Transcription Factor KLF11 Integrates Progesterone Receptor Signaling and Proliferation in Uterine Leiomyoma Cells

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Abstract
Uterine leiomyoma is the most common tumor of the female genital tract and the leading cause of hysterectomy. Although progesterone stimulates the proliferation of uterine leiomyoma cells, the mechanism of progesterone action is not well understood. We used chromatin immunoprecipitation (ChIP)–cloning approach to identify progesterone receptor (PR) target genes in primary uterine leiomyoma smooth muscle cells. We identified 18 novel PR-binding sites, one of which was located 20.5 kb upstream of the transcriptional start site of the Krüppel-like transcription factor 11 (KLF11) gene. KLF11 mRNA levels were minimally downregulated by progesterone but robustly upregulated by the progesterone antagonist RU486. Luciferase reporter assays showed significant baseline and RU486-inducible promoter activity in the KLF11 basal promoter or distal PR-binding region, both of which contained multiple Sp1-binding sequences but lacked classic progesterone response elements. RU486 stimulated recruitment of Sp1, RNA polymerase II, PR, and the coactivators SRC-1 and SRC-2 to the distal region and basal promoter. siRNA knockdown of PR increased KLF11 expression, whereas knockdown of KLF11 increased leiomyoma cell proliferation and abolished the anti-proliferative effect of RU486. In vivo, KLF11 expression was significantly lower in leiomyoma tissues compared with adjacent myometrial tissues. Taken together, using a ChIP-cloning approach, we uncovered KLF11 as an integrator of PR signaling and proliferation in uterine leiomyoma cells. Cancer Res; 70(4): 1722–30. ©2010 AACR.

Introduction
Uterine leiomyomata are the most common gynecologic tumors in women of reproductive age. Growing evidence from basic and clinical studies indicates that progesterone and the progesterone receptor (PR) play key roles in uterine leiomyoma growth and development (1). Several investigators have shown an increased concentration of both PR isoforms (PR-A and PR-B) in leiomyoma tissue compared with adjacent myometrium (2, 3). Mitotic activity in leiomyoma tissue was also increased relative to the adjacent myometrial tissue during the luteal phase and after treatment with medroxyprogesterone acetate (4, 5).

Progesterone suppresses apoptosis and stimulates proliferation of leiomyoma cells in culture, whereas PR modula-
similar genome-wide binding pattern of PR and consequent regulation of human genes through distant binding sites have been suspected but not yet shown.

To better understand the effects of progesterone on human uterine leiomyoma growth, we sought to identify a larger number of PR target genes. We used a ChIP-cloning strategy for unbiased identification of regulatory targets of progesterone/PR in leiomyoma. Interestingly, among the cloned targets, endogenous PR was found to bind in intact cells to a region located 20.5 kb upstream of the transcriptional start site of the Krüppel-like transcription factor 11 (KLF11) gene. Here, we report that this region may interact with the basal KLF11 promoter through recruitment of common transcription factors in response to the antiprogestin RU486. We also showed a potential role for KLF11 in protecting against leiomyoma growth and mechanism for the therapeutic action of antiprogestins.

Materials and Methods

**Tissue collection and cell culture.** Human uterine leiomyoma and matched myometrium were collected from 56 premenopausal women undergoing hysterectomy, following the protocol approved by the institutional review board for Human Research of Northwestern University. The subjects had not received any hormonal treatment during the 6 mo before surgery. The leiomyoma smooth muscle (LSM) cells were cultured as previously described (27). Cells used in these experiments were passaged once or twice.

**ChIP and ChIP cloning.** We performed the ChIP-cloning procedure as described previously except that the chromatin fragments were immunoprecipitated using anti-PR antibody (Abcam; ref. 25). Antibodies used for standard ChIP included anti-phosphorylated RNA PolII, anti-Sp1, anti–SRC-1 (Santa Cruz Biotechnology). Primers used for ChIP on SLC7A8 and SOX8 are available upon request. Primers used for ChIP at 20.5 kb upstream of the KLF11 transcription start site were 5′-GACCACTCGGAAATGG-3 (forward) and 5′-GGAGGACACGTGACTTTT-3 (reverse). Real-time PCR primers used for fold enrichment of distal regulatory sites were identical, and real-time PCR primers for basal promoter region were 5′-GGTGCAAGGACACGTATCTG-3 (forward) and 5′-GGAGGGACACGGCTTAGAG-3 (reverse). In this instance, real-time PCR was performed using ABI SYBR Green Master kit (ABI).

**RNA preparation and real-time quantitative PCR.** Total RNA from LSM cells and tissues of leiomyoma and matched myometrium was extracted using TRI-reagent (Sigma-Aldrich). cDNA was prepared with Superscript III first-Strand Synthesis System (Invitrogen). KLF11, SOX8, SLC7A8, PR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were amplified by real-time PCR using the ABI TaqMan Gene Expression system (ABI) and the ABI 7900 Sequence Detection System. All gene expressions were normalized to GAPDH.

**Plasmids, transfection, and luciferase assays.** PR-binding region (−19,369 bp/−21,192 bp) identified by ChIP cloning for KLF11 was amplified by PCR using primers 5′-ATGAGG-GACCACTCGGAAATGG-3 (forward) and 5′-GGAGGACACGTGACTTTT-3 (reverse), and cloned into the pGL3 promoter vector (Promega). The proximal 5′-flanking region (−549 bp/+171 bp) was amplified by PCR using primers 5′-ATGTCACCTAGAAGCGG-3′ (forward) and 5′-GTGGTGCATGTGCAGCAG-3′ (reverse), and cloned into the pGL3 basic vector (Promega).

T47D cells (American Type Culture Collection) were transfected with 2 μg reporter plasmid and 0.2 μg PRL-TK-Luc using FuGene HD transfection reagent (Roche). Twenty-four hours after transfection, cells were treated with vehicle (ethanol) or RU486 (10−6 mol/L) for 48 h, and cell extracts were prepared using passive lysis buffer (Promega). Illustrated results are normalized to PRL-TK-Luc using a dual luciferase reporter assay system (Promega).

**Immunostaining for bromo-2-deoxyuridine.** The effect of KLF11 on cell proliferation was determined by bromo-2-deoxyuridine (BrdUrd) incorporation assay as described previously except that BrdUrd (20 μmol/L) was added during the final 4 h of cell treatment (28). The result was expressed as the BrdUrd labeling index (percentage of BrdUrd-labeled cells in total cells counted).

**Small interfering RNA.** To knock down the expression of endogenous PR and KLF11, LSM cells were transfected with PR siRNA or KLF11 siRNA (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen). Nontargeting control siRNA (Dharmacon) was transfected as a negative control.

**Immunoblotting.** Cell lysates were analyzed by immunoblotting as described previously (29) using monoclonal anti-PR (kindly provided by Dr. Edwards, Baylor College of Medicine, Houston, TX), anti-PR-BrdUrd (Abcam), and anti–proliferating cell nuclear antigen (PCNA; GenScript Corp.) antibodies. Equal loading was confirmed using anti–β-actin antibody (Sigma-Aldrich). The intensity of bands was quantified using ImageJ software.

**Statistical analysis.** Differences between groups were analyzed by Student’s t test or one-way ANOVA analysis followed by Fisher’s protected least significance difference test. Significance was accepted at P < 0.05.

Results

**Identification of PR genomic targets in human LSM cells.** To identify regulatory regions that recruit PR within the entire genome of human LSM cells, we performed ChIP using anti-PR antibody in LSM cells treated with progesterone for 1 hour. Twenty-one cloned fragments were mapped to the entire genome. Among these, 17 fragments were localized within an intron of a gene; two fragments were located 5′ or 3′ to the most proximally located genes; the other two fragments were mapped to an exon of a gene. Because two genomic regions were associated with multiple ChIP fragments, a total of 18 novel PR-binding sites were identified (Supplementary Table S1).

These PR-binding sites were distributed genome-wide to chromosomes 1, 2, 5, 7, 10, 14, 15, 16, 17, 19, and 20 (Supplementary Fig. S1). Interestingly, binding sites were not...
distributed evenly between the chromosomes. For example, four binding sites were located on chromosome 1 and two sites were located on each of chromosomes 7, 14, 17, and 20, whereas no binding to 11 chromosomes was noted, suggesting specificity for genomic distribution.

Of the 18 novel sites, we identified the 16 genes that had a PR-binding site within an intron or an exon. We also identified a pair of the most proximal genes that flanked each of the remaining two PR-binding sites (Supplementary Table S1). One binding site was located 5′ upstream of both KLF11 and AILT75830, which are transcribed in opposite directions, whereas the remaining binding site was 3′ downstream of both SOX8 and FLJ32252 genes, also transcribed in opposite directions (Supplementary Table S1).

We next selected 11 well-characterized genes from Supplementary Table S1 and investigated their progesterone responsiveness. We treated LSM cells for 2 and 4 hours with progesterone in the presence or absence of the progesterone antagonist RU486 and measured mRNA levels. Treatment with progesterone in the presence or absence of RU486 altered mRNA levels in 5 of 11 (45%) genes (Supplementary Table S2). In particular, we observed that SLC7A8 mRNA levels were increased after progesterone treatment, reaching statistical significance at 4 hours (Fig. 1A). This increase was blocked by RU486. SLC7A8 encodes a key amino acid transporter protein that may be an important regulator of leiomyoma growth (30). SOX8 mRNA levels were not affected by progesterone but were downregulated significantly at 2 hours in the presence of RU486 (Fig. 1A). SOX8 encodes a transcription factor that inhibits skeletal muscle differentiation (31).

Conversely, KLF11 mRNA levels were slightly but significantly downregulated by progesterone treatment. However, they were robustly and significantly increased by 2.3-fold at 2 hours and 3.3-fold at 4 hours in the presence of RU486 (Fig. 1A). Similarly, mRNA levels of the genes PRDM16 and
MAN1C1 were upregulated by RU486 (Supplementary Table S2). As shown in Supplementary Table S1, PRDM16 encodes a transcription factor that may play an important role in the leukemogenic transformation of the cells (32), whereas MAN1C1 plays an important role in N-glycan biosynthesis (33).

The mild but significant downregulation of KLF11 by progesterone was also observed independently in a gene microarray experiment using the Illumina platform; progesterone treatment for 4 hours downregulated KLF11 mRNA by 16.5% (P < 0.05, data not shown). We also showed that R5020, a synthetic progesterone agonist, significantly decreased the KLF11 mRNA level by ~30% (Fig. 1B). R5020 induced a more robust reduction in KLF11 mRNA levels compared with progesterone (Fig. 1B).

These results suggested that the distant PR-binding modules were functionally active and differentially modulated progesterone and antiprogestin responsiveness of the genes identified in the ChIP-cloning screen. We next confirmed the recruitment of PR to the distant PR-binding sites of three selected genes, SLC7A8, SOX8, and KLF11, and further investigated whether these sites interacted with RNA PolII using a conventional ChIP assay. Independent pools of immunoprecipitated chromatin fragments from LSM cells treated with progesterone were cloned into the pGL3 promoter vector. As shown in Fig. 1C, PR and RNA PolII were recruited to PR-binding sites in the SLC7A8, SOX8, and KLF11 genes.

Regulation of KLF11 mRNA and protein levels by RU486 in LSM cells. RU486 and other antiprogestins have been shown to reduce leiomyoma size in a number of clinical trials (12, 13). Mechanisms responsible for this therapeutic effect are unknown. KLF11 is a known tumor suppressor (34) and was significantly upregulated in the presence of RU486 as shown in Fig. 1A. Therefore, we focused our investigation on KLF11 regulation and function in LSM cells as a possible mediator of the therapeutic effects of antiprogestins on uterine leiomyoma. Cells were treated with different doses of RU486 (10^{-10}–10^{-6} mol/L) for 6 hours and KLF11 mRNA levels were determined. As shown in Fig. 2A, RU486 (10^{-8}–10^{-6} mol/L) significantly induced KLF11 expression. RU486 at 10^{-6} mol/L also stimulated comparable increases in KLF11 protein levels after 72 hours of treatment (Fig. 2B).

Next, we knocked down endogenous PR using siRNA to establish a functional role for PR in mediating KLF11 expression in LSM cells. Transfection of LSM cells with PR siRNA reduced PR mRNA and protein levels (Fig. 2C). PR knockdown increased KLF11 mRNA by 2.1-fold compared with control siRNA–transfected cells (Fig. 2D).

Stimulation of KLF11 promoter activity by RU486. To determine whether the novel PR-binding region identified by ChIP cloning confers promoter activity, we cloned this fragment (~19,369 bp/–21,192 bp) into the pGL3 promoter vector. To determine whether the proximal 5′-flanking region of KLF11 gene was also involved in RU486-regulated gene transcription, the ~549 bp/–171 bp sequence was cloned into the pGL3 basic vector. Individual constructs were transfected into T47D breast cancer cells because this cell line with high levels of endogenous PR could be transfected reproducibly with both vectors (21). As shown in Fig. 3A, the 1,823-bp fragment bearing the distal PR-binding region showed significant promoter-enhancing activity in the absence or presence of RU486. The basal promoter region also conferred significant
transcriptional activity that was induced by RU486 (Fig. 3B). These results indicated that both the distal region and basal promoter of the KLF11 gene have transcriptional regulatory activity.

**Recruitment of transcription factors and steroid receptor coactivators to KLF11 basal promoter and distal PR-binding region.** As the PR-binding region is located 20.5 kb upstream of the KLF11 transcription start site, we sought to elucidate whether this distal region was associated with the basal transcriptional multimeric complex. There did not seem to be any classic, palindromic PRE consensus sequences in either the basal promoter or the 20.5-kb distal PR-binding region. However, we found several half PREs in the distal region and several GC-rich Sp1 sites in both regions, and hypothesized that ligand-bound PR may associate with Sp1 and regulate KLF11 expression by mediating an interaction between the distal and basal regulatory sites. To test this hypothesis, we designed primers flanking the basal promoter and the distal region. We then evaluated simultaneous recruitment of RNA PolII, Sp1, and PR to the distal PR-binding region and the basal promoter of KLF11 using real-time PCR.

We also characterized whether RU486 stimulated KLF11 expression through recruiting coactivators to its basal promoter and the distal PR-binding region. It was reported that PR action selectively required the steroid receptor coactivator SRC-3 in breast; in contrast, it required SRC-1 as a coactivator in uterus (35). SRC-1 and SRC-2 have a similar pattern of expression that is distinct from the pattern of expression of SRC-3, indicating that these two proteins play cooperative roles in regulating steroid hormone action in the murine uterus (36). We thus evaluated the recruitment of SRC-1 and SRC-2 to KLF11 basal promoter and the distal regulatory region.

In the basal promoter region, RU486 significantly increased the association of RNA PolII, Sp1, PR (Fig. 4A), SRC-1, and SRC-2 (Fig. 4B) with this region of the KLF11 gene. In the distal regulatory region, however, RU486 significantly induced the recruitment of only SRC-2 to this region (Fig. 4B). We observed trends that RU486 enhanced the interaction of RNA PolII, Sp1, PR, and SRC-1 to the distal regulatory region, but none of them reached statistical significance (Fig. 4A and B).

To further verify that PR is necessary for the recruitment of transcriptional complexes, we determined the effects of PR knockdown on SRC-2 recruitment in the presence or absence of RU486. As expected, RU486 induction of PR recruitment to both the distal region and the basal promoter was abolished by PR siRNA (data not shown). PR knockdown significantly reduced the baseline recruitment of SRC-2 to the distal region but did not cause significant change of its association with the basal promoter region (Fig. 4C). RU486-induced SRC-2 recruitment to the basal promoter was decreased robustly by ∼2.4-fold in the presence of PR siRNA, whereas PR siRNA strikingly decreased RU486-induced SRC-2 binding to the distal region by ∼7-fold (Fig. 4C).

These data suggest that RU486 regulates KLF11 expression through simultaneous recruitment of PR, its coactivators, and general transcription factors to its basal promoter and distal regulatory region. These findings also suggest that the distal PR-binding region regulates KLF11 expression through an interaction with the basal transcriptional multimeric complex.

**Knockdown of KLF11 is associated with increased LSM proliferation.** We performed knockdown of endogenous KLF11 to investigate how it may be involved in progesterone- or RU486-regulated LSM cell proliferation, as indicated by PCNA level and the rate of BrdUrd incorporation. Real-time PCR analysis confirmed that KLF11 siRNA reduced endogenous KLF11 expression by ∼80% (Fig. 5A). As shown in Fig. 5B, KLF11 siRNA or the pure progesterone agonist R5020 decreased KLF11 protein levels and increased PCNA levels. In contrast, RU486 decreased PCNA levels and knockdown of KLF11 blunted this inhibitory effect (Fig. 5C). Alterations in LCM cell proliferation were verified by the BrdUrd incorporation assay. Figure 5D shows that RU486 significantly decreased the BrdUrd labeling index from 7.1% to 3.8%. Knockdown of KLF11 increased the BrdUrd labeling index to 17.6% (P < 0.01, compared with vehicle-treated and control siRNA–transfected cells), even in the presence of RU486.
(16.3%). These results suggest that regulation of LSM cell proliferation by progesterone agonists or antagonists may be mediated, at least in part, by KLF11.

**KLF11 expression in leiomyoma and matched myometrial tissues.** To understand the in vivo relevance of progesterone and antiprogestin-regulated KLF11 expression in leiomyoma, we analyzed mRNA and protein levels of KLF11 in human leiomyoma and matched adjacent normal myometrial tissues using real-time PCR and Western blot. KLF11 mRNA levels in 17 subjects were lower in leiomyoma than in myometrial tissues, and only one subject had higher KLF11 mRNA levels in leiomyoma compared with myometrial tissues. As shown in Fig. 6A, the mean expression level of KLF11 in leiomyoma tissues was approximately half of that of matched myometrial tissues \((n = 18, P < 0.001)\). Similarly, mean KLF11 protein level was significantly lower in leiomyoma than matched myometrial tissues from six subjects (Fig. 6B).

**Discussion**

A significant volume of work has focused on identifying estrogen receptor binding sites using computational genomics (37–39), promoter microarrays (26), CpG island libraries (40), ChIP cloning (25, 26), and ChIP-on-chip methods (24). Although progesterone has important and complex effects on the female reproductive tract, comparatively little is known about genome-wide PR-binding sites. Using ChIP-cloning technology and progesterone-responsive primary leiomyoma cells, we identified 18 novel PR-binding sites, which corresponded to 20 genes proximal to these sites. Specifically, we showed that RU486 enhanced recruitment of PR, its coactivators SRC-1 and SRC-2, as well as key general transcription factors RNA PolII and Sp1, to both distal PR-binding region and basal promoter of the KLF11 gene and induced its expression. We also found that KLF11 is a robust suppressor of leiomyoma growth.

We did not identify any PR-binding sites within a basal promoter. Five percent of PR-binding sequences were located in far distal 5′ regions, 5% were in 3′ regions, and 80% of binding sites lay within an intron of a gene. Considering that introns comprise ∼40% of the genome, this suggests a higher affinity of PR to coding regions of its target genes. This is consistent with our previous findings indicating that 46% of ERα binding sites were located within an intron of a gene in breast cancer cells (25).

None of the reported PR target genes was pulled out in this study (14–22). Some unavoidable limitations of the current

![Figure 4.](image-url)
ChIP-cloning strategy, such as the affinity of the antibody used to pull down chromatin and the on-and-off interaction pattern of transcription factors with DNA, may cause the incomplete cloning of all target genes (25, 26, 41, 42). The low endogenous PR levels in cultured LSM cells may also account for the low number of PR-binding sites cloned in this study (43).

Regulation of half of the genes located proximately to these distant PR-binding sites by progesterone or RU486 suggested that these sequences are functionally active. PR-binding fragments located distal to genes or within introns might function through long-range interactions that involve looping of chromatin to bring the regulatory elements within the proximity of basal promoters.

Both proximal and distal regulatory regions of the KLF11 gene recruited not only PR and Sp1 but also RNA PolII upon RU486 treatment. Various models have been proposed to explain how a distal enhancer communicates with a proximal promoter: the looping, the tracking, and the linking models (44, 45). With regard to the distal-proximal communication of the KLF11 gene, our data support a looping model. We plan to use a chromosome conformation capture assay to investigate whether the KLF11 distal region and proximal promoter physically interact in intact cells.

RU486 stimulated the basal promoter activity but not the enhancer function of the distal region. RU486 also enhanced PR binding to the basal promoter but not to the distal region. The distal region, however, exhibits significant basal enhancer function, basal PR-binding activity, and RU486-induced SRC-2 recruitment, which is PR dependent (Fig. 4C). Taken together, these findings are suggestive that the major function of the distal PR-binding site is the stabilization of the RU486/PR-dependent enhancer complex at the KLF11 promoter. It seems that an interaction between the basal promoter and the distal PR-binding region would be necessary for RU486-dependent regulation of KLF11 expression.

We found that, at least for the target genes studied here, RU486 was a much more robust regulator compared with progesterone. Knockdown of PR or treatment with RU486 significantly increased KLF11 expression, whereas the native progesterone hormone minimally suppressed its expression. The synthetic progesterone agonist R5020, however, markedly suppressed KLF11 levels (Figs. 1B and 5B). The difference between the effects of progesterone and R5020 may be due to possible rapid metabolization of progesterone in these cells.

Consistent with the notion that coactivators play major roles in determining PR activity (46), we found that, through interacting with PR, RU486 enhanced the binding of SRC-1 and SRC-2 to the basal promoter and the distal PR-binding region, which gave rise to gene induction. The function of RU486 as an inducer of gene transcription is not without precedent. Shatnawi and colleagues showed that RU486 can stimulate expression of genes encoding folate receptor type α, p21, and p27 in a specific promoter context that has G/C-rich Sp1 binding elements but no classic PRE sites (22). In line with this observation, we found that both distal PR-binding region and the basal promoter of the KLF11 gene, with
growth in vitro and in vivo, and inhibits neoplastic transformation (34). Consistent with these reports, we found that KLF11 expression was significantly downregulated in leiomyoma tissues compared with adjacent matched myometrial tissues. Moreover, KLF11 inhibited LSM cell growth. Although the mechanism involved in KLF11-regulated cell proliferation needs to be further clarified, our studies provide an initial insight into the mechanism by which progesterone or RU486 regulates leiomyoma growth and development. Given that progesterone exerts a number of essential functions in inhibition of estrogen-dependent endometrial carcinogenesis and promotion of breast cancer development, it is tempting to explore the regulation and function of KLF11 in these malignancies (47–49).

In summary, using an unbiased approach, we uncovered KLF11 as a novel PR target responsive to progesterone antagonist RU486 in LSM cells. KLF11 mediated the antiproliferative effects of RU486 in leiomyoma cells. These studies reveal a potential mechanism underlying the modulation of progesterone- and antiprogestin-regulated differentiation and proliferation in LSM cells. The PR-dependent downregulation of KLF11 expression in LSM cells may represent a missing link in steroid signaling pathways that explains the pathogenesis of uterine leiomyoma. Furthermore, the identification of direct targets of KLF11 will be important to elucidate its role in uterine leiomyoma biology.

Disclosure of Potential Conflicts of Interest

S.E. Bulun: consultant, GSK, Meditrina, and Novartis. The other authors disclosed no potential conflicts of interest.

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Figure 6. mRNA and protein levels of KLF11 in human leiomyoma and matched myometrial tissues. A. 36 samples from 18 subjects were analyzed for mRNA; 18 samples were obtained from leiomyomas and 18 from adjacent myometrial tissues. To allow comparisons of data obtained from samples from different subjects, mRNA levels in the myometrial tissues were normalized to 1. B. 12 samples from six subjects were analyzed for KLF protein levels. Immunoblot densities were quantified with Image J software. M, myometrial tissue; L, leiomyoma tissue.

References


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