MYH9 binds to IncRNA gene PTCSC2 and regulates FOXE1 in the 9q22 thyroid cancer risk locus

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A locus on chromosome 9q22 harbors a SNP (rs965513) firmly associated with risk of papillary thyroid carcinoma (PTC). The locus also comprises the forkhead box E1 (FOXE1) gene, which is implicated in thyroid development, and a long noncoding RNA (lncRNA) gene, papillary thyroid cancer susceptibility candidate 2 (PTCSC2). How these might interact is not known. Here we report that PTCSC2 binds myosin-9 (MYH9). In a bidirectional promoter shared by FOXE1 and PTCSC2, MYH9 inhibits the promoter activity in both directions. This inhibition can be reversed by PTCSC2, which acts as a suppressor. RNA knockdown of FOXE1 in primary thyroid cells profoundly interferes with the p53 pathway. We propose that the interaction between the lncRNA, its binding protein MYH9, and the coding gene FOXE1 underlies the predisposition to PTC triggered by rs965513.

Intronic RNA | MYH9 | transcriotional regulation | bidirectional promoter | thyroid cancer

Thyroid cancer is the most common endocrine malignancy. Based on the data from the National Cancer Institute for 2016, in total 64,300 new patients will be diagnosed with thyroid cancer, accounting for 3.8% of all cancer patients in the United States (https://seer.cancer.gov/statfacts/html/thyro.html). Thyroid cancer is classified into four main types: papillary, follicular, medullary, and anaplastic. Papillary thyroid carcinoma (PTC) accounts for 85–90% of all thyroid cancers (1). In efforts to explain the genomic background to PTC, genome-wide association studies (GWAS) have disclosed an SNP marker, rs965513, in 9q22.33, strongly associated with PTC in European populations [odds ratio (OR) = 1.75; \( P = 1.7 \times 10^{-22} \)] (2, 3). The association has been observed in other populations as well (4–8). Additionally, rs965513 has been reported to be associated with levels of thyroid-related hormones, hypothyroidism, goiter, and other abnormal thyroid functions (2, 3, 9). SNP rs965513 resides in an intron of a recently detected long noncoding RNA (IncRNA), papillary thyroid cancer susceptibility candidate 2 (PTCSC2), which is located between two coding genes, DNA damage recognition and repair factor (XPAT) and forkhead box E1 (FOXE1) (Fig. S1).

FOXE1, also known as thyroid transcription factor 2, is a single exon coding gene belonging to the forkhead/winged helix-domain protein family (10). FOXE1 knockout mice were born alive but exhibited an ectopic or completely absent thyroid gland and severe cleft palate, accompanied by up-regulated TSH and down-regulated free thyroxine hormone levels (11). FOXE1 is essential for thyroid gland development and the maintenance of thyroid differentiated status (12, 13). It also plays a role in the predisposition and development of thyroid cancer (14, 15). Moreover, somatic mutational inactivation of FOXE1 has been found in PTC, suggesting FOXE1 may contribute to tumorigenesis in a subset of thyroid cancers (16).

PTCSC2 was discovered in the region harboring rs965513 on 9q22 (15). Similar to some 25% of all known lncRNA genes (17), PTCSC2 has one unspliced isoform and several spliced isoforms, all of which display thyroid-specific expression. In PTC tumors, the risk allele [A] of rs965513 is significantly associated with low expression of both PTCSC2 and FOXE1 (15). Three enhancer elements are located in a 33-kb linkage disequilibrium (LD) block within PTCSC2. Previous studies showed that PTCSC2 is transcribed in the opposite direction of FOXE1. Exon 1 and intron 1 of PTCSC2 isoform C overlap with the FOXE1 promoter region (15). Although the promoter region shared by PTCSC2 and FOXE1 was found to be regulated via long-range looping interactions (18), the detailed mechanisms and their bearing on thyroid cancer need to be explored.

In the present study, we identified myosin-9 (MYH9) as an lncRNA binding protein that targets the FOXE1 promoter region through interactions with PTCSC2 and performs its regulatory function in thyroid cancer via downstream pathways. These findings provide a better defined description of the complex mechanisms involved in the 9q22 thyroid cancer locus.

**Results**

**Identification of MYH9 as an lncRNA Binding Protein.** To begin to unravel the underlying mechanisms by identifying proteins potentially interacting with PTCSC2, we performed biotin-labeled RNA pull-down assays with protein lysate from normal thyroid tissue. Two PTCSC2 isoforms—isoform C (1,947 nt) and isoform D (1,804 nt)—were used as baits by generating both sense and antisense (negative control) RNA probes for each of them (Fig. S1). As a result, a strand-specific binding protein was discovered between

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Readers: B.H., University of Colorado School of Medicine; and S.K., National Cancer Institute/National Institutes of Health. The authors declare no conflict of interest.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE83919).

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B

ptcsc2 isoform C was used as the negative control. The arrows indicate the bonding protein bands −226 KD and 42 KD in size, respectively. (B) Information for the identification of MYH9 by MS. The full length of MYH9 protein is 1,960 amino acids, as shown in the lower diagram. Blue boxes indicate the peptides identified by MS analysis. (C) qRT-PCR detection of the indicated RNAs retrieved by MYH9 antibody (RIP assay) in the BCPAP cell line with stable PTCSC2 isoform C overexpression. IgG was used as a negative control. The relative fold change was normalized to the IgG control. **p < 0.01. Student’s t test.

150 KD and 250 KD in size (Fig. L1 and Fig. S2). This protein was identified as MYH9, being 226 KD in size by mass spectrometry (MS) (Fig. 1A). Another protein band that was noticed between 37 KD and 50 KD in size was identified as beta-actin (ACTB) (Fig. L1 and Figs. S2 and S3). As MYH9 is an ACTB binding partner participating in important cellular processes (19), ACTB was not studied further here.

The interaction between PTCSC2 isoform C and MYH9 was confirmed by RNA immunoprecipitation (RIP) assay in BCPAP cells with PTCSC2 isoform C stable overexpression (Fig. 1C). PTCSC2 was readily enriched by MYH9 antibody compared with IgG-negative controls (>sixfold), and this enrichment was significantly higher than in the 18S RNA controls (P < 0.01), demonstrating the specificity of the assay.

MYH9 Binds to the FOXE1 Promoter Region. To determine whether the PTCSC2 binding protein MYH9 is involved in the transcriptional expression of the FOXE1 gene, four overlapping regions (R1, R2, R3, and R4) that cover the transcription factor enriched region (from ENCODE ChIP sequencing data) in the 5′UTR of FOXE1 were chosen for ChIP analysis using MYH9 antibody (Fig. 2A). Of note, the previously reported thyroid cancer risk-associated SNP rs1867277 (14) is located in the R3 genomic region. In the KTC1 cell line, significant enrichment was found in the R1 and R3 regions (Fig. 2B). The enrichment was confirmed in non-tumorous thyroid tissue (Fig. 2C), suggesting the DNA binding activity of MYH9 is in the FOXE1 promoter, perhaps more specifi- cally in regions R1 and R3.

MYH9 Functions as a Suppressor in the Promoter Region of PTCSC2 and FOXE1 with Bidirectional Activity. To investigate the role of MYH9 binding to the FOXE1 promoter region, luciferase assays were performed in cells transiently transfected with FOXE1 promoter or empty constructs (without promoter), together with MYH9 expression or empty expression vectors.

As PTCSC2 and FOXE1 are transcribed in opposite directions (15) and there is only 174 bp between their transcription start sites (TSSs), we postulated that they are transcribed by the same pro- moter with bidirectional activity. To test this hypothesis, a shorter (1,607 bp) and a longer (2,520 bp) fragment cloned from the FOXE1 promoter region were used for luciferase reporter assay in the BCPAP cell line with both forward (Promoter-S and Promoter-L) and inverted (Promoter-Inv-S and Promoter-Inv-L) orientation, as indicated (Fig. 3A). Although the inverted promoters showed lower activity compared with their corresponding forward ones, the fragment with both orientations exhibited a significantly higher level of promoter activity in comparison with the no pro- moter control. Moreover, cotransfection with the MYH9 expression vector showed that MYH9 can significantly inhibit the luciferase activity in both the forward and inverted promoter fragments. That MYH9 functions as a suppressor was disclosed in both the shorter and longer promoter fragments (Fig. 3B and C).

Thus, MYH9 had an impact on the FOXE1 gene, but its role in the presence of IncRNA PTCSC2 was not known. We transiently cotransfected the PTCSC2 expression vector in the BCPAP cell line in the presence or absence of the MYH9 expression construct. Because there was no significant difference in promoter activity between the two fragments of different sizes, only the longer pro- moter sets were used. Interestingly, an increase in promoter lucif- erase activity was observed with cotransfection of the PTCSC2 expression vector, irrespective of MYH9 overexpression. The ef- fect was significant on the forward Promoter-L, whereas it was not significant on the inverted Promoter-Inv-L, suggesting a rescuing effect on the inhibition caused by MYH9 (Fig. 4A and B).

To further validate the roles of MYH9 and PTCSC2 in regu- lating the endogenous FOXE1 promoter, a transient transfection assay with MYH9 and PTCSC2 expression vectors was performed in BCPAP and TPC1 cells. The expression of the endo- genous FOXE1 gene showed a significant decrease in the presence of MYH9 overexpression, which is consistent with the role of MYH9 as an inhibitor in our luciferase assay. This inhibitory effect becomes nonsignificant in the presence of PTCSC2, indicating that FOXE1 is regulated by PTCSC2 and MYH9 differentially (Fig. 4C and D). When we examined the PTCSC2 transcript levels and protein levels of MYH9 and FOXE1 in 6 thyroid cancer cell lines and in nontumorous thyroid tissue, we did not notice a correlation among the expression levels of these three genes (Fig. S4).

SNP rs1867277 is in partial LD with rs965513 and is reported as a functional variant in the regulation of FOXE1 expression and confers thyroid cancer susceptibility (14). As rs1867277 is located in the MYH9 interacting region R3 (Fig. 2A), we tested the regulatory effects of MYH9 and PTCSC2 on the FOXE1 promoter with either the wild-type allele (G) or the thyroid cancer risk allele (A). BCPAP cells were transiently transfected with either MYH9 or both MYH9 and PTCSC2 constructs using promoters with opposite orientations. The promoter containing the A allele exhibited significantly lower activity than the promoter with the G allele, and a similar difference was found in both forward and inverted promoters (Fig. S5).

FOXE1 Is Involved in the P53 Pathway in Human Primary Thyroid Cells. FOXE1 is an important transcriptional regulator in the physiological functions of the thyroid as well as in thyroid cancer. Lower expression level of FOXE1 is associated with PTC risk in the presence of the risk allele of SNP rs965513 (15). To identify genes involved in FOXE1-mediated signaling pathways and transcriptional regulation in thyroid, knockdown of FOXE1 was performed with siRNAs in nontumorous human primary thyroid cells (20). Nontumorous human primary thyroid cells from the same patient sample transfected with scrambled siRNAs were used as the control. Knockdown of FOXE1 resulted in numerous changes in gene expression determined by RNA deep sequencing.
(fold change > 1.5, P < 0.001). Of the dysregulated genes (total N = 107; up-regulated n = 80), 89.7% were coding genes (Table S1). Biological functional analysis using Ingenuity Pathway Analysis (IPA) software showed that cancer was in the top category of “Diseases and Disorders” with the second lowest P value (Fig. S6). The top five categories of “Molecular and Cellular Functions”—namely, cellular movement, cell death and survival, cellular development, cellular growth and proliferation, and cell signaling—suggested its possible relevance to pathways related to cell apoptosis, migration, or invasion (Table 1). These effects have been widely reported as the major effects of the well-known p53 signaling pathway (21, 22). Of the top 25 dysregulated genes, the thrombospondin 1 (THBS1, also known as TSP-1) and insulin-like growth factor binding protein 3 (IGFBP3) genes were found to be up-regulated in FOXE1 knockdown cells (Fig. S4 and Table S1). This inverse relationship was subsequently confirmed by quantitative RT-PCR (qRT-PCR) in primary thyroid cells (Fig. S5A) and also in the BCPAP and TPC1 cell lines (Fig. S7). We also analyzed gene expression data from 59 pairs of tumor/nontumor PTC samples available in the TCGA data portal (https://tcga-data.nci.nih.gov/docs/publications/tcga). In this analysis, the expression of FOXE1 was significantly (P = 2.02 × 10−15) higher in nontumorous tissue, whereas the expression of both THBS1 and IGFBP3 are significantly higher in tumor tissue (P = 1.73 × 10−10 and P = 4.19 × 10−15, respectively) (Table S2). This is consistent with our data and further supports the putative role of FOXE1 as a suppressor of THBS1 and IGFBP3 in thyroid. Both THBS1 and IGFBP3 are important members of the p53 pathway involved in apoptosis, inhibition of the IGF1/mTOR pathway, and inhibition of angiogenesis and metastasis, suggesting interactions between FOXE1 and the p53 pathway in thyroid cells (Fig. 5C).

To further assess the function of FOXE1 in thyroid cells, we performed cell viability and apoptosis assays in FOXE1 knockdown BCPAP cells. The down-regulation of FOXE1 led to reduced cell viability in CellTiter-Glo assay (Fig. 5D). Consistently, apoptosis assay with flow cytometric analysis indicated that increases of apoptotic cells at both early and late apoptosis stages were found in the FOXE1 knockdown cells (Fig. 5E).

Discussion

LncRNAs are important regulators of tissue physiology and disease processes including cancer. The regulatory effectiveness of lncRNAs is dependent on their expression. Many lncRNAs show tissue-specific expression patterns. Several thyroid tissue- and cancer-associated lncRNAs were recently discovered (23). Only a few lncRNAs related to thyroid and thyroid cancer have been well characterized, including PTSC2, PTSC3, and NAMA (15, 24, 25), but even in these cases, the relevant molecular mechanisms are not known. It is of special interest that these lncRNAs are involved in the genetic predisposition to thyroid cancer (germ-line involvement). Therefore, their impact on nontumorous thyroid tissue needs to be explored, which we here have endeavored to accomplish. We identified MYH9 as a noncoding RNA binding partner, which had not been reported before.

MYH9 protein is a member of the nonmuscle myosin II (NMII) group, which belongs to the myosin II subfamily (26). As a subunit of myosin II heavy chains, MYH9 takes part in the generation of cell polarity, cell migration, cell–cell adhesion processes, and maintaining cytoskeleton structure by binding to actin filaments (19). Recently, additional functions of MYH9 have been reported. For example, MYH9 affects the expression of PAX5 by interacting with Thy28 (27), and it can activate AKT through RAC1 and PAK1 (28), suggesting a role in gene regulation. In 2014, a role for MYH9 as a tumor suppressor gene was discovered in squamous cell carcinomas (SCCs). This report implicated MYH9 in tumor development by regulating posttranscriptional p53 stabilization, but the underlying mechanism is still unknown (29). Our results are consistent with this finding and provide further evidence for a regulatory model by which a specific noncoding RNA, PTSC2, and MYH9 work together by regulating FOXE1 promoter activity. Knockdown of FOXE1 in primary human thyroid cells revealed that FOXE1 can regulate events in the p53 pathways.

As a common occurrence, bidirectional transcription of two protein coding genes has been discovered and studied during the past decades (30, 31). More recently, many noncoding RNAs that are transcribed in the proximity of coding genes and driven by the same bidirectional promoter have been reported (32, 33). Some noncoding transcripts exert repression of their nearby coding genes by competing for the same polymerases and accessory factors or by transcriptional interference or histone modification (34–36). A number of highly expressed antisense transcripts derived from bidirectional TSSs are able to enhance the expression of the corresponding protein coding gene.
in a tissue-specific manner (37). The PTCSC2–FOXE1 pair in our study further supports this regulatory model, which can be directed by the same inhibitor. Considering the complexity of the 9q22 region including nearby CpG islands (38), it is reasonable to assume that other transcription factors and some epigenetic modifications might be involved in the regulation of this bidirectional promoter, in addition to MYH9 and PTCSC2. It will be of interest to learn whether other regulatory factors have any impact on MYH9, or vice versa.

Cell line models were used in most previous reports on FOXE1 in the thyroid (39, 40). Because several thyroid markers including lncRNAs lose their expression in most cell lines (41), we consider thyroid primary cell culture to be a superior model for pathway and network analysis. In this study, we use human thyroid primary cell cultures that were recently established (20). Two important members of the p53 pathway, THBS1 and IGFBP3, have been reported to be involved in various cancer types. The regulation of tumor growth and metastasis by THBS1 has been widely described (42). THBS1 is regulated by BRAF V600E in thyroid cancer cells. The silencing of THBS1 results in decreased cell proliferation, adhesion, migration, and invasion (43). Moreover, THBS1 silencing can also cause changes in the levels of integrin receptors and anaplastic thyroid cancer cell morphology (44). THBS1 promotes human follicular thyroid carcinoma cell invasion mainly through up-regulation of urokinase plasminogen activator (PLAU) (45). This is consistent with our RNA-seq results, as PLAU is also one of the top 25 dysregulated genes in FOXE1 knockdown primary thyroid cells (Fig. 5 and Table S1). IGFBP3 inhibits tumor growth by promoting apoptosis and inhibiting cell proliferation in some cancer types, but in other circumstances, it increases cell survival and stimulates proliferation (46). Our data further emphasize the roles played by these two genes (THBS1 and IGFBP3) in thyroid cancer.

In conclusion, our study addresses the mechanisms and provides a biological characterization of the widely reported GWAS locus 9q22 in thyroid cancer. We propose MYH9 as a binding protein of thyroid-specific noncoding RNA that may underlie the predisposition to thyroid cancer.
Materials and Methods

The study was approved by the Institutional Review Board at The Ohio State University (OSU), and all subjects gave written informed consent before participation.

Cell Lines and Thyroid Tissue Samples. The human thyroid carcinoma cell lines used in this study were incubated in antibiotic-free RPMI medium 1640 (KTC1, BCPAP, C643, SW1736) or DMEM (TPC1, FTC133) supplemented with 10% (vol/vol) FBS (Gibco) at 37 °C in humidified air with 5% (vol/vol) CO₂. Thyroid nontumorous samples were snap-frozen in liquid nitrogen and kept at −80 °C after being obtained from patients with PTC during surgery. All cases were histologically diagnosed as PTC; clinical information on these samples will be made available on request.

In Vitro RNA Probe Synthesis and RNA Pull-Down. In vitro RNA synthesis procedures using MEGAscript T7 Transcription Kit for target probe and MEGAscript SP6 Transcription Kit for antisense control probe (Life Technologies) were performed according to the manufacturers’ instructions. The whole-protein lysate from the human nontumorous thyroid sample was extracted using T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific). Protein concentration was determined by using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) kit. RNA pull-down assay was performed using Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) according to the standard instructions. A detailed description can be found in SI Materials and Methods.

RIP Assay. In total, 2 × 10⁴ BCPAP cells with PTCS2 isoform C overexpression were used for RIP lysis preparation using Harsh Lysis Buffer of Imprint RIP Kit (Sigma-Aldrich) following the instructions. Briefly, 5 μg antibody of rabbit anti-MYH9 (Santa Cruz, sc-98978) or rabbit IgG (Sigma, I5006-1MG) was used to incubate with the cell lysate in 1 mL RIP wash buffer with Protease Inhibitor Mixture (Roche) and also RNase Inhibitor overnight at 4 °C after ligation to Magnetic Beads. The RIP products were harvested by washing 1 mL washing buffer up to five times. Finally, the washed product resuspended in 200 μL buffer was subjected to the purification step using Taqman ligation to Magnetic Beads. The quantification of target RNA in the final RIP products was determined by real-time qRT-PCR using the Taqman method.

CHIP Assay. A detailed description of the chromatin immunoprecipitation (CHIP) assays to determine the degree of MYH9 enrichment on the KTC1 cell line and frozen thyroid tissue samples can be found in SI Materials and Methods. The primer sequences are provided in Table S3.

Table 1. Molecular and cellular functions of the dysregulated genes in FOXE1 knockdown cells by IPA analysis

<table>
<thead>
<tr>
<th>Function</th>
<th>P value</th>
<th>Molecules</th>
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<tbody>
<tr>
<td>Cellular movement</td>
<td>1.30 × 10⁻⁴–7.65 × 10⁻¹⁰</td>
<td>42</td>
</tr>
<tr>
<td>Cell death and survival</td>
<td>1.27 × 10⁻⁴–8.74 × 10⁻¹⁰</td>
<td>46</td>
</tr>
<tr>
<td>Cellular development</td>
<td>1.22 × 10⁻⁴–1.20 × 10⁻⁸</td>
<td>53</td>
</tr>
<tr>
<td>Cellular growth and proliferation</td>
<td>1.13 × 10⁻⁴–1.20 × 10⁻⁸</td>
<td>47</td>
</tr>
<tr>
<td>Cell signaling</td>
<td>9.58 × 10⁻⁴–6.75 × 10⁻⁸</td>
<td>19</td>
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Transfection and Dual Luciferase Reporter Assay. For the luciferase reporter assay, BCPAP cells were transiently transfected with reporter plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, cells were seeded in a 24-well plate at 0.7 × 10^4 cells per well and cultured overnight. The following day, 250 ng luciferase reporter plasmids and 250 ng effector plasmids were cotransfected along with 1.25 ng Renilla plasmid pRL-TK (Promega) as a control for each well. Cells were lysed with 100 μL passive lysis buffer (Promega) for luciferase activity analysis using the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection. A 25-μL aliquot of cell lysate was then assayed for luciferase activity using GloMax 96 Microplate Luminometer (Promega).

Cell Viability Assay and Cell Apoptosis Assay. Cell viability in the BCPAP cell line transfected with FOXE1 siRNA or negative control siRNA at different time points (24, 48, and 72 h) was analyzed by using the CellTiter-Glo assay.


