Molecular Therapy
Original Article

Upregulation of lncRNA ADAMTS9-AS2 Promotes Salivary Adenoid Cystic Carcinoma Metastasis via PI3K/Akt and MEK/Erk Signaling

Shule Xie,[1,2,7] Xin Yu,[1,2,7] Yingru Li,[3,4,7] Hanyu Ma,[6] Song Fan,[1,2] Weixiong Chen,[1,2] Guokai Pan,[1,2] Weiwei Wang,[5] Hanqing Zhang,[1,2] Jinsong Li,[1,2] and Zhaoyu Lin[1,2]

[1]Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China; [2]Department of Oral and Maxillofacial Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China; [3]Department of Gastroenterology, Hernia and Abdominal Wall Surgery, the Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; [4]Guangdong Provincial Key Laboratory of Colorectal and Pelvic Floor Diseases, the Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; [5]Department of Stomatology, Zibo Center Hospital, Zibo, China; [6]Department of Pathology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Neurotropic infiltrative growth and distant metastasis are the main causes of death in salivary adenoid cystic carcinoma (SACC) patients. Long noncoding RNAs (lncRNAs) are involved in many human neoplasms, however, their potential roles in SACC are unclear. In our study, we found that ADAM metallopeptidase with thrombospondin type 1 motif, 9 (ADAMTS9) antisense RNA 2 (ADAMTS9-AS2) was significantly upregulated in SACC patients with metastasis and SACC-lung metastasis (LM) cells. Moreover, ADAMTS9-AS2 expression was closely associated with the prognosis and distant metastasis in SACC patients. Next, we found that *c-myc* could specifically bind to the promoter of ADAMTS9-AS2 and activated its transcription. Knockdown of ADAMTS9-AS2 significantly inhibited migration and invasion of SACC cells *in vitro* and distant lung metastasis *in vivo*. Furthermore, ADAMTS9-AS2, which mainly expressed in the cytoplasm, shared microRNA (miRNA) response elements with Integrin α6 (ITGA6). Overexpression of ADAMTS9-AS2 competitively bound to miR-143-3p that inhibited ITGA6 from miRNA-mediated degradation, and thus it activated the activity of PI3K/Akt and MEK/Erk signaling and facilitated SACC metastasis. In summary, ADAMTS9-AS2 promotes migration and invasion in SACC by competing with miR-143-3p. This sheds a new insight into the regulation mechanism of ADAMTS9-AS2, and it provides a possible application for the SACC treatment.

INTRODUCTION

Salivary adenoid cystic carcinoma (SACC) is the most common tumor that primarily arises from the salivary duct epithelium, accounting for a quarter of malignant salivary gland tumors. SACC exhibits many specific characteristics, including neurotropic infiltrative growth and distant lung metastasis.[1–3] Distant metastasis is a crucial prognostic factor for SACC patients.[4,5] Therefore, demonstrating the mechanisms that regulate perineural invasion and distant metastasis of SACC is necessary for the development of successful therapeutic strategies to improve SACC patient survival rates.

Annotation of the results of large-scale sequencing of the human genome has indicated that only 2% of our genomes are actively transcribed to protein-coding RNAs and the majority of remaining transcripts are noncoding RNAs.[6] Long noncoding RNAs (lncRNAs) are a type of noncoding RNA with more than 200 nt. They have no protein-coding capacity and have been identified as key mammalian transcription regulators. Accumulating studies have revealed that the dysregulation of lncRNAs plays an important role in a wide range of biological processes and likely contributes to the development of human cancer.[7] The lncRNAs may act as oncogenes or tumor inhibitors through the modulation of cell apoptosis, invasion, and proliferation and by mediating epigenetic modifications and regulating transcriptional activity.[8,9] Many functions of lncRNAs in cancer biology have been discovered and investigated, but only a small fraction of lncRNAs have been examined in detail. lncRNAs can modulate gene expression at the transcriptional or post-transcriptional level, and they can interact with DNA, RNA, or proteins to exert their gene-regulating functions and affect the behavior of cancer cells.[10,11] Additionally, cancer-specific lncRNA expression can also be used as an independent prognostic biomarker for diagnosis of several cancers, including head and neck cancer.[12] However, the molecular

Received 4 February 2018; accepted 19 August 2018;
https://doi.org/10.1016/j.ymthe.2018.08.018.

[7]These authors contributed equally to this work.

Correspondence: Zhaoyu Lin, Department of Oral and Maxillofacial Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107 Yanjiang West Road, Guangzhou 510120, China.
E-mail: linzhy2008@163.com
Correspondence: Jinsong Li, Department of Oral and Maxillofacial Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107 Yanjiang West Road, Guangzhou 510120, China.
E-mail: lijinsong1967@163.com

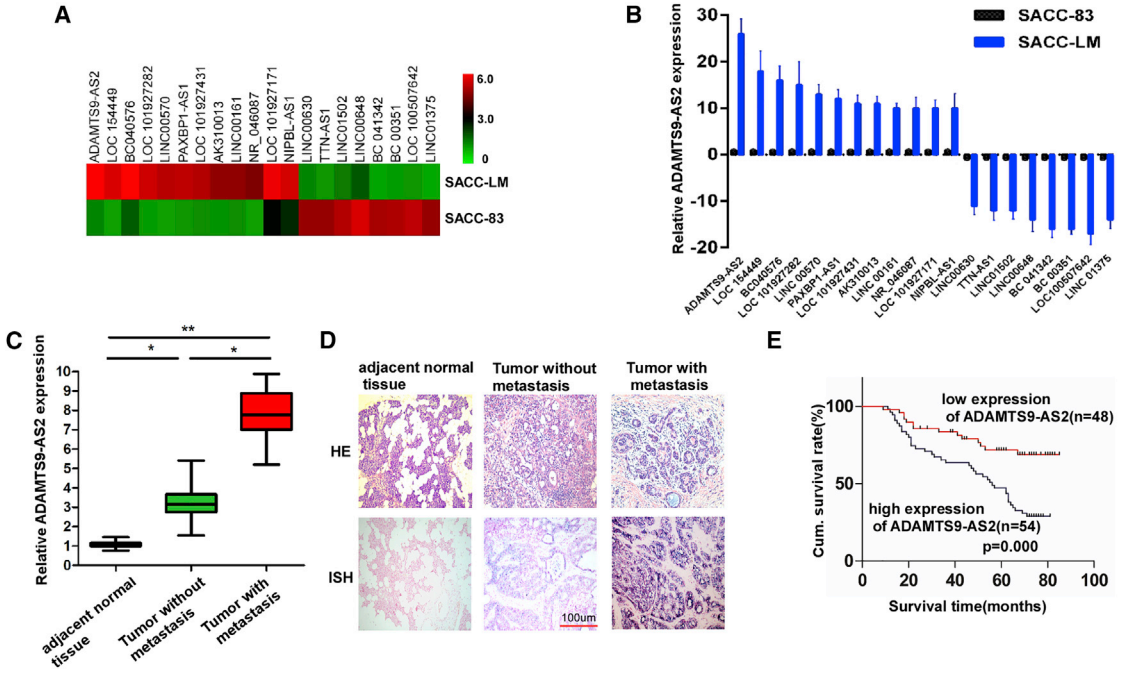

Figure 1. ADAMTS9-AS2 Is Upregulated in SACC Tissues and Correlated with a High Metastasis Rate and Poor Prognosis

(A) A heatmap depicting lncRNAs for which >2-fold change in expression was found in SACC-LM and SACC-83 cells. (B) The expression levels of several significantly differentially expressed lncRNAs were validated by qRT-PCR. (C) ADAMTS9-AS2 expression levels were detected by qRT-PCR in clinical SACC patients with metastasis or without metastasis and adjacent normal salivary tissues (*$p < 0.05$, **$p < 0.01$). (D) Representative images of *in situ* hybridization for ADAMTS9-AS2 in paraffin-embedded SACC tissues and adjacent normal salivary tissues (200×). (E) Kaplan-Meier curves for SACC patients from Sun Yat-sen Hospital comparing ADAMTS9-AS2 expression and survival ($p < 0.001$).

mechanisms by which lncRNAs regulate biological processes in SACC are still unclear.

In our study, using lncRNA microarray analysis, we discovered that lncRNA ADAM metallopeptidase with thrombospondin type 1 motif, 9 (ADAMTS9) antisense RNA 2 (ADAMTS9-AS2) was significantly upregulated in SACC-lung metastasis (LM) cells and also in SACC tissues with metastasis. High expression levels of ADAMTS9-AS2 were correlated with metastasis rate and poor prognosis in SACC patients. Meanwhile, the upregulation of ADAMTS9-AS2 was transcriptionally activated by a key oncogene, *c-myc*. Furthermore, we identified that ADAMTS9-AS2 promoted SACC metastasis and invasion by acting as a competing endogenous RNA (ceRNA) to increase the expression of Integrin α6 (ITGA6) and enhance the activity of PI3K/Akt and MEK/Erk signaling, contributing to SACC metastasis. Therefore, lncRNA ADAMTS9-AS2 may be a potential marker and therapeutic target for SACC patients.

RESULTS

ADAMTS9-AS2 Is Upregulated in SACC Tissues, and It Is Correlated with High Metastasis Rate and Poor Prognosis

To uncover lncRNAs involved in SACC metastasis, we used an lncRNA microarray to screen for differentially expressed lncRNAs in SACC-LM cells and parental SACC-83 cells, and a heatmap was constructed (Figure 1A). Among all the differentially expressed lncRNAs in SACC-LM cells, the most dramatic increase was observed for ADAMTS9-AS2, and the accuracy of lncRNA microarray was further validated via qRT-PCR (Figure 1B). Using a rapid amplification of cDNA ends (RACE) assay, we identified ADAMTS9-AS2 as a 2,286-nt antisense transcript of ADAMTS9 that is identical to uc003dml in the University of California, Santa Cruz (UCSC) database (Figure S1). Next, we observed that ADAMTS9-AS2 was highly expressed in SACC samples compared with the adjacent salivary tissues. Interestingly, ADAMTS9-AS2 expression was also increased in SACC patients with metastasis compared with those without metastasis (Figure 1C). *In situ* hybridization further confirmed that ADAMTS9-AS2 was highly expressed in SACC samples with metastasis compared with those without metastasis, and it revealed that ADAMTS9-AS2 was mainly located in the cytoplasm (Figure 1D).

To explore the clinical significance of ADAMTS9-AS2, we analyzed the relationship between ADAMTS9-AS2 expression and the clinicopathological factors of 102 SACC patients (Table S1). Reduced ADAMTS9-AS2 expression in SACC patients was closely associated with tumor size ($p = 0.017$), clinical stage ($p = 0.033$), and distant metastasis ($p = 0.035$). However, no significant association was found between ADAMTS9-AS2 expression and gender or age. In addition, Kaplan-Meier analysis indicated that increased ADAMTS9-AS2

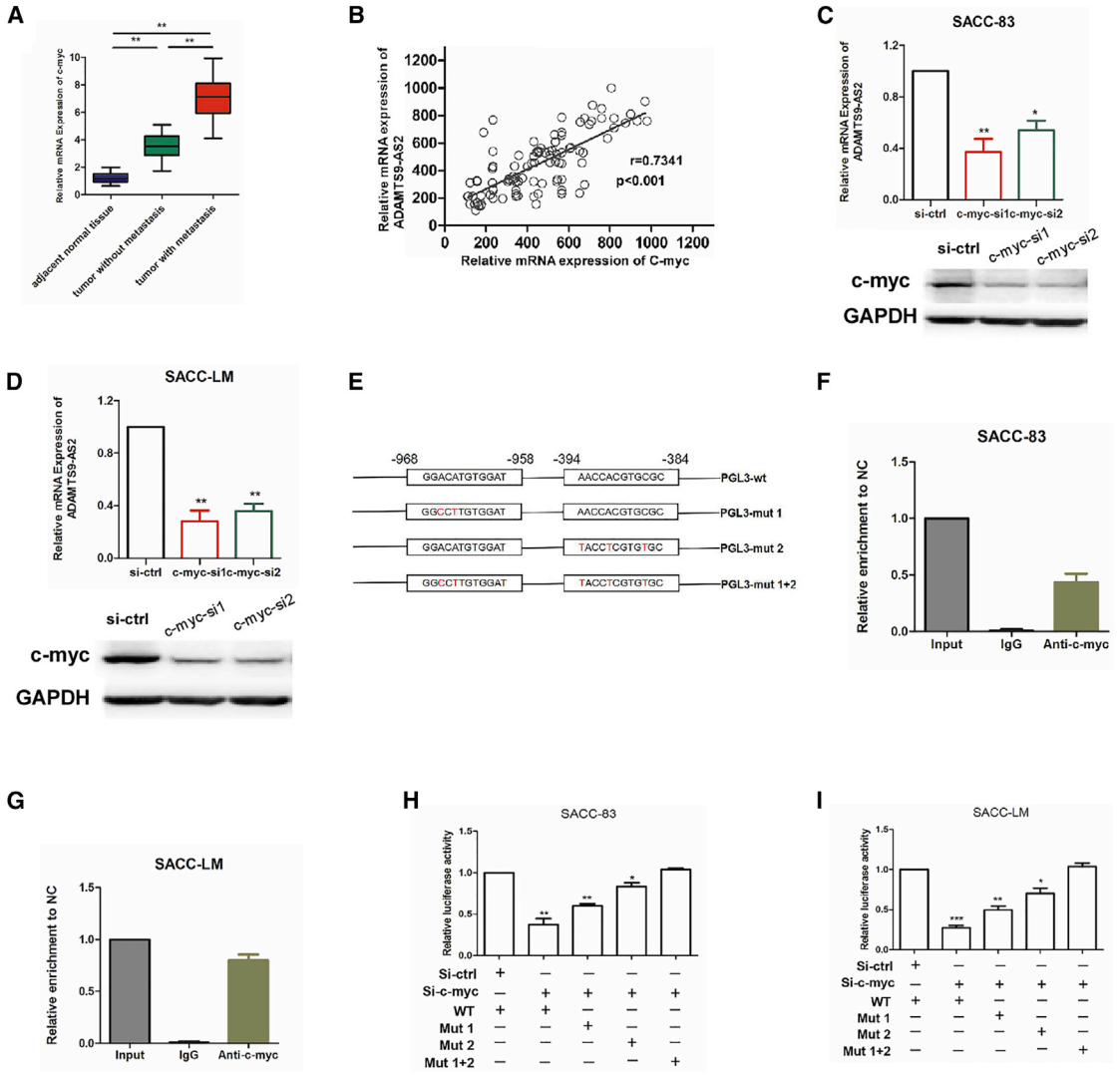

Figure 2. ADAMTS9-AS2 Is Regulated by the Transcription Factor c-*myc*

(A) mRNA expression level of c-*myc* in 102 samples of SACC tissues. (B) Expression correlation analysis between ADAMTS9-AS2 and c-*myc* in SACC tissues. (C) Expression levels of ADAMTS9-AS2 in SACC-83 cells after knockdown of c-*myc*. (D) Expression levels of ADAMTS9-AS2 in SACC-LM cells after knockdown of c-*myc*. (E) Schematic representation of constructs for ADAMTS9-AS2 promoter reporter. (F and G) ChIP assay in SACC-83 cells (F) and SACC-LM cells (G), followed by qPCR amplification of binding site within ADAMTS9-AS2 promoter region. Genomic DNA input was 1%. (H and I) Luciferase activities of wild-type and mutant plasmids after knockdown of c-*myc* were detected in SACC-83 (H) and SACC-LM (I) cells. Data are presented as means ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

expression in SACC was significantly associated with decreased overall survival (Figure 1E). These results suggested that ADAMTS9-AS2 may be a potential diagnosis and prognosis marker of SACC patients and associated with the regulation of distant metastasis in SACC patients.

ADAMTS9-AS2 Is Activated by c-*myc*

To explore the underlying mechanism of ADAMTS9-AS2 upregulation in SACC, two bioinformatical software programs (JASPAR and PROMO) were used to search possible transcription factors binding to the promoter region of ADAMTS9-AS2. The result demonstrated that there were two putative c-*myc*-binding sites at 968 and 394 bp upstream of ADAMTS9-AS2. Next, we examined the expression of c-*myc* in 102 SACC specimens using qRT-PCR. Interestingly, we found that c-*myc* was highly expressed in SACC specimens compared with normal adjacent tissues and especially upregulated in SACC patients with metastasis. Meanwhile, c-*myc* expression was positively correlated with ADAMTS9-AS2 in SACC tissues (Figures 2A and 2B). Then, we examined whether the mRNA expression levels of ADAMTS9-AS2 were regulated by c-*myc*. Our results revealed that the depletion of c-*myc* significantly reduced ADAMTS9-AS2 expression in

SACC-83 (Figure 2C; Figure S2E) and SACC-LM cells (Figure 2D; Figure S2F).

To confirm whether ADAMTS9-AS2 is a direct transcriptional target of c-*myc*, the promoter region of ADAMTS9-AS2 was cloned into luciferase reporter plasmids. Then two potential c-*myc*-binding sites in the promoter region of ADAMTS9-AS2 were mutated, either respectively or combined, and then the luciferase activity was tested after co-transfection (Figure 2E). Upon c-*myc* inhibition, SACC cells transfected with a reporter containing the wild-type ADAMTS9-AS2 promoter regions exhibited dramatically decreased luciferase activity. However, individual mutation in the c-*myc*-binding site rescued the effects of c-*myc* on the transcriptional activity of ADAMTS9-AS2 (Figures 2H and 2I). In addition, chromatin immunoprecipitation (ChIP) assay demonstrated that c-*myc* could directly bind to the promoter region of ADAMTS9-AS2 (Figures 2F and 2G). Briefly, our findings suggest that the upregulation of ADAMTS9-AS2 is transcriptionally activated by c-*myc* in SACC.

ADAMTS9-AS2 Binds miR-143-3p and Depresses Its Expression

Numerous studies have reported that lncRNAs could act as microRNA (miRNA) sponges, to regulate endogenous miRNAs for their binding to the target mRNAs, and inhibit the expression of these target mRNAs.[13] Using LncBase Predicted version (v.)2 of DIANA tools, we chose 50 potential miRNAs with higher miTG scores for our research objects. Previous studies have shown that six miRNAs, miR-2392, miR-362-5p, miR-193a-5p, miR-143-3p, miR-493-5p, and miR-600, were closely associated with invasion and cancer metastasis.[14–19] Then we used qRT-PCR to validate the expression levels of six miRNAs in SACC cells after transfection. Our results showed that the expression levels of miR-193a-5p and miR-143-3p were significantly upregulated in SACC-LM cells when ADAMTS9-AS2 was deleted, yet miR-193a-5p and miR-143-3p were downregulated in SACC-83 cells when ADAMTS9-AS2 was overexpressed, indicating a potentially competing relationship between the ADAMTS9-AS2 and miR-193a-5p or miR-143-3p (Figures S2A and S2B).

Subsequently, we performed pull-down assays to further explore to which miRNAs ADAMTS9-AS2 could directly bind. Fortunately, only miR-143-3p obtained a great enrichment in the ADAMTS9-AS2 pulled-down pellets compared with the negative control group (Figures 3A and 3B). Using LncBase Predicted v.2 of DIANA tools, we predicted that there are three latent binding sites for ADAMTS9-AS2 at the regions of 666–671, 1,808–1,814, and 1,793–1,800 on miR-143-3p (Figure 3C). We created wild-type ADAMTS9-AS2 luciferase plasmids containing potential miR-143-3p-binding sites or the mutant of each site. The luciferase plasmids were co-transfected with miR-143-3p mimics into SACC cells, and luciferase activity was examined. As shown in Figure 3D, miR-143-3p inhibited the activity of wild-type ADAMTS9-AS2, mutant 1 and mutant 2, but it did not impact mutant 3, indicating that miR-143-3p bound to the ADAMTS9-AS2 transcript at the position of 1,793–1,800. Through qRT-PCR analysis, we found that the expression levels of miR-143-3p were significantly downregulated in SACC patients with metastasis and in SACC-LM cells (Figure 3E).

To confirm whether miR-143-3p is a target of ADAMTS9-AS2, we first inhibited ADAMTS9-AS2 expression in SACC-LM cells and then upregulated ADAMTS9-AS2 expression in SACC-83 cells. The transfection efficacy of ADAMTS9-AS2 plasmids and small interfering RNAs (siRNAs) was validated by qRT-PCR analysis (Figure S2C). The expression levels of miR-143-3p were elevated when we knocked down ADAMTS9-AS2 expression in SACC-LM cells, but an opposite trend was observed in SACC-83 cells after transfected with ADAMTS9-AS2 plasmids (Figure 3F). Second, we found that the expression of miR-143-3p was remarkably inhibited by wild-type, but not by mutant, ADAMTS9-AS2 after we transfected with wild-type ADAMTS9-AS2 and mutant plasmids (Figure 3H). Third, we investigated whether overexpression or knockdown of miR-143-3p in SACC cells could cause any change in expression level of ADAMTS9-AS2, and the results (Figure S2D) indicated that miR-143-3p was indeed a downstream inhibitory gene of ADAMTS9-AS2. In an analysis of clinical samples, we also observed a reverse correlation between the expression level of ADAMTS9-AS2 and that of miR-143-3p (Figure 3G; $r = -0.547$, $p < 0.001$). In general, our results supported the proposal that miR-143-3p is an inhibitory target of ADAMTS9-AS2 in SACC cells and tissue samples.

ADAMTS9-AS2 Promotes SACC Cell Migration and Invasion

To explore the biological function of ADAMTS9-AS2 in SACC cell migration and invasion, we transfected SACC-83 cells with ADAMTS9-AS2 plasmids, yet we decreased ADAMTS9-AS2 expression in SACC-LM cells via ADAMTS9-AS2 siRNA transfection. Knockdown of ADAMTS9-AS2 in SACC-LM cells significantly inhibited cell invasion and migration by 64% and 75%, respectively (Figure 4A). In contrast, overexpression of ADAMTS9-AS2 promoted invasion and migration of SACC-83 cells. The number of SACC-83 cells that passed through the chamber increased by 2.5-fold (invasion assay) and 3.4-fold (migration assay) (Figure 4A). In brief, increased ADAMTS9-AS2 levels enhanced the migration and invasion abilities of SACC cells.

Next, we examined whether elevated expression of miR-143-3p inhibited the migration and invasion abilities of SACC cells. The transfection efficiency of miR-143-3p mimics or miR-143-3p inhibitors was confirmed by qRT-PCR (Figure 4C). Overexpression of miR-143-3p significantly inhibited SACC-LM cell invasion and migration by 61% and 72%, respectively (Figure 4B). But chamber assays indicated that inhibition of miR-143-3p in SACC-83 cells led to enhanced invasion and migration of these cells, which increased by 2.4-fold and 3.2-fold, respectively (Figure 4B). Consequently, ADAMTS9-AS2 overexpression contributed to enhanced invasion and migration ability of SACC cells, yet miR-143-3p upregulation inhibited invasion and migration ability of SACC cells.

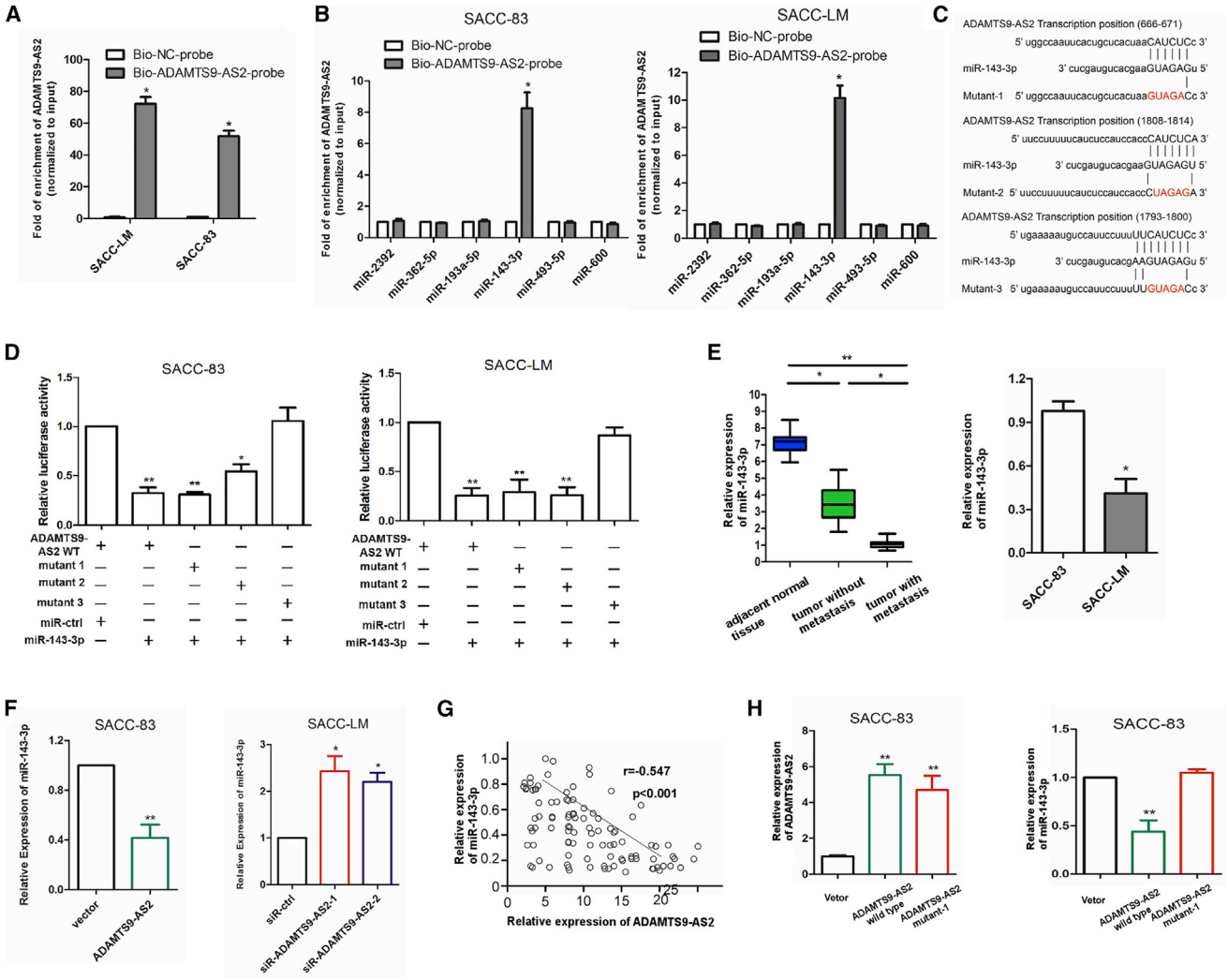

Figure 3. ADAMTS9-AS2 Binds to miR-143-3p and Represses Its Expression

(A) Detection of ADAMTS9-AS2 using qRT-PCR in the samples pulled down by biotinylated ADAMTS9-AS2 and negative control (NC) probe. (B) Detections of miR-2392, miR-362-5p, miR-193a-5p, miR-143-3p, miR-493-5p, and miR-600 using qRT-PCR in the same samples pulled down by biotinylated ADAMTS9-AS2 and NC probe. Input was used for normalization. *$p < 0.05$ compared with respective control. (C) Schematic illustration of the predicted binding sites between ADAMTS9-AS2 and miR-143-3p and mutation of potential miR-143-3p-binding sequence in ADAMTS9-AS2. (D) Luciferase assays in SACC-83 and SACC-LM cells transfected with wild-type or mutant ADAMTS9-AS2 and miR-143-3p. (E) Expression levels of miR-143-3p determined by qRT-PCR in clinical samples from SACC patients and in SACC cells. (F) miR-143-3p expression was examined with qRT-PCR in SACC-LM cells transfected with siR-ADAMTS9-AS2 or in SACC-83 cells with overexpressed ADAMTS9-AS2. (G) Reverse correlation between the expression levels of ADAMTS9-AS2 and those of miR-143-3p in SACC samples. (H) Expression levels of ADAMTS9-AS2 and miR-143-3p were examined with qRT-PCR in SACC-83 cells transfected with wild-type, mutant ADAMTS9-AS2 plasmids, or empty vector. Data are presented as means ± SD. *$p < 0.05$ and **$p < 0.01$.

miR-143-3p Targets ITGA6 and Inhibits PI3K/Akt and MEK/Erk Signaling

Next, we identified potential targets of miR-143-3p using TargetScan and MiRanda tools, and we found a miR-143-3p recognition site in the 3′ UTR of ITGA6 (Figure 4D). To determine whether ITGA6 is a target gene of miR-143-3p, we generated wild-type and mutant luciferase reporter plasmids encoding the ITGA6 3′ UTR, and we co-transfected cells with these plasmids and miR-143-3p in SACC cells. The results demonstrated that luciferase activity was reduced when we co-transfected cells with miR-143-3p and the wild-type 3′ UTR of ITGA6, but the mutant 3′ UTR of ITGA6 did not cause any change in luciferase activity (Figure 4F). These results suggested that miR-143-3p suppressed ITGA6 expression in SACC cells. Furthermore, western blot analysis showed that ITGA6 levels in SACC-LM cells were reduced by miR-143-3p overexpression but up-regulated in SACC-83 cells by miR-143-3p inhibition (Figure 4G).

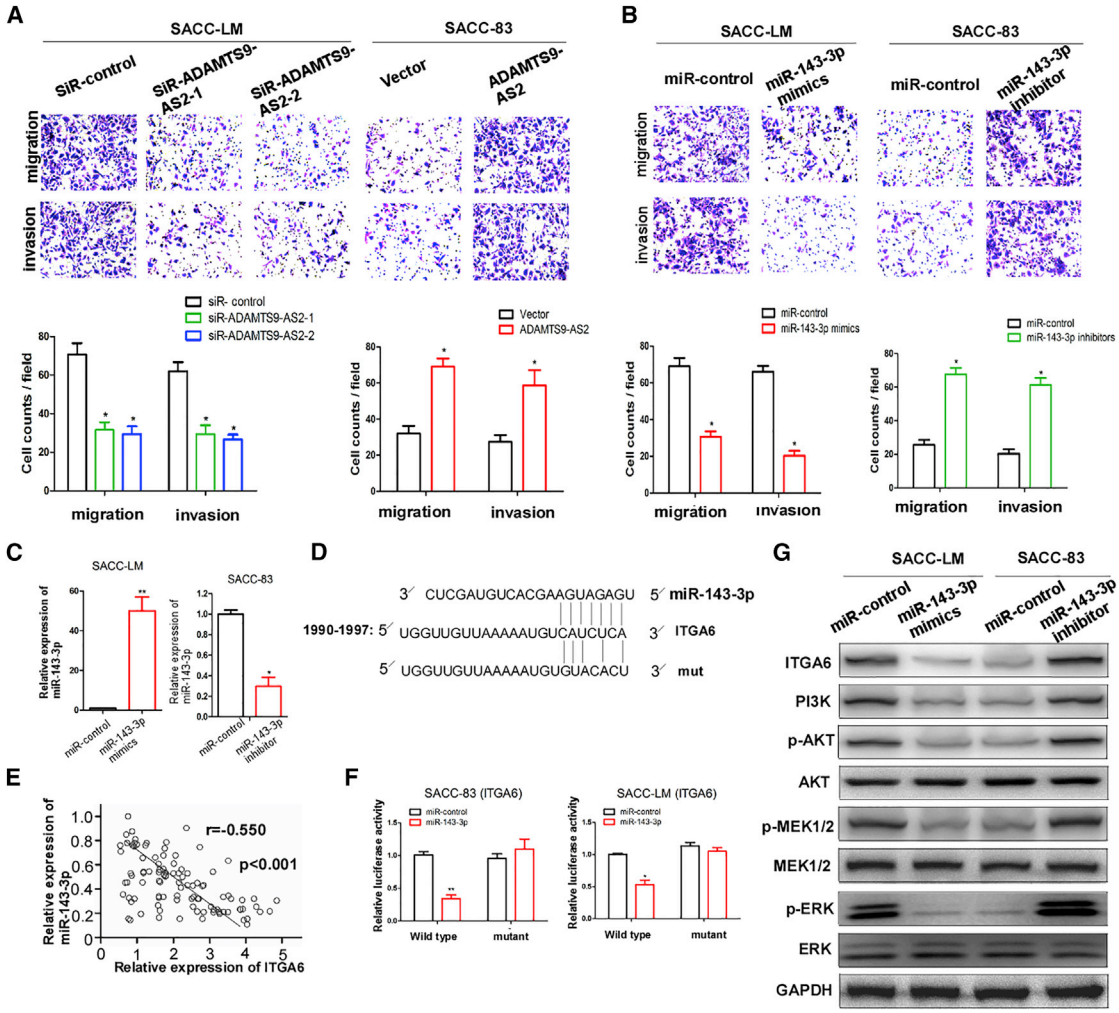

Figure 4. ADAMTS9-AS2 Knockdown and miR-143-3p Overexpression Repress SACC Cell Migration and Invasion

(A) Chamber assays were performed with SACC-LM cells transfected with siR-ADAMTS9-AS2 and SACC-83 cells with overexpressed ADAMTS9-AS2. (B) Chamber assays were performed with SACC-LM cells transfected with miR-143-3p mimics and SACC-83 cells transfected with miR-143-3p inhibitors. (C) miR-143-3p expression levels were examined with qRT-PCR in SACC cells after transfection with miR-143-3p mimics or inhibitors. (D) Target sequence of miR-143-3p in ITGA6 3′ UTR predicted by TargetScan as well as the mutated sequence. (E) Reverse correlation between the expression levels of ITGA6 and those of miR-143-3p in SACC samples. (F) Luciferase reporter assays were performed in SACC cells co-transfected with miR-143-3p-mimics and reporter vectors carrying an ITGA6 3′ UTR with a wild-type (ITGA6 3′ UTR) or mutated miR-143-3p (ITGA6 3′ UTR mut) response element. (G) ITGA6 expression as well as downstream Erk and Akt phosphorylation was assessed by western blot in SACC cells. Data are presented as means ± SD. *$p < 0.05$ and **$p < 0.01$.

Two vital pathways, the PI3K/Akt- and MEK/Erk-signaling pathways, are known to be involved in cancer metastasis and invasion.[20] In SACC cells, ITGA upregulated the expression of PI3K, p-AKT, p-MEK1/2, and p-ERK, suggesting the activation of PI3K/Akt- and MEK/Erk-signaling pathways (Figures S4A and S4B). Overexpression of miR-143-3p enhanced phosphorylation of Akt and Erk in SACC-LM cells, but a reverse trend was observed in SACC-83 cells after transfection with miR-143-3p inhibitors (Figure 4G). Clinical SACC samples further exhibited an inverse correlation between miR-143-3p and ITGA6 expression (Figure 4E; $r = -0.550$, $p < 0.001$).

To explore whether miR-143-3p inhibits SACC cell metastasis by targeting ITGA6, a rescue experiment in which SACC-LM cells were co-transfected with miR-143-3p mimic and wild-type ITGA6 or mutant ITGA6 plasmids was performed. When we rescued ITGA6 expression in the presence of miR-143-3p mimics, the invasion and migration abilities of SACC-LM cells were restored (Figure 5A). When miR-143-3p mimics and ITGA6 mutant plasmids were co-expressed in SACC-LM cells, ITGA6 expression, as well as Akt and Erk phosphorylation, was restored, but these parameters were unchanged by co-transfection with wild-type ITGA6 plasmid and miR-143-3p mimic (Figure 5B). Taken together, these results

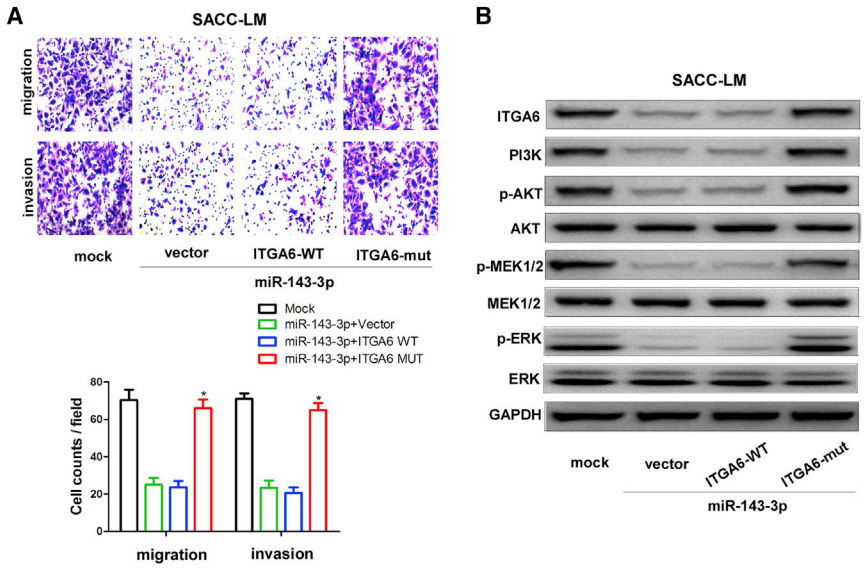

Figure 5. miR-143-3p Inhibits Migration and Invasion of SACC Cells by Targeting ITGA6

(A) Chamber assays were performed with SACC-LM cells transfected with miR-143-3p mimics alone or with a combination of either pcDNA3.1+ (vector) or pcDNA3.1+ containing a wild-type (ITGA6) or mutant (ITGA6 mut) ITGA6 expression cassette of the miR-143-3p response element. (B) ITGA6 expression and downstream Erk and Akt phosphorylation were detected by western blot in SACC-LM cells transfected with miR-143-3p mimic alone or with a combination of either pcDNA3.1+ (vector) or pcDNA3.1+ containing a wild-type (ITGA6) or mutant (ITGA6 mut) ITGA6 expression cassette of the miR-143-3p response element.

suggested that miR-143-3p regulates SACC cell metastasis by targeting ITGA6 and inhibiting PI3K/Akt and MEK/Erk signaling.

ADAMTS9-AS2 Promotes SACC Cell Migration and Invasion via miR-143-3p-Mediated Regulation of PI3K/Akt and MEK/Erk Signaling

Based on our previous results, we hypothesized that ADAMTS9-AS2 could promote SACC cell migration and invasion via the suppression of miR-143-3p expression and consequent promotion of ITGA6 and downstream PI3K/Akt and MEK/Erk signaling. To confirm this hypothesis, we performed another rescue experiment. Chamber assays showed that an ADAMTS9-AS2 decrease in SACC-LM cells significantly inhibited their migration and invasion, but simultaneous miR-143-3p knockdown could reverse the repression of cell migration and invasion, indicating that upregulation of miR-143-3p was essential for the decreased cell migration and invasion induced by ADAMTS9-AS2 downregulation (Figure 6A; Figure S3A). By contrast, miR-143-3p overexpression could also reverse the promotion of migration and invasion caused by ADAMTS9-AS2 overexpression in SACC-83 cells (Figure 6B; Figure S3B).

Importantly, we also determined whether ADAMTS9-AS2 regulated ITGA6 expression and the activity of the PI3K/Akt and MEK/Erk pathways and whether this regulation was dependent on miR-143-3p. As expected, knockdown of ADAMTS9-AS2 led to reduced ITGA6 and PI3K/Akt and MEK/Erk phosphorylation levels, presumably via upregulation of miR-143-3p. Moreover, miR-143-3p knockdown reversed the inhibition of ITGA6 and p-Akt and p-Erk expression (Figure 6C). A reverse expression pattern of ITGA6 and p-Akt and p-Erk was found in SACC-83 cells (Figure 6D). Collectively, these data supported our hypothesis that ADAMTS9-AS2 promoted SACC cells migration and invasion via miR-143-3p-mediated regulation of ITGA6 and downstream PI3K/Akt and MEK/Erk signaling.

Knockdown of ADAMTS9-AS2 Expression Inhibits SACC-LM Xenograft Growth and Metastasis

Next, we examined the effect of ADAMTS9-AS2 *in vivo*. To verify the role of ADAMTS9-AS2 in regulating SACC growth, 1×10^6 SACC-LM cells infected with lentivirus containing sh-ADAMTS9-AS2 or with sh-vector as a control were subcutaneously injected into nude mice. As shown in Figures 7A–7C, decreased ADAMTS9-AS2 expression in SACC-LM cells slowed down the tumor growth and reduced tumor weight in BALB/c-nu mice. To validate the effect of ADAMTS9-AS2 on tumor metastasis, we injected 4×10^5 SACC-LM cells stably expressing small hairpin RNA (shRNA) empty vector or sh-ADAMTS9-AS2 vector into the tail vein of nude mice. 8 weeks later, lung tissues were extracted to observe whether there was a tumor metastasis. As shown in Figure 7D, the number of lung metastasis nodules in the sh-ADAMTS9-AS2 group was less than that in the sh-vector group, and H&E staining of lung also showed the tumor metastasis in the lung of sh-vector mice. Therefore, ADAMTS9-AS2 downregulation significantly inhibited metastasis and growth of SACC-LM xenografts.

DISCUSSION

Dysregulation of lncRNA has been widely reported in different types of human neoplasms, and various lncRNAs play crucial roles in tumor occurrence, invasion, and metastasis and can be considered prognostic biomarkers in several cancers.[21] In our study, we screened out lncRNA ADAMTS9-AS2 using an lncRNA microarray, and we explored its potential functions in the regulation of metastasis and invasion in SACC. We examined the expression levels of ADAMTS9-AS2 in SACC clinical specimens and cell lines, and we found that ADAMTS9-AS2 was significantly increased in SACC samples with distant metastasis and in highly metastatic SACC cells. *In situ* hybridization showed that ADAMTS9-AS2 was primarily located in the cytoplasm of SACC cells and was highly expressed in SACC samples compared to paired adjacent normal tissues. ADAMTS9-AS2 is an antisense transcript of the protein-coding gene ADAMTS9, which is an anti-angiogenesis factor that inhibits tumor progression and metastasis.[22] Only a few studies have reported that ADAMTS9-AS2

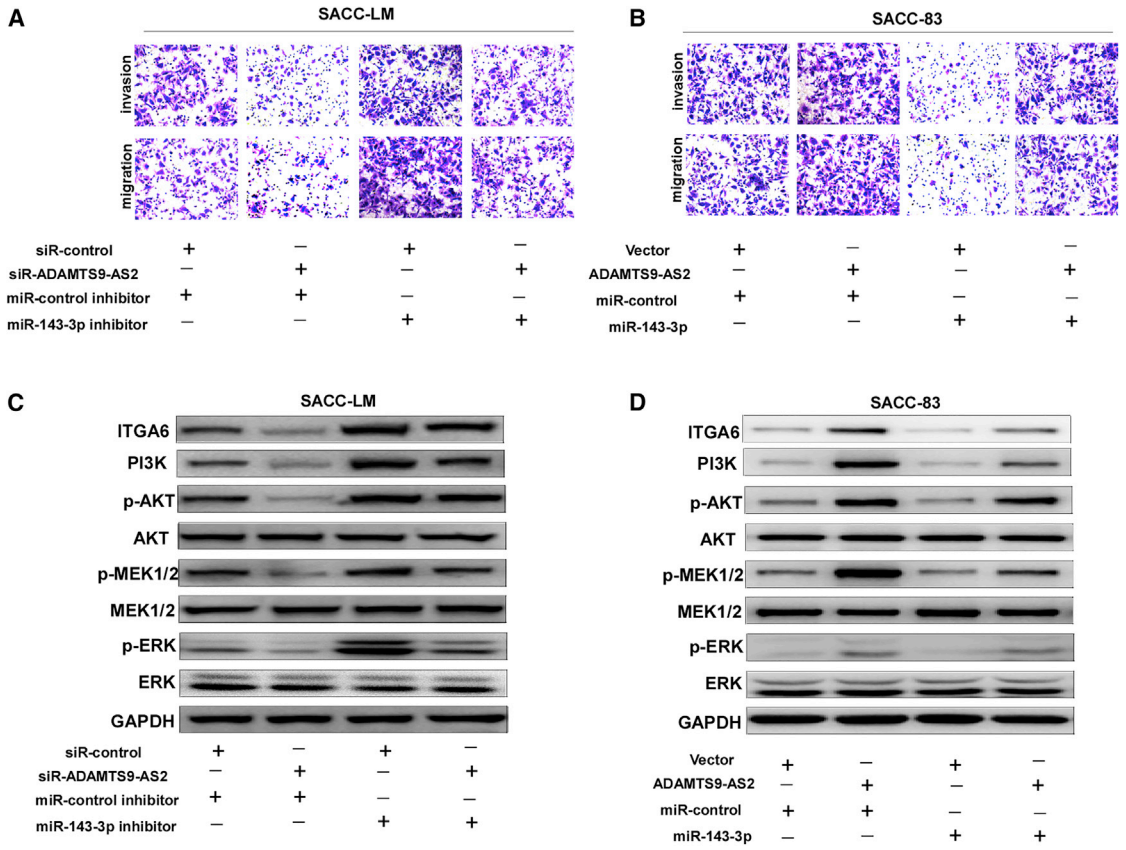

Figure 6. Regulation of SACC Cell Metastasis and PI3K/Akt and MEK/Erk Signaling by ADAMTS9-AS2 Requires miR-143-3p

(A) The migration and invasion abilities of SACC-LM cells transfected with siR-ADAMTS9-AS2 or siR-control simultaneously with miR-143-3p inhibitors or miR-controls were examined with chamber assays. (B) The transwell assays were performed by SACC-83 cells transfected with ADAMTS9-AS2 plasmids or vectors simultaneously with miR-143-3p mimics or miR-controls. (C) The ITGA6 protein level and inhibition of downstream PI3K/Akt and MEK/Erk signaling were determined by western blot analysis in SACC-LM cells. (D) The ITGA6 protein level and inhibition of downstream PI3K/Akt and MEK/Erk signaling were determined by western blot analysis in SACC-83 cells.

is involved in human malignancies, and it was urgent for us to explore the underlying mechanism by which ADAMTS9-AS2 modulates cancer progression, especially in SACC.[23]

To explore the underlying mechanism of ADAMTS9-AS2 in SACC, we first indicated that c-*myc* could directly interact with the promoter and activated transcription of ADAMTS9-AS2 through the binding sites located at −968 to −958 nt and −394 to −384 nt. c-*myc* is always reported as an amplifierof transcription, and it is overexpressed or amplifed in different kinds of human cancers.[24] However, previous studies mainly focused on the transcriptional levels of coding transcripts regulated by c-*myc*. Whether lncRNAs could be activated by c-*myc* have only begun to be explored. Recent studies showed that lncRNA CASC11 was near c-*myc* and could be transcriptionally activated by c-*myc* in human colorectal cancer.[25] The regulatory function of c-*myc* on a large scale of lncRNAs opens up a new area of c-*myc* effect on tumorigenesis.

Previous studies have confirmed that lncRNAs with miRNA response elements (MREs) could function as endogenous miRNA sponges, influencing the expression of target mRNAs at the post-transcriptional level.[13,26] Here, using qRT-PCR and fluorescence *in situ* hybridization (FISH) assays, we identified that lncRNA ADAMTS9-AS2 was located in the cytoplasm, which suggested that ADAMTS9-AS2 may compete with miR-143-3p. Numerous studies have proven that lncRNAs possess the ability for sponging miRNA to exert functions in tumorigenesis and tumor progression, especially competing with miR-143-3p.[27] Liu et al.[28] reported that lncRNA HOTAIR competed with miR-143-3p through regulating BCL2 and promoted cervical cancer progression. lncRNA MALAT1 also regulated ZEB1 expression by sponging miR-143-3p and promoted hepatocellular carcinoma progression.[29] Using bioinformatics tools (LncBase Predicted v.2 of DIANA tools) and RNA pull-down and luciferase reporter assays, we confirmed that miR-143-3p was a direct target of lncRNA ADAMTS9-AS2. Furthermore, the reverse ADAMTS9-AS2 and miR-143-3p expression levels were observed in both SACC clinical samples and cell lines, which further validated the targeting regulatory relationship between the two noncoding RNAs. lncRNA ADAMTS9-AS2 knockdown combined with miR-143-3p inhibition significantly rescued the reduced cell migration

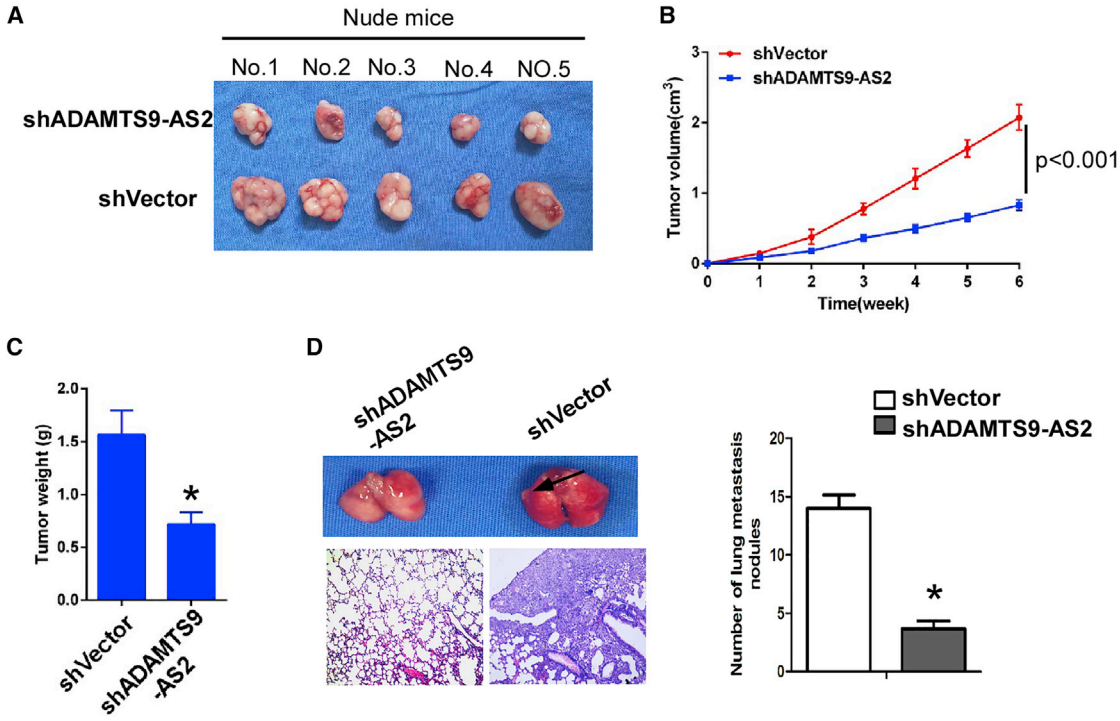

Figure 7. Ectopic Expression of ADAMTS9-AS2 Inhibits SACC-LM Xenograft Growth and Metastasis in BALB/c-nu Mice

(A) Representative photomicrographs of xenografts from the sh-ADAMTS9-AS2 group or sh-vector group at day 36 (200×). (B) Tumor growth curves for SACC-LM tumors transfected with sh-ADAMTS9-AS2 or sh-vector (*$p < 0.05$ versus sh-vector). (C) Weight of SACC-LM tumors transfected with sh-ADAMTS9-AS2 or sh-vector (*$p < 0.05$ versus sh-vector). (D) Photomicrographs and H&E staining of lung metastases (200×) in nude mice. The number of lung metastatic nodules was determined (*$p < 0.05$ versus sh-vector).

and invasion induced by ADAMTS9-AS2 knockdown alone in SACC cells. These results showed that ADAMTS9-AS2, competing with miR-143-3p, inhibited the migration and invasion in SACC cells. However, the underlying mechanism by which miR-143-3p suppresses SACC metastasis is still unclear.

The involvement of miR-143-3p in tumorigenesis and tumor development has been studied by several research teams, and the resulting reports indicate that miR-143-3p might function as a tumor suppressor. miR-143-3p was reported to be upregulated in H. pylori-positive gastric cancer, and it suppressed tumor proliferation, migration, and invasion by directly targeting AKT2.[17] However, miR-143-3p was also found to be downregulated in esophageal squamous cell carcinoma and ovarian carcinoma, and it contributed to cancer progression.[17,30,31] miR-143-3p was induced by p53, and an excess of miR-143-3p prevented the induction of MDM2, BTG2, and CDKN1A mRNA and/or protein.[32,33] Nevertheless, there have been a few literature reports addressing the regulatory mechanism of miR-143-3p in SACC. Our results appear to support the idea that miR-143-3p acts as a tumor suppressor due to its effects on cell metastasis and invasion via targeting ITGA6. Jin et al.[34] also indicated that miR-143-3p could suppress tumor growth and angiogenesis by targeting ITGA6 and PLGF through the PI3K/AKT pathway in gallbladder carcinoma.

ITGA6 is a transmembrane glycoprotein adhesion receptor that regulates cell-cell adhesion and is widely upregulated in many types of tumors.[35] A number of studies have reported that ITGA6 contributes to tumor processes, including cell adhesion, migration, invasion, and survival.[36,37] However, there have not been any studies demonstrating a relationship between ITGA6 and SACC invasion. Accumulated evidence shows that integrins regulate many intracellular signaling pathways, including the PI3K/Akt and MAPK/Erk pathways.[38,39] Here, our study confirmed that ITGA6 could regulate PI3K/Akt and MAPK/Erk signaling in SACC cells. Although our research did not provide direct evidence regarding whether the inhibition of PI3K/Akt and MEK/Erk signaling was required for the modulation of SACC cell metastasis and invasion by ADAMTS9-AS2 and miR-143-3p, many studies have implicated PI3K/Akt and MEK/Erk signaling in the regulation of cell metastasis and invasion.[40,41] Based on our work, it is reasonable to conclude that ADAMTS9-AS2 promotes SACC metastasis and invasion via miR-143-3p-mediated downregulation of ITGA6 and downstream PI3K/Akt and MEK/Erk signaling. According to our clinical characteristics analysis, SACC patients exhibiting high ADAMTS9-AS2 expression had a significantly shorter overall survival rate and higher risk of distant metastasis than patients with low ADAMTS9-AS2 expression, which suggested that ADAMTS9-AS2 might serve as an effective prognostic marker in advanced SACC patients. However, lncRNA regulatory

networks are complicated, and other potential regulatory mechanisms of ADAMTS9-AS2 should be further investigated in SACC.

In conclusion, we first demonstrated that the upregulation of lncRNA ADAMTS9-AS2 was specifically mediated by an oncogene, c-*myc*. We confirmed a signaling cascade involving ADAMTS9-AS2 and miR-143-3p that modulated ITGA6 expression and the activity of PI3K/Akt and MEK/Erk signaling, thus regulating SACC cell migration and invasion. Furthermore, we verified that the reduction of elevated miR-143-3p expression using an miRNA inhibitor abolished the biological processes induced by ADAMTS9-AS2 knockdown, and this showed that miR-143-3p is required for ADAMTS9-AS2-mediated regulation of ITGA6 and downstream PI3K/Akt and MEK/Erk signaling, which contribute to SACC cell migration and invasion (Figure S5). Our study may provide a potential therapeutic approach for SACC treatment.

MATERIALS AND METHODS

Cell Lines

The SACC-83 and SACC-LM cells used in our study were purchased from Peking University (Beijing, China). SACC-LM is a highly metastatic cell line derived from lung metastases of SACC-83 xenografts, respectively. All cells were maintained in RPMI-1640 medium (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2.

lncRNA Microarray Analysis

Analyses using Human lncRNA Microarray v.3.0 were performed with SACC-83 and SACC-LM cells as described previously.[42] The heatmap demonstrating the expression levels of total lncRNAs that are differentially expressed in SACC-83 versus SACC-LM cells was generated with DMVS 2.0 software (Shanghai Biotechnology, Shanghai, China).

Cell Transfection

The siRNA targeting lncRNA ADAMTS9-AS2 and c-*myc* and control siRNAs were obtained from RiboBio (Guangzhou, China). The indicated cells were transfected with 50 nM siRNAs using Lipofectamine 3000 (Invitrogen). miR-143-3p mimics, miR-143-3p inhibitors, and miR-control were purchased from GenePharma (Shanghai, China). miRNA mimics, miRNA inhibitors, and miR-control transfected cells with a concentration of 40 nM using Lipofectamine 3000 (Invitrogen). ITGA6 cDNA carrying a wild-type 3′ UTR or a 3′ UTR containing mutated seed sequence for miR-143-3p (ITGA6 mut) was cloned into pcDNA 3.1 for rescue experiments.

Stable ADAMTS9-AS2 Knockdown Cell Lines

The plasmids containing specific shRNAs targeting ADAMTS9-AS2 and empty vector pLKO.1 were obtained from GenePharma (Shanghai, China). To establish stable knockdown cell lines, the PLKO.1-shADAMTS9-AS2 vector and control shRNA vector plasmid was transfected into the 293FT cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Then SACC-LM cells were infected with lentivirus containing PLKO.1-shADAMTS9-AS2 vector and control plasmids and selected with 2 μg/mL puromycin for 7 days.

5′ and 3′ RACE

We used a SMARTer RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA) to find the transcriptional initiation and termination sites of the lncRNA, according to the manufacturer's instructions. PCR products were obtained and then cloned into pEASY-T3 (TransGen Biotech, Beijing, China) for further sequencing.

RNA Extraction and Real-Time qPCR

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using a Prime Script RT reagent kit according to the manufacturer's instructions (Takara Biotechnology, China). Real-time qPCR was performed using a LightCycler 480 system (Roche, Basel, Switzerland) in triplicate in three independent experiments, and the relative expression of ADAMTS9-AS2 or miR-143-3p was normalized to that of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6. Primers used for qRT-PCR are shown in Table S2.

Western Blot Analysis

Total protein was extracted from tissue and cell samples using RIPA lysis buffer (Beyotime) supplemented with protease inhibitor mixture (Sigma-Aldrich). An equal amount of each protein sample was loaded onto a 10% SDS-PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), blocked with 5% (w/v) skim milk at room temperature (RT) for 1 hr, and then incubated with primary specific antibodies overnight (Table S3). Peroxidase-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (Proteintech, USA) was used as a secondary antibody. Finally, the antigen-antibody reaction was visualized using enhanced chemiluminescence reagent (ECL, Thermo Fisher Scientific, Rockford, IL, USA).

Patients and Specimens

In this study, adenoid cystic carcinoma samples were obtained from the salivary gland of 102 recruited patients. None of the patients received any preoperative chemotherapy or radiotherapy before surgery, and all patients were histopathologically and clinically diagnosed at the Department of Oral and Maxillofacial Surgery, Sun Yat-sen Hospital, Sun Yat-sen University, between 2003 and 2012. Informed consent was obtained from all patients. The clinicopathological features of the patients are summarized in Table S1. This study was approved by the Institutional Ethics Committee of the Sun Yat-sen Hospital, Sun Yat-sen University, China.

In Situ Hybridization

An *in situ* hybridization assay was performed according to the manufacturer's protocol (Exiqon, Vedbaek, Denmark). Briefly, after dewaxing, SACC specimens were hybridized to ADAMTS9-AS2 using a 5′-DigN/AGATCACATAGGCACTGAA GAA-3′ probe.

Then, the digoxigenin was recognized by a specific anti-DIG antibody to which alkaline phosphatase was conjugated. Cell nuclei were counterstained with hematoxylin. A total of 5 × 200 tumor cells were counted randomly in each section (high expression, positive cells ≥30%; low expression, positive cells <30%).

Boyden Chamber Assay

Cell migration and invasion abilities were examined using transwell inserts with 8-μm pores (BD Biosciences, San Jose, CA, USA). For the invasion assays, 1×10^5 cells were added to the upper level of a Boyden chamber that was pre-coated with Matrigel (Corning, New York, NY, USA), and 1×10^5 cells were placed into the upper chamber without Matrigel pre-coating for the migration assay. After 24 hr, cells were fixed, stained, photographed, and counted.

ChIP Assay

The ChIP assay was performed using the EZ-ChIP kit (Millipore, Billerica, MA) according to the manufacturer's instructions. The following antibodies were utilized to immunoprecipitate crosslinked protein-DNA complexes: rabbit anti-c-*myc* (Ab56, Abcam) and normal rabbit IgG (12-370, Millipore). The specific primer for ChIP assays was as follows: sense 5′-AGAAACCACGTGCGCA CTAC-3′; anti-sense 5′-AGAAACCACGTGCGCACTAC-3′.

Luciferase Assay

5,000 cells were placed on a 96-well plate. After 24 hr, cells were co-transfected with a mixture of 5 ng pRL-CMV Renilla luciferase reporter, 50 ng firefly luciferase reporter, and 5 pmol small RNA (siRNA or miRNA mimics). After 48 hr of transfection, firefly and Renilla luciferase activities were detected with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). After 48 hr of transfection, firefly and Renilla luciferase activities were detected with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) read by a microplate reader of a multi-wavelength measurement system (Bio-Rad, USA).

RNA Pull-Down Assays

The pcDNA3.1 plasmids containing the full sequence of ADAMTS9-AS2 or a negative control sequence with a T7 promoter were obtained from IGE (Guangzhou, China) The ADAMTS9-AS2 plasmids and negative plasmids were linearized using restriction enzyme Xho I and Xba I, respectively. Biotin-labeled RNAs were reversely transcribed using Biotin RNA Labeling Mix (Roche, USA) and T7 RNA polymerase (Takara Biomedical Technology). The products were purified with the Thermo GeneJET RNA Purification Kit (Invitrogen) and treated with RNase-free DNase I (Roche, USA). Then pull-down assays were performed as previously described.[43]

In Vivo Mouse Xenograft Assay

The 4-week-old BALB/c mice were obtained from the Central Laboratory of Animal Science at Sun Yat-sen University (Guangzhou, China). The animal experiments were approved by The Institute Research Medical Ethics Committee of Sun Yat-sen University. To verify the role of ADAMTS9-AS2 in regulating SACC growth, 1×10^6 SACC-LM cells infected with lentivirus containing sh-ADAMTS9-AS2 or with sh-vector as a control were subcutaneously injected into the left or right flank of mice (n = 5 per group). The size and weight of tumors were measured every week. For the metastasis model, 4×10^5 SACC-LM cells were injected into the tail vein of nude mice (n = 5 per group); 8 weeks later, the mice were sacrificed, and their liver tissues were removed and fixed in formalin. All the harvested tissues were imaged after sacrifice and then analyzed with H&E staining.

Accession Number

The accession number for the array data reported in this paper is GEO: GSE117575.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA). All data are expressed as the group mean ± SD. The $\chi 2$ test was used to analyze relationships between ADAMTS9-AS2 expression and clinicopathological features. Kaplan-Meier survival curves were plotted, and the log-rank test was used. All experiments were performed at least three times. The results of the experiments are expressed as the mean ± SD; $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and three tables and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.08.018.

AUTHOR CONTRIBUTIONS

S.X., X.Y., Y.L., and H.M. performed the *in vitro* assays. S.F., W.C., and G.P. performed *in vivo* experiments. W.W. and H.Z. collected and analyzed the data. Z.L. wrote the manuscript. Z.L. and J.L. designed the study. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (81602379 to Z.L.; 816726768, 81472521, and 81872194 to J.L.; and 81402251 to S.F.); the Guangdong Provincial Natural Science Foundation (2017A030311011 to J.L.); the Guangdong Province Medical Research Fund (A2016183 to Z.L.); the Specialized Research Fund for the Doctoral Program of Higher Education (20110171110068 to J.L.); the Science and Technology Project of Guangzhou City (201803010060 to J.L.); grant [2013]163 from the Key Laboratory of Malignant Tumor Molecular Mechanism and Translational Medicine of Guangzhou Bureau of Science and Information Technology; and grant K1809001 from the Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of Guangdong Higher Education Institutes.

REFERENCES

1. Dodd, R.L., and Slevin, N.J. (2006). Salivary gland adenoid cystic carcinoma: a review of chemotherapy and molecular therapies. Oral Oncol. *42*, 759–769.
2. Tang, Y., Liang, X., Zheng, M., Zhu, Z., Zhu, G., Yang, J., and Chen, Y. (2010). Expression of c-kit and Slug correlates with invasion and metastasis of salivary adenoid cystic carcinoma. Oral Oncol. *46*, 311–316.
3. Szanto, P.A., Luna, M.A., Tortoledo, M.E., and White, R.A. (1984). Histologic grading of adenoid cystic carcinoma of the salivary glands. Cancer *54*, 1062–1069.
4. Gupta, G.P., and Massagué, J. (2006). Cancer metastasis: building a framework. Cell *127*, 679–695.
5. Rapidis, A.D., Givalos, N., Gakiopoulou, H., Faratzis, G., Stavrianos, S.D., Vilos, G.A., Douzinas, E.E., and Patsouris, E. (2005). Adenoid cystic carcinoma of the head and neck. Clinicopathological analysis of 23 patients and review of the literature. Oral Oncol. *41*, 328–335.
6. Derrien, T., Johnson, R., Bussotti, G., Tanzer, A., Djebali, S., Tilgner, H., Guernec, G., Martin, D., Merkel, A., Knowles, D.G., et al. (2012). The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. *22*, 1775–1789.
7. Sánchez, Y., and Huarte, M. (2013). Long non-coding RNAs: challenges for diagnosis and therapies. Nucleic Acid Ther. *23*, 15–20.
8. Adams, B.D., Parsons, C., Walker, L., Zhang, W.C., and Slack, F.J. (2017). Targeting noncoding RNAs in disease. J. Clin. Invest. *127*, 761–771.
9. Schmitz, S.U., Grote, P., and Herrmann, B.G. (2016). Mechanisms of long noncoding RNA function in development and disease. Cell. Mol. Life Sci. *73*, 2491–2509.
10. Peng, Z., Zhang, C., and Duan, C. (2016). Functions and mechanisms of long noncoding RNAs in lung cancer. OncoTargets Ther. *9*, 4411–4424.
11. Li, C.H., and Chen, Y. (2013). Targeting long non-coding RNAs in cancers: progress and prospects. Int. J. Biochem. Cell Biol. *45*, 1895–1910.
12. Yang, Q.Q., and Deng, Y.F. (2014). Long non-coding RNAs as novel biomarkers and therapeutic targets in head and neck cancers. Int. J. Clin. Exp. Pathol. *7*, 1286–1292.
13. Tay, Y., Rinn, J., and Pandolfi, P.P. (2014). The multilayered complexity of ceRNA crosstalk and competition. Nature *505*, 344–352.
14. Li, J., Li, T., Lu, Y., Shen, G., Guo, H., Wu, J., Lei, C., Du, F., Zhou, F., Zhao, X., et al. (2017). MiR-2392 suppresses metastasis and epithelial-mesenchymal transition by targeting *MAML3* and *WHSC1* in gastric cancer. FASEB J. *31*, 3774–3786.
15. Ni, F., Zhao, H., Cui, H., Wu, Z., Chen, L., Hu, Z., Guo, C., Liu, Y., Chen, Z., Wang, X., et al. (2015). MicroRNA-362-5p promotes tumor growth and metastasis by targeting CYLD in hepatocellular carcinoma. Cancer Lett. *356* (2 Pt B), 809–818.
16. Xie, F., Hosany, S., Zhong, S., Jiang, Y., Zhang, F., Lin, L., Wang, X., Gao, S., and Hu, X. (2017). MicroRNA-193a inhibits breast cancer proliferation and metastasis by downregulating WT1. PLoS ONE *12*, e0185565.
17. Wang, F., Liu, J., Zou, Y., Jiao, Y., Huang, Y., Fan, L., Li, X., Yu, H., He, C., Wei, W., et al. (2017). MicroRNA-143-3p, up-regulated in H. pylori-positive gastric cancer, suppresses tumor growth, migration and invasion by directly targeting AKT2. Oncotarget *8*, 28711–28724.
18. Zhao, L., Feng, X., Song, X., Zhou, H., Zhao, Y., Cheng, L., and Jia, L. (2016). miR-493-5p attenuates the invasiveness and tumorigenicity in human breast cancer by targeting FUT4. Oncol. Rep. *36*, 1007–1015.
19. Zhang, P., Zuo, Z., Wu, A., Shang, W., Bi, R., Jin, Q., Wu, J., and Jiang, L. (2017). miR-600 inhibits cell proliferation, migration and invasion by targeting p53 in mutant p53-expressing human colorectal cancer cell lines. Oncol. Lett. *13*, 1789–1796.
20. Nakamura, K., Terai, Y., Tanabe, A., Ono, Y.J., Hayashi, M., Maeda, K., Fujiwara, S., Ashihara, K., Nakamura, M., Tanaka, Y., et al. (2017). CD24 expression is a marker for predicting clinical outcome and regulates the epithelial-mesenchymal transition in ovarian cancer via both the Akt and ERK pathways. Oncol. Rep. *37*, 3189–3200.
21. Jiang, C., Li, X., Zhao, H., and Liu, H. (2016). Long non-coding RNAs: potential new biomarkers for predicting tumor invasion and metastasis. Mol. Cancer *15*, 62.
22. Demircan, K., Gunduz, E., Gunduz, M., Beder, L.B., Hirohata, S., Nagatsuka, H., Cengiz, B., Cilek, M.Z., Yamanaka, N., Shimizu, K., and Ninomiya, Y. (2009). Increased mRNA expression of ADAMTS metalloproteinases in metastatic foci of head and neck cancer. Head Neck *31*, 793–801.
23. Yao, J., Zhou, B., Zhang, J., Geng, P., Liu, K., Zhu, Y., and Zhu, W. (2014). A new tumor suppressor LncRNA ADAMTS9-AS2 is regulated by DNMT1 and inhibits migration of glioma cells. Tumour Biol. *35*, 7935–7944.
24. Lin, C.Y., Lovén, J., Rahl, P.B., Paranal, R.M., Burge, C.B., Bradner, J.E., Lee, T.I., and Young, R.A. (2012). Transcriptional amplification in tumor cells with elevated c-Myc. Cell *151*, 56–67.
25. Zhang, Z., Zhou, C., Chang, Y., Zhang, Z., Hu, Y., Zhang, F., Lu, Y., Zheng, L., Zhang, W., Li, X., and Li, X. (2016). Long non-coding RNA CASC11 interacts with hnRNP-K and activates the WNT/β-catenin pathway to promote growth and metastasis in colorectal cancer. Cancer Lett. *376*, 62–73.
26. Zhu, K.P., Ma, X.L., and Zhang, C.L. (2017). LncRNA ODRUL Contributes to Osteosarcoma Progression through the miR-3182/MMP2 Axis. Mol. Ther. *25*, 2383–2393.
27. Yoon, J.H., Abdelmohsen, K., and Gorospe, M. (2013). Posttranscriptional gene regulation by long noncoding RNA. J. Mol. Biol. *425*, 3723–3730.
28. Liu, M., Jia, J., Wang, X., Liu, Y., Wang, C., and Fan, R. (2018). Long non-coding RNA HOTAIR promotes cervical cancer progression through regulating BCL2 via targeting miR-143-3p. Cancer Biol. Ther. *19*, 391–399.
29. Chen, L., Yao, H., Wang, K., and Liu, X. (2017). Long Non-Coding RNA MALAT1 Regulates ZEB1 Expression by Sponging miR-143-3p and Promotes Hepatocellular Carcinoma Progression. J. Cell. Biochem. *118*, 4836–4843.
30. Zhang, H., and Li, W. (2016). Dysregulation of micro-143-3p and BALBP1 contributes to the pathogenesis of the development of ovarian carcinoma. Oncol. Rep. *36*, 3605–3610.
31. He, Z., Yi, J., Liu, X., Chen, J., Han, S., Jin, L., Chen, L., and Song, H. (2016). MiR-143-3p functions as a tumor suppressor by regulating cell proliferation, invasion and epithelial-mesenchymal transition by targeting QKI-5 in esophageal squamous cell carcinoma. Mol. Cancer *15*, 51.
32. Cabrita, M.A., Bose, R., Vanzyl, E.J., Pastic, A., Marcellus, K.A., Pan, E., Hamill, J.D., and McKay, B.C. (2017). The p53 protein induces stable miRNAs that have the potential to modify subsequent p53 responses. Gene *608*, 86–94.
33. Cabrita, M.A., Vanzyl, E.J., Hamill, J.D., Pan, E., Marcellus, K.A., Tolls, V.J., Alonzi, R.C., Pastic, A., Rambo, T.M., Sayed, H., and McKay, B.C. (2016). A Temperature Sensitive Variant of p53 Drives p53-Dependent MicroRNA Expression without Evidence of Widespread Post-Transcriptional Gene Silencing. PLoS ONE *11*, e0148529.
34. Jin, Y.P., Hu, Y.P., Wu, X.S., Wu, Y.S., Ye, Y.Y., Li, H.F., Liu, Y.C., Jiang, L., Liu, F.T., Zhang, Y.J., et al. (2018). miR-143-3p targeting of ITGA6 suppresses tumour growth and angiogenesis by downregulating PLGF expression via the PI3K/AKT pathway in gallbladder carcinoma. Cell Death Dis. *9*, 182.
35. Hehlgans, S., Haase, M., and Cordes, N. (2007). Signalling via integrins: implications for cell survival and anticancer strategies. Biochim. Biophys. Acta *1775*, 163–180.
36. Landowski, T.H., Gard, J., Pond, E., Pond, G.D., Nagle, R.B., Geffre, C.P., and Cress, A.E. (2014). Targeting integrin α6 stimulates curative-type bone metastasis lesions in a xenograft model. Mol. Cancer Ther. *13*, 1558–1566.
37. Wewer, U.M., Shaw, L.M., Albrechtsen, R., and Mercurio, A.M. (1997). The integrin alpha 6 beta 1 promotes the survival of metastatic human breast carcinoma cells in mice. Am. J. Pathol. *151*, 1191–1198.
38. Weng, L.X., Wang, G.H., Yao, H., Yu, M.F., and Lin, J. (2017). Epigallocatechin gallate inhibits the growth of salivary adenoid cystic carcinoma cells via the EGFR/Erk signal transduction pathway and the mitochondria apoptosis pathway. Neoplasma *64*, 563–570.
39. Hu, K., Li, S.L., Gan, Y.H., Wang, C.Y., and Yu, G.Y. (2009). Epiregulin promotes migration and invasion of salivary adenoid cystic carcinoma cell line SACC-83 through activation of ERK and Akt. Oral Oncol. *45*, 156–163.

40. Liu, X., Zhang, W., Guo, H., Yue, J., and Zhuo, S. (2016). miR-98 functions as a tumor suppressor in salivary adenoid cystic carcinomas. OncoTargets Ther. *9*, 1777–1786.

41. Xiong, J., Li, Z., Zhang, Y., Li, D., Zhang, G., Luo, X., Jie, Z., Liu, Y., Cao, Y., Le, Z., et al. (2016). PRL-3 promotes the peritoneal metastasis of gastric cancer through the PI3K/Akt signaling pathway by regulating PTEN. Oncol. Rep. *36*, 1819–1828.

42. Fan, L.J., Han, H.J., Guan, J., Zhang, X.W., Cui, Q.H., Shen, H., and Shi, C. (2017). Aberrantly expressed long noncoding RNAs in recurrent implantation failure: A microarray related study. Syst Biol Reprod Med *63*, 269–278.

43. Gu, P., Chen, X., Xie, R., Han, J., Xie, W., Wang, B., Dong, W., Chen, C., Yang, M., Jiang, J., et al. (2017). lncRNA HOXD-AS1 Regulates Proliferation and Chemo-Resistance of Castration-Resistant Prostate Cancer via Recruiting WDR5. Mol. Ther. *25*, 1959–1973.