Original Article
Hypoxia-induced IncRNA CASC9 enhances glycolysis and the epithelial-mesenchymal transition of pancreatic cancer by a positive feedback loop with AKT/HIF-1α signaling

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Abstract: Increasing evidence indicates the dysregulations and pivotal roles of IncRNAs in the development and progression of various cancers, including pancreatic cancer. Enhanced glycolytic flux and epithelial-to-mesenchymal transition (EMT) have been considered as important factors in driving the malignance of pancreatic cancer. Here, we sought to evaluate the biological role and involved mechanism of IncRNA CASC9 (CASC9) in pancreatic cancer. Our present study showed that CASC9 was upregulated in various pancreatic cancer cell lines. Loss- and gain-of function of CASC9 demonstrated its critical roles in promoting the glycolysis and EMT phenotypes of pancreatic cancer. Moreover, knockdown of CASC9 inhibited the tumorigenicity and metastasis in vivo. Additionally, our findings showed that hypoxia induced the expression of CASC9 and enhanced the binding of HIF-1α to its promoter. We also demonstrated that the positive feedback loop of CASC9 and the AKT/HIF-1α signaling cascade partially mediated this biological process. Altogether, our results suggest that CASC9 promotes the glycolysis and EMT of pancreatic cancer by a positive feedback loop with AKT/HIF-1α signaling, which is synergistically enhanced by the tumor hypoxic niche. Our study will provide potential therapeutic targets for treating pancreatic cancer.

Keywords: CASC9, HIF-1α, AKT, hypoxia, glycolysis, EMT, pancreatic cancer

Introduction
Pancreatic cancer is one of the most lethal digestive malignancies with a poor outcome globally [1, 2]. At the time of diagnosis, most patients with pancreatic cancer are already at an advanced stage, and the cancer is unresectable. Unfortunately, chemotherapy also exhibits weak efficacy to treat pancreatic cancer due to profoundly inherent or acquired drug resistance [3]. Despite great improvements in diagnostic and therapeutic approaches in recent years, the prognosis of pancreatic cancer remains dismal, with an overall 5-year survival rate of 8% [1]. Hence, it is of great significance to elucidate the driving factors and mechanisms involved in order to improve the prognosis of pancreatic cancer.

It is well known that metabolic reprogramming is one of the key hallmarks of pancreatic cancer, and the most representative metabolic change is the “Warburg effect”, which is characterized by increased glycolytic flux even with normal oxygen supply [4]. The main and branches from the glycolytic process not only produce the required energy quickly but also provide biomass that supports cellular survival and
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other malignant behaviors [5]. A line of evidence suggests that many key enzymes that regulate glycolytic flux are highly expressed in pancreatic cancer and have an inverse correlation with patient prognosis [6, 7]. Another important driving mechanism for pancreatic cancer is epithelial-to-mesenchymal transition (EMT), which occurs even at a very early stage of neoplastic development and confers cells with enhanced abilities of migration, invasion, early dissemination, and chemoresistance [8-10]. A previous study has shown that upregulation of glycolysis promotes the EMT phenotypes of pancreatic cancer cells [11], while the EMT promoting factor Twist facilitates the reprogramming of glucose metabolism [12], suggesting that both mechanisms collaborate in promoting tumor malignant phenotypes. Thus, elucidating the regulatory mechanism of glycolysis and EMT will contribute to achieving a better therapeutic effect in patients with pancreatic cancer.

Long noncoding RNAs (lncRNAs), defined as a class of transcripts longer than 200 nucleotides with no protein-coding potential, have been shown to be able to regulate both protein-coding and noncoding genes extensively [13]. Recently, accumulating evidence suggests that a number of lncRNAs are aberrantly expressed and involved in the development and progression of pancreatic cancer. For example, IncRNA-BX111 was validated to be overexpressed and promote tumor proliferation, invasion, and metastasis [14]. Moreover, IncRNA XLOC_006390 was shown to enhance glutamate metabolism and promote pancreatic carcinogenesis by stabilizing c-Myc [15]. Nevertheless, the dysregulated lncRNAs and the relevant functions in pancreatic cancer have not been fully elucidated.

LncRNA Cancer Susceptibility Candidate 9 (CASC9), has been reported to be overexpressed in different types of cancers, including esophageal, nasopharyngeal, hepatocellular, colorectal cancer, etc. [16-19]. A recent study has suggested that CASC9 is abnormally regulated in pancreatic cancer and promotes cell proliferation and invasion [20], but the mechanism involved is still unclear. Hypoxia is a well-known common condition in pancreatic cancer, and our preliminary study found that CASC9 could be induced by hypoxia treatment in pancreatic cancer cells. The hypoxic niche has been widely considered to promote the process of glycolytic flux and EMT phenotypes through hypoxia inducible factors (HIFs) in various cancers [21]. On the other hand, CASC9 was shown to activate HIF-1α and promote the glycolysis of nasopharyngeal carcinoma [17], and HIF-1α could also positively regulate CASC9 expression in lung cancer [22], suggesting a potential positive feedback loop between CASC9 and HIF-1α. PI3K/AKT signaling is a frequently activated oncogenic pathway in pancreatic cancer [23, 24], which can be aggravated and mediated by HIF-1α activation [25]. Moreover, it has been revealed that CASC9 promotes the tumor progression of oral squamous cell carcinoma by the activation of the AKT/mTOR pathway [26]. Therefore, we hypothesized that hypoxia augmented the positive feedback loop of the CASC9 and AKT/HIF-1α pathway, thus enhancing the malignant phenotypes of pancreatic cancer.

In this study, we demonstrated that CASC9 promoted glycolytic metabolism and EMT in pancreatic cancer by a positive feedback loop with AKT/HIF-1α signaling, which was synergistically enhanced by the hypoxic niche. Our data indicate that blocking this signaling cascade may suppress the malignant phenotype, thus providing a potential target for pancreatic cancer treatment.

Materials and methods

Cell culture and treatments

The human pancreatic cancer cell lines (SW-1990, PANC-1, BxPC-3, and MiaPaCa-2) and normal pancreatic ductal cells (HPDE6-C7) were both obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and incubated under optimal conditions (5% CO₂, 37°C). For the hypoxia condition, cancer cells were incubated with 1% O₂ for different durations, balanced by nitrogen and CO₂. To investigate the role of AKT on CASC9-induced glycolysis and EMT, pancreatic cancer cells were transfected with pcDNA-CASC9 plasmid vector, followed by 20 μM AKT inhibitor LY294002 (Beyotime Biotechnology, Shanghai, China) treatment for 24 h. To mimic the effect of hypoxia on the expression of CASC9, cancer cells were treated with 100 μM CoCl₂ (Sigma-Aldrich, St. Louis, MO, USA) for 24 h.
Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was performed as described previously [27]. CASC9 expression was normalized to that of GAPDH. Primer sequence (5’ to 3’): CASC9-F: GACACA-TTTGCTGCTTCCATTCC, CASC9-R: TGGCATCTGTT-GATTATCTTTTCC; GAPDH-F: GACGCTGGGGCT-GGCAATTG; GAPDH-R: GCTGGTGGTCCAGGGGTC.

Glucose uptake and lactate release assays

The glucose uptake and lactate release assay was carried out as described previously [11]. Briefly, after different treatments, the culture medium of pancreatic cancer cells was replaced with glucose-free medium for 2 h incubation. The fluorescent-labeled glucose analog 2-NBDG was utilized as a probe to measure the ability of glucose uptake according to the protocol of the glucose uptake cell-based assay kit (Cayman Chemical, Ann Arbor, MI, USA). In addition, the lactate produced and secreted into the culture medium was analyzed as per the manufacturer's protocol by the lactic acid assay kit (Nanjing Jiancheng Bio. Nanjing, China). The lactic acid level in each sample was normalized to total protein.

Western blot analysis

Western blot analysis was performed as previously described [28]. Total cell lysates were obtained and separated by sodium dodecyl sulfate polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). Then, the stained membranes were blocked with 5% nonfat dry milk, and incubated sequentially with primary antibodies at 4°C overnight and secondary horseradish peroxidase-coupled antibody (Aspen, Wuhan, China) for 1 h at room temperature. The immunoblot was visualized by utilizing ECL substrate (Thermo Fisher, Waltham, MA, USA). Antibodies against HK2, GLUT4, LDHA, Snail, Vimentin, N-Cadherin, E-Cadherin, HIF-1α, p-AKT, and GAPDH were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Transfection

Transfection of siRNA was performed by using Lipofectamine™ 2000 (Invitrogen, Shanghai, China) as described previously [29]. The siRNA targeting CASC9, HIF-1α, and matched negative controls (Si-CASC9, Si-HIF-1α, and Si-NC), were obtained from Ribobio Co. (Guangzhou, China). Overexpression plasmid pcDNA-CASC9 and the control vector were purchased from GeneChem (Shanghai, China). Transfection of siRNA and plasmid DNA was accomplished using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. The lentiviral vector containing the sequence of Si-CASC9 and the appropriate negative control (LV-Si-CASC9 and LV-Si-NC) were synthesized from GeneChem (Shanghai, China) and transfected according to the provided protocols.

Transwell migration/invasion assay

The migration and invasion assay were conducted in Transwell chambers (Costar, Corning, Cambridge, MA, USA) as described previously [28].

Chromatin immunoprecipitation (ChIP) assay

The binding of HIF-1α to the promoter of CASC9 was examined by utilizing the ChIP Kit (Millipore, Billerica, MA, USA) as previously described [29]. Corresponding rabbit IgG antibody (Millipore) was used as a negative control. The bound DNA was amplified by PCR with the specifically designed primers, followed by electrophoretic separation on agarose gel (2%). Primer sequence (5’ to 3’): Target 1-F: CATTCATTT-CATCTTCC; Target 1-R: AAGAATGTATAACTAGAA; Target 2-F: GGACCCTCTGAGCCAGGT; Target 2-R: CGAGGCTTGCCTCACCCTT.

Tumor xenografts

Tumor xenografts were established as previously described [28]. After being transfected with LV-Si-CASC9 or LV-Si-NC, SW1990 cells were harvested, and approximately 5 × 10^6 cells suspended in 100 μL of phosphate-buffered saline (PBS) were subcutaneously injected into the right flank of athymic 3- to 4-week-old mice (n = 5 mice per group; HFK Bioscience Co., Beijing, China). Tumor volume was measured every 4 days by using the formula: volume = 1/2 × length × width^2. After 24 days, the mice were euthanized and the xenografts were removed and weighed. Then, the tumor xenografts were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into slices at 5 μm. Immunohistochemical staining was used to detect the expression of HK2, GLUT4, LDHA, Snail, Vimentin, N-Cadherin, and E-Cadherin in
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xenograft tissues. The proliferative index of tumor cells was determined by Ki-67 immunostaining, while apoptosis was detected by the TUNEL immunofluorescence assay. All the animal protocols were approved by the “Guide for the Care and Use of Animals in Wuhan University”.

In vivo lung metastasis assay

After treatment, approximately $4 \times 10^6$ SW1990 cells (suspended in 0.2 mL of PBS) were injected into the lateral tail vein of athymic mice (7 to 8 weeks old; HFK Bioscience Co.; n = 5 per group). The mice were euthanized after approximately 4 weeks. The lungs of mice were removed, fixed, and photographed. Paraffin sections were made and stained with H&E. Then, the number of metastases in the lungs was determined under the light microscope.

Statistical analysis

The data are presented as the mean ± standard deviation. The difference between two groups was compared by Student’s t-test. $P < 0.05$ was considered to be statistically significant.

Results

CASC9 enhances the glycolysis of pancreatic cancer cells

A previous study has suggested that CASC9 is highly upregulated in pancreatic cancer tissues [20]; however, its exact role is still unclear. We first validated the relative expression in different pancreatic cancer cell lines. As shown in Figure 1A, compared with a normal pancreatic duct cell line (HPDE6-C7), the expression level of CASC9 was significantly higher in various types of pancreatic cancer cell lines. We then selected two cancer cell lines, namely, SW1990 and PANC-1, for the subsequent experiments. CASC9 has been suggested to promote the glycolysis of nasopharyngeal carcinoma [17]. To confirm the similar effect of CASC9 on glucose metabolism in pancreatic cancer, RNA interference was applied to silence CASC9. As revealed in Figure 1B, knockdown of CASC9 effectively inhibited the expression of CASC9. Consequently, the ability of glucose uptake was dramatically attenuated after inhibition of CASC9 in both cell lines (Figure 1C), as indicated by detecting 2-NBDG, a fluorescently labeled deoxyglucose analog. Moreover, CASC9 suppression markedly inhibited the production of lactic acid (Figure 1D). Notably, a reduction in the protein levels of the key glycolytic enzymes HK2, GLUT4, and LDHA was also observed in both cell lines (Figure 1E).

To further elucidate the role of CASC9 in glycolytic metabolism, we applied a plasmid vector to overexpress CASC9. As shown in Figure 1F, transfection with the pcDNA-CASC9 vector could effectively upregulate the level of CASC9. As a result, ectopic expression of CASC9 significantly promoted the ability of glucose uptake and lactic acid production (Figure 1G, 1H). Moreover, the key glycolytic enzymes HK2, GLUT4, and LDHA were dramatically increased after CASC9 overexpression (Figure 1I). Altogether, our data suggested that CASC9 enhanced the glycolytic flux of pancreatic cancer cells.

CASC9 enhances the EMT phenotype of pancreatic cancer cells

EMT is a reversible process during which epithelial cells switch to motile mesenchymal cells, thus conferring cells with aggressive traits, such as mobility, invasion, and metastasis [30, 31]. We thus examined the effect of CASC9 on the EMT phenotype. As shown in Figure 2A, silencing CASC9 significantly inhibited the protein level of molecular markers of EMT, such as Snail, Vimentin, and N-Cadherin, while promoting the expression of E-Cadherin in both cell lines. Conversely, ectopic overexpression of CASC9 upregulated the expression of Snail, Vimentin, and N-Cadherin, whereas it downregulated E-Cadherin expression (Figure 2D). In line with these molecular changes, the migratory and invasive abilities were also attenuated after CASC9 knockdown (Figure 2B, 2C), while these were enhanced after CASC9 overexpression in two pancreatic cancer cell lines (Figure 2E, 2F). Altogether, CASC9 promoted the EMT phenotype of pancreatic cancer cells.

CASC9 inhibition suppresses the tumorigenicity and metastasis of pancreatic cancer in vivo

Since CASC9 has been verified to enhance the glycolysis and EMT phenotypes of pancreatic cancer cells, we further determined its effects on tumorigenicity and metastasis in vivo.
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Figure 1. CASC9 enhances the glycolysis of pancreatic cancer cells. A. The relative expression of CASC9 was compared between different pancreatic cancer cell lines (BxPC-3, MiaPaCa-2, PANC-1, and SW1990) and a normal pancreatic duct cell line (HPDE6-C7) by using qRT-PCR. SW1990 and PANC-1 cell lines were transfected with Si-CASC9 or Si-NC. B. The relative mRNA level of CASC9 was detected by qRT-PCR. C. The ability of relative glucose uptake was measured by the glucose uptake assay. D. The relative production of lactate in each culture medium was determined by the lactate release assay. E. Western blot analysis was used to detect the protein levels of the key glycolytic enzymes HK2, GLUT4, and LDHA. Then, two pancreatic cancer cell lines were transfected with the pcDNA-CASC9 or pcDNA-NC plasmid. F. The mRNA level of CASC9 was determined by qRT-PCR. G and H. The relative abilities of glucose uptake and lactate production were measured by glucose uptake and lactate release assays, respectively. I. The protein levels of HK2, GLUT4, and LDHA were detected by western blot analysis. The results shown are from three independent experiments. The mRNA value was normalized to GAPDH. The lactate level was normalized to total protein of each sample. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

test the biological role of CASC9 in vivo, SW1990 cells were transfected with lentivirus containing Si-CASC9 (LV-Si-CASC9) or its control vector (LV-Si-NC). As shown in Figure 3A,
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3B, the group transfected with LV-Si-CASC9 showed reduced Ki-67 and enhanced TUNEL in the group transfected with LV-Si-CASC9, when compared with the LV-Si-NC group (Figure 3D, 3E). Further, decreased staining of HK2, GLUT4, LDHA, Snail, Vimentin, and N-Cadherin and increased staining of E-Cadherin in mouse tumors were observed in the LV-Si-CASC9 group transfected with LV-Si-CASC9 and then subjected to the Transwell migration and invasion assays. A. The relative protein levels of Snail, Vimentin, N-Cadherin, and E-Cadherin were measured by western blot analysis. B. Representative images of migrated and invaded cells are shown on the membrane of the lower chamber. C. The relative abilities of migration and invasion are presented by quantification of stained cells. D. Two cell lines were transfected with the pcDNA-CASC9 or pcDNA-NC plasmid, and western blot analysis was used to detect the expression of Snail, Vimentin, N-Cadherin, and E-Cadherin. E and F. After transfection with pcDNA-CASC9 or pcDNA-NC, the relative migratory and invasive abilities in the two cell lines were determined by Transwell migration and invasion assays, respectively. The graphs presented are from three independent assays. Scale bar, 100 μm. **, P < 0.01.
Figure 3. CASC9 inhibition suppresses the tumorigenicity and metastasis of pancreatic cancer in vivo. LV-Si-CASC9- and LV-Si-NC-transfected SW1990 cells were implanted in the right flank of nude mice subcutaneously. After approximately 24 days, the mice were euthanized, and the tumor was completely removed. A. Representative photographs
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of mice and tumors are shown. B. The volume of the tumor was measured every 4 days, and the growth curve was delineated accordingly. C. The tumor weight was measured in each group. Then, the tumor tissue sections of xenografts in different groups were used for further analysis. D-F. The expression of the apoptotic marker TUNEL was stained using an immunofluorescence assay; the staining of Ki-67, HK2, GLUT4, LDHA, Snail, Vimentin, N-Cadherin, and E-Cadherin was analyzed by immunohistochemistry (scale bar, 50 μm). After being transfected with LV-Si-CASC9 or LV-Si-NC, SW1990 cells were injected into the lateral tail vein of mice. G. Representative photographs of resected lungs in each group are revealed. H. Representative H&E staining images of lungs are shown (scale bar, 200 μm). The metastatic nodules are indicated by arrows. I and J. The number of mice with lung metastasis and metastatic nodules were calculated in the two groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

group (Figure 3D, 3F). To examine the change in the ability of distant metastasis, the in vivo lung metastasis assay was carried out. As shown in Figure 3G-J, the ability of cancer cells to metastasize to the lung was attenuated after CASC9 knockdown. Altogether, our results demonstrated that inhibition of CASC9 suppressed the tumorigenicity and metastasis of pancreatic cancer in vivo.

HIF-1α activation partially mediates CASC9-induced glycolysis and EMT phenotype

HIF-1α is commonly activated in pancreatic cancer and plays an important role in the process of both glycolytic metabolism and EMT [32, 33]. We next examined the change in HIF-1α activation upon CASC9 overexpression and its role in the enhanced glycolysis and EMT phenotype. As revealed in Figure 4A, upregulation of CASC9 by transfection with the pcDNA-CASC9 plasmid significantly promoted the protein level of HIF-1α in both pancreatic cancer cell lines. To further elucidate the role of HIF-1α in CASC9-induced glycolysis and EMT, two cell lines were cotransfected with CASC9 overexpression plasmid and HIF-1α siRNA vector. After HIF-1α knockdown, CASC9-induced HIF-1α expression was dramatically inhibited (Figure 4A). Accordingly, the protein levels of the key glycolytic enzymes HK2, GLUT4, and LDHA were significantly attenuated (Figure 4A). Moreover, inhibiting HIF-1α suppressed both the glucose uptake ability and lactate production enhanced by CASC9 (Figure 4B, 4C). We next investigated the changes in EMT phenotype. Similarly, the expression of the EMT-associated markers Snail, Vimentin, and N-Cadherin induced by CASC9 overexpression was reduced after HIF-1α knockdown, while the E-Cadherin level was increased (Figure 4A). In line with these changes, inhibition of HIF-1α also markedly blocked the CASC9-enhanced migratory and invasive abilities (Figure 4D-F). Altogether, these altered molecules and associated behaviors consistently suggested that HIF-1α activation plays an important part in CASC9-induced glycolysis and EMT phenotype.

AKT activation participates in the enhancement of glycolytic flux and EMT partially mediated by CASC9-induced HIF-1α activation

AKT has been reported to be universally activated and plays an important role in the malignancy of pancreatic cancer [34]. We investigated the change in p-AKT (Ser 473) in pancreatic cancer cells as an activated status of AKT. As shown in Figure 5A, overexpression of CASC9 significantly promoted the expression of p-AKT in both pancreatic cancer cell lines. To determine whether activated AKT participates in the enhanced glycolysis and EMT induced by CASC9, we transfected pancreatic cancer cells with the pcDNA-CASC9 plasmid, combined with 20 μM LY294002 (an AKT inhibitor) treatment. Remarkably, the expression of p-AKT induced by CASC9 was reduced (Figure 5A). Moreover, western blot analysis revealed that the protein levels of the key glycolytic enzymes HK2, GLUT4, and LDHA were significantly inhibited (Figure 5A). Furthermore, CASC9-enhanced glucose uptake ability and lactate production were blocked (Figure 5B, 5C). We further determined the changes in EMT in pancreatic cancer cells. After being treated with LY294002, the protein levels of Snail, Vimentin, and N-Cadherin induced by CASC9 were suppressed, while E-Cadherin expression was increased (Figure 5A). Along with the above changes, the CASC9-enhanced migratory and invasive abilities were also attenuated (Figure 5D-F). In addition, AKT inhibition obviously suppressed CASC9-induced HIF-1α expression in all pancreatic cancer cells tested (Figure 5A). These results demonstrated that activated AKT participates as an important mediator in CASC9-induced HIF-1α and subsequent glycolysis and EMT.
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Hypoxia potentiates HIF-1α-regulated CASC9 transcriptionally

The hypoxic niche is a ubiquitous microenvironment in pancreatic cancer and has been reported to promote the process of glycolysis and EMT [35, 36]. We detected the change in CASC9 with hypoxia treatment. As shown in Figure 6A, 1% O$_2$ treatment on two pancreatic cancer cell lines for 6-9 h dramatically promoted the expression of CASC9. Then, we treated cells with CoCl$_2$ to stabilize HIF-1α and mimic a hypoxic microenvironment. The results showed that CoCl$_2$ also obviously upregulated the expression of CASC9 in both cell lines (Figure 6B). These findings demonstrated that CASC9 is a hypoxia-induced IncRNA. To further determine the role of HIF-1α in the hypoxia-induced CASC9, two pancreatic cancer cell lines were transfected with HIF-1α siRNA and then exposed to a 1% O$_2$ environment. As revealed in Figure 6C, silencing HIF-1α significantly inhibited the protein level of HIF-1α induced by hypoxia. The expression of p-AKT was also dramatically suppressed. Moreover, hypoxia-induced CASC9 expression was markedly reduced after HIF-1α inhibition (Figure 6D).

To further clarify the underlying mechanism of HIF-1α in upregulating CASC9 induced by
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Figure 5. AKT activation participates in the enhancement of glycolytic flux and EMT partially mediated by CASC9-induced HIF-1α activation. SW1990 and PANC-1 cells were transfected with pcDNA-CASC9 or pcDNA-NC, followed by treatment with 20 μM LY294002 for 24 h. A. Western blot analysis was used to detect the protein levels of HK2, GLUT4, LDHA, Snail, Vimentin, N-Cadherin, E-Cadherin, HIF-1α, and p-AKT. B and C. The relative abilities of glucose uptake and lactate release were determined by the glucose uptake and lactate release assays, respectively. D-F. The relative migratory and invasive abilities of the two cell lines were measured by the Transwell migration and invasion assays, respectively. The graphs shown are from three independent assays. The lactate value was normalized to total protein of each sample. Scale bar, 100 μm. *, P < 0.05; **, P < 0.01.

Discussion

Hypoxia has been considered as a hallmark in pancreatic cancer. A line of evidences suggest that hypoxia contributes to the process of glycolytic metabolism and EMT, which collaboratively promote the development and progression of pancreatic cancer [38]. Numerous lncRNAs have been shown to be dysregulated and play important roles in malignant phenotypes of pancreatic cancer [39]; however, the regulatory mechanisms and functions involved are still not fully understood. In the present study, we demonstrated that CASC9 was upreg-
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Recent research has indicated that CASC9 is frequently upregulated and plays a critical role in supporting the malignant behaviors of various tumors, including pancreatic cancer [16, 20, 26]. In the present study, we demonstrated that CASC9 was overexpressed in different pancreatic cancer cell lines. In addition, our gain- and loss-of-function studies of CASC9 thus providing potential therapeutic targets for pancreatic cancer.
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verified its pivotal role in facilitating the traits of EMT exhibiting both the changes in molecular markers as well as migratory and invasive abilities, which might explain the mechanism of enhanced invasion in previous report [20]. Glycolysis has been suggested to have a close correlation with the EMT process [40]. Our data also revealed that ectopic overexpression of CASC9 increased the key glycolytic enzymes HK2, GLUT4, and LDHA, as well as the abilities of glucose uptake and lactic acid production, indicating the enhanced glycolytic flux. Further, depletion of CASC9 in vivo exhibited a suppressive effect on the tumor growth and metastasis of pancreatic cancer cells. Thus, our data suggest that CASC9 acts as an oncogenic role in promoting pancreatic cancer glycolysis and EMT phenotypes.

HIF-1α is a pivotal transcription factor in promoting tumor metabolic reprogramming in response to hypoxia [21]. It has also been implicated in regulating cellular invasion and metastasis in pancreatic cancer [41]. A recent report has shown that CASC9 could activate HIF-1α and target genes, thus promoting the glycolysis in nasopharyngeal cancer [17]. In the present study, we also observed a similar effect in pancreatic cancer cells. Further, knockdown of HIF-1α significantly attenuated CASC9-induced key glycolytic enzymes, as well as the abilities of glucose uptake and lactic acid level, indicating a critical role of HIF-1α in mediating CASC9-induced glycolytic flux. In addition, we revealed that depletion of HIF-1α impaired enhanced expression of EMT-related markers and the migratory and invasive abilities induced by CASC9. Collectively, our findings demonstrate that HIF-1α, serving as the downstream factor, plays an important role in mediating CASC9-induced glycolysis and EMT phenotypes.

PI3K/Akt is a frequently aberrant activated signaling pathway in pancreatic cancer, which is closely involved in tumor metabolism and aggressive malignance maintenance [34]. Accumulative evidence suggested that it contributes to the EMT phenotype in a variety of ways [42]. In our study, we showed that CASC9 could promote the activation of AKT, which was consistent with the observations on the enhanced characteristics of glycolytic flux and EMT in pancreatic cancer cells. Further, inhibition of AKT dramatically weakened these increased effects, suggesting that activation of AKT mediates CASC9-induced glycolysis and EMT in pancreatic cancer. In addition, we observed that AKT inhibition obviously downregulated the level of HIF-1α induced by CASC9. This finding indicated a new regulatory mechanism of HIF-1α by CASC9 in which it was reported that CASC9 could bind to and stabilize HIF-1α in nasopharyngeal cancer [17]. In addition, our results revealed that knockdown of HIF-1α significantly impaired hypoxia-induced activation of AKT. Our study provides evidence of a potential mutual activation of AKT and HIF-1α in which both signaling pathways intertwined in mediating the malignant behaviors in response to CASC9 overexpression. Altogether, our data emphasize an important promoting role of the activation of AKT in facilitating CASC9-induced HIF-1α and subsequently enhanced glycolysis and EMT phenotypes.

Hypoxia, a ubiquitous microenvironment in pancreatic cancer, is deemed as a critical promoting factor in tumor malignances from many aspects [21]. Our previous data have shown that hypoxia reinforced gemcitabine-induced stemness in pancreatic cancer cells [28]. In the present results, we revealed that hypoxia promoted the expression of CASC9, indicating that it might be a hypoxia-inducible IncRNA. Given the vital role of HIF-1α in the hypoxia-mediated effect, we further speculated whether CASC9...
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could be reciprocally regulated by HIF-1α. Our results showed that hypoxia-induced CASC9 was significantly attenuated after HIF-1α knockdown. In addition, we validated that the proximal promoter of CASC9 could bind with HIF-1α at several putative sites, which was enhanced by hypoxia treatment. This observation was in line with published findings and might explain the explicit mechanism involved [22]. Further, hypoxia has been shown to facilitate the activation of AKT, an effect that was blocked by HIF-1α inhibition. Collectively, our findings present evidence supporting a positive feedback loop of CASC9 and the AKT/HIF-1α signal pathway in response to hypoxia in pancreatic cancer cells.

Conclusions

In conclusion, our studies present a novel mechanism of enhanced glycolytic flux and EMT phenotypes in pancreatic cancer via the positive feedback loop of CASC9 and the AKT/HIF-1α signaling cascade, which is reinforced by the tumor hypoxic niche. Our data provide new insights for reversing the malignant phenotypes and improving the prognosis of patients with pancreatic cancer by targeting such relevant pathways.

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Disclosure of conflict of interest

None.

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References

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