Molecular Medicine

Signaling Pathways in Leiomyoma: Understanding Pathobiology and Implications for Therapy

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Uterine leiomyomas are the most common tumors of the female genital tract, affecting 50% to 70% of females by the age of 50. Despite their prevalence and enormous medical and economic impact, no effective medical treatment is currently available. This is, in part, due to the poor understanding of their underlying pathobiology. Although they are thought to start as a clonal proliferation of a single myometrial smooth muscle cell, these early cytogenetic alterations are considered insufficient for tumor development and additional complex signaling pathway alterations are crucial. These include steroids, growth factors, transforming growth factor-beta (TGF-β)/Smad; wingless-type (Wnt)/β-catenin, retinoic acid, vitamin D, and peroxisome proliferator-activated receptor γ (PPARγ). An important finding is that several of these pathways converge in a summative way. For example, mitogen-activated protein kinase (MAPK) and Akt pathways seem to act as signal integrators, incorporating input from several signaling pathways, including growth factors, estrogen and vitamin D. This underlines the multifactorial origin and complex nature of these tumors. In this review, we aim to dissect these pathways and discuss their interconnections, aberrations and role in leiomyoma pathobiology. We also aim to identify potential targets for development of novel therapeutics.

INTRODUCTION

Uterine leiomyomas are the most common tumors of the female genital tract and 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 burdens. In fact, the lifetime incidence of leiomyomas ranges from 50% to 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 fibroids is not clear. This leads to an 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. An overview of the signaling pathways relevant to leiomyoma development and growth is presented in Box 1.

STEROID SIGNALING

Estrogen

Uterine leiomyomas have classically been considered estrogen-dependent tumors as they tend to grow during reproductive years and regress 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

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Box 1. Overview of signaling pathways in uterine leiomyoma.

A. Steroid signaling
  a. Estradiol
    i. Estrogen receptors
      1. Nuclear: ERα and ERβ
      2. Membrane bound: mERα, mERβ and GPR30
    ii. Signaling
      1. Transcriptional (genomic)
      2. Rapid (nongenomic)
    iii. Aberrations in estradiol signaling in leiomyoma pathobiology
    iv. Interaction with other signaling pathways
    v. Targeting in leiomyoma therapy
  b. Progesterone
    i. Progesterone receptors
    ii. Signaling
    iii. Interaction with other signaling pathways
    iv. Targeting in leiomyoma therapy
B. Growth factor signaling
  a. Growth factor receptors: RTKs
  b. Growth factor signaling pathways
    i. RTK/Ras/Raf/MEK/ERK pathway
    ii. PI3K/Akt/mTOR pathway
    iii. Smad signaling
  c. Role of individual growth factors
    i. IGF-1
    ii. EGF
    iii. PDGF
    iv. VEGF
    v. FGF
    vi. Transforming growth factors
  d. Targeting in leiomyoma therapy
C. Other signaling pathways
  a. Wnt/β-catenin
  b. RA
  c. Vitamin D
  d. ECM
  e. PPARγ

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 the 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 (or classical) estrogen receptors (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 expressed mainly in uterus and breast, ERβ is more broadly distributed in ovary, brain, bone and other organs. However, both ERα and ERβ are coexpressed 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% to 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 mERβ) (19,20). On the other hand, the transmembrane G protein-coupled receptor 30 (GPR30), first described in the 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 (E_2) binds to ERs and then E_2-ER complexes regulate transcriptional activities. Although unbound ERs were initially thought to be mostly cytosolic and to 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 E_2 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). A number of coactivators such as steroid receptor coactivator 1 (SRC-1) and corepressors such as nuclear receptor corepressor (NCoR) also regulate the transcription of these target genes (25). Another mode of action of the classic pathway is the 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, for example, epidermal growth factor receptors (19). As a G protein-coupled receptor, GPR30 is involved in rapid signaling events including cyclic adenosine monophosphate (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 generally are 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.

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 with 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 with 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
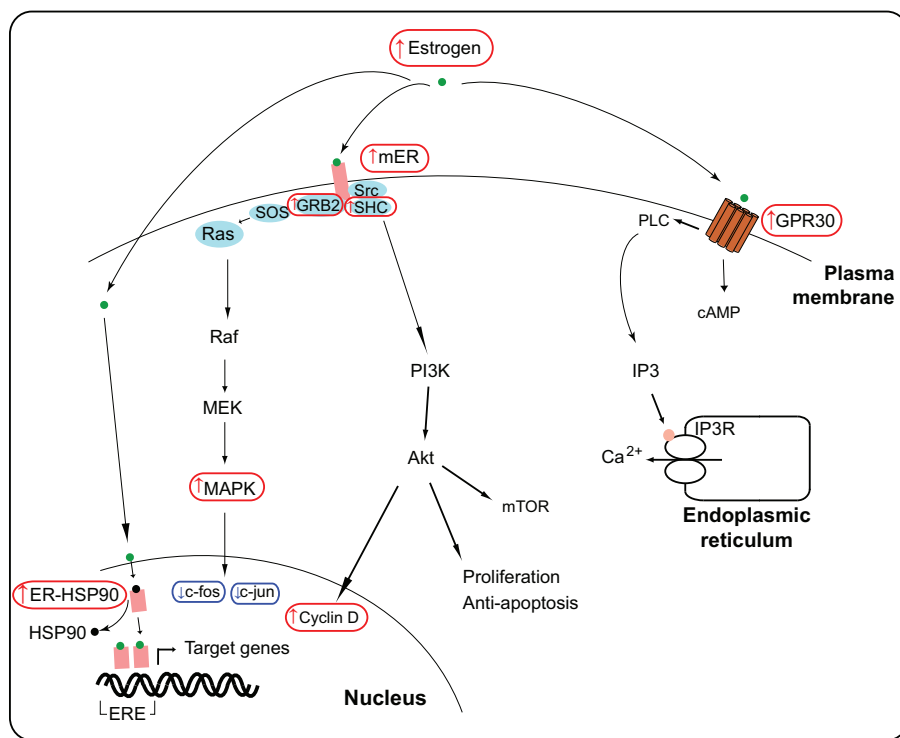

Figure 1. Schematic presentation of estrogen signaling pathways in uterine leiomyoma. ↑ and ↓ denote increased (red) or decreased (blue) level or function, respectively. ER: estrogen receptor; mER: membrane-bound estrogen receptor; PLC: phospholipase C; IP3: inositol triphosphate; IP3R: inositol triphosphate receptor.

for 1, 5 and 30 min triggers rapid activation of phosphatidylinositide 3-kinases (PI3K), mitogen-activated protein kinase (MAPK) and phosphoinositide phospholipase Cγ (PLCγ) (32). In addition, Nierth-Simpson and colleagues (33) showed that E_2 treatment for 5 min activates protein kinase Cα (PKCα) in both human leiomyoma cells and human smooth muscle cells. Furthermore, they noted that it increases phosphorylated MAPK in human leiomyoma cells but not human smooth muscle cells. Therefore, they concluded that aberrant and rapid MAPK 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 with normal myometrium. In addition, Kasai and colleagues (38) demonstrated that leiomyoma tissue overexpresses type I 17β-hydroxysteroid dehydrogenase (17β-HSD) compared with 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 sig-

nificant side effects such as loss of bone mineral density, it can only be used for a short period. Because of this, it is almost conclusively used only preoperatively. 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 (an SERM) effects on uterine fibroids are conflicting (41,42). Similarly, a Cochrane review (43) summarized three randomized controlled trials using raloxifene (another SERM) in uterine fibroids 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 with proliferative (estrogen-dominant) phase of the menstrual cycle. Furthermore, leiomyoma xenograft animal models supported the necessity of progesterone for growth of uterine leiomyoma. 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 a 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 predominant 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 path-

ways. 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 described previously, 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 factor signaling. The Maruo group in Japan found that progesterone downregulates expression of insulinlike growth factor-I (IGF-1) in human leiomyoma cells (57). They also demonstrated that progesterone upregulates 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 and proliferation in uterine leiomyoma cells. From all this, it seems that progesterone signaling is an integrated part of a complex signaling network in leiomyoma.

Progesterone signaling as a therapeutic target in leiomyoma treatment.

Fiscella and colleagues (62) randomized 42 women with symptomatic uterine fibroids into treatment with progesterone antagonist mifepristone or placebo for 26 wks. 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 a 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 wks of treatment, there was a significant improvement in uterine bleeding and a reduction in the 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 the 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 in myometrial cells. Furthermore, they demonstrated that asinoprisnil decreases ex-
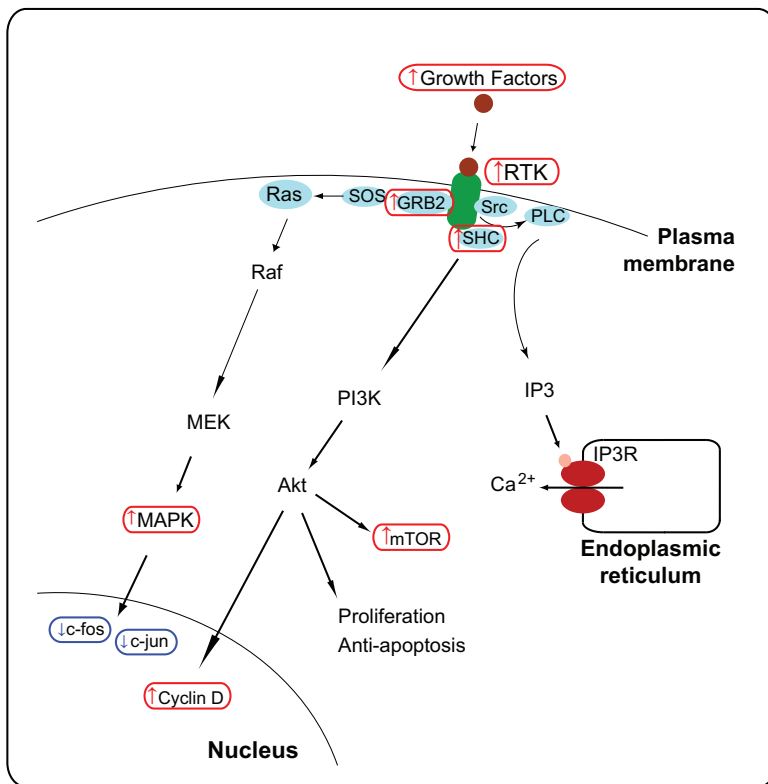

Figure 2. Schematic presentation of growth factor signaling pathways in uterine leiomyoma. ↑ and ↓ denote increased (red) or decreased (blue) level or function, respectively. IP3: inositol triphosphate; IP3R: inositol triphosphate receptor.

pression 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

Growth factors are relatively small proteins secreted into extracellular spaces to bind to cell membrane receptors of target cells. This results in activation of intracellular signal transduction pathways that 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), several growth factors have been characterized. Perturbation of growth factor signaling plays a significant role in the development and growth of many tumors (66).

GROWTH FACTOR SIGNALING

Substantial evidence exists to suggest that aberrations of certain growth factors and their receptors or signaling pathways play a significant role in growth and development of uterine fibroids (68–71). These include IGF-1 (72,73), platelet-derived growth factor (PDGF) (71), vascular endothelial growth factor (VEGF) (74), EGF (75) and fibroblast growth factor (FGF) (76). Figure 2 presents a simplified illustration of growth factor signaling.

Receptor Tyrosine Kinases

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 three parts: an extracellular ligand binding domain; a transmembrane helix; and an intracellular domain that contains the tyro-

sine 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 a growing body of evidence for the 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 overphosphorylated in leiomyomas compared with myometrial tissues. In addition, Swartz and colleagues (79) found that 17β estradiol treatment leads an increase in 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). MAP kinases are serine/threonine-specific protein kinases that regulate many cellular functions including proliferation, survival and apoptosis. They include several subfamilies, for example, 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 an Shc protein linker) working as adapter or bridge proteins. The Sos works as a guanine nu-

cleotide exchange factor (GEF) to stimulate the exchange of a GDP for a GTP molecule to (and therefore activation of) the small guanine nucleotide-binding protein (G protein) Ras. Thereafter, activated Ras recruits and activates the Raf kinases that subsequently phosphorylate (and therefore activate) MEK protein that 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 lead to a complex series of nuclear events modulating transcription of several target genes (83). Of note, Lessi and colleagues (84) and Gustavsson and his group (85) independently discovered lower mRNA levels of c-Fos and c-Jun in leiomyoma compared with myometrium (see Figure 2).

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 the ERK pathway through GPCR as well as transactivation of EGFR (86). In addition, estrogen can activate ERK through the pathway involving the non-receptor tyrosine kinase c-src (87–89). On the other hand, growth factors can modulate the response to steroids through effects of ERK on transcriptional activity of steroid receptors (90).

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 with 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 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 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 and phosphoinositide-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 (91).

There is recent evidence of aberrant PI3K/Akt/mTOR signaling in uterine leiomyoma. Karra and colleagues (92) found increased expression of glycogen synthase kinase 3 (GSK3) and cyclin D_2 in leiomyoma compared with myometrium, with interaction between phosphorylated GSK3 and Akt. In addition, Jeong and colleagues (93) 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 in myometrial cells. Finally, Crabtree and colleagues (94) found upregulated mTOR pathways 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.

Smad Signaling

Smads are intracellular proteins that transmit signals from several cell membrane receptors to the nucleus (95). They transduce signaling from receptors of transforming growth factor-β, activin, mystatin, BMP and others. In vertebrates, eight Smads have been identified and named Smad1 through Smad8. Upon ligand binding to Smad-coupled receptors, two receptor-activated Smads (R-Smads) are phosphorylated and then heterotrimerize with one common Smad (Smad4). The resulting complex translocates to nucleus to act as a transcription factor for target genes (95). TGF-β, activin and myostatin all converge on Smads to regulate cellular proliferation and extracellular matrix (ECM) formation at the transcriptional level. A simplified cartoon describing Smad signaling is presented in Figure 3. There is evidence that leiomyomas demonstrate aberrant Smad signaling. Chegini and colleagues (96), using immunohistochemistry, Western blotting and RE-PCR, found leiomyomas overexpress Smad3, Smad4 and phosphorylated Smad3 (pSmad3) compared with myometrium. In addition, they found that the levels of phosphorylated Smad2/3 (pSmad2/3) in leiomyoma and myometrium are lower after GnRH-agonist treatment as compared to controls (96). Therefore, it seems that Smad signaling can be a potential therapeutic target in leiomyoma treatment.

Role of Individual Growth Factors in Leiomyoma Pathobiology

Insulinlike growth factors (IGFs). Several studies point to a role for IGFs in leiomyoma pathobiology. For example, Peng and colleagues (73) found that one third of uterine leiomyomas demonstrate dysregulation in IGFs. They obtained uterine leiomyoma samples from hysterectomy cases and 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 levels. In ad-

Figure 3. Schematic presentation of transforming growth factor-β, activin, myostatin and Smad signaling in uterine leiomyoma. ↑ and ↓ denote increased (red) or decreased (blue) level or function, respectively. TGF-β: transforming growth factor-β; TβRI: transforming growth factor-β receptor I; TβRII: transforming growth factor-β receptor II; ALK4: activin-like kinase-4 (activin receptor type-1B); ActRII: activin receptor type II.

dition, they noted that IGF-1 levels correlate with activation of AKT. Interestingly, they noted that overexpression of IGF-1 and p-AKT correlate with fibroid size.

Burroughs and colleagues (72) used the Eker rat leiomyoma model to demonstrate that IGF-1 is expressed in leiomyoma tissues 7.5 times higher than 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, is four-fold higher in leiomyoma as 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-1 and Myb (a transcription factor promoting cell cycle progression) genes.

EGF and PDGF. Fayed and colleagues (97) studied specific binding of EGF,

PDGF and insulin in leiomyoma and myometrium. They found that EGF, PDGF and insulin stimulated protein synthesis in both leiomyoma and myometrial cells. This denotes that these signaling pathways are operational in leiomyoma and myometrium.

More recently, Liang and colleagues (98) demonstrated that human leiomyomas expresses PDGF more than the surrounding myometrium. In addition, they found that PDGF increases expression of PCNA and collagen α1 in leiomyoma cells compared with myometrial cells. Therefore, it seems reasonable to conclude that PDGF signaling is increased in leiomyoma and that it may contribute not only to proliferation but also to excessive deposition of ECM (98).

Rossi and colleagues (75) demonstrated using immunohistochemical studies that myometrial cells express EGF, PDGF, EGFR and PDGFR-β. More
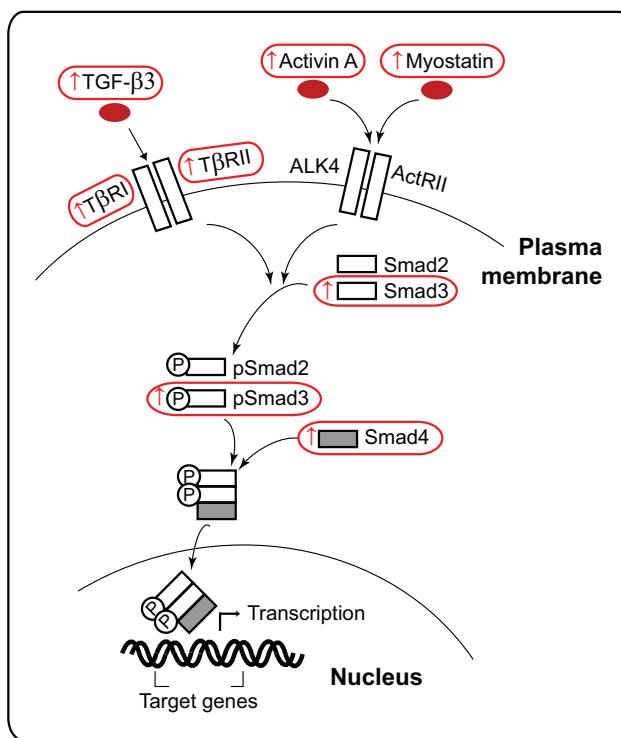
recently, Ren and colleagues (99) demonstrated that EGF stimulates DNA synthesis in leiomyomas but not myometrial smooth muscle cells. In addition, they found that EGF-induced effects can be blocked by AG1478, an EGFR inhibitor as well as by PD98059, a MEK1/2 inhibitor. However, they noted that both leiomyoma and myometrial cells express EGFR equally. Importantly, they found that downstream signaling induced by EGF stimulation is different in leiomyoma versus myometrial cells, suggesting fundamental alterations in EGF signaling in leiomyomas.

VEGF. VEGF and its receptors (VEGFR-1 and VEGFR-2) were demonstrated to be expressed both in myometrium and leiomyoma tissue (100–102). Using immunohistochemical studies, Gentry and colleagues found that vascular endothelial growth factor-A (VEGF-A) was overexpressed significantly in leiomyoma tissue as compared to the adjacent myometrium (103). It was demonstrated that VEGF is necessary for the growth of leiomyoma xenografts *in vivo* (104). VEGF represents a potential therapeutic target in leiomyoma, and Xu and colleagues demonstrated that it is downregulated by the selective progesterone receptor modulator CDB-2914 (105).

Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF). aFGF, also known as FGF-1, is expressed in both myometrium and leiomyoma tissue, with overexpression in leiomyoma (106,107). Similarly, basic fibroblast growth factor (bFGF), also known as FGF-2, was found to be expressed in both myometrium and leiomyoma tissue (100). Anania and colleagues demonstrated changes in the expression of basic fibroblast growth factor type 1 receptors in leiomyoma-associated abnormal bleeding compared with normal women (108).

Transforming growth factors. 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 the ECM 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 leads to receptor heterotetrameric complex formation and phosphorylation, which in turn phosphorylates Smads. These, in turn, translocate into the nucleus and regulate transcription of several target genes. In addition, intracellular signaling activated by TGF-β also includes non-Smad pathways. Ikushima and Miyazono (109) as well as Elliott and Blobe (110) provide detailed reviews of TGF-β signaling pathways in human tumors. Aberrations of TGF-β signaling are considered central in tumorigenesis and tumor progression (111). The role of TGF-β signaling in tumors is complex and is context- and type-specific (109). Figure 3 presents a simplified illustration of TGF-β, activin, myostatin and Smad signaling.

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 (112) found that TGF-β3 mRNA was five-fold higher in leiomyoma compared with normal myometrium. In addition, they demonstrated that leiomyomas are refractory to the antiproliferative 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 these cells resistant to the antiproliferative effects of TGF-β (112). Arici and Sozen (113) found that leiomyoma tissue overexpresses TGF-β3 as compared to myometrial tissue. In addition, they found that TGF-β3 induces fibronectin secretion by leiomyoma cells. However, in contrast to Lee and Nowak (112), they found that TGF-β3 stimulates proliferation in both leiomyoma and myometrial cells. Again,

this underlines the complex and context-dependent nature of the outcome of TGF-β3 signaling. Furthermore, Salama and colleagues (114) 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 tissue was found to overexpress TGFBR1, TGFBR2, Smad3, Smad4 and phosphorylated Smad3 as compared to the myometrium (96). All of these studies point to a potential role for aberrant TGF-β/Smad signaling in leiomyoma (see Figure 3).

Modulating growth factor signaling as a potential target in leiomyoma treatment. Growth factor signaling pathways represent a potential target for leiomyoma treatment (115). For example, Di Lieto and colleagues (116) demonstrated that reduction of uterine volume after GnRHa treatment was associated with a reduction in the expression of 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 to induce apoptosis of leiomyoma cells, also decreases expression of EGF, IGF-1, TGF-β mRNA and protein. In addition, it decreases expression of EGFR, IGF-1R α and TGFRII protein in leiomyoma but not in myometrial cells. Therefore, they suggested that the potential therapeutic effects of asoprisnil are mediated through downregulating the expression of growth factors and their receptors in leiomyoma cells. Finally, Borahay and colleagues (117) demonstrated that simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, inhibits ERK phosphorylation in leiomyoma cells. They also demonstrated that this effect is associated with the inhibition of both proliferation and induction of apoptosis. All these findings support the notion that growth factor signaling pathways present a potential therapeutic target in leiomyoma therapy.

Modulating the PI3K/Akt/mTOR pathway seems to be an intriguing target

in leiomyoma therapy. For example, Sefton and colleagues (118) 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 (119).

The TGF-β/Smad signaling pathway represents a potential target for therapeutics development. Chegini and colleagues (96) 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 (114) found that 2-methoxyestradiol (an estrogen metabolite with antitumor properties) inhibits TGF-β3 profibrotic effects in leiomyoma cells through Smad- and non-Smad-mediated pathways. Furthermore, De Falco and colleagues (120) used tissues obtained from hysterectomy patients and demonstrated that both TGF-β3 and connective tissue growth factor are overexpressed in leiomyoma compared with myometrium.

OTHER PATHWAYS

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 an accumulation of β-catenin in the cytoplasm and its subsequent translocation into the nucleus and subsequent activation of transcription factors (121).

There are several recent reports of the role of Wnt/β-catenin in leiomyoma growth and development. For example, Mangioni and colleagues (122) demonstrated that the 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 the initiation of transcription. Aberrations of MED12 were found in 70% uterine leiomyomas (123). In addition, Markowski and colleagues found that leiomyomas with MED12 mutations overexpress the Wnt protein family member Wnt4 (124). They further hypothesized that estrogen along with MED12 mutations lead to Wnt/β-catenin activation with leiomyoma-like lesions in murine models. In addition, Tanwar and colleagues demonstrated that constitutively expressing activated β-catenin in a Cre-recombinase mouse model leads to myometrial hyperplasia and the development of leiomyoma-like uterine tumors (125).

More recently, Ono and colleagues (126) demonstrated that Wnt/β-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 that, through paracrine effects, leads to nuclear translocation of β-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 in leiomyomas is modulated, at least in part, through Wnt expression by mature leiomyoma cells and its paracrine response on β-catenin signaling in LMSP.

Retinoic Acid Signaling

Retinoic acid (RA), the active metabolite of vitamin A (retinol), is involved in several functions, especially those related to growth and development (127). It acts as the ligand for the nuclear retinoic acid receptors (RARs: alpha, beta and gamma) that bind 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) (128). Interestingly, RXRs acts as heterodimers for several nuclear receptors, including the vitamin D receptor (VDR), the peroxisome proliferator-activated receptor (PPAR) and the thyroid hormone receptor (TR). In addition, RXRs can directly bind 9-*cis* retinoic acid (9-*cis*-RA) and other retinoids (128).

There is strong evidence that aberrations in the RA-signaling pathway play a role in leiomyoma development and growth. First, Boettger-Tong and colleagues (129) 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 (129).

More recently, Zaitseva and colleagues (130) used microarray analysis and found differential gene expression of several proteins, enzymes and receptors in the RA-signaling pathway affecting leiomyoma as 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 (131). Catherino and Malik found lower expression of several genes in the RA pathway, including alcohol dehydrogenase-1, aldehyde dehydrogenase-1, cellular retinol binding protein-1 (CRBP-1) and retinoic acid binding protein-1 in leiomyoma, as compared to the adjacent myometrium (132). Furthermore, they found that cytochrome P450, which catabolizes RA, is upregulated in leiomyoma. Therefore, they concluded that leiomyoma displays a molecular pattern associated with lower exposure to RA. Importantly, there is evidence that leiomyoma dis-
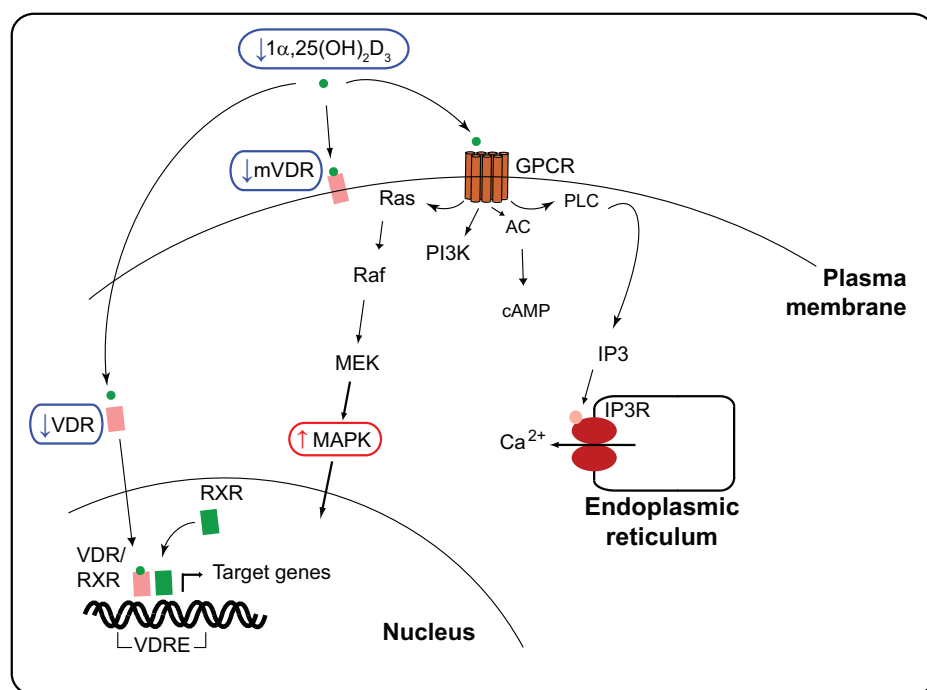
Figure 4. Schematic presentation of vitamin D signaling in uterine leiomyoma. ↑ and ↓ denote increased (red) or decreased (blue) level or function, respectively. mVDR: membrane-bound vitamin D receptor; PLC: phospholipase C; AC: adenylate cyclase; IP3: inositol triphosphate; IP3R: inositol triphosphate receptor.

association between serum vitamin D deficiency and increased risk of uterine fibroids. The Al-Hendy group first reported on the association between lower serum vitamin D levels and increased susceptibility to uterine fibroids in 2012 in a cohort of black and white women in North Africa (138,139). This was followed by two other major studies including a cohort of women from the eastern United States in 2013 by Baird and colleagues (140) and another cohort of Italian women in 2013 by Paffoni and colleagues (141). The high prevalence of vitamin D deficiency in African Americans may explain, at least in part, the high prevalence of uterine fibroids in this patient population (142).

In addition to the epidemiologic evidence, Halder and colleagues (143) found lower expression of VDRs in 60% of fibroids compared with myometrium. In addition, treatment with vitamin D was demonstrated to inhibit leiomyoma cells proliferation *in vitro* (144) and to shrink fibroid tumors in the Eker rat animal model (145). Furthermore, Sharan and colleagues (146) demonstrated that the antiproliferative 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 antiproliferative effects, Halder and colleagues (147) demonstrated that vitamin D reduces the 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 the ECM in uterine fibroids (143,148) 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.

plays different levels of RXR alpha than myometrium (133) and that this difference can be due to alteration in its degradation and transcriptional activity (134).

In addition to the *in vitro* evidence (129), Gamage and colleagues (135) 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 associated with calcium metabolism, including bone mineralization (136), there has been recent strong evidence for an association with several other biological processes, including tumorigenesis (137).

Similar to other steroids discussed in this review, $1\alpha,25(OH)_2D_3$ (the active metabolite of vitamin D) activates two main cellular signaling pathways: classic nuclear and rapid, nongenomic. In the classic nuclear pathway, $1\alpha,25(OH)_2D_3$ forms a complex with VDR and RXR that binds vitamin D response elements (VDREs) to modulate expression of target genes. In the rapid, nongenomic pathway, $1\alpha,25(OH)_2D_3$ binds to membrane-bound vitamin D receptors (mVDRs), including GPCRs, to activate several signaling pathways, including Ras-Raf-MEK-ERK, PLCγ-PKC and AC-cAMP-PKA pathways (137). Vitamin D signaling pathways seem to modulate several processes, including G0/G1 progression, apoptosis, differentiation and angiogenesis (137). A simplified illustration of vitamin D signaling in leiomyoma is presented in Figure 4.

Recent evidence from three independent research groups in populations in North Africa, eastern United States and central Europe clearly demonstrates an

Extracellular Matrix

The ECM in uterine leiomyomas is not only excessive, but also disordered with alterations in its composition (149). ECM proteins formed by leiomyoma cells include collagens, proteoglycans and fibronectin. In addition to alterations in composition, the ultrastructure of collagen fibrils in leiomyoma is altered in both structure and orientation compared with normal myometrium (150). In fact, this disordered matrix represents the major part of the tumor (151) and is responsible for most of the symptoms (152).

The ECM in leiomyomas does not appear to be a passive nonfunctioning (that is, purely structural) component. On the contrary, there is strong evidence that the ECM directly stimulates intercellular signaling. Because of the abnormal composition, structure, fluid content and stiffness of the tumor, there is increased tissue tension (153). This increased tissue tension and stretch induces mechanical signaling transmitted from collagen and other fibers in the ECM to intracellular components through transmembrane receptors including integrins and cadherins (153,154). This complex mechanical signaling network involves alterations in cell shape and cytoskeleton in addition to altered stiffness and ECM (155).

Altered ECM and mechanical signaling in leiomyoma represents targets for the development of novel therapeutic agents. For example, Islam and colleagues demonstrated that Tranilast, an antiallergic drug, can inhibit ECM production by leiomyoma cells (156). Therefore, it can be a potential therapeutic agent. Also, Levy and colleagues demonstrated that liarozole, an agent that modulates RA metabolism, can inhibit ECM formation in leiomyoma 3-D culture by inhibiting TGF-β3 expression (157).

Peroxisome Proliferator-Activated Receptor γ

PPARs are members of the ligand-activated nuclear transcription factors superfamily (158). 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 the RXR to regulate the transcription of several genes involved in lipid metabolism, adipogenesis, proliferation and other important cellular processes (159). Thiazolidinediones (TZDs), insulin sensitizers used in the treatment of type 2 diabetes, are among the more well known PPARγ ligands (159).

There is growing evidence for a role of PPARγ signaling in leiomyoma development and growth. For example, Jeong and colleagues (93) found higher PPARγ levels in leiomyoma compared with myometrium. In addition, they found that $17\beta\text{-}E_2$ treatment increases levels of PPARγ in leiomyoma, but not myometrial cells. These findings point to an underlying aberrant PPARγ signaling in leiomyoma. In addition, Houston and colleagues (160) demonstrated that leiomyoma cells express PPARα, PPARβ/δ and PPARγ. In addition, they found that one pan-PPAR ligand and several PPARγ ligands inhibit $17\beta E_2$-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 (161) found that leiomyoma cells are more sensitive than myometrium cells to the proliferation-inhibiting effect of ciglitizone, a member of the TZD 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 (161,162).

CONCLUSIONS

The hallmarks of uterine leiomyoma development and growth are proliferation of leiomyoma cells along with a deposition of excessive disordered ECM. These two processes are tightly regulated through a complex network of interconnected signaling pathways. Interestingly, signaling pathways are not only intracellular, but also include extracellular and intercellular pathways. For example, transforming growth factors are secreted into and reside in the ECM in an inactive state. Once activated by tissue proteases, they bind to cell surface receptors and activate intercellular signaling.

Signaling pathways in leiomyoma have two main characteristics: interconnectedness and convergence. In fact, they are not only interconnected, but also reciprocally active in many situations. The complex relationship between leiomyoma cells and ECM is an interesting example. Mechanical signals from the stiff ECM are transmitted intracellularly through transmembrane receptors such as integrins. At the same time, cells secrete transforming growth factors into extracellular space, which increases the production of collagen and therefore affects ECM stiffness. As discussed previously, certain research compounds, for example, liarozole, take advantage of this relationship.

From our discussion, it is clear that several signal transduction pathways converge into a common final pathway. For example, growth factors and the membrane-bound receptors of estradiol, progesterone and vitamin D converge to activate Ras/Raf/MEK/ERK pathway. This phenomenon of convergence potentially has important therapeutic consequences where the treatment of different pathways can be additive. This represents the conceptual basis of dual targeting or multitargeting, where more than one signaling pathway is targeted simultaneously. This can lead to additive or even synergistic effects. This approach of multifocal signal modulation is currently considered for cancer treatment of some cancers such as prostate cancer (163). We believe that with better understanding of the pathobiology of signaling pathways in leiomyoma, multifocal targeting can be an area of interest in future research.

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DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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