LncRNA HOXD-AS1 functions as a ceRNA to promote hepatocellular carcinoma metastasis in zebrafish xenograft models

Ying Zhang (zy13952047319@126.com)
Nanjing University School of Medicine https://orcid.org/0000-0002-5500-3036
Xiaolu Wang
Jiangsu Provincial Traditional Chinese Medicine: Jiangsu Province Academy of Traditional Chinese Medicine
Feng Qin
Jingling Hospital
Shaochang Jia
Jingling Hospital

Primary research

Keywords: HOXD-AS1, zebrafish xenograft, ceRNA, miR-130-3p, metastasis, HCC

Posted Date: February 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-232919/v1

Abstract

Background: A few studies have shown that long noncoding RNA (lncRNA) HOXD cluster antisense RNA 1 (HOXD-AS1) plays an important role in hepatocellular carcinoma (HCC) metastasis as a competing endogenous RNA (ceRNA), but there is little *in vivo* evidence. This study aims to explore the zebrafish HCC xenograft as an *in vivo* metastasis model to verify the ceRNA network of HOXD-AS1.

Methods: The quantitative reverse transcription PCR (qRT-PCR) assay was used to assess the expression level of HOXD-AS1 in HCC cell lines. Knockdown of HOXD-AS1 or miR-130a-3p was performed by transfecting small interfering RNA (siRNA) or microRNA (miRNA) inhibitor, respectively. The proliferation and invasion of HCC cells *in vitro* were analyzed by CCK-8 and transwell assays. The growth and metastasis of HCC cells *in vivo* were assessed by zebrafish xenograft models.

Results: We verified that HOXD-AS1 was overexpressed in all tested HCC cell lines than the normal hepatic cells. Silence of HOXD-AS1 suppressed cell proliferation and invasion in Hep3B and Huh7 HCC cell lines *in vitro*. In zebrafish xenograft models, knockdown of HOXD-AS1 also reduced the growth and metastasis of the two HCC cells. Moreover, downregulation of miR-130a-3p not only increased the HCC metastasis, but also rescued the metastasis which inhibited by silence of HOXD-AS1 *in vitro* and *in vivo*.

Conclusions: Our study demonstrates the metastasis role of the HOXD-AS1/miR-130a-3p ceRNA network in HCC cells *in vitro* and *in vivo*, and these findings suggest that zebrafish xenograft model could be used for ceRNA mechanism verification in tumor metastasis.

Background

Zebrafish xenograft model has been developed rapidly for human cancers in recent years [1-3]. So far, kinds of zebrafish cell line derived xenograft (zCDX) models have been established, such as colorectal cancer, melanoma, hepatocellular carcinoma, breast cancer, lung adenocarcinoma, and so on [1-6]. Moreover, a few of zebrafish patient derived xenograft (zPDX) models also can be generated successfully [1, 2, 7-9]. Compared with the mouse xenograft model, the transparent zebrafish larva xenograft can be used to observe the transplanted cancer cells at the cellular level in intact animals, which provides an *in vivo* strategy to study the tumor growth and metastasis simultaneously, even the fine tumor cell behavior [1, 10, 11]. Thus, with the help of zebrafish xenograft model, metastasis of tumor cells could be assessed in more precise way in a few days, even for personalized clinical testing of metastasis inhibition.

HCC has an incidence of approximately 800,000 new cases annually, and it is the third most common cause of cancer-related death worldwide [12]. Although kinds of innovative therapeutic strategies are used for HCC treatment, survival rate of HCC patients is still poor [13, 14]. It is mainly due to the high rates of recurrence and metastasis after surgical resection, and the metastatic HCC cells to other parts of the body are the prominent cause of cancer-related death [15]. To understand the underling mechanisms of HCC metastasis and develop the corresponding clinical treatment would reduce the rates of cancer-related death, which depends on good *in vivo* metastasis models.

LncRNAs are a class of non-coding transcripts (>200 nucleotides) and have been thought to be “noise” previously [16]. In the past few decades, increasing evidences indicate that lncRNA play an important role in diverse physiological and pathological processes [17]. In HCCs, many lncRNAs have been reported which is crucial in tumor growth, metastasis and drug resistance [18]. Several recent studies have demonstrated the ceRNA mechanism of different lncRNAs in HCC metastasis, such as HULC/miR-372/PRKACB, LINC00974/miR-642/KRT19, AGAP2-AS1/miR-16-5p/ANXA11, and so on [19-22]. Zebrafish xenograft can offer the fast and intuitive metastasis model in cancer research for lncRNA study [23], which prompts us to explore its application in verification of ceRNA network in cancer metastasis.

LncRNA HOXD-AS1 (also named HAGLR) has been first reported as a marker of neuroblastoma progression [24]. It is transcribed from the HOXD cluster on human chromosome 2q31.2 in an antisense manner, and it contains eight exons [25]. HOXD-AS1 has been proved that it is closely associated with the progression of several tumors [26]. Among these tumors, HOXD-AS1 mainly functions as a ceRNA which binding with miRNAs in different cancers, such as HCC, lung cancer, ovarian cancer, glioma, breast cancer, colorectal cancer, cholangiocarcinoma and cervical cancer [27-35]. So far, most of these studies lack *in vivo* metastasis models of HOXD-AS1, and no study offers the *in vivo* evidence of its ceRNA network. Considering this, we attempt to establish a feasible *in vivo* strategy by using zebrafish xenograft models.

In this study-, we first verified the high expression of HOXD-AS1 in different HCC cell lines. Next, we assessed the proliferation and invasion by silencing the HOXD-AS1 in Hep3B and Huh7 cell lines both in the cell culture systems and zebrafish xenograft models. Then, we inspected the metastasis role of miR-130a-3p which is competitively bound with HOXD-AS1. Further, we verified that miR-130a-3p inhibition could rescue the HCC metastasis which was decreased by HOXD-AS1 knockdown *in vitro* and *in vivo*.

Materials And Methods

Zebrafish husbandry

Adult zebrafish were maintained in a fish auto culture system (Haishen, China) at 28°C, and the light cycle was 14-hours (hrs) on and 10-hrs off. Zebrafish embryos were cultured in 10% Hank’s solution which contained 140 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$ (pH 7.2). AB wild-type and Tg(fli1a:EGFP) transgenic zebrafish were used in this study. The zebrafish handling procedures were approved by Nanjing University School of Medicine.

Cell culture

The human HCC cell lines HepG2, Hep3B, Huh7, and the normal hepatic cell line LO2 were used in this study. All cell lines were obtained from CUNMAI Biotechnologies (Shanghai, China) in 2019 and all cell lines were authenticated by STR test. And all cell lines were tested for mycoplasma contamination by PCR. LO2 cells were cultured in 1640. HepG2, Hep3B, and Huh7 cells were cultured in DMEM. Both media

were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin and then cultured in a humidified air atmosphere containing 5% carbon dioxide at 37 ℃.

RNA extraction and qRT-PCR

Total RNA was extracted from cell lines using TRIzol reagent. Total RNA was reverse transcribed to cDNA using 1st Strand cDNA Synthesis SuperMix for qPCR kit (Takara). We performed real-time PCR analyses using SYBR Green Master Mix kit (Takara) following the instructions to detect the expression levels of HOXD-AS1 in different liver cell lines. Data were analyzed based on the ΔΔCT method and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The sequences for gene-specific primers were HOXD-AS1-F: 5´-ATTCGTCTGACTTGGCTCTT-3´and HOXD-AS1-R: 5´-CCTGTTTTGACCTTTTCCTG-3´. The GAPDH sequences were GAPDH-F: 5´-GGGAGCCAAAAGGGTCAT-3´and GAPDH-R: 5´-GAGTCCTTCCACGATACCAA-3´.

Cell transfection of siRNA and miRNA inhibitor

HepG2, Hep3B and Huh7 cell lines were cultured in six-well plates, then specific siRNAs or miRNA inhibitor were transfected after 24 hrs using Lipofectamine 3000 reagents (Thermo Fisher Scientific, USA). We purchased two HOXD-AS1 siRNAs, miR-130a-3p inhibitor and negative control (NC) siRNA from General Biosystems (China). The HOXD-AS1 siRNA sequences [36] are as follow: si1-HOXD-AS1: 5’-GAAAGAAGGACCAAAGTAA-3’, si2-HOXD-AS1: 5’-GCACAAAGGAACAAGGAAA-3’, and the NC siRNA sequence are 5’-TTCTCCGAACGTGTCACGT-3’. The sequence of miR-130a-3p inhibitor is AUGCCCUUUUAACAUUGCACUG [28]. Cells were harvested at 24 hrs after transfection, and silencing efficiencies were analyzed by qRT-PCR.

Cell proliferation assays

Cell proliferation assays were performed using Cell Counting Kit-8 (CCK-8, DOJINDO, Japan). HOXD-AS1 and NC siRNA were transfected into Hep3B and Huh7 cell lines, then cultured in 6-well plates for 24 hrs. A total of 2×10^3 cells were placed in each well of 96-well plates. Cell proliferation was monitored by measuring the optical density (OD) at 450 nm every 24 hrs according to the manufacturer's instructions, from 0 to 96 hrs, then we analyzed data of each group.

Cell invasion assays

Hep3B and Huh7 were transfected with HOXD-AS1 and NC siRNA. After 24 hrs, transfected cells were plated in 24-well plates with 8-mm-pore size chamber inserts. 2.0×10^4 or 6.0×10^4 cells were plated into the upper chambers, which was diluted with the serum-free culture medium. Then the transwell was put into the lower chamber, which including 800 μL medium with 10% FBS. After 24 hrs, the cells migrated to the bottom surface of the membrane, then the cells were fixed with methanol, and stained with 0.1% crystal violet for 30 minutes. The images were taken under the microscope for analysis.

Zebrafish xenograft models

Before injection, cancer cells were labeled with fluorescent dye CM-DiI (Invitrogen, USA). The detailed protocol was as follows: cells were collected, then washed three times with HBSS. The cells were then labeled with CM-DiI at 37 ℃ for 5 min, following by 15 min at 4 ℃. Next, the unincorporated dye was removed by rinsing three times with HBSS and the labeled cells were prepared for injection. The labeled cancer cells were injected into the perivitelline space (PVS) of 48-hpf (hrs post fertilization) zebrafish larvae. Each zebrafish larvae were mounted in 1.2% low-melting gel, and about 300-400 cells were implanted into PVS by the micro-injector. After injection, the zebrafish larva xenografts were cultured at 34℃ until the end of experiments. At 1 day post injection (dpi), the successfully injected xenografts with similar tumor size were selected for the following experiments.

In vivo imaging and quantitative analysis

At 4 dpi, the zebrafish larvae were mounted in 1.2% low-melting gel, and the images were taken by stereotype microscope (MVX10, Olympus, Japan) or confocal microscope using a 20X water-immersion objective lens (Fluoview 3000, Olympus, Japan). The spatial resolution of the images was 1600×1200 (MVX10) or 1024×1024 pixels (Fluoview 3000). The images taken by stereotype microscope were quantitatively analyzed by ImageJ software.

Statistical analysis

Statistical analysis was performed using unpaired Student's *t*-tests. A level of $p<0.05$ was considered to be statistically significant. Results were displayed as the mean ±SEM.

Results

HOXD-AS1 was highly expressed in HCC cell lines

We first examined the expression level of HOXD-AS1 in three HCC cell lines (HepG2, Hep3B and Huh7) using qRT-PCR via comparisons with the normal human liver cell line LO2. Among these cell lines, we found HOXD-AS1 was highly expressed in all tested HCC cell lines, and the values were 4.5~65.6-fold higher than that of the LO2 cell line (Fig. 1a). To evaluate the function of HOXD-AS1 in HCC progression, we tried to silence the expression of HOXD-AS1 in the three HCC cell lines by transfecting the siRNAs [36]. At 24 hrs later, we collected the transfected cells to analyze the knockdown efficiency of HOXD-AS1 by qRT-PCR. Compared with NC, the efficiency of first siRNA (si1-HOXD-AS1) was 40.9%, 96.6%, 75.7% in HepG2, Hep3B and Huh7 cells respectively, and the second siRNA (si2-HOXD-AS1) was 3.9%, 41.0% and 45.2%, respectively (Fig. 1b-d). Based on knockdown efficiency data, we chose Hep3B and Huh7 cell lines for further studies.

Knockdown of HOXD-AS1 suppressed growth and metastasis of Hep3B cells *in vitro* and *in vivo*

We next examined the cell proliferation in Hep3B cell by CCK-8 assay and found that the viability of Hep3B was significantly decreased after transfected with si1-HOXD-AS1, but not si2-HOXD-AS1 (Fig. 2a). Base on it, we only examined the cell invasion by silencing HOXD-AS1 using si1-HOXD-AS1, and we found

that the invasion of Hep3B cell was repressed after knockdown of HOXD-AS1 compared with the control (Fig. 2b and Additional file 1: Fig. 1). To determine the roles of HOXD-AS in HCC progression *in vivo*, we transplanted the Hep3B cells with si1-HOXD-AS1 transfection into zebrafish embryos. These transplanted cells were firstly transfected with si1-HOXD-AS1for 24 hrs, and then were labeled with CM-DiI. About 400 cells were inoculated into the PVS of 2-dpf Tg(fli1a:EGFP) zebrafish larvae, which the vascular endothelial cells were labeled by EGFP. At 4 dpi, the yolk and trunk of the zebrafish larva samples were imaged by stereomicroscopy and confocal microscopy. We first quantified the area with CM-DiI positive signals in yolk which represented the cell proliferation, and found knockdown of HOXD-AS1 decreased the growth of Hep3B cells compared with the control (Fig. 2c-e and Additional file 2: Fig. 2). Then we quantified the area with CM-DiI positive signals in trunk which represented the metastasis, and we also found silence of HOXD-AS1 decreased the metastasis of Hep3B cells (Fig. 2f-h and Additional file 3: Fig. 3). These results show that HOXD-AS1 plays important role in the progression of Hep3B cells.

Knockdown of HOXD-AS1 suppressed growth and metastasis of Huh7 cells *in vitro* and *in vivo*

We did the same experiments in Huh7 cells. Different from Hep3B cells, knockdown of HOXD-AS1 only slightly decreased the proliferation of Huh7 cells at 72-hr post-transfection (Fig. 3a), but the invasion of Hep3B cell was significantly repressed (Fig. 3b and Additional file 4: Fig. 4). In zebrafish xenograft models, the silence of HOXD-AS1 dramatically decreased the growth and metastasis of Huh7 cells (Fig. 3c-h, Additional file 5: Fig. 5 and Additional file 6: Fig. 6). These results not only confirm the oncogenic role of HOXD-AS1 in HCC, but also indicate its critical function in HCC metastasis.

Knockdown of miR-130a-3p increased the metastasis of HCC cells *in vitro* and *in vivo*

There are a few studies which reported that HOXD-AS1 functioned as a ceRNA to facilitate HCC metastasis [27, 28]. To prove this role, we chose one of its competitive binding miRNA miR-130a-3p for verification, because there were lot of evidence that miR-130a-3p specifically inhibited metastasis in multiple cancer cells [28, 31, 37-40]. We firstly verified the metastasis function of miR-130a-3p in cultured Huh7 cells. After knockdown of miR-130a-3p by its inhibitor, we found that the invasion of Huh7 cells was significantly increased compared with the control (Fig. 4a and Additional file 7: Fig. 7). We also transplanted the miR-130a-3p inhibitor transfected cells in zebrafish larvae, and the CM-DiI positive cells were also dramatically increased in trunk (Fig .4b and Additional file 8: Fig. 8). These results demonstrate that miR-130a-3p plays an important role in the inhibition of HCC metastasis.

Knockdown of miR-130a-3p rescued the HCC metastasis which was decreased by silence of HOXD-AS1 *in vitro* and *in vivo*

To investigate whether miR-130a-3p involved in HOXD-AS1-mediated metastasis in HCC cells, we co-transfected HOXD-AS1 siRNA and miR-130a-3p inhibitor and the results of transwell assay showed that the miR-130a-3p inhibitor rescued the invasion which was decreased by HOXD-AS1 knockdown in Huh7 cell (Fig. 5a and Additional file 9: Fig. 9). To demonstrate it *in vivo*, we implanted Huh7 cells transfected with si1-HOXD-AS1 and miR-13a-3p inhibitor, and we found knockdown of miR-130a-3p also rescued the

metastasis which was inhibited by silence of HOXD-AS1 (Fig. 5b and Additional file 10: Fig. 10). The results indicate that HOXD-AS1 promotes HCC metastasis through competitively binding with miR-130a-3p.

Discussion

HOXD-AS1 has been revealed that it could regulate the proliferation, migration, invasion, apoptosis and cycle progression through different pathways in HCC [27, 28, 41, 42]. Among these studies, HOXD-AS1 has been proved that it can function as a ceRNA by binding with miR-19a, miR-130a-3p and miR-326 [27, 28, 41]. In this present study, we verified that HOXD-AS1 functioned as an oncogenic lncRNA in growth and metastasis in HCC cell lines *in vitro* and *in vivo*. We also showed that miR-130a-3p, which could bind with HOXD-AS1, has an inhibition role in HCC metastasis. Moreover, the rescue experiments also indicate HOXD-AS1 might regulate the HCC metastasis via miR-130a-3p. These findings firstly demonstrate that the zebrafish xenograft model is a feasible model for study the biological function of the ceRNA network in cancer.

Mouse metastasis models are widely used for the study of human cancers. It has been reported that HOXD-AS1 promotes the HCC cells metastasis by using mouse metastasis models [27, 41]. At 28 days after tail vein injecting or at 42 days after intra-spleen injecting in the nude mouse, overexpressed HOXD-AS1 in HCCLM3 cells shows increased metastasis in liver and lung [27]. Consistent with these results, our present study shows that knockdown of HOXD-AS1 decreases the metastasis of HCC cells in zebrafish xenograft models. These data suggest that zebrafish xenografts could be a reliable metastasis model for human cancers.

Moreover, zebrafish xenograft models show more advantages in tumor biology. First, we accessed the metastasis role of HOXD-AS1 by zebrafish xenograft in only 4 days after transplantation, whereas mouse metastasis xenograft models require at least 4 weeks. Second, although we mainly focused on metastasis of HCC cells in the present study, we could analyze the HCC proliferation by the same xenograft samples. Third, by combing different transgenic zebrafish lines, we also can study the microenvironment of HCC cells, such as angiogenesis by Tg(fli1a:EGFP) transgenic line. In the present study, we did not found any angiogenic differences in our experiments, but we found the growth difference between Hep3B and Huh7 cells which Huh7 cells preferred growing in a more concentrated way than Hep3B (Fig. 2c and Fig. 3c). As more and more oncologists use zebrafish as a model organism, more specific and more precise xenograft models will be developed.

Recent studies have demonstrated that some of the lncRNAs, as ceRNAs, can regulate the expression levels of miRNA targets to impact the progression of tumors by competitive binding with miRNAs [43, 44]. In our study, we verified knockdown of miR-130a-3p promoted HCC metastasis. In addition, knockdown of HOXD-AS1 decreased metastasis of HCC cells, and this downregulation could be rescued by miR-130a-3p inhibitor in zebrafish xenograft. These findings show that zebrafish xenograft could be feasible *in vivo*

model for verifying the ceRNA pathway in human cancers. In total, zebrafish xenografts could offer reliable cancer models, which will be gradually developed in more applications.

Conclusion

In conclusion, our study demonstrates that HOXD-AS1 plays an important role in HCC metastasis in zebrafish xenograft models. Additionally, silencing miR-130a-3p can rescue the HCC metastasis which is decreased by knockdown of HOXD-AS1. Our study suggests that zebrafish xenograft models are the feasible metastasis model for ceRNA research.

Abbreviations

CCK-8: Cell Counting Kit-8; ceRNA: Competing endogenous RNA; dpf: Days postfertilization; dpi: Days postinjection; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HCC: Hepatocellular carcinoma; hpf: Hours post fertilization; HOXD-AS1: HOXD cluster antisense RNA 1; lncRNA: Long non-coding RNA; miRNA: MicroRNA; NC: Negative control; PVS: Perivitelline space; qRT-PCR: Quantitative reverse transcription-PCR; siRNA: Small interfering RNA; STR: Short tandem repeat; zCDX: Zebrafish cell line derived xenograft; zPDX: Zebrafish patient derived xenograft.

Declarations

Acknowledgements

We thank Nanjing XinJia Medical Technology Co., Ltd. for helping with zebrafish xenograft.

Authors' contributions

YZ conceived and designed this study; YZ and XW performed the cell culture experiments, YZ and FQ performed the zebrafish transplant and imaging experiments; YZ analyzed all results; YZ wrote the manuscript with inputs from SJ; all authors read and approved the final manuscript.

Funding

This work was supported by the Nanjing Science and Technology Development Plan Project (No. 201715061).

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal studies were approved by the Ethics Committee and carried out in compliance with the Medicine Institutional Guidelines of Nanjing University School of Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

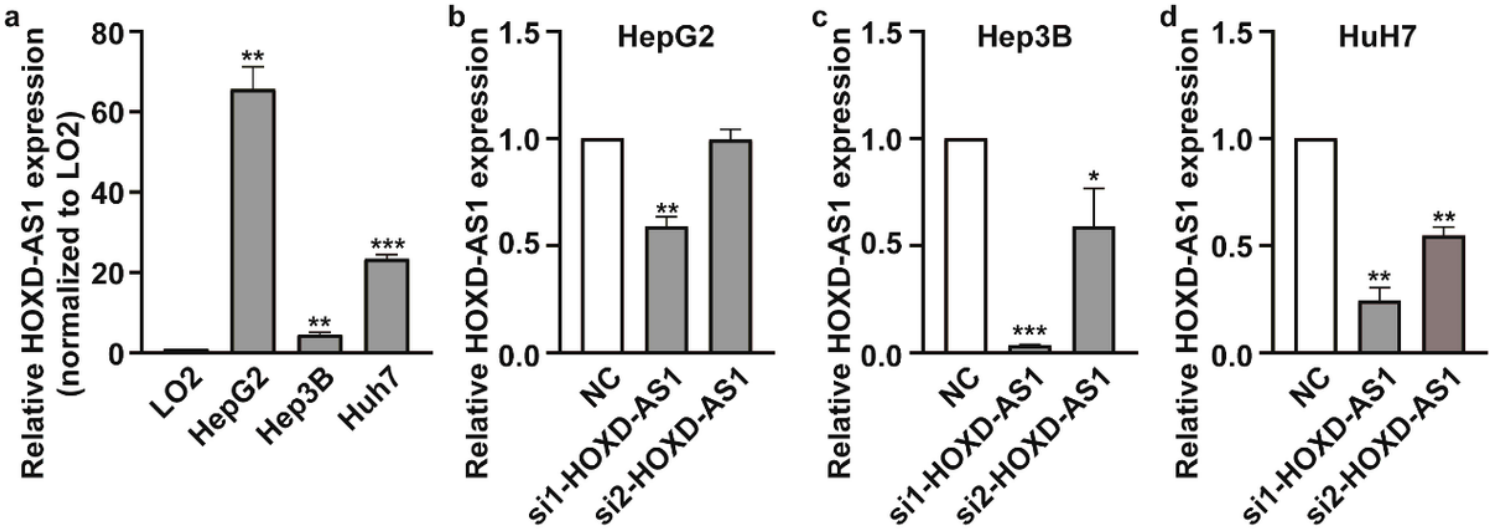

Figure 1

HOXD-AS1 is highly expressed in HCC cell lines. a HOXD-AS1 expression levels in 3 HCC cell lines (HepG2, Hep3B, Huh7) compared with normal hepatocytes cell line (LO2) were analyzed by qRT-PCR. b-d The HOXD-AS1 mRNA expression levels in HepG2 (b), Hep3B (c), Huh7 (d) cells after transfected with two HOXD-AS1 siRNAs (si1-HOXD-AS1 and si2-HOXD-AS1) were compared with NC siRNA. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

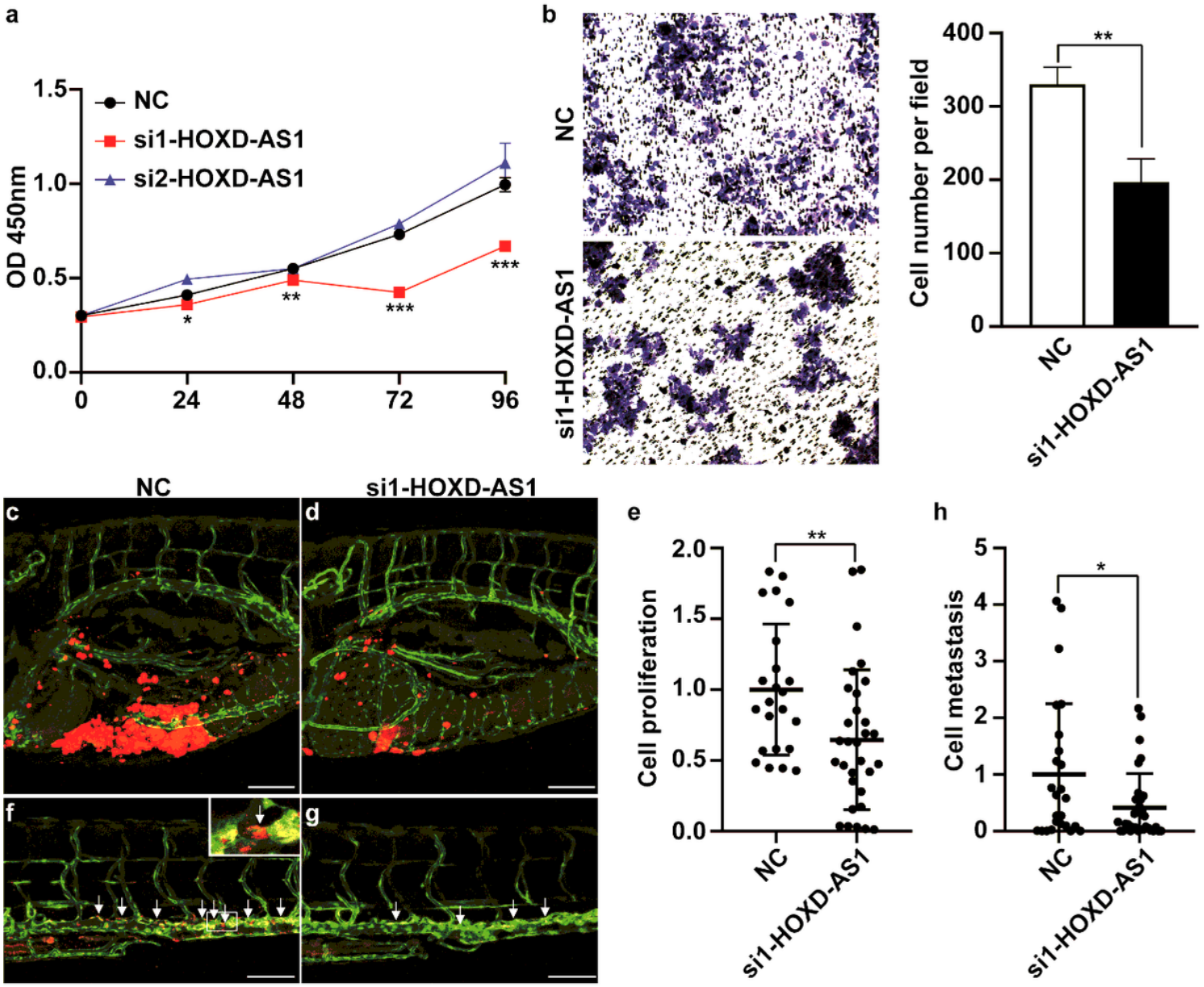

Figure 2

Knockdown of HOXD-AS1 decreases the growth and metastasis of Hep3B cells. a CCK-8 assays were performed to detect the cell proliferation of Hep3B after transfection with HOXD-AS1 or NC siRNAs. b Transwell assays were performed to detect the cell invasion of Hep3B after transfection with HOXD-AS1 or NC siRNAs. c-h Hep3B cells transfected with HOXD-AS1 or NC siRNAs were injected into the PVS of 2-dpf Tg(fli1a:EGFP) zebrafish larvae. Images of yolk were taken using a confocal microscope at 4 dpi. CM-DiI-positive areas in the yolk were quantified for proliferation (c, d). Statistical analysis of proliferation when knocking down HOXD-AS1 in Hep3B cells (e). f-g Images of trunk were taken using a confocal microscope at 4 dpi. CM-DiI-positive areas in the trunk were quantified for metastasis. The arrows represent some metastatic cells. The enlarged view of the area, which is outlined by the rectangles in f, shows the typical metastatic cell (inset in f). Statistical analysis of metastasis (h) when knocking down HOXD-AS1 in Hep3B cells was compared with NC group. Scale: 100 μm. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

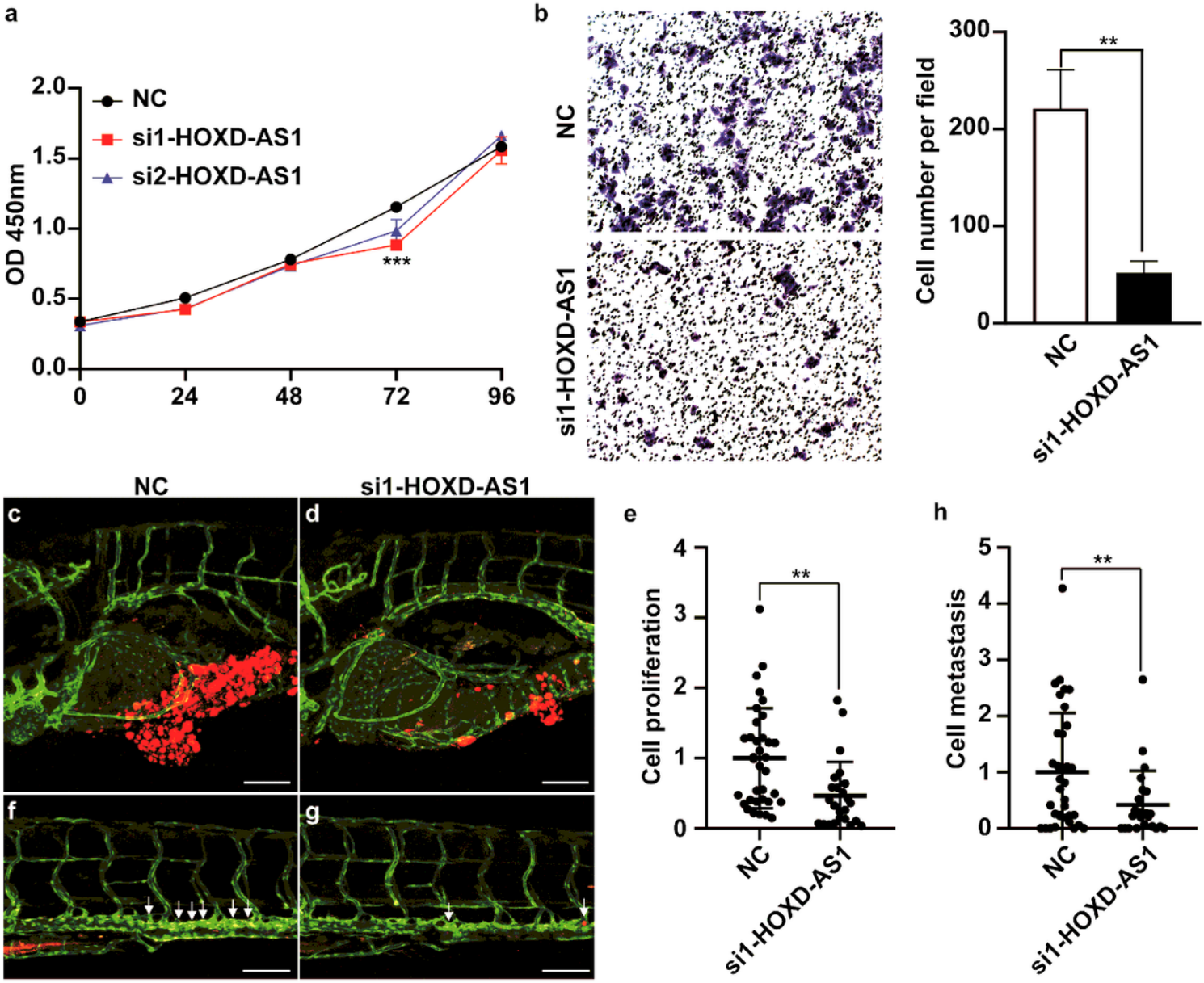

Figure 3

Knockdown of HOXD-AS1 decreases the growth and metastasis of Huh7 cells. a CCK-8 assays were performed to detect the cell proliferation of Huh7 after transfection with HOXD-AS1 or NC siRNAs. b Transwell assays were performed to detect the cell invasion of Hep3B after transfection with HOXD-AS1 or NC siRNAs. c-h Huh7 cells transfected with HOXD-AS1 or NC siRNA were injected into the PVS of 2-dpf Tg(fli1a:EGFP) zebrafish larvae. Images of yolk were quantified for proliferation (c, d). Statistical analysis of proliferation when knocking down HOXD-AS1 in Huh7 cells (e). f-g Images of trunk were quantified for metastasis. The arrows represent some metastasis cells. Statistical analysis of metastasis when knocking down HOXD-AS1 in Huh7 cells (h). Scale: 100 μm. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

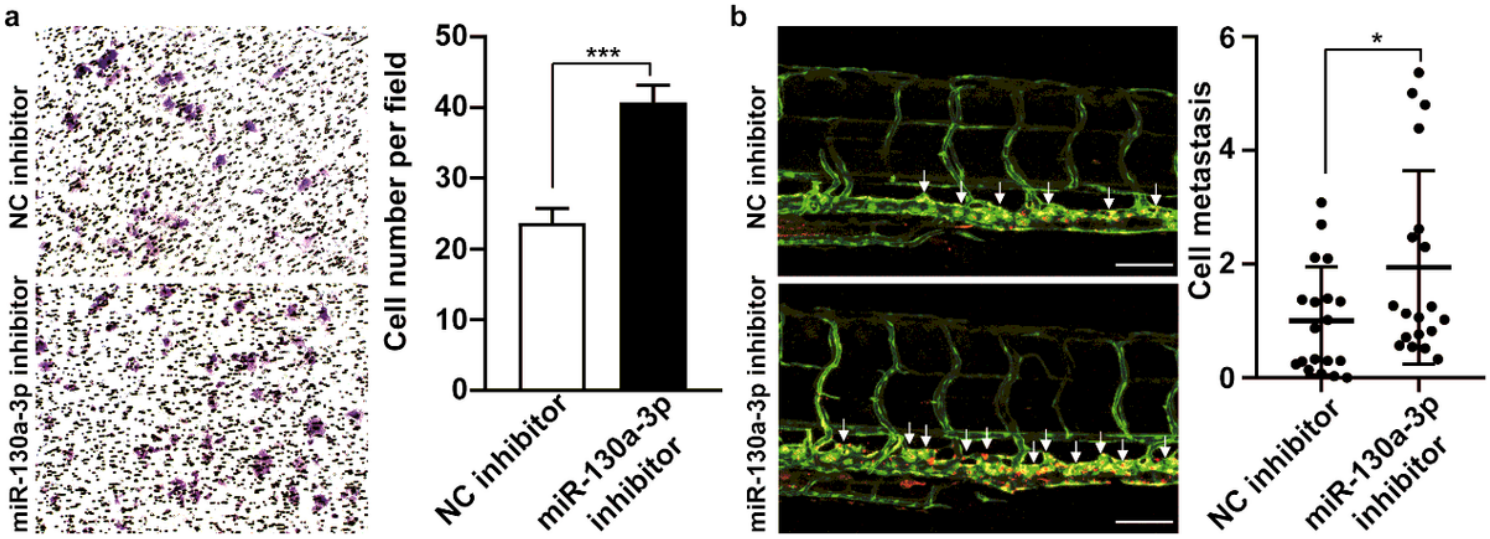

Figure 4

Knockdown of miR-130a-3p increases the metastasis of Huh7 cells. a Transwell assays were performed to detect the cell invasion of Huh7 after transfection with miR-130a-3p inhibitor or NC inhibitor. b Huh7 cells transfected with miR-130a-3p inhibitor or NC inhibitor were injected into the PVS of 2-dpf Tg(fli1a:EGFP) zebrafish larvae. Images of trunk were quantified for metastasis. The arrows represent some metastatic cells. Scale: 100 μm. *: p<0.05, **: p<0.01.

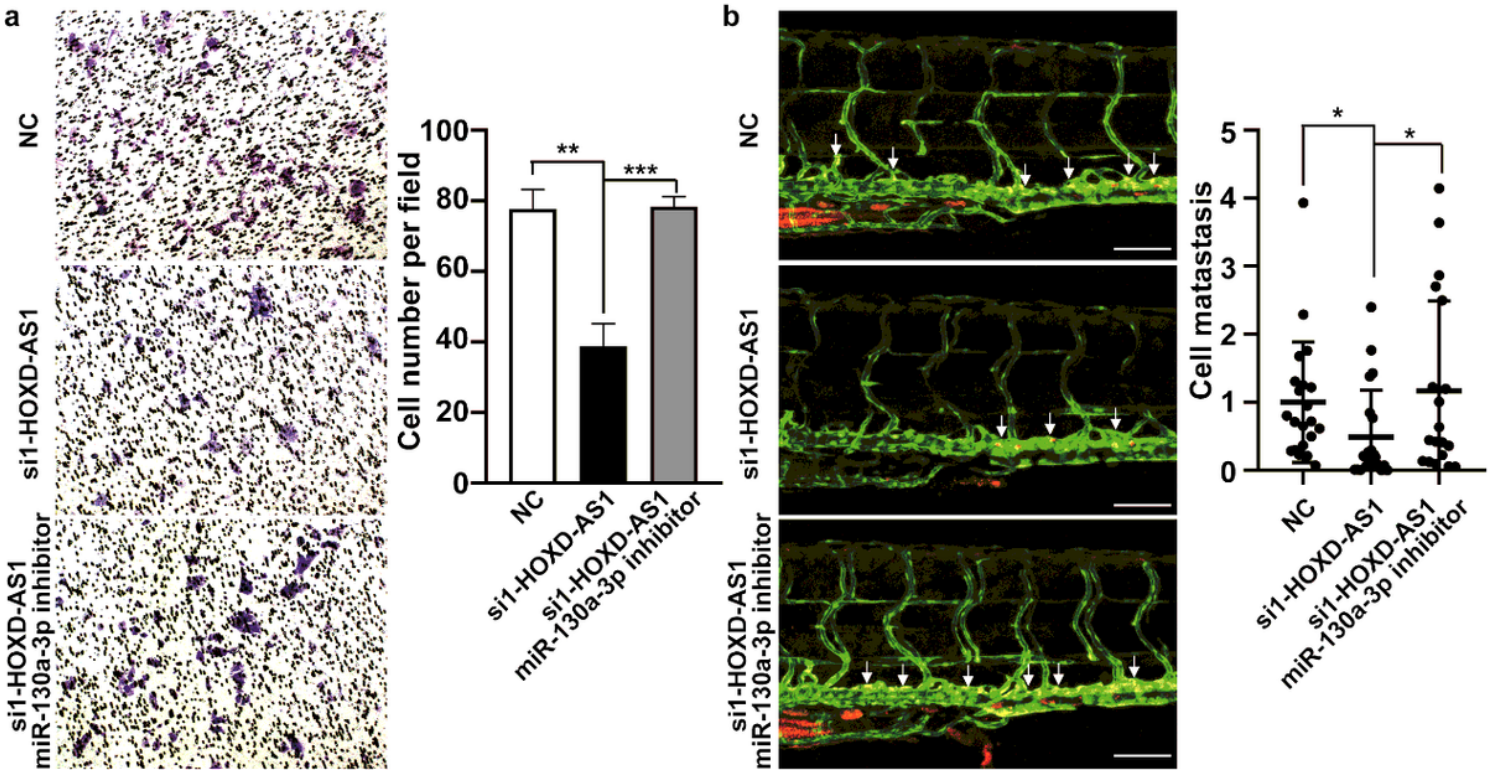

Figure 5

Knockdown of miR-130a-3p rescue the metastasis which inhibited by knockdown of HOXD-AS1 in Huh7 cells. a Transwell assays were performed to detect the cell invasion of Huh7 after transfection with si1-

HOXD-AS1, si1-HOXD-AS1plus miR-130a-3p inhibitor or NC siRNA. b Huh7 cells transfected with si1-HOXD-AS1, si1-HOXD-AS1plus miR-130a-3p inhibitor or NC siRNA were injected into the PVS of 2-dpf Tg(fli1a:EGFP) zebrafish larvae. Images of trunk were quantified for metastasis. The arrows represent some metastatic cells. Scale: 100 μm. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Supplementary Files

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