) and sub-G0/G1 (apoptotic) population (H) at each simvastatin concentration. *p<0.05; **p<.0.01 versus 0 μM.

SIMVASTATIN INDUCES APOPTOSIS OF LEIOMYOMA CELLS

We next examined whether simvastatin induces apoptotic cell death. As shown in figure 6A, simvastatin induces a dose-dependent increase in cell death as demonstrated by percentage of propidium iodide positive cells. We next examined if simvastatin-induced cell death was apoptotic in nature by measuring caspase-3 enzymatic activity. As shown in figure 6B, simvastatin treatment resulted in a dose-dependent and robust increase in caspase-3 activation which was evident at concentrations as low as 0.1μM. These results are consistent with the effects on morphology, proliferation, and cell cycle progression, suggesting that simvastatin potently induces leiomyoma cell death at therapeutically relevant concentrations.

SIMVASTATIN INCREASES EXPRESSION OF BIM

We next wanted to further elucidate the underlying mechanism of simvastatin-induced apoptosis and to examine if it is linked to its proliferation-inhibitory effect. It is known that ERK1/2 promotes degradation of the Bim spliceoform Bim_{EL} (197). Bim_{EL} is a pro-apoptotic BH3-only member of the Bcl-2 family that induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-xL. Bim_{EL} expression and activity goes up following decreases in ERK/MAPK activity, and thus is a cytosolic pro-apoptotic sensor of growth factor deprivation. Therefore, we examined the effect of simvastatin treatment on Bim_{EL} protein levels. As shown in Figure 3C, simvastatin treatment increases Bim_{EL} protein levels, consistent with decreased ERK

activation (figure 4F) and the induction of apoptosis (figure 6B-C). The doses at which we observe increased Bim_{EL} expression are somwhat lower than those observed for pERK (figure 4F). However, the effects of simvastatin on ERK activity and expression are complex, with even the lowest dose (0.1μM) increasing total ERK protein levels suggesting ERK inhibition at this dose and concomitant compensatory increase in ERK protein levels. Regardless of the mechanism, simvastatin dose-dependently increases Bim_{EL} levels with direct implications for apoptotic signaling.

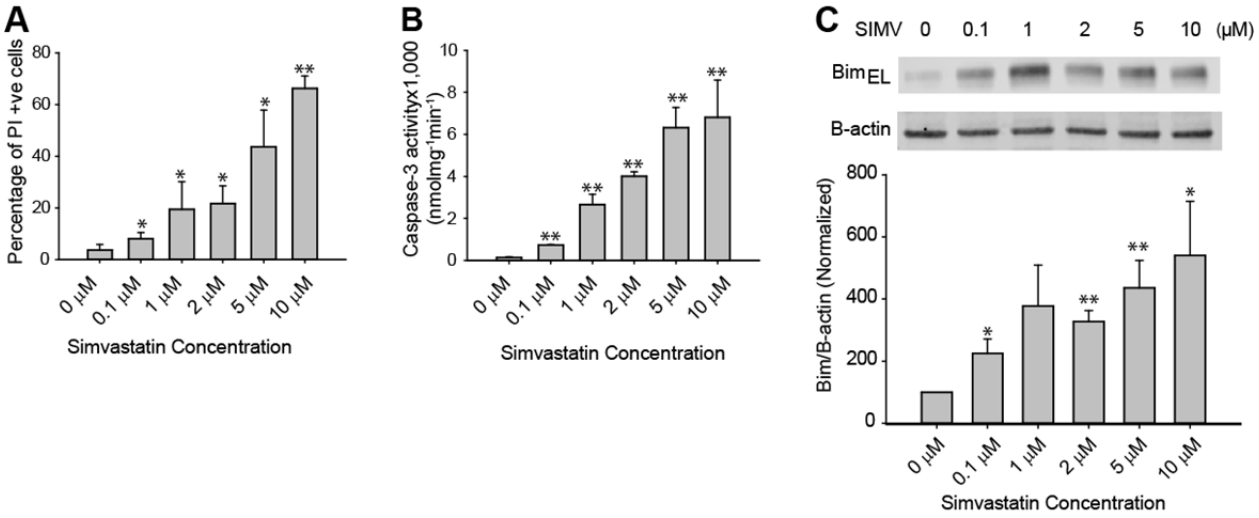

Figure 6: Apoptosis-inducing effects of simvastatin on human leiomyoma cells.

A, Blinded quantification of percentage of propidium iodide (PI) positive cells showing dose-dependent apoptosis induction after simvastatin treatment for 48 hours. B, Caspase-3 enzymatic activity showing that simvastatin treatment for 48 hours dose-dependently increases apoptotic signaling in human leiomyoma cells. C, Western blotting showing effects of SIMV treatment for 48 hours on Bim expression, along with quantification. Actin is used as a loading control. *p<0.05; **p<.0.01 versus 0 μM.

SIMVASTATIN INDUCES CYTOSOLIC CALCIUM RELEASE

We next sought to further determine the mechanism by which simvastatin induces apoptosis of leiomyoma cells. Given reports that statins can affect cellular calcium homeostasis (198) and the central role of calcium in many apoptotic paradigms (194), we determined whether simvastatin-mediated apoptotic cell death is associated with engagement of the calcium signaling machinery. Treatment with 0.1μM simvastatin for 24h increased resting cytosolic calcium in HuLM cells to an extent consistent with the activation of calcium-dependent apoptosis in other paradigms (figure 7A; (199)). Chronically elevated cytosolic calcium during apoptosis as shown in figure 7A results in mitochondrial accumulation of calcium, mitochondrial depolarization, and release of pro-apoptotic factors. Consistent with apoptotic calcium release, we found that treatment with 0.1μM simvastatin for 24h significantly increased mitochondrial calcium levels (figure 7B) and associated mitochondrial depolarization (figure 7C). Many apoptotic stimuli induce calcium release very early in the apoptotic program (minutes to hours; (200);), and this is useful to discriminate if the calcium elevations are causative or a consequence of the apoptotic program (201). To measure the effects of simvastatin early in the apoptotic program, we measured cytosolic calcium continuously for the first 5 hours after simvastatin administration using the genetically encoded calcium indicator GCaMP6s (195). Simvastatin dose-dependently increased the spiking activity of huLM cells, consistent with apoptotic calcium release (figure 7D-F). Finally, to determine if cytosolic calcium release is absolutely required for simvastatin-induced apoptosis, we chelated intracellular calcium by loading the cells with BAPTA-AM. Loading HuLM cells with BAPTA dramatically and dose-dependently protected HuLM cells against simvastatin-induced morphologic changes (figure 7H), prevented simvastatin-induced induction of caspase-3 activity (figure 7I) and cell death (figure 7J). Thus, simvastatin-induced cytosolic calcium elevations are required for leiomyoma cell apoptosis.

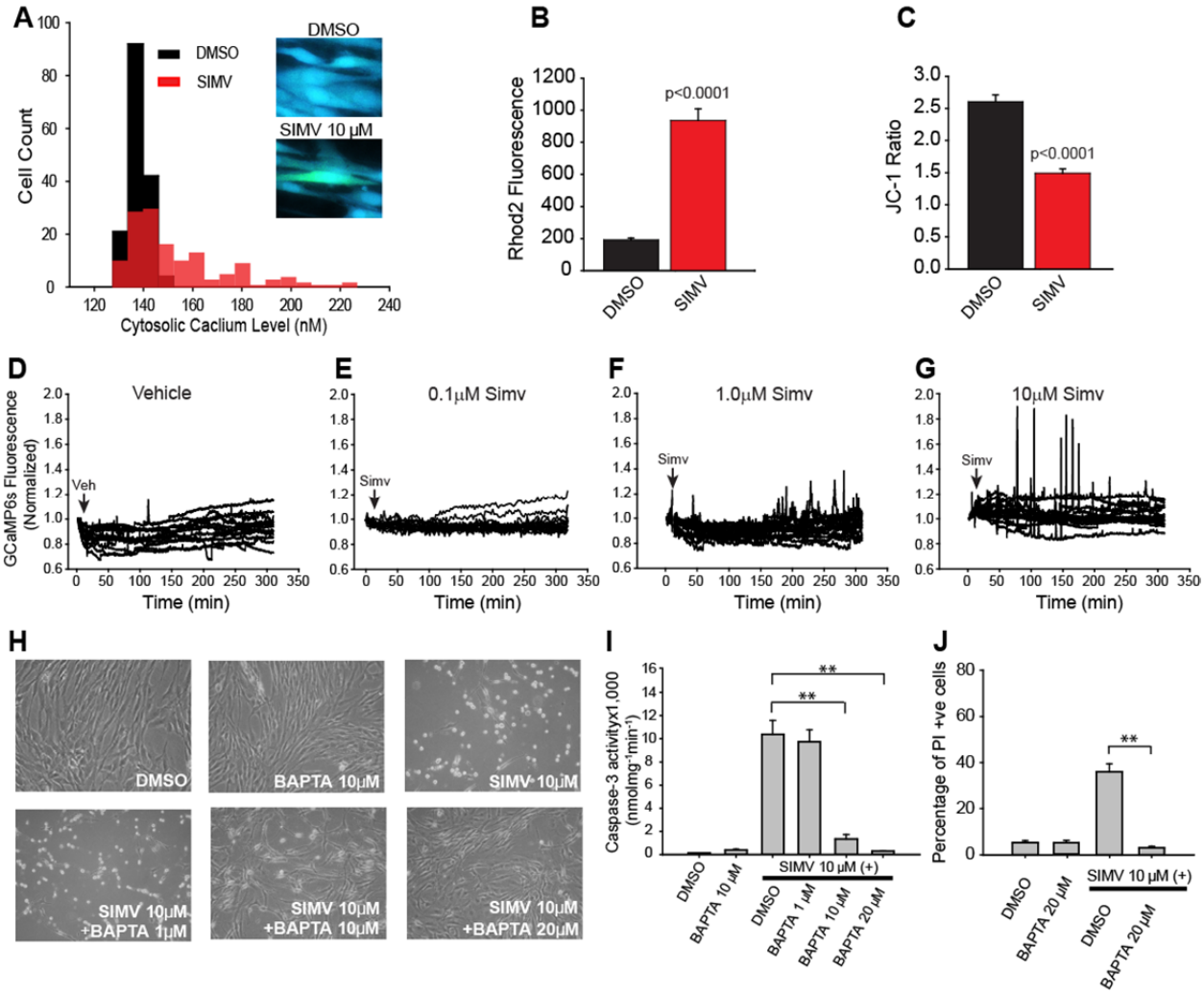

Figure 7: Effects of simvastatin on cytosolic calcium release in human leiomyoma cells.

A, Histogram showing cytosolic calcium levels in control (DMSO-black) cells and 0.1 µM simvastatin treated (SIMV-red) cells for 24 hours. Inset of panel shows representative pseudocolored Fura-2 ratio images from control (DMSO) and simvastatin-treated (SIMV) cells. Low calcium is represented by "cool" colors such as blue, and higher calcium is indicated by "warmer" colors such as green. B, Mitochondrial calcium as measured by Rhod-2 fluorescence after treatment with vehicle (DMSO) or 0.1µM simvastatin for 24 hours. C, Mitochondrial polarization as determined by the ratio of JC-1 fluorescence (see methods) after treatment with DMSO or 0.1µM simvastatin for 24

hours. A decrease in the ratio is indicative of depolarization. D-G, Dynamic changes in cytosolic calcium after the first five hours of exposure to simvastatin as determined by GCaMP6s fluorescence. Concentrations are indicated at the top of each panel. Graphed are the responses of all cells on a single coverslip. These experiments were repeated three times with essentially identical results. H, Simvastatin-induced morphologic changes in human leiomyoma cells are calcium dependent. Pre-loading cells with calcium chelator BAPTA-AM (lower 3 panels) prevents simvastatin-induced morphologic changes in a dose-dependent fashion. I and J, Simvastatin-induced apoptosis in human leiomyoma cells is calcium-dependent. Pre-loading cells with calcium chelator BAPTA-AM prevents simvastatin-induced apoptosis as demonstrated by decreased caspase-3 activity (I) and percentage of propidium iodide (PI) positive cells (J). Effects are evident at 10-20 μM BAPTA-AM concentrations. **$p<0.01$ versus 10μM SIMV.

SIMVASTATIN-INDUCED APOPTOSIS IS NOT MEDIATED BY IP3RS

As calcium-mediated apoptosis is most commonly associated with inositol 1,4,5-trisphosphate receptor (IP_3R)-mediated calcium release from the endoplasmic reticulum (ER) (194, 200, 202, 203), we examined the role of IP_3R in simvastatin-induced apoptosis. We used xestospongin C, which is a cell membrane-permeable inhibitor of IP_3R and the endoplasmic-reticulum Ca^{2+} ATPase (204, 205). We found that xestospongin C did not prevent simvastatin-induced apoptosis (Figure 8A). To further confirm that IP_3Rs do not contribute to simvastatin-induced cell death, we used the cell membrane permeable phosphoinositide-specific phospholipase C (PLC) inhibitor U73122 (206, 207). We found that U73122 has no effect on simvastatin-induced apoptosis (figure 8B). Thus, the PLC/IP_3R pathway does not appear to be involved in simvastatin-induced apoptosis of HuLM cells.

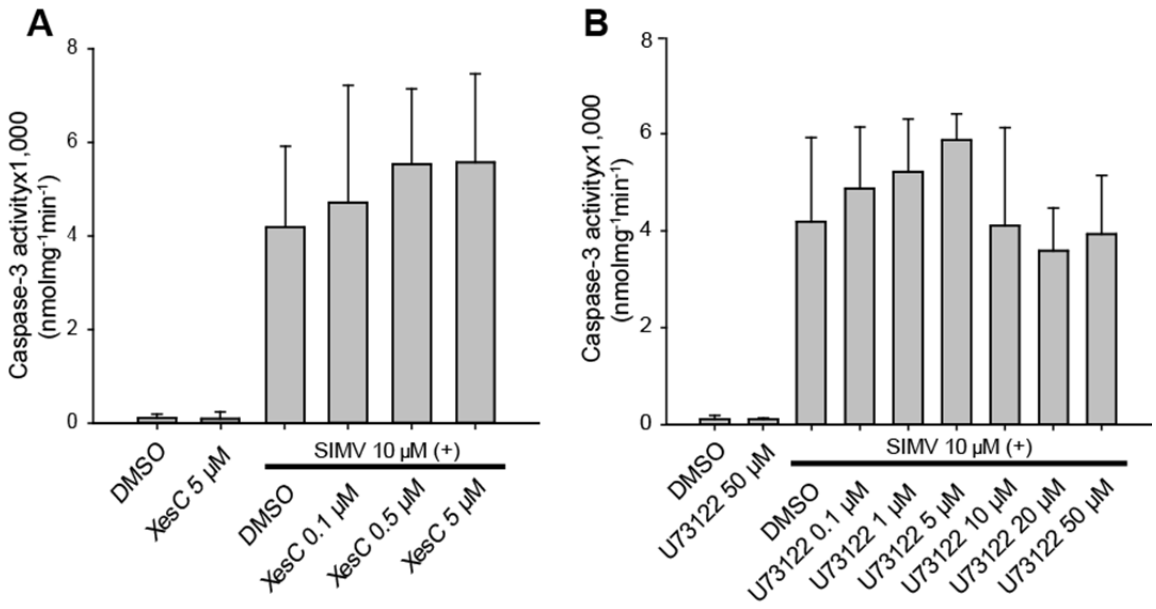

Figure 8: Simvastatin-induced apoptosis in human leiomyoma cells is not dependent on ER calcium release.

A and B, Pre-treating cells with inositol 1,4,5-trisphosphate receptor (IP_3R) inhibitor Xestospongin C (XesC) (A) or cell membrane permeable phosphoinositide-specific phospholipase C (PI-PLC) inhibitor U73122 (B) does not prevent simvastatin-induced apoptosis as measured by caspase-3 activity.

SIMVASTATIN-INDUCED APOPTOSIS REQUIRES VOLTAGE-GATED CALCIUM CHANNEL ACTIVITY

We next examined whether activation of plasma membrane calcium channels contribute to simvastatin-induced apoptosis. Myometrial smooth muscle cells express TRP channels (208) and several classes of voltage-gated channels including T- and L-type calcium channels (209-211). To examine the potential contribution of these channels, we used mibefradil, a non-selective voltage-gated calcium channel blocker (212), and SKF96365 (SKF), a widely used inhibitor of TRP channels (213) which can also potently inhibit both L- and T-type calcium channels (214). We found that pretreatment with both mibefradil and SKF dose-dependently prevented simvastatin-

induced caspase-3 activation (figure 9A-B). Together, these results suggest that simvastatin-induced apoptosis requires the activation of L- or T-type calcium channels (or both).

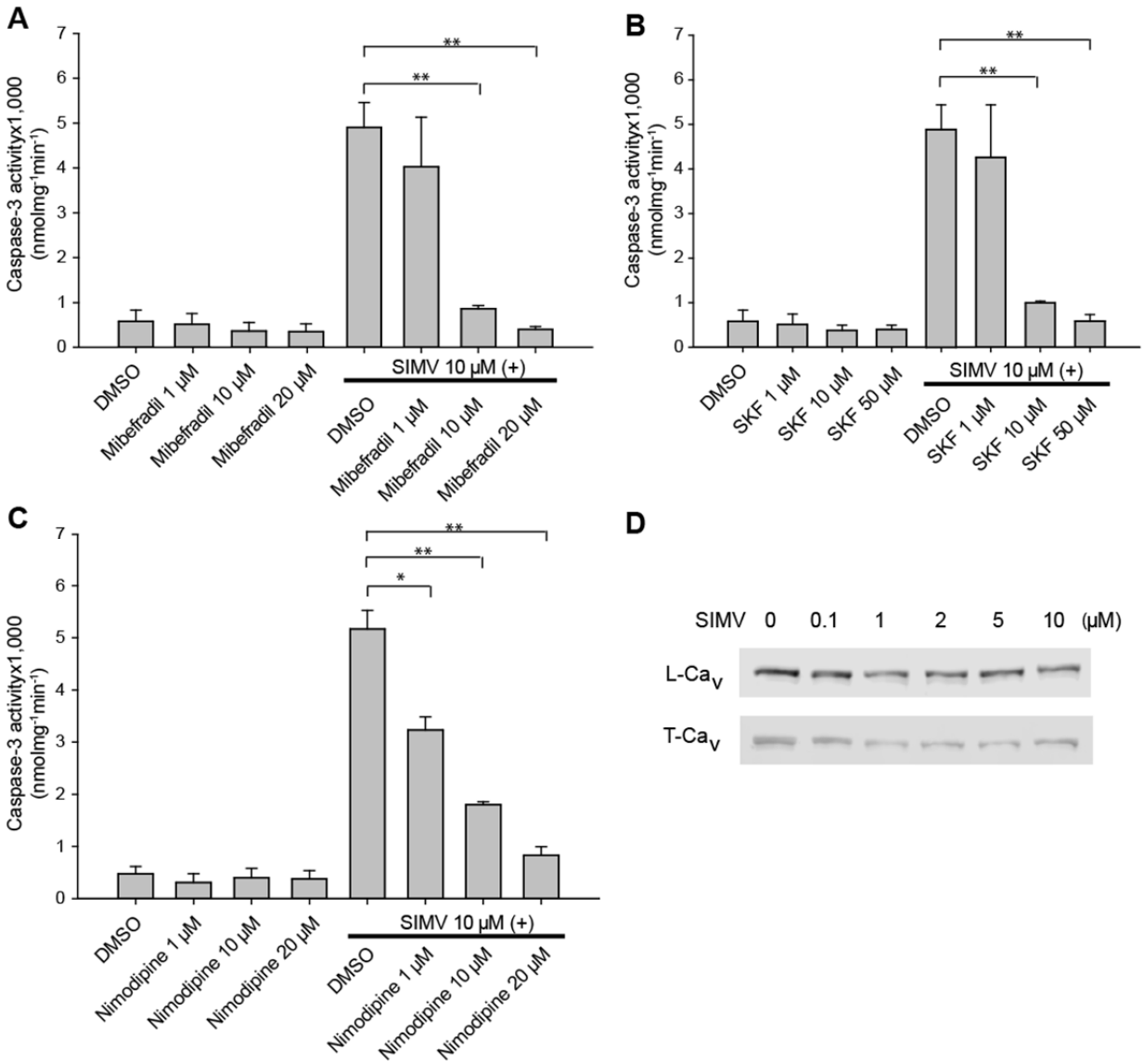

Figure 9: Simvastatin-induced apoptosis is inhibited by non-selective voltage gated calcium channel inhibitors.

A and B, Pre-treating cells with the voltage-gated calcium channels blockers mibefradil (A) or SKF96365 (SKF) (B) prevents simvastatin-induced apoptosis as measured by

caspase-3 activity. C, Pretreatment with the highly selective L-type voltage gated calcium channel inhibitor nimodipine prevents simvastatin-induced apoptosis as measured by caspase-3 activity. *$p<0.05$; **$p<0.01$ versus 10μM SIMV. C, Western blotting for expression of L-type (top blot) and T-type (lower blot) voltage gated calcium channels in human leiomyoma cells. We observed no significant changes in expression levels after simvastatin treatment.

SIMVASTATIN-INDUCED APOPTOSIS REQUIRES L-TYPE VOLTAGE-GATED CALCIUM CHANNEL ACTIVITY

We further sought to determine whether simvastatin-induced apoptosis requires L- or T-type channels. Treatment with L-type selective voltage-gated calcium channel blocker nimodipine potently inhibited simvastatin-induced apoptosis in a dose-dependent manner (figure 9C). Treatment of cells with T-type selective voltage-gated calcium channel blocker NNC 55-0396 by itself lead to rapid detachment and death of cells, which precluded analysis.

SIMVASTATIN TREATMENT DOES NOT AFFECT THE EXPRESSION OF L- OR T-TYPE VOLTAGE-GATED CALCIUM CHANNELS

We next examined whether the effects of simvastatin are mediated through modulating expression of L- or T-type voltage-gated calcium channels. Western blotting showed that treatment with simvastatin does not significantly affect the expression of either L- or T-type channels (figure 9D). This is consistent with simvastatin increasing the activity of existing L-type calcium channels (either directly or indirectly) to increase apoptotic calcium release.

DISCUSSION

The results of this study demonstrate that simvastatin inhibits the proliferation of human uterine leiomyoma cells. In addition, we have shown that inhibition of proliferation is associated with inhibition of ERK phosphorylation, a common pathway for growth factor signaling. Furthermore, our results show that simvastatin causes cell cycle progression arrest and induces calcium-dependent apoptosis in human leiomyoma cells. We were not able to determine whether the effects of simvastatin were specific for leiomyoma cells *versus* normal myometrium, as there are no reliable models for the culture and propagation of human myometrial smooth muscle. However, several lines of evidence suggest that statins may have specific anti-tumor effects *in vivo*. First, unlike normal myometrium, leiomyoma growth is dependent upon autocrine production of steroids (35, 37, 40), which would be expected to be dramatically reduced in response to statin treatment. Second, leiomyoma growth, also unlike normal myometrium, is dependent upon continual stimulation with a variety of growth factors such as insulin-like growth factor-I (72, 73), platelet-derived growth factor (71) and epidermal growth factor (75). This growth factor "addiction" would make the tumor more susceptible to statin-dependent inhibition of Ras/ERK/MAPK signaling. Future *in vivo* studies will examine these possibilities.

The anti-tumor effects of statins have been described in a number of neoplasms, including breast, ovarian, prostate, colon and leukemia (147-150, 153-159). However, the exact mechanism of these anti-tumor properties is not completely understood. In the present study, we found that calcium release is absolutely required for these effects, and is mediated by voltage-gated calcium channels. Importantly, calcium chelation with BAPTA or incubation with calcium channel blockers completely suppressed the effects of simvastatin suggesting that cytosolic calcium elevation is necessary for inducing leiomyoma cell death. T-type calcium channels, which are a class of low-voltage

activated channels, are known to be up-regulated in tumors and are thought to be pro-proliferative (215). In contrast, the high voltage-activated L-type calcium channels have been shown to contribute to cell death in multiple models by promoting mitochondrial calcium uptake and cytochrome c release (Figure 10; (216-218)).

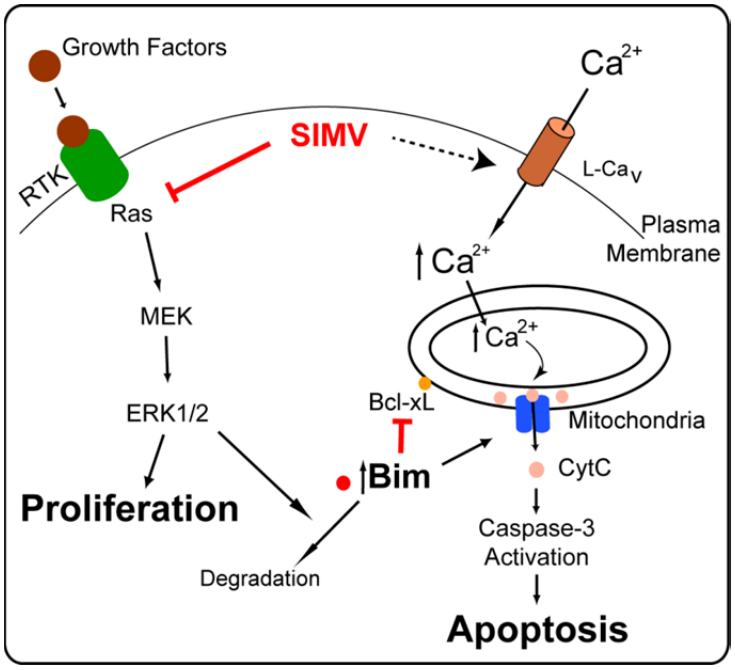

Figure 10: Cartoon demonstrating proposed mechanism of simvastatin inhibition of proliferation and induction of calcium-dependent apoptosis in human leiomyoma cells.

Proximal effects include inhibition of growth factors signaling. Downstream effects include increased expression of the pro-apoptotic Bcl-2 family member protein Bim through decreased ERK-mediated degradation. This leads to increased Bim_{EL} activity and mitochondrial leakage of apoptosis-initiating proteins including cytochrome C. Permeabilization of mitochondria also requires activation of L-type voltage-gated calcium channels and calcium influx into the mitochondria. RTK: Receptor Tyrosine Kinase. MEK: mitogen-activated protein kinase kinase. ERK: Extracellular signal-regulated kinases. CytC: Cytochrome C. L-Ca_V: L-type Voltage-gated calcium channels.

Our results demonstrating that nimodipine potently suppresses simvastatin-induced toxicity (Figure 9C) suggest that simvastatin increases the activity of L-type calcium channels (Figure 10, dashed arrow). This leads to mitochondrial calcium influx and increased release of pro-apoptotic factors such as cytochrome c. Interestingly, it has been hypothesized by others that statins can directly increase the expression of L-type calcium channels in vascular smooth muscle cells (219). In our experimental paradigm, simvastatin did not alter total protein levels of L- or T-type channels. Future work will examine directly the contribution of L-type calcium channels in leiomyoma cell death and potential mechanisms of activation.

We have found that simvastatin decreases ERK activation, which may be related to the ability of statins to decrease isoprenylation of certain proteins such as Ras. Thus, statin-induced leiomyoma cell death may represent an example of "trophic factor deprivation cell death" as previously demonstrated (220) and supported by our findings of decreased ERK activation and increased Bim expression. As mentioned above, multiple growth factors affect the development and growth of uterine leiomyomas (69) including insulin-like growth factor-I (72, 73), platelet-derived growth factor (71) and epidermal growth factor (75). Increased signaling through the Raf-MEK-ERK pathway can drive tumor growth (80). In addition, it is known that ERK phosphorylates the pro-apoptotic BH3-only protein Bim_{EL} which leads to its degradation (197, 221, 222). Bim_{EL} is a central regulator of cell death induced by trophic factor deprivation (223), and is negatively regulated by ERK activity (197, 221, 222). Thus, Bim_{EL} activation after simvastatin treatment is a plausible mechanism for increased mitochondrial calcium, membrane depolarization, cytochrome c release, and cell death (Figure 10).

In addition to effects on ERK signaling, simvastatin effects can be explained by modulating other pathways including Akt, JNK, and p38. Importantly, these pathways are involved in regulating apoptotic cellular machinery and, therefore, can explain at least partially simvastatin effects. In Appendix A, we examined effects of simvastatin

treatment on Akt pathway. Future studies can address effects of simvastatin treatment on phosphorylation of p38 and JNK.

In our experiments, we found that adding the T-type voltage-gated calcium blocker NNC 55-0396 to cells lead to rapid cellular detachment and death. This phenomenon can be attributed to necessity of T-type channel activity for cell survival. Alternatively, it can be attributed to cell-detachment induced apoptosis (anoikis). In this phenomenon, when anchorage-dependent cells get detached e.g. from extracellular matrix, apoptosis is induced. This represents a form of cell-matrix interaction necessary for cellular survival. Therefore, when NNC 55-0396 (or other pharmacologic agent) interferes with cellular adhesion (through modulating calcium-dependent cellular functions), cells become detached and therefore apoptotic machinery gets activated.

In this chapter, we examined in vitro effects of simvastatin on leiomyoma cells using primary patient-derived leiomyoma cells and immortalized human leiomyoma cell line. To confirm that these findings are characteristics of leiomyoma cells in general and not specific to these 2 cells lines, we performed additional experiments using the rat-derived leiomyoma cell line ELT-3. Details of these additional experiments are presented in Appendix A.

CHAPTER 3: EFFECTS OF SIMVASTATIN ON UTERINE LEIOMYOMA PATIENT-DERIVED XENOGRAFT MOUSE MODEL

Modified from: Novel Effects of Simvastatin on Uterine Leiomyoma:
In vitro and Patient-Derived Xenograft Mouse Model Study

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INTRODUCTION

We previously demonstrated that simvastatin inhibits proliferation, interrupts cell cycle progression, and induces apoptosis through a calcium-dependent mechanism in human leiomyoma cells in vitro. In this study, we proceeded to investigate in vivo effects of simvastatin on a patient-derived xenograft leiomyoma murine model. We report, for the first time to the best of our knowledge, that simvastatin treatment is associated with tumor growth inhibition in leiomyoma xenograft animal models and thus possibly has therapeutic potential for treating uterine leiomyoma.

EXPERIMENTAL PROCEDURES

MATERIALS

The simvastatin was purchased from Cayman Chemical (Ann Arbor, MI). Rabbit polyclonal anti-Ki67 antibody, used for immunohistochemistry, was purchased from Abcam (Cambridge, MA). A Matrigel™ basement membrane matrix was purchased from BD Biosciences (San Jose, CA). Sixty-day release pellets with 17ß-Estradiol (0.05 mg) + Progesterone (50 mg) were purchased from Innovative Research of America (Sarasota, FL).

SIMVASTATIN ACTIVATION

The simvastatin was activated prior to use to convert the prodrug (lactone) to an active (beta-hydroxyacid) form. Activation was done as previously described (224). In brief, 25 mg of simvastatin was dissolved in 625 µL of absolute ethanol and then added to 935 µL of 0.1 NaOH. After mixing, the solution was placed in a water bath at 50^{o}C for 2 hours and then diluted with water to the required concentration. Finally, the solution was sterile filtered and kept at 4^{o}C until use.

TUMOR SAMPLES

Approval from the Institutional Review Board (IRB) at the University of Texas Medical Branch (UTMB) was obtained, and verbal consents were obtained from patients. Leiomyoma samples (otherwise discarded) from patients undergoing hysterectomies were obtained and immediately transported to the laboratory under sterile conditions.

ANIMALS

Approval from the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch (UTMB) was obtained. All animal handling was performed in accordance with IACUC and other appropriate guidelines.

We modified a previously described leiomyoma xenograft animal model (225). Six-week-old female immunodeficient NOG (NOD/Shi-*scid*/IL-2Rγ^{null}) mice were purchased from Taconic (Hudson, NY). These mice have multiple immunodeficiencies and were originally developed by the Central Institute for Experimental Animals (CIEA) in Japan in 2000 from 3 mice strains: NOD/Shi, SCID, and IL-2Rγ^{null}. These combined defects rendered the mice more appropriate as human xenograft models (226). All procedures were performed under sterile precautions and, the mice were kept in an appropriately isolated environment.

ESTROGEN-PROGESTERONE PELLET INSERTION

Mice were anesthetized by isoflurane (1%–2%), administered by mask. At least 5 days before leiomyoma xenograft placement, 1 17ß-estradiol (0.05 mg)/ progesterone (50 mg) 60-day pellet was placed subcutaneously (SQ) in each animal. For pain control, buprenorphine (0.05–0.1 mg/kg SQ twice daily, then as needed) was administered.

TISSUE PROCESSING AND IMPLANTATION

Leiomyoma tumors obtained from patients were immediately processed under sterile conditions. Uniform 2 x 2 x 3 mm cylinders were obtained from the tumor using a 2 mm Keyes' biopsy punch and then dipped in the Matrigel™ basement membrane matrix. The tumors were then inserted subcutaneously into 20 mice through a small skin incision in their flanks, which were then closed with sterile surgical staples (2 tumors per mouse). The animals were closely observed for pain and signs of infection after procedures. One week after xenograft placement, the staples were removed and treatment was initiated. The twenty animals were randomly assigned to treatment (n=10) or control (n=10) groups.

ANIMAL TREATMENT AND SACRIFICE

Animals were treated by a daily subcutaneous injection of simvastatin (20 μg/ gm body weight/ day) or vehicle control for 28 days. Animals were closely observed on a daily basis, and tumor sizes were measured weekly by caliper. After 28 days, tumor sizes were measured by calipers for last time and by high-resolution sonography (Vevo 2100, Visualsonics, Toronto, Canada). Then, animal euthanasia was performed using an isofluorane overdose followed by cervical dislocation. Skin incisions were made and the tumors were removed. Representative images of the tumors by ultrasound and at sacrifice are shown in figure 11. Tumor sizes were measured again by calipers and wet tumor weights were obtained. Finally, tumors were placed in a 10% buffered formalin solution and kept at 4^{o}C until immunohistochemistry (IHC). The research personnel administering treatment and measuring tumor size were blinded in regard to treatment vs control groups.

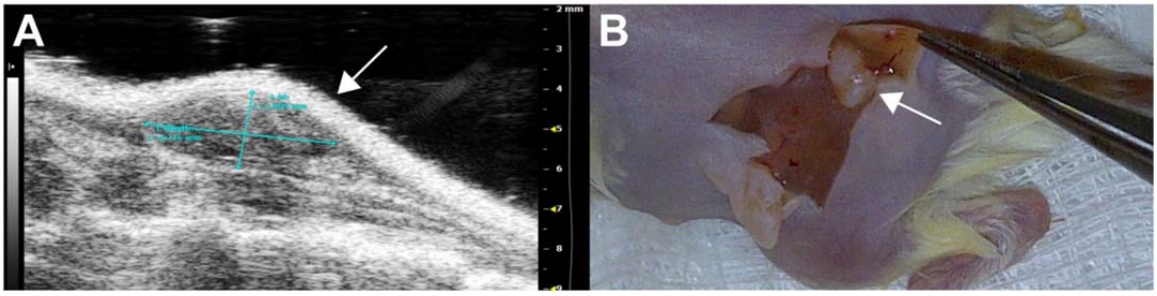

Figure 11: Leiomyoma xenograft animal model.

A, Ultrasound image of tumor. B, Macroscopic photo of tumor after animal sacrifice. Arrows demonstrate tumor.

IMMUNOHISTOCHEMICAL STUDIES

Tumor tissues obtained from the animals were fixed using 10% buffered formalin, and blocks were used to prepare tissue sections. These sections were next stained with hematoxylin and eosin (H&E) to examine the tissue morphology. Further sections were used for immunostaining for the proliferation marker Ki67, using the immunoperoxidase method with DAB as the chromogen. Immunostaining was then quantitated using the Image-Pro Plus software (Media Cybernetics, Rockville, MD). This analysis software generates arbitrary numbers using a computer grading algorithm that takes into account the tissue area, the percentage of positively-stained cells to the total number of cells, and the intensity of the staining. This was performed in 10 separate high-power fields (20x) per slide. The pathologist who read the slides was blinded in regard to treatment vs control groups.

STATISTICAL ANALYSIS

In vitro experiments were performed in triplicates and independently repeated at least 3 times. Data were checked for normality using the Shapiro–Wilk test. Whenever applicable, data were presented as mean ± SEM (standard error of the mean). For in vivo

experiments, the Student's t-test was used to compare tumor size measurements and IHC results between the treatment group and control. A 2-sided alternative to the null hypothesis of no difference was used. *P* values less than .05 were considered statistically significant. We used SigmaPlot software (Systat Software Inc, San Jose, CA) for statistical analysis.

RESULTS

SIMVASTATIN TREATMENT INHIBITS LEIOMYOMA XENOGRAFT GROWTH IN THE ANIMAL MODEL

To determine the antitumor efficacy of simvastatin in a patient-derived xenograft leiomyoma model, mice were treated with a daily dose of 20 μg/kg simvastatin (treatment group, *n*=10) or vehicle control (control group, *n*=10). Since each animal received 2 tumor xenografts, we had 20 tumors in each group (total of 40 tumors). The sizes of tumors were measured weekly by calipers. At the time of sacrifice, the tumor sizes were measured by both calipers and ultrasound. As graphically presented in figure 12 (A and B), tumor sizes were 37.9% and 49.1% smaller in the treatment group compared to controls at weeks 3 and 4 (*P*=.003 and .002, respectively). In addition, as measured by ultrasound prior to sacrifice, the mean tumor size was 43.1% smaller in the treated group compared to the control group (*P*=.043%) (figure 12C). Finally, the mean wet tumor weight was 28.5% smaller in treated group compared to the control; however, this difference did not reach significance (figure 12D; *P*=.17). In future experiments, we are considering using dry weight as it is expected to be more accurate compared to wet weight.

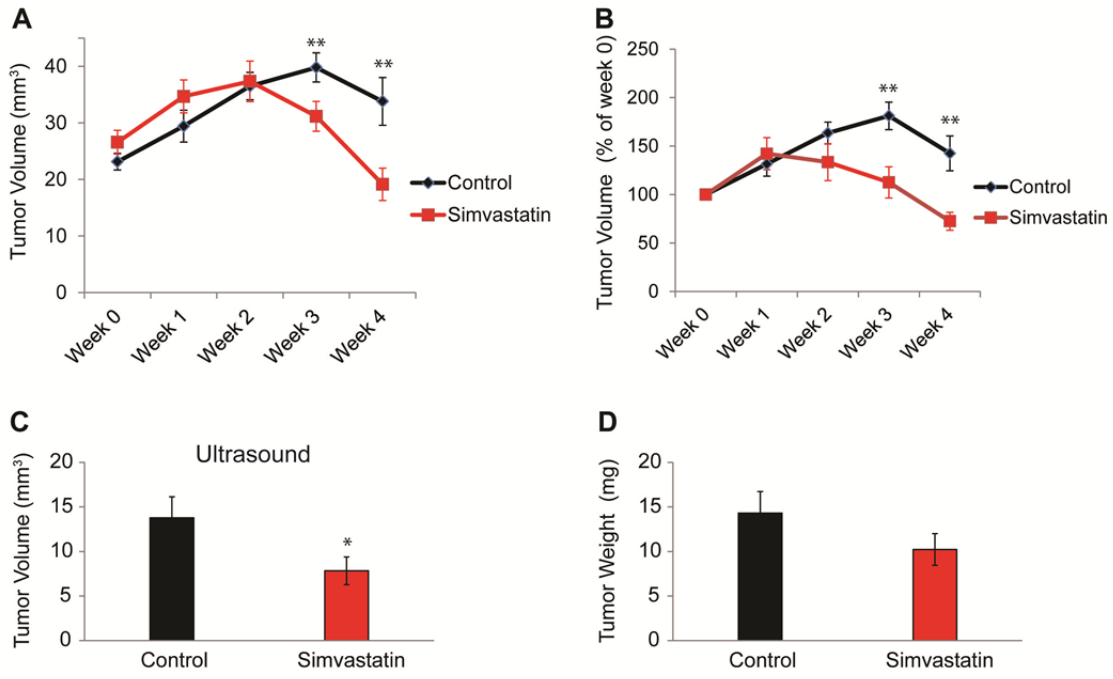

Figure 12: Effect of simvastatin treatment of patient-derived leiomyoma xenograft animal model on tumor volume.

Treated animals (*n*=10) received 20 µg/ gm body weight/ day subcutaneously for 28 days while controls (*n*=10) received vehicle. This figure shows tumor volumes in control and simvastatin groups during the study period. A, Tumor volume in mm^3 as measured weekly by calipers. B, Tumor volumes as percentage of week 0 as measured weekly by calipers. C, Tumor size at sacrifice as measured by ultrasound. D, Tumor weight immediately after sacrifice. Data are presented as means ± SEM. * $P<.05$; ** $P<.01$ vs control.

SIMVASTATIN TREATMENT INHIBITS LEIOMYOMA CELL PROLIFERATION IN XENOGRAFTED TUMORS

Finally, we wanted to examine the effect of simvastatin treatment on proliferation markers in vivo. Xenograft tumors were removed and fixed in formalin after animal sacrifice. Thereafter, immunohistochemistry was done to examine the expression of the

proliferation marker Ki67. Using computer-aided quantitation, Ki67 expression was measured and statistically analyzed. As graphically presented in figure 13, simvastatin treatment was associated with a significant reduction of the expression of the proliferation marker Ki67 by 43.7% (*P*=.015).

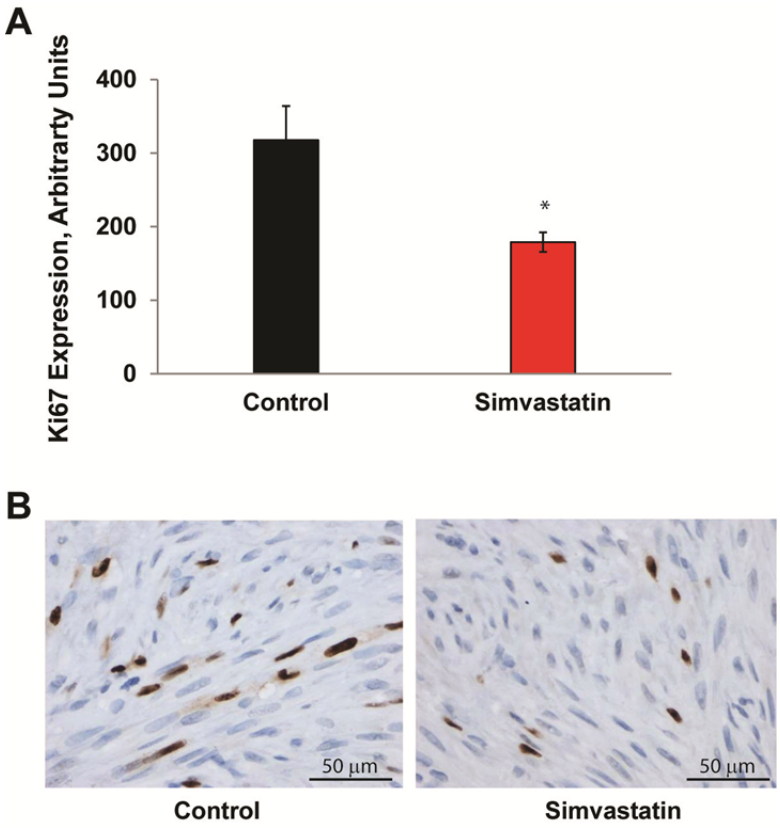

Figure 13: Effect of simvastatin treatment of patient-derived leiomyoma xenograft animal model on expression of Ki67 by immunohistochemistry.

Animals were treated as described in Figure 12. Tumor tissues obtained after sacrifice were placed in 10% buffered formalin and then processed to prepare tissue sections. These sections were next used for immunostaining with the proliferation marker Ki67, using DAB as a chromogen (original magnification 40x). Expression was then graded using an arbitrary grading system generated by the image analysis software, which correlates with the percentage of positively staining cells and the intensity of the staining

in 10 separate high power field (20x) per slide. The expression is presented by arbitrary numbers. Data are presented as means ± SEM from 20 independent slides from the treatment group and 20 slides from control group. *P<.05 vs control.

DISCUSSION

The results of this study demonstrated that simvastatin induces apoptosis and inhibits proliferation of leiomyoma cells in vitro. In addition, it inhibits leiomyoma tumor growth in a patient-derived xenograft animal model.

This study has several strong points. First, using simvastatin in the treatment of uterine leiomyoma is innovative as simvastatin is a commonly used, FDA-approved drug with well-known pharmacokinetics and safety profile (227). Second, we used an innovative animal model in which we modified a previously described protocol (225). Instead of using estrogen-only pellets, as previously described, we used estrogen-progesterone pellets, which is important as progesterone has been demonstrated to be critical for maintenance and growth of uterine leiomyoma (51). A third advantage of this model is the use of human leiomyoma tissue since the extrapolation of findings will be more reasonable. Also, the subcutaneous location of tumors allows more accurate and relatively easier measurement. Finally, the tumor growth rate in our model is closer to a clinical pattern than NSG mice models in which tumors grow 6–7 times larger (51, 225). On the other hand, the study has some weaknesses. First, we have not used a myometrial control to determine differential effects of simvastatin on leiomyomas vs normal myometrium or to detect any myometrial toxicity. Second, we have not done a drug pharmacokinetics study to evaluate plasma and tumor drug levels. However, we plan to use myometrial control and study pharmacokinetic in future works.

Among the available statins, we picked simvastatin for this study because of its favorable chemical and pharmacokinetic properties. Statins are classified as lipophilic (e.g. simvastatin, lovastatin, atorvastatin) or hydrophilic (e.g. pravastatin, fluvastatin, rosuvastatin). This solubility profile significantly affects the hepatoselectivity of a certain statin. While lipophilic statins passively diffuse to hepatic and non-hepatic tissues, the hepatic uptake of hydrophilic statins is active and carrier-mediated through membrane transporters, including organic anion-transporting polypeptide (OATP). Therefore, hydrophilic statins are more hepatoselective (concentrated in the liver), while lipophilic statins (including simvastatin) can reach hepatic and non-hepatic tissues (228-230).

In this study, we based our formulation and administration of simvastatin on previous studies utilizing rodent models where simvastatin has been administered subcutaneously (231, 232), intra-peritoneally (143), or by oral gavage (233-235). In published animal studies, oral dosages ranged from 5–140 μg/ gm body weight/ day (233-235) while subcutaneous and intraperitoneal dosages ranged from 2–40 μg/ gm/ day (143, 231, 232).

In the in vitro part of this study, we used simvastatin concentrations of 0.1–10 μM. We extensively reviewed literature regarding bioavailability, hepatic metabolism, serum levels (including peak plasma levels [C_{max}]), and maximum tolerated dosages of simvastatin. After an oral dosage of 80 mg/ day (used for hypercholesterolemia) in an average adult, simvastatin C_{max} is around 0.3 μM (190, 191, 236). In addition, the maximum tolerated dosage of simvastatin in humans was noted to be 15 mg/ kg/ day (15 μg/ gm/ day) (192). Furthermore, in humans, bioavailability of simvastatin is about 5% of the orally administered dosage (191, 237). Therefore, we picked our concentrations by taking into account a future need to increase local tissue concentrations of simvastatin to reach higher doses using technologies discussed later.

Simvastatin pharmacokinetics and pharmacodynamics in mice and rats are different than in humans. Rodents require higher doses of simvastatin to achieve the same

effects and plasma levels as humans. This is due to several factors, including the hepatic uptake and clearance of statins and feedback regulation of HMG-CoA reductase expression after statin administration (238, 239). In fact, it was demonstrated that mice treated with 25, 100, and 400 μg/ gm of body weight resulted in plasma levels 1, 4, and 8 times the mean human plasma level observed after 80 mg oral dosage, respectively (240). Similarly, another study (241) showed that oral administration of lovastatin (very similar molecular weight, lipophilicity and pharmacokinetics to simvastatin) to mice at a dosage of 25 μg/ gm was associated with a peak lovastatin acid plasma level of 0.4 μM (similar to human levels at 80 mg/day). As simvastatin undergoes extensive uptake by the liver on the first pass (more than 95%), we decided to administer it parenterally. The dosage used in this study is somewhat in the middle compared to doses used in other simvastatin animal experiments (2–40 μg/ gm/ day using subcutaneous and intraperitoneal routes) (143, 231, 232).

Simvastatin exists in a lactone or acid form. The lactone form is a prodrug and is converted to the active (beta-hydroxy acid) form by opening the lactone ring by hydrolysis. The lactone form is more readily extracted by the liver, and is metabolically cleared faster than the acid form (242, 243). Therefore, it is logical to use the lactone form when the liver is targeted, e.g. treating hypercholesterolemia. However, when extra-hepatic tissues are targeted, e.g. treating extra-heptic tumors, activation and conversion to an acid form is more appropriate. Therefore, we activated simvastatin prior to use in this study.

In conclusion, simvastatin seems to have a potential therapeutic utility in uterine leiomyoma. However, further studies are required. In future studies, we plan to use a myometrial control to examine for differential effect against leiomyoma and any myometrial toxicity. To achieve higher tumor drug concentrations while minimizing systemic side effects, localized/targeted delivery approaches can be explored, such as medicated intra-uterine devices or targeted simvastatin-loaded nanoparticles (244, 245).

We plan to develop these targeted delivery systems in our future work. In addition, we plan to study pharmacokinetics of simvastatin in our animal model by measuring drug plasma and intra-tumor levels as previously described (246).

CHAPTER 4: CONCLUSION

We conducted this study to determine the effects of simvastatin, a semisynthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on uterine leiomyomas. The study included two parts; the first is an in vitro study of the effects of simvastatin on leiomyoma cells. The second part included study of in vivo effects of simvastatin using a patient-derived leiomyoma xenograft mouse model.

In the vitro study, we used both primary human leiomyoma cells (obtained from hysterectomy patients) and immortalized human leiomyoma cells (HuLM) which were previously immortalized through telomerase induction and have been fully characterized.

We found that simvastatin induces morphologic cellular changes consistent with proliferation inhibition and cellular death. To confirm these findings, we proceeded with further experiments where, using MTT assay, simvastatin was confirmed to inhibit cellular proliferation in a dose-dependent and time-dependent fashion. We further demonstrated that simvastatin decreases phosphorylation of ERK and expression of PCNA, both are associated with cellular proliferation.

Next, we proceeded to examine effects of simvastatin on cell cycle. Modulation of cell cycle progression seems to be a possible mechanism for the inhibition of proliferation by simvastatin. We treated synchronized (by serum starvation) human leiomyoma cells with simvastatin and examined cell cycle using flow cytometry after using propidium iodide for DNA staining. We found that simvastatin induces a dose-dependent decrease in cells in S phase and a concomitant increase in the sub-G0/G1 (apoptotic) cell population.

We next sought to examine the effects of simvastatin on apoptosis. We started with effect on apoptosis using propidium-iodide (PI) staining. We found that simvastatin induces dose-dependent increase in the percentage of PI +ve cells. To confirm this

finding, we used fluorometric caspase-3 activity assay. We found that simvastatin treatment induces dose-dependent increase in caspase-3 activity.

Uterine leiomyomas are known to express aromatase enzyme, and therefore are capable of synthesizing their own estrogen locally. When we started this work, one of our working hypotheses was that as simvastatin inhibits HMG-CoA reductase inhibitor, it decreases synthesis of cholesterol and, therefore, synthesis of cholesterol-dependent steroid hormones including estrogen and progesterone. To this end, we treated leiomyoma cells with simvastatin in addition to control. We then examined the levels of sex steroids in culture medium. This included 17β-estradiol, progesterone, testosterone, and dehydroepiandrosterone sulfate (DHEA-S). These assays were done by ELISA. We didn't find any significant effect of treatment. We repeated this experiment several times, including using serum-free medium; however, no effect was found. Therefore, we thought that after all this might not be a contributing mechanism of action.

Therefore, we moved to explore other potential mechanisms. There were previous reports of statins affecting cytosolic calcium levels. Therefore, we proceeded and examined effects of simvastatin treatment on cytosolic calcium level using ratiometric Fura-2 dye assay and the genetically encoded calcium indicator protein GCaMP6. Our results demonstrated that simvastatin treatment is associated with cytosolic calcium release. To further examine if this calcium release Is necessary for simvastatin-induced apoptosis, we pretreated cells with the cell-permeable calcium chelator BAPTA. This prevented simvastatin induced apoptosis as demonstrated by lack of morphologic changes and prevention of simvastatin-induced caspase-3 activation and increase of propidium-iodide +ve cells

We further examined if cytosolic calcium release was associated with an increase in mitochondrial calcium level and if this elevation is associated with mitochondrial depolarization. Using Rhod-2 AM dye, we found that simvastatin treatment is associated

with an increase in mitochondrial calcium level. In addition, mitochondrial depolarization in response to simvastatin treatment was found as measured using cationic dye JC-1.

Next, we wanted to determine the source of cytosolic calcium released in response to simvastatin treatment. First, we thought it is inositol 1,4,5-trisphosphate receptor (IP3R)-mediated calcium release from the endoplasmic reticulum (ER) as this is one of the most common apoptosis-related cytosolic calcium release mechanisms. To examine this, we pretreated cells with xestospongin C which is a cell membrane-permeable inhibitor of IP3R and the endoplasmic-reticulum Ca^{2+} ATPase. This didn't prevent simvastatin-induced apoptosis. To confirm this, we pretreated cells with the cell membrane permeable phosphoinositide-specific phospholipase C (PLC) inhibitor U73122. Again, this did not prevent simvastatin-induced apoptosis.

From all these findings, it appeared that PLC/IP3T pathway is not involved in simvastatin-induced apoptosis. We next wanted to determine if these calcium currents are mediated through voltage-gated calcium channels. We pretreated cells with mibefradil, a non-selective voltage-gated calcium channel blocker, and SKF96365 (SKF), an inhibitor of TRP channels and both L- and T-type calcium channels. We found that both mibefradil and SKF prevented simvastatin-induced apoptosis in a dose-dependent fashion. We next sought to determine if L-type or T-type channels are involved. Therefore, we pretreated cells with the L-type channel inhibitor nimodipine and the T-type inhibitor NNC 55-0396. We found that nimodipine prevents simvastatin-induced apoptosis in a dose-dependent fashion. However, treating cells with the T-type channel inhibitor NNC 55-0396 lead to rapid cell detachment, clumping and death, precluding any analysis. Therefore, it appears that while L-type channels are involved in simvastatin-induced apoptosis; T-type channels are involved in cellular integrity and survival.

In the in vivo study, we used a patient-derived xenograft leiomyoma mouse model. First, we obtained approval from the institutional animal care and use committee (IACUC) at the University of Texas Medical Branch. Next, we purchased female NOG

immunodeficient mice at six weeks of age. This mouse strain combines several immunodeficiencies and therefore provides an excellent platform for xenograft models. Mice were kept in sterile environment and all manipulations and procedures were done under aseptic conditions. First, we subcutaneously placed 60-day continuous release estrogen/progesterone pellets to provide the estrogen and progesterone necessary to tumor growth. Five days later, we placed leiomyoma xenografts from patients who underwent hysterectomy. Animals were then randomized into treatment and control groups. Treatment group received 20 μg/gm body weight daily subcutaneous injections for 28 days while control group received vehicle. Weekly measurements of the tissue size using calibers showed that treatment groups had significantly smaller tumors. Furthermore, tumor sizes were measured by ultrasound prior to animal sacrifice confirmed that tumors in the treatment group were significantly smaller. Finally, tumors removed from mice were examined by immunohistochemistry and expression of proliferation marker Ki67 was decreased in the treatment compared to control group.

POPULATION STUDY

Statins have been in clinical use for decades. Therefore, it is intriguing to examine incidence of uterine leiomyomas, and leiomyoma-related clinical problems among statin users and compare it to non-users. To this end, we collaborated with the biostatistics division at the public medicine and community health department (PMCH) department at the University of Texas Medical Branch (UTMB). The team of biostatisticians provided the scientific support in the design of the study and writing the SAS code (statistical software used to analyze large databases). We used united healthcare database which is a claims-based administrative database that include de-identified data for about 50 million patients in the US. The database includes patient's demographics, medical diagnoses, outpatient visits, hospital admissions, medications, and surgical procedures. It has previously been validated in several studies.

Almost all statin users have hyperlipidemia. Therefore, if we simply compare statin users to non-users, we will be essentially comparing simvastatin-treated hyperlipidemia patients to normal individuals. This will represent a major study confounder. To avoid this, we used a nested case-control study design. In this design, we compared statin users to non-users within a cohort of hyperlipidemia patients, significantly eliminating hyperlipidemia as a confounding factor. We identified women 18-65 years old from 1996-2011 with diagnosis of hyperlipidemia and at least 2 years of continuous records. This formed the initial cohort of our study. Thereafter, we identified cases diagnosed with uterine leiomyomas during this time. The date when the patient was diagnosed with uterine leiomyoma was called the *index date*. For each case, we identified 3 age-matched controls. Thereafter, we used regression analysis to assess statin exposure in the 2 years prior to the index date in cases versus controls. We found that incidence of uterine leiomyomas in statin users was less than that in statin non-users. We adjusted for geographic area and comorbidities and the findings persisted. Preliminary results are tabulated in table 1.

Table 1: Crude and Adjusted Associations between Statin Use and the Risk of Uterine Leiomyoma in Study Population.

	Leiomyoma Patients	Controls	Unadjusted		Adjusted †	
Statin use	*No. of subjects (%)*		Odds Ratio (95% CI) ‡	P Value	Odds Ratio (95% CI)	P Value
Yes	12692 (26.60)	42488 (29.68)	0.855 (0.835-0.875)	<.0001	0.852 (0.831-0.874)	<.0001
No	35021 (73.40)	100651 (70.32)				

† Analyses were adjusted for geographic region and comorbidity.

‡ CI denotes confidence interval.

Next, we analyzed use of individual statins using regression analysis models. Preliminary results showed that simvastatin was the most effective statin. Data are in table 2.

Table 2. Effect of Different Types of Statin on the Risk of Uterine Leiomyoma.

Statin type	Unadjusted		Adjusted†	
	Odds Ratio (95% CI) ‡	P Value	Odds Ratio (95% CI)	P Value
Atorvastatin	0.949 (0.919-0.98)	0.0015	0.938 (0.907-0.969)	0.0002
Fluvastatin	1.137 (0.94-1.375)	0.1870	1.162 (0.958-0.969)	0.1270
Lovastatin	1.421 (0.95-2.125)	0.0868	1.219 (0.8-1.856)	0.3567
Pitavastatin	1.016 (0.106-9.781)	0.9888	0.999 (0.104-9.628)	0.9994
Pravastatin	0.879 (0.825-0.935)	<.0001	0.879 (0.825-0.937)	<.0001
Rosuvastatin	0.889 (0.845-0.935)	<.0001	0.856 (0.813-0.902)	<.0001
Simvastatin	0.832 (0.804-0.86)	<.0001	0.853 (0.823-0.883)	<.0001

† Analyses were adjusted for geographic region and comorbidity.

‡ CI denotes confidence interval.

We further proceeded to evaluate for the effects of statin use on clinical symptoms caused by leiomyomas and therapeutic procedures performed for statins. As shown in table 3, statin use was associated with decrease in menorrhagia (heavy uterine bleeding) and pelvic pain which are the commonest symptoms of uterine leiomyomas. Most importantly, statin use was associated with significant reduction in the myomectomy procedure (surgical removal of uterine leiomyomas). However, hysterectomy was not changed, probably as there are several non-leiomyoma related indications for hysterectomy.

Table 3: Effect of Statins on the Risk of Menorrhagia, Pelvic Pain, Anemia, Myomectomy and Hysterectomy.

Symptoms and Procedures	Unadjusted		Adjusted†	
	Odds Ratio (95% CI) ‡	P Value	Odds Ratio (95% CI)	P Value
Menorrhagia	0.870 (0.834-0.907)	<.0001	0.876 (0.838-0.916)	<.0001
Pelvic Pain	0.86 (0.799-0.926)	<.0001	0.86 (0.796-0.929)	0.0001
Anemia	0.986 (0.902-1.079)	0.7649	0.944 (0.855-1.044)	0.2624
Myomectomy	0.77 (0.669-0.886)	0.0003	0.856 (0.74-0.992)	0.0382
Hysterectomy	1.101 (1.039-1.168)	0.0012	1.049 (0.986-1.116)	0.1294

† Analyses were adjusted for geographic region and comorbidity.

‡ CI denotes confidence interval.

In conclusion, the findings from our epidemiologic study are strongly supportive of the hypothesis that statins have anti-uterine leiomyoma effects and further support our in vitro and in vivo findings.

MYOMETRIAL CONTROL

One area of future study is to look for effects of simvastatin on normal myometrial tissue. This is important to demonstrate any differential effect where simvastatin affects only the neoplastic tissues (leiomyoma) while spares the normal (myometrium). This will also demonstrate any myometrial toxicity. This study can be accomplished in vitro by treating primary and immortalized myometrial cells with simvastatin and examining effects as apoptosis and proliferation. In addition, we can use xenograft myometrial mouse model where immunodeficient mice are xenografted by normal myometrial tissue and are treated by simvastatin in a manner similar to the in vivo leiomyoma study we performed.

RESCUE EXPERIMENT

Given our finding that treatment of leiomyoma cells with simvastatin was not associated with changes in hormonal levels, it seemed that modulating estrogen biosynthesis plays a minor role, if any, to mediate simvastatin effects. Therefore, we currently believe that the likely mechanism of action of simvastatin is through modulating the signaling of growth factors. As known, isoprenoids (products of mevalonate pathway downstream of site of action of statins) are necessary for activation of Ras. This activation step is critical for the signaling of growth factors. Therefore, it is meaningful to examine which depleted molecule that is needed to reverse effects of simvastatin and prevent apoptosis. Candidates include mevalonate, Qualene, CoQ10, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). Our results show that geranylgeranyl pyrophosphate (GGPP) completely prevents simvastatin-induced apoptosis and other effects.

RAS ACTIVITY ASSAY

As we already discussed in the introduction section, Ras is a small G protein that is crucial for growth factor- receptor tyrosine kinase (RTK) signaling. Activated Ras is GTP-bound while deactivated Ras is GDP-bound. Ras is essentially switching between on and off. We believe that the simvastatin-induced apoptosis is mostly mediated through trophic-deprivation. Using Western blot analysis for Ras, we found that simvastatin doesn't induce changes in the level of Ras expression in leiomyoma cells. Therefore, it is reasonable to think the effects are mediated via inhibiting Ras prenylation, and subsequently, inhibiting Ras activation. To examine this, we plan to use Ras activation assay kit. This previously validated pull-down assay uses purified GST-Raf1 Ras-binding domain (RBD) to isolate active (GTP) Ras.

RNA SEQUENCING

Some of the effects of simvastatin in this study can't be completely explained by inhibiting growth factor signaling through mevalonate pathway inhibition. For example, in our Western blot analysis evaluating effects of simvastatin on ERK phosphorylation, we noted an increase in phosphorylation at 0.1 μM simvastatin concentration. In our experiments, this effect persisted despite rescuing cells by replacing farnesyl pyrophosphate (FPP). This finding suggests that simvastatin may exert effects not mediated through mevalonate pathway. To examine this hypothesis, we will pretreat cells with FPP (to negate mevalonate-mediated effects) and then treat with simvastatin. Cells pre-treated by FPP, then vehicle control will be used as control. RNA will be isolated. Total RNA sequencing (RNA-Seq) will be done and analyzed to examine effects on additional pathways.

CALCIUM CHANNEL BLOCKERS

During our experiments, we examined the effects of selective L-type and T-type voltage-gated calcium channel blockers. While we found that the L-type channel blocker nimodipine prevented simvastatin-induced caspase-3 activation, the addition of T-type channel blocker NNC 55-0396 dihydrochloride to leiomyoma cell culture dish lead to rapid (within 20-30 min) cell detachment, clumping and death. This can be an indicator that T-type calcium channels are necessary for leiomyoma cellular homeostasis, or another unclear mechanism. Therefore, it is intriguing to examine the effects of another T-channel blocker (e.g. ML 218 hydrochloride). This will help determine if this effect is related to T-type channel blockage in general, or something specific to NNC 55-0396 dihydrochloride. Next, it would be reasonable to examine the effect of T-type blockers on other leiomyoma cell lines (e.g. the rat-derived ELT-3 cells) and normal myometrial cells (primary or immortalized). According to results, further investigations can address the

role of voltage-gated calcium channel blockers in leiomyoma development and proliferation.

STATINS AND VOLTAGE-GATED CALCIUM CHANNEL BLOCKERS IN UTERINE LEIOMYOMA

Our results demonstrated that statin treatment of leiomyoma cells leads to an increase in cytosolic calcium level. This increase was found to be necessary for calcium-induced apoptosis as cytosolic calcium chelation by BAPTA prevents simvastatin-induced apoptosis. Furthermore, we found that this this cytosolic calcium release is not mediated by IP3, but rather through voltage-gated calcium channels. This opens the door for studying the effects of simvastatin on voltage gated channels. Our Western blotting results show that simvastatin doesn't affect expression level of these channels. Therefore, it seems that it rather modulates its activity. How does simvastatin modulate activity of voltage-gated calcium channels? Is this through mevalonate pathway inhibition or a direct effect? This appears to be an area for future possible research.

DIFFERENT STATINS

Although statins are structurally and functionally similar, they have individual differences. For example, some are lipophilic (e.g. simvastatin, lovastatin, atorvastatin) while others are hydrophilic (e.g. pravastatin, fluvastatin, rosuvastatin). This chemical difference affects it pharmacokinetics where hydrophilic statins are more hepatoselective (concentrated in liver) while lipophilic statins (including simvastatin) target both hepatic and non-hepatic tissues. It is intriguing to examine whether the effects noted in this study are shared with other statins or are specific to simvastatin. We are currently examining the effects of several statin on leiomyoma cells in vitro. Our preliminary results demonstrate that pravastatin doesn't seem to induce apoptosis. However, lovastatin, fluvastatin and atorvatstatin seem to induce apoptosis, although not as potently as simvastatin. These experiments are very early and we plan to elaborate on this area.

PHARMACOKINETIC STUDY

One of the critical points in the efficacy of simvastatin is it pharmacokinetics. We do see in vitro anti-proliferative properties for simvastatin as low as 0.1 μM, however, apoptosis induction was notable at 1 μM. After an oral dosage of 80 mg/ day (used for hypercholesterolemia) in an average adult, simvastatin Cmax is around 0.3 μM. In addition, the maximum tolerated dosage of simvastatin in humans was noted to be 15 mg/ kg/ day (15 μg/ gm/ day).

Therefore, it makes sense to study pharmacokinetics of drug delivery in our animal model. In future experiments, we plan to examine simvastatin drug level in plasma and in the tumor itself. This will be done to study distribution of simvastatin and its tumor penetration.

DRUG DELIVERY

In our animal experiments, we used a dosage of 20 μg/ gm body weight/ day subcutaneously. We would like to further increase the dosage of simvastatin delivered to the tumor without increasing the systemic exposure. To accomplish this, we can use nanoparticles to specifically deliver simvastatin at leiomyomas or use a local delivery method, e.g. a simvastatin-medicated intrauterine system (IUS). For the nanoparticles, we started an experiment in collaboration with Dr. Bulent Ozpolat from MD Anderson cancer. We developed and used simvastatin-nanoliposomal delivery system and administered it to our leiomyoma mouse model. Our preliminary results show that this formulation inhibits tumor growth. However, this is only a preliminary result and we will need to repeat it. Regarding the local delivery method, we are considering a specific group of collaborators to develop the intra-uterine delivery system. However, we will further proceed with both projects only after performing and standardizing our pharmacokinetic assay. This is critical so that we can assess efficacy of tumor targeting approach.

HUMAN TRIAL

Next step in our study is to examine in vivo effects of simvastatin on uterine leiomyomas in humans. This can be done through an IRB-approved randomized controlled trial. Patients scheduled for hysterectomy for uterine leiomyomas would be randomized to receive simvastatin or placebo prior to surgery. Thereafter, tumor size will be monitored. In addition, tumors removed during surgery will be examined for markers of proliferation and apoptosis. In addition, simvastatin levels can be measured in tumor and compared to simultaneous plasma levels. This will lead to a better understanding of the fraction of drug reaching tumor.

Our ultimate aim will be to study the effects of simvastatin on uterine leiomyomas patients not scheduled for a hysterectomy. Ideally, this will be a multi-institutional IRB-approved randomized controlled trial (RCT). Patients (as well as controls receiving placebo) will be administered simvastatin and followed over a pre-determined period of time. Outcomes will include tumor size as measured by ultrasound and quality of life of the patients. Quality of life will include heavy bleeding, pelvic pain, anemia and need for hospitalization.

APPENDIX A: IN VITRO EXPERIMENTS USING RAT-DERIVED LEIOMYOMA CELL LINE

In chapter 2, we examined in vitro effects of simvastatin on leiomyoma cells using primary patient-derived leiomyoma cells and immortalized human leiomyoma cell line. To confirm that these findings are characteristics of leiomyoma cells in general and not specific to these 2 cells lines, we used the rat-derived leiomyoma cell line ELT-3 as an additional cell type.

EXPERIMENTAL PROCEDURES

MATERIALS

The simvastatin was purchased from Cayman Chemical (Ann Arbor, MI). A complete protease inhibitor cocktail without EGTA was purchased from Roche Applied Science (Indianapolis, IN). Z-DEVD-R110 used for the caspase-3 assay was purchased from the American Peptide Company (Sunnyvale, CA). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was purchased from Calbiochem (EMD Millipore, Merck KGaA, Darmstadt, Germany). Monoclonal anti-phospho Akt and anti-total Akt antibodies, used for Western blotting, were purchased from Cell Signaling (Danvers, MA).

CELLS

The Eker rat leiomyoma cell line (ELT-3) was a kind gift from Dr. Cheryl Walker, professor and director at the Texas A&M Health Science Center Institute of Biosciences and Technology in Houston, TX. These cells were established and have been fully characterized (247). ELT-3 leiomyoma cells were cultured and maintained in a DF8

medium as previously described (247). Cells were incubated in a 5% CO_2 atmosphere under 37 ^{o}C and split once 70%–80% confluent.

PROLIFERATION ASSAY

An MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to monitor cellular proliferation. ELT-3 cells were seeded into 96-well plates. After 24 hours, the medium was replaced with a medium containing 0, 0.1, 0.5, 1, 5, and 10 μM of simvastatin. 48 hours later, the MTT assay was performed as previously described (189).

CASPASE-3 ASSAY

Caspase-3 activity was measured using a quantitative fluorometric assay as previously described (194). In brief, cells were seeded in 10 cm dishes. After 24 hours, the medium was replaced with a medium containing 0, 0.1, 0.5, 1, 5, and 10 μM of simvastatin. After 48 hours, cells were harvested and cell lysates were obtained. Cell lysates containing equal amounts of proteins were loaded in a 96-well plate in addition to a reaction mixture containing Z-DEVD-R110 (caspase-3 substrate). Caspase-3 activity was measured fluorometrically over a period of 60 minutes.

STATISTICAL ANALYSIS

In vitro experiments were performed in triplicates and independently repeated at least 3 times. Data were checked for normality using the Shapiro–Wilk test. Whenever applicable, data were presented as mean ± SEM (standard error of the mean). The Student's t-test was used to compare individual treatment groups to control. A 2-sided alternative to the null hypothesis of no difference was used. *P* values less than .05 were considered statistically significant. We used SigmaPlot software (Systat Software Inc, San Jose, CA) for statistical analysis.

RESULTS

SIMVASTATIN INDUCES APOPTOSIS IN LEIOMYOMA CELLS IN VITRO

To investigate the effects of simvastatin on apoptosis, we treated ELT-3 cells with different concentrations of simvastatin for 48 hours, followed by a fluorometric caspase-3 activity assay using cell lysates. Statistical analysis showed significant differences at 5 and 10 μM compared to controls (*P*=.005 and *P*=.025, respectively) (figure 14).

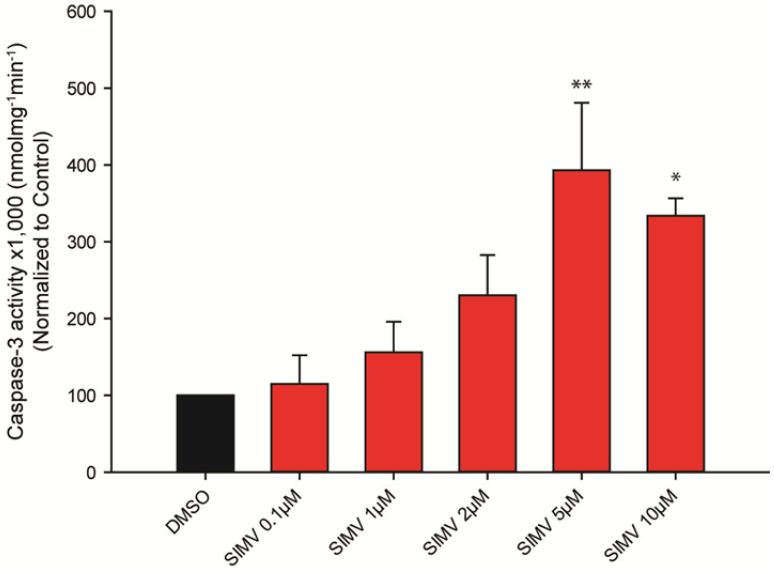

Figure 14: Effect of simvastatin treatment of rat leiomyoma cells on apoptosis.

ELT-3 leiomyoma cells were treated with the indicated doses of simvastatin (SIMV) for 48 hours, followed by fluorometric caspase-3 activity assay. The data were normalized to control and presented as means ± SEM of independently repeated 3 experiments. *P<.05; *** P <.001 vs control.

SIMVASTATIN INHIBITS PROLIFERATION OF LEIOMYOMA CELLS IN VITRO

Next, we proceeded to determine if simvastatin affects proliferation of leiomyoma cells. After treating ELT-3 cells for 48 hours, we performed an MTT assay. Statistical

analysis showed that simvastatin significantly inhibits proliferation of cells at 10 μM (*P*=.021) (figure 15).

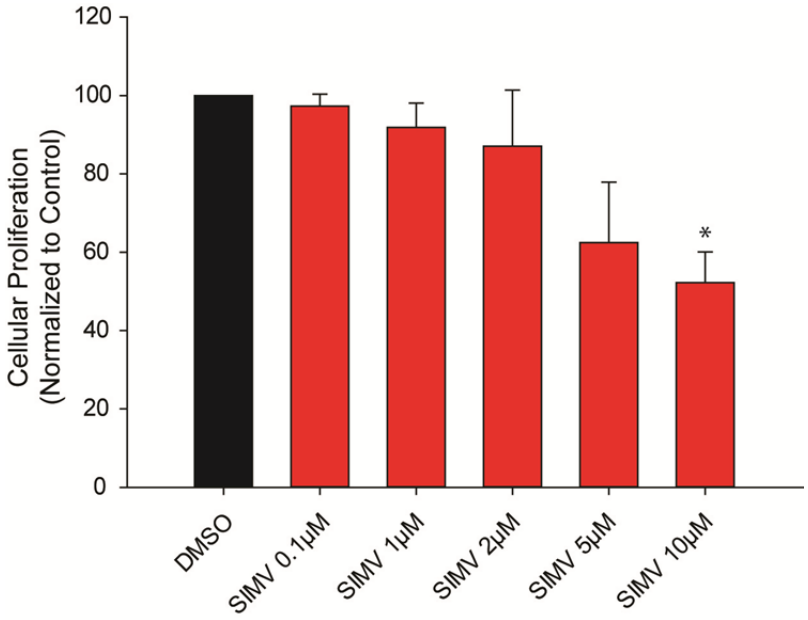

Figure 15: Effect of simvastatin treatment of rat leiomyoma cells on cellular proliferation. ELT-3 leiomyoma cells were treated with the indicated doses of simvastatin (SIMV) for 48 hours, followed by an MTT assay. The data were normalized to control and presented as means ± SEM of independently repeated 3 experiments. * P <.05; *** P <.001 vs control.

SIMVASTATIN INHIBITS AKT SIGNALING PATHWAY IN LEIOMYOMA CELLS IN VITRO

We next sought to examine the effects of simvastatin on proliferation signaling pathways. To this end, we used Western blotting to examine the phosphorylation of Akt protein. Phosphorylation was calculated by dividing the expression of phosphorylated fraction by the total Akt protein. Statistical analysis showed that simvastatin significantly inhibits phosphorylation of Akt at 2, 5, and 10 μM (*P*=.049, .017, and .014, respectively) (figure 16).

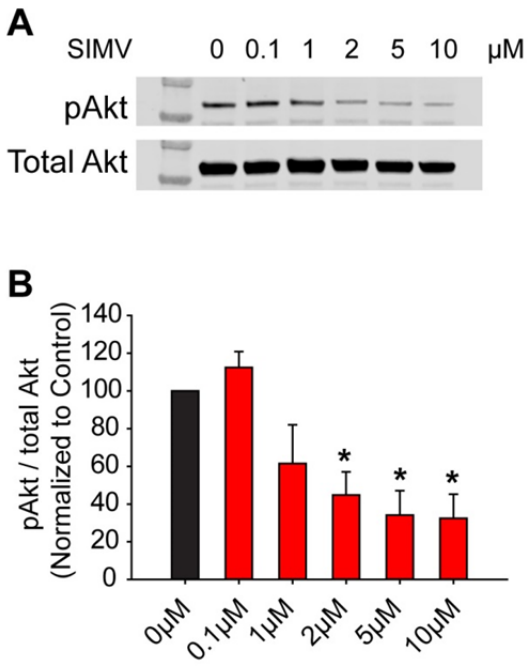

Figure 16: Effect of simvastatin treatment of leiomyoma cells on activation of Akt cellular signaling pathway.

HuLM leiomyoma cells were treated with the indicated doses of simvastatin (SIMV) for 48 hours, followed by western blotting of cell lysates for active (phosphorylated) Akt and total Akt protein. Protein levels were quantified and data presented as the ratio of phosphorylated to total Akt. The data were normalized to control and presented as means ± SEM of independently repeated 3 experiments. * P <.05; ** P <.01 vs control.

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