**DGUIDOK-AS1 acts as a tumor promoter through regulating miR-204-5p/IL-11 axis in breast cancer**

Yiran Liang,1,6 Fangzhou Ye,1,6 Yajie Wang,1 Yalu Li,2 Yaming Li,1 Xiaojin Song,1 Dan Luo,3 Li Long,4 Dianwen Han,1 Ying Liu,1 Zekun Wang,1 Bing Chen,3 Wenjing Zhao,3 Lijuan Wang,3 and Qifeng Yang1,3,5

Breast cancer is one of the most lethal malignancies among women; however, the underlying molecular mechanism involved in the progression and metastasis of breast cancer remains unclear. Numerous studies have confirmed that long noncoding RNAs are abnormally expressed in breast cancer and play crucial roles in cell proliferation and metastasis. In the study, we evaluated the functional role and detailed mechanism of DGUK-AS1 in breast cancer progression and metastasis. DGUK-AS1 knockdown suppressed proliferation, migration, and invasion of breast cancer cells in vitro and in vivo. Mechanistically, miR-204-5p was identified as an inhibitory target of DGUK-AS1, which served as a tumor suppressor in breast cancer. Significantly, we found that the ectopic expression of miR-204-5p could counteract DGUK-AS1-mediated promotion of cell proliferation and metastasis in breast cancer. Moreover, IL-11 was found to be the downstream target of miR-204-5p, and DGUK-AS1 could protect IL-11 from miR-204-5p-mediated degradation. DGUK-AS1 overexpression promoted breast cancer cell migration, angiogenesis, and macrophage migration, mediating by the increased secretion of IL-11, which was extremely important for cancer progression. Collectively, our studies reveal that DGUK-AS1/miR-204-5p/IL-11 axis plays a significant role in the progression and metastasis of breast cancer, and DGUK-AS1 might be a novel biomarker and therapeutic target for breast cancer.

**INTRODUCTION**

Breast cancer is one of the most threatening cancers with the highest incidence for women worldwide, causing a large amount of cancer-related death.1 In China, with the up-trend of both incidence and mortality, breast cancer has become the leading cause of cancer-related death among women younger than 45 years.2 It has been discovered that breast cancer is a heterogenous malignancy and can be mainly classified into four subtypes with different prognoses and therapeutic strategies, including Luminal A, Luminal B, HER-2-positive, and triple-negative breast cancer. Although great progress has been made in the diagnosis and treatment of breast cancer, the mechanism of carcinogenesis and progression of breast cancer still remains unclear. Moreover, the patients with metastatic breast cancer suffer a worse outcome; the 5-year survival rate is only 25%.3 Therefore, it is urgent to reveal the molecular mechanism of the progression and metastasis of breast cancer, providing vital evidence for searching for effective therapeutic targets to suppress the metastasis and relapse of breast cancer.

Except for a small portion of genome encoding proteins, at least 75% of the genome is transcribed into noncoding RNAs.4 Among them, long noncoding RNAs (lncRNAs) are defined as transcripts with more than 200 nucleotides in length. Various studies have suggested that lncRNAs could regulate gene expression at the transcriptional, posttranscriptional, and posttranslational levels, participating in various cancer processes, such as carcinogenesis, metastasis, stemness, and drug resistance in a tissue-specific manner.5,6 For example, lncRNA HDAC2, which was overexpressed in hepatocellular carcinoma cells, could activate hedgehog signaling through transcriptionally inhibiting the expression of PTCH1, leading to enhanced self-renewal of liver CSCs.7 LncRNA TROJAN was specifically highly expressed in estrogen receptor (ER)-positive breast cancer, upregulating the expression of CDK2 through blocking the association of AKRFB and RELA, and subsequently induced drug resistance to CDK4/6 inhibitor.8 LncRNA RAMS11 recruited CBX4 and then activated Top2α during transcription, consequently enhancing the aggressiveness and causing poor prognosis of colorectal cancer.9 On the other hand, lncRNAs could also function as tumor suppressing factors.
LncRNA MALAT1 could bind to the metastatic transcription factor TEAD and block its interaction with YAP, as well as its target gene promoter region, thereby inhibiting lung metastasis of breast cancer. However, due to the variety and complicated regulating mechanisms, more investigations are needed to further reveal the roles of lncRNAs in the progression and metastasis of breast cancer.

Previous studies have suggested that lncRNAs could regulate gene expression through various manners. For example, lncRNAs can guide chromatin-modifying enzymes to their target genes, constitute the transcriptional coactivator or corepressor complexes, and serve as scaffolds for RNA-binding proteins. Significantly, lncRNAs can also act as competing endogenous RNAs (ceRNAs), binding with microRNAs (miRNAs) to prevent the degradation of the target genes. LncRNA PVT1 competitively absorbs miR-619-5p and consequently microRNAs (miRNAs) to prevent the degradation of the target genes. LncRNA NEAR1 could promote breast cancer progression through protecting miR-16-5p to preserve the expression of SMAD5, resulting in an immunosuppression effect served as scaffolds for RNA-binding proteins. Significantly, lncRNAs can also act as competing endogenous RNAs (ceRNAs), binding with microRNAs (miRNAs) to prevent the degradation of the target genes. LncRNA PVT1 competitively absorbs miR-619-5p and consequently upregulates the expression of Pygo2 and ATG14 in pancreatic cancer, which activates Wnt/b-catenin and autophagy signals and promotes the gemcitabine resistance. LncRNA NEAR1 could promote breast cancer progression through protecting miR-133b-mediated degradation of TIMM17A. LncRNA SNHG16, released from breast cancer cell-derived exosomes, could bind with miR-16-5p to preserve the expression of SMAD5, resulting in an immunosuppression effect through upregulating CD73 expression in T regulatory cells.

Our previous study identified a novel lncRNA DGUOK-AS1 based on bioinformatic analysis, whose expression was upregulated in breast cancer tissues and correlated with poor prognosis of breast cancer patients. However, the underlying regulatory mechanism and biological function of DGUOK-AS1 was unclear. In the present study, we took a deeper look at the functional role of DGUOK-AS1 using in vitro and in vivo experiments. Our results found that DGUOK-AS1 promoted the progression and metastasis of breast cancer through sponging miR-204-5p, which may provide a promising molecular target for breast cancer therapy.

RESULTS
DGUOK-AS1 knockdown suppresses cell proliferation, migration/invasion in breast cancer cells
DGUOK-AS1 is composed of two exons with a full length of 563 nt, which is widely expressed in most of the tissues (Figure S1). To evaluate the role of DGUOK-AS1 in breast cancer, we knocked down DGUOK-AS1 with small interfering RNAs (siRNAs) in different breast cancer cells (Figure 1A). The results of MTT showed that DGUOK-AS1 knockdown significantly inhibited cell proliferation (Figure 1B). In agreement with these results, colony formation assay showed that DGUOK-AS1 knockdown obviously decreased the colony number of breast cancer cells (Figure 1C). Moreover, DGUOK-AS1 knockdown also inhibited the DNA synthesis activities and sternness of breast cancer cells (Figures 1D and 1E). Then, the wound healing assay and transwell assay indicated that the migratory and invasive capacity of breast cancer cells transfected with siRNAs against DGUOK-AS1 led to decreased tube formation ability of human umbilical vein endothelial cells (HUVECs) (Figure 2C). Consistently, ectopic expression of DGUOK-AS1 promoted cell growth, migration, and invasion in breast cancer cells (Figures S2 and S3). These results collectively revealed that DGUOK-AS1 exerts a promotional role in the progression of breast cancer.

DGUOK-AS1 could competitively bind with miR-204-5p in breast cancer cells
Accumulated evidence demonstrated that lncRNAs could act as ceRNAs to regulate gene expression. To explore whether DGUOK-AS1 could function as an miRNA sponge, we first evaluated the subcellular location of DGUOK-AS1 in breast cancer cells. The results of nuclear/cytosol fractionation assay and fluorescence in situ hybridization (FISH) assay indicated that DGUOK-AS1 was predominantly distributed in the cytoplasm (Figures 3A and 3B), which is the prerequisite for acting as an miRNA sponge. According to the DIANA-LncBase v2 database, we identified miR-204-5p as a potential target of DGUOK-AS1, and there were two putative binding sites (Figure 3C). In order to explore the specific binding sites between DGUOK-AS1 and miR-204-5p, we constructed three wild-type DGUOK-AS1 reporters and three mutant reporters. Our results indicated that miR-204-5p could significantly reduce the luciferase activity of the wild-type DGUOK-AS1 reporters (Figure 3D), and the inhibitory effect of miR-204-5p on Fragment 1 and Fragment 2 reporters was attenuated compared with that on the full-length reporter. Moreover, miR-204-5p had no influence on the luciferase activity of the mutant reporters with two mutant sites, but miR-204-5p only partly inhibited that of the mutant reporters with a single mutant site. These results indicated that miR-204-5p could bind with DGUOK-AS1 through these two sites, and the effect of the site 2 seemed to be more significant. Moreover, the results of RNA immunoprecipitation (RIP) assay showed that significantly more DGUOK-AS1 and miR-204-5p were pulled down with anti-AGO2 antibodies than with anti-immunoglobulin (Ig)G (Figure 3E). Moreover, the expression of miR-204-5p was lower in breast cancer cells compared with normal breast cells (Figure 3F). Then, we determined the effect of DGUOK-AS1 on miR-204-5p expression. The results showed that knockdown of DGUOK-AS1 increased miR-204-5p expression, whereas overexpression of DGUOK-AS1 led to decreased miR-204-5p expression (Figure 3G). In summary, DGUOK-AS1 could act as an endogenous sponge for miR-204-5p in breast cancer.

Overexpression of miR-204-5p could reverse the tumor-promoter role of DGUOK-AS1 in breast cancer cells
Previous study indicated that miR-204-5p could inhibit proliferation, migration, and invasion of breast cancer cells. Therefore, we performed rescue experiments in breast cancer cells to explore whether miR-204-5p could influence the biological function of DGUOK-AS1. Overexpression of miR-204-5p led to decreased expression of DGUOK-AS1, indicating a reciprocal suppressive relationship between them (Figure 4A). Moreover, miR-204-5p mimics could partly abrogate the DGUOK-AS1 overexpression-mediated promotion on proliferation, migration, and invasion in breast cancer cells (Figures 4B–4E). These results indicated that miR-204-5p served as a tumor
suppressor and partly abolished the oncogenic effect of DGUOK-AS1 in breast cancer cells.

**DGUOK-AS1 upregulates interleukin-11 expression via competitively binding with miR-204-5p**

Based on the miRTarBase, Starbase, TargetScan, and miRDB databases and previous reports, we selected 11 candidates as potential targets of DGUOK-AS1/miR-204-5p axis. We first evaluated the expression of these 11 genes after knockdown or overexpression of DGUOK-AS1 in MDA-MB-231 and MDA-MB-468 cells. According to the results of qRT-PCR, the changes of interleukin (IL)-11 expression was the most significant (Figures 5A, S4A, and S4B). Furthermore, the expression of IL-11 was also remarkably decreased by miR-204-5p overexpression (Figure 5B). ELISA was used to confirm the alterations of secreted IL-11 proteins. We found that IL-11 expression in the 24-h cultured supernatant from DGUOK-AS1-knockdown MDA-MB-231 and MDA-MB-468 cells was reduced compared with the medium from the control cells, whereas DGUOK-AS1 overexpression led to increased secreted IL-11 proteins (Figure 5C). Moreover, the IL-11 expression in the 24-h conditioned medium from miR-204-5p overexpression MDA-MB-231 and MDA-MB-468 cells was also decreased compared with the medium from the control cells (Figure 5D). Using The Cancer Genome Atlas (TCGA) database, we found that the expression of IL-11 was positively associated with the expression of DGUOK-AS1 in breast cancer tissues (Figure 5E), and the expression of IL-11 was elevated in breast cancer tissues compared with normal tissues (Figure 5F). Moreover, previous study reported that miR-204-5p could inhibit the expression of IL-11 in esophageal squamous cell carcinoma.20 Thus, we chose IL-11 as a putative target of DGUOK-AS1 for further observation. There were two putative binding sites between miR-204-5p and 3′UTR of IL-11 that were far away from each other (Figure 5G); therefore, we constructed two reporters, respectively, containing these two sites and two corresponding mutant reporters. The luciferase assays showed that overexpression of miR-204-5p decreased the luciferase activity of the wild-type IL-11 reporter but not the mutant reporter (Figure 5H), indicating that miR-204-5p could bind with the 3′UTR of IL-11 through these two sites to further regulate the expression of...
IL-11. RIP assay also validated that more IL-11 was pulled down with anti-AGO2 antibodies than anti-IgG antibodies (Figure 5I), indicating that IL-11 could be regulated in an AGO2 manner. Significantly, while DGUOK-AS1 overexpression led to increased expression of IL-11, simultaneous miR-204-5p overexpression was able to reverse the elevated expression of IL-11 (Figures 5J, 5K, and S4C). These results suggest that DGUOK-AS1 could protect IL-11 expression from miR-204-5p-mediated degradation.

DGUOK-AS1 aggravated malignant behaviors of breast cancer cells by regulating IL-11 expression

Previous studies reported that IL-11 played important roles in tumor migration, invasion, proinflammation, and angiogenesis in various cancers, such as hepatocellular carcinoma,21 endometrial carcinoma,22 and breast cancer.23 To confirm the role of IL-11 in DGUOK-AS1 regulated malignant behaviors of breast cancer cells, we performed IL-11 rescue experiments. DGUOK-AS1 knockdown led to inhibited migrative abilities of breast cancer cells, while IL-11 overexpression could partly reverse the suppressive effect of DGUOK-AS1 knockdown in breast cancer cells (Figure 6A). Moreover, the migration and tube formation assays indicated that the promoted migration ability of MDA-MB-231 cells and enhanced tubular structure formation of HUVECs in conditioned medium from DGUOK-AS1-overexpressing MDA-MB-231 cells were partly rescued by the supplement of IL-11 neutralizing antibodies (Figure 6B). Cancer cells could recruit monocyte to cancer tissues through secreting cytokines and chemokines,24 and the infiltration of macrophage is significantly associated with the clinical outcomes and drug resistance in breast cancer. Therefore, we further evaluated the effect of DGUOK-AS1 on the recruitment of monocytes. Our results indicated that the supernatants from DGUOK-AS1-knockdown cells led to decreased migration ability (Figure S5A) and inhibited chemotaxis (Figure S5B) of THP1 cells. Consistently, DGUOK-AS1 overexpression promoted the migration (Figure S5C) and showed enhanced chemotaxis of THP1 cells (Figure S5D). Moreover, supplement of IL-11 neutralizing antibodies could partly abrogate the promoted effect of the supernatants from DGUOK-AS1-overexpressing cells on THP1 migration (Figure 6B). According to the above findings, we confirmed that IL-11 is essential to the DGUOK-AS1-induced breast cancer progression.
DGUOK-AS1 promotes tumor growth and metastasis in vivo

We then sought to determine whether DGUOK-AS1 could promote tumor growth in vivo. The stable DGUOK-AS1-overexpressing or control MDA-MB-231 cells were injected into the left flank of BALB/c nude mice. The results showed that overexpression of DGUOK-AS1 significantly increased the tumor volume and tumor weight (Figures 7A–7C). H&E staining was used to evaluate the morphology of the tumors (Figure 7D). Immunohistochemical (IHC) analysis also showed that increased ki67 and IL-11 levels were identified in the DGUOK-AS1-overexpressing groups (Figure 7E). In addition, we evaluated the role of DGUOK-AS1 in cancer metastasis in vivo using a pulmonary metastasis model. Two of the five mice (2/5) injected with control breast cancer cells and all five mice (5/5) injected with DGUOK-AS1-overexpressing breast cancer cells exhibited metastatic foci in their lungs (Figure 7F). H&E staining was performed to pathologically confirm the metastatic nodules in the lungs (Figure 7G). Significantly, the size and number of lung metastatic nodules were increased in the DGUOK-AS1-overexpressing group compared with those in the control group. Moreover, the subcutaneous xenograft model also showed that DGUOK-AS1 overexpression led to increased number of microvessels (CD31 positive) in the xenograft tissues (Figure 7H). We also constructed stable DGUOK-AS1-knockdown cells to further evaluate the effect of DGUOK-AS1 on breast cancer in vivo. The xenografts derived from breast cancer cells with DGUOK-AS1 knockdown showed significantly reduced tumor volume and tumor weight (Figures 7I–7L). The IHC results indicated that DGUOK-AS1 knockdown could reduce the expression of its target protein IL-11 and cell proliferation-related protein Ki67 (Figure 7M). Furthermore, stable DGUOK-AS1-knockdown or control breast cancer cells were intravenously injected into the tail vein of BALB/c nude mice. Two mice (2/5) in the control group exhibited metastatic foci in their lungs, while no metastatic foci were identified in DGUOK-AS1-knockdown group (Figure 7N). H&E staining was used to pathologically confirm the metastatic nodules in the lungs.
Collectively, these results suggest that DGUOK-AS1 facilitated tumor growth and metastasis in vivo.

**DISCUSSION**

Although there are current innovations in diagnosis and treatment, the prognosis of breast cancer remains unsatisfactory. Therefore, more research is needed to further explore the mechanism of breast cancer progression and provide novel therapeutic targets. Recently, various studies verified that the lncRNAs play significant roles in human physiological and pathophysiological processes. Moreover, dysregulation of lncRNAs is highly relevant to the tumorigenesis, progression, and treatment resistance of breast cancer. The overexpression of lncRNA HUMT was associated with poor prognosis of breast cancer patients, which could promote lymph node metastasis via activating FOXK1 transcription and further regulate VEGFC expression. LncRNA HOST2 was upregulated in TNBC cell line and tissues, and promoted STAT3-mediated proliferation and migration abilities of TNBC cells by decoying let-7b. Previous study revealed that DGUOK-AS1 was upregulated in breast cancer tissues and was correlated with poor prognosis of breast cancer patients, indicating that DGUOK-AS might be a promising prognosis predictor and therapeutic target. Moreover, previous study has reported that DGUOK-AS1 could promote the cervical cancer cell proliferation through regulating miR-653-5p/EMSY pathway. However, the role of DGUOK-AS1 in breast cancer has not been fully elucidated. In this study, we investigated the function and molecular mechanisms of DGUOK-AS1 through in vitro and in vivo experiments. We found that DGUOK-AS1 could induce breast cancer proliferation, migration, and invasion, indicating a critical role of DGUOK-AS1 in breast cancer progression and metastasis.

**Figure 4.** DGUOK-AS1 regulated the proliferation, migration, and invasion of breast cancer cells by targeting miR-204-5p

Transfected MDA-MB-231 and MDA-MB-468 cells were divided into pcDNA3.1 + NC, DGUOK-AS1+NC, pcDNA3.1+miR-204-5p mimics, and DGUOK-AS1+ miR-204-5p mimic groups. (A) The expression of DGUOK-AS1 in breast cancer cells was detected by qRT-PCR. (B and C) MTT (B) and EdU (C) assays were used to detect the effect on cell proliferation. Scale bar, 200 μm. (D and E) Transwell assays (D) and tube formation assays (E) were used to evaluate the effect on cell migration and invasion. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 7O.** Collectively, these results suggest that DGUOK-AS1 facilitated tumor growth and metastasis in vivo.
the miR-153-5p/ARHGAP18 axis. In this study, we found that DGUOK-AS was mainly located in the cytoplasm and exerted its oncogenic function via sponging miR-204-5p. Previous study reported that miR-204-5p served as a tumor suppressor in various cancers, including head and neck squamous cell carcinoma, gastric cancer, and breast cancer. Although the abnormal expression of miR-204-5p has a potentially important role in cell proliferation, migration, and invasion, whether DGUOK-AS1 could affect the progression of breast cancer through direct interaction with miR-204-5p remains to be explored. The results of luciferase reporter assay and RIP assay indicated that DGUOK-AS1 exerted its regulation function via binding to miR-204-5p as an miRNA sponge. Furthermore, functional assays further demonstrated that miR-204-5p overexpression alleviated the promotive effect of DGUOK-AS1 overexpression on cell proliferation, migration, and invasion. Moreover, our results also revealed the negative regulatory effect of DGUOK-AS1 on the expression of miR-204-5p in breast cancer cells. Previous studies reported a mechanism of miRNA decay mediated by the targeted term target-directed miRNA degradation (TDMD). This process requires the base pairing with the seed sequence at the 5’ end of miRNA, complementarity between the 3’ end of the miRNA and the target, as well as central mismatches, leading to further tailing (addition of nucleotides at the 3’ end) and trimming (shortening). However, the detailed mechanisms and the effector proteins are still obscure. Although we did not find the additional complementarity between the 3’ end of the miR-204-5p and the DGUOK-AS1, DGUOK-AS1 might mediate the degradation of miR-204-5p through regulating the expression or function of trans-acting factors responsible for TDMD. Moreover, previous studies reported that some nuclear-localized lncRNAs could regulate the expression or maturation of miRNAs through binding with transcription factors, chromatin, or the stem-loop sequence of pri-miRNAs. Although DGUOK-AS1 is mainly located in the cytoplasm, we also detected its nuclear distribution. It is possible to speculate that DGUOK-AS1 inhibited the expression of miR-204-5p through sponging miR-204-5p.

Figure 5. IL-11 was a downstream target of miR-204-5p
(A) The qRT-PCR was used to detect the expression of IL-11 in DGUOK-AS1 knockdown or overexpression cells. (B) The qRT-PCR was used to detect the expression of IL-11 in miR-204-5p overexpression cells. (C) ELISA assay was used to evaluate the expression of IL-11 in the conditioned medium of DGUOK-AS1 knockdown or overexpression cells. (D) ELISA assay was used to evaluate the expression of IL-11 in the conditioned medium of miR-204-5p overexpression cells. (E) The expression of IL-11 was positively associated with DGUOK-AS1 using the TCGA database. (F) The expression of IL-11 was increased in breast cancer tissues compared with normal tissues using the TCGA database. (G) Schematic diagram represents the predicted binding sites for miR-204-5p in IL-11 3’ UTR and mutant sequences of the potential miR-204-5p binding sites. (H) The binding capacity between miR-204-5p and IL-11 was confirmed by luciferase reporter assay. Fragment 1 refers to reports containing the sequence of 3’UTR of IL-11 with wild-type first putative binding site; Fragment 2 refers to reports containing the sequence of 3’UTR of IL-11 with wild-type second putative binding site; MUT 1 refers to reports containing the sequence of 3’UTR of IL-11 with mutant first putative binding site; MUT 2 refers to reports containing the sequence of 3’UTR of IL-11 with mutant second putative binding site. (I) RIP assay was used to confirm expression of IL-11 in RISC complex. (J and K) The qRT-PCR (J) and ELISA (K) assays were conducted to detect the expression levels of IL-11 in transfected cells. *p < 0.05, **p < 0.01, ***p < 0.001.
miRNAs are endogenous small noncoding RNAs, which could regulate gene expression through the partial base pairing between the “seed” region of an miRNA (nucleotides 2 to 8) and the sequence in the 3′UTR of their target mRNA and further destabilizing them in an AGO2/RISC-dependent method. In the present study, we identified IL-11 as a putative target of miR-204-5p. Previous study reported that IL-11 is a member of the IL-6 family of cytokines with multiple functions, including hemopoiesis and thrombopoiesis stimulation, macrophage differentiation regulation, and intestinal mucosal protection. Moreover, IL-11 was found to exert oncogene effects in various cancers, such as colorectal cancer, liver cancer, and non-small cell lung cancer. Recently, various studies indicated that IL-11 was upregulated in breast cancer tissues compared with normal tissues, and high expression of IL-11 was associated with poor prognosis in breast cancer patients. Further study revealed that IL-11 plays a significant role in breast cancer progression, especially the well-documented capacity to promote breast cancer bone metastasis. One study reported that IL-11 could activate the JAK1/STAT3 signaling pathway to induce the expression of c-MYC, and thus play a significant role in breast cancer bone metastasis. Another study reported that CUL1 regulated EZH2 expression to promote the production of IL-11 and significantly aggravated the breast cancer cell metastasis and angiogenesis through activating the PI3K-AKT-mTOR signaling pathway. However, the regulatory mechanism involved in the expression and function of IL-11 in breast cancer has not been fully elucidated. In our study, we observed that IL-11 expression was upregulated by DGUOK-AS1 through sponging miR-204-5p. The dual-luciferase reporter assay and RIP assay indicated that IL-11 was a direct target gene of miR-204-5p. The IL-11 expression was positively associated with DGUOK-AS1 expression in breast cancer tissues, and DGUOK-AS1 knockdown led to significantly downregulated IL-11 expression. Moreover, DGUOK-AS1 overexpression led to increased expression of IL-11, which could be partially reversed by miR-204-5p overexpression. These results indicated a DGUOK-AS1/miR-204-5p/IL-11 axis in breast cancer.

In conclusion, our study illustrates that DGUOK-AS1 functions as an oncogene to facilitate breast cancer cell proliferation, migration, and invasion through regulating the miR-204-5p/IL-11 axis. These results indicate that DGUOK-AS1 is a critical molecule for breast cancer.
Figure 7. The effect of DGUOK-AS1 on breast cancer cell growth and metastasis in vivo

(A) Tumor xenograft model in nude mice. DGUOK-AS1-overexpressed MDA-MB-231 cells and control cells were inoculated to the flank of nude mice. Scale bar, 1 cm. (B) The tumor weight and TV were analyzed. (C) The expression of DGUOK-AS1 in xenograft tumor tissues. (D) H&E staining showed the tissue morphology. (E) Representative images of Ki67 and IL-11 staining in the tumor tissues. (F) Representative images of lung metastatic nodules. DGUOK-AS1 overexpression resulted in an increased number of lung metastatic colonies. (G) H&E staining of lungs isolated from mice injected with DGUOK-AS1-overexpressed or control cells. (H) Representative immunohistochemistry staining...
progression and might serve as a novel biomarker and potential therapeutic target for breast cancer.

MATERIALS AND METHODS

Cell culture

Breast cancer cell lines (MDA-MB-231, MDA-MB-468, and MCF-7) and HUVECs were purchased from ATCC (Manassas, VA). The cells were characterized by Genetic Testing Biotechnology Corporation (Suzhou, China) using short tandem repeat markers and tested for negative mycoplasma contamination using the Mycoplasma Detection Kit (Sigma). The cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were grown in 5% CO₂ in an incubator at 37°C.

Cell transfection

The full length of DGUOK-AS1 or IL-11 was cloned into pcDNA3.1 (Invitrogen, USA) to generate pcDNA3.1-DGUOK-AS1 or pcDNA3.1-IL-11 constructs. The primers used for vector construction are shown in Table S1. The empty vector and overexpression plasmids were transfected into breast cancer cell lines respectively using Lipofectamine 2000 reagent (Invitrogen, USA). The stably transfected cells were constructed as previously reported. After transfection with the overexpression vectors or empty vectors, the cells were cultured with G418 (2 mg/mL) for 3 to 4 weeks, and the nontransfected cells or transiently transfected cells would die. The surviving cell clones were selected for further amplification, and the qRT-PCR assay was performed to confirm the expression of DGUOK-AS1 in each cell clone. Finally, the satisfactory cell lines were regarded as stably transfected cells and used for subsequent in vivo experiments. To obtain stable DGUOK-AS1-knockdown cells, short hairpin RNAs (shRNAs) against DGUOK-AS1 were constructed, which inserted siRNA into the pLKO.1 vector. Puromycin (2 µg/mL) was used for 3 to 4 weeks to select stably transfected cell lines. Furthermore, clonal selection and qRT-PCR analysis were performed to confirm the DGUOK-AS1 expression in individual clones. All the mimics and siRNAs were purchased from Applied Biological Materials (ABM, Canada).

RNA extraction and quantitative real-time PCR

Total RNA from transfected cells was extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer’s protocol. RT-PCR was performed using PrimeScript reverse transcriptase reagent kit (TaKaRa, Japan). qRT-PCR was performed using SYBR Green PCR Master Mix (TaKaRa, Japan). Actin was used as the reference gene for IncRNA DGUOK-AS1 or IL-11. U6 served as the internal reference for miR-204-5p. The relative expression of indicated genes was normalized to the expression of corresponding reference gene and calculated using the relative standard curve method (2^(-ΔΔCt)). The sequences of primers are listed in Table S2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The transfected cells were seeded into 96-well plate at a density of 1,500 cells per well. After incubation for the indicated time, 20 µL MTT (5 mg/mL) was added into the wells in light-resistant condition. After incubation for 4 to 6 h, the supernatant was removed and 100 µL DMSO was added into each well. The absorbance results were obtained using Microplate Reader (Bio-Rad, USA) at 570 nm.

Colon formation assay

The transfected cells were seeded into a 6-well plate at a density of 500 cells per well and incubated for over 2 weeks. Then, the cells were washed with PBS three times, fixed with methanol, and stained by 0.2% crystal violet. The stained colonies were photographed and counted.

EdU incorporation assay

The transfected cells were seeded into a 96-well plate at a density of 10,000 cells per well. Then, the cells were incubated in 50 mM EdU for 2.5 h and 4% paraformaldehyde was used to fix the cells. Apollo Dye Solution and Hoechst were used to stain proliferative cells and nucleic acid of all cells respectively using EdU incorporation assay kit (RiboBio, China). Images were obtained using the fluorescence microscope (Nikon, Japan).

Sphere formation assay

A total of 500 cells/mL/well were seeded onto 96-well Ultra-Low Attachment Plates containing DMEM/F2 medium supplemented with B27 (1:50), 20 ng/mL human EGF, 20 ng/mL basic fibroblast growth factor, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5 µg/mL insulin. After 7 days, the size and number of primary tumor spheres were measured using an inverted Olympus microscope.

Wound healing assay

Transfected cells were plated into 24-well plates until reaching 90% confluence. Then, a sterile tip (10 µL) was used to scratch the cell monolayer. PBS was used to remove the detached cells and cells were grown in serum-free DMEM. An Olympus microscope was used to record the scratch wounds at the indicated time.

Transwell assay

For migration assay, 700 µL medium containing 20% fetal bovine serum was added into the 24-well plate as lower insert, and then 80,000 transfected cells were added into the upper insert of the transwell chamber (pore size 8 µm; Costar Corporation, USA). After incubation for 24 to 48 h, the cells migrated to the lower insert were fixed with methanol for 15 min and stained by 0.2% crystal violet for 20 min. Then the stained cells were photographed and the relative cell number was calculated.
Tube formation assay
Transfected cells were incubated with 2 mL fresh serum-free medium for 24 h and the conditioned medium was collected. A 48-well plate was precoated with 200 μL Matrigel (BD Biosciences, USA) and kept at 37°C for 0.5 h. Then 1 × 10⁴ HuVECs were suspended in 200 μL of indicated conditioned medium and seeded in the plate. After incubation for 4 to 6 h, the complete tubular structures were imaged and quantified.

Subcellular fractionation
Nuclear and cytoplasmic separation was performed using the PARIS Kit (Life Technologies, USA) according to the manufacturer’s instructions.

FISH assay
The localization of DGUOK-AS1 in cells was detected using FISH assay (GenePharma, China) according to the instructions of the manufacturer. Briefly, cells were collected and fixed in formaldehyde (4%) at room temperature. After 30 min, cells were permeabilized and incubated with specific probes at 37°C in a moist chamber overnight (dark). The cell nuclei were stained with DAPI. Finally, the images were obtained under an inverted fluorescence microscope (Nikon, Japan).

Luciferase reporter assay
The wild-type or mutant DGUOK-AS1 or 3’UTR of IL-11 was amplified by PCR and inserted into pmirGLO vector (Invitrogen, USA) separately. The wild-type or mutant luciferase plasmids and miR-204-5p or control miRNA were cotransfected into HEK293T cells separately. The wild-type or mutant luciferase plasmids and miR-200 or control miRNA were cotransfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, USA). The Dual-Luciferase Reporter Assay System (Promega, USA) was used to measure the luciferase activities.

RIP assay
The interaction among DGUOK-AS1 and miR-204 was detected using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). Anti-AGO2 and control IgG (Millipore, USA) were used as antibodies for RIP assay. The expression of specific molecules was detected using qRT-PCR.

Western blot assay
Protein samples were extracted by RIPA lysis buffer (Beyotime, China) with PMSF, and then subjected to 10% to 12% SDS-PAGE gels according to the concentration of protein. After separating in the gels, proteins were transferred onto 0.22 μm PVDF membranes (Millipore, USA). The membrane was then blocked with 5% skim milk in TBST and incubated with primary antibody of target protein under 4°C overnight. After washing, the membrane was incubated with corresponding secondary antibody for 2 h. The protein blots were finally detected using the ECL detection system.

ELISA
The IL-11 concentration in the culture supernatant of indicated cells was measured by human IL-11 ELISA kits (CUSABIO, China) according to the manufacturer’s instructions. Briefly, 3 × 10⁵ indicated cells were seeded in a 12-well plate and cultured overnight in a 5% CO₂ incubator at 37°C. After cell attachment, the culture medium was changed into DMEM with no fetal bovine serum and the cells were cultured for 24 h. Then, the cell medium was collected and centrifuged at 3,000 × g for 15 min at 4°C to obtain the culture supernatant; 100 μL of supernatant was added to the ELISA plate and successively incubated with biotin-labeled IL-11 antibody for 1 h at 37°C and horseradish peroxidase-labeled avidin for 1 h at 37°C. After washing and spinning, 90 μL of transmembrane domain substrate was added to each well in sequence and incubated for 15 to 30 min in the dark. Then, 50 μL of stop solution was added to each well in the same sequence and the absorbance value at 450 nm was measured using Microplate Reader (Bio-Rad, USA). The concentration of IL-11 was calculated according to the standard curve.
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AUTHOR CONTRIBUTIONS
Y.R.L., F.Z.Y., and Q.F.Y. designed the experiments; Y.R.L., F.Z.Y., Y.J.W., Y.L.L., Y.M.L., X.J.S., D.L., and L.L. carried out most of the experiments; D.W.H. and Y.L. collected samples; Y.R.L., F.Z.Y., Z.K.W., Y.J.W., Y.L.L., Y.M.L., X.J.S., D.L., and L.L. prepared the figures and tables; Y.R.L., F.Z.Y., and Q.F.Y. wrote and revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no conflict of interest.

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