Loss of the repressor REST in uterine fibroids promotes aberrant G protein-coupled receptor 10 expression and activates mammalian target of rapamycin pathway

Binny V. Varghese, Faezeh Koohestani, Michelle McWilliams, Arlene Colvin, Sumedha Gunewardena, William H. Kinsey, Romana A. Nowak, Warren B. Notchick, and Varghese M. Chennathukuzhi

Departments of *Molecular and Integrative Physiology and 3Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS 66160; and 1Department of Animal Sciences, University of Illinois, Urbana–Champaign, IL 61801

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Uterine fibroids (leiomyomas) are the most common tumors of the female reproductive tract, occurring in up to 77% of reproductive-aged women, yet molecular pathogenesis remains poorly understood. A role for atypically activated mammalian target of rapamycin (mTOR) pathway in the pathogenesis of uterine fibroids has been suggested in several studies. We identified that G protein-coupled receptor 10 (GPR10, a putative signaling protein upstream of the phosphoinositide 3-kinase–protein kinase B/AKT–mammalian target of rapamycin (PI3K/AKT–mTOR) pathway) is aberrantly expressed in uterine fibroids. The activation of GPR10 by its cognate ligand, prolactin, prolactin releasing peptide, promotes PI3K/AKT–mTOR pathways and cell proliferation specifically in cultured primary leiomyoma cells. Additionally, we report that RE1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF), a known tumor suppressor, transcriptionally represses GPR10 in the normal myometrium, and that the loss of REST in fibroids permits GPR10 expression. Importantly, mice overexpressing human GPR10 in the myometrium develop myometrial hyperplasia with excessive extracellular matrix deposition, a hallmark of uterine fibroids. We demonstrate previously unrecognized roles for GPR10 and its upstream regulator REST in the pathogenesis of uterine fibroids. Importantly, we report a unique genetically modified mouse model for a gene that is misexpressed in uterine fibroids.

In a focused effort aimed at finding putative signaling molecules upstream of mTOR that may regulate cell growth and tumorigenesis in leiomyomas, we identified GPR10 as the most highly upregulated G protein coupled-receptor (GPCR) in human fibroid samples. GPR10, also known as the prolactin releasing hormone receptor (PRLHR), is the receptor for prolactin releasing peptide (PrRP), although evidence for its specific role in pituitary PRL production is tenuous (16, 17). Normal expression and function of GPR10 have been shown to be limited to several regions of the hypothalamus (18, 19). The mechanism of hypothalamic GPR10 action that evokes stress hormone release (19) from the pituitary is not well understood and its function in the periphery, either in normal tissues or in disease, has not been reported. The near-ubiquitous overexpression of GPR10, a normally neuronal specific G protein coupled receptor (GPCR), in human leiomyomas is unparalleled among genes shown to be dysregulated in fibroids. This increased expression of GPR10 in uterine fibroids is particularly relevant because its activation by PrRP has been shown to promote the proliferation of cultured cells (20).

The RE1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) has been shown to transcriptionally repress GPR10 in cell lines (21). Particularly relevant to the role that PI3K/AKT appears to play in the pathogenesis of leiomyomas, the activation of GPR10 as well as the loss of REST/NRSF, is shown to trigger PI3K/AKT signaling and proliferation in tumor cell lines (20, 22–24). REST is an important transcriptional repressor that silences a multitude of genes in the periphery through epigenetic mechanisms (25). The loss of this tumor suppressor through proteasomal degradation has been shown to result in oncogenic transformation of human mammary epithelial cells (24). Mutations or alternative splicing that result in dominant negative forms of REST have also been associated with lung and colon cancers (24, 26, 27). A tumor suppressor role for REST in the uterus has not been described previously. We demonstrate that the loss of REST permits overexpression of GPR10 in leiomyomas and this misexpression functionally promotes tumor cell proliferation contributing to the pathogenesis of uterine fibroids. Our study describes unique roles for GPR10 and for the loss of REST in the development of fibroids.


The authors declare no conflict of interest.

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Data deposition: The Affymetrix gene expression data from the siRNA knockdown experiments reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE41386).


1To whom correspondence should be addressed. E-mail: vchennathukuzhi@kumc.edu.

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Results

Aberrant Expression of GPR10 in Uterine Fibroids. In an effort to identify drug targetable, signaling molecules upstream of the mTOR pathway that might be aberrantly regulated in uterine fibroids, we analyzed gene expression profiling data available from the GEO database. Analysis of human leiomyoma and normal myometrial samples from dataset GSE13319 revealed that GPR10, a GPCR with known functions in PI3K/AKT pathway activation, was the most highly dysregulated GPCR in fibroids (Fig. 1A). TaqMan RT-PCR was used to confirm the expression of GPR10 mRNA in fibroids (Fig. 1B). Expression of GPR10 was significantly up-regulated in 13 out of 14 patient samples. The aberrant expression of GPR10 was further confirmed by Western blotting (Fig. 1C). Expression of the receptor protein was evident in all of the fibroid tissue samples tested, whereas expression was either absent or negligible in matched normal myometrial tissues (Fig. 1C). Because fibroid tumor growth is known to be associated with activation of the PI3K/AKT pathway leading to the activation of mTOR, we compared the expression of GPR10 to the status of AKT phosphorylation. The AKT phosphorylation levels in individual patient samples reflected the level of expression of GPR10 in those samples (Fig. 1D), indicating that the aberrant expression of GPR10 and PI3K/AKT pathway activation may be related.

Activation of GPR10 Leads to PI3K/AKT–mTOR Pathway Activation and Leiomyoma Cell Proliferation. Next we tested whether the activation of GPR10 leads to regulation of the PI3K/AKT–mTOR pathways in primary myometrial and leiomyoma SMCs cultured in vitro. Abundant expression of GPR10 was maintained in cultured leiomyoma cells (Fig. 2A, LC1–LC3), whereas in normal myometrial SMCs GPR10 expression was negligible (Fig. 2A, MC1–MC3). Persistence of differential GPR10 expression in cultured primary cells enabled us to study the effect of its activation by PrRP. The cells were serum starved overnight and were then treated with 1 μM PrRP-31 peptide for up to 1 h and analyzed by Western blotting. Treatment of leiomyoma cells with PrRP-31 peptide resulted in robust phosphorylation of AKT within 1 h (Fig. 2B), whereas phosphorylation of AKT was not affected by PrRP treatment in myometrial cells. Knockdown of GPR10 in leiomyoma cells using siRNA resulted in the loss of AKT phosphorylation upon treatment with PrRP peptide (Fig. 2 C and D and Fig. S1), indicating that the effect of PrRP is transduced by the activation of GPR10. Furthermore, addition of 1 μM PrRP to cultured leiomyoma cells resulted in the mobilization of intracellular Ca2+ (Fig. S2). Functional coupling of GPR10 to G-protein subunit Gq and to intracellular calcium is known to occur (28). Familial mutations to the GPR10 gene resulting in altered calcium homeostasis have also been reported (29).

We hypothesized that the activation of GPR10 and ensuing AKT phosphorylation in leiomyoma cells may trigger the mTOR pathway that is usually activated in uterine fibroids. Leiomyoma SMCs treated with the ligand PrRP showed increased mTOR phosphorylation at Ser2448 and activation of p70S6K and 4EBP1 (Fig. 2E). Phosphorylation of mTOR, p70S6 kinase (p70S6K), and 4E-binding protein 1 (4EBP1) were unchanged in normal SMCs treated with PrRP. These results indicate that GPR10 activation may trigger the mTOR pathway specifically in leiomyoma cells. Activation of GPR10 in leiomyoma cells had significant mitogenic effects compared with that in normal myometrial cells in culture as indicated by BrdU incorporation (Fig. 2F). Under serum starvation, vehicle-treated control myometrial and leiomyoma SMCs showed equivalent BrdU incorporation in 24 h compared with vehicle-treated cells (Fig. 2F). The mitogenic effect of PrRP on myometrial cells was significantly lower and reflected the lower level of GPR10 expression (Fig. 2F, Inset).

Transgenic Overexpression of hGPR10 in Mouse Myometrium Leads to Leiomyoma Phenotype. Activation of aberrantly expressed GPR10 triggering PI3K–AKT–mTOR pathways and leiomyoma cell
expression. Values are average from the two male founder lines (TG2, TG3, lanes 2 and 5 in WT. (Fig. 3).

Using histomorphometry, total myometrial area of 20 cross-sections CaBP9K estimates is plotted. (Fig. 3).

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expression to myometrium (30) to drive simian virus 40 (SV40) large T-anti- 

overexpression of hGPR10 in the myometrium would result in 

uterine cross-sections using anti-GPR10 antibodies showing myometrial ex- 

tions showing excessive collagen deposition (blue) in the transgenic tissue. We stained sections of the uteri also 

showing hGPR10 

hGPR10 transgenic mice. Control experiments 

were normal in the transgenic mice. Our preliminary data also 

indicated that the female transgenic mice expressing hGPR10 in the 

myometrium have severely reduced fertility. (Fig. 3L).

Loss of REST Permits GPR10 Expression. An earlier report suggested that REST/NRSF-mediated repression precludes peripheral GPR10 expression (21). Because GPR10 expression in leiomyomas is widespread and abundant, we tested the status of REST/ NRSF expression in uterine fibroid samples. Interestingly, REST/ NRSF mRNA expression was unchanged in leiomyomas compared with that in normal myometrium. The results also corroborated the expression data for GPR10 in the REST GSE13319. Ubiquitinylation and proteasomal degradation of REST mediated by beta-transducin repeat containing protein (β-TRCP), contributing to oncogenic transformation in cell lines, has been reported recently (23). REST protein levels, determined by Western blotting, indicated that compared with normal myo-

mertial tissues, patient-matched leiomyoma samples expressed markedly reduced levels of the protein (Fig. 4A and Fig. S5). This result was further confirmed by immunofluorescence staining of REST in tissue sections from normal myometrium and leiomyo-

mas (Fig. 4B, panels 1–4). Normal myometrial samples showed intense REST staining, whereas the fibroid samples showed lower levels of REST. Further, in leiomyoma samples the residual level of REST protein present was predominantly cytoplasmic (Fig. 4B, panels 4 and 6), suggesting that REST-mediated repression of a multitude of genes could be compromised in fibroids. Our results thus indicated a potentially unique role for REST, a major transcriptional repressor and known tumor suppressor, in the pathogenesis of fibroids. Additionally, GFF-REST protein expression from a transfected construct was less stable in leiomyoma cells compared with that in myometrial cells (Fig. S6 A and B). This led us to test whether REST corepressor 1 (CoREST) or β-TRCP, known regulators of REST, were dysregulated in uterine fibroids. Our data indicated that the levels of expression of Co-REST and β-TRCP were not significantly altered in fibroids (Fig. S6 C and D). Altered protein–protein interactions of REST

proliferation in vitro convinced us to test whether transgenic overexpression of hGPR10 in the myometrium would result in a fibroid-like phenotype in vivo. We used a proximal promoter sequence from the rat calbindin-D9K (CaBP9K) promoter that has been reported to drive simian virus 40 (SV40) large T-antigen expression to myometrium (30) to drive hGPR10 cDNA expression (Fig. 3).

We obtained three founder mice (one female, two male) following pronuclear injection of the transgenic construct as determined by PCR (Fig. 3B). All three of the mouse lines expressed hGPR10 in the uterus (Fig. 3C). Expression of the transgene mRNA was specific to the myometrial compartment of the uterus as indicated by in situ hybridization using a locked nucleic acid (LNA) probe (Fig. 3J). Additionally, immunofluorescence microscopy using anti-GPR10 antibodies demonstrated myometrial expression of GPR10 protein in the transgenic mice (Fig. 3K). Interestingly, histomorphometric analysis of uterine cross-sections revealed that the transgenic mice had a twofold increase in the myometrial cross-sectional area compared with littermate WT controls (Fig. 3D). Increase in overall uterine thickness was noticeable in all of the adult transgenic mice compared with estrus cycle stage matched control littersmates (representative cross-sections at equal magnification are shown in Fig. 3J). We further analyzed the uterine cross-sections for the number of mitotic cells present using Ki-67 antigen immunofluorescence staining. Average numbers of Ki-67 positive cells per cross-section in 3- to 5-mo-old WT and the transgenic mice were 1.42 ± 0.99 and 6.17 ± 2.29, respectively. Extent of mitotic activity present in the transgenic myometrium was comparable to the reported level of mitotic activity in uterine leiomyomas (31).

Because uterine leiomyomas are known to be associated with increased TGFB signaling and with altered expression of a number of genes that encode proteins of smooth muscle cells and the extracellular matrix, we tested the expression of collagens Co- 

lla1 and Col3A1, Tgf3, dermatopontin (Dpt), and alpha smooth muscle actin (Acta2) in uteri from 2- to 4-mo-old mice. Our results indicated that the transgenic mice expressed significantly higher levels of Col1A1, Col3A1, Tgf3, and Acta2 and a lower level of Dpt compared with WT mice (Fig. 3E–I). Dysregulation of these genes in the CaBP9K-hGPR10 mice represent a phenotype similar to leiomyomas. We stained sections of the uteri also

littermates of 6-mo-old mice using Masson’s trichrome to visualize collagen in the extracellular matrix. Collagen deposition in the transgenic uterus was dramatically increased compared with that in the uterus of wild-type littermates (Fig. 3L).

Myometrial tissue sections from transgenic mice stained with hematoxylin and eosin were morphologically identical to those of human leiomyoma samples (Fig. S3). In addition, uteri from 6- to 9-mo-old transgenic mice contained SMC tumors with excessive collagen deposition (Fig. S4). Histological sections of the ovaries were normal in the transgenic mice. Our preliminary data also indicated that the female transgenic mice expressing hGPR10 in the myometrium have severely reduced fertility.
REST may mediate the expression of GPR10 in myometrial and leiomyoma SMCs. (A) Western blotting for REST in matched myometrial and leiomyoma tissues showing down-regulation of REST protein in leiomyomas (additional patient data in Fig. S5). (B) Immunofluorescence staining of myometrial and leiomyoma tissues (panels 1–4) and SMCs (panels 5 and 6) with anti-REST (green) antibody. Nuclei are stained in panels 1–4 with EthD-1 (red). (C) Chromatin immunoprecipitation of REST with GPR10 promoter and acetylated histone H3 in matched myometrial and leiomyoma SMCs. (D) Quantitative RT-PCR on REST and GPR10 mRNA in myometrial SMCs silenced with siREST and scrambled sRNA (n = 3). (E) Expression of REST and GPR10 in myometrial SMCs silenced with siREST and scrambled sRNA for 24 and 48 h. (F) Working model depicting the link between loss of REST and the overexpression of GPR10 in myometrium. Error bars indicate ± SD. *P < 0.05.

with Co-REST or with β-TRCP may also influence its repressor function or stability. Our results suggest that the mechanism of loss of REST in leiomyomas may be unique.

We carried out chromatin immunoprecipitation experiments in primary myometrial and leiomyoma SMCs to test whether REST was associated with the GPR10 promoter. Our results indicated that REST was associated with the Gpr10 promoter in normal myometrial cells (Fig. 4C). Conversely, in leiomyoma cells, the RE1 element in the Gpr10 promoter was not associated with REST but was specifically associated with acetylated histone H3, indicating that the chromatin is permissive to transcription (Fig. 4C). Additionally, sRNA knockdown of REST in normal myometrial cells led to expression of GPR10 mRNA (Fig. 4D) and protein expression (Fig. 4E), indicating that the loss of REST may mediate the expression of GPR10 in myometrial SMCs as illustrated (Fig. 4F).

Because the loss of REST could potentially lead to derepression of a large number of its targets in the periphery, we used data mining to identify additional REST target genes expressed in leiomyomas. In fact, several of the most aberrantly expressed genes in fibroids, including glutamate receptor, ionotropic, AMPA 2 (GRIA2); stathmin-like 2 (STMN2); glutamate receptor, ionotropic, N-methyl D-aspartate 2A (GRIN2A); neurofilament heavy polypeptide (NEFH); sal-like protein 1 (SALL1); secretogranin II (SG2); and cerebellin 1 (CBLN1) are known REST-repressed targets (Fig. S7A; dataset GSE13319). Pathway analysis (using IPA; Ingenuity Systems, www.ingenuity.com) of the gene expression dataset also revealed that two additional genes down-regulated in leiomyoma samples, PRICKLE1 and HBEGF with REST regulatory but not direct transcriptional target and REST (Fig. S7A). PRICKLE1 (also known as RILP for REST/NRSF-interacting LIM-domain protein) has been shown to influence nuclear localization of REST (32). The aberrant expression of a number of REST target genes in addition to GPR10 supports a role for the loss of this tumor suppressor in fibroid pathogenesis.

Because down-modulation of REST in cultured primary myometrial cells leads to GPR10 expression, we further queried whether additional REST target genes with potential functions in leiomyomas show concomitant changes in expression. Gene expression profiling using Affymetrix microarrays (U133 Plus 2.0) indicated that several REST target genes with functions in connective tissues were also up-regulated in myometrial cells after knockdown of REST (Fig. S7B, GEO dataset GSE41386). Interestingly, functional analysis (using IPA; Ingenuity Systems) of REST regulated gene networks that are altered in leiomyoma tissue samples also revealed that the loss of REST could affect a number of genes, including type 1 and 3 collagens, and the TGFβ family of proteins with crucial connective tissue functions (Fig. 5C). Our results indicate that the ectopic expression of GPR10 in the uteri of CaB9K-ΔGPR10 transgenic mice or that resulting from the loss of REST in human uterine leiomyomas could lead to identical changes in genes that are markers of uterine fibroid pathogenesis (Fig. 5E–I and Fig. S7C).

Discussion

The mechanisms that initiate uterine leiomyoma growth and pathogenesis are still not completely understood. Here we provide evidence that the loss of tumor suppressor REST and the ensuing derepression of GPR10 play a role in the pathogenesis of uterine fibroids. We propose that the degradation of REST acts as a trigger for the proliferation of quiescent SMCs in the myometrium. We further provide evidence that the aberrant expression of GPR10 and its activation lead to P38/AKT–mTOR pathway activation in uterine SMCs. Crucially, we show that transgenic mice expressing hGPR10 in the myometrium develop a uterine fibroid phenotype, validating the role of this signaling protein in the pathogenesis of fibroids in vivo.

GPR10 has central functions in stress hormone release and feeding behavior (16, 33–35). It is now widely accepted that, whereas the ligand PrRP is expressed in the periphery, normal function of the receptor GPR10 is limited to various parts of the brain (16, 36). Although GPR10 expression or function in uterine leiomyomas have not been reported previously, activation of the P38/AKT pathway by GPR10 in cultured rat pituitary tumor derived GH3 cells (22), and its influence on rat pheochromocytoma (PC-12) cell proliferation have been reported (20). We hypothesized that aberrantly expressed GPR10 may act upstream of the mTOR signaling pathway, known to be dysregulated in leiomyomas. Aberrant GPR10 expression was present in the vast majority (>90%) of leiomyoma samples in sharp contrast to other growth factor receptor pathways including insulin-like growth factor 2 pathway, that are up-regulated in roughly a third of uterine fibroids (11). The expression levels of GPR10 also reflected the extent of phospho-AKT levels in tissue samples (Fig. 1C and D), suggesting that the underlying mechanisms of GPR10 expression and P38/AKT–mTOR pathway activation in fibroids may be interrelated. Activation of Akt/mTOR and ensuing phosphorylation of the downstream targets p70S6K and 4E-BP1 are known to promote cell growth and proliferation (37–39). Results from our experiments using cultured primary myometrial and leiomyoma cells confirm that the activation of GPR10 by PrRP results in the activation of P38/AKT–mTOR pathway, mobilization of intracellular calcium, and mitogenic responses in leiomyoma cells, revealing a unique role for GPR10 in leiomyoma cell signaling and cell proliferation.
Our results indicated that transgenic mice carrying CabP9K–hGPR10 display myometrial-specific robust GPR10 expression. The transgenic mice showed increased myometrial thickness, altered uterine smooth muscle gene expression, enhanced extracellular matrix production, and fibroid tumor formation, indicating that aberrantly expressed GPR10 plays an important role in the pathogenesis of fibroids. Available literature shows that, whereas mice genetically modified for known tumor suppressors and oncogenes exist as models for uterine fibroids, this is a unique gain-of-function model for a gene aberrantly expressed in uterine fibroids.

The presence of an REI element in the promoter of GPR10 has been suggested to preclude the expression of this GPCR in nonneuronal cells (21). Thus, its widespread expression in uterine fibroids is very fascinating and suggested to us that REST/NRSF may be dysregulated in uterine fibroids. In addition to GPR10, a number of other known targets of REST including GRIA2, GRIN2A, DCX, STMN2, SGC2, SALL1, and CBLN1 were among the most significantly up-regulated genes in uterine fibroids (Fig. S7A), strongly suggesting that the function of tumor suppressor REST is severely compromised in uterine fibroids.

Our results indicated that, whereas REST/NRSF mRNA levels were comparable in normal myometrium and leiomyomas, REST protein levels were markedly reduced in leiomyomas (Fig. A and B). Fibroid cells showed decreased chromatin condensation and gene knockdown experiments (Fig. 4 C–E) confirmed that REST regulates GPR10 expression in the myometrium and that loss of REST leads to aberrant expression of GPR10 in leiomyoma cells. The loss of this master regulator of epigenetic long-term gene silencing provides a compelling mechanism for the pathogenesis of fibroids and links the activation of PI3K/AKT pathway (24) and the proposed regulation of REST target genes by estrogen (40) to uterine leiomyomas.

REST binds to a 21- to 23-bp repressor element (REI) preferentially found in ~2000 gene promoters within the human genome (41, 42). Through the recruitment of numerous corepressors and gene regulatory proteins to promoter sites, REST epigenetically silences target genes (26). Overexpression of alternatively spliced forms of REST as function that dominant negatives has also been shown to promote tumorigenesis (26, 27). Importantly, down-regulation of REST has been shown to enable gene expression that promotes vascular SMC proliferation (43). The role of REST in myometrial SMCs or its loss in the pathogenesis of uterine fibroids has not been reported previously. We found that β-TRCP, the ubiquitin ligase known to regulate REST stability, is down-regulated in fibroids (23) and Co-REST, an important regulator of REST activity (44), were expressed at comparable levels in leiomyomas and myometrium (Fig. S6 C and D). Elucidation of the exact mechanism leading to the accelerated REST degradation in leiomyomas requires further studies. The results of our wound-healing studies (Fig. S6 E and F) support a role for the loss of REST in myometrial SMC proliferation and/or cell migration. It is conceivable that the loss of REST protein triggers myometrial cell proliferation and transformation of the cells to a fibroid phenotype. Loss of REST is known to result in mitotic arrest deficient-like 1 (MAD2L2)-mediated genomic instability in tumor-derived cell lines (45). Recurring chromosomal translocations and gene mutations have also been shown to exist in uterine fibroids (46).

Because REST is known to interact with MED12 (47), a gene frequently mutated in fibroids (6, 48), we investigated whether such somatic mutations resulted in altered protein–protein interactions that may contribute to improper tumor suppressor function of REST. We were unable to detect altered REST–MED12 interactions or aberrant subcellular localization of MED12 in leiomyoma cells. Somatic missense mutations occurring in MED12 at high frequencies indicate upstream mechanisms that trigger initial steps of myometrial cell proliferation, considering that the adult normal myometrium is essentially quiescent. Based on our results showing the overexpression of REST target genes in leiomyomas, it will be important to test whether MED12 mutation occurs as a “second hit” that leads to the activation of these genes. Further studies are needed to determine if the loss of REST leads to MED12 mutations or if missense mutations in MED12 may influence REST function.

Pathway analysis of genes dysregulated in uterine fibroids revealed that several connective tissue genes including type 1 and type 3 collagens and TGFβ are potential downstream targets of REST and that GPR10 (PRLHR) may act as a mediator of this pathway (Fig. S7C). Results from our small interfering RNA mediated REST knockdown also indicated that several connective tissue genes were derepressed when REST is down-modulated (Fig. S7B). Because the loss of REST appears to regulate a number of connective tissue genes and pathways implicated in scar formation, we hypothesized that myometrial SMCs may down-regulate REST during the wound-healing process. In vitro wound-healing assays demonstrated that the cells migrating/proliferating into the wound area expressed significantly lower levels of REST in their nuclei (Fig. S6E). Additionally, siRNA knockdown of REST accelerated the wound healing process in cultured primary myometrial cells (Fig. S6F). Thus, the loss of REST may initiate or accelerate SMC proliferation during the pathogenesis of leiomyomas. Our finding of loss of REST in uterine fibroids could have an extraordinary impact on our understanding of uterine fibroid pathogenesis. Further studies using a REST conditional KO mouse model, currently being developed in our laboratory, will determine the specific role of this tumor suppressor in the pathogenesis of fibroids. It may also be important to understand whether accumulation of mutations in MED12, TSC2 (13), HMGAA2 (49, 50), or REST pathway genes occur after the loss of REST and resultant chromosomal instability. Our finding that GPR10 plays an important role as an effector downstream of REST in the pathogenesis of uterine fibroids provides an excellent opportunity for the development of brain secreting small molecule antagonists for the treatment of fibroids. The CabP9K–hGPR10 transgenic mice will facilitate preclinical development of such GPR10 antagonists.

Materials and Methods

Chemicals and Reagents. Dulbecco’s Modified Eagle’s medium (DMEM), penicillin–streptomycin, and L-glutamine were purchased from BioWhittaker. Dulbecco’s PBS modified, FBS, and bovine calf serum (BCS) were purchased from HyClone. Ethidium homodimer (EthD-1), Alexa Fluor-conjugated secondary antibodies, and collagenase were obtained from Life Technologies. Anti-GPR10 antibody (catalog no. NB81-00854, rabbit polyclonal) was obtained from Novus Biologicals. Anti-REST antibodies (catalog nos. 90-019, 05-1477, and 17-641 for Western blotting, immunofluorescence, and ChiP assays, respectively) were obtained from EMD Millipore. Phospho specific antibodies for mTOR (Ser2448), p70S6K (Thr389), and 4E-BP1 (Thr37/46) were from Cell Signaling Technology Inc. (Danvers, MA), FuGene 6 transfection reagent and colorimetric Brdu cell proliferation ELISA kit were from Roche Applied Science. Anti-CaBP9K (Abcam) siRNA oligos were obtained from Life Technologies Applied Biosystems and IDT. Locked nucleic acid probes and controls for mRNA in situ hybridizations were obtained from Exiqon. PrRP peptide was obtained from Phoenix Pharmaceuticals.

Tissue Collection and Cell Culture. Leiomyoma samples were obtained by R.A.N. under institutional review board-approved protocols from the University of Illinois at Urbana-Champaign and from Carle Hospital from premenopausal women undergoing hysterectomy at Carle Foundation Hospital (Urbana, IL). Smooth muscle cells were prepared from the samples and were cultured as described previously (51). The cells were serum starved for 24 h in the presence of 0.1% charcoal stripped FBS before treatment with PrRP (0.1–2 μM) ligand. Serum-starved cells were treated for 24 h with Brdu labeling reagent in the presence of PrRP and were analyzed using the Brdu labeling detection ELISA kit (Roche).

RNA Isolation and qRT-PCR Analyses. Total RNA was isolated from tissue samples or cultured cells stored in RNAlater (Qiagen) using RNeasy mini kit (Qiagen) according to manufacturer protocol. After quantitation using Nanodrop spectrophotometer, aliquots of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Applied Biosystems). TaqMan assays for PREL1/P9GPR10 (Hs00244465_s1; ABI), Co1A1 (Mm.PT.47.6999992; IDT), Col3A1 (Mm.PT.47.7777198; IDT), Acta2 (Mm.PT.47.7024549; IDT), Tgfβ3 (Mm.PT.10648587; IDT), and Dpt (Mm.PT.47.17098032; IDT) were used to quantify gene expression using the delta CT method in comparison with 18s.

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Generation of CaBP9K–hGPR10 Transgenic Mice. The rat calbindin D9K (−117 to +365 nt) promoter and the ORF of hGPR10 cDNA were used to generate the transgenic construct. Further details about the CaBP9K–hGPR10 mouse model are provided in SI Materials and Methods. The transgenic founder mice were bred to C57BL/6J females to obtain hemizygous transgenic mice used in the study.


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_002448) 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 AvaI 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 TGC GAT GAC GCC ATT TTT CTC CTC CCC ACA TCA TC–3′. The PCR product was cloned into a vector upstream of the AvaI site of GPR10 as shown in Fig. S3. 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 antibodies SSC, incubated with alkaline phosphatase CTG CTC CTC CCC ACA TCA TC. A pool (L-006466-00-0005; Dharmacon, GEO dataset f6 o f6 bp 80466) to amplify a 119-bp μP using DharmaFECT REST. CAC CAG CTG CAG GCT CTT GGC TGC end and were designed based on the genomic sequence hGPR10 transgenic mouse mice. We obtained three

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 0.1% fetal bovine serum (FBS) overnight followed by GPR10 ligand treatment proline releasing peptide [(PrRP) 1 μM] in FBS 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). SMCs were transfected 12 h after plating and RNA and protein expression were analyzed 24 h and 48 h after transfection. The results were further confirmed by using ON-TARGETplus SMARTpool (L-006466-00-0005; Dharmacon, ThermoFisher) siRNAs to REST. Transfection reagents and controls were the same as described above for GPR10.

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-fuchsin, 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 proteinase-K (25 μg/mL in PBS) at 37 °C for 15 min followed by three washes in diethyl pyrocarbonate-treated water. The sections were treated at 60 °C for 5 min with digoxigenin-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 digoxigenin-labeled locked nucleic acid antibodies (37 °C, 1 h) followed by nitro-blue tetrazolium/3-bromo-4-chloro-3-indophenol reagent to visualize target mRNA. Custom LNA antisense probe for hGPR10 (probe sequence: 5DiG/NACG TTC AGG AGG AGG ATG ACCA3/Dig_N) or custom LNA control sense probe (probe sequence: 5DiG/NTTG TCA TCC TCC TCT TCT ACTGCT/3Dig) was used for the hybridization. Additional LNA mRNA detection control probe (product number 300514-15, 5DiG/NTTG TAA GTC GTC CAT ACCG 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 cells 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 80348–80466) to amplify a 119-bp PCR product. The primer sequences were: 5′ CTG GCC TGC AGC GCC CTC A and 5′ CTG 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 ≥2.5, P value ≤0.05) differentially expressed genes between myometrial cell siREST and myometrial control cell (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

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were incubated (37 °C, 5% CO₂) 18 h after wounding, and chamber slides were formalin fixed and analyzed by immunofluorescence using anti-REST antibodies. In the experiments in which REST/NRSF 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 ([Ca²⁺] as described earlier) (6). PrRP (1 μM) or vehicle (PBS) was added during the live confocal imaging of the cells.


![Graph](image-url)

**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. S5. 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).