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[a], Faezeh Koohestani[a], Michelle McWilliams[a], Arlene Colvin[a], Sumedha Gunewardena[a], William H. Kinsey[b], Romana A. Nowak[c], Warren B. Nothnick[a], and Vargheese M. Chennathukuzhi[a,1]

Departments of [a]Molecular and Integrative Physiology and [b]Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS 66160; and [c]Department of Animal Sciences, University of Illinois, Urbana–Champaign, IL 61801

Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved November 28, 2012 (received for review September 12, 2012)

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 releasing peptide, promotes PI3K–AKT–mTOR pathways and cell proliferation specifically in cultured primary leiomyoma cells. Additionally, we report that RE1 suppressing 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.

Uterine fibroids (leiomyomas) are benign smooth muscle cell (SMC) tumors of the myometrium. Leiomyomas represent the most frequent clinical indication for hysterectomy that prematurely ends a woman's reproductive capability (1). However, despite this, there are currently no approved drugs that can provide effective, long-term treatment for these tumors. In the year 2010, the estimated annual cost of uterine fibroid tumors in the United States was $5.9–34.4 billion (2). Although uterine fibroids are the most common tumors of the female reproductive tract and a major quality of life issue for a significant percentage of women, the mechanisms that initiate leiomyoma growth and pathogenesis are still not well understood.

Several genetic and environmental risk factors have been suggested to influence the etiology of leiomyomas (1, 3–5). Although, rare germ-line mutations that give rise to heritable leiomyomas are known, such mutations being rare do not account for the vast majority of uterine fibroids. The occurrence of high-frequency somatic missense mutations in the second coding exon of the mediator gene *MED12* was reported in fibroid tumors in a recent study (6). The functional significance of missense mutations to this mediator complex protein and its developmental origins are not currently known. Alternative mechanisms including epigenetic variations, microRNA-mediated posttranscriptional gene regulation, and altered cell signaling have recently gained traction as putative disease initiating events (7–12). Several recent studies have demonstrated a central role for a dysregulated phosphoinositide 3-kinase–protein kinase B/AKT (PI3K/AKT) pathway leading to the activation of mammalian target of rapamycin (mTOR) in the pathogenesis of leiomyomas (13–15). There is a need to identify signaling molecules upstream of mTOR that differentially activate this pathway in fibroids compared with normal myometrium.

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.

Author contributions: V.M.C. designed research; B.V.V., F.K., M.M., A.C., S.G., W.H.K., R.A.N., W.B.N., and V.M.C. performed research; R.A.N. contributed new reagents/analytic tools; B.V.V., F.K., M.M., A.C., S.G., W.H.K., W.B.N., and V.M.C. analyzed data; and B.V.V., F.K., M.M., S.G., W.H.K., R.A.N., W.B.N., and V.M.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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).

See Commentary on page 1980.

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

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1215759110/-/DCSupplemental.

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. 1*A*). TaqMan RT-PCR was used to confirm the expression of GPR10 mRNA in fibroids (Fig. 1*B*). 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. 1*C*). 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. 1*C*). 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. 1*D*), 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. 2*A*, LC1–LC3), whereas in normal myometrial SMCs GPR10 expression was negligible (Fig. 2*A*, 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. 2*B*), 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 Ca^{2+} (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. 2*E*). 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. 2*F*). Under serum starvation, vehicle-treated control myometrial and leiomyoma SMCs showed equivalent BrdU incorporation. Leiomyoma SMCs treated with 2 μM PrRP showed a greater than 2.5-fold increase in BrdU incorporation in 24 h compared with vehicle-treated cells (Fig. 2*F*). The mitogenic effect of PrRP on myometrial cells was significantly lower and reflected the lower level of GPR10 expression (Fig. 2*F*, *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

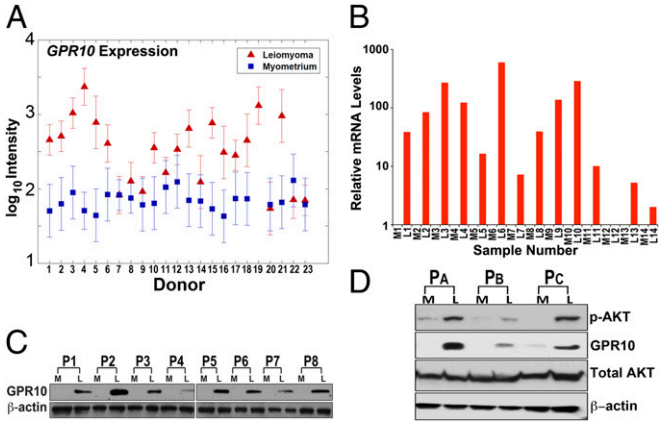

Fig. 1. GPR10 is aberrantly expressed in uterine leiomyomas. (*A*) Analysis of *GPR10* expression in leiomyoma samples compared with that in matched normal myometrial samples from the gene expression dataset GSE13319. (*B*) TaqMan qRT-PCR analysis of *GPR10* expression in 14 pairs of matched normal myometrial and leiomyoma samples. (*C*) Western blot analysis of protein extracts from patient (P1–P8) samples comparing GPR10 expression in normal myometrium to matched leiomyoma samples. *Lower* panel shows β-actin expression used as protein loading control. (*D*) Western blot analysis of phospho-AKT (Ser473) and GPR10 expression in three patient (P_A, P_B, and P_C)-matched myometrial and leiomyoma samples. Total AKT and β-actin were used as loading controls.

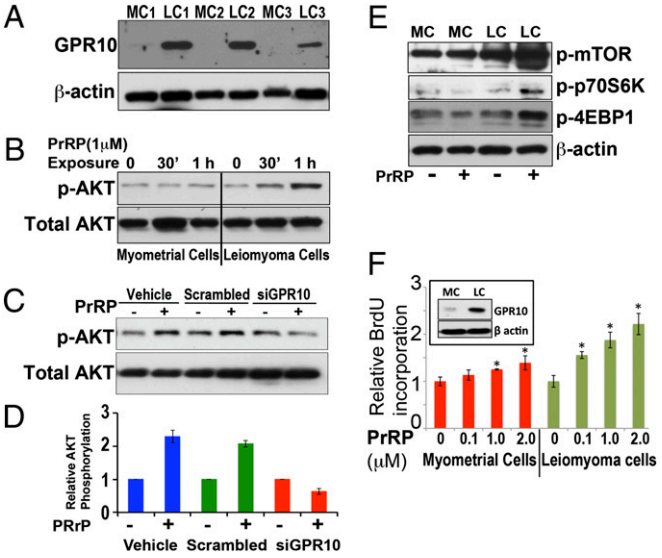

Fig. 2. Cultured primary leiomyoma cells express functional GPR10. (*A*) GPR10 expression in primary myometrial ($MC_{1–3}$) and autologous leiomyoma SMCs ($LC_{1–3}$). β-Actin was used as loading control. (*B*) Anti–phospho-AKT (s473) Western blot showing that the activation of GPR10 using PrRP (1 μM) leads to the phosphorylation of AKT in leiomyoma SMCs. Total AKT was used as loading control. (*C* and *D*) Representative anti–p-AKT (s473) Western blot and densitometric analysis of Western blots from three independent experiments, respectively, showing that siRNA knockdown of GPR10 leads to a loss of PrRP response on AKT phosphorylation. (*E*) PrRP treatment of leiomyoma SMCs leads to mTOR pathway activation. β-Actin was used as loading control. (*F*) Mitogenic effect of PrRP on myometrial and leiomyoma cells after serum starvation (0.1% FBS for 24 h followed by PrRP treatment for 24 h). Levels of BrDU incorporation in relation to vehicle-treated controls were plotted. (*Inset*) Western blot analysis of GPR10 in the same SMCs used in proliferation assay. In all Western blots, 30 μg of total cell lysates from cells in third passage were used. Error bars indicate ± SD, $*P < 0.05$.

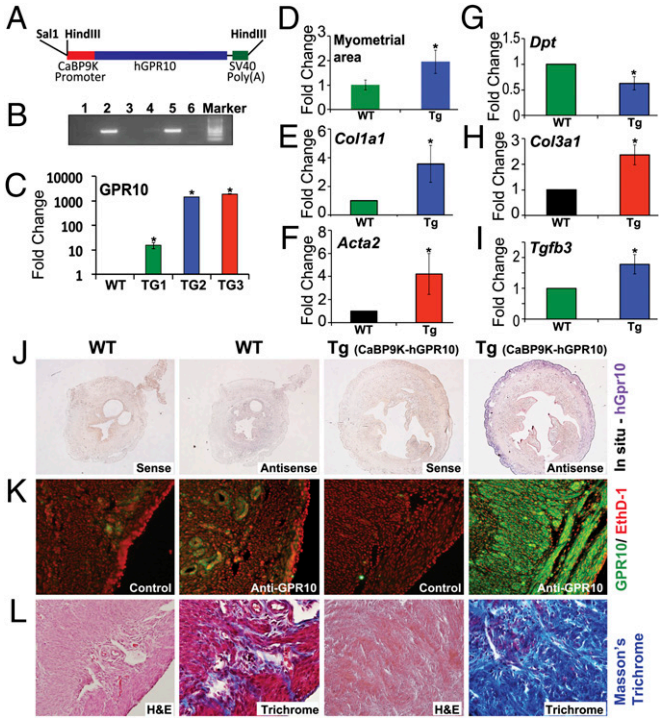

Fig. 3. Transgenic mice expressing *hGPR10* cDNA in the myometrium develop a uterine fibroid phenotype. (*A*) CaBP9K–hGPR10 transgenic construct. (*B*) PCR genotyping of transgenic founder mice showing the presence of three transgenic mice (lanes 2, 4, and 5). (*C*) TaqMan RT-PCR analysis of uterine RNA of F_0 female founder (TG1, lane 4 in *B*, 6 mo old) and F_1 females (3 mo old) derived from the two male founder lines (TG2, TG3, lanes 2 and 5 in *B*) showing *hGPR10* expression. Values are average ± SD for three mice in each group except for the F_0 female founder (TG1) where the average ± SD of three independent RT-PCR estimates is plotted. (*D*) Transverse cross-sections of uteri from 4-mo-old CaBP9K–hGPR10 transgenic mice and WT littermates (four each) were stained with H&E. Using histomorphometry, total myometrial area of 20 cross-sections in each genotype were measured and represented as relative values to that of WT. (*E–I*) TaqMan RT-PCR analysis of gene expression in the uteri from four CaBP9K–hGPR10 and four WT mice (3–4 mo old), measuring Col1A1, Acta2, Dpt, Col3A1, and Tgfb3, respectively. All samples were analyzed in triplicates. (*J*) In situ hybridization using an antisense LNA probe to *hGPR10* showing myometrial expression of the transgene. LNA oligo corresponding to the sense strand sequence was used as control. (*K*) Immunofluorescence staining of uterine cross-sections using anti-GPR10 antibodies showing myometrial expression of GPR10 (green) in the CaBP9K–hGPR10 mice. Control experiments used no primary antibodies. Sections were costained with EthD-1 (red) to visualize nuclei. (*L*) Masson's trichrome staining of uterine myometrial sections showing excessive collagen deposition (blue) in the transgenic tissue. Adjacent sections were stained with H&E for comparison. Error bars indicate ± SD, $*P < 0.05$.

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*A*).

We obtained three founder mice (one female, two male) following pronuclear injection of the transgenic construct as determined by PCR (Fig. 3*B*). All three of the mouse lines expressed *hGPR10* in the uteri (Fig. 3*C*). 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. 3*J*). Additionally, immunofluorescence microscopy using anti-GPR10 antibodies demonstrated myometrial expression of GPR10 protein in the transgenic mice (Fig. 3*K*). 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. 3*D*). Increase in overall uterine thickness was noticeable in all of the adult transgenic mice compared with estrus cycle stage matched control littermates (representative cross-sections at equal magnification are shown in Fig. 3*J*). 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 cells in uterine leiomyomas (31).

Because uterine leiomyomas are known to be associated with increased TGFβ 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 *Col1A1* and *Col3A1*, *Tgfb3*, 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*, *Tgfb3*, *and Acta2* and a lower level of *Dpt* compared with WT mice (Fig. 3 *E–I*). Dysregulation of these genes in the *CaBP9K-hGPR10* mice represent a phenotype similar to leiomyomas. We stained sections of uteri from 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. 3*L*). 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.

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 the normal myometrium. The results also corroborated the expression data for *REST* from GEO dataset 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 myometrial tissues, patient-matched leiomyoma samples expressed markedly reduced levels of the protein (Fig. 4*A* and Fig. S5). This result was further confirmed by immunofluorescence staining of REST in tissue sections from normal myometrium and leiomyomas (Fig. 4*B*, 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. 4*B*, 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, GFP-REST protein expressed 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

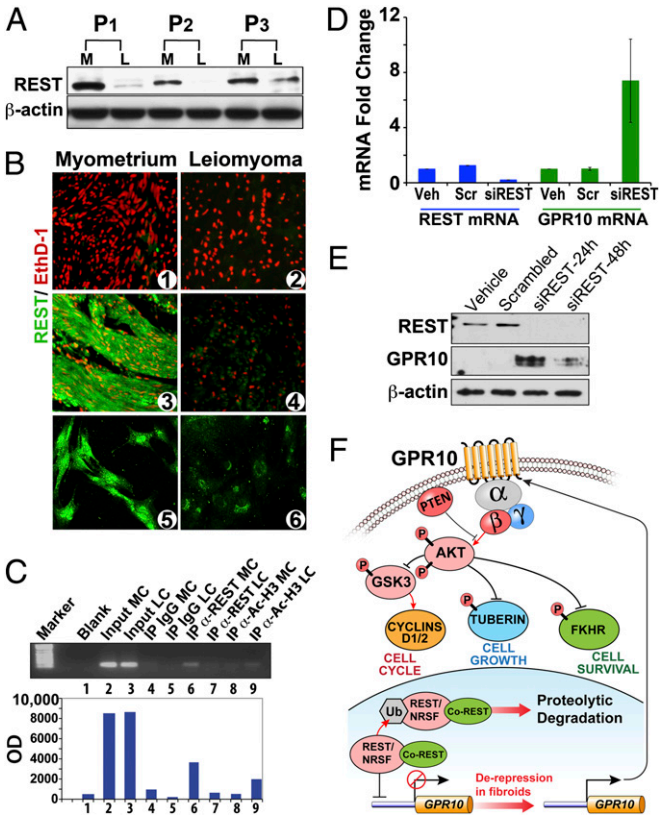

Fig. 4. Loss of REST enables GPR10 expression in leiomyomas. (*A*) Western blotting for REST in matched myometrial and leiomyoma tissues showing down-regulation of REST protein in leiomyomas (additional patient data in Fig. S5). (*B*) Immunofluoresence staining of myometrial and leiomyoma tissues (panels 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 siRNA ($n = 3$). (*E*) Expression of REST and GPR10 in myometrial SMCs silenced with siREST and scrambled siRNA 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. 4*C*). 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. 4*C*). Additionally, siRNA knockdown of REST in normal myometrial cells led to expression of *GPR10* mRNA (Fig. 4*D*) and protein expression (Fig. 4*E*), indicating that the loss of REST may mediate the expression of GPR10 in myometrial SMCs as illustrated (Fig. 4*F*).

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 (*SCG2*); and cerebellin 1 (*CBLN1*) are known REST-repressed targets (Fig. S7*A*; 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* were REST related but not direct transcriptional targets of REST (Fig. S7*A*). 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. S7*B*, 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. S7*C*). Our results indicate that the ectopic expression of GPR10 in the uteri of *CaB9K–hGPR10* 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. 3 *E–I* and Fig. S7*C*).

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 PI3K/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 PI3K–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. 1 *C* and *D*), suggesting that the underlying mechanisms of GPR10 expression and PI3K–AKT–mTOR pathway activation in fibroids may be interrelated. Activation of AKT/mTOR and ensuing phosphorylation of the downstream targets p70S6K and 4EBP1 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 PI3K/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 RE1 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*, *SCG2, SALL1*, and *CBLN1* were among the most significantly up-regulated genes in uterine fibroids (Fig. S7*A*), 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 fibroids (Fig. 4 *A* and *B* and Fig. S5). Furthermore, results from chromatin immunoprecipitation 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 (RE1) predicted to occur in ∼2,000 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 that function as 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 in tumor cells (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 (MAD2)-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. S7*C*). Results from our small interfering RNA mediated *REST* knockdown also indicated that several connective tissue genes were derepressed when REST is down-modulated (Fig. S7*B*). 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. S6*E*). Additionally, siRNA knockdown of REST accelerated the wound healing process in cultured primary myometrial cells (Fig. S6*F*). 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* (6, 48), *TSC2* (13), *HMGA2* (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 sparing 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. NBP1-00854, rabbit polyclonal) was obtained from Novus Biologicals. Anti-REST antibodies (catalog nos. 09-019, 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. TaqMan primer-probe sets and siRNA reagents 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 *PRLHR/GPR10* (Hs00244685_s1; ABI), Col1A1 (Mm.PT.47.6999992; IDT), Col3A1 (Mm.PT.47.9778198; IDT), Acta2 (Mm.PT.47.7024949; IDT), Tgfb3 (Mm.PT.47.10648587; IDT), and Dpt (Mm.PT.47.17098032; IDT) were used to quantify gene expression using the delta delta C(T) method in comparison with 18s.

Generation of CaBP9K–hGPR10 Transgenic Mice. The rat calbindin D9K (−117 to +365 nt) promoter and the ORF from 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.

ACKNOWLEDGMENTS. We thank Dr. Melissa Larson and the Transgenic and Gene-Targeting Institutional Facility, University of Kansas Medical Center for help with the transgenic mice; Stan Fernald for illustrations; and Dr. Greg Kopf for critical comments. This project was supported by National Center for Research Resources Grant P20 RR016475, National Institute of General Medical Sciences Grant P20 GM103418 from the National Institutes of Health (to V.M.C.), and Grant P01HD057877 (to R.A.N.).

1. Walker CL, Stewart EA (2005) Uterine fibroids: The elephant in the room. *Science* 308 (5728):1589–1592.
2. Cardozo ER, et al. (2011) The estimated annual cost of uterine leiomyomata in the United States. *Am J Obstet Gynecol* 206(3):211, e1–e9.
3. Stewart EA, Morton CC (2006) The genetics of uterine leiomyomata: What clinicians need to know. *Obstet Gynecol* 107(4):917–921.
4. Taran FA, Brown HL, Stewart EA (2009) Racial diversity in uterine leiomyoma clinical studies. *Fertil Steril* 94(4):1500–1503.
5. Gross KL, et al. (2004) Involvement of fumarate hydratase in nonsyndromic uterine leiomyomas: Genetic linkage analysis and FISH studies. *Genes Chromosomes Cancer* 41(3):183–190.
6. Mäkinen N, et al. (2011) MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. *Science* 334(6053):252–255.
7. Navarro A, et al. (2012) Genome-wide DNA methylation indicates silencing of tumor suppressor genes in uterine leiomyoma. *PLoS ONE* 7(3):e33284.
8. Pan Q, Luo X, Chegini N (2010) microRNA 21: Response to hormonal therapies and regulatory function in leiomyoma, transformed leiomyoma and leiomyosarcoma cells. *Mol Hum Reprod* 16(3):215–227.
9. Luo X, Chegini N (2008) The expression and potential regulatory function of microRNAs in the pathogenesis of leiomyoma. *Semin Reprod Med* 26(6):500–514.
10. Pan Q, Luo X, Chegini N (2008) Differential expression of microRNAs in myometrium and leiomyomas and regulation by ovarian steroids. *J Cell Mol Med* 12(1):227–240.
11. Peng L, et al. (2009) Expression of insulin-like growth factors (IGFs) and IGF signaling: Molecular complexity in uterine leiomyomas. *Fertil Steril* 91(6):2664–2675.
12. Wei JJ, et al. (2006) Ethnic differences in expression of the dysregulated proteins in uterine leiomyomata. *Hum Reprod* 21(1):57–67.
13. Crabtree JS, et al. (2009) Comparison of human and rat uterine leiomyomata: Identification of a dysregulated mammalian target of rapamycin pathway. *Cancer Res* 69 (15):6171–6178.
14. Cook JD, Walker CL (2004) The Eker rat: Establishing a genetic paradigm linking renal cell carcinoma and uterine leiomyoma. *Curr Mol Med* 4(8):813–824.
15. Karra L, et al. (2010) Changes related to phosphatidylinositol 3-kinase/Akt signaling in leiomyomas: possible involvement of glycogen synthase kinase 3alpha and cyclin D2 in the pathophysiology. *Fertil Steril* 93(8):2646–2651.
16. Gu W, Geddes BJ, Zhang C, Foley KP, Stricker-Krongrad A (2004) The prolactin-releasing peptide receptor (GPR10) regulates body weight homeostasis in mice. *J Mol Neurosci* 22(1–2):93–103.
17. Lin SH (2008) Prolactin-releasing peptide. *Results Probl Cell Differ* 46:57–88.
18. Lawrence CB, Celsi F, Brennand J, Luckman SM (2000) Alternative role for prolactin-releasing peptide in the regulation of food intake. *Nat Neurosci* 3(7):645–646.
19. Samson WK, et al. (2003) Prolactin-releasing peptide and its homolog RFRP-1 act in hypothalamus but not in anterior pituitary gland to stimulate stress hormone secretion. *Endocrine* 20(1-2):59–66.
20. Samson WK, Taylor MM (2006) Prolactin releasing peptide (PrRP): An endogenous regulator of cell growth. *Peptides* 27(5):1099–1103.
21. Kemp DM, Lin JC, Ubeda M, Habener JF (2002) NRSF/REST confers transcriptional repression of the GPR10 gene via a putative NRSE/RE-1 located in the 5′ promoter region. *FEBS Lett* 531(2):193–198.
22. Hayakawa J, et al. (2002) Regulation of the PRL promoter by Akt through cAMP response element binding protein. *Endocrinology* 143(1):13–22.
23. Westbrook TF, et al. (2008) SCFbeta-TRCP controls oncogenic transformation and neural differentiation through REST degradation. *Nature* 452(7185):370–374.
24. Westbrook TF, et al. (2005) A genetic screen for candidate tumor suppressors identifies REST. *Cell* 121(6):837–848.
25. Weissman AM (2008) How much REST is enough? *Cancer Cell* 13(5):381–383.
26. Coulson JM (2005) Transcriptional regulation: Cancer, neurons and the REST. *Curr Biol* 15(17):R665–668.
27. Palm K, Metsis M, Timmusk T (1999) Neuron-specific splicing of zinc finger transcription factor REST/NRSF/XBR is frequent in neuroblastomas and conserved in human, mouse and rat. *Brain Res Mol Brain Res* 72(1):30–39.
28. Langmead CJ, et al. (2000) Characterization of the binding of [(125)I]-human prolactin releasing peptide (PrRP) to GPR10, a novel G protein coupled receptor. *Br J Pharmacol* 131(4):683–688.
29. Bhattacharyya S, et al. (2003) Association of polymorphisms in GPR10, the gene encoding the prolactin-releasing peptide receptor with blood pressure, but not obesity, in a U.K. Caucasian population. *Diabetes* 52(5):1296–1299.
30. Romagnolo B, et al. (1996) Tissue-specific and hormonal regulation of calbindin-D9K fusion genes in transgenic mice. *J Biol Chem* 271(28):16820–16826.
31. Kawaguchi K, et al. (1991) Immunohistochemical analysis of oestrogen receptors, progesterone receptors and Ki-67 in leiomyoma and myometrium during the menstrual cycle and pregnancy. *Virchows Arch A Pathol Anat Histopathol* 419(4):309–315.
32. Shimojo M, Hersh LB (2006) Characterization of the REST/NRSF-interacting LIM domain protein (RILP): Localization and interaction with REST/NRSF. *J Neurochem* 96(4): 1130–1138.
33. Watanabe A, et al. (2007) Altered emotional behaviors in the diabetes mellitus OLETF type 1 congenic rat. *Brain Res* 1178:114–124.
34. Bechtold DA, Luckman SM (2006) Prolactin-releasing peptide mediates cholecystokinin-induced satiety in mice. *Endocrinology* 147(10):4723–4729.
35. Laurent P, et al. (2005) The prolactin-releasing peptide antagonizes the opioid system through its receptor GPR10. *Nat Neurosci* 8(12):1735–1741.
36. Nieminen ML, Brandt A, Pietilä P, Panula P (2000) Expression of mammalian RF-amide peptides neuropeptide FF (NPFF), prolactin-releasing peptide (PrRP) and the PrRP receptor in the peripheral tissues of the rat. *Peptides* 21(11):1695–1701.
37. Distefano G, et al. (2009) Polycystin-1 regulates extracellular signal-regulated kinase-dependent phosphorylation of tuberin to control cell size through mTOR and its downstream effectors S6K and 4EBP1. *Mol Cell Biol* 29(9):2359–2371.
38. Fingar DC, Salama S, Tsou C, Harlow E, Blenis J (2002) Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev* 16(12): 1472–1487.
39. Ruvinsky I, Meyuhas O (2006) Ribosomal protein S6 phosphorylation: From protein synthesis to cell size. *Trends Biochem Sci* 31(6):342–348.
40. Bronson MW, Hillenmeyer S, Park RW, Brodsky AS (2010) Estrogen coordinates translation and transcription, revealing a role for NRSF in human breast cancer cells. *Mol Endocrinol* 24(6):1120–1135.
41. Liu Z, Liu M, Niu G, Cheng Y, Fei J (2009) Genome-wide identification of target genes repressed by the zinc finger transcription factor REST/NRSF in the HEK 293 cell line. *Acta Biochim Biophys Sin (Shanghai)* 41(12):1008–1017.
42. Abrajano JJ, et al. (2010) Corepressor for element-1-silencing transcription factor preferentially mediates gene networks underlying neural stem cell fate decisions. *Proc Natl Acad Sci USA* 107(38):16685–16690.
43. Cheong A, et al. (2005) Downregulated REST transcription factor is a switch enabling critical potassium channel expression and cell proliferation. *Mol Cell* 20(1):45–52.
44. Lunyak VV, et al. (2002) Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* 298(5599):1747–1752.
45. Guardavaccaro D, et al. (2008) Control of chromosome stability by the beta-TrCP-REST-Mad2 axis. *Nature* 452(7185):365–369.
46. Nibert M, Heim S (1990) Uterine leiomyoma cytogenetics. *Genes Chromosomes Cancer* 2(1):3–13.
47. Ding N, et al. (2008) Mediator links epigenetic silencing of neuronal gene expression with x-linked mental retardation. *Mol Cell* 31(3):347–359.
48. McGuire MM, et al. (2012) Whole exome sequencing in a random sample of North American women with leiomyomas identifies MED12 mutations in majority of uterine leiomyomas. *PLoS ONE* 7(3):e33251.
49. Hunter DS, et al. (2002) Aberrant expression of HMGA2 in uterine leiomyoma associated with loss of TSC2 tumor suppressor gene function. *Cancer Res* 62(13): 3766–3772.
50. Velagaleti GV, et al. (2010) Fusion of HMGA2 to COG5 in uterine leiomyoma. *Cancer Genet Cytogenet* 202(1):11–16.
51. Mesquita FS, et al. (2010) Reactive oxygen species mediate mitogenic growth factor signaling pathways in human leiomyoma smooth muscle cells. *Biol Reprod* 82(2): 341–351.