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Loss of the repressor REST affects progesterone receptor function and promotes uterine leiomyoma pathogenesis

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Uterine leiomyomas (UL) are benign tumors that arise in the myometrial layer of the uterus. The standard treatment option for UL is hysterectomy, although hormonal therapies, such as selective progesterone receptor modulators, are often used as temporary treatment options to reduce symptoms or to slow the growth of tumors. However, since the pathogenesis of UL is poorly understood and most hormonal therapies are not based on UL-specific, divergent hormone signaling pathways, hallmarks that predict long-term efficacy and safety of pharmacotherapies remain largely undefined. In a previous study, we reported that aberrant expression of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes activate UL growth due to the near ubiquitous loss of REST. Here, we show that ablation of the *Rest* gene in mouse uterus leads to UL phenotype and gene-expression patterns analogous to UL, including altered estrogen and progesterone signaling pathways. We demonstrate that many of the genes dysregulated in UL harbor *cis*-regulatory elements bound by REST and progesterone receptor (PGR) adjacent to each other. Crucially, we identify an interaction between REST and PGR in healthy myometrium and present a putative mechanism for the dysregulation of progesterone-responsive genes in UL ensuing in the loss of REST. Using three *Rest* conditional knockout mouse lines, we provide a comprehensive picture of the impact loss of REST has in UL pathogenesis and in altering the response of UL to steroid hormones.

uterine leiomyoma | fibroids | progesterone receptor | REST/NRSF

Significance

Ablation of the *Rest* gene in the mouse uterus, modeling the loss of repressor element 1 silencing transcription factor (REST) in uterine fibroids, results in tumor formation and gene-expression patterns analogous to human uterine leiomyomas, including altered estrogen and progesterone receptor pathways. The current study provides a putative mechanism for the aberrant function of progesterone receptor in uterine leiomyomas.

Uterine fibroids (uterine leiomyoma, UL) are the most common reproductive tumors in women, with an annual estimated cost of $5.9 to 34.3 billion in the United States (1, 2). These tumors are found in the myometrial layer of the uterus and are characterized by increased proliferation of disordered smooth muscle cells (SMCs), enhanced estrogen sensitivity, and excessive deposition of extracellular matrix (ECM) (1, 3). Unfortunately, the heterogeneous nature of fibroids has limited our understanding of the pathogenesis of UL, and as a result hindered the development of effective pharmacotherapies (4).

Genetic, epigenetic, and chromosomal abnormalities, as well as dysregulations in key signaling pathways involved in cell proliferation, apoptosis, ECM deposition, and sex steroid hormone response have been known to play a role in UL development and growth (1, 4). Patterns of inherited genetic risk factors that predispose women to UL are extremely rare, whereas chromosomal abnormalities occur in 40 to 50% of UL tumors (5). In addition to chromosomal instability, a somatic mutation in mediator complex subunit 12 (*MED12*) has been reported to occur in up to 70% of UL, but not in the surrounding myometrium (6, 7). The molecular mechanisms that trigger these chromosomal events in the quiescent myometrial tissue are unknown.

Previously, we have shown that the aberrant widespread expression of GPR10 (PRLHR) led to uterine SMC proliferation through the activation of the PI3K/AKT-mammalian target of rapamycin (mTOR) pathway (8). PRLHR is normally repressed in the periphery by the transcriptional repressor element-1 (RE1) silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) (9, 10). REST acts as a tumor suppressor in mammary epithelial cells and is shown to be down-regulated in breast cancer, lung cancer, and colon cancer (11–14). We reported that the loss of REST protein, but not mRNA, in UL promotes aberrant expression of REST target genes (8).

Ovarian steroid hormones, estrogen, and progesterone are essential for UL tumor growth (15). An assortment of mechanisms that elevate estrogen signaling, including local estrogen production, increased expression of estrogen receptor 1 (ESR1), or progesterone receptor (PGR) and altered phosphorylation of ESR1 have been proposed as putative pathways that promote UL growth (16). Normally, progesterone suppresses ESR1 expression and cell proliferation in the myometrium through PGR (17). Molecular pathways that confer an aberrant mitogenic role for progesterone in UL are not well

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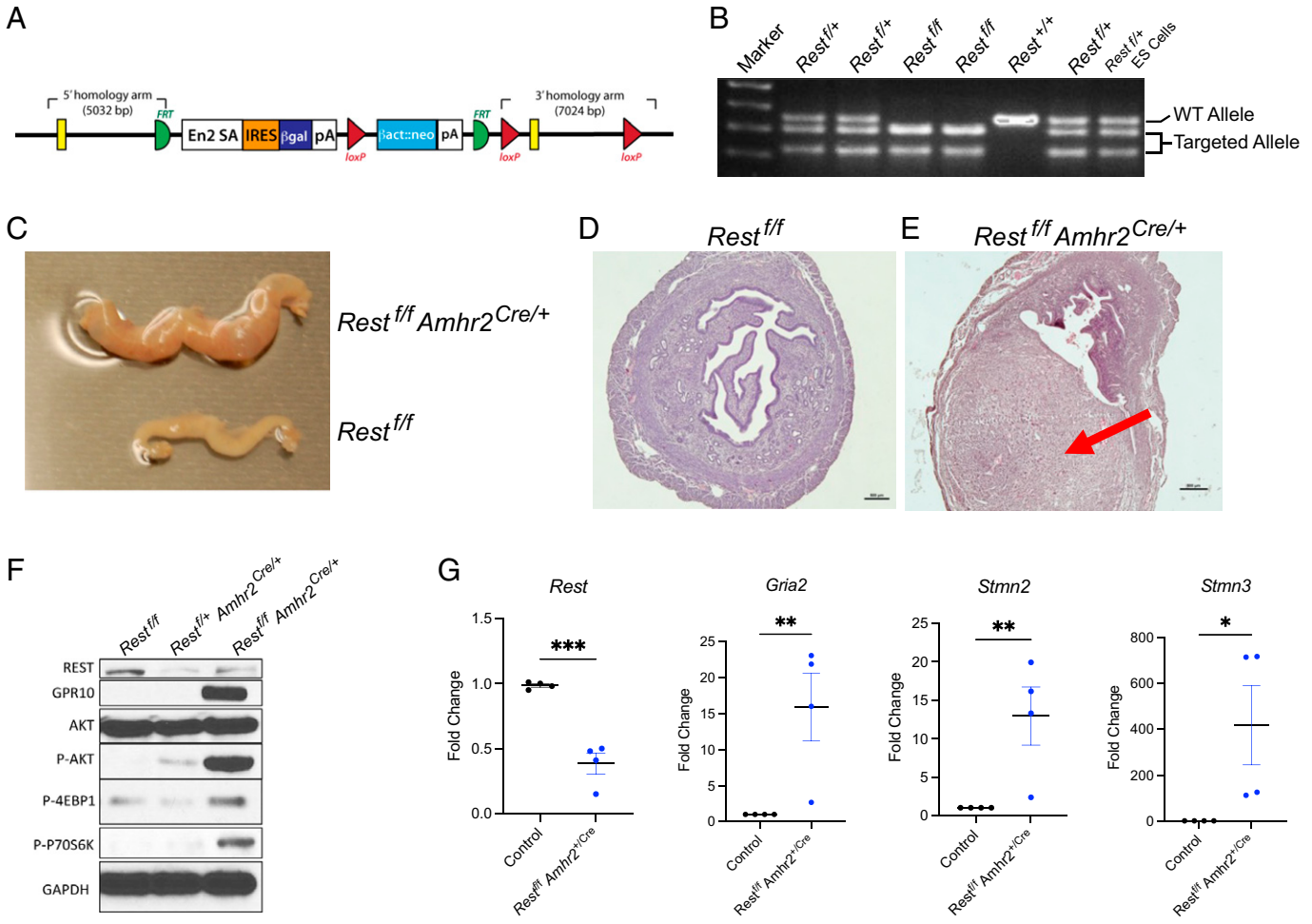

Fig. 1. Loss of *Rest* under the *Amhr2* promoter displays a UL phenotype. (*A*) *Rest*$^{f/f}$ targeting construct. (*B*) PCR genotyping of *Rest*$^{fl/fl}$ mice (lanes 4 and 5) *Rest* heterozygous mice (lanes 2, 3, and 7), and control mice (lane 6). (*C*) Representative image showing increased uterine size in 6-mo-old *Rest*$^{f/f}$ *Amhr2*$^{Cre/+}$ cKO mouse compared with control mouse during diestrus. (*D*) H&E stain of control mouse uterus. (Scale bar, 500 μm.) (*E*) H&E stain of *Rest*$^{f/f}$ *Amhr2*$^{Cre/+}$ cKO with a tumor indicated by the red arrow. (Scale bar, 500 μm.) Images are at 4× magnification. (*F*) Western blot showing increased GPR10 expression and activation of downstream tumorigenic signaling proteins in total uterine tissue of *Rest*$^{f/f}$ *Amhr2*$^{Cre/+}$ cKO mice compared with control and *Rest*$^{fl/+}$ *Amhr2*$^{Cre/+}$ mice. GAPDH was used as a protein loading control. (*G*) Gene-expression analysis of *Rest* and Rest targets, *Gria2*, *Stmn2*, and *Stmn3* in *Rest*$^{f/f}$ *Amhr2*$^{Cre/+}$ compared with control ($n = 4$). Error bars represent ± SEM. Student's *t* test was performed, *$P < 0.05$, **$P < 0.01$, ***$P = 0.0001$.

understood and may be key to developing long-term pharmacotherapies for UL in the future (18).

We developed three UL-relevant conditional knockout (cKO) mouse models to understand the role of *Rest* in the uterus. We demonstrate that the loss of REST in myometrium permits overexpression of its target genes, leading to a UL phenotype consisting of SMC tumors and excess ECM production. Additionally, we provide evidence for the presence of conserved *cis* elements associated with REST and PGR adjacent to each other and a critical REST–PGR interaction in normal myometrium, which are essential for proper regulation of progesterone responsive genes. Absence of REST–PGR interaction due to the loss of REST alters PGR function and steroid hormone response in UL. Based on the role that REST plays in steroid hormone sensitivity and UL tumorigenesis in the uterus, *Rest* cKO mouse models represent a crucial set of preclinical tools for the future development of pharmacotherapies for UL.

Results

cKO of *Rest* in Mouse Uterus Leads to Leiomyoma Phenotype. Previously, we had shown that the loss of REST protein in leiomyoma patient samples leads to derepression of its target genes, including *GPR10* (8). To investigate the loss of REST in vivo, we developed a cKO of *Rest* in the mouse uterus since the conventional *Rest* KO is embryonically lethal (19). Chimeric founder mice were generated using embryonic stem cell clones in which exon 3 of *Rest* was floxed (Fig. 1 *A* and *B*) and homozygous reproductive tract-specific *Rest*$^{f/f}$ *Amhr2*$^{Cre/+}$ cKO mice were generated, as described in *SI Appendix, Supplemental Methods*. The *Rest*$^{f/f}$ *Amhr2*$^{Cre/+}$ cKO mice had an increased uterine size compared with the control (Fig. 1*C* and *SI Appendix*, Fig. S1). Uteri of *Rest*$^{f/f}$ *Amhr2*$^{Cre/+}$ mice were consistently hypertrophic, contained cystic glands, and had abnormal uterine morphology compared with control mice (*SI Appendix*, Fig. S1). In addition, the cKO mice developed distinct fibroid tumors within the uterus that were not seen in control mice (Fig. 1 *D* and *E*). Immunostaining of mice uteri showed an increase in myometrial thickness and excessive deposition of ECM components, including collagen 3A1 (*SI Appendix*, Fig. S2).

Similar to the aberrant expression of GPR10 in a UL specimen in which REST was lost (8), Western blot analysis showed the ablation of *Rest* in the cKO mouse uterus resulted in an increase in GPR10 expression (Fig. 1*F*). In addition, the cKO mouse uteri contained higher levels of phosphorylated forms of AKT, 4EBP1, and p70S6K, which are known mediators (8, 20) of the

dysregulated PI3K/AKT-mTOR pathway in UL (Fig. 1*F*). qRT-PCR confirmed a significant decrease in *Rest* mRNA expression in the $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO mice (Fig. 1*G*). Additionally, REST target genes *Gria2*, *Stmn3*, and *Stmn2* were all significantly overexpressed in the cKO mouse (Fig. 1*G*), confirming our earlier findings that loss of REST leads to overexpression of its target genes, including *GRIA2*, *STMN2*, and *STMN3* (8). Many putative REST target genes that have known roles in the uterus and significantly dysregulated in human UL, were also dysregulated in our $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO mice, although species-specific differences in gene regulation were present (*SI Appendix*, Table S1).

To further characterize the $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO mice, gene-expression profiles of the cKO and control mice in similar estrous cycle stage (diestrus) were analyzed using RNA sequencing (GSE178141, token qrcdqusibfaxjub). Ingenuity Pathway Analysis (IPA, Qiagen) revealed significant similarities in dysregulated gene-expression profiles in human UL and the $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO mouse model, with most significant disease pathways in the KO mouse uteri being analogous to leiomyomatosis, smooth muscle tumor, benign neoplasia, and leiomyoma (Table 1). Furthermore, the gene-expression profile of the cKO mouse showed similarities to dermatological disorders (Table 1), hepatic fibrosis, and retinoic acid signaling (*SI Appendix*, Figs. S3 *A–C*), diseases of the reproductive and nervous systems, all bearing overlapping cellular and molecular characteristics with UL (21–23). Finally, the IPA confirmed REST to be down-regulated and its network of targets to be significantly dysregulated, as anticipated in the cKO model (*SI Appendix*, Fig. S3 *B* and *D*).

A highly relevant prediction of the IPA was how the loss of REST affected its upstream regulators, such as estrogen signaling (*SI Appendix*, Fig. S3*B*). IPA identified β-estradiol as a highly significant upstream regulator of genes expressed in the $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO, showing an activation of estrogen receptor signaling in the absence of REST. Indeed, the RNA sequencing on the cKO mouse uteri identified ESR1-associated genes as significantly ($P < 0.05$) dysregulated in these mice (*SI Appendix*, Fig. S4). Moreover, we observed an increase in uterine size and hypertrophy in the cKO mice during diestrus when estrogen and progesterone were low and high, respectively (Fig. 1*C* and *SI Appendix*, Table S3).

Loss of *Rest* in Mouse Uterus Leads to Changes in Uterine Architecture. To study the impact of loss of REST in the uterus, we performed single-cell RNA-sequencing using $Rest^{f/f}PR^{Cre/+}$ cKO mice, instead of the $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO mice, mainly due to the frequent embryonic lethality of *Rest* cKO due to leaky Cre expression under the *Amhr2* promoter, as reported by others (25, 26). While the complete phenotypic characterization of the $Rest^{f/f}PR^{Cre/+}$ is still ongoing, preliminary studies showed an increase in the overall uterine size in 6-mo-old mice compared with age-matched control uteri (*SI Appendix*, Fig. S5). Additionally, the uteri of the $Rest^{f/f}PR^{Cre/+}$ cKO mice were hypertrophic with cystic glands and abnormal lumen structure (*SI Appendix*, Fig. S5), similar to $Rest^{f/f}$ $Amhr^{Cre/+}$ cKO mice.

Results from Seurat single-cell RNA-sequencing data analysis (27) on uteri from 5-mo-old $Rest^{f/f}PR^{Cre/+}$ cKO mice and littermate $Rest^{f/f}$ controls (GSE178141), which passed the quality-control markers before being analyzed (*SI Appendix*, Fig. S6), identified 19 different clusters in the uterus based on their expression profiles (Fig. 2*A*). Cell types were determined by gene-expression profiles in each cluster using the SingleR software (28) and expert curation. A χ^2 test of homogeneity comparing control and $Rest^{f/f}PR^{Cre/+}$ cKO single-cell RNA-sequencing cell counts revealed significant differences in population of neutrophils, epithelial, stromal, and myometrial cells (Fig. 2*B* and *SI Appendix*, Fig. S7). The results showing an increased presence of neutrophils corroborates with reported up-regulation of these cells in human UL (29). Based on conserved gene expression, a cluster of cells representing SMCs (*Acta2*, *Cnn1*, *Myh11*, *Actg2*, *Myl9*, *Tpm2*, *Pcp4*, *Mylk*, and *Tagln*) (*SI Appendix*, Fig. S8), as well as uterine stromal, myometrial fibroblast lineage (*Slco5a1*, *Col5a2*, *Col6a3*, *Dpt*, *Ddr2*, *Adamts4*, *Ifi205*, *Tgfb2*, and *Cd34*) (*SI Appendix*, Fig. S9) were identified. Analysis of top TCA features (average cluster expression) of genes showed up-regulation of ECM components in myometrial and stromal fibroblasts, as well as SMCs (clusters 0 to 4, 8, and 17) in $Rest^{f/f}$ $PR^{Cre/+}$ mice (*SI Appendix*, Fig. S10).

Uteri of $Rest^{f/f}PR^{Cre/+}$ cKO mice showed increases in REST target gene expression, including overexpression of *Gria2* (*SI Appendix*, Fig. S11*A*), *Stmn3* (*SI Appendix*, Fig. S11*B*), and *Stmn2* (*SI Appendix*, Fig. S11*C*). Additionally, up-regulation of REST target genes was found in the stromal, epithelial, and myometrial cell clusters as represented in a violin plot (*SI Appendix*, Fig. S12). Moreover, genes downstream of estrogen receptor α (ERα) signaling, including *Mmp24* (matrix metallopeptidase 24) and *Snap25* (synaptosome associated protein 25), were activated in the $Rest^{f/f}PR^{Cre/+}$ cKO mice (*SI Appendix*, Fig. S11 *D* and *E*), indicating enhanced estrogen signaling upon the loss of REST. IPA analysis of stromal and myometrial cells (Cluster 0, fibroblast lineage) in cKO uteri showed gene-expression profiles similar to those found in benign solid tumors (*SI Appendix*, Fig. S13*A*). In addition, estrogen signaling genes were altered in this cluster (*SI Appendix*, Fig. S13*B*). IPA also identified an adenomyosis phenotype in this cluster, which needs to be further explored (*SI Appendix*, Fig. S13*C*). Finally, the $Rest^{f/f}PR^{Cre/+}$ cKO mice had higher levels of collagens and *Acta2* compared with control uteri (*SI Appendix*, Figs. S14 and S15). This phenotype is consistent with data from $Rest^{f/f}$ $Amhr^{Cre/+}$ cKO mice.

Table 1. Pathway analysis of functions or predicted diseases associated with dysregulated genes in UL and cKO mice

Diseases or functions	*P* value
Benign connective or soft tissue neoplasm	4.85E-17
Benign neoplasia	1.74E-15
Smooth muscle tumor	5.24E-14
Leiomyomatosis	1.63E-13
Benign neoplasm of female genital organ	1.99E-13
Connective or soft tissue tumor	3.34E-12
Uterine leiomyoma	8.04E-12
Skin lesion	3.65E-11

Disease predications made by IPA software based off genes which were found to be dysregulated in both human UL (GEO dataset GSE13319; $n = 23$) and RNA-sequencing results (GEO dataset GSE178141 (24); $n = 3$) of 4-mo-old $Rest^{f/f}$ $Amhr2^{+/Cre}$ cKO in diestrus.

Myometrial-Specific Deletion of *Rest* Leads to Leiomyoma Phenotype. To faithfully recapitulate the loss of REST in human UL, we next developed a cKO mouse model in which REST was specifically deleted in the myometrial layer of the uterus. Using a proximal promoter sequence from the rat calbindin-D9K (*CaBP9K*) promoter, which drives transgene expression specifically in myometrium (8, 30), we generated myometrial-specific Cre recombinase transgenic mice (CaBP9K-iCre, Myometrial-specific M-iCre or MiC) (Fig. 3). Myometrial specificity of iCre expression was confirmed by crossing MiC mice with Rosa mt/mG reporter mice (Fig. 3*B* and *SI Appendix*, Fig. S16). The

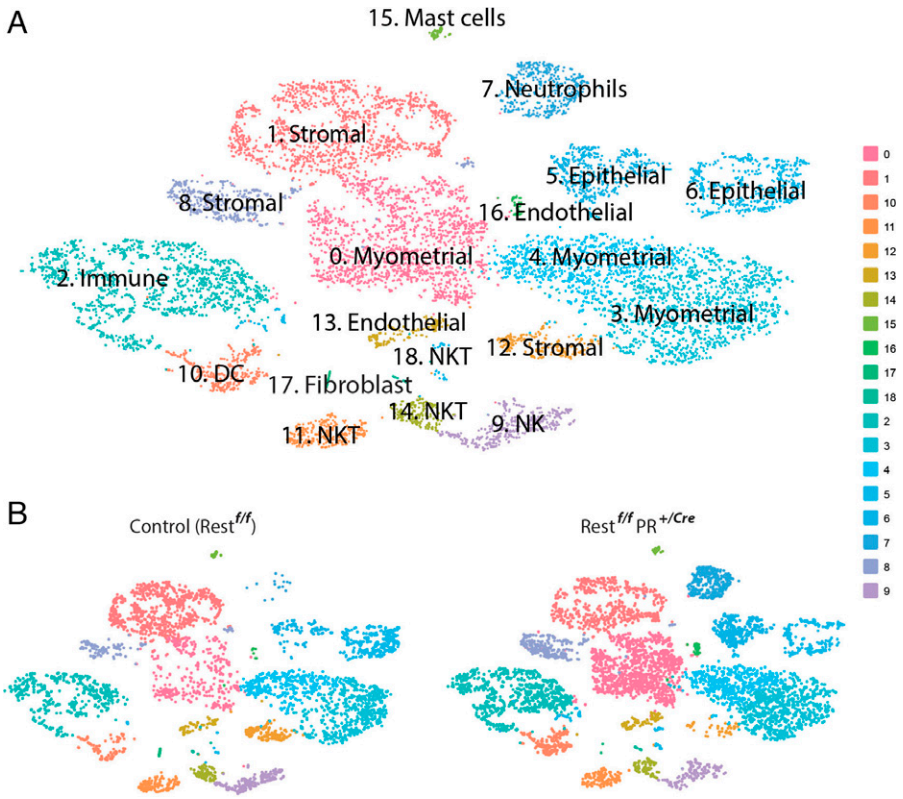

Fig. 2. Single-cell RNA-sequencing analysis. (*A*) A t-distributed stochastic neighbor embedding (t-SNE) plot of the hierarchical clustering from uteri of 5-mo-old control and *Rest*$^{f/f}$ *PR*$^{Cre/+}$ cKO mice showed 19 distinct clusters. Populations of cells included epithelial, neutrophils, mast, immune, myometrial, endothelial, natural killer (NK), and stromal cells. (*B*) Individual t-SNE plots of control and *Rest*$^{f/f}$ *PR*$^{Cre/+}$ cKO showed differences in subpopulations.

Rest cKO mouse, *Rest*$^{f/f}$ M-iCre cKO, was generated by breeding the *Rest*$^{f/+}$ *MiCre* mice with the *Rest*$^{f/f}$ mice.

Similar to the *Rest*$^{f/f}$ *Amhr2*$^{+/Cre}$ mice, the *Rest*$^{f/f}$ M-iCre cKO mice had increased uterine size, cystic glands, and abnormal uterine morphology compared with control uteri in diestrus (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S17). Additionally, we observed SMC tumors expressing αSMA, originating in the myometrial layer of the uterus of the *Rest*$^{f/f}$ M-iCre cKO mice (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S18). Furthermore, REST target genes *Stmn3*, *Stmn2*, and *Gria2* were all significantly overexpressed in the *Rest*$^{f/f}$ M-iCre cKO mouse, confirming the loss of REST function (Fig. 3*G*).

ULs are known to be associated with altered expression of genes involved in transforming growth factor (TGF)-β signaling, SMCs, and ECM components (31). Using uteri from 6-mo-old mice, we tested the expression of *Tgfb3*, dermatopontin (*Dpt*), α-smooth muscle actin (*Acta2*), and collagens *Col1A1* and *Col3A1* to determine if they contributed to the increased uterus size. Results indicated that the *Rest*$^{f/f}$ M-iCre cKO mice expressed higher levels of *Col3A1*, and statistically significantly higher levels of *Dpt*, *Col1A1*, *Tgfb3*, and *Acta2* compared with control mice (*SI Appendix*, Fig. S19). Dysregulation of these genes in the *Rest*$^{f/f}$ M-iCre cKO mice—except for *Dpt*, which showed an increase in expression unlike the decrease reported in UL—represents a phenotype similar to human UL.

REST Interacts with Progesterone Receptor. Our three *Rest* cKO models showed altered signaling, including activation of the ER pathway as well as uterine hypertrophy during diestrus (Fig. 1*C*), even when progesterone was present. To understand how REST regulated this phenotype, we investigated whether REST had a direct relationship with the steroid hormone receptors, ER and progesterone receptor (PGR). Using available chromatin immunoprecipitation-sequencing (ChIP-seq) datasets for REST, PGR-A, PGR-B, and ERα (*SI Appendix*, Table S2) [GSE62475, GSE36455 (32, 33)], we identified a high frequency of conserved RE1 sites associated with REST within 100 bp of PGR-A binding sites (Fig. 4*A*). This conserved relationship was found on roughly 200 REST target genes. Additionally, analysis of binding sites of PGR-B and REST in available ChIP-seq data identified conserved binding sites within 300 bp of each other (*SI Appendix*, Fig. S20). Interestingly, there was no overlap or proximal relationship between ERα and RE1 binding sites (Fig. 4*B*). These results indicate that the interaction between REST and PGR is unique.

Further analysis of available ChIP-seq data revealed that there were 1,598 PGR-A ChIP binding sites with at least one REST ChIP binding site in its vicinity. There were 2,215 PGR-B ChIP binding sites with at least one REST ChIP binding site in its vicinity. Of these sites, 1,135 sites were common to both PGR-A and PGR-B (Datasets S1 and S2). We analyzed the putative roles of REST/PGR-A and REST/PGR-B target genes using gene ontology (GO) enrichment analysis. First, top enriched GO-terms for genes with PGR-A/PGR-B and REST binding sites within 10k from their transcription start sites (TSSs) were plotted (*SI Appendix*, Figs. S21 and S22). Then, we analyzed the top-level GO-biological processes associated with genes having a PGR-A/PGR-B and REST binding sites within 10k from their TSSs (*SI Appendix*, Figs. S23 and S24). As expected, GO terms related to distinct pathways were enriched (significantly associated) with REST/PGR-A and REST/PGR-B regulated genes.

We next hypothesized that the absence of interaction between REST and PGR due to the loss of REST could

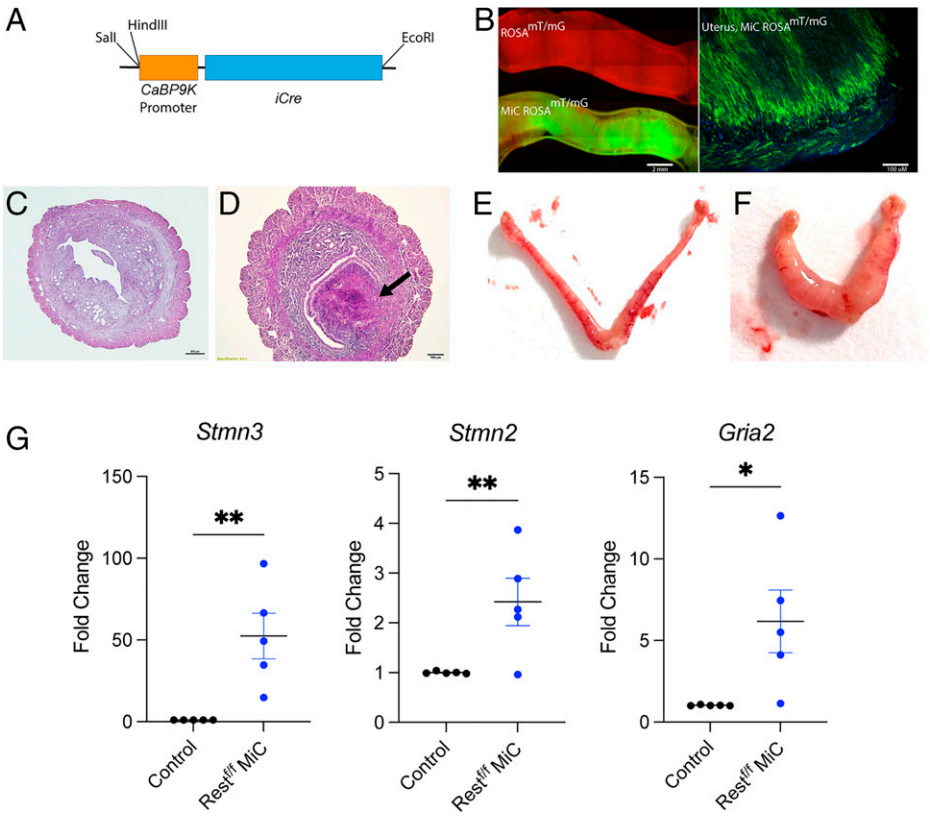

Fig. 3. Generation and characterization of *Rest* cKO mouse under a myometrial-specific iCre (MiC) ablation. (*A*) Transgenic construct for the myometrial-specific iCre expression using the proximal region of the rat *Cabp9k* promoter. (*B*, *Left Upper*) ROSA$^{mT/mG}$ mouse uterine horn expressing tdTomato. (*Left Lower*) MIC ROSA$^{mT/mG}$ mouse uterine horn expressing EGFP in the myometrium after tdTomato was removed using the myometrial-specific iCre, magnification at 4× (Scale bar, 2 mm). (*Right*) EGFP expression is specific to the mouse myometrium, magnification at 10×, (Scale bar, 100 μm). (*C*) H&E stain of uterine horn section in a control mouse. (Scale bar, 500 μm.) (*D*) H&E stain of uterine horn showing changes in morphology of *Rest$^{f/f}$* MiC mouse. Tumor formation in the *Rest$^{f/f}$* MiC mouse indicated by arrow. Magnification at 4×. (Scale bar, 500 μm.) (*E* and *F*) Representative image of increased uterus size of *Rest$^{f/f}$* MiC mouse compared with control uterus during diestrus. (*G*) Gene expression of known Rest targets *Stmn3*, *Stmn2*, and *Gria2* in 6-mo-old *Rest$^{f/f}$* MiC mice compared with control ($n = 5$). Error bars represent ± SEM. Student's *t* test was performed, *$P < 0.05$, **$P < 0.01$.

account for altered sex steroid hormone signaling seen in UL and in our mouse models. Results from coimmunoprecipitation (co-IP) studies with PGR antibodies on healthy human myometrial tissue and paired UL specimen showed association of REST with PGR in myometrial samples (Fig. 4*C*). This interaction was reduced in the leiomyoma sample where REST expression was low (Fig. 4*C*). Reciprocal IP with REST antibodies confirmed IP results with PGR antibodies (Fig. 4*D*). These results indicate that REST and PGR interact in the healthy myometrium.

To further study the interaction between REST and PGR, expression of REST–PGR target genes that contained conserved binding sites were further investigated. Many of these targets were found to be dysregulated in the *Rest$^{f/f}$ Amhr2$^{+/Cre}$* cKO mice (*SI Appendix*, Table S4). Among the top 24 dysregulated REST–PGR targets, which were also dysregulated in UL (*SI Appendix*, Table S5), the cell migration inducing hyaluronidase 1 (CEMIP, KIAA1199) contained both RE1 and PGR binding sites within 1,000 bp of each other (Fig. 5*A*). We also found CEMIP to be significantly up-regulated in human UL specimens compared with a healthy myometrial specimen (Fig. 5*B*) and correlated with low levels of REST (Fig. 5*B*). Similarly, *Cemip* was found to be overexpressed in the *Rest$^{f/f}$ Amhr2$^{+/Cre}$* cKO, and significantly overexpressed in the *Rest$^{f/f}$* M-iCre cKO mice uteri, at both the mRNA and protein levels (Fig. 5 *C* and *D*). Finally, in order to investigate the effect of the loss of REST on PGR binding to its target sequences, we performed ChIP-PCR experiments in uterine tissue from *Rest$^{f/f}$* and *Rest$^{f/f}$ PR$^{Cre/+}$* mice (three pairs each, 6-mo old). Interestingly, ChIP-PCR results indicated that in the absence of REST there was significant increase in PGR binding to a PGRE/RE1 locus upstream of the TSS and a potential trend in the same direction in a locus within the first intron of CEMIP (*SI Appendix*, Fig. S25). A detailed picture of REST-dependent PGR binding and regulation of its target genes may emerge in the future from sequencing of ChIP samples generated in the above study. Our results indicate that, upon the loss of REST, PGR binding to its target genes could be altered in a gene environment-dependent manner and may lead to aberrant expression of PGR–REST target genes. (Fig. 4*E*).

Discussion

Despite the long-recognized roles of epigenetic and environmental factors that predispose women to UL, molecular events that lead to the initiation of UL growth and pathogenesis are still not well understood (34–36). Here we provide evidence that the loss of REST, a known tumor suppressor and a major epigenetic regulator of gene expression, plays a significant role in UL pathogenesis. We provide evidence that the loss of REST function leads to aberrant expression of REST target genes that contribute to cell proliferation and ECM accumulation in the uterus. We further provide evidence that loss of *Rest* in tissue-specific cKO mouse models leads to UL formation, and changes in uterine morphology and gene-expression profiles with extensive similarities to human UL. Crucially, we show *Rest* cKO mice have altered responses to endogenous

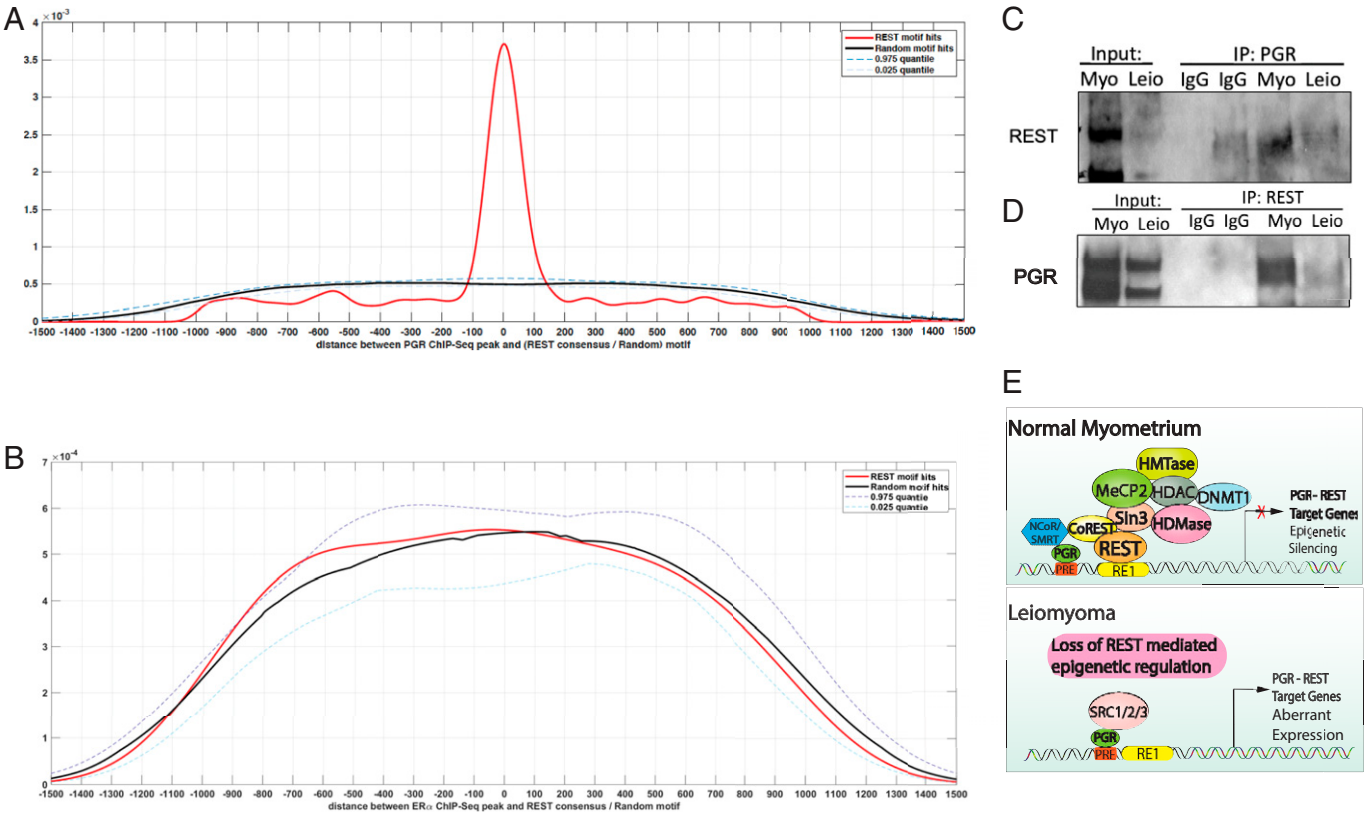

Fig. 4. REST's interactions with steroid hormone receptors. (*A*) A conserved frequency of occurrence of REST binding sites (0) within 100 bp of PGR-A binding sites. The *x* axis represents distance in base pairs and *y* axis represents density. (*B*) No overlap or proximal relationship between REST binding sites (0) and ERα binding sites. The *x* axis represents distance in base pairs and *y* axis represents density. (*C* and *D*) Representative image of co-IP of REST and PGR in human myometrial tissue and decreased interaction in leiomyoma tissue. (*E*) Working model depicting a REST–PGR interaction in the normal myometrium and loss of REST's impact in leiomyoma.

hormones, estrogen, and progesterone, due to a novel interaction between REST and PGR.

REST is a master epigenetic silencer that represses neuronal genes in nonneuronal cells (37) through binding to a 21- to 23-bp repressor element sequence (RE1 site) located on an estimated 2,000 target genes within the human genome (38). Previously, we demonstrated that loss of REST leads to derepression of *GPR10* (*PRLHR*), which plays a role in UL pathogenesis by activating the PI3K/AKT-mTOR pathway (8, 39, 40). Additionally, *PRLHR* was shown to be overexpressed ubiquitously in a UL specimen carrying wide range of known mutations or translocations (4), indicating that loss of REST may be a crucial upstream event in uterine fibroids (8). Our data demonstrate that loss of *Rest* in vivo leads to tumorigenesis in uterine SMCs and accumulation of ECM.

Preclinical models that accurately recapitulate altered steroid hormone pathways in UL, which are necessary for the development of safe and efficacious treatments for UL, are nonexistent in the field. Although Eker rats, which harbor a mutation in the *Tsc2* gene (41, 42), develop spontaneous, estrogen-sensitive UL tumors, they also develop fatal renal and liver cancers (43). Additionally, UL growth in this model can only be induced by estrogen and not progesterone (44). In addition, formation of UL in another model in guinea pig is also dependent on estrogen (45). These tumors do not have similar histological features to human UL and are inhibited by progesterone treatment (3). Although a recently developed xenograft model of UL shows tumor growth in the presence of estrogen and progesterone (46), this model is not useful to study initiation and development of UL. There is, therefore, an urgent need for UL-relevant animal models which are sensitive to estrogen and progesterone, and represent cellular, molecular, and genetic features of human UL; the $Rest^{f/f}$ M-iCre cKO and $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO mouse models fulfill this unmet need in the field.

Specifically, the *Rest* cKO mice showed increased uterine size, SMC proliferation, and ECM deposition, which are key features of human UL tumors. Similar to increased sensitivity of human UL to estrogen and the abnormal expression of estrogen-responsive genes during the luteal phase (1), the cKO mice showed increases in uterine size during diestrus when estrogen and progesterone were both present. Moreover, results from the single-cell RNA sequencing of the $Rest^{f/f}$ $PR^{Cre/+}$ cKO mice provided compelling evidence that loss of REST has significant effects on uterine tissue architecture. While $Amhr2^{Cre/+}$ and $PR^{Cre/+}$ drivers are traditionally used to delete floxed genes in the reproductive tract, we developed and utilized a myometrial-specific Cre driver to delete *Rest* in the myometrium, to further confirm a distinct role of REST in UL pathogenesis. This Cre driver (CaBP9K-iCre, M-iCre) will be invaluable for future studies involving myometrial-specific gene deletion.

Importantly, our data show that the loss of REST can lead to alterations in sex steroid hormone signaling and UL development. Our results show: 1) REST and PGR interact in the heathy myometrium, 2) loss of REST may influence PGR interaction with its target sequences, and 3) REST and PGR target genes are dysregulated in UL. One of the novel targets of REST, CEMIP, is significantly overexpressed in both human UL and in our cKO mouse models. CEMIP has been shown to induce fibrosis in arthrofibrosis (47), and its up-regulation in several cancer types is linked to cell proliferation, migration, and changes in cell

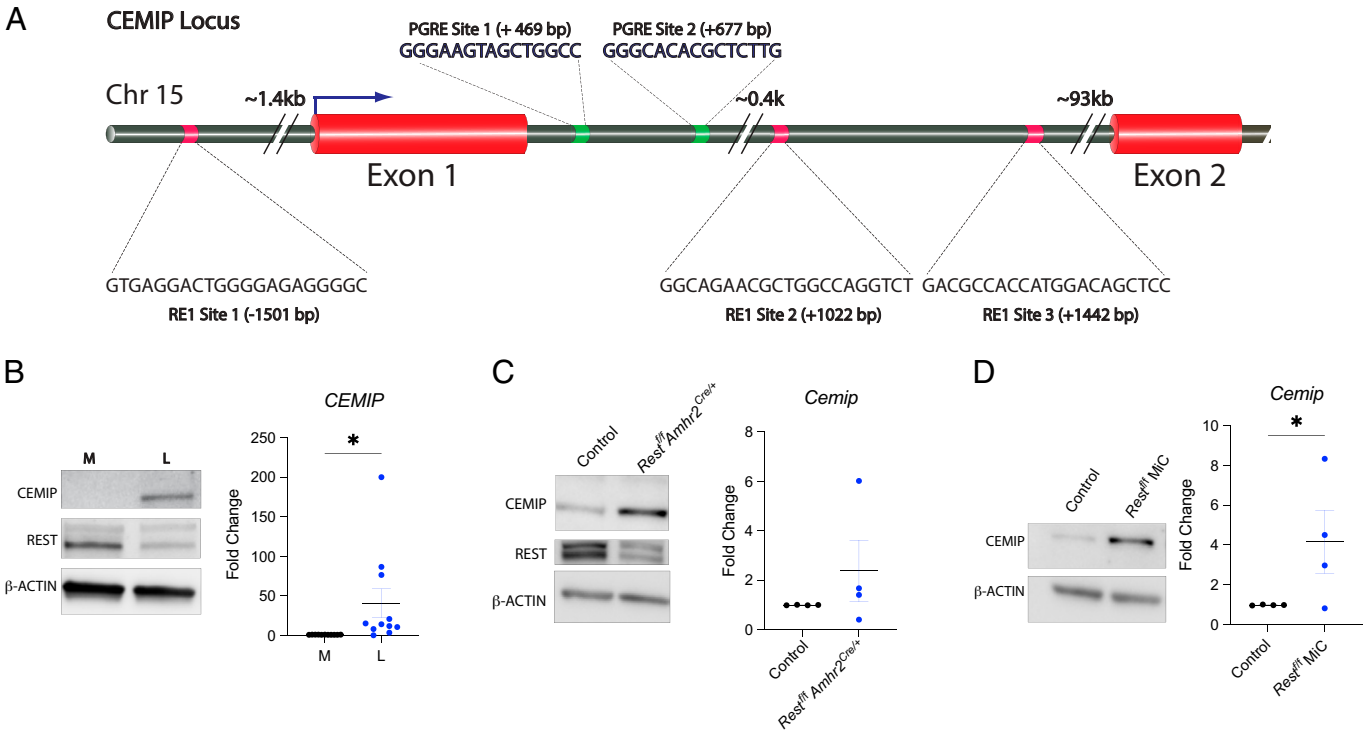

Fig. 5. Overexpression of CEMIP in uterine leiomyoma and *Rest* cKO mice. (*A*) Human *CEMIP* gene locus showing REST binding sites (RE1 sites) and PGR binding sites (PGRE), and their locations relative to the TSS (+1). (*B*, *Left*) Western blot showing CEMIP overexpression and loss of REST in leiomyoma patient samples compared with control myometrium. (*Right*) TaqMan qRT-PCR of *CEMIP* in leiomyoma patient samples compared with control myometrium ($n = 11$). (*C*, *Left*) Western blot showing CEMIP overexpression and loss of Rest in $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO compared with control. (*Right*) TaqMan qRT-PCR of *Cemip* in $Rest^{f/f}$ $Amhr2^{Cre/+}$ cKO compared with control ($n = 4$). (*D*, *Left*) Cemip overexpression shown by Western blot in $Rest^{fl/fl}$ M-iCre cKO compared with control. (*Right*) TaqMan qRT-PCR of *Cemip* in $Rest^{f/f}$ M-iCre cKO compared with control ($n = 4$). β-Actin was used as a loading control for Western blots. Error bars represent ± SEM. Student's nonparametric *t* test was performed, $^*P < 0.05$.

signaling, such as PI3K/AKT (48, 49), which is overactivated in UL. In addition, CEMIP is an HA binding protein involved in HA depolymerization (50, 51). HA is ubiquitously present in the ECM and provides structural integrity to the uterus. It has also been shown that under certain pathological conditions, HA degradation is enhanced, and the lower molecular weight molecules, resulting from HA degradation, can contribute to tumor growth and angiogenesis (52). We recently showed preferential association of REST with RE1 sites within the CEMIP locus as well as aberrant expression of CEMIP in the absence of REST in breast cancer cells (53). Based on our results showing loss of REST leads to overexpression of CEMIP, it will be important to test what role CEMIP plays in UL pathogenesis and how PGR and progesterone affect this gene.

Current hormone therapies for UL suffer from poor safety profiles, precluding them from long-term use (54, 55). Current generation of selective progesterone receptor modulators, which are developed based on PGR antagonism, reduce UL tumor size in randomized control trials (16, 56). However, side effects of endometrial hyperplasia have been reported and concerns of their effects on nontargeted tissues (breast, ovary, liver) exist (56). Our work provides a link between the altered response to progesterone and loss of REST in UL. We have found that REST interacts with PGR and influences the expression of target genes. Future studies are needed to identify isoform selective effects of REST–PGR-A and REST–PGR-B interactions in the uterus since these isoforms are known to have functionally distinct roles in the uterus. We believe this unique relationship between REST and PGR presents a mechanism that can be targeted to develop a new generation of selective progesterone receptor modulators. In addition to the myometrium, the role of REST in other progesterone-responsive tissues, including mammary epithelium and uterine endometrium, needs to be investigated in the future.

Methods

Study Approval. All human studies were approved by and performed in accordance with the University of Kansas Medical Center Policies and Procedures Relating to Human Subjects (IRB#: 5929). All patients gave informed consent when donating their tissue samples to the University of Kansas Cancer Center's Biospecimen Repository Core Facility. All mouse experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee protocols (2019-2508) and adhere to NIH guidelines for care and use of laboratory animals.

Tissue Collection and Cell Culture. Matched myometrium and leiomyoma tissue samples were obtained from premenopausal women undergoing hysterectomies at the University of Kansas Hospital (Kansas City, KS). Criteria for patients in this study exclude women undergoing hysterectomy for a primary condition other than UL and those taking hormone therapy in the 3 mo preceding surgery. Myometrial primary cells were prepared from samples as described previously (8).

Generation of Mouse Models, Gene-Expression Analysis. Details of the generation of mouse models and gene and protein expression analysis are described in *SI Appendix, Supplemental Methods*.

Data, Materials, and Software Availability. Gene expression and single-cell RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo [accession no. GSE178141 (24)].

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