Supporting Information

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SI Materials and Methods

Transgenic Construct and Generation of CaBP9K–hGPR10 Mice. A full-length cDNA encoding human G-protein coupled receptor 10 (hGPR10) (NM_004248) was obtained from Open Biosystems. The rat calbindin-D9K (CaBP9K) short (−117 to +365 bp) promoter (reference sequence GenBank X16635.1) was PCR amplified with primers containing HindIII site at the 5′ end and a reverse primer containing overlapping GPR10 sequence up to a unique Apal site close to the start ATG. Forward (CaBP9K) primer: GAG TCT TAA GCT TGG TCT CAG, reverse hybrid primer: 5′ CTG GGG CCC CGA GTG GTC GAT GAG GCC ATT TTT CCG TCC TGG TAT ATG G (overlapping GPR10 sequence including the Apal site is underlined). The PCR product was cloned into a vector upstream of the Apal site of GPR10 as shown in Fig. 34. The construct was used to generate the CaBP9K–hGPR10 transgenic mouse mice. We obtained three (two male, one female) positive founders (Fig. 3B). These transgenic founders were bred to WT C57BL6 mice to obtain F1 progeny. The mice were genotyped by PCR using a rat CaBP9K forward primer (5′ CCA CTA ATG CTT CGA CCT GTC) and a GPR10 reverse primer (5′ CAC CAG CTG CAG CTG GAA G).

siRNA Knockdown of GPR10 and REST. Cultured primary leiomyoma SMCs were transfected with On-TARGETplus SMARTpool siRNAs (J-005524-05, J-005524-06, J-005524-07, and J-005524-08, Dharmacon, ThermoFisher) to GPR10 using DharmaFECT 2 transfection reagent according to manufacturer protocol. Control experiments included ON-TARGETplus nontargeting scrambled siRNA 2 (D-001810-02-05). After 24 h, transfected cells were serum starved in 1% fetal bovine serum (FBS) overnight followed by GPR10 ligand treatment prolactin releasing peptide [(PrRP) 1 μM in PBS] for 1 h. Protein extracts from the cells were then analyzed for Western blotting. siRNA knockdown of RE-1 suppressing transcription factor (REST) in primary myometrial SMCs was performed using Silencer Select siRNA (s11934) from Ambion (Life Technologies) using Lipofectamine RNAiMAX transfection reagent (Invitrogen/LifeTechnologies). Scrambled siRNA (D-001810-02-05). After 24 h, transfected cells were serum starved in 1% fetal bovine serum (FBS) overnight followed by GPR10 ligand treatment prolactin releasing peptide [(PrRP) 1 μM in PBS] for 1 h. Protein extracts from the cells were then analyzed for Western blotting.

H&E, Masson’s Trichrome, and Immunofluorescence Staining of Tissue Sections. Uterine tissues were fixed in paraformaldehyde (4% (wt/vol) in PBS) and processed for paraffin embedding. Tissue sections (5-μm thickness) were deparaffinized in xylene, rehydrated through a series of ethanol, and stained with H&E. For Masson’s trichrome staining, the deparaffinized tissue sections were made mordant in Bouin’s fixative, stained with Weigert’s iron hematoxyline, Biebrich scarlet acid-fuchsain, and aniline blue solutions according to the Accustain Trichrome protocol (Sigma-Aldrich). For immunofluorescence, rehydrated tissue sections were subjected to antigen retrieval by heating in citrate buffer (Vector Laboratories). When cells cultured on chamber slides (Tissue Tek) were used, they were fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 0.3% Triton X-100 before immunofluorescence experiments. The slides were washed with PBS (5 min, three times), blocked for 30 min in blocking agent [5% (vol/vol) normal goat serum] followed by primary antibody in blocking buffer overnight at 4 °C. The slides were washed with PBS (5 min, three times) and incubated with Alexa Fluor 488 or Alexa Fluor 555 labeled secondary antibodies (Invitrogen, Life Technologies) for 1 h at 37 °C. The slides were treated with nuclear dye (EthD-1, 5 μM in PBS) for 5 min at room temperature and washed in PBS (5 min, three times). The slides were mounted using 50% glycerol in PBS and visualized under fluorescence microscope.

RNA in Situ Hybridization. Deparaffinized sections (5 μm) were digested with protease-K (25 μg/mL in PBS) at 37 °C for 15 min followed by three washes in diethylpyrocarbinate-treated water. The sections were treated at 60 °C for 5 min with di-goxigenin-labeled locked nucleic acid (LNA) probes and hybridized to the probes overnight at 37 °C in humid chamber as described previously (1). The next day, slides were washed with 0.2% BSA in 0.2x SSC, incubated with alkaline phosphatase conjugated antidigoxigenin antibodies (37 °C, 1 h) followed by nitro-blue tetrazolium/3-bromo-4-chloro-3′-indolylphosphate reaction to visualize target mRNA. Custom LNA antisense probe for hGPR10 (probe sequence: 5′DigN/ACG TAA GAC AGG AGG ATG ACCA3Dig_N) or custom LNA control sense probe (probe sequence: 5′DigN/TGG TCA TCC TCC TGT CTT ACCGT/3Dig) was used for the hybridization. Additional LNA mRNA detection control probe (product number 300514-15, 5′DigN/TGG TAA GAC GTG CAC GTC TAT ACG CCCA/3Dig_N; Exiqon) was used to confirm assay specificity.

Protein Extraction and Western Blotting. Frozen tissue samples were homogenized in a hypotonic buffer (40 mM NaCl, 10 mM KCl, 20 mM Tris-HCl pH 7.4, 0.1% Triton X-100, 0.1% Tween 20) supplemented with protease and phosphatase inhibitor mixtures (Sigma-Aldrich). The homogenates were centrifuged at 12,000 × g at 4 °C. The postnuclear supernatants were combined with extracts from the nuclear pellets, solubilized using the above buffer supplemented with 300 mM NaCl. Western blots were performed as described previously (2).

Chromatin Immunoprecipitation and PCR. ChIP assays of cultured primary myometrial and leiomyoma SMCs were performed as described by Yin et al. (3). Anti-REST ChIPAb+ antibodies (Millipore) were used according to the manufacturer’s protocol. PCR primers spanning the conserved RE1 element in the promoter of GPR10 were designed based on the genomic sequence (GenBank: AL356865.19, bp 80348–bp 80466) to amplify a 119-bp PCR product. The primer sequences were: 5′ CTT GGC TGC AGC GCC GTC CTC A and 5′ CTT GTC CTC CTC CCC ACA TCA TC.

Data Mining and Ingenuity Pathway Analysis. GEO dataset GSE13319 was analyzed for the expression of REST associated/regulated genes in myometrial and leiomyoma samples as described (4). Briefly, the dataset was preprocessed for analysis using the robust multichip averages procedure (RMA). Statistical analysis was performed on biological triplicates. Biological functional and pathway analysis were performed using Ingenuity Systems pathway analysis software IPA, version 7.6; Ingenuity Systems (www. ingenuity.com) on the significantly (fold change ≥1.5, P value ≤0.05) differentially expressed genes between myometrial cell siREST and myometrial cell control (4).

Wound-Healing Assay. Myometrial SMCs were grown on chamber slides (Lab-Tek, Nunc, Thermo Scientific) or in six-well plates to full confluence and wounds were introduced after serum starvation (0.1% FBS, 24 h) as described by Rodriguez et al. (5). The cells
were incubated (37 °C, 5% CO\textsubscript{2}) 18 h after wounding, and chamber slides were formalin fixed and analyzed by immunofluorescence using anti-REST antibodies. In the experiments in which REST/NR5F was knocked down using gene-specific siRNAs, the cells were transfected just before serum starvation. Images were recorded at 0, 6, 12, and 24 h to assess cell migration.

Calcium Imaging. Myometrial and leiomyoma SMCs, grown on chamber slides, serum starved as described above, and incubated with Fura2-AM and fluorometric imaging techniques, were used to monitor the intracellular calcium concentration ([Ca\textsuperscript{2+}] as described earlier) (6). PrRP (1 μM) or vehicle (PBS) was added during the live confocal imaging of the cells.


Fig. S1. siRNA knockdown of GPR10 in leiomyoma SMCs. TaqMan qPCR analysis of GPR10 mRNA after siRNA knockdown. Replicates of samples treated with control vehicle, scrambled siRNA, and siGPR10 for 24 h were serum starved for an additional 24 h (0.1% FBS) and tested for PrRP activation of AKT (Fig. 2C). Error bars indicate ±SD, *P < 0.05.
Fig. S2. PrRP induces intracellular calcium release in leiomyoma SMCs. Fluorometric analysis of intracellular calcium release in leiomyoma SMCs upon treatment with 1 μM PrRP-31 peptide.

Fig. S3. Myometrial tissue in GPR10 transgenic mice is identical to human leiomyomas. Tissue sections from human myometrial and leiomyoma (A and B), WT, and transgenic mouse myometrium (C and D) were stained with hematoxylin & eosin for morphological comparison.

Fig. S4. CaBP9K-hGPR10 Tg mice develop fibroid tumors. (A) H&E and (B), Masson’s trichrome stained cross-sections showing a submucosal smooth muscle tumor protruding into the cervix of CaBP9K-hGPR10 Tg mouse (6 mo old).
Fig. 55. Leiomyomas express reduced levels of REST. (A) Western blot showing lower REST expression in leiomyoma (L) samples compared with matched myometrium (M) from additional patients (P4–P7). (B) Densitometric analysis of Western blots showing significantly lower relative REST levels in leiomyomas. Error bars indicate ±SD, *P < 0.05.
Fig. S6. Altered stability of REST in leiomyoma SMCs. (A and B) Western blot and densitometric analysis showing the accelerated degradation of GFP-REST in leiomyoma SMCs. (C and D) Western blots showing that Co-REST and β-TRCP are not significantly altered in leiomyomas. (E) Immunofluorescence image showing the down-regulation of REST (green) in myometrial SMCs migrating into the wound. (F) siRNA knockdown of REST leads to accelerated wound healing in myometrial SMCs.
Fig. S7. REST target genes are overexpressed in leiomyomas. (A) Expression of known REST-related genes in uterine fibroids from dataset GSE13319, pathway analysis (using IPA; Ingenuity Systems). Note that REST mRNA expression is unchanged. (B) Gene expression analysis using Affymetrix microarray (U133 Plus 2.0, GEO dataset GSE41386) show that siRNA knockdown of REST in myometrial SMCs leads to overexpression of REST-repressed targets. (C) REST-related gene networks are dysregulated in uterine fibroids (dataset GSE13319, pathway analysis using IPA).