

# LncRNA-HOST2 regulates cell biological behaviors in epithelial ovarian cancer through a mechanism involving microRNA let-7b

Yuan Gao<sup>1,2</sup>, Hao Meng<sup>3,4</sup>, Shupeng Liu<sup>2</sup>, Jingjing Hu<sup>2</sup>, Yemin Zhang<sup>2</sup>, Tingting Jiao<sup>2</sup>, Yujie Liu<sup>2</sup>, Jun Ou<sup>2</sup>, Dan Wang<sup>2</sup>, Lin Yao<sup>2</sup>, Shanrong Liu<sup>2</sup> and Ning Hui<sup>2,\*</sup>

<sup>1</sup>Chinese PLA General Hospital, Beijing 100853, China, <sup>2</sup>Changhai Hospital of Second Military Medical University, Shanghai 200433, China, <sup>3</sup>Air Force General Hospital, Beijing 100142, China and <sup>4</sup>Beijing Military Region General Hospital, Beijing 100700, China

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Recently, a large number of long non-coding RNAs (lncRNAs) have been reported in mammalian genomes and are evolutionarily conserved and presumably function in many biological events, especially in the pathogenesis of diverse human cancers. A lncRNA, named HOST2 (human ovarian cancer-specific transcript 2), was once reported to specifically be expressed at high level in human ovarian cancer. However, how HOST2 acts to regulate gene functions in ovarian carcinogenesis has remained enigmatic. Here we report, for the first time, that HOST2 promotes tumor cell migration, invasion and proliferation in epithelial ovarian cancer by working in key aspects of biological behaviors. In the present study, bioinformatics analysis indicated that HOST2 binds with microRNA let-7b, a potent tumor suppressor, which was then verified to target HOST2. Our results showed that HOST2 harbors a let-7b binding site and modulates let-7b availability by acting as a molecular sponge. HOST2 inhibits let-7b functions, which post-transcriptionally suppress the expression of targets, including some oncogenes that regulate cell growth and motility. Additionally, understanding HOST2/let-7b-dependent regulation may lead to alternative approaches for the diagnosis and cure of this deadly disease.

## INTRODUCTION

Ovarian cancer (OC) is one of the most common gynecological malignancies, accounting for the fourth major cause of cancer mortality in women (1). Most worldwide OC cases are presented in an advanced stage when finally diagnosed due to the absence of specific signs/symptoms in the early phase as well as the lack of effective or sensitive clinical screening methods (1). Additionally, a high tumor recurrence rate and poor prognosis as a consequence of metastatic spread of this devastating disease are common and serious problems with low overall cure rates, increasing its incidence. Furthermore, its mechanism remains unclear (2). Successfully eradicating this disease therefore crucially requires deep research and a better understanding of how OC initiates and progresses to explain its serious clinical course. Epithelial ovarian cancer (EOC) represents ~85% of OC, and this histological subtype is the most

serious threat to the health of women, with a 5-year mortality of ~70% and a high incidence of metastasis (1). Due to the absence of specific, early phase signs or symptoms and the lack of effective or sensitive clinical screening methods, most EOC cases present in an advanced stage when they are finally diagnosed (1). This devastating disease is also characterized by a low overall cure rate, high tumor recurrence rate, poor prognosis, increasing incidence and unclear mechanism (2). Therefore, successfully treating this disease requires more extensive research and a better understanding of how it initiates and progresses.

To date, a large set of non-protein-coding RNAs (ncRNAs) have been recognized to control nearly every level of gene expression, including the activation and repression of homeotic genes and the targeting of chromatin-remodeling complexes (3). Foremost among these are those termed microRNAs (miRNAs), which is a large family of short, non-coding RNA

\*To whom correspondence should be addressed at: 6A No. 6 Building, 168 Changhai Road, Shanghai 200433, China. Tel: +86 13386273691; Fax: +86 2181873592; Email: huining324@aliyun.com

molecules of ~22–25 nt involved in a range of developmental and physiological approaches {(4)}. Alterations in the expression of miRNAs appear important for carcinogenesis, and their profile might identify pivotal tumorigenic pathways {(5)}; however, most have yet to be systematically studied. Although a plenty of studies have comprehensively analyzed alterations in the miRNome that are associated with malignant transformation of OC {(6)}, the underlying mechanism of OC is still poorly demonstrated. Accumulating evidence and efforts have indicated a vast, hidden but surprisingly prominent layer of regulatory macro ncRNAs, named long non-coding RNAs (lncRNAs) {(7)}. LncRNAs, which are >200 nt in length, constitutes the majority of genomic programming and implicated in many types of gene regulation {(8)}, including epigenetic, transcriptional or post-transcriptional regulation and so forth, to sequentially induce the development of cancers and other diseases {(9)}. Furthermore, recent molecular studies have demonstrated that plentiful lncRNAs play critical roles in the progression of prostate cancer, colorectal cancer, breast cancer, liver cancer and many other types of human solid tumors {(10–12)}.

Recently, it has been demonstrated that five transcripts, named human ovarian cancer-specific transcripts (HOSTs), are rarely expressed in normal or cancer tissues, except for OC, and are specifically expressed in all four major, clinicopathologic subtypes of ovarian malignancy {(13)}. Among these transcripts, the protein-coding genes HOST1, 3 and 5 protein-coding transcripts were found to be closely associated with the biological behaviors of OC cells, including more proliferative, aggressive, differential and migratory tumor phenotypes {(14,15)}. One attraction is that HOST2 is now known as a novel member of the family of long non-coding mRNA-like genes, 2.9 kb in length, without an obvious open reading frame (ORF) {(13)}. A line of evidence has supported that aberrant expression of lncRNAs may play an important role in cancer biology {(16)}. However, only preliminary data on the role of HOST2 in OC exists, and how HOST2 functions is not well understood up to now. It would be extremely crucial to further investigate the potential roles of HOST2 in the etiology of malignant ovarian tumors.

The major role of lncRNAs in disease processes creates an urgency to understand the mechanisms by which these RNAs seek their targets{(17)} and control the epigenetic trajectories underlying their ontogeny{(18)}. Possible RNA-targeting schemes include sequence-specific recognition (RNA–RNA){(19)}, RNA–DNA hybrids {(20)}, structure-mediated interactions {(21)} and protein-mediated interactions {(22)}. In some cases, many lncRNAs are processed to yield small RNAs or, conversely, modulate how other RNAs are processed {(23)}. Many reports have suggested that the oncogenic activity of some lncRNAs might be attributed to miRNAs. For example, lncRNA H19 acts as a molecular ‘sponge’ for the let-7 family of miRNAs, which are well-characterized tumor suppressors. Let-7 suppresses target-gene expression by binding to imperfect complementary sequences in the mRNA, resulting in translational repression and mRNA degradation {(24)}. Therefore, we considered that lncRNA-HOST2 must serve some key regulatory roles in EOC.

In the present study, bioinformatics analysis indicated that HOST2 bound with miRNA let-7b, a potent tumor suppressor, which was then verified to target HOST2. Because decreased let-7b function has been associated with increased tumor

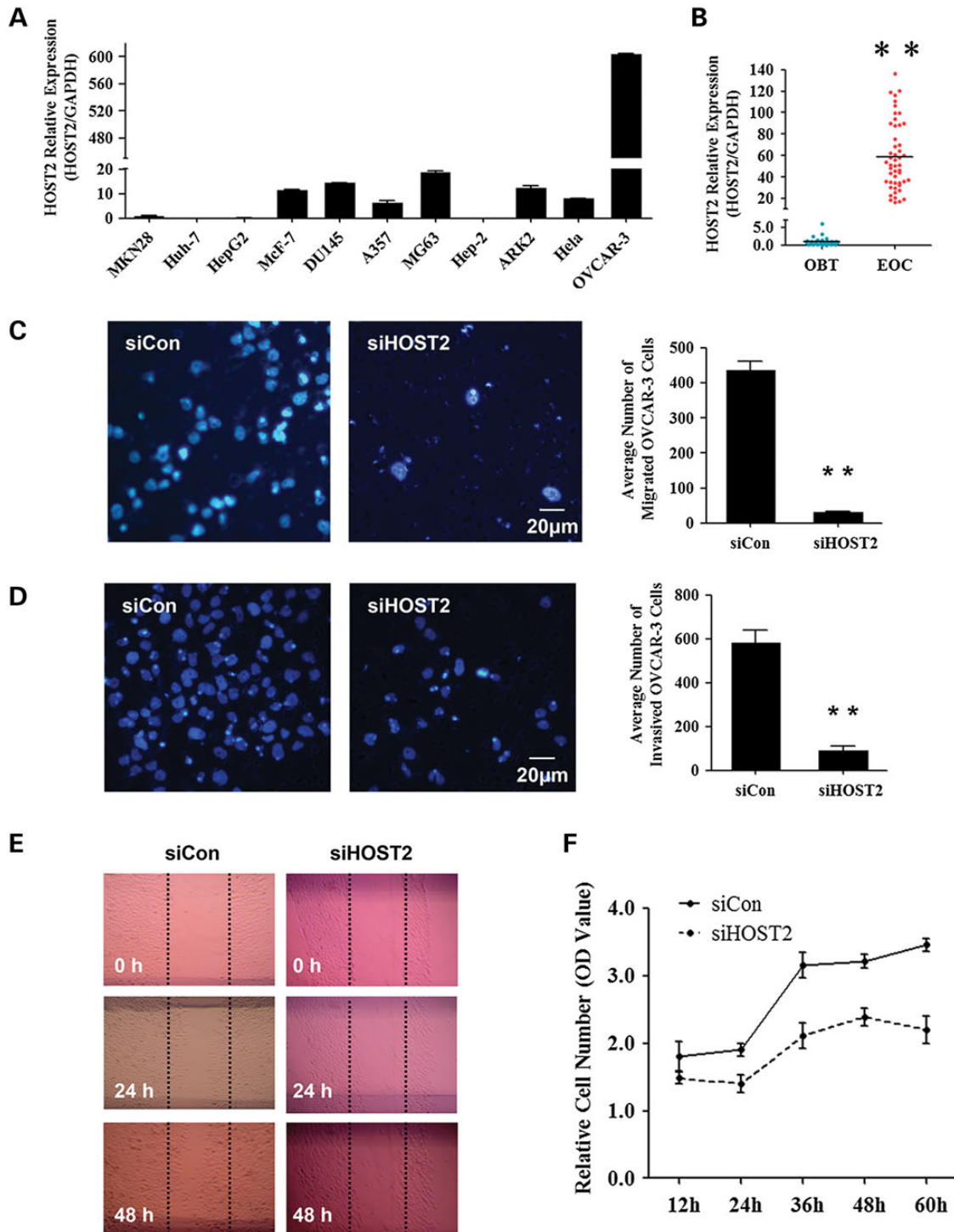
metastasis and poor prognosis {(25–28)}, we hypothesized that HOST2 may promote EOC metastasis, in part through impeding let-7b function. We demonstrate that HOST2 promotes tumor cell migration, invasion and proliferation by inhibiting let-7b to maintain the growth of tumor cells in epithelial ovarian cancer by working in key aspects of biological behaviors. Our results showed that let-7b functions to post-transcriptionally suppress the expression of oncogenes that regulate cell growth and motility. Additionally, this HOST2/let-7b-dependent regulation may address alternative approaches for diagnosis and cure of this deadly disease.

## RESULTS

### Specific and high-expression of HOST2 promotes EOC cell migration, invasion and proliferation

The roles of HOST2 in EOC remain unclear, therefore, to first verify the specificity of HOST2 in EOC that was shown by Rangel, we used the quantitative real-time PCR (qRT-PCR) to test the HOST2 mRNA levels in multiple human cell lines of common solid tumors. The data showed that HOST2 was obviously upregulated in an EOC-derived cell line, OVCAR-3, compared with the other tested cell lines, including MKN28, Huh-7, HepG2, MCF-7, DU145, A357, MG63, Hep-2, ARK2 and HeLa (Fig. 1A). Meanwhile, we examined the expression differences of HOST2 in the following clinical samples; ovarian epithelial benign tumors (OBT,  $n = 30$ ) and EOC ( $n = 50$ ), the former served as a control group. As Figure 1B shows, HOST2 levels were dramatically elevated in tissue samples of EOC patients when compared with normal controls ( $P < 0.01$ ). Then, we utilized RNA interference (RNAi)-mediated silencing *in vitro* by, respectively, transfecting OVCAR-3 cells with four distinct small interfering RNA (siRNA) sequences that targeted non-overlapping regions of the HOST2 transcript and determined that ‘siRNA2’ had the relatively best interference efficiency (renamed siHOST2), amounting to a mean decrease of 79% when compared with the negative control (renamed siCon) transfection ( $P < 0.01$ ) (Supplementary Material, Fig. S1A). Then, we down-regulated HOST2 using siHOST2 in OVCAR-3 cells that endogenously expressing a high level of HOST2 (Fig. 1A) and examined the effects on cell migration, invasion, proliferation and apoptosis. As shown by the loss-of-function analysis, HOST2 depletion led to a significant decrease in migration (Fig. 1C) and invasion (Fig. 1D) using *in vitro* Transwell migration and invasion assays. To further identify the regulation of HOST2 on cell migration, we performed a wound healing assay and found that HOST2 knockdown inhibited the migration rate of OVCAR-3 cells (Fig. 1E). A CCK-8 proliferation assay showed that HOST2 depletion significantly attenuated the cell proliferation potential of OVCAR-3 cells ( $P < 0.01$ ) (Fig. 1F). However, HOST2 had no significant effect on the rate of cell apoptosis ( $P = 0.12$ ), as measured by Annexin V-FITC/PI staining (Supplementary Material, Table S1).

Having documented that decreasing HOST2 levels significantly played a crucial role in the down-regulation of cell migration, invasion and proliferation in EOC, we next attempted to demonstrate whether decreasing HOST2 expression could inhibit EOC tumor growth *in vivo*. We administered a series of treatments when established OVCAR-3 tumors were enlarged



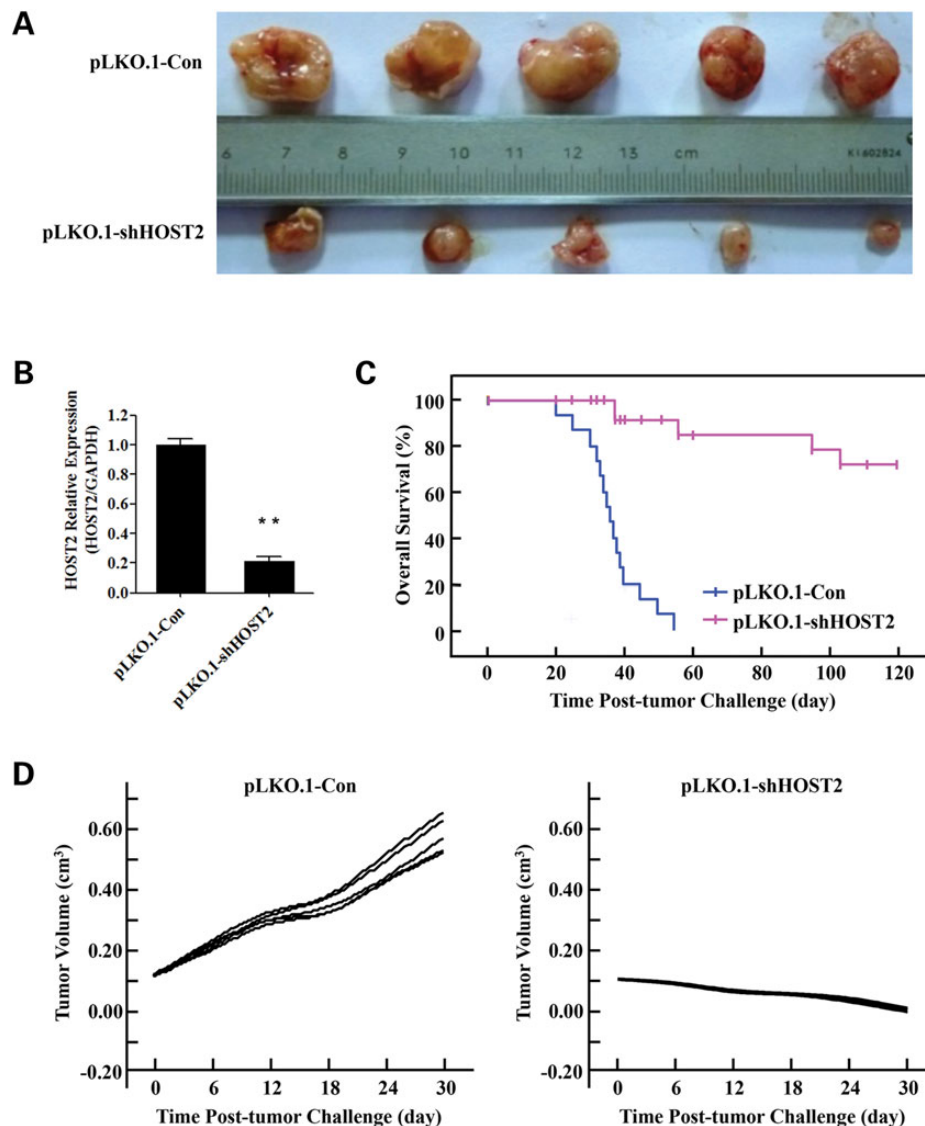
**Figure 1.** The specific, highly expressed HOST2 regulates EOC cell migration, invasion and proliferation. (A) HOST2 expression in various human cancer cell lines, including MKN28 gastric cancer, Huh-7 hepatocarcinoma, HepG2 hepatocarcinoma, McF-7 breast adenocarcinoma, DU145 prostate cancer, A357 melanoma, MG63 osteosarcoma, Hep-2 throat squamous carcinoma, ARK2 uterine serous papillary carcinoma, HeLa cervical cancer and OVCAR-3 epithelial ovarian cancer cell lines. (B) HOST2 expression in EOC ( $n = 50$ ) was detected using qRT-PCR and compared with those of OBT samples ( $n = 30$ ). (C) OVCAR-3 cells were transfected with siCon (control siRNA) or siHOST2 (siRNA specific for HOST2), and cell migration was analyzed 24 h post-transfection. Representative DAPI-stained images of migrated cells are presented. (D) OVCAR-3 cells were transfected with siCon or siHOST2, and cell invasion was analyzed 48 h post-transfection. Representative DAPI-stained images of invaded cells are presented. (E) Silencing HOST2 induced OVCAR-3 cell migration at the invasive front in a wound healing migration assay. Closure of the scratch-wounded cell monolayer was measured and analyzed 0, 24 and 48 h after wounding using photography. (F) OVCAR-3 cells were incubated in 96-well plates in serum-free DMEM medium, and cell proliferation was measured using a colorimetric assay (CCK-8, *Dojindo*).

to 5 mm in diameter by intratumorally injecting plasmids pLKO.1-shHOST2 ( $n = 5$ ) and pLKO.1-Con ( $n = 5$ ) (100  $\mu\text{g}/500$  g) twice a week for 4 weeks, as described previously ((29)). All of the mice were sacrificed, and the tumors were isolated and a patch of each tumor was used to perform pathology test to diagnose with EOC (Supplementary Material, Fig. S1B). As shown in Figure 2A, compared with those in pLKO.1-Con-treated mice, tumors in the pLKO.1-shHOST2-treated group were significantly smaller. The levels of HOST2 expression in the tumors were measured using qRT-PCR, and the results indicated that HOST2 was dramatically reduced in the tumors of mice in the pLKO.1-shHOST2-treated group (Fig. 2B). We found that pLKO.1-shHOST2 intratumoral injection ( $n = 15$ ) significantly prolonged the survival of the treated mice compared

with pLKO.1-Con-treated mice ( $n = 15$ ) (Fig. 2C). The growth curves of the tumors in the two groups indicated that all mice in the pLKO.1-Con-treated group showed progressive tumor growth, whereas the mice treated with pLKO.1-shHOST2 showed markedly repressive tumor growth (Fig. 2D). Collectively, these data showed that down-regulation of HOST2 could inhibit EOC tumor growth *in vivo*.

### LncRNA-HOST2 interacts with miRNA let-7b in EOC

One of most important regulation mechanisms of RNA–RNA is that miRNAs regulate mRNAs through semi-complementarily pairing with their untranslated regions (3'-UTR or 5'-UTR) ((30),31)). Therefore, we wanted to determine whether some

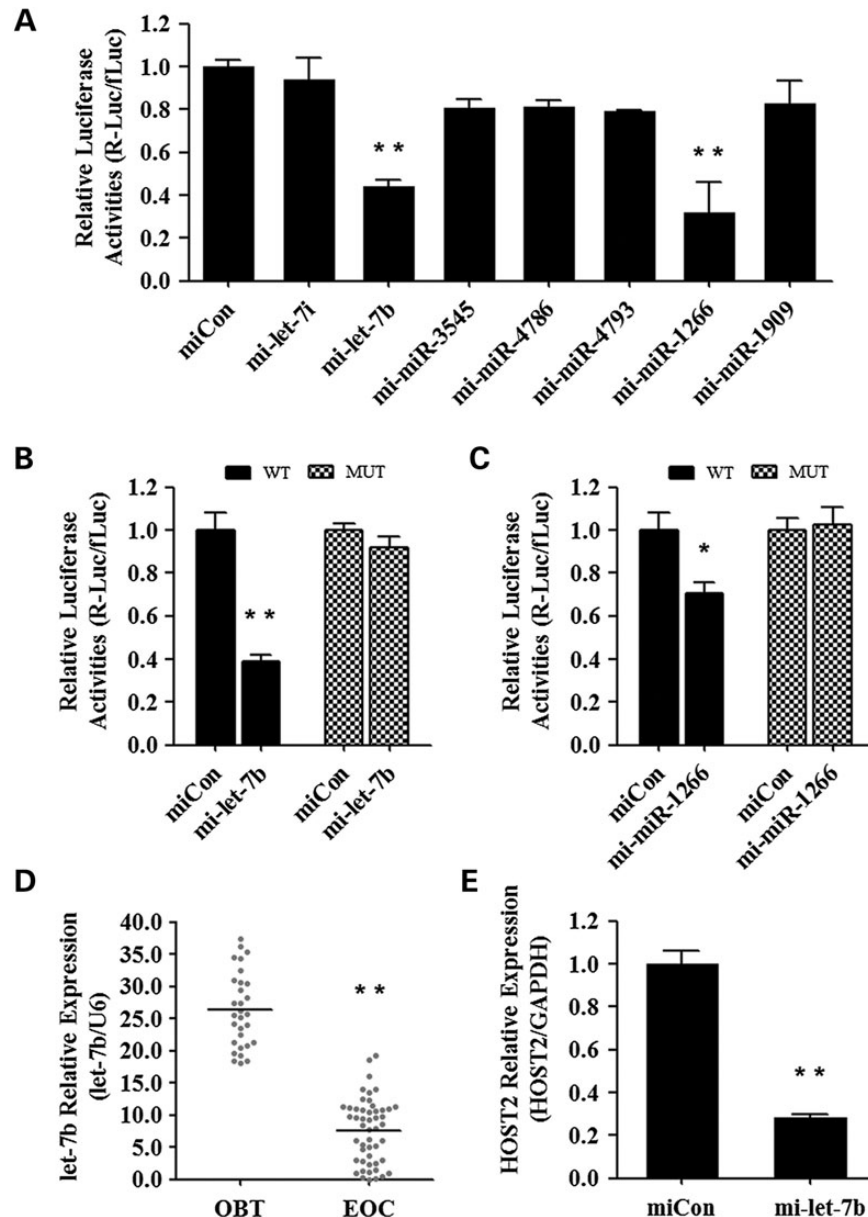


**Figure 2.** Repression of HOST2 expression significantly inhibited EOC growth *in vivo*. (A) On Day 50 post-treatment, tumors in pLKO.1-Con- or pLKO.1-shHOST2-injected mice were excised and measured using a ruler. (B) HOST2 expression in EOC xenograft tumors *in vivo* was measured using qRT-PCR. Tumor cells were, respectively, harvested from pLKO.1-Con- or pLKO.1-shHOST2-injected mice. (C) Kaplan–Meier survival plot. Overall survival curves for nude mice with pLKO.1-Con or pLKO.1-shHOST2 injections. The total experimental period was 120 days post-injection. Seventy percent of pLKO.1-shHOST2-treated mice survived until the endpoint of the study. (D) OVCAR-3 cells were inoculated and EOC tumors were established in nude mice. Tumor growth curves were plotted for different intratumoral injections with pLKO.1-Con or pLKO.1-shHOST2 at regular intervals. At the indicated times, the tumor volume was determined.



or certain miRNAs could combine with lncRNA-HOST2. The online software miRBase (<http://mirbase.org/search.shtml>, last accessed on 6 October 2014) was utilized to find the functional molecule that interacts with HOST2. Seven candidate human miRNAs were predicted by matching them to the HOST2 sequence, and those directly binding to complementary sites were chosen and are listed in Supplementary Material, Table S2. A luciferase reporter vector encoding 1500–2000 nt of the HOST2 sequence (harboring all of complementary

positions of seven miRNAs, renamed pGL3-HOST2-wt) and a Renilla luciferase (Rluc) gene in the 3' UTR and a downstream, constitutively expressed firefly luciferase gene, which served as an internal control for normalization, were constructed. These vectors were then sequentially co-transfected with mimics of the above-mentioned, predicted miRNAs and a scramble mimic (as the control, renamed miCon) into OVCAR-3 cells (Supplementary Material, Table S3). As shown in Figure 3A, among the seven candidate miRNAs, let-7b and miR-1266 effectively

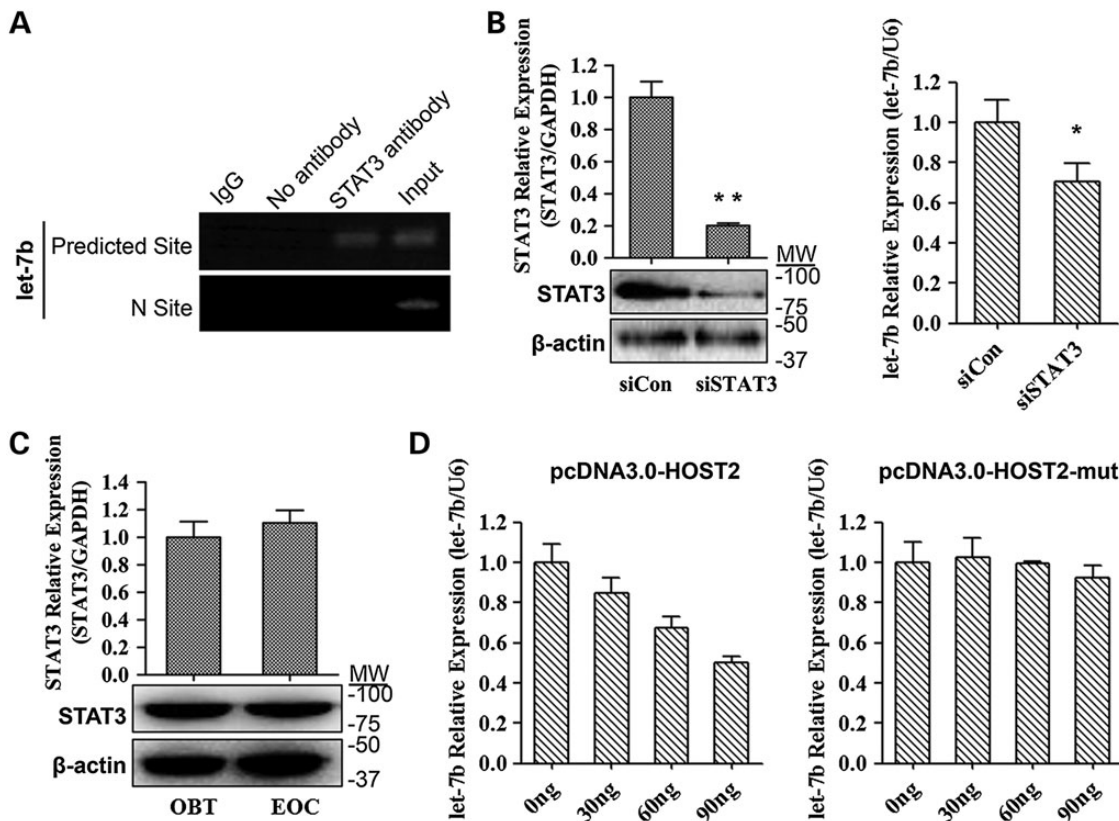


**Figure 3.** Let-7b is a predicted combining miRNA of HOST2 and is down-regulated in EOC. (A) Luciferase activity of pGL3-HOST2-wt in OVCAR-3 cells was detected after transfection with mimics of let-7i, let-7b, miR-3545, miR-4786, miR-4793, miR-1266 or miR-1909. Relative luciferase activities are presented after normalization against that of the miCon group. (B) Predicted duplex combination between pGL3-HOST2-wt (WT) or pGL3-HOST2-mut (MUT) and let-7b. Luciferase activity of WT or MUT in OVCAR-3 cells was detected after transfection with the let-7b mimic (mi-let-7b) or miCon. The deleted nucleotides in MUT are from 1710 to 1722 nt of HOST2 sequences. (C) Predicted duplex combination between pGL3-HOST2-wt (WT) or pGL3-HOST2-mut (MUT) and miR-1266. Luciferase activity of WT or MUT in OVCAR-3 cells was detected after transfection with miR-1266 mimic or miCon. (D) let-7b was detected in tissues of OBT ( $n = 30$ ) or EOC ( $n = 50$ ) by qRT-PCR. (E) OVCAR-3 cells were transfected with mi-let-7b or miCon and then assayed for HOST2 expression by qRT-PCR. Relative RNA levels are presented after normalization against that of the miCon group.

inhibited the luciferase activity of pGL3-HOST2-wt (WT), whereas little to no significant reduction in luciferase activity was observed when the cells were co-transfected with the other five predicted miRNAs. This finding indicated that the 1500–2000 nt of the HOST2 sequence contains motifs with sequence complementary to let-7b and miR-1266. To further confirm this result, the 1500–2000 nt fragment of the HOST2 sequence containing a mutated putative let-7b or miR-1266 binding sequence were also cloned into a luciferase reporter construct and were named pGL3-HOST2-mut (MUT). The data indicated that mutating the seed regions for let-7b and miR-1266 barely abrogated its luciferase activity (Fig. 3B and C). We also found that the inhibition effect of let-7b was more stable than that of miR-1266. Given that let-7 regulates target-gene expression by binding to imperfect complementary sequences in messenger RNAs (mRNAs), leading to translational repression and/or mRNA destabilization [(32)], and because we knew nothing about miR-1266, we only focused on the regulatory role of let-7b for the HOST2 transcript in the further work. Meanwhile, the expression of let-7b was detected in the 30 OBT and 50 EOC tissue samples and was shown to be lower in EOC than in OBT ( $P < 0.01$ ) (Fig. 3D). After transfecting the let-7b mimic into OVCAR-3 cells, HOST2 expression was observed to be markedly down-regulated at the mRNA level when compared

with that in cells transfected with miCon (Fig. 3E). These results indicated that let-7b repressed HOST2 expression in EOC, but the reason for let-7b expression being lower in EOC than in OBT was uncertain.

We next set out to investigate how the transcription of let-7b was controlled and whether the level of transcription influenced the expression of let-7b under our experimental settings. To this end, we searched the MAPPER database (A web-based system designed and implemented by Harvard Medical School. It is a platform for the computational identification of transcription factor (TFs) binding sites in multiple genomes) to identify candidate transcriptional factors targeting let-7b. During the subsequent computational screen (<http://microrna.sanger.ac.uk>, last accessed on 6 October 2014) using nucleotide sequences that included 1 kb upstream of the let-7b gene, a STAT3 binding site which has been reported to regulate diverse biological responses involved in neoplasia [(33)], was found within the let-7b gene transcriptional element. To further determine whether STAT3 helps to transcribe miRNA let-7b, we amplified the sequence from using OVCAR3 cells using PCR, performed chromatin immunoprecipitation (ChIP) assays and confirmed the binding of STAT3 in the chromatin immunoprecipitates rather than in the negative location (N site) (Fig. 4A). Subsequently, we transfected either a designed-siRNA against STAT3 (siSTAT3)



**Figure 4.** Let-7b expression might be regulated by sponge lncRNA-HOST2 rather than its transcription factor. (A) Schematic of the indicated binding site of the STAT3 transcription factor, including the complementary precursor sequence of let-7b. ChIP assay presenting the amount of let-7b in the predicted site in OVCAR-3 cell extracts. (B) OVCAR-3 cells were transfected with siCon or siSTAT3, and the STAT3 mRNA and protein were analyzed using qRT-PCR and western blotting, respectively. The indicated protein levels relative to those of the siCon group (arbitrarily set as 100%) after normalization against  $\beta$ -actin loading controls are marked. (C) OVCAR-3 cells were transfected with siCon or siSTAT3. Let-7b was analyzed by qRT-PCR. (D) The let-7b mimic was transfected into HEK 293 cells with 0, 30, 60 or 90 ng of sponge plasmid (pcDNA3.0-HOST2 or pcDNA3.0-HOST2-mut).

{(34)} or siCon into OVCAR-3 cells, and qRT-PCR and western blotting of the off-target effects confirmed the inhibition of the STAT3 mRNA and protein, respectively, (Fig. 4B, left panel) while the level of let-7b was shown to be reduced by ~80% using qRT-PCR (Fig. 4B, right panel). However, to our surprise, no aberrant differences in the expression of STAT3 at the mRNA or protein levels across two cohorts (OBT and EOC tissue samples) were observed by qRT-PCR and western blotting, respectively, (Fig. 4C). The reason for the apparently lower expression of let-7b in EOC was unknown; however, given that lncRNAs can act as sponges to bind specific miRNAs and regulate their function, we suspected that HOST2 might bind let-7b and interfere with its expression and even its functions. Therefore, we transfected pcDNA3.0-HOST2, expressing full-length human HOST2, into human embryonic kidney 293 (HEK293) cells, which do not express endogenous HOST2 but do express appreciable levels of let-7b {(35)}. With increasing amounts of pcDNA3.0-HOST2, the expression of let-7b decreased in a dose-dependent manner (Fig. 4D, left panel), suggesting that ectopically expressed HOST2 specifically sequestered endogenous let-7b. To confirm this effect, a mutant plasmid (pcDNA3.0-HOST2-mut) in which the predicted let-7b interaction site was mutated was also tested. Remarkably, pcDNA3.0-HOST2-mut no longer elicited the above-mentioned effect (Fig. 4D, right panel). In short, above results, to some degree, point to the role of HOST2 as a molecular sponge for let-7b.

### HOST2 enhances the endogenous expression of metastasis-promoting genes that are targeted by let-7b

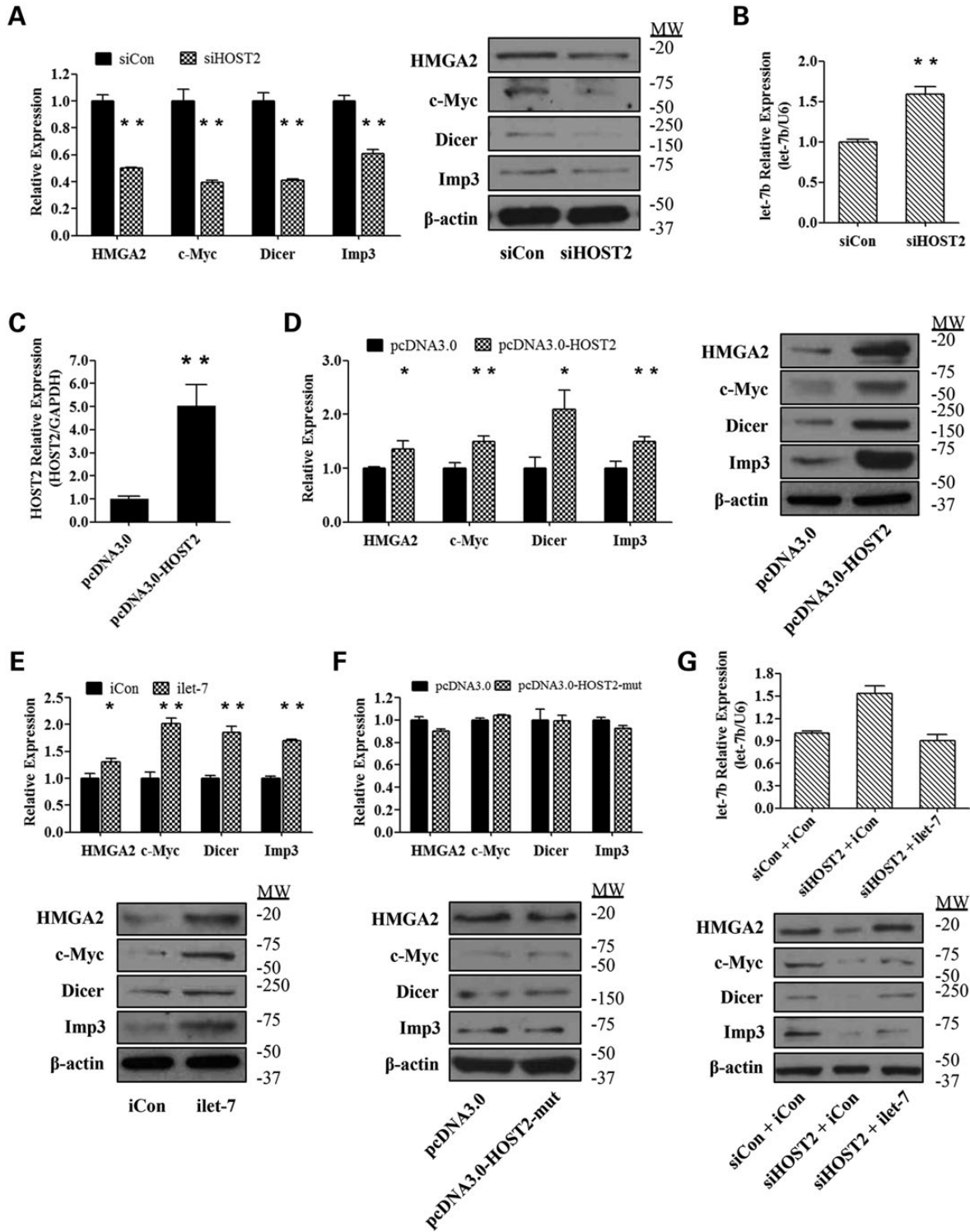
Based on our speculation that HOST2 inhibits let-7b by acting as a molecular sponge and transfection of siHOST2 into OVCAR-3 cells contributes to the up-regulation of let-7b expression, we wished to determine whether HOST2 sequesters let-7b and then the HOST2-dependent inhibition of let-7b affects the endogenous levels of four known let-7b target genes, HMGA2, c-Myc, Dicer and Imp3, which might be related to the observed effects in EOC. When HOST2 was down-regulated, the expression of HMGA2, c-Myc, Dicer and Imp3 were also significantly reduced at the RNA and protein level (Fig. 5A), which was consistent with an increase in let-7b expression (Fig. 5B). Concomitantly, cell migration (Fig. 1C and E), invasion (Fig. 1D) and proliferation (Fig. 1F) were all inhibited, similar to what we have shown. Thus, the expression of the four targets were analyzed using reciprocal, HOST2 over-expressed experiments, where pcDNA3.0-HOST2 and a control empty vector (pcDNA3.0) were, respectively, transfected into HEK293 cells to increase the endogenous HOST2 level. As shown in Figure 5C, cells transfected with pcDNA3.0-HOST2 expressed HOST2 5-fold higher than cells transfection with pcDNA3.0. Significant increases of HMGA2, c-Myc, Dicer and Imp3 at the mRNA and protein levels in response to HOST2 over-expression were all observed (Fig. 5D). Together, these HOST2 loss- and gain-of-function studies suggest that ectopically expressed HOST2 sequesters endogenous let-7b and inhibits its functions, leading to derepression of the target genes of let-7b. To confirm that these effects were indeed due to down-regulated let-7b, we performed transfection experiments using *ilet-7*. As expected, increased mRNA as well as protein levels of HMGA2, c-Myc,

Dicer and Imp3 were all observed in *ilet-7*-treated HEK293 cells when compared with iCon-treated cells (Fig. 5E). Furthermore, when pcDNA3.0-HOST2-mut was used, increased HMGA2, c-Myc, Dicer and Imp3 mRNA and protein levels were not observed (Fig. 5F). Taken together, these results strongly suggest that up-regulation of the endogenous let-7b targets HMGA2, c-Myc, Dicer and Imp3 was due to a direct sponge effect of HOST2. To provide further evidence supporting the HOST2/let-7b axis in regulating tumor cell functions, we performed HOST2 knockdown experiments in combination with the let-7-specific inhibitor *ilet-7*. Given that inhibiting endogenous HOST2 promotes let-7b expression, we wanted to determine whether the effects of HOST2 down-regulation would be attenuated by *ilet-7*. We transfected siCon with iCon, siHOST2 together with iCon or siHOST2 together with *ilet7* into OVCAR-3 cells, in which endogenous HOST2 typically rises. The level of endogenous let-7b was positively affected by HOST2 down-regulation, but fell back in the presence of *ilet7* (Fig. 5G, upper panel). Obvious down-regulation of HMGA2, c-Myc, Dicer and Imp3 were shown after transfection with siHOST2 and iCon, while co-transfection with siHOST2 and *ilet-7* apparently relieved this inhibition (Fig. 5G, lower panel). Finally, functional rescue by *ilet-7* was further supported by cell migration and invasion analyses (Supplementary Material, Fig. S2). All in all, these results strongly point to the notion that HOST2 knockdown releases more let-7b, leading to heightened let-7b function, and the HOST2/let-7b axis contributes to regulating tumor cell growth and biological functions.

### DISCUSSION

In the present study, we found that a significant network mediated by HOST2 contributes much to EOC carcinogenesis. In this complex network, let-7b is transcribed using the promoter STAT3, which is well known to play a significant role in tumorigenesis {(33)}. However, STAT3's involvement in the network was not different between EOC and OBT tissue samples, while let-7b was expressed at a lower level in EOC. Based on our findings, we put forward a model that states that let-7b is negatively regulated by its sponge lncRNA, resulting in highly expressed HOST2 in EOC. Inhibited let-7b action thereby contributes to increased expression of its targets, which includes many oncogenes. Although we still cannot present the predominant mechanism of HOST2 up-regulation in EOC, our findings suggested that the level of HOST2 is one of most important keys to determine the balance involved in the malignant transformation of the ovary.

In this report, we confirmed that the HOST2 lncRNA is extremely highly expressed in EOC tissues and cell line, which is consistent with data of a previous publication {(13)}. Our results suggested that HOST2 likely plays a role in controlling EOC growth *in vitro* and *in vivo*. More importantly, a reduction in the levels of HOST2 significantly retarded tumor growth in our *in vivo* transplantation experiment. Our findings may provide an attractive therapeutic approach by deleting this potential biomarker, which will achieve better clinical outcomes for EOC patients. Recently, a new class of endogenously expressed lncRNAs, called circular RNAs, has been demonstrated to act as potent miRNA sponges {(36)}. In our study,



**Figure 5.** The HOST2/let-7b axis operates for many tumor metastasis-promoting genes. (A) OVCAR-3 cells were transfected with siCon or siHOST2. HMGA2, c-Myc, Dicer and Imp3 mRNA and proteins levels were then analyzed by qRT-PCR and western blotting, respectively. (B) qRT-PCR was used to detect the let-7b expression levels in OVCAR-3 cells transfected with siCon or siHOST2. (C) HEK293 cells were transfected with empty vector (pcDNA3.0) or HOST2-expressing plasmid (pcDNA3.0-HOST2). qRT-PCR was used to detect the expression of HOST2 in these groups. (D) HEK293 cells were transfected with pcDNA3.0 or pcDNA3.0-HOST2, followed by qRT-PCR and western blotting to detect the expression of HMGA2, c-Myc, Dicer and Imp3. (E) OVCAR-3 cells were transfected with iCon or ilet-7. The mRNA and proteins of HMGA2, c-Myc, Dicer and Imp3 were then analyzed using qRT-PCR and western blotting, respectively. (F) HEK293 cells were transfected with pcDNA3.0 or pcDNA3.0-HOST2-mut, followed by qRT-PCR and western blotting to detect the expression of HMGA2, c-Myc, Dicer and Imp3. (G) OVCAR-3 cells were transfected with the control mixture siCon + iCon, siHOST2 + iCon or siHOST2 + ilet-7. qRT-PCR was performed to detect let-7b expression; and western blotting was used to measure the proteins levels of HMGA2, c-Myc, Dicer and Imp3 which were normalized against  $\beta$ -actin loading controls.



we displayed, for the first time, that the abundantly expressed lncRNA-HOST2, which acts as a molecular sponge, binds to let-7b and inhibits its functions in EOC. More importantly, our findings contributed to the expansion of the repertoire of endogenous miRNA sponges. Given the abundant expression pattern of HOST2 in EOC, we postulated that HOST2/let-7b regulation might contribute to EOC growth and development. Therefore, transcriptional and/or post-transcriptional regulation of let-7b target genes by the HOST2 lncRNA contributing to EOC growth remains possible. It is therefore tempting to speculate that the down-regulation of HOST2 may heighten let-7b activity, which, in turn, would lead to increased expression of HMGA2, c-Myc, Dicer and Imp3, thereby contributing to the EOC phenotype. miRNA-mediated repression generally involves translational repression followed by RNA degradation, and we observed increased mRNA and protein levels of HMGA2, c-Myc, Dicer and Imp3, which were regulated by inhibited let-7b action.

We only identified a single let-7b-binding site in the HOST2 sequence, but it showed appreciable levels of sponge activity when using both reporter and endogenous genes as readouts under different conditions. We had demonstrated that positions 1500 and 2000 of HOST2 contain functional let-7b binding sites, but still have not proven the more accurate binding sites 1654 and 1675, which were predicted by bioinformatic analysis. Although we know the putative complementary sequences for let-7b in human HOST2 (Supplementary Material, Table S3), it is necessary to perform further bioinformatic analyses to reveal possible seedless let-7b sites that have strong base-pairing in the seed region of human HOST2 sequence and their conservation scores. Findings on non-canonical seed sites suggest many functions, because non-conserved seed sites outnumber conserved seed sites by approximately one order of magnitude [\(31\)](#), which means that canonical and non-canonical sites may function in target regulation. In addition, HOST2 contains putative binding sites for additional miRNAs (Supplementary Material, Table S2), suggesting that HOST2 may also regulate other miRNAs, perhaps in tissue, cell and developmental stage-dependent manners.

It should be noted that this is the first time that it is shown that, besides negatively regulating mRNA, miRNAs could also repress lncRNA expression through semi-complementarily pairing with its sequence in EOC. The Central Dogma of Molecular Biology manifests that genetic information is transmitted by way of 'DNA → RNA → Protein' [\(37\)](#). With the development of epigenetics, the classical Central Dogma of Genomics has been now enriched. As summarized in Supplementary Material, Figure S3, the involvement of non-protein-coding genes in human physiology and disease was fully illustrated. Among the ncRNAs, miRNAs are involved as post-transcriptional regulators that bind to complementary sequences of mRNAs, leading to gene silencing that affects both the stability and translation of mRNAs [\(31\)](#). It is clear that mammalian genomes encode numerous lncRNAs, and alternative expression of lncRNAs has been previously observed in several cancers [\(8\)](#). lncRNAs can mediate epigenetic changes by recruiting chromatin-remodeling complexes to specific genomic loci, some lncRNAs have been shown to regulate transcription, whereas a few lncRNAs are anti-sense transcripts, that may regulate mRNA dynamics at a post-transcriptional level [\(38\)](#). Our study identified a potential

mechanism by which let-7b epigenetically modulates the tissue-specific lncRNA-HOST2 in EOC through the same manner. Our work highlights the inter-relationship between two large classes of ncRNAs and epigenetic regulation of gene expression, which supplements and enriches The Central Dogma of Genomics.

It is noteworthy that we chose the let-7b target genes HMGA2, c-Myc, Dicer and Imp3 for the following reasons. All of these genes contain functional let-7b binding sites and are validated targets of let-7b [\(26,39–41\)](#). Among these, HMGA2, c-Myc, and Imp3 have been shown to promote tumor cell migration and invasion. The HMGA2 protein regulates pro-metastatic genes through epigenetic modification to promote cancer cell metastasis [\(28\)](#). c-Myc drives tumorigenesis and metastasis by directly stimulating transcription of the Lin28 gene [\(42\)](#), which blocks processing of let-7 precursors to mature forms [\(43\)](#). Imp3 is often expressed in many aggressive cancer types, including those of the female reproductive tissues, and enhances the motility and invasiveness of tumor cells by post-transcriptionally regulating the expression of pro-migration/invasion genes [\(44\)](#). In summary, HMGA2, c-Myc and Imp3 have been demonstrated to promote metastasis via regulating various downstream effector genes. In the case of Dicer, a microRNA (miRNA) biogenesis factor, its silencing has been demonstrated to alter cell proliferation, migration and invasion in tongue squamous cell carcinoma [\(45\)](#) and melanoma [\(46\)](#).

In conclusion, our study demonstrated, for the first time, that the EOC-specific lncRNA-HOST2, which acts as a miRNA sponge, sequesters let-7b to maintain the expression of oncogenes and further maintains EOC biological functions. Moreover, it is worth noting that our contribution to the field appears to be the first to report that miRNA regulates a lncRNA in EOC, and our findings might open up new research avenues for EOC diagnostics and treatment.

## MATERIALS AND METHODS

### Collection of tissue samples

Samples of EOC and OBT tissues were excised surgically from patients with informed consent in the Department of Gynecology, Changhai Hospital, Second Military Medical University (SMMU), during the period from August 2010 to January 2012. Each sample was divided into three portions and immediately frozen in liquid nitrogen after resection. All patients had never been given chemotherapy or biotherapy before, and the diagnoses were confirmed pathologically in all cases. Specimen collection and archiving of patient data were performed with written informed consent and approved by the ethical committee of the hospital.

### Cell lines and cell culture

All cell lines were obtained from ATCC (American Type Culture Collection, USA) and maintained according to the supplier's recommendation. The OVCAR3 cell line was originally established from the ascites of a patient with poorly differentiated papillary epithelial OC [\(47\)](#). OVCAR3 cells were routinely cultured in RPMI-1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine at 37°C in a humidified

incubator with an atmosphere of 5% CO<sub>2</sub> atmosphere. The medium was changed every 2 days.

### RNA isolation, reverse transcription reaction and qRT-PCR

Total RNA was extracted and purified from tissues or cell lines using the standard Trizol Reagent (Invitrogen, USA) procedure. After the quality and quantity of the extracted total RNA were confirmed by NanoDrop 1000 spectrophotometer (Thermo Scientific, USA), complementary DNA (cDNA) was synthesized using a reverse transcription kit (TaKaRa, Japan) according to the manufacturer's protocol. In brief, a master mixture was prepared on ice that contained 1 µl cDNA sample, 10 µl SYBR Green qRT-PCR Master Mix (TaKaRa, Japan) and 1 µl primers. The final volume was then adjusted to 20 µl with RNase-free water. All reactions were performed on an ABI StepOne™ Real-time PCR system (Applied Biosystems, USA) with the primers listed in Supplementary Material, Table S4 and were carried out under the following cycling conditions: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 s. After amplification, the products were subjected to a temperature gradient from 60 to 95°C at 0.2°C per second with continuous fluorescence monitoring to produce a melting curve of the products. Relative expression was calculated using the 2<sup>-ΔC<sub>t</sub></sup> method. Each PCR amplification was performed in triplicate to verify the results. The specificity of every qRT-PCR was assessed with melting a curve in a tentative test.

### Cell migration and invasion assays

These were carried out using Transwell chambers (8 µm pores) placed into a 24-well plate (Millipore, USA). Briefly, the lower chamber was filled with 600 µL RPMI 1640 containing 20% FBS. Cells were trypsinized, counted and re-suspended in serum-free RPMI. For migration assay, 3 × 10<sup>4</sup> cells in 200 µL serum-free RPMI were added to the upper chamber. The cells were allowed to migrate for 24 h at 37°C before fixing. For invasion assay, 100 µL Matrigel (BD Biosciences, USA), 1:3 diluted in serum-free RPMI, was coated onto the upper chamber and incubated at 37°C for 60 min. Cells were seeded into the upper chamber at a concentration of 5 × 10<sup>4</sup>/200 µL and incubated for 48 h at 37°C before fixing. The non-migrated cells were removed from the upper surface of the membrane by scraping with a cotton swab. Cells on the bottom surface of the membrane were fixed with 95% ethanol at RT for 30 min, gently rinsed with 1 × phosphate-buffered saline (PBS), stained with DAPI for 5 min, and photographed using a Zeiss (Melville, USA) microscope system. Migration/invasion was assessed by counting the number of stained cell nuclei from 10 random fields per filter in each group at ×100 magnification. The experiments were conducted in triplicate. Cell counts were expressed as the mean number of cells per field of view.

### Protein extraction and western blot analysis

After the cells were washed, scraped and lysed, the cell lysates were centrifuged, the supernatants were collected, and the protein concentration was measured using the bicinchoninic acid (BCA) assay and then stored at -80°C. Total soluble

proteins (80 µg) was resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene fluoride membranes. The blots were blocked with 5% skim milk, incubated with antibody specific for the targeted protein under the optimized dilution rate, incubated with goat anti-rabbit or anti-mouse secondary antibody (Cell Signaling Technologies, USA) and visualized using an enhanced chemiluminescence kit (Thermo, USA). The antibodies are listed in Supplementary Material, Table S5.

### Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed in OVCAR-3 cells using the ChIP Assay Kit (Catalog # 17-295, Millipore) according to the manufacturer's instructions. The STAT3 rabbit antibody (sc-482, Santa Cruz Biotechnology, USA) was used. qRT-PCR reactions were performed in triplicate with 1 ml of precipitated DNA. DNA recovered from samples containing an antibody was compared with no-antibody negative controls performed on aliquots from the same chromatin preparation.

### Transient transfection

Plasmids, RNAi oligos, miRNA mimics or miRNA inhibitor were mixed with Lipofectamine 2000 (Invitrogen, USA) in OPTI-MEM medium for transfection according to the recommendations of the manufacturer. Cells grown to a confluency of ~50% were transfected with the mixture. The medium was replaced with fresh growth medium without antibiotics 4–6 h after transfection. One to two days later, the cells were harvested for subsequent analysis. To verify the knockdown efficiency, mRNA and protein of the transiently transfected cells were harvested for qRT-PCR and western blot analysis as described above. Verification of knockdown was determined by normalizing the levels of the target gene to that of the control. All of the siRNAs had little complementarity with other transcripts encoded by the human genome. Sequences for the specific siRNAs and mimics are shown in Supplementary Material, Tables S6 and S3.

### Construction of expression vectors and reporter plasmids

To construct RNAi expression vectors for the human HOST2 gene, oligos encoding HOST2-siRNA2 (annealed mixture of two DNA oligomers: forward 5'-CCG GTG ACT AAA CAA GGT CTT AAT TTC TCG AGA AAT TAA GAC CTT GTT TAG TCT TTT TG-3' and reverse 5'-AAT TCA AAA AGA CTA AAC AAG GTC TTA ATT TAT CGA GAA ATT AAG ACC TTG TTT AGT CA-3') were ligated into the *Age*I-*Eco*RI site of pLKO.1 to generate plasmid pLKO.1-shHOST2. pLKO.1-Con was produced by Addgene, USA. To construct the firefly luciferase reporter vector, 1500–2000 nt of the HOST2 sequence was inserted into pGL3 at the *Hind*III site to yield plasmid pGL3-H&C sameSeq (Primers: forward 5'-GGG GTA CCC CCC TGT TCT GTT TCT CTC TGA CCA C-3' and reverse 5'-CCC AAG CTT GGG CAG ATG TCC GGA CTC CAA GT-3'). To construct an expression vector for human HOST2 (pcDNA3.0-HOST2), the HOST2 sequence was cloned using HOST2 expression primers and inserted into

the pcDNA3.0 vector. The empty plasmid (pcDNA3.0) was used as the control.

### Ovarian tumor xenograft model and *in vivo* treatment experiments

Female BALB/c-nu mice (4–6 weeks old) were purchased from the B&K Universal Group Limited (China). All of the mice were bred and maintained in a pathogen-free facility, and all of the experimentation procedures involving mice conformed to the institutional guidelines for animal care developed by the Animal Research Center at SMMU. OVCAR-3 cells in FBS-free DMEM medium (Gibco) were inoculated subcutaneously into the dorsal flanks of the nude mice, and tumor size was measured twice weekly. After the OVCAR-3 tumors grew to 5 mm in diameter, the mice were divided into two groups (respectively, injected with plasmids pLKO.1-Con and pLKO.1-shHOST2,  $n = 15$ /group, 100  $\mu$ g of plasmid /100  $\mu$ l PBS). We randomly chose five mice from each group and euthanized them to detect other molecules of concern. The remaining 15 mice in each group were injected intratumorally with the corresponding recombinant plasmids at above-mentioned dosage twice a week for 4 weeks as previously described.

### Statistical methods

Statistical analysis was performed using SPSS 13.0 statistic software (SPSS, USA). All numerical data were expressed as mean  $\pm$  SEM for multiple samples. Differences/correlations between two groups were assessed by the two-tailed Student's *t*-test. Survival was calculated with the log-rank test. *P* values at 0.05 or less were considered significant.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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