Functional evidence for two distinct mechanisms of action of progesterone and selective progesterone receptor modulator on uterine leiomyomas

Gabriela Milewska, M.D.,[a] Donata Ponikwicka–Tyszko, Ph.D.,[b] Piotr Bernaczyk, M.D., Ph.D.,[c] Oana Lupu, M.Sc.,[a] Michal Szamatowicz, M.D., Ph.D.,[a] Maria Sztachelska, Ph.D.,[b] Agata Pilaszewicz-Puza, M.D.,[c] Mariusz Koda, M.D., Ph.D.,[d] Tomasz Bielawski, Ph.D.,[b] Monika Zbucka-Kretowska, M.D., Ph.D.,[a] Adam Pawelczyk, M.D.,[e] Jakub Tomaszewski, M.D., Ph.D.,[f] Xiangdong Li, Ph.D.,[a,g] Ilpo Huhtaniemi, M.D., Ph.D.,[h,i] Slawomir Wolczynski, M.D., Ph.D.,[a,b] and Nafis A. Rahman, M.D., Ph.D.[a,i]

[a] Department of Reproduction and Gynecological Endocrinology, Medical University of Bialystok, Poland; [b] Department of Biology and Pathology of Human Reproduction, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland; [c] Department of Medical Pathomorphology, Medical University of Bialystok, Poland; [d] Department of General Pathomorphology, Medical University of Bialystok, Poland; [e] Division of General and Transplant Surgery, Department of General, Vascular and Transplant Surgery, Poznan University of Medical Sciences, Poznan, Poland; [f] Tomaszewski Medical Center of Obstetrics and Gynecology; Bialystok, Poland; [g] State Key Laboratory of Agrobiotechnology, China Agricultural University; Bejing, People's Republic of China; [h] Institute of Reproductive and Developmental Biology, Department of Metabolism, Digestion and Reproduction, Imperial College London, United Kingdom; [i] Institute of Biomedicine, University of Turku, Finland

Objective: To study the specific mechanisms through which progesterone and selective progesterone receptor modulators impact the growth, synthesis, and accumulation of the extracellular matrix in uterine leiomyomas.
Design: Laboratory study.
Setting: Academic Research Institutions.
Patients (s): This study involved reproductive-age women diagnosed with infertility associated uterine leiomyomas who underwent myomectomy either after selective progesterone receptor modulator ulipristal acetate (UA) treatment or without any pharmacological pretreatment. Control samples included healthy myometrium tissue (n = 100). Specimens were obtained from the Department of Reproduction and Gynecological Endocrinology and Biobank, Medical University of Bialystok, Poland.
Interventions: Daily (5 mg/d) UA treated for 2 months (n = 100) and untreated (n = 150) patients with uterine leiomyomas or normal healthy myometrium (n = 100) tissue samples immediately after surgery were collected for transcriptional analysis and assessments.
Main Outcome Measures: Progesterone-induced activation of the signaling pathways related to uterine leiomyomas extracellular matrix synthesis, deposition, and growth, as well as the expression profile of progesterone receptors in uterine leiomyomas, were assessed.
Results: The results indicated that progesterone activated the transforming growth factor-β and SMAD3 signaling pathways and promoted proliferation, growth, and extracellular matrix remodeling in uterine leiomyomas by up-regulating SMAD3, transforming growth factor-β (TGF-β) receptor type 1 and II, Ras homolog A, vascular endothelial growth factor, or increasing the fibrosis-related gene collagen, type I, α-1, and procollagen, type I, α-1 production. In contrast, UA had inhibitory effects on these processes. The study also showed that both nuclear and membrane progesterone receptors play distinct roles in uterine leiomyoma pathobiology.
Conclusions: We showed that both nuclear and membrane progesterone receptors were relevant in the treatment of uterine leiomyomas, especially when combined with selective progesterone receptor modulators. Novel therapeutic approaches combining selective progesterone receptor modulators with or without direct and indirect extracellular matrix targeting through selected specifically

Received September 26, 2023; revised January 20, 2024; accepted February 27, 2024; published online February 29, 2024.
G.M. and D.P-T. should be considered similar in author order.
Partly supported by the Polish National Science Centre grant 2016/23/B/NZ5/01477 (to N.A.R.).
Partly supported by the Medical University of Bialystok grant SUB/1/DN/19/001/1104 (to G.M.).
Partly supported by the Academy of Finland 254366 (to N.A.R.).
Correspondence: Nafis A. Rahman, M.D., Ph.D., Institute of Biomedicine, University of Turku, 20520 Turku, Finland (E-mail: nafis.rahman@utu.fi).

Fertil Steril® Vol. 122, No. 2, August 2024 0015-0282

https://doi.org/10.1016/j.fertnstert.2024.02.046

TGF-β and SMAD3 (SMAD3, TGF-β receptor types 1 and II, Ras homolog A, vascular endothelial growth factor, collagen, type I, α-1) signaling pathways could therefore be a treatment option for uterine leiomyomas. (Fertil Steril® 2024;122:341–51. ©2024 by American Society for Reproductive Medicine.)

El resumen está disponible en Español al final del artículo.

Key Words: Leiomyoma, progesterone, selective progesterone receptor modulator, extracellular matrix, growth factors

Uterine leiomyomas (ULs) are common gynecological tumors in reproductive-age women, with a range of occurrence from 217–3,745 cases per 100 000 women per year, and they are a major indication for hysterectomy (1, 2). Uterine leiomyomas may negatively affect embryo implantation in the uterus and increase the risk of miscarriage (3, 4) or cause heavy menstrual bleeding, anemia, and pelvic pain, thus decreasing the quality of life of women (2). Somatic mutations are thought to underlie the primary cause of ULs (5). Uterine leiomyomas' development and growth are highly dependent on ovarian steroid hormones, growth factors, and cytokines (5), which further influence smooth muscle cell proliferation, followed by excessive production of extracellular matrix (ECM) (5). Although estrogen has long been considered the most important initiating factor, recent research indicates that progesterone (P4) plays a key role in UL growth and development (6), as suggested by the increased expression of nuclear progesterone receptors (PRs) in ULs (7). Progesterone may also play a role in the epigenetic changes that lead to mutations in normal uterine muscle cells (3). There is still a data gap in the characterization and profiling of all the PRs in ULs.

Transforming growth factor β (TGF-β) regulates the expression of genes involved in tissue remodeling and ECM regulation (8). Up-regulated TGF-β signaling in ULs has been suggested as a mechanism for the development of their fibrotic phenotype (9). It has also been shown that P4 upregulates TGF-β expression in ULs (10). The TGF-β signaling complex is usually composed of the canonical SMAD 2, 3, and 4 transcription factor-dependent pathway or noncanonical pathway activating the small Ras homolog GTPase Ras homolog family member A (RhoA) (8). Ras homolog family member A may induce the growth of ULs via interactions with cytoskeleton proteins (11). Transforming growth factor β and SMAD3 pathways have been also shown to upregulate vascular endothelial growth factor (VEGF) expression, and SMAD3 mediates TGF-β1 induction of VEGF production in several tissues (12, 13). However, there exists inadequate functional data on the interactions of the above-mentioned factors with P4, as well as more generally on the mechanisms underlying P4 action in the regulation of UL proliferation, apoptosis, and ECM synthesis (14).

Among the selective P4 receptor modulators (SPRMs), ulipristal acetate (UA) successfully modulates PRs' action through its combined agonistic and antagonistic activity (15). Ulipristal acetate decreases UL cell viability, suppresses their expression of growth factors, and induces apoptosis, but does not affect the function of normal myometrial cells (15). Clinical studies have shown that treatment with UA significantly shrinks ULs and controls the frequency and abundance of uterine bleeding (16). Functional data on the precise mechanisms underlying the interference of UA with P4 action in the regulation of UL proliferation, apoptosis, and ECM synthesis and remodeling remain obscure. Our present study was therefore conducted to characterize the PR profile in ULs and to investigate the molecular mechanisms underlying the action of SPRM UA and its putative differential P4-induced activation of the signaling cascades involved in UL in the deposition and growth of ECM. Finally, to explore the novel molecular mechanisms and signaling pathways that may enhance the therapeutic effects on ULs.

MATERIALS AND METHODS

Study design

This was a controlled laboratory experiment to investigate the molecular mechanisms underlying the action of the SPRM UA and how it differs from the P4-induced activation of the signaling cascades involved in UL in the deposition and growth of the ECM. We wanted to investigate additionally the mechanisms that explain the therapeutic effects of UA in the control of UL growth and progression.

For the assessment and transcriptional analysis of human ULs, we used patient-derived UL tissue samples, primary UL cells, and UL explant cultures. To study the UA and P4 molecular mechanisms of action, we assessed the PR profile in UA-treated and UA-untreated (control) ULs in vivo as well as in explant culture in vitro. After establishing the PR profile in ULs, we tested the impact of P4 and UA on the activation of the TGF-β signaling pathway and SMAD3 signaling. For this, patient-derived UL explants were treated without or with inhibitors of TGF-β type I and II receptors (TGF-βRI and II) and SMAD3. To investigate the SMAD3 translocation, UL primary cell cultures were treated with UA. The therapeutic effects of UA on UL cell proliferation, growth, and ECM accumulations were investigated in vitro and in vivo. The sample numbers are stated in the figure legends, and replicates are described below. The statistical methods are described in separate sections and indicated in the figure legends.

Uterine leiomyoma tissue samples

Fresh ULs (n = 250) or healthy myometrium (n = 100) tissue samples were collected immediately after surgery at the Department of Reproduction and Gynecological Endocrinology, Medical University of Bialystok, Poland, during the years 2017–2021. The Local Human Investigation Ethics Committee approved the study (R-I-002/482/2017,

APK.002.4.2021). Written informed consent was obtained from all the patients (before inclusion) before the surgery. Uterine leiomyomas were collected from patients (aged 25–45 years) who underwent myomectomy because of infertility associated with ULs or had serious clinical symptoms like heavy menstrual bleeding and severe intermenstrual bleeding associated with lower abdominal pain. Patients were divided into two groups: the UA-treated (UA-L) group (n = 100) and the UA-untreated (NT-L) group (n = 150) before the surgery. Preoperative UA therapy consisted of the administration of UA (5 mg/d) for 3 months. A myomectomy was performed in the first phase of the menstrual cycle, 1 month after the end of the therapy. The type, size, and location of ULs were different. In patients with multiple myomas, tumors (10–40 mm in diameter) from all (UA-L and NT-L) groups were randomly selected. Myomas (>40 mm) were not selected to avoid any potential degeneration and necrosis that may have occurred in them.

Qualification criteria for the study, drugs, and inhibitors, ULs and myometrium explants and their primary cell culture, cell viability, real-time quantitative polymerase chain reaction (qPCR), cytokines measurement, and immunohistochemical and immunocytochemistry analyses have been shown in the Supplemental Materials (available online).

Study approval

The Local Human Investigation Ethics Committee approved the study (R-I-002/482/2017, APK.002.4.2021). The research was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). Written informed consent was obtained before the surgery from all patients' prior inclusion.

Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was assessed using one-way ANOVA with the post hoc Bonferroni test and two-way ANOVA with the post hoc Bonferroni test using GraphPad PRISM v. 7.0 (GraphPad Software, Inc). $P \leq .05$ was considered statistically significant.

RESULTS

Progesterone and UA modulate the PRs subtype expression profile in ULs

As P4 is pivotal for UL development and growth (1, 8), we characterized the expression profile of the nuclear progesterone receptors (PGRs) and all membrane P4 subtypes (membrane P4 receptor α [mPRα], mPRβ, mPRγ, P4 receptor membrane component 1 [PGRMC1], and P4 receptor membrane component 2 [PGRMC2]) in UA-treated (n = 100) and nontreated control (n = 150) UL samples, as well as in normal myometrium (n = 100) tissue samples. The expression levels of PGRA and B, PGRB, mPRα, mPRβ, and PGRMC1 were significantly higher in the nonUA-treated UL tissue samples compared with normal myometrium (Fig. 1A). In contrast, only PGRA and B expression remained up-regulated in the UA-treated UL samples, whereas those of PGRB, mPRα, mPRβ, and PGRMC1 were similar to those in normal myometrium. The expression levels of mPRγ and PGRMC2 were similar in all three types of samples (Fig. 1A). The expression level of PGRA and B was significantly up-regulated after P4 and UA treatments of leiomyoma explants, but P4 showed no additive effects to the UA action (Supplemental Fig. 1A, available online). In contrast, PGRB expression was up-regulated by P4 but down-regulated by UA treatment. Progesterone did not abolish this UA-induced effect (Supplemental Fig. 1B, available online). Similarly, the expression levels of mPRα, mPRβ, mPRγ, and PGRMC1 were significantly up-regulated after P4 but down-regulated after UA treatments. Progesterone did not reverse this UA action (Supplemental Fig.1 C to F, available online). Progesterone and UA treatments did not affect PGRMC2 expression level in leiomyoma explants (Fig. 1G, available online).

Immunohistochemical studies demonstrated abundant cytoplasmic staining for mPRα, mPRβ, mPRγ, and PGRMC1 in the nontreated control ULs tissues (NT-L) group (Fig. 1B to D and Supplemental Fig. 2D and E, available online), whereas traceable or weak staining was found in the UA-treated ULs tissues (UA-L) group (Fig. 1E to G). Densitometric quantification and optical density (OD) evaluation revealed a significantly decreased percentage ratio of mPRα, mPRβ, mPRγ and PGRMC1 expression in the UA-L group compared with the NT-L group (Fig. 1H to J and Supplemental Fig. 2F, available online). Ulipristal acetate treatment did not affect the strong PGR nuclear (Supplemental Fig. 2A and B, available online) and PGRMC2 staining of ULs (UL-L vs. NT-L groups) (Supplemental Fig. 2G, H, available online).

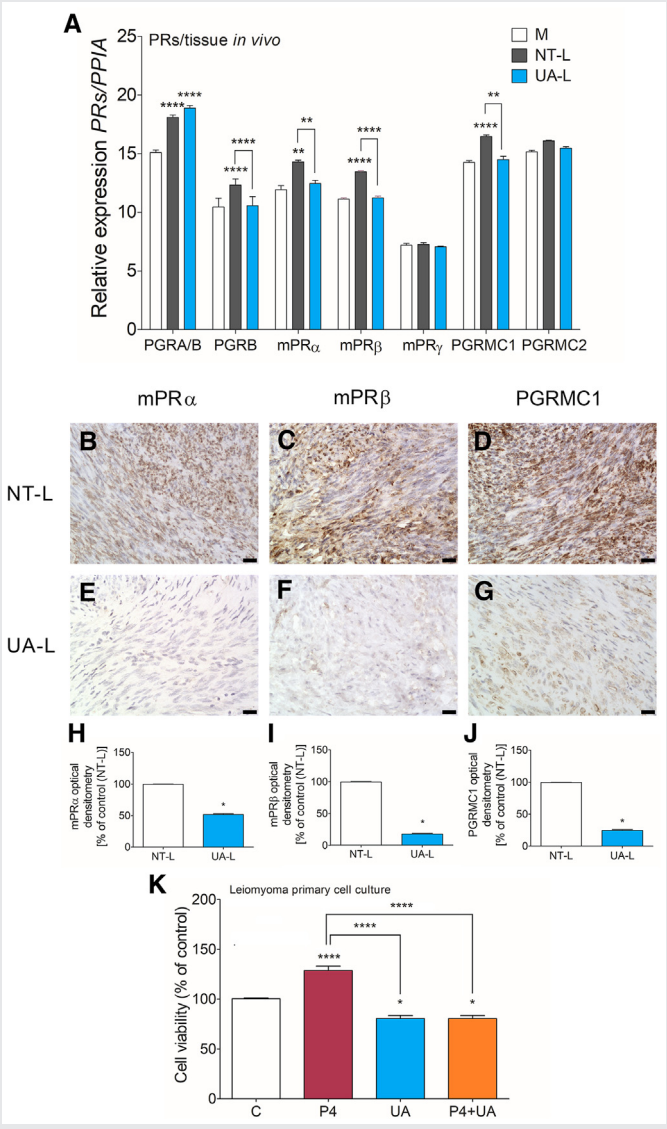
Next, we analyzed the effects of P4 (0–1 μM) and UA (0–100 μM) on the viability of primary UL cells in vitro. Progesterone (0.01–1 μM) significantly and dose-dependently stimulated (Fig. 1K and Supplemental Fig. 3A, available online), whereas UA (1 μM) or higher doses inhibited UL cell viability (Fig. 1K and Supplemental Fig. 3B, available online). We also found that UA itself had no effect on primary UL cell viability but inhibited the stimulatory effects of P4 (Fig. 1K).

Progesterone and UA activate differently the TGF-β signaling pathway in ULs

The TGF-β superfamily pathways are regarded as a potential stimulus for tumor progression (9, 17). Therefore, we characterized the expression profiles of the TGF-β family members in UL tissues. Transforming growth factor β1, *TGF-β3*, *SMAD2*, and *SMAD3* expression levels in ULs were significantly up-regulated compared with normal myometrium (Fig. 2A to D). This response was abolished by UA treatment in TGF-β1, TGF-β3, TGF-βR2, and SMAD3 in UL tissues and explants (Fig. 2A to D). In accordance, the secretion levels of TGF-β1 and TGF-β3 were significantly higher in ULs compared with normal myometrium, whereas UA treatment significantly suppressed these levels in ULs but not in normal myometrium (Fig. 2E and F).

To investigate further the TGF-β signaling pathway involvement affected by the UA or P4 actions in ULs, a

FIGURE 1

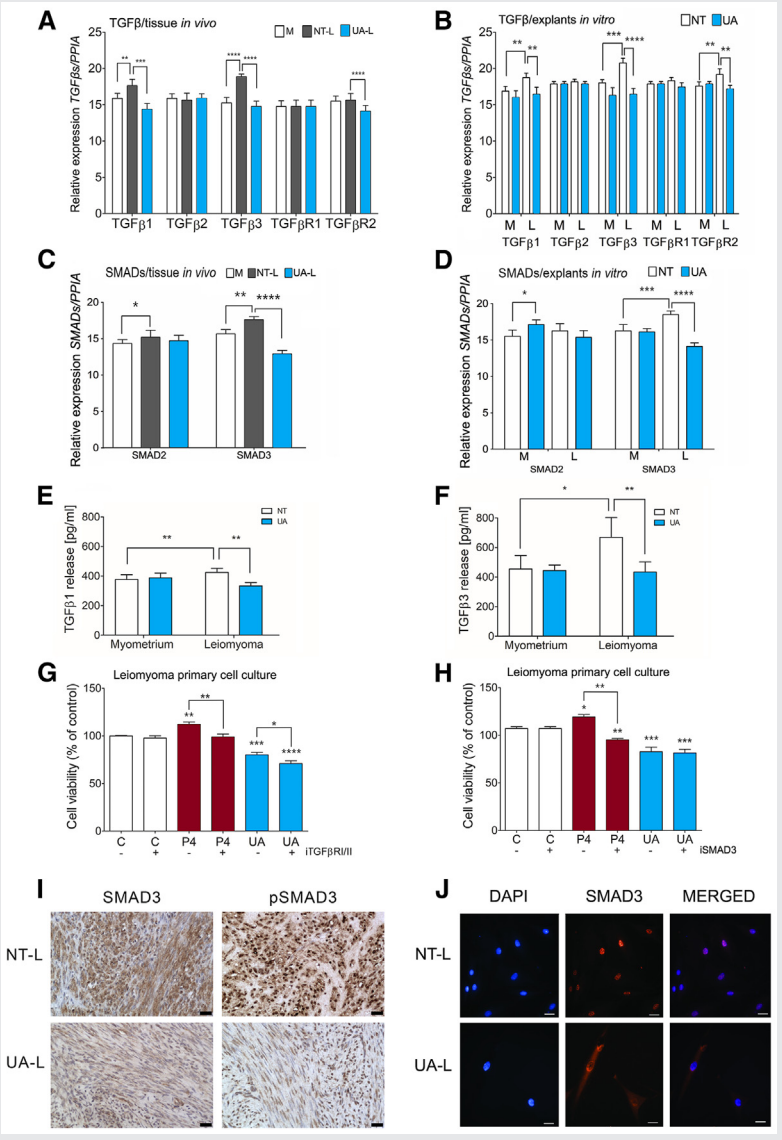


Characterization of the progesterone receptors (PRs) expression profile in uterine leiomyomas (ULs) after ulipristal acetate (UA) and progesterone (P4) treatment. The quantitative polymerase chain reaction gene expression profile of nuclear progesterone receptor isoforms (PGRA and PGRA/B) and membrane progesterone receptors (mPRα, mPRβ, PGRMC1, and PGRMC2) in normal myometrium (M) (n = 100) and ULs tissues of nontreated (NT-L) (n = 150) and UA-treated (UA-L) women (n = 100) (**A**). Immunohistochemical (IHC) staining for mPRα, mPRβ, and PGRMC1 in nontreated UL tissues (NT-L) (n = 150) (**B** to **D**) and UA-treated UL tissues (UA-L) (n = 100) (**E** to **G**). Quantification of UL tissue IHC staining of mPRα (**H**), mPRβ (**I**), and PGRMC1 (**J**) using Fiji (Image J). The results are presented as percentages of the controls compared with the nontreated control. Scale bar, 200 μm. The viability of cells after UA, P4, and UA + P4 treatments of ULs primary cell culture (n = 30) was measured using the MTT assay (**K**). The cell viability level of the treated groups is presented as a percentage of the control group, considered to be 100%.
Each bar represents the ratio of the investigated gene to the housekeeping gene (*PPIA* ± SEM). *Statistically significant differences between nontreated (NT-L) and treated (UA-L) groups (*, $P<.05$; ****, $P<.0001$) (one-way ANOVA with the post hoc Bonferroni's test).
C/NT-L = control/nontreated ULs; M = normal myometrium; mPRα = membrane progesterone receptor α; mPRβ = membrane progesterone receptor β; mPRγ = membrane progesterone receptor γ; MTT assay = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; NT-L = nontreated ULs; P4 = progesterone treated; PGRA = nuclear progesterone receptor isoform A; PGRA/B = nuclear progesterone receptor isoform A/B; PGRMC1 = progesterone receptor membrane component 1; PGRMC2 = progesterone receptor membrane component 2; UA = ulipristal acetate treated; UA-L, ulipristal acetate treated ULs; ULs = uterine leiomyomas.

Milewska. P4/SPRM actions on uterine leiomyoma. Fertil Steril 2024.

TGF-βRI and II inhibitor (iTGF-βRI and II) was used (Fig. 2G). Coculture of iTGF-βRI and II with UA or P4 significantly enhanced the inhibitory effects of UA on the UL primary cell viability and inhibited P4-stimulated leiomyoma cell viability (Fig. 2G). Furthermore, iTGF-βRI and II alone did not affect UL cell viability (Fig. 2G). When the SMAD3 inhibitor (iSMAD3) was added to P4 or UA, it abolished the P4-dependent stimulation of the primary UL cell viability but showed no additive effects on the UA action (Fig. 2H).

FIGURE 2



Ulipristal acetate (UA) and progesterone (P4) treatment effects on the transforming growth factor (TGF-β) superfamily signaling pathway in uterine leiomyomas (ULs). The quantitative polymerase chain reaction (qPCR) gene expression profile of TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) and their receptors (TGF-βR1, TGF-βR2) in normal myometrium (M) (n =100) and ULs tissues of nontreated (NT-L) (n = 150) and UA-treated (UA-L) (n = 100) women (**A**), as well as in normal myometrium (n = 50) and ULs explants (n = 50) before and after UA treatment (**B**). The qPCR gene expression profile of signal transducers (SMAD2, and SMAD3) in normal myometrium (M) (n = 100) and ULs tissues of nontreated (NT-L) (n = 150) and UA-treated (UA-L) (n = 100) women (**C**), as well as in healthy myometrium (n = 50) and ULs explants (n = 50) before and after UA treatment (**D**). Release of TGF-β1 (**E**) and TGF-β3 (**F**) to the medium by normal myometrium (n = 50) and ULs explants (n = 50) with or without UA treatment. Effects of UA and P4 with or without the SMAD3 inhibitor (**G**) or with or without the TGF-β receptor I and II inhibitor (**H**) on ULs primary cell (n = 30) viability after 24 hours of treatment were measured using the MTT assay. The cell viability of the treated groups is presented as the percentage of the control, considered to be 100%. Immunohistochemical staining of SMAD3 and phosphorylated SMAD3 (pSMAD3) in ULs from nontreated (NT-L) (n = 150) and SMAD3 and pSMAD3 in ULs from UA-treated (UA-L) (n = 100) women (**I**). Scale bar, 200 μm. Immunocytochemical localization of SMAD3 without or with UA treatment in ULs primary cells (n = 30) (**J**). Scale bar, 20 μm.
Each bar represents the ratio of the investigated gene to the housekeeping gene (*PPIA* ± SEM). *Statistically significant differences between nontreated and treated groups (*, $P<.05$; **, $P<.01$; ***, $P<.001$; ****, $P<.0001$) (one-way ANOVA with the post hoc Bonferroni's test).
C/NT-L = control/nontreated ULs; iSMAD3 = SMAD3 inhibitor; iTGF-βRI/II = TGF-β receptor I and II inhibitors; NT-L = nontreated ULs; M, healthy myometrium; MTT assay = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; P4 = progesterone treated; UA-L = ulipristal acetate treated ULs; ULs = uterine leiomyomas.

Milewska. P4/SPRM actions on uterine leiomyoma. Fertil Steril 2024.

Ulipristal acetate affects nuclear translocation of SMAD3 in ULs

Transforming growth factor β may promote tumor growth through SMAD-dependent and/or independent pathways (9). To determine whether P4 and UA activate TGF-β1-SMAD signaling, we investigated their effects on the ratio of phosphorylated SMAD3 (pSMAD3) and nonphosphorylated SMAD3 in ULs. The nontreated (NT-L) group showed abundant expression of SMAD3 and pSMAD3, whereas the UA-L group samples demonstrated weaker cytoplasmic SMAD3 and nuclear pSMAD3 staining (Fig. 2I). Automatic quantification and OD evaluation revealed significantly decreased SMAD3 and pSMAD3 expression in the UA-L group compared with the NT-L group (Supplemental Fig. 4A to C, available online). In accordance, immunocytochemical localization of pSMAD3 could be found only in the NT-L cell nuclei, and no pSMAD3 translocation was found in the UA treatment samples (Fig. 2J).

ULIPRISTAL ACETATE AND P4 REGULATE VEGF AND INTERLEUKIN-6 EXPRESSION IN ULs

To screen for other potentially relevant factors affecting UL biology, we checked the expression of VEGF, interleukin 6 (IL-6), and collagen 1A1 (COL1A1) (5, 18–23). Abundant VEGF staining was observed in the NT-L group, whereas VEGF expression was very weak in the UA-L group (Fig. 3A). Automatic quantification and OD evaluation revealed a significantly decreased VEGF expression in the UA-L group compared with the NT-L group (Supplemental Fig. 5A, available online). Human recombinant VEGF (0–50 ng/mL) stimulation showed no effect on UL cell viability (Supplemental Fig. 5B, available online). Vascular endothelial growth factor A levels did not differ from healthy myometrium in nontreated UL (NT-L) tissue, was unaffected by P4, but was suppressed by UA (UA-L) (Fig. 3B). Vascular endothelial growth factor B level was up-regulated in NT-L tissue vs. myometrium, and P4 did not affect UL, whereas UA suppressed its levels (Supplemental Fig. 6A, available online). *VEGFC* and *VEGFR2* were down-regulated in NT-L tissue vs. myometrium, and P4 up-regulated *VEGFC* but had no effect on VEGFR2, whereas UA suppressed both genes (Supplemental Fig. 6B and C, available online). UA inhibited the VEGF release from UL explants, whereas iSMAD3 abolished this UA-induced effect (Fig. 3D).

Nontreated UL tissue showed abundant IL-6 staining, whereas this expression was weak in UA-L tissue (Fig. 3A). Automatic quantification and OD evaluation revealed significant IL-6 expression in UA-L tissue compared with NT-L tissue (Supplemental Fig. 7A, available online). Treatment with human recombinant IL-6 (0–30 ng/mL) significantly up-regulated UL cell viability (Supplemental Fig. 7B, available online). In UL explants, IL-6 and IL-6R were significantly up-regulated after P4 treatment and significantly down-regulated after UA treatment (Fig. 3C and, Supplemental Fig. 7A and C, available online). Ulipristal acetate treatment of UL explants significantly decreased their VEGF and IL-6 production, whereas the addition of iSMAD3 partially abolished this UA effect (Fig. 3D to E and Supplemental Fig. 8B, available online). Furthermore, iSMAD3 also reduced the P4-stimulated IL-6 release (Fig. 3E).

The overexpression of COL1A1 and excessive production of procollagen I α-1 (proCOLIA1) have been associated with fibrosis and were potentially regulated by P4 (19). In UL explants, P4 treatment increased *COL1A1* expression as well as the release of proCOLIA1, whereas UA treatment decreased collagen type I expression and proCOLIA1 release (Fig. 3F and g). Furthermore, iSMAD3 significantly suppressed P4-stimulated *COL1A1* expression but showed no additive or synergistic effects with UA (Fig. 3F).

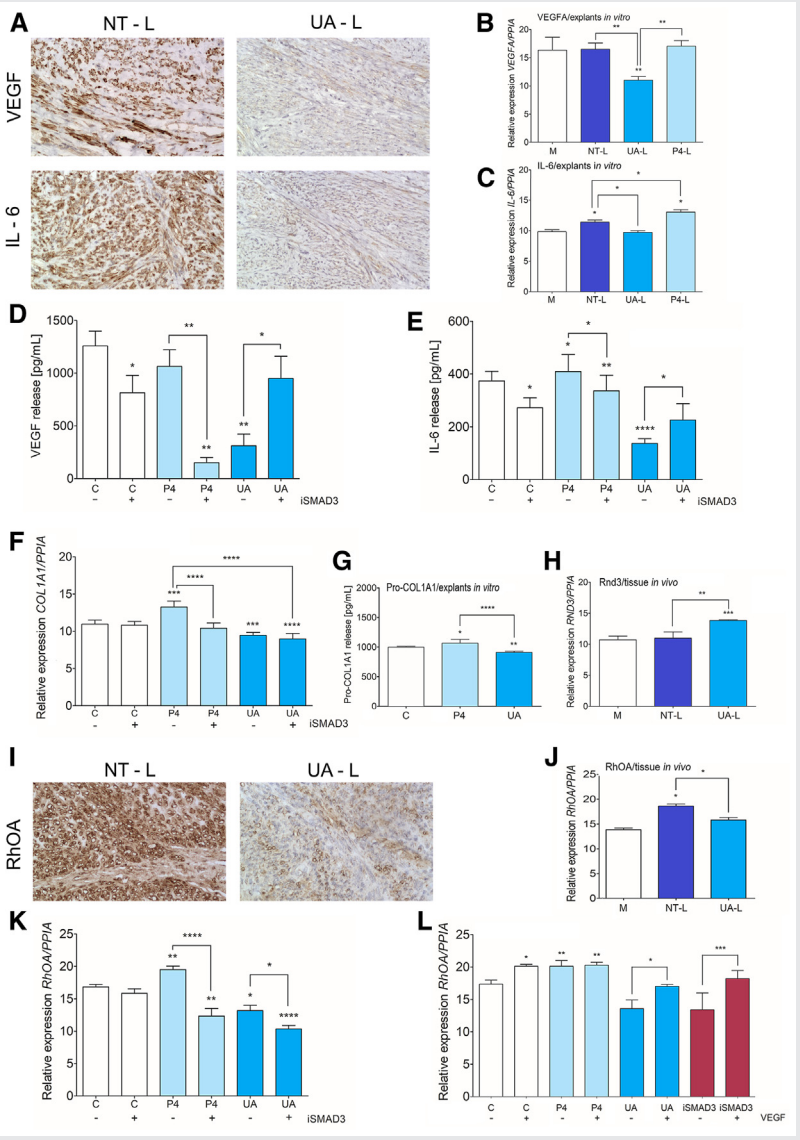
Progesterone and UA regulate the RhoA pathway in ULs

Ras homolog family member A, a key regulator of ECM accumulation, is activated by TGF-β and VEGF in several fibrotic or neoplastic diseases (20–22). We found abundant RhoA staining in the NT-L tissue, whereas only weak expression and levels were found after UA treatment (Fig. 3I). Automatic quantification and OD evaluation revealed significantly decreased RhoA expression in UA-L tissue compared with NT-L tissue (Supplemental Fig. 4C, available online). In addition, UA significantly down-regulated RhoA expression levels in UL tissues and explants (Fig. 3J and K). Progesterone up-regulated RhoA expression in UL explants, and this effect could be abolished by iSMAD3 (Fig. 3K). The addition of iSMAD3 to UA showed an additive effect on the down-regulation of RhoA expression in UL explants (Fig. 3K). Co-treatment of VEGF with P4 did not enhance the stimulating effects of P4 (Fig. 3I). VEGF abolished the UA and iSMAD3-dependent down-regulation of RhoA (Fig. 3I). Furthermore, UA treatment up-regulated the RhoA antagonist Rnd3 expression levels in UL tissues (Fig. 3H).

DISCUSSION

The functional implications of P4 actions through activation of the nuclear and membrane PRs are still rather poorly understood in the biology of ULs, although up-regulated PGR expression levels in them have been demonstrated (24). Among the two distinct isoforms of the human progesterone receptor (PGRA and PGRB), the transcriptional effects of PGRA and PGRB on progestin-responsive promoters differ (25). In general, PGRB functions as a transcriptional activator of progesterone-responsive genes, whereas PGRA is an inhibitor of steroid hormone receptors, as well as for the PGRB isoform (26). The current study showed that the expression of *PGRB* was down-regulated after UA, and the expression of total *PGRA/B* was up-regulated, indirectly suggesting that UA increases the expression of the *PGRA* isoform in ULs. Our results, therefore, suggested that UA may inhibit the transcriptional action of PGRB through an antagonistic effect. Conversely, UA might activate the inhibitory effect of PGRA as its agonist, causing double inhibition of the activity of PGR in ULs. Moreover, UA showed no effect on PGR and PGRMC2 but down-regulated the mPRα, mPRβ, mPRγ, and PGRMC1 membrane receptors at gene and/or protein levels in ULs. These novel findings suggested that not only PGRs but also the membrane P4 receptors may play a role in UL

FIGURE 3



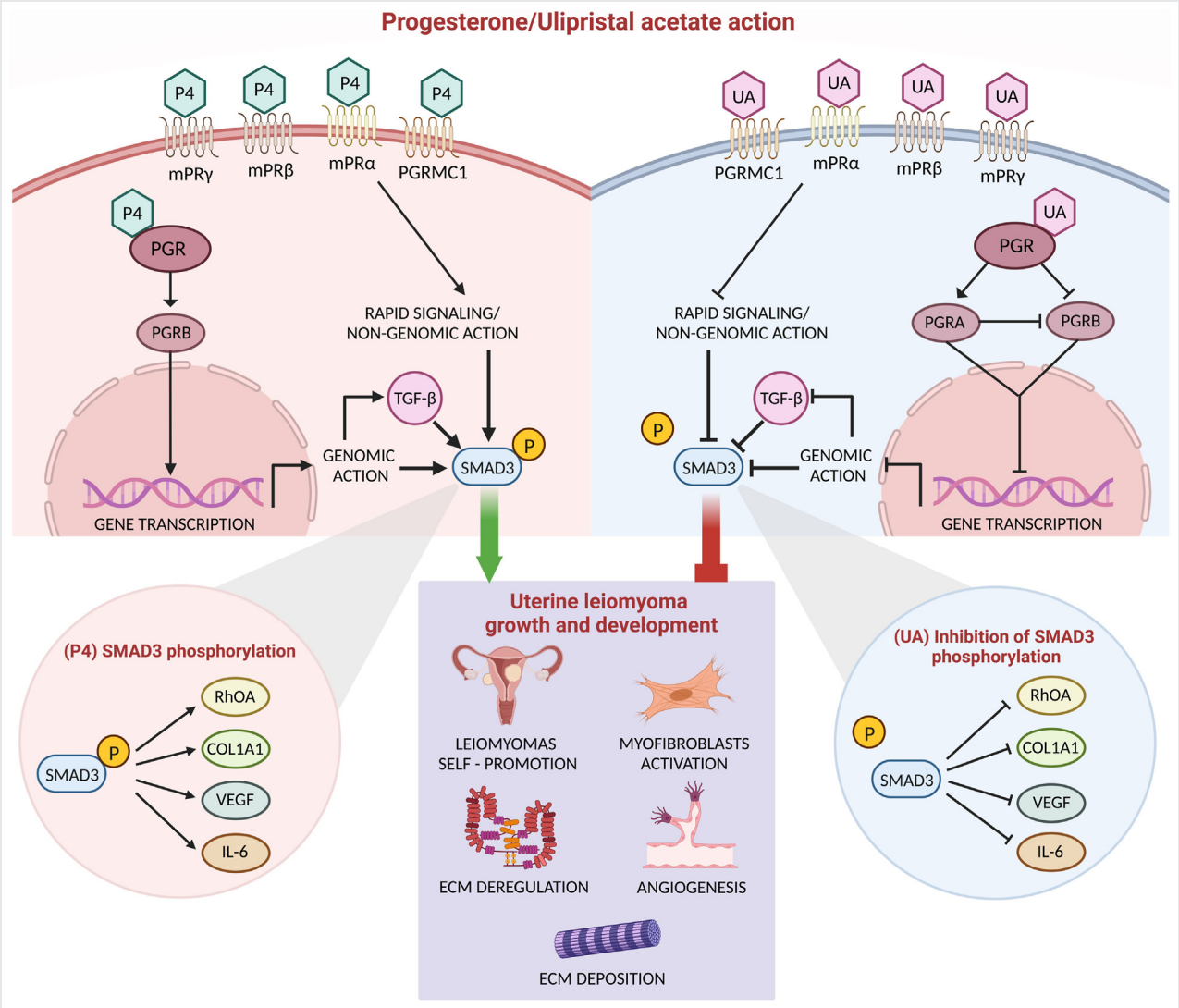
The effect of ulipristal acetate (UA) and progesterone (P4) treatments on vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), collagen type I α-1 chain (COL1A1), and Ras homolog family member A (RhoA) expression and on the VEGF, IL-6, and proCOL1A1 release by uterine leiomyoma (UL) cells and explants. Immunohistochemical (IHC) staining of VEGF and IL-6 (**A**) in ULs from nontreated (NT-L) (n = 150) and UA-treated (UA-L) (n = 100) women. The quantitative polymerase chain reaction (qPCR) analysis of VEGFA (**B**) and IL-6 (**C**) expression in NT-L, UA-L, and P4-treated (P4-L) ULs explants (n = 50). Release of VEGF (**D**) and IL-6 (**E**) levels in the control, P4, and UA with or without iSMAD3 in ULs cells (n = 30). The qPCR gene expression analysis of COL1A1 in ULs primary cells (n = 30) after treatment with UA, P4, and iSMAD3 (**F**). Release of proCOL1A1 (**G**) levels in the NT-L, P4-, and UA-treated ULs explants (n = 50). The qPCR gene expression analysis of Rnd3 in normal myometrium (M) (n = 100), ULs tissue from nontreated (NT-L) (n = 150), and UA-treated (UA-L) (n = 100) (**H**) women. Immunohistochemical staining of RhoA (**I**) in ULs from nontreated (NT-L) (n = 150) and UA-treated (UA-L) (n = 100) women. The qPCR gene expression analysis of RhoA in UL tissue from nontreated (NT-L) (n = 150) and UA-treated (UA-L) (n = 100) (**J**) women. The qPCR gene expression analysis of RhoA in UL explants (n = 50) after treatment with UA, P4, iSMAD3 (**K**), and VEGF (**I**).
Each bar represents the ratio of the investigated gene to the housekeeping gene (*PPIA* ± SEM). * Statistically significant differences between nontreated control and treated groups (*, $P<.05$; **, $P<.01$; ***, $P<.001$; ****, $P<.0001$) (one-way ANOVA with the post hoc Bonferroni's test). C/NT-L, control/nontreated ULs; iSMAD3, an inhibitor of SMAD3; M, healthy myometrium; P4, progesterone treated ULs; RhoA, Ras homolog family member A; UA-L, ulipristal acetate treated ULs. Scale bar, 200 μm.

Milewska. P4/SPRM actions on uterine leiomyoma. Fertil Steril 2024.

pathobiology, which might be of future importance in UL treatment strategies along with UA.

Excessive accumulation of ECM components plays an important role in the formation of ULs (5). P4 has been shown to activate the signaling pathways involved in the control of ECM deposition and cell proliferation (5). The present study emphasized a major role for P4 in ULs in the regulation of the TGF-β signaling pathway. Besides SMADs, TGF-β can

FIGURE 4



Overview of progesterone (P4) and ulipristal acetate (UA) action via genomic and nongenomic signaling in uterine leiomyomas (UL) cells. Progesterone induces the classical signaling by binding to PGR located within the cytoplasm. This conformation activates genomic action by triggering the transcription of target genes in UL cells. One of the effects of genomic P4 action is SMAD3 phosphorylation, which controls tumor growth and development. Nontreated ULs have high gene and protein expression of mPRs such as mPRα, mPRβ and PGRMC1. Progesterone initiates a rapid nonclassical signaling of mPRs by activating downstream targets, leading to the tumor's growth and development. One of these targets is SMAD3, whose phosphorylation is stimulated by P4. On the contrary, UA demonstrated specific antagonistic effects on PRs. Ulipristal acetate down-regulates the expression of mPRα, mPRβ, mPRγ, and PGRMC1 membrane receptors, but shows no effect on PGR and PGRMC2 at gene and/or protein levels in ULs. Through PR blockage, UA inhibited UL proliferation, growth, or ECM accumulation via the TGF-β and SMAD3 signaling pathway and downstream specific targets. Figure 4 was created with BioRender.com.
P4 = progesterone; PR = progesterone receptor; mPR = membrane progesterone receptor.

Milewska. P4/SPRM actions on uterine leiomyoma. Fertil Steril 2024.

activate other signaling molecules, modulating cellular downstream responses (27, 28). Transforming growth factor β receptors may regulate the Rho GTPases like Ras, RhoA, Cdc42, and Rac1 nonSMAD factors through phosphorylation or direct interaction (29). However, these kinases can also activate the canonical SMAD pathway (27). Ras homolog family member A plays an important role in different processes, including cell growth, proliferation, and modulation of the actin cytoskeleton and ECM (30, 31). We demonstrated the overexpression of RhoA in ULs. Progesterone and UA regulated differentially RhoA expression in an SMAD3-dependent manner, P4 with up-regulation and UA with down-regulation, which suggested RhoA action in ULs may also be regulated by canonical SMAD signaling. It has been demonstrated that SMAD3 regulates RhoA activation and cytoskeletal reorganization by controlling neuroepithelial

cell transforming 1 in TGF-β1-induced cells (32). Interestingly, in the present study, UA up-regulated the RhoA antagonist Rnd3 in ULs, which was a novel finding with future target application potential.

Overexpression of collagen subtypes, an abnormal collagen structure (5), and TGF-β3 are the main factors for the induction of collagen expression in ULs (23). Additionally, the SMAD3 mediation is known to play an important role in TGF-β-induced transcription of fibrosis-related genes, including collagens (33, 34). Hereby, we found that P4 increased *COL1A1* expression and proCOL1A1 production by UL explants; both were suppressed by UA. It has been shown that SMAD3 down-regulation may decrease the gene expression of procollagen types I and III in fibroblasts and reduce the ECM deposition and fibrosis processes in keloid disease fibroblasts (35). Our findings provide additional evidence that UA treatment might affect several central fibrotic factors, such as collagens, potentially influencing the abnormal ECM deposition in ULs.

Besides TGF-β, other cytokines, like interleukins, may regulate the pathobiology of ULs (18). It has been shown that IL-6 may regulate ECM remodeling and induce collagen production (36). Our study revealed significantly up-regulated IL-6 and IL-6R expression after P4 treatment but no effects on IL-6 release. Interleukin-6 down-regulation by UA highlighted the involvement of this interleukin in UL biology. In addition, *SMAD3* silencing has been shown to attenuate IL-6-induced collagen synthesis in dermal fibroblasts (36). Thus, the inhibition of IL-6 release after SMAD3 blockage may suggest that the canonical TGF-β signaling pathway regulates its activity in ULs.

Because of excessive production and accumulation of ECM, ULs have been considered to possess poor vascularization (37). We hereby show that UA treatment down-regulated the high VEGF expression and their release in ULs. Activation of the canonical TGF-β pathway has been shown to play a key role in the regulation of VEGF release (12, 13). We did not observe any P4-induced up-regulation of VEGF production in ULs. However, when we blocked SMAD3, it significantly inhibited the P4-support of VEGF release, suggesting that P4 may sustain VEGF activation via the TGF-β-SMAD3 pathway. In addition, VEGF has been shown to stimulate angiogenesis in a Rho GTPase-dependent manner in endothelial cells. The VEGFA and VEGF2 axes stimulate the activation of RhoA, Cdc42, and Rac1, leading to vascular development and the formation of cytoplasmic migratory structures (20, 21, 38). Our present analysis revealed that VEGF up-regulated RhoA expression in UL explants, and UA inhibited this effect. The mechanistic sequence of events might involve the release of VEGF by the ULs to stimulate intratumor angiogenesis, which was enhanced by P4 through SMAD3 signaling. The RhoA pathway was likely involved not only in the ECM reorganization but also in its regulation of vascular processes inside the ULs. Extracellular matrix deposition and structure are crucial for fibrotic tumor formation (39).

A better mechanistic understanding of the synthesis and accumulation of ECM is critical for the development of further novel therapeutic strategies for ULs. However, for clinical translation, several key points still need to be addressed (like combining UA with direct and indirect ECM targeting with SMAD3, TGF-βRI and II, RhoA, VEGF, or COL1A1). This proof of the principal and concept experiments could be a limitation of this study. The most important next step would be to investigate the combination therapy of UA with RhoA and ROCK inhibitors, as well as with VEGF or IL-6 inhibitors, to determine their effectiveness in the regulation of ECM synthesis and accumulation in ULs in vitro, followed by in vivo studies. Subsequently, the tolerance and safety of these therapies should be assessed. We assert that identifying and targeting fibrogenic signaling hubs like RhoA could serve as a strategy for the design, characterization, and translation of new antineoplastics against ULs and other neoplasms with excessive fibrosis.

CONCLUSIONS

In summary, P4 activated the TGF-β and SMAD3 signaling pathways and stimulated proliferation, growth, and ECM remodeling in ULs (Fig. 4). On the contrary, SPRM-presentative UA showed novel opposite effects on UL proliferation and growth or ECM accumulations through the TGF-β and SMAD3 signaling pathways, namely negatively acting through SMAD3, TGF-β RI and II, RhoA, VEGF, or decreasing the fibrosis-related gene *COL1A1* and p*roCOLIA1* production. Our present findings therefore highlight a novel treatment option for ULs through combining selected TGF-β and SMAD3 signaling pathway members with SPRMs.

CRediT Authorship Contribution Statement

Conceptualization: G.M., D.P.T., P.B., O.L., M.Sz., M.S., A.P.P., M.K., T.B., M.Z.K., J.T.; Investigation: G.M., D.P.T., P.B., O.L., M.Sz., M.S., A.P.P., M.K., T.B., M.Z.K., A.P., J.T.; Funding acquisition: N.A.R., G.M.; Supervision: G.M., D.P.T., X.L., I.H., S.W., N.A.R.; Writing – original draft: G.M., D.P.T., S.W., I.H., N.A.R.; Writing – review and editing: G.M., D.P.T., S.W., I.H., and N.A.R.

Declaration of Interests

G.M. has nothing to disclose. D.P.T. has nothing to disclose. P.B. has nothing to disclose. O.L. has nothing to disclose. M.Sz. has nothing to disclose. M.S. has nothing to disclose. A.P.P. has nothing to disclose. M.K. has nothing to disclose. T.B. has nothing to disclose. M.Z.K. has nothing to disclose. A.P. has nothing to disclose. J.T. has nothing to disclose. X.L. has nothing to disclose. I.H. has nothing to disclose. S.W. has nothing to disclose. N.A.R. has nothing to disclose.

REFERENCES

1. Stewart EA, Cookson CL, Gandolfo RA, Schulze-Rath R. Epidemiology of uterine fibroids: a systematic review. BJOG 2017;124:1501–12.
2. Flynn M, Jamison M, Datta S, Myers E. Health care resource use for uterine fibroid tumors in the United States. Am J Obstet Gynecol 2006;195:955–64.
3. Stewart EA. Uterine fibroids. Lancet 2001;357:293–8.
4. Pritts EA, Parker WH, Olive DL. Fibroids and infertility: an updated systematic review of the evidence. Fertil Steril 2009;91:1215–23.

5. Islam MS, Ciavattini A, Petraglia F, Castellucci M, Ciarmela P. Extracellular matrix in uterine leiomyoma pathogenesis: a potential target for future therapeutics. Hum Reprod Update 2018;24:59–85.
6. Kim JJ, Sefton EC. The role of progesterone signaling in the pathogenesis of uterine leiomyoma. Mol Cell Endocrinol 2012;358:223–31.
7. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update 2015;21:155–73.
8. Massague J. TGFbeta signalling in context. Nat Rev Mol Cell Biol 2012;13:616–30.
9. Lewis TD, Malik M, Britten J, Parikh T, Cox J, Catherino WH. Ulipristal acetate decreases active TGF-beta3 and its canonical signaling in uterine leiomyoma via two novel mechanisms. Fertil Steril 2019;111:806–15.e1.
10. Arici A, Sozen I. Transforming growth factor-beta3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. Fertil Steril 2000;73:1006–11.
11. Malik M, Segars J, Catherino WH. Integrin beta1 regulates leiomyoma cytoskeletal integrity and growth. Matrix Biol 2012;31:389–97.
12. Noma K, Smalley KS, Lioni M, Naomoto Y, Tanaka N, El-Deiry W, et al. The essential role of fibroblasts in esophageal squamous cell carcinoma-induced angiogenesis. Gastroenterology 2008;134:1981–93.
13. Seystahl K, Tritschler I, Szabo E, Tabatabai G, Weller M. Differential regulation of TGF-beta-induced, ALK-5-mediated VEGF release by SMAD2/3 versus SMAD1/5/8 signaling in glioblastoma. Neuro Oncol 2015;17:254–65.
14. Cordeiro Mitchell CN, Islam MS, Afrin S, Brennan J, Psoter KJ, Segars JH. Mechanical stiffness augments ligand-dependent progesterone receptor B activation via MEK 1/2 and Rho/ROCK-dependent signaling pathways in uterine fibroid cells. Fertil Steril 2021;116:255–65.
15. Yoshida S, Ohara N, Xu Q, Chen W, Wang J, Nakabayashi K, et al. Cell-type specific actions of progesterone receptor modulators in the regulation of uterine leiomyoma growth. Semin Reprod Med 2010;28:260–73.
16. Donnez J, Tatarchuk TF, Bouchard P, Puscasiu L, Zakharenko NF, Ivanova T, et al. Ulipristal acetate versus placebo for fibroid treatment before surgery. N Engl J Med 2012;366:409–20.
17. Ciebiera M, Wlodarczyk M, Wrzosek M, Meczekalski B, Nowicka G, Lukaszuk K, et al. Role of transforming growth factor beta in uterine fibroid biology. Int J Mol Sci 2017;18:2435.
18. Islam MS, Protic O, Stortoni P, Grechi G, Lamanna P, Petraglia F, et al. Complex networks of multiple factors in the pathogenesis of uterine leiomyoma. Fertil Steril 2013;100:178–93.
19. Nallasamy S, Yoshida K, Akins M, Myers K, Iozzo R, Mahendroo M. Steroid hormones are key modulators of tissue mechanical function via regulation of collagen and elastic fibers. Endocrinology 2017;158:950–62.
20. El Baba N, Farran M, Khalil EA, Jaafar L, Fakhoury I, El-Sibai M. The Role of Rho GTPases in VEGF Signaling in Cancer Cells. Anal Cell Pathol (Amst) 2020;2020:2097214.
21. Zahra FT, Sajib MS, Ichiyama Y, Akwii RG, Tullar PE, Cobos C, et al. Endothelial RhoA GTPase is essential for in vitro endothelial functions but dispensable for physiological in vivo angiogenesis. Sci Rep 2019;9:11666.
22. Monaghan-Benson E, Wittchen ES, Doerschuk CM, Burridge K. A Rnd3/p190RhoGAP pathway regulates RhoA activity in idiopathic pulmonary fibrosis fibroblasts. Mol Biol Cell 2018;29:2165–75.
23. Ignotz RA, Endo T, Massague J. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. J Biol Chem 1987;262:6443–6.
24. Szydlowska I, Grabowska M, Nawrocka-Rutkowska J, Piasecka M, Starczewski A. Markers of cellular proliferation, apoptosis, estrogen/progesterone receptor expression and fibrosis in selective progesterone receptor modulator (ulipristal acetate)-treated uterine fibroids. J Clin Med 2021;10:562.
25. Horwitz KB, Alexander PS. In situ photolinked nuclear progesterone receptors of human breast cancer cells: subunit molecular weights after transformation and translocation. Endocrinology 1983;113:2195–201.
26. Wen DX, Xu YF, Mais DE, Goldman ME, McDonnell DP. The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells. Mol Cell Biol 1994;14:8356–64.
27. Zhang YE. Non-Smad pathways in TGF-beta signaling. Cell Res 2009;19:128–39.
28. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003;425:577–84.
29. Moustakas A, Heldin CH. Non-Smad TGF-beta signals. J Cell Sci 2005;118:3573–84.
30. Amano M, Nakayama M, Kaibuchi K. Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity. Cytoskeleton (Hoboken) 2010;67:545–54.
31. Clements RT, Minnear FL, Singer HA, Keller RS, Vincent PA. RhoA and Rho-kinase dependent and independent signals mediate TGF-beta-induced pulmonary endothelial cytoskeletal reorganization and permeability. Am J Physiol Lung Cell Mol Physiol 2005;288:L294–306.
32. Lee J, Moon HJ, Lee JM, Joo CK. Smad3 regulates Rho signaling via NET1 in the transforming growth factor-beta-induced epithelial-mesenchymal transition of human retinal pigment epithelial cells. J Biol Chem 2010;285:26618–27.
33. Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, et al. p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. Hepatology 2003;38:879–89.
34. Cao Q, Mak KM, Lieber CS. DLPC decreases TGF-beta1-induced collagen mRNA by inhibiting p38 MAPK in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 2002;283:G1051–61.
35. Wang Z, Gao Z, Shi Y, Sun Y, Lin Z, Jiang H, et al. Inhibition of Smad3 expression decreases collagen synthesis in keloid disease fibroblasts. J Plast Reconstr Aesthet Surg 2007;60:1193–9.
36. O'Reilly S, Ciechomska M, Cant R, van Laar JM. Interleukin-6 (IL-6) trans signaling drives a STAT3-dependent pathway that leads to hyperactive transforming growth factor-beta (TGF-beta) signaling promoting SMAD3 activation and fibrosis via Gremlin protein. J Biol Chem 2014;289:9952–60.
37. Farrer-Brown G, Beilby JO, Tarbit MH. The vascular patterns in myomatous uteri. J Obstet Gynaecol Br Commonw 1970;77:967–75.
38. Shimizu A, Zankov DP, Kurokawa-Seo M, Ogita H. Vascular endothelial growth factor-A exerts diverse cellular effects via small G proteins, Rho and Rap. Int J Mol Sci 2018;19:1203.
39. Bonnans C, Lohela M, Werb Z. Real-time imaging of myeloid cells dynamics in ApcMin/+ intestinal tumors by spinning disk confocal microscopy. J Vis Exp 2014:51916.

Evidencia funcional de dos mecanismos de acción distintos de progesterona y un modulador selectivo del receptor de progesterona en los leiomiomas uterinos

Objetivo: Estudiar los mecanismos específicos a través de los cuales la progesterona y los moduladores selectivos del receptor de progesterona impactan el crecimiento, síntesis y acumulación de la matriz extracelular en los leiomiomas uterinos.

Diseño: Estudio de laboratorio.

Lugar: Instituciones de Investigación Académica.

Paciente(s): Este estudio involucró a mujeres en edad reproductiva con diagnóstico de infertilidad asociada a leiomiomas uterinos que se sometieron a miomectomía ya sea después de un tratamiento con el modulador selectivo del receptor de progesterona acetato de ulipristal (UA) o sin ningún tratamiento previo farmacológico. Las muestras control incluyeron tejido de miometrio sano (n = 100). Las muestras se obtuvieron del Departamento de Reproducción y Endocrinología Ginecológica y del Biobanco de la Universidad Médica de Bialystok, Polonia.

Intervención(es): Se recolectaron muestras de tejido miometrial inmediatamente después de cirugía para su análisis y evaluación transcripcional de pacientes con leiomiomas uterinos tratadas durante dos meses con UA diario (5mg/d) (n = 100) y de pacientes que no recibieron tratamiento (n = 150) o miometrio sano normal (n = 100).

Principal(es) Medida(s) de Resultado(s): Se evaluó la activación inducida por progesterona de las vías de señalización relacionadas con la síntesis, el depósito y el crecimiento de la matriz extracelular de los leiomiomas uterinos, así como el perfil de expresión de los receptores de progesterona en los leiomiomas uterinos.

Resultado(s): Los resultados indicaron que la progesterona activó las vías de señalización del factor de crecimiento transformador-β y SMAD3 y promovió la proliferación, el crecimiento y la remodelación de la matriz extracelular en los leiomiomas uterinos mediante la regulación a la alta de SMAD3, el receptor del factor de crecimiento transformador-β (TGF-β) tipo I y II, el homólogo A de Ras, el factor de crecimiento vascular endotelial, o aumentando la producción del gen de colágeno relacionado con fibrosis, tipo I, -1, y procolágeno, tipo I, -1. Por el contrario, el UA tuvo efectos inhibidores sobre estos procesos. El estudio también mostró que los receptores de progesterona tanto nucleares como de membrana desempeñan funciones distintas en la biopatología del leiomioma uterino.