Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.054

RESEARCH ARTICLE

lncRNA 28672 Facilitates Canine Mammary Tumor Progression and is Associated with H2BC11 Expression and JAK1/STAT1 Activation

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ARTICLE HISTORY (25-988)

Received: October 16, 2025
Revised: March 01, 2026
Accepted: March 05, 2026
Published online: March 08, 2026

Key words:
Canine mammary tumor
H2BC11
JAK1/STAT1 signaling
lncRNA 28672

ABSTRACT

Canine mammary tumor (CMT) is prevalent in dogs, which severely threatens dogs' life and health due to the limitations of treatment except for surgery. Growing evidences demonstrate that long noncoding RNAs (lncRNAs) play a critical role in multiple cancer progression. Here, we focused on a novel lncRNA downregulated by doxorubicin to explore its functions and underlying mechanism. First, a significantly high expression of lncRNA 28672 was observed in clinical CMT tissues. Subsequently, functional experiments demonstrate that overexpression of lncRNA 28672 significantly promoted the growth and metastasis of CMT cells, and the growth of tumors in xenograft models. Finally, mechanistic studies reveal that lncRNA 28672 upregulates the expression of H2BC11 and activates the JAK1/STAT1 pathway. Collectively, lncRNA 28672 acts as an oncogene that drives CMT progression specifically by activating the JAK1/STAT1 pathway, which may serve as a potential diagnostic biomarker and a targeted therapeutic agent for the treatment of CMTs.

To Cite This Article: Diao H, Zhang Y, Chen S, Tao Q, Zhang Y, Liu S, Yan S and Su R, 2026. lncRNA 28672 facilitates canine mammary tumor progression and is associated with H2BC11 expression and JAK1/STAT1 activation. Pak Vet J, 46(3): 653-661. http://dx.doi.org/10.29261/pakvetj/2026.054

INTRODUCTION

Breast cancer presents as a major contributor to mortality in both women and female dogs. According to GLOBOCAN statistics in 2024, the incidence of female breast cancer is 11.6%, which is the second highest among global tumors (Bray *et al*., 2024). Notably, the incidence of CMT is much higher than that of human breast cancer. Epidemiological studies find that CMTs are prevalent in older intact female dogs, accounting for approximately 50% of all tumors, and 51.59 to 70% are malignant (Vascellari *et al*., 2016; Zheng *et al*., 2022). Due to the limitations of clinical management and the aggressiveness of malignant CMTs, the prognosis for dogs remains poor (Gedon *et al*., 2021; Carvalho *et al*., 2024). Numerous studies in genomics, transcriptomics, and metabolomics have been conducted to improve the early diagnosis rate and treatment efficacy of CMTs. Nevertheless, no reliable tumor biomarkers for early and effective detection are currently available.

Long noncoding RNAs (lncRNAs) represent an emerging class of regulatory molecules implicated in metabolic processes, tumorigenesis, and immune system modulation (Liu *et al*., 2020; Ma *et al*., 2022; Chen and Kim, 2024). To date, 20,130 human lncRNA genes have been registered in the GENCODE database, while research on lncRNAs in dogs has also gradually gained traction. Hoeppner *et al*. (2014) initially documented approximately 7200 intergenic multi-exon transcripts with no coding capacity, which are considered probable candidates for long intergenic noncoding RNAs. In 2022, 68 differentially expressed lncRNAs were identified between CMTs and adjacent non-tumor tissue (Lu *et al*., 2022). Increasing evidence demonstrated that lncRNAs play a pivotal regulatory role in CMTs: for instance, lnc34977 promotes cell growth and tumor progression in xenograft mouse models (Lu *et al*., 2022); lncRNA LOC610012 targets PTGS2 to regulate EP3 and GSK-3β expressions, thereby promoting oxidative damage in CMTs (Zhang *et al.*, 2025); and the novel lnc_025370 correlates with NRG1 expression and facilitates CMTs growth and metastasis (Diao *et al*., 2024). Notably, certain lncRNAs exhibit tissue-specific and cell-specific expression patterns during tumorigenesis (Le Béguec *et al.*, 2018; Peltier *et al*., 2022), which underscores their potential for enabling precision medicine in future clinical applications.

As a key mediator of transmembrane signal transduction in cells, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway has aroused great attention in cell proliferation, division, apoptosis, metabolism, immune regulation, and carcinogenesis (Xue *et al*., 2023; Xu *et al*., 2025). Therapeutic strategies targeting the JAK/STAT pathway include antibodies against cytokines, STAT inhibitors, and JAK inhibitors. Importantly, some agents have entered clinical trials and even obtained FDA approval for clinical use, such as Danvatirsen (investigated in multiple types of cancers), tofacitinib (indicated for rheumatoid arthritis), and oclacitinib (used for canine allergic dermatitis) (Little *et al*., 2015; Hu *et al*., 2023). Increasing evidence further indicates that dysregulation of the JAK/STAT pathway is associated with lncRNA-mediated regulation of key pathway factors. In particular, in renal cell carcinoma, lncRNA MIAT forms complexes with ETS1 and the promoters of JAK3, activating the JAK3/STAT3 pathway and thereby alleviating $CD8^+$ T cell exhaustion (Zhang *et al*., 2025). In glioma, lncRNA-PVT1 impairs sensitivity to temozolomide via the JAK/STAT pathway, promoting tumor chemoresistance (Chen *et al*., 2023). Additionally, Xiang *et al*. demonstrated that in acute myeloid leukemia, lncRNA LINC-PINT positively regulates SUZ12 by acting as a sponge for miR-767-5p, leading to reduced STAT3 phosphorylation (Xiang *et al*., 2025). However, research on lncRNAs in canine tumors remains limited, and currently, no reported studies have examined the association between lncRNAs and the JAK/STAT pathway.

Based on our previous transcriptomic analysis (Zhang *et al.*, 2024), we focused on a novel lncRNA 28762. Herein, we identify that lncRNA 28672 is markedly upregulated in canine mammary tumor tissues when compared with normal tissues. Functional experiments *in vitro* and *in vivo* demonstrated that overexpression of lncRNA 28672 accelerates tumor growth and metastasis. Furthermore, we confirm that the antitumor activity of lncRNA 28672 is associated with the high expression of H2BC11 and the activation of the JAK1/STAT1 pathway. Our findings provide evidence supporting lncRNA 28672 as a potential therapeutic target of CMTs.

MATERIALS AND METHODS

Institutional Review Board Statement: Animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee at Fujian Agriculture and Forestry University (No. PZCASFAFU23006).

Cells: Canine mammary tumor cells were generously provided by the University of Tokyo. CHMp originated inflammatory adenocarcinoma in a 12-year-old female mixed dog with T4N1+M1 stage, and CIPp originated adenocarcinoma in a 10-year-old female Shih Tzu dog with T1cN1+M1 stage. The characteristics of these two cell lines were identified by Uyama (Uyama *et al.*, 2006). The 293T cells were preserved in our laboratory.

Clinical Sample Collection: Eight pairs of CMT tissues and adjacent normal mammary tissues were obtained from our Veterinary Teaching Hospital. The tumors were surgically excised and stained with Hematoxylin and Eosin (H&E) for histopathological examination.

RNA Extraction and RT-qPCR: RNA extraction and reverse transcription into complementary DNA (cDNA) were performed according to the Kit introductions (RNA Isolation Kit: Foregene, China; cDNA Synthesis Kit: TransGen Biotech, China). Quantitative real-time polymerase chain reaction (RT-qPCR) was conducted with GAPDH as the reference gene. All reactions were performed in three biological replicates, and primer sequences are provided in Table 1.

Table 1: The prime sequences of lncRNA 28672, *H2BC11*, and *GAPDH*

Gene	Sequence	(5'→3')
lncRNA 28672	Forward	CTCCGAGTAATTGCCCTTGC
	Reverse	GCTTCACGTTGTATTGCAGGAG
H2BC11	Forward	GCAGAAGAAGGACGGCAAGA
	Reverse	ATGGTCGAGCGCTTGTTGTA
GAPDH	Forward	GTCGGAGTGAACGGATTTG
	Reverse	CTTCTGGGTGGCAGTGAT

Overexpression of lncRNA 28672 in Cells: Lentiviruses encapsulating the overexpression plasmids PNL-28672 and PNL-EGFP (NC) were constructed by transfecting 293T cells with Lipo8000 (Beyotime, China) along with pNL-VSVG and Package plasmids. Subsequently, the virus-containing supernatant was collected and transduced CHMp and CIPp (2×10^5 cells/well) for 2 hours by centrifugation (2100rpm), then cultured in 10% FBS medium. Finally, verified the stable cell overexpressing lncRNA 28672 (PNL-28672) and control cells (NC) of CIPp and CHMp.

Cell viability assay: PNL-28672 and NC cells were seeded in 96-well plates at 1×10^3 (CHMp) and 2×10^3 (CIPp) cells/well. Cell Counting Kit-8 (10μL/well) (Fuzhou Nahai Biology Technology Co., Ltd., China) was added at different time points. The optical density (OD) was measured by a microplate reader. Each experimental group included five biological replicates.

Colony formation assay: PNL-28672 and NC cells were seeded at 300 cells/well and cultured for 8 to 10 days. The colonies were then stained and quantified using Image J (version 1.53c, USA). Each experimental group included three biological replicates.

Cell migration assay: PNL-28672 and NC cells were seeded at 2×10^5 cells/well. A uniform wound was created after attachment. Then, the cells were cultured with 2% FBS DMEM. Images of the wounded areas were captured, and the migration rate was quantified using Image J software (version 1.53c, USA). Each experimental group included three biological replicates.

Invasion assay: PNL-28672 and NC cells were seeded into the upper chamber, which was pre-coated with Matrigel (BD Biosciences, USA), with 2×10^5 cells in 100μL of FBS-free DMEM. The lower chamber was filled with complete medium. After 48 hours of incubation, invading cells were stained and quantified using Image J software (version 1.53c, USA). Each experimental group included three biological replicates.

Mouse xenografts: The animal studies and research protocols were approved by the Institutional Animal Care and Use Committee at Fujian Agriculture and Forestry University under the license number PZCASFAFU23006. Ten female BALB/c nude mice (5 weeks; SPF Biotechnology Co., Ltd.) were randomized into two groups (N=5) and injected with 5×10^6 CHMp PNL-28672 and NC cells, respectively. The weight and volume of tumors were monitored for 15 days. Finally, mice were euthanized, and

the tumor masses were collected for histopathological examination and immunohistochemical analysis to investigate the expression of Ki-67 in a blind manner (anti-Ki-67 antibody, 27309-1-AP, Proteintech, China, 1:1400). The remaining tumor tissues were used for the detection of lncRNA 28672 expression.

Western blotting: Western blotting was performed by a previous study (Liu *et al*., 2024), and the primary antibodies were HIST1H2BJ (bs-17409R, Bioss, China, 1:500), JAK1 (66466-1-Ig, Proteintech, China,1:1000), Phospho-JAK1 (Tyr1034+Tyr1035) (bs-3238R, Bioss, China, 1:500), STAT1(10144-2-AP, Proteintech, China, 1:500), Phospho-STAT1 (Ser727) (28977-1-AP, Proteintech, China, 1:500), and β-Actin (HC201-01, Transgen Biotech, China, 1:8000), secondary antibodies were HRP-conjugated anti-rabbit/mouse (SA00001-2, SA00001-1, Proteintech, China, 1:2000). Membranes were visualized and immunoblotting signals were quantified using densitometry with ImageJ software (version 1.53c, USA).

Statistical Analysis: Data are expressed as the mean±standard deviation. Student's t-test and ANOVA were conducted with GraphPad Prism 8. A significance level was defined as *P<0.05, **P<0.01, and ***P<0.001.

RESULTS

The lncRNA expression profiles under the treatment of doxorubicin: In our previous study, doxorubicin inhibited the proliferation of CMT cells, and RNA sequencing (BioProject ID: PRJNA1067318) was conducted to explore the underlying mechanism (Zhang *et al*., 2024). The heat map and volcano map of partially differentially expressed lncRNAs are shown in Fig. 1A and B, respectively. Based on the expression levels and P-values of the lncRNAs, we selected lncRNA 28672 for further investigation. Subsequently, we examined the expression of lncRNA 28672 following treatment with doxorubicin (1μg/mL) and confirmed it as a target of doxorubicin. As illustrated in Fig. 1C and D, doxorubicin significantly downregulated the expression of lncRNA 28672. Therefore, lncRNA 28672 is identified as one of the target molecules of doxorubicin.

lncRNA 28672 is upregulated in the CMT tissue: Clinically, eight cases of canine mammary tumors were collected and stained with H&E staining to determine the histological stage. Among the eight cases, there was one case of simple adenocarcinoma, six cases of tubular adenocarcinoma, and one case of tubulopapillary adenocarcinoma. Representative images of the tumor tissues were shown in Fig. 2A-C, while representative images of normal mammary tissues were shown in Fig. 2D and E. Additionally, the expression levels of lncRNA 28672 in normal mammary tissues and tumor tissues were compared using RT-qPCR in Fig. 2F, suggesting that lncRNA 28672 may function as a potential oncogenic molecule in CMTs.

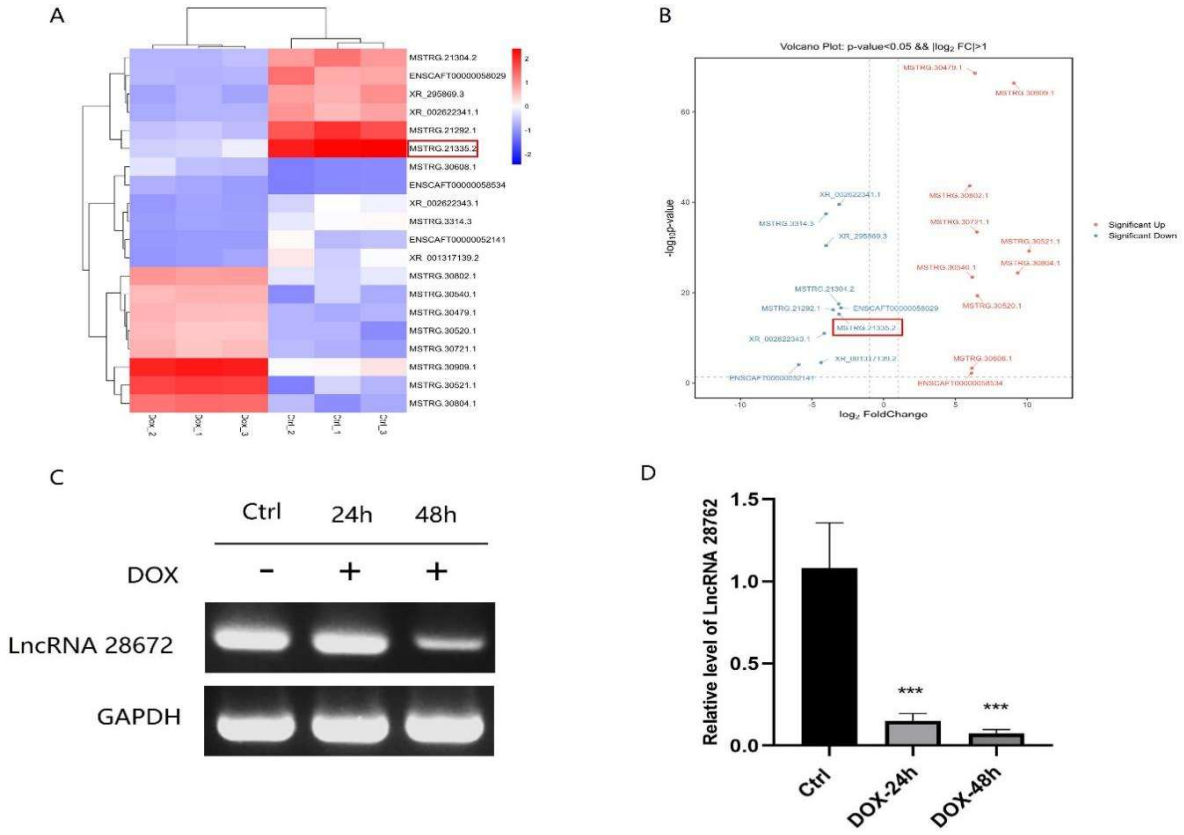

Fig. 1: RNA-Seq identifies lncRNA 28672 as a key downstream target of doxorubicin. (A) Heatmap plot of a subset of lncRNAs. Red indicates high relative expression, while blue indicates low relative expression. (B) Volcano plots of a subset of lncRNAs; (C) Expression of lncRNA 28672 by RT-PCR; (D) Relative expression of lncRNA 28672 after treatment with doxorubicin for 24 and 48 hours. The data were analyzed from three independent experiments and presented as the Mean±SD. ***P<0.001.

lncRNA 28672 promoted canine mammary tumor cells proliferation: As shown in Fig. 3A-C, RT-PCR and RT-qPCR analyses confirmed that lncRNA 28672 was successfully overexpressed in CHMp and CIPp, and the morphologies of the cells remained consistent. Fig. 3D-F revealed that the comparisons of growth in PNL-28672 cells and NC cells; the proliferation rate of PNL-28672 cells significantly increased since 48h compared to NC cells. Similarly, a higher number of colony formations was observed in PNL-28672 cells. Collectively, lncRNA 28672 enhanced the viability of CMT cells.

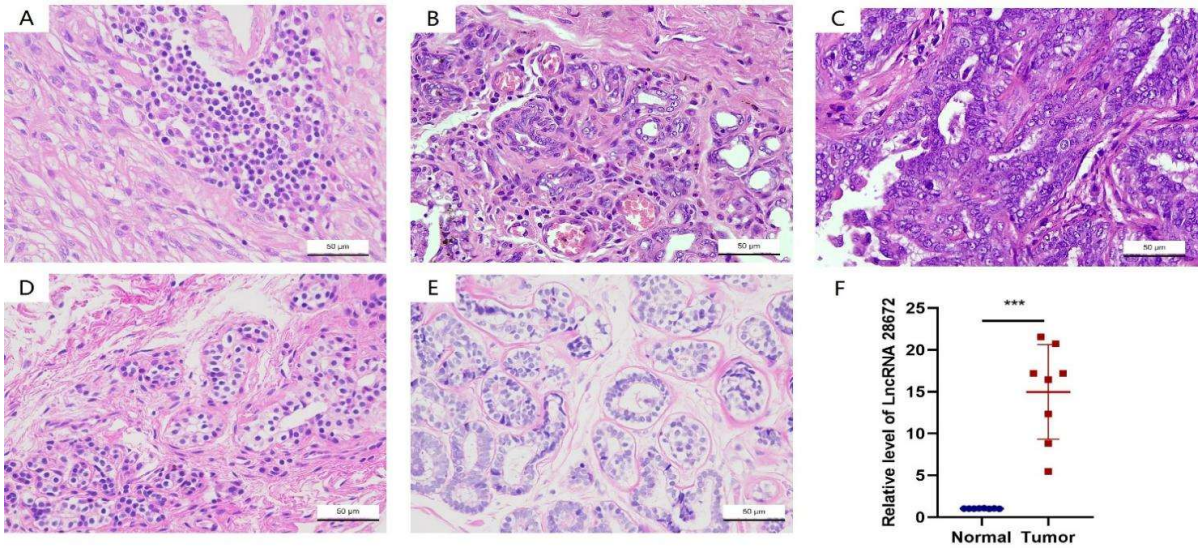

Fig. 2: Histopathological analysis of canine mammary tumors and the expression of lncRNA 28672 in these tumor tissues. (A-C) Representative images of H&E staining in canine mammary tumor tissues, showing simple adenocarcinoma, tubular adenocarcinoma, and tubulopapillary adenocarcinoma. (D) and (E) Representative images of H&E staining in normal canine mammary tissues. Magnification×40, scale bar=50μm. (F) Expression of lncRNA 28672 in canine mammary tumor tissues analyzed by RT-qPCR. ***P<0.001.

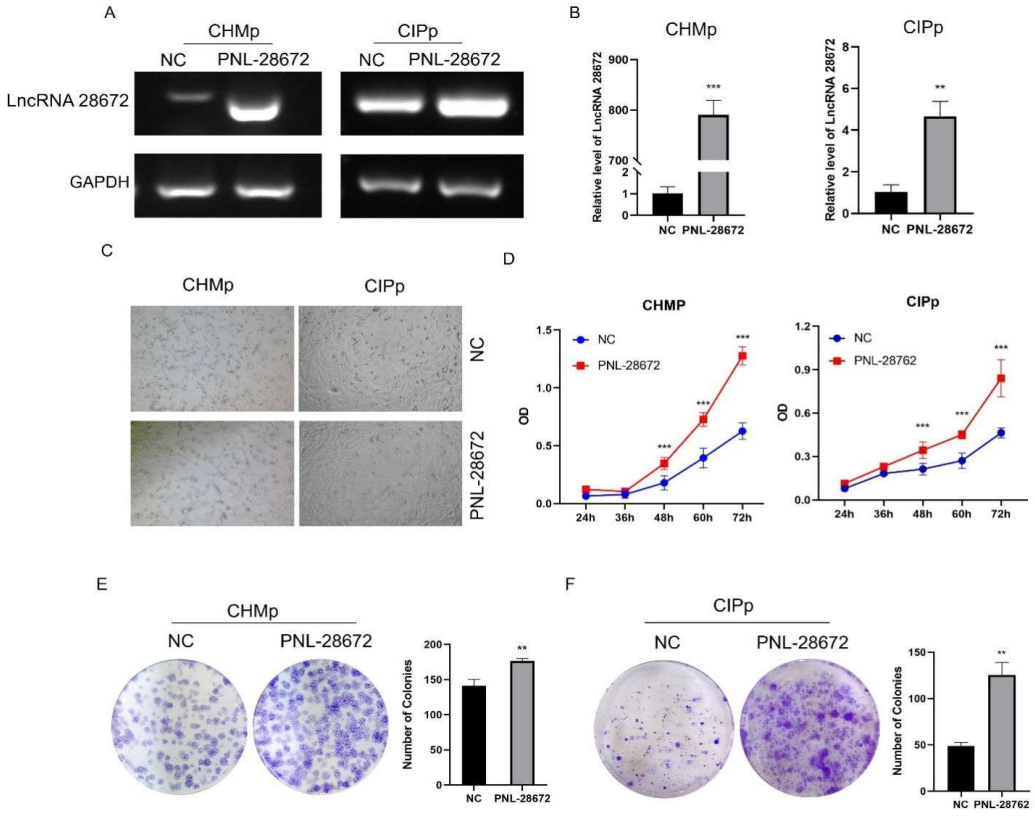

Fig. 3: Overexpression of lncRNA 28672 promoted canine mammary tumor cells proliferation. The expression of lncRNA 28672 and GAPDH was determined with RT-PCR (A) and RT-qPCR (B) in cells. (C) Morphological features of PNL-28672 and NC cells. (D) Growth curves of PNL-28672 and NC cells. (E, F) Colony formation assays of PNL-28672 and NC cells. The data were analyzed from three independent experiments and presented as the mean±SD. **P<0.01, and ***P<0.001.

lncRNA 28672 promoted canine mammary tumor cells metastasis: The abilities of motility and invasion are critical contributors to cancer metastasis. The results of the wound-healing assay in Fig. 4A revealed that the percentages of area recovery were significantly higher in PNL-28672 cells compared to NC cells. The transwell assay results in Fig. 4B showed a significantly greater number of cells invaded in the PNL-28672 cells. Collectively, lncRNA 28672 enhanced the migration and invasion ability of CMT cells.

lncRNA 28672 promoted xenograft tumor growth *in vivo*: To evaluate the effect of lncRNA 28672 *in vivo*, a subcutaneous xenograft tumor model was successfully established by PNL28672 and NC cells of CHMp in Fig. 5A. The pathology of the tumor derived from CHMp PNL28672 and NC cells are shown in Fig. 5B. These cells demonstrated extensive proliferation, with significant variability in size and morphology, marked pleomorphism, an increased nuclear-to-cytoplasmic ratio, and numerous prominent mitotic figures. Fig. 5C, D, and E showed the tumor growth in tumor-bearing mice; tumors in the PNL28672 group grew faster than those in the NC group, with significantly higher tumor size and weight. Additionally, the percentage of Ki-67, a proliferation marker, was explored in both groups. As shown in Fig. 5F and G, Ki-67 positive cells (stained in brown) were significantly higher in the PNL28672 group. lncRNA 28672 expression was significantly higher in the PNL28762 group, see Fig. 5H. Collectively, lncRNA 28672 enhances the tumorigenic potential of canine mammary tumor cells *in vivo*.

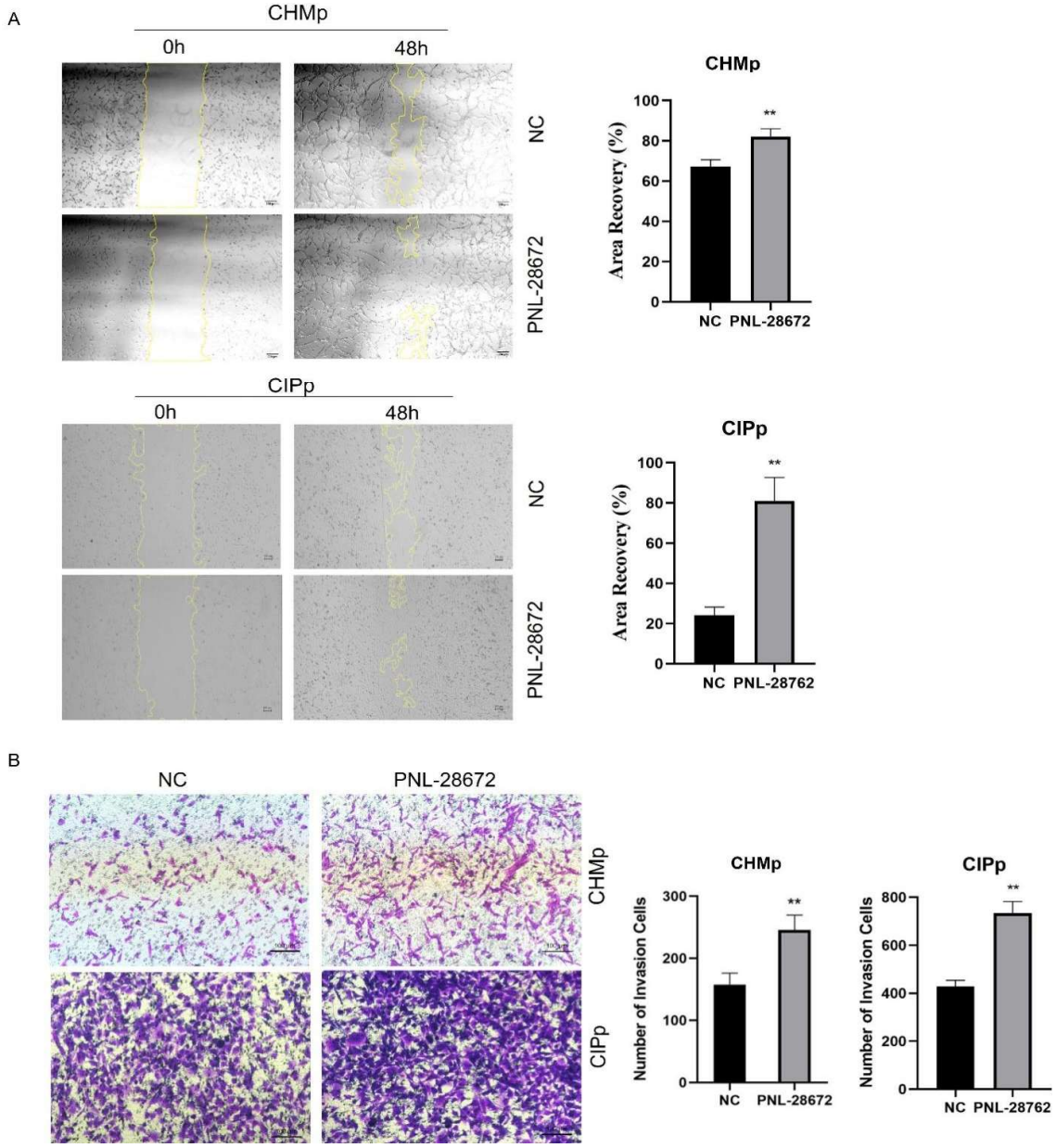

Fig. 4: Overexpression of lncRNA 28672 promoted canine mammary tumor metastasis *in vitro*. (A) Wound-healing assay. Percent area recovery of PNL-28672 and NC cells. Magnification×4, scale bar=100μm. (B) Invasion assay. Invading cells were stained with 0.1% (w/v) crystal violet. Magnification ×10, scale bar=100μm. The data were analyzed from three independent experiments and presented as the Mean±SD. **P< 0.01.

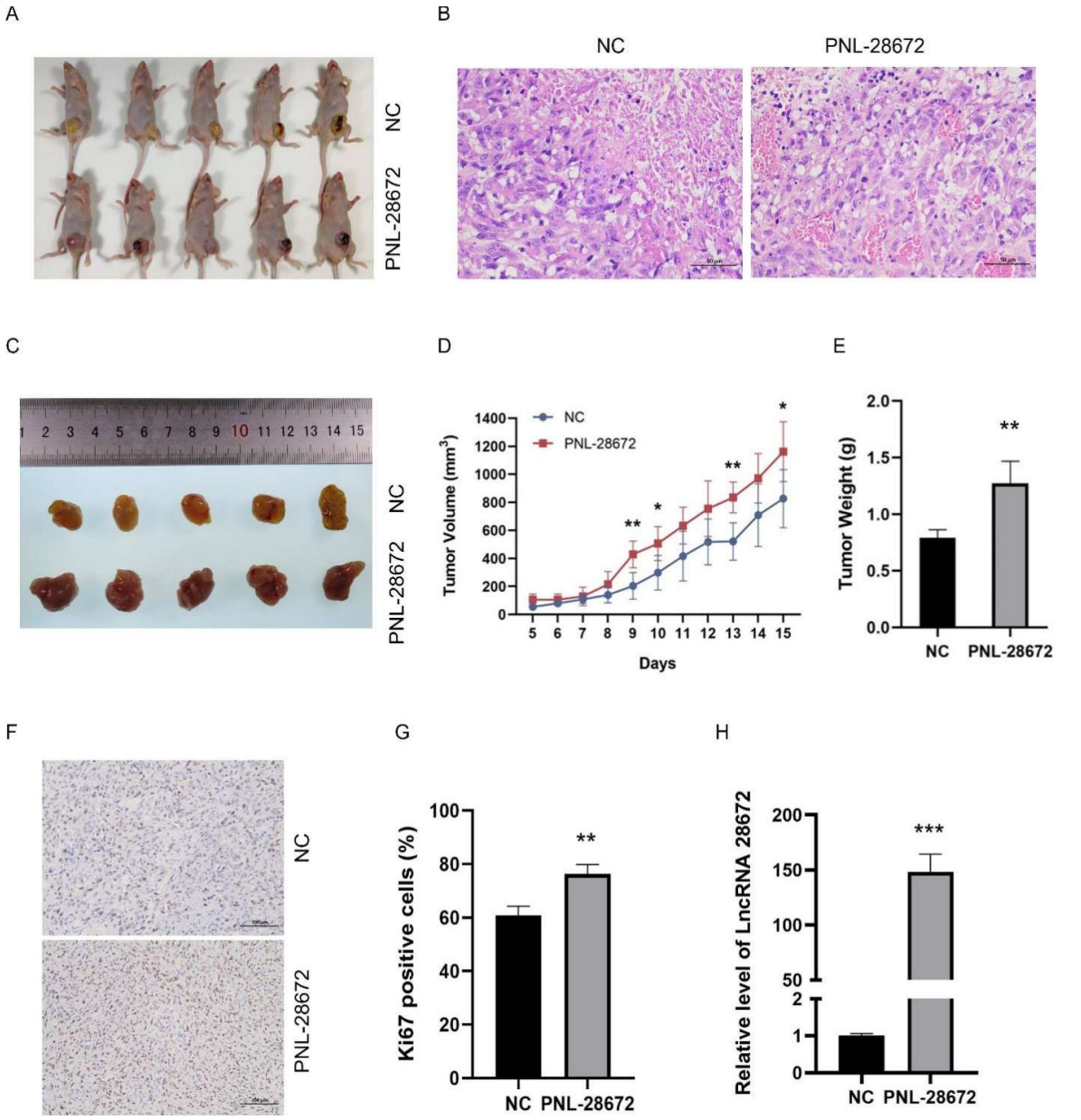

Fig. 5: lncRNA 28672 promoted xenograft tumor growth. (A) Mice bearing subcutaneously transplanted tumors; (B) Hematoxylin and eosin staining of the tumor masses (Scale bar=50μm); (C) Photographs of tumors after the experiment; (D) Tumor volumes; (E) Weight measurements of the subcutaneously transplanted tumors; (F) Representative images of immunohistochemistry (IHC) staining for Ki-67 expression (Scale bar=100μm), with a quantitative analysis of Ki-67 staining(G). (H) The expression of lncRNA 28672 in tumor tissues. The data were analyzed from three independent experiments and presented as the Mean±SD. *P<0.05, **P<0.01, ***P<0.001.

lncRNA 28672 promoted canine mammary tumor progression by regulating H2BC11 and the JAK1/STAT1 signaling pathway: A potential regulatory relationship between H2BC11 and lncRNA 28672 was identified through cis-acting target gene prediction. As shown in Fig. 6A-D, *H2BC11* mRNA levels were significantly upregulated in PNL28672 cells relative to NC cells, and the protein expression of H2BC11 exhibited a similar trend. Further mechanistic investigations were shown in Fig. 6E-F; the expression levels of p-JAK1, JAK1, p-STAT1, and STAT1 were elevated in PNL28672 cells, with significant differences in the phosphorylation levels of p-JAK1 and p-STAT1, indicating the involvement of the JAK1/STAT1 pathway. Collectively, lncRNA 28672 promotes the survival and metastasis of CMT cells, which correlates with H2BC11 and the activation of the JAK1/STAT1 path.

DISCUSSION

CMTs are a kind of tumor associated with a high incidence and malignancy in female dogs. Currently, surgery remains the primary treatment, but substantial bodily trauma and a high rate of recurrence cannot be ignored (Stratmann *et al*., 2008; Yu *et al*., 2022). Hence, early diagnosis is beneficial for both treatment and prevention of CMT progression. Additionally, dogs and

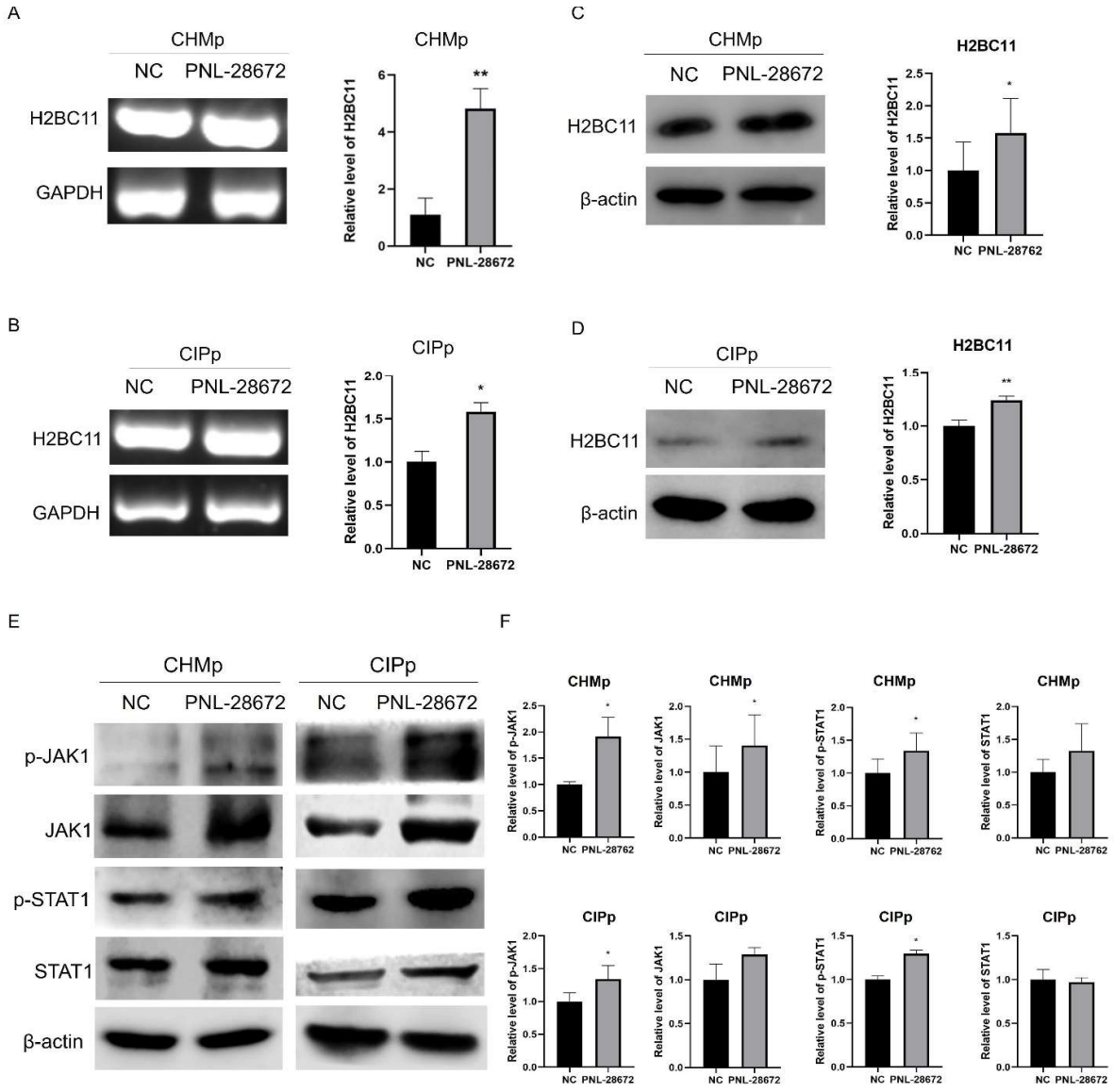

Fig. 6: lncRNA 28672 promoted canine mammary tumor progression, with a possible association with H2BC11, by regulating JAK1/STAT1 signaling. (A, B) The mRNA expression of *H2BC11* in PNL-28672 cells and NC cells. (C, D) The protein expression and densitometric quantification of H2BC11 in PNL-28672 cells and NC cells. (E) The protein expression of p-JAK1, JAK1, p-STAT1, and STAT1 in PNL-28672 cells and NC cells. (F) Densitometric quantification of p-JAK1, JAK1, p-STAT1, and STAT1 levels in E. The data were analyzed from three independent experiments and presented as the Mean±SD. *P<0.05, **P< 0.01.

humans live in the same environment, and spontaneous CMTs, as an ideal model of human breast cancer, exhibit similarities to human breast cancer in histopathology, epigenetics, subtype classification, and physiology(Gray *et al*., 2020; Kwon *et al*., 2023). Therefore, exploring the diagnostic biomarkers and therapeutic targets of CMTs is also meaningful in comparative oncology.

lncRNAs are unable to encode protein, but can function to encode small peptides or interact with other genes. For instance, Chi *et al.* demonstrate that lncRNA PCBP1 encodes a small peptide that participates in cellular immunity (Chi *et al*., 2024). Another study in CMTs finds that lncRNA-42060 interacts with miR-204-5p, thereby impacting the expression of SOX4, which in turn regulates tumor cell progression and resistance to tamoxifen (Xu *et al*., 2021). Besides, a study also finds that lncRNAs exhibit 44% tissue-specific expression in CMTs (Le Béguec *et al*., 2018), which highlights their potential value in CMT diagnosis and prognosis assessment. In this study, lncRNA 28672 is significantly overexpressed in CMT tissues compared to normal mammary tissues; functional validation reveals that overexpression of lncRNA 28672 enhances the proliferation and metastatic capacity of CMT cells. Subsequently, cis-acting target gene prediction suggests that H2BC11 (also known as HIST1H2BJ) might be a potential target. H2BC11, a member of the histone H2B family, has already been confirmed to contribute to the regulation of transcription, DNA repair, and maintain the stability of chromosomal DNA (Jia *et al*., 2022). Studies reveal that H2BC11 is significantly overexpressed in colorectal and breast cancer patients, and has negative correlates with breast cancer patient prognosis (Zhou *et al*., 2022; Shi *et al*., 2023), which highly indicates its potential in prognosis prediction for multiple types of cancer. Further

study also reports that H2BC11 is involved in regulating cellular immunity by influencing macrophages, T helper 2 cells, neutrophils, eosinophils, and immune infiltration (Jia *et al*., 2022). The above data suggest H2BC11 plays an oncogenic role in various types of cancer. Our study demonstrates that H2BC11 is significantly upregulated in CHMp PNL-28672 cells, suggesting that the tumor-promoting effect of lncRNA 28672 in CMTs is linked to the expression of the oncogene H2BC11, though the specific mechanism governing their interaction remains to be studied.

Numerous studies demonstrate that JAK1 contributes to tumor progression by phosphorylating STAT proteins. For instance, Sexl *et al.* (2003) found that transplanting Jak1-deficient, v-abl-transformed cell lines into mice resulted in higher tumorigenicity than transplanting wild-type cells. Furthermore, leukemic mice that receive the treatment of the JAK1 I inhibitor INCB18424 have smaller tumors and longer survival time than control mice (Lo *et al*., 2013). Conversely, either JAK1 mutation or overexpression contributes to tumorigenesis. STAT1, a downstream target of JAK1, has accumulated evidence that indicates it can function as a tumor contributor in recent years. Studies have demonstrated that high levels of STAT1 and pSTAT1 enhance cancer cell invasiveness and metastatic capacity, leading to poor prognosis and reduced patient survival time (Khodarev *et al*., 2009; Hix *et al*., 2013). Additionally, STAT1 overexpression induces resistance to chemotherapy and radiation by upregulating interferon-responsive genes (IRGs), which is beneficial for cancer cell survival and immune exhaustion (Meissl *et al*., 2017). Kovacic *et al.* also confirmed that STAT1 serves as a positive regulator in leukemia progression (Kovacic *et al*., 2006). In this study, overexpressing lncRNA 28672 upregulates p-JAK1, JAK1, STAT1, and p-STAT1 levels, indicating activation of the JAK1/STAT1 pathway and confirming the oncogenic role of STAT1 in CMTs. However, the precise molecular mechanism by which lncRNA 28672 modulates the JAK1/STAT1 pathway warrants further investigation. Future studies should also clarify the regulatory link between H2BC11 and this pathway.

Conclusions: This study demonstrates that lncRNA 28672, which is highly expressed in tumor tissues, significantly enhances the CMT cells' growth and progression *in vitro* and *in vivo*, and this is associated with upregulation of H2BC11 and the activation of the JAK1/STAT1 axis. As an oncogene, lncRNA 28672 drives CMT progression, representing a potential biomarker and therapeutic target for this tumor.

Author Contributions: HD conceived, supervised, and designed the study. YZ, SC, and QT executed the experiment. YZ contributed to the data collection. SY and SL provided technical support and assisted with data interpretation. RS supervised the entire project.

Funding: This research was supported by the National Natural Science Foundation of China (No. 32302946) and Fujian Provincial Natural Science Foundation of China (No. 2024J01384).

Data Availability Statement: The original data can be provided by the corresponding author.

Acknowledgments: We are grateful to the University of Tokyo for the donation of CMT cell lines.

Conflicts of Interest: No conflicts of interest.

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