CRTC1-MAML2 fusion-induced IncRNA LINC00473 expression maintains the growth and survival of human mucoepidermoid carcinoma cells

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Abstract

Mucoepidermoid carcinoma (MEC) arises in many glandular tissues and contributes to the most common malignant salivary gland cancers. MEC is specifically associated with a unique t(11;19) translocation and the resulting CRTC1-MAML2 fusion is a major oncogenic driver for MEC initiation and maintenance. However, the molecular basis underlying the CRTC1-MAML2 oncogenic functions remain very limited. Through gene expression profiling analysis, we observed that LINC00473, a long noncoding RNA (lncRNA), was the top down-regulated target in CRTC1-MAML2-depleted human MEC cells. LncRNAs belong to a new class of non-coding RNAs with emerging roles in tumorigenesis and progression, but remain poorly characterized. In this study, we investigated the role of LINC00473 in mediating CRTC1-MAML2 oncogenic activity in human MEC. We found that LINC00473 transcription was significantly induced in human
CRTC1-MAML2-positive MEC cell lines and primary MEC tumors, and was tightly correlated with the CRTC1-MAML2 RNA level. LINC00473 induction was dependent on the ability of CRTC1-MAML2 to activate CREB-mediated transcription. Depletion of LINC00473 significantly reduced the proliferation and survival of human MEC cells in vitro and blocked the in vivo tumor growth in a human MEC xenograft model. RNA in situ hybridization analysis demonstrated a predominantly nuclear localization pattern for LINC00473 in human MEC cells. Furthermore, gene expression profiling revealed that LINC00473 depletion resulted in differential expression of genes important in cancer cell growth and survival. LINC00473 likely regulates gene expression in part through its ability to bind to a cAMP signaling pathway component NONO, enhancing the ability of CRTC1-MAML2 to activate CREB-mediated transcription. Our overall results demonstrate that LINC00473 is a downstream target and an important mediator of the CRTC1-MAML2 oncoprotein. Therefore, LINC00473 acts as a promising biomarker and therapeutic target for human CRTC1-MAML2-positive MECs.

Keywords
mucoepidermoid carcinoma; CRTC1-MAML2 fusion; lncRNA; diagnostic biomarker; therapeutic target

Introduction

Human mucoepidermoid carcinoma (MEC) is a group of histologically distinct cancer arising in many glandular tissues and accounts for the most frequent epithelial subtype of salivary gland tumors\(^1\)\(^2\). MEC tumors consist of mucin-secreting cells, squamous cells, and intermediate cells with varying degrees of cyst structure. Currently, patients with advanced, metastatic MEC have limited treatment options and poor clinical outcomes. Lack of clinical trials and a limited understanding of the molecular pathogenesis of MEC are the major challenges to the development of effective therapeutics.

A high frequency of MEC cases contain a unique t(11;19) (q14-21;p12-13) translocation\(^3\)\(^-\)\(^8\), strongly suggesting that this genetic rearrangement is the driving oncogenic event in MEC pathogenesis. This translocation joins the exon 1 of the \(CRTC1\) gene at 19p13 and the exons 2–5 of the \(MAML2\) gene, creating a new \(CRTC1-MAML2\) fusion gene\(^9\). The CRTC1-MAML2 fusion contains the 42-aa CREB-binding domain (CBD) of CRTC1 and the 983-aa transcriptional activation domain (TAD) of MAML2. Previously, we demonstrated that the \(CRTC1-MAML2\) fusion has oncogenic activity based on the colony focus assays in RK3E epithelial cells in vitro. The RK3E cells transformed by CRTC1-MAML2 gave rise to tumors after being implanted subcutaneously in immunocompromised mice\(^10\)\(^-\)\(^12\). Moreover, shRNA-mediated knockdown of CRTC1-MAML2 fusion expression reduced the proliferation and survival of human MEC cells in vitro and the MEC xenograft growth\(^13\), demonstrating that the CRTC1-MAML2 fusion is essential for the maintenance of MEC tumors. Therefore, these lines of evidence suggest that CRTC1-MAML2 fusion is a key oncogenic driver for human MEC.

The \(CRTC1-MAML2\) fusion gene encodes a nuclear protein that functions as a transcriptional co-activator. A major molecular function of the CRTC1-MAML2 product is
through its interaction with the CREB transcription factor and constitutive activation of the CREB transcriptional program, which at least partially contributes to the oncogenic activity of the CRTC1-MAML2 oncogene\textsuperscript{14, 15}. The CRTC1-MAML2 fusion was also shown to interact with AP-1 and MYC that potentially contribute to MEC cell growth\textsuperscript{16, 17}. However, the essential downstream effectors for CRTC1-MAML2 transforming activity in MEC remain incompletely understood. To elucidate the downstream target genes and signaling pathways of the CRTC1-MAML2 fusion that are critical for MEC generation and progression, we have performed gene expression profiling and identified differentially expressed genes in control vs. CRTC1-MAML2 fusion-depleted MEC cells\textsuperscript{18}. Unexpectedly, a long non-coding RNA (lncRNA), LINC00473 (\textit{C6orf176}) emerged as the top differentially regulated showing the greatest down-regulation after the depletion of CRTC1-MAML2 fusion. LncRNAs are a group of transcripts with a length of over 200 nucleotides and no protein-coding potential. More than 10,000 lncRNAs were found in human genome but only a few lncRNAs have been characterized\textsuperscript{19–21}. LncRNAs are novel regulators of gene expression through diverse mechanisms\textsuperscript{22–24}. Growing evidence indicates that certain lncRNAs are associated with cancer development and progression, and could act as diagnostic biomarkers and treatment targets\textsuperscript{25–27}. However, lncRNA functions and mechanisms remain an understudied area. Currently, it remains to be determined whether LINC00473 mediates the CRTC1-MAML2 oncogenic activity and contributes to MEC tumorigenesis.

In this study, we examined the significance of LINC00473 in human CRTC1-MAML2 fusion-positive MEC. Our gene expression, functional, and molecular mechanistic studies indicate that the CRTC1-MAML2 fusion aberrantly activates expression of lncRNA LINC00473 that supports MEC cell growth and survival. Our findings identify LINC00473 as a promising target for diagnosis and therapy of human CRTC1-MAML2-positive MEC.

**Results**

**LINC00473 expression is elevated in CRTC1-MAML2 fusion-positive human MEC cell lines and primary tumors**

LncRNA LINC00473 (NR_026860, 1822 nt) was the top differentially down-regulated target (with a fold-change of $-37.12$ and $p<1\times10^{-16}$) after the depletion of the CRTC1-MAML2 fusion expression in human H3118 MEC cells in an expression profiling analysis\textsuperscript{18}. This gene resides at the chromosome 6q27 locus and encodes an intergenic lncRNA. To understand whether LINC00473 has a role in MEC, we first evaluated the expression levels of LINC00473 in both human MEC cell lines and primary MEC tumors. Western blotting confirmed the expression of endogenous CRTC1-MAML2 fusion protein in four fusion-positive human MEC cell lines (HMC3A, HMC3B and H3118 from salivary gland MECs and H292 from lung MEC), but not in the fusion-negative human pleomorphic adenoma cell line (HPA-1) (Figure 1a). Through qRT-PCR analysis, we found significantly enhanced LINC00473 expression in fusion-positive MEC cell lines but low or undetectable expression in the fusion-negative cells (Figure 1b). Furthermore, we observed significantly elevated LINC00473 expression in fusion-positive primary MEC tumors (n=6) in comparison with fusion-negative tumors (n=6) (Figure 1c). Pearson’s correlation analysis
showed that the expression levels of LINC00473 had a significant positive correlation with that of CRTC1-MAML2 (n=12, r=0.785157) (Figure 1d). Therefore, LINC00473 expression is up-regulated in fusion-positive MEC cell lines and primary MEC tumors and its level positively correlates with CRTC1-MAML2 fusion expression, indicating that CRTC1-MAML2 may up-regulate LINC00473 expression.

**LINC00473 is a novel target gene directly activated by CRTC1-MAML2 fusion**

To determine whether CRTC1-MAML2 fusion is able to induce LINC00473 transcription, we examined the effects of the depletion or over-expression of CRTC1-MAML2 fusion on LINC00473 transcript levels. Two lentiviral-based shRNAs, shM2-1 and shM2-3 that target the respective 3-untranslated region and the transcriptional activation domain (TAD) region of CRTC1-MAML2 fusion and MAML2, were used to deplete fusion/MAML2 expression in fusion-positive MEC cells and MAML2 in fusion-negative cells. We observed that the depletion of both the fusion and MAML2 expression significantly reduced LINC00473 expression in fusion-expressing H3118 and H292 MEC cell lines (Figure 2a and 2b, Supplementary Figure S1) whereas the depletion of MAML2 expression in the fusion-negative HPA-1 and HTB-41 cell lines did not affect LINC00473 expression (Supplementary Figure S2). Thus, the knockdown of CRTC1-MAML2 fusion expression decreased LINC00473 transcript levels. We next investigated whether exogenous CRTC1-MAML2 fusion could restore LINC00473 expression after the depletion of endogenous fusion. The endogenous fusion was first depleted using lentiviral-based shRNAs (shM2-1) that targeted the 3′ UTR of the CRTC1-MAML2 fusion in fusion-expressing H3118 MEC cells and then exogenous FLAG-tagged CRTC1-MAML2 was reintroduced. This exogenous CRTC1-MAML2 expression construct did not contain the 3′ UTR and consequently was resistant to shM2-1 knockdown. We observed that expression of exogenous CRTC1-MAML2 was capable of restoring LINC00473 expression in endogenous fusion-depleted cells (Figure 2c). Moreover, expression of FLAG-tagged CRTC1-MAML2 significantly increased LINC00473 transcript levels in fusion-negative HEK293T cells (Figure 2d). Therefore, these data indicate that CRTC1-MAML2 fusion positively regulates LINC00473 transcription in fusion-positive MEC.

**CRTC1-MAML2 fusion induces LINC00473 expression through CREB co-activation**

We next investigated the molecular mechanism underlying CRTC1-MAML2 regulation of LINC00473 expression. Previously, we demonstrated that the CRTC1-MAML2 fusion binds to the CREB transcription factor via the CRTC1 CBD domain and constitutively activates CREB-mediated transcriptional program via the MAML2 TAD domain. Since the LINC00473 proximal promoter contains two half-sites of cAMP-responsive element (CRE) in its proximal promoter region and is activated by CRTC-CREB signaling in lung cancer when the tumor suppressor gene LKB1 becomes inactivated, we therefore explored whether the activation of LINC00473 transcription is via the CRTC1-MAML2 co-activation of the CREB transcription factor. We found that shRNA-mediated CREB depletion significantly reduced LINC00473 expression in fusion-expressing H3118 MEC cells (Figure 3a, b). Using a LINC00473 promoter luciferase reporter containing the proximal promoter which contains the two CRE sites (−523 to +88), we found that ectopic fusion expression markedly increased the LINC00473 promoter reporter activity in fusion-negative HEK293T
cells (Figure 3c). Furthermore, we demonstrated that CRTC1-MAML2 fusion and CREB were significantly associated with the LINC00473 gene promoter region that contains the two CRE sites through chromatin immunoprecipitation (ChIP) analysis (Figure 3d). These data indicate that the CRTC1-MAML2/CREB interaction in fusion-positive MEC cells directly induces LINC00473 expression.

Knockdown of LINC00473 expression reduced the growth and survival of the fusion-positive MEC cells in vitro and in vivo

To determine whether aberrant high LINC00473 expression plays a role in regulating MEC, we inspected the consequence of LINC00473 knockdown on the growth and survival of fusion-positive MEC cells. Human H3118 MEC cells were infected with LINC00473-targeting shRNAs or scrambled control shRNA lentiviruses for 96 hours. The transduced cells were then assayed for LINC00473 depletion, cell proliferation, and apoptosis in vitro. We observed that two independent LINC00473 shRNAs (shLnc473-2 and -4) effectively knocked down LINC00473 expression and that LINC00473 knockdown decreased the proliferation and increased the apoptosis of MEC cells (Figure 4a–c, Supplementary Figure S3a–d). Similar effects were also observed in an independent fusion-positive MEC cell line HMC3A (Supplementary Figure S3e–g). Moreover, transduction of two fusion-negative, LINC00473-low cell lines (HPA-1 and HTB-41) with these LINC00473 shRNAs had no significant effects on the cell proliferation and survival (Supplementary Figure S4). Conversely, exogenous LINC00473 expression in fusion-negative HPA-1 cells moderately increased cell proliferation (Supplementary Figure S5).

We subsequently probed the functional impact of LINC00473 knockdown on the growth of H3118 MEC xenografts in vivo. The luciferase-expressing H3118 MEC cells were infected with LINC00473 shRNAs or scrambled control shRNA lentiviruses for 96 hours. The same number of LINC00473-knockdown or -control cells were next injected subcutaneously to NOD.SCID mice. The tumor growth monitoring was performed through bioluminescent imaging and direct measurement after cancer cell injection. We found that LINC00473 knockdown significantly inhibited the growth of MEC xenograft tumors as evidenced by tumor size and weight (Figure 4d–g). TUNEL IHC analysis revealed that LINC00473-knockdown MEC xenograft tumors contained an increased number of cells that were stained positive for apoptotic DNA fragmentation (Figure 4h). Therefore, these in vitro and in vivo data demonstrate that LINC00473 expression is crucial for the cell proliferation and survival programs in human CRTC1-MAML2 fusion-expressing MEC cells.

LINC00473 regulates gene expression in MEC in a transcriptomic analysis and interacts with NONO enhancing CRTC1-MAML2/CREB transcription

To explore the molecular mechanisms underlying LINC00473 regulation of cell proliferation and survival in human MEC cells, we then investigated the subcellular localization of LINC00473 in MEC cells. We performed RNA fluorescence in situ hybridization (by RNA-FISH) using LINC00473-specific oligonucleotide probes and detected 1–2 intense dot-like and relatively weak diffuse signals in the nuclear compartment of H3118 MEC cells (Figure 5a). However, the LINC00473 RNA signals were undetectable when RNase treatment was included prior to the hybridization (Figure 5a), indicating that these signals are RNA-
dependent. Moreover, RNAscope RNA ISH assays revealed positive nuclear dot-like staining in tumor cells using formalin-fixed paraffin embedded sections (FFPE) of H292 and H3118 MEC xenograft tumors (Figure 5b, Supplementary Figure S6) and primary fusion-positive MEC tumor (Figure 5c). The LINC00473 nuclear dot-like signals likely represent the polymerase firing at the LINC00473 loci, as we observed similar LINC00473 gene copy number for four fusion-positive, LINC00473-high MEC cells (H3118, H292, HMC3A and HMC3B) and one fusion-negative, LINC00473-low HPA-1 cells (Supplementary Figure S7).

To investigate whether LINC00473 regulates gene expression, we performed gene expressing profiling of LINC00473-depleted and control MEC cells to identify LINC00473-regulated genes and pathways. Briefly, human H3118 MEC cells were infected with LINC00473-targeting shRNA or scrambled control shRNA lentiviruses. At 96 hours post-infection, RNAs were isolated and subjected to the gene expression profiling analysis using Affymetrix human transcriptome array 2.0 (HTA 2.0). With the cut-off criteria of an absolute fold-change greater than or equal to 2.0 and a p-value less than 0.05, we identified a total of 645 down-regulated genes and 675 up-regulated genes in LINC00473-depleted cells (Figure 6a, b and Supplementary Table S1). We have further validated multiple LINC00473-regulated target genes by qRT-PCR (Supplementary Figure S8). Ingenuity Pathway Analysis (IPA) was carried out on the down-regulated genes to identify the biological functions and molecular pathways associated with these genes. The top 6 molecular pathways that are associated with LINC00473-regulated genes include organismal development; cell death and survival; cell growth and proliferation; cellular assembly and organization, cellular function and maintenance; DNA replication, recombination, repair, nucleic acid metabolism, small molecule biochemistry; and cell cycle (Figure 6c). Therefore, these data support that LINC00473 likely regulates specific gene programs that are essential for the growth and survival of fusion-expressing MEC cells.

LINC00473 was previously found to physically interacts with NONO protein in lung cancer cells via RNA pull-down assay. NONO is known to functionally interact with CRTC coactivators and is required for cAMP-induced activation of CREB transcription. To investigate whether LINC00473 might interact with NONO influencing CRTC-MAML2-induced CREB-mediated transcription in MEC cells, we performed RNA immunoprecipitation (RNA-IP) and observed that LINC00473, but not the negative control ASNS, was significantly enriched in the NONO immunoprecipitates (Figure 6d). Mammalian two-hybrid assays showed that LINC00473 overexpression enhanced the binding of Gal4-NONO and CRTC1-MAML2, as evidenced by enhanced Gal4 promoter reporter activities (Figure 6e). Moreover, NONO knockdown via two independent shRNAs (shNONO-1 and -3) or LINC00473 knockdown via two shRNAs (shLnc473-2 and -4) reduced the ability of CRTC1-MAML2 to activate the cAMP response element (CRE) luciferase reporter (pCRE-luc) (Figure 6f). These data strongly suggest that LINC00473 regulates gene expression in part through facilitating the interaction of CRTC1-MAML2 and NONO and promoting the activation of CRTC1-MAML2/CREB target genes.
Discussion

The discovery of the CRTC1-MAML2 fusion as the major oncogenic driver for human MEC has important implications, as it is crucial for understanding the molecular pathogenesis of human MEC and developing new approaches for MEC diagnosis and therapy. In this study, we demonstrated that the IncRNA LINC00473 is potently induced by the CRTC1-MAML2 fusion and is essential for the MEC cell growth and survival in vitro and in vivo. Our findings support a model in which the oncogenic CRTC1-MAML2 fusion interacts with and co-activates the CREB transcription factor, constitutively inducing expression of LINC00473. LINC00473 subsequently regulates a new transcriptional program that supports MEC cell proliferation and survival (Figure 6g). Our study indicates that LINC00473 could serve as a promising biomarker and therapeutic target for human fusion-positive MEC.

Emerging evidence supports the important functions of IncRNAs in multiple human cancers and their relevance in cancer diagnosis and treatment is an active area of ongoing research. LINC00473 is a cAMP/CREB target gene and can be transiently activated by enhanced cAMP levels in human ocular ciliary smooth muscle and endometrial stromal cells. Currently, its normal functions are unclear. We recently reported that LINC00473 is the consistently and highly induced target in lung cancer cells with the inactivated tumor suppressor gene LKB1, one of the most frequent genetic alterations in non-small cell lung cancer. LKB1 inactivation results in aberrant de-phosphorylation and nuclear translocation of CRTC co-activators, leading to the activation of CREB-mediated gene transcription program including LINC00473 up-regulation. More recently, LINC00473 was shown to be up-regulated in fibrolamellar carcinoma, a rare liver cancer with a fusion event between DNAJB1 and PRKACA. PKA is a cAMP/CREB pathway component and DNAJB1-PRKACA fusion likely results in constitutive PKA activation subsequent promoting CREB pathway. In this study, we discovered that LINC00473 can be induced by an alternative mechanism – the CRTC1-MAML2 oncoprotein in human mucoepidermoid carcinoma. Thus, the CRTC1-MAML2 oncogene, the inactivated tumor suppressor LKB1 gene, and potentially DNAJB1-PRKACA fusion, can converge on the activation of the CRTC/CREB transcriptional program leading to elevated LINC00473 expression.

The identification of LINC00473 as the critical downstream effector of CRTC1-MAML2 has potential clinical application for diagnosis and treatment of MECs. Accurate diagnosis of MEC remains challenging because of its cellular heterogeneity and the extent of the histologic overlap with other salivary gland tumors. The t(11;19) translocation and its encoded CRTC1-MAML2 fusion have been applied as a clinical biomarker for MEC diagnosis due to the unique and high frequency of t(11;19) translocation in up to 82% of human MEC cohorts in several studies. Current approaches of detecting the t(11;19) translocation consists of the use of CRTC1-MAML2 fusion transcript-specific RT-PCR, which requires good quality of RNA, and fluorescence in situ hybridization with MAML2 split probes for MAML2 gene rearrangement status, which is a technically challenging and time-consuming with relative high expense. The latter assay only provides MAML2 rearrangement status but no information on functional CRTC1-MAML2 expression or activity. LINC00473 expression is a specific functional readout for the CRTC1-MAML2 fusion activity and the unique nuclear dot-like LINC00473 signals can be detected and are...
easily distinguishable in FFPE MEC by RNA in situ hybridization. Therefore, LINC00473 has potential to serve as a vastly improved diagnostic marker for CRTC1-MAML2-positive MEC. In addition, a CRTC3-MAML2 fusion variant was reported in up to 6% of MEC tumors and the presence of CRTC3-MAML2 and CRTC1-MAML2 was mutually exclusive. The CRTC3 and CRTC1 exon 1 domains are highly homologous and consists of the CREB binding domain and it is unclear whether CRTC3-MAML2 could activate LINC00473 expression in a manner similar to CRTC1-MAML2. The follow-up LINC00473 RNA ISH studies of the defined fusion-positive and −negative MEC cases are needed to evaluate the utility of LINC00473 expression as a surrogate for fusion status in MEC.

The CRTC1-MAML2 fusion gene encodes a nuclear protein without any known enzymatic activity; therefore, it is challenging to develop approaches of interfering its function. Therefore, a more rational approach is to identify and target its downstream mediators. Here, we observed that CRTC1-MAML2-positive MEC depends on LINC00473 expression for tumor cell growth and survival, revealing a new critical role of LINC00473 in maintaining MEC malignant phenotypes. LINC00473 expression is low or undetectable in normal human tissues. We thus predict that suppressing LINC00473 expression or activity likely represent an effective therapeutic approach for blocking MEC tumor growth. Targeting cancer-associated IncRNAs is a promising approach in cancer treatment in multiple proof-of-principle studies. For instance, RNAi-mediated depletion of HOTAIR, a lncRNA highly expressed in cancer, significantly reduced the renal carcinoma cell proliferation and survival in vitro and in vivo36. Locked nucleic acid GapmeRs, a modified version of antisense oligonucleotides (ASO) targeting the IncRNA SAMMON, were shown to block melanoma cell growth both in vitro and in patient-derived xenograft models37. Moreover, the use of CRISPR/CAS9 technology to knock out IncRNA UCA1 attenuated malignant phenotypes38. Therefore, these strategies including siRNAs, ASOs and CRISPR/Cas9 can be investigated in the future to target LINC00473 expression in blocking MEC. Alternatively, novel upstream regulators and downstream effectors of LINC00473 can be investigated for new means of inhibiting LINC00473 expression or activity.

In summary, we discovered that LINC00473 expression is directly regulated by the CRTC1-MAML2 fusion oncoprotein and its expression level highly correlates with the that of CRTC1-MAML2 fusion in human MEC cells and primary MEC tumors. LINC00473 has a role in regulating gene expression, in part through the interaction with NONO and enhancing the CRTC1-MAML2 activation of the CREB target genes. LINC00473 is required for the growth and survival of human fusion-positive MEC cells both in vitro and in vivo. Collectively, our studies reveal that LINC00473 is a promising biomarker for detecting CRTC1-MAML2-expressing MEC and that targeting LINC00473 expression or function will be an effective strategy for blocking MEC.

Materials and Methods

Plasmids

The LINC00473 luciferase reporter containing the LINC00473 promoter (−523 to +88) in the pGL3 luciferase reporter were previously described 29. The lentiviral-based pLKO.1 MAML2 shRNA (RHS4533-NM_032427), NONO shRNA (RHS4533-EG4841) and CREB
shRNA (RHS4533-NM_004379) constructs were purchased from GE Dharmacon. The pLKO.1-scrambled shRNA vector was purchased from Addgene. The lentiviral-based pLKO.1 constructs containing LINC00473 shRNA sequences were used, including shLnc473-2 (AAGTGGATCTTTGCAGACAGG); shLnc473-3 (AAAGATCCAGTTTAATACAGA) and shLnc473-4 (AAGAACCCAAGTCATATTCAT). The pCMV-based CRTC1-MAML2 expression construct expresses the CRTC1-MAML2 fusion with a N terminal FLAG tag.

Cell culture, viral production and transduction

Human salivary gland-derived MEC cell lines (UM-HMC3A and -3B) and a benign human pleomorphic adenoma (UM-HPA-1) were kindly provided by Dr. Jacques Nör’s lab. Salivary MEC H3118 and pulmonary MEC H292 cell lines were previously described. HTB-41 cells were originally derived from salivary gland epidermoid carcinoma and obtained from ATCC. These cell lines, as well as HEK293T and HEK293FT cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 1% antibiotics (Corning Cellgro) and 10% FBS (Sigma) at 37 °C with 5% CO₂.

Lentiviral production was carried out by transfecting HEK293FT cells with the lentiviral constructs and packaging plasmids psPAX2 and pMD2.G using the Effectene Transfection Reagent (QIAGEN). Lentiviruses were harvested at 48, 72 and 96 hours after transfection. Target cells were infected on three consecutive dates using the viruses in the complete medium containing 8 μg/ml Polybrene (Sigma) for 6 to 8 hours and then replaced with fresh culture medium.

Western blotting

Western blotting was performed as previously described. The antibodies were used in this study including anti-MAML2 (CST-4618, Cell Signaling Technology), anti-CREB (06-863, Millipore), anti-β-ACTIN (SC-47778, Santa Cruz) and anti-β-TUBULIN (1799-1, Epitomics).

Quantitative RT-PCR (qRT-PCR)

RNA isolation and qRT–PCR analysis was carried out as previously described. The primer sequence information was shown in Supplemental Table S2.

Luciferase reporter assays

Cells were plated in 24-well plates and were transfected on the second day with pGL3-LINC00473 promoter luciferase reporter and pCMV_FLAG-tagged CRTC1-MAML2 or empty pCMV vector along with internal control Renilla luciferase construct (pEF-RL). The Effectene Transfection Reagent (Qiagen) was used. Cells were harvested at 48-hour post-transfection which were subjected the Dual-luciferase reporter assays (Promega).

Chromatin immunoprecipitation (ChIP)

ChIP was carried out as previously described. The CREB antibodies (06-863, Millipore), MAML2 TAD antibodies (CST-4618, Cell Signaling Technology) or control immunoglobulin G (SC-2027, Santa Cruz) were used to immunoprecipitate the chromatin.
complex. The chromatin DNA was purified and used for qRT-PCR assays with LINC00473 specific primers flanking the two CREB binding half sites (forward, 5′-AGCAGCCTTGCCAAAGGTC-3′; revers, 5′-TTTCCCTTTAAGCCGGAGAT-3′).

**RNA fluorescence in situ hybridization (RNA-FISH)**

RNA-FISH was carried out on human MEC cells using twenty-seven LINC00473 Stellaris® FISH probes that were labeled with Quasar 570 dyes (Biosearch Technologies). LINC00473 signal was observed under Leica DM6000B fluorescence microscope. Cells treated with 50 μg/mL RNase A at 37°C for 1 hour before the hybridization step were included as negative controls.

**RNA in situ hybridization (RNA ISH)**

RNA ISH was performed on FFPE tissue sections with the RNAscope® 2.0 HD Detection Kit (Advanced Cell Diagnostics) and custom probe set Hs-LINC00473 as per the manufacturer’s instructions. Hematoxylin counterstaining was performed. The stained tissue sections were scanned using the Aperio Imagescope (Leica).

**LINC00473 copy number evaluation**

The genomic DNAs were isolated from cultured cells using DNeasy Blood& Tissue kit (Qiagen) and subjected to qRT-PCR analysis for the LINC00473 locus as well as the GAPDH locus as the internal reference control42. The copy number for LINC00473 was normalized relative to that of GAPDH and was expressed as a relative value compared to that of the fusion-negative HPA-1 cells which was set as 1. The primers for the LINC00473 locus are 5′-CTTTTGCGAGAAGATCGGCG-3′ (forward) and 5′-CCATGGAGAACTGCGCAAAG-3′ (reverse). The primers for the GAPDH locus were 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (forward) and 5′-AGCCTTCTCCATGGTGTTGAAGAC-3′ (reverse).

**RNA immunoprecipitation (RNA-IP)**

MEC cells were crosslinked with UV and then lysed in RIPA buffer containing the proteinase and Rnase inhibitors. Cell lysates were collected for immunoprecipitation overnight at 4°C using anti-NONO antibodies (A300-587A, Bethyl Laboratories) control and IgG control (SC-2027, Santa Cruz) and protein A/G beads. The beads that bound with protein-RNA complex were then washed with RIPA buffer for 5 times and digested with Proteinase K at 45°C for 45 minutes to release the RNA from the beads. Then the TRIzol-isolated RNAs were analyzed by qRT-PCR as previously described29.

**Microarray gene expression profiling**

H3118 MEC cells were infected with LINC00473 shRNA or control shRNA lentiviruses for 96 hours. The total RNAs were isolated with a RNasy RNA extraction kit (Qiagen). RNAs were then subjected to Affymetrix human transcriptome array 2.0 (HTA 2.0) analysis and duplicate biological repeats were set up. Differentially expressed genes (with an absolute fold change of ≥2 and FDR p-value < 0.05) were further analyzed using Ingenuity Pathway
Analysis software (IPA; Ingenuity Systems Inc). The microarray data (GEO Series GSE81960) were deposited in the NCBI Gene Expression Omnibus.

**Cell growth and apoptosis assays**

Cells were transduced with LINC00473 shRNA or scrambled shRNA control lentiviruses for 72 hours and then cultured at 5 X 10^5/well in 6-well plates for additional 96 hours. Cells were then harvested for cell viability via trypan blue exclusion assay and Annexin V-FITC Apoptosis Detection assay (BD Pharmingen). Triplicate assays were set up.

**Mouse xenograft studies**

NOD.SCID mice (NOD.CB17-Prkdscid/J) at age of about 2 months were randomly divided into three cohorts. Both sexes were used with similar matched genders in each cohort. The mice were subcutaneously injected with 2 million luciferase-expressing H3118 cells transduced with control scrambled shRNA (shCtl) or LINC00473 shRNA (shLnc473-2 and -4) lentiviruses for 96 hours (n=5 for shCtl; n=5 for shLnc473-2; and n=4 for shLnc473-4). No blinding was done. Tumor volume measurement were performed by Dial Caliper and by bioluminescence imaging as previously described. The experiment followed a protocol that was approved by the Institutional Animal Care and Use Committee, the University of Florida.

**TUNEL assay**

TUNEL assay was performed on FFPE xenograft sections using the ApopTag® Peroxidase In Situ Apoptosis Detection kit (S7100, Millipore).

**Statistical analysis**

Student’s t-tests were carried out to assess statistical differences. A p-value of less than 0.05 was considered to be statistically significant. The biological repeats were indicated in the figure legends for each experiment.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. LINC00473 expression is highly elevated in human CRTC1-MAML2 fusion-positive MEC cell lines and primary tumors
(a) Western blotting analysis of the expression of CRTC1-MAML2 fusion in human MEC cell lines. The HPA-1 cell line derived from a benign human pleomorphic adenoma was used as a fusion-negative control. An anti-MAML2 TAD antibody was used to detect the MAML2 and CRTC1-MAML2 fusion proteins. Blotting with anti-β-ACTIN was used as a protein loading control. (b) The qRT-PCR analysis showed that 4 CRTC1-MAML2 fusion-positive human MEC cell lines (HMC3A, HMC3B, H3118 and H292) express much higher levels of LINC00473 transcripts than the fusion-negative cells (HPA-1). (n=3, **p<0.001 and ***p<0.0001. (c) LINC00473 expression levels were higher in human fusion-positive MEC primary tumors (n=6) in comparison with fusion-negative MEC tumors (n=6). RNAs isolated from human MEC tumors were subjected to qRT-PCR analysis. (d) Expression levels of LINC00473 and CRTC1-MAML2 fusion were positively correlated (r=0.785157) in human MEC tumors (n=12). The ΔCt values (normalized to GAPDH) were subjected to Pearson correlation analysis.
Figure 2. LINC00473 expression is directly regulated by the CRTC1-MAML2 fusion oncoprotein
(a) The qRT-PCR analysis showed that the transcript levels of LINC00473 were significantly reduced in CRTC1-MAML2 fusion-positive H3118 cells after knockdown the expression of fusion by two independent lentiviral shRNAs (shM2-1 and -3) as compared with the scramble shRNA control (shCtl) (n=3, *p<0.05 and **p<0.001). (b) LINC00473 expression was reduced after fusion knockdown in a second fusion-positive MEC cell line (H292) (n=3, **p<0.001). (c) Exogenous CRTC1-MAML2 expression restored LINC00473 expression levels in H3118 cells depleted of the endogenous fusion gene expression (n=3, *p<0.05 and ***p<0.0001). (d) LINC00473 expression was enhanced in the HEK293T cells after transfection with FLAG-tagged CRTC1-MAML2 expression constructs (n=3, *p<0.05 and ***p<0.0001).
Figure 3. CRTC1-MAML2 fusion activates LINC00473 expression though co-activating the transcription factor CREB.

(a) Western blotting analysis showed that CREB expression was knocked down by two independently lentiviral-mediated CREB shRNAs in fusion-positive H3118 cells. (b) The LINC00473 expression was significantly reduced in the CREB-depleted fusion-positive H3118 cells by qRT-PCR assays (n=3, *p<0.05 **p<0.001). (c) A LINC00473 promoter region reporter containing two conserved CRE half binding sites was cloned into pGL3 luciferase reporter plasmid. LINC00473 promoter luciferase activities were increased after ectopic expression of CRTC1-MAML2 fusion in HEK293T cells (n=3, **p<0.001). (d) ChIP analysis indicated that CREB and CRTC1-MAML2 were significantly enriched on the LINC00473 promoter encompassing the two CRE half sites in H292 cells. The ChIP assays were performed in fusion-positive H292 cells by using anti-CREB antibodies or anti-MAML2 antibodies to immune-precipitate the bound LINC00473 promoter. The immunoglobulin G (IgG) was used as a negative control (n=3, *p<0.05).
Figure 4. Depletion of LINC00473 expression in CRTC1-MAML2 fusion-positive MEC H3118 cells results in a reduction in cell proliferation and survival in vitro and blocks the growth of human MEC xenografts in vivo

(a) H3118 cells were transduced with two independent lentiviral shRNAs against LINC00473 (shLnc473-2 and -4) or the scramble shRNA control (shCtl) on 3 consecutive days. Transduced cells were collected 96 hours after first infection. The LINC00473 transcript levels were significantly reduced as compared with shRNA control by qRT-PCR assay (n=3, *p<0.05 and **p<0.001).

(b and c) Transduced cells at 96-hours post-transduction were cultured at 5 X 10^5 per well in 6-well plates for another 96 hours to perform cell proliferation and survival assay. Viable cells were counted using Trypan blue assay and (b) the apoptotic cells of LINC00473-knockdown and control cells were determined by Annexin V/PI staining followed by FACS analysis (c) (n=3, *p<0.05 and **p<0.001).

(d–g) LINC00473 knockdown reduced the growth of MEC xenograft tumors. Approximately 2 X 10^6 luciferase-expressing H3118 (H3118-luc) cells after transduction with shLINC00473 or shRNA control for 96 hours were implanted subcutaneously to the dorsal flanks of NOD-SCID mice. Tumor sizes were measured overtime and tumor volumes were calculated in three cohorts of mice (shCtl, n=5; shLnc473-2, n=5, shLnc473-4, n=4) (*p<0.05).

(d). A representative bioluminescent image showed mice injected with shCtl, shLnc473-2 or shLnc473-4 H3118-luc cells, respectively (e). The image was taken at day 18 after the transduced H3118-luc cells injection at the end point study. The excised tumors (f) and the tumor weights (g) at the end points were shown. (*p<0.05 and ***p<0.0001).

(h) Immunohistochemical staining of control and shLINC00473 xenograft tumor sections by a TUNEL assay. Scale bars: 100 μm (left panels), 20 μm (right panels).
Figure 5. Specific nuclear distribution of LINC00473 was revealed in CRTC1-MAML2 fusion positive human MEC cells and primary tumors

(a) Dot-like nuclear signals of LINC00473 were detected by RNA-FISH in H3118 cells. LINC00473 RNA-FISH probe sets were labeled with Quasar 570 dyes (red) and nuclei were labeled with the DNA dye DAPI (blue). RNase treatment was used as a negative control. (b) Predominantly nuclear localization of LINC00473 was detected based on RNAscope detection in H292 MEC xenograft. (c) One CRTC1-MAML2 fusion-positive human MEC tumor sample exhibited the nuclear localization of LINC00473 based on the RNAscope analysis. The housekeeping gene PPIB was used for sample RNA quality control.

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Figure 6. LINC00473 regulates gene expression in MEC in a transcriptomic analysis and interacts with NONO enhancing CRTC1-MAML2/CREB transcription

(a,b) Heatmap and volcano plot show differentially expressed genes in shLnc473 knockdown in compared with shRNA control in fusion-positive H3118 cells. The cutoff criteria were absolute fold-change of ≥2 and p<0.05. This analysis led to the identification of a total of 1320 LINC00473-regulated candidate coding genes, with 675 up-regulated and 645 down-regulated genes.

(c) Functional classification of LINC00473 down-regulated genes was performed using Ingenuity Pathway Analysis (IPA). The top 6 molecular and cell functions were ranked based on p-value and activation z-score. Negative z-score indicates inhibition.

(d) The qRT-PCR analysis showed that LncRNA LINC00473 was significantly enriched in the NONO immunoprecipitates relative to the IgG control in H3118 MEC cells. ASNS was used as a negative control (n=3, ***p<0.0001).

(e) Overexpression of LINC00473 in HEK293T cells significantly increased the interaction of CRTC1-MAML2 and NONO by a Gal4-NONO reporter assay (n=3, *p<0.05).

(f) Knockdown of NONO or LINC00473 expression significantly reduced the pCRE-luc reporter activities in HEK293T cells with CRTC1-MAML2 overexpression (n=3, *p<0.05 and **p<0.001).

(g) A model for the molecular basis of LINC00473 induction by CRTC1-MAML2 and function in fusion-positive MEC cells.