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RESEARCH ARTICLE

Gambogenic Acid Inhibited Stemness and Malignant Biological Behavior of Canine Osteosarcoma Cells by Regulating KLF5 Expression

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ABSTRACT

Canine osteosarcoma (OS) stem cells are crucial factors for the formation, recurrence, and distant metastasis of osteosarcoma. Gambogenic acid (GNA), a broad-spectrum anti-tumor drug, has the advantage of low side effects. Whether GNA can intervene in the inhibition of stem cells to suppress the progression of canine OS still needs to be explored. This study targeted the stem cells regulatory factor - KLF transcription factor 5 (KLF5), to evaluate the functional impact of GNA on canine OS cells. In this study, the canine OS cell line McKinley was used to investigate the effects on proliferation, migration, and invasion, as well as stem cell transformation and stem cell characteristics after cancer stem cell sphere screening. The IC_{50} of GNA on McKinley was 0.28 μM, and it exhibited dose-dependent inhibition of proliferation, migration, and invasion. GNA significantly inhibited the stemness transformation, stemness maintenance, and EMT of canine OS stem cells, while also inducing differentiation potential. KLF5 was highly expressed in canine OS cells and tissues. GNA significantly inhibited the expression and nucleation of KLF5. KLF5 over-expression promoted McKinley proliferation, migration, and invasion, enhancing the stemness transformation and stemness maintenance of stem cells, and reversed the inhibitory effect of GNA on the malignant biological behavior and stemness factors. This study found that KLF5 is a crucial factor in GNA against canine OS. This further improves the pharmacological mechanism of GNA anti-tumor and provides new insights and experimental basis for canine OS stem cell research.

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INTRODUCTION

Osteosarcoma (OS) is a malignant tumor that originates from mesodermal connective tissue, characterized by tumor cells that produce bone or osteoid material (Gill and Gorlick, 2021). The disease predominantly affects older canines, typically between the ages of 8 and 10 years, with a predisposition observed in medium- to large-sized breeds such as Golden retrievers and German shepherds, which are often utilized as working dogs. Furthermore, OS primarily targets limb bones, seriously jeopardizing the survival quality of affected dogs. Affected animals are susceptible to pulmonary metastasis and drug-resistant recurrence, resulting in mortality rates as high as 85% (Roessner *et al*., 2021). Despite surgical resection of the primary tumor combined with adjuvant chemotherapy, achieving long-term survival exceeding one year remains challenging (Mason, 2020). The presence of canine OS stem cells (CSCs) is associated with distal metastasis, resistance to radiation and chemotherapy, and tumor recurrence, all of which contribute to a poor prognosis. CSCs exhibit robust resistance to both radiation and chemotherapeutic agents, such as doxorubicin (ADM) (Tanabe *et al*., 2016). When CSCs tend to divide symmetrically, tumours rapidly deteriorate within a short period, subsequently driving the

generation of differentiated state cells with more aggressive or resistant phenotypes (Lytle *et al*., 2018). Furthermore, CSCs promote epithelial-mesenchymal transition (EMT) to reduce tumor cell adhesion and enhance invasive motility (Radisky and LaBarge, 2008). Therefore, targeting CSC subpopulations and elucidating the mechanisms underlying stemness regulation are essential for effectively controlling the malignant behavior of OS.

KLF transcription factor 5 (KLF5) functions as a transcriptional activator within the Krüppel-like factor (KLF) family, which is associated with embryonic development, bone and cartilage formation, angiogenesis, and lung morphogenesis (Parisi and Russo, 2011). Its expression is often elevated in pathological conditions, particularly in a range of cancers. Dysregulated transcriptional activation of KLF5 typically results in hyperactivation of target genes associated with proliferation, anti-apoptosis, and stemness, which ultimately facilitates the progression of malignancies, such as OS, colorectal cancer, breast cancer, and gliomas. KLF5 proteins are predominantly expressed in diverse types of stem cells (McConnell *et al*., 2011) and are involved in the maintenance of stemness (Cartwright *et al*., 2005; Niwa *et al*., 2009). Given the significant involvement of KLF5 in CSCs, extensive research has been conducted to explore the therapeutic potential of targeting KLF5 with agents such as mifepristone, metformin, and the small molecule tyrosine kinase inhibitor DCC-2036, all of which aim to inhibit triple-negative breast cancer stem cells (Liu *et al*., 2016; Shi *et al*., 2017; Shen *et al*., 2022). Additionally, the KLF5 small molecule inhibitor ML264 has demonstrated considerable anticancer efficacy in OS cells and models, indicating that targeting KLF5 may represent a vital strategy for overcoming the bottleneck of canine OS therapy (Huang *et al*., 2020).

Gambogenic acid (GNA) exhibits significant bioactivities, including anti-inflammatory, antiviral, and antitumor properties. In comparison with its structural homologue, gambogic acid (GNA) is considered safer, demonstrates superior antitumor efficacy, and possesses a longer half-life. In addition, GNA displays a broad antitumor spectrum, effectively inhibiting the proliferation of various tumor, including colon cancer, cervical cancer, and melanoma, through mechanisms such as the induction of apoptosis and ferroptosis, as well as the inhibition of tumor angiogenesis and CSC self-renewal. In colorectal cancer, GNA interferes with the Wnt/β-catenin pathway, inducing G0/G1 phase cell cycle arrest and affecting the self-renewal ability of CSCs (Li *et al*., 2022). In OS cells, GNA accelerates the ubiquitination-mediated degradation of the stem cell-associated factor SOX2 protein, consequently affecting OS cell proliferation and promoting apoptosis (Chen *et al*., 2020).

In this research, the pursuit of KLF5 as a potential target for drug development or exploration may effectively reduce the stemness characteristics of OS cells and lower the risk of metastatic recurrence. This research aimed to elucidate the pharmacological mechanisms of GNA in combating tumor and to establish an experimental foundation for the targeted treatment of canine osteosarcoma stem cells.

MATERIALS AND METHODS

Cell lines and clinical samples: The canine OS cell line McKinley was donated by the Flint Animal Cancer Centre. The canine renal cell line MDCK and canine primary osteoblasts were extracted by our group. Tissue samples were used for related research with the consent of the owners of the affected dogs. The cells were cultured with DMEM containing 10% serum (Vivacell, Israel) in 5% CO_2 at 37°C. Drug-treated cells are usually cultured in media supplemented with 2% serum.

Cell proliferation and toxicity assay: The cells were inoculated into 96-well plates with different GNA concentrations for 48h. CCK-8 (Meilunbio, China) working solution was added, and the absorbance was measured at 450nm (Wu Y *et al*., 2023).

Plate colony formation experiment: The cells were seeded into 6-well plates and incubated with different concentrations of GNA until colonies formed. The cells were fixed with 4% paraformaldehyde and photographed after they were stained with 0.1% crystal violet.

Cell scratch assay: The cell mixture was added to a 12-well plate, and a line was created in the centre of each well. The cells were incubated with different GNA solutions for 12 h, and photographs were taken at different time points.

Transwell migration and invasion assays: The cell mixture was added to a Transwell chamber (Corning, USA) for 24h. The cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet solution; the excess dye was removed, and the results were collected. In the Transwell invasion assay, the Matrigel (Mogengel, China) was deposited in the chamber (Wu *et al*., 2023).

Cancer stem cell spheroid formation assay: Agarose solution was added to the plate. The cells were mixed with cancer stem cell medium and added to a low-adhesion 6-well plate for 6 days.

Immunofluorescence staining: The cells were fixed in 4% paraformaldehyde and blocked in 5% goat serum, after which they were incubated with the primary antibody overnight. The sections were incubated with the secondary antibody. Finally, DAPI was added to stain the nuclei, after which they were observed under a fluorescence microscope.

Alkaline phosphatase with alizarin red staining: The cancer stem cells were cultured in osteogenic induction medium and stained with an ALP kit (Meilunbio, China) at 6d. The cells were stained with ARS staining solution (Beyotime Biotechnology, China) at 21d (Li *et al*., 2022).

Western blotting: Total protein and nuclear proteins were extracted from cells and tissue. Denatured protein samples were separated by SDS-PAGE and transferred to the PVDF membrane. Finally, the bands were visualized using a fully automated gel imager (Tanon, China) and

analysed with ImageJ. All primary antibody sources are shown in Table 1.

Table 1: Primary antibody sources for Western blotting

Antibody name	Source	Dilution ratio
NANOG	HY-P80237, MCE, USA	1:500
Oct-4	HY-P80770, MCE, USA	1:500
SOX2	HY-P84348, MCE, USA	1:500
Slug	WL01508, Wanleibio China	1:1000
TWIST	WL00997, Wanleibio China	1:1000
E-Cadherin	CSB-PA005034MA01HU, Cusabio, China	1:500
N-Cadherin	13116, CST, USA	1:1000
RUNX2	WL03358, Wanleibio China	1:1000
Osteocalcin	23418-1-AP, Proteintech, USA	1:1000
OPN	WL02378, Wanleibio China	1:1000
KLF5	21017-1-AP, Proteintech, USA	1:1000
GAPDH	60004-1-Ig, Proteintech, USA	1:50000
β-Actin	66009-1-Ig, Proteintech, USA	1:50000
β-Tubulin	66240-1-Ig, Proteintech, USA	1:50000

Real-time PCR: The Total RNA Extraction Kit and the cDNA reverse transcription Kit (Promega, USA) were used to extract cell RNA and cDNA. Then, cDNA, RNA primers, and 2 × SYBR Green qPCR Master Mix Fluorescent Dye (Bimake, USA) were added to the reaction plates, and RNA amplification was performed on a Light Cycler® 480 II Detection System (Roche, Switzerland). All the PCR primer sequences are shown in Table 2. The data were analysed using the following method.

$\Delta CT = CT\ (target\ gene) - CT\ (GAPDH)$
$\Delta\Delta CT = \Delta CT\ (treated\ group) - \Delta CT\ (control\ group)$
$2^{-\Delta\Delta CT} = mRNA$

Public data acquisition: GSE57884 contained 3 cases of oncogenicity and 3 cases of non-oncogenic primary canine osteosarcoma cell lines, and GSE126209 included 11 cases of human OS patient tumor tissue with para-cancerous tissue. Datasets were downloaded from the GEO.

Table 2: Primers for real-time PCR

Gene name	Primer sequences（5'-3'）	
SOX2	F	GGAGAACCCCAAGATGCACA
	R	GCTTCTCCGTCTCCGACAAA
NANOG	F	AAGCCATGGTGTCCTACAGC
	R	GGACACTATCGAGGCAGAGC
Oct-4	F	AGCTTGACCAGAGCACCATC
	R	ACGAGCAAAGGCATCATCCA
Slug	F	AACTACGGCGAACTGGACAC
	R	CGGTAATGGGGCTGTATGCT
TWIST	F	CAACAGCACTAGGAGCGACA
	R	CCAGGAAGGGAGGCTGTTG
E-Cadherin	F	TCCTCGCTCTACTAATCCTGA
	R	ACATTGTCCCGGGTGTCATC
N-Cadherin	F	TCGGGTAATCCCCCGAAATC
	R	ACATCAGAACGAGGATGAGCA
KLF5	F	CTTCCACAGCAGGCCACTTA
	R	TGGATGCGTCGTTTCTCCAA
GAPDH	F	TGCCTCCTGCACCACCAAC
	R	CCGTCACGCCACATCTTCCC

Cell transfection: The KLF5 overexpression plasmid was transfected into cells with PolyFast transfection reagent (MCE, USA).

Statistical analysis: Statistical analysis of the data obtained from this experiment was performed by one-way ANOVA and t-test via SPSS software, and the graphics were created via GraphPad Prism 10. The results are expressed as the means±standard deviations (means±SD).

RESULTS

GNA inhibited the proliferation, migration, and invasion of canine OS cells: As shown in Fig. 1A, 5000 cells were incubated in 96-well plates for 48h under optimal conditions, and this density was used in subsequent experiments. GNA inhibited McKinley cells in a dose-dependent manner with an IC_{50} of 0.28 μM (Fig. 1B). At this concentration, MDCK cell viability remained above 90% (Fig. 1C). The experiments categorized cells into the OSc group, OSc-GNA(L) group, OSc-GNA(M) group, and OSc-GNA(H) groups based on cell viability, with respective values of 100%, 70%, 50%, and 30%. After treatment with GNA, the apoptosis rate of McKinley cells increased (Fig. 1D), and the clonogenesis (Figure 1E), migration (Fig. 1F-G), and invasion (Fig. 1H) decreased in a dose-dependent manner. In summary, GNA suppressed the proliferation, migration, and invasion of McKinley cells in a dose-dependent manner.

GNA impaired stemness maintenance in canine OS stem cells: The results of the canine OS stem cell spheroids showed that the OSc group was dense, full, and had a large volume. After GNA intervention, the stem cell spheroids' diameter failed to meet the criteria, with a loose shape and reduced volume. Furthermore, the number of spheroids decreased in a dose-dependent manner. Positive results for CD133 and CD117 in the stem cell spheroids were identified as canine OS stem cells (Fig. 2B). Compared with the OSc group, stemness-related markers NANOG, Oct-4, and SOX2 mRNA and protein expressions were higher than those of the OSC-stem group (Fig. 2C-D). The results indicated that GNA inhibited the expression of stemness-related markers in canine OS stem cells and impaired stemness maintenance.

GNA inhibited EMT and promoted the osteogenic differentiation of canine OS stem cells: CSCs exhibit elevated expression of EMT-related markers, correlating with enhanced invasive metastasis. As shown in Fig. 3A, the OSc-stem group displayed significantly increased mRNA levels of Slug, TWIST, and N-cadherin compared with the OSc group, while E-cadherin mRNA levels were significantly reduced. The expressions of the protein and immunofluorescence assays were consistent with the mRNA expressions (Fig. 3B-C). Following promoting the osteogenic differentiation of CSC, the ALP and ARS contents and protein expressions of the osteogenic differentiation-related factors, including RUNX2, osteocalcin, and OPN, were significantly upregulated in the OSc-stem group in comparison with the OSc group. (Fig. 3D-E). In summary, GNA inhibited EMT, promoted the differentiation of canine OS stem cells into osteoblasts, and increased their differentiation potential.

GNA inhibits KLF5 expression and nuclear translocation in canine OS cells: The GSE57884 dataset demonstrated that the oncogenicity group presented higher KLF5 expression than the non-oncogenicity group

(Fig. 4A). Meanwhile, the GSE126209 dataset demonstrated that KLF5 expression was upregulated in OS tissues (Fig. 4B). Further, KLF5 expression was significantly increased in canine OS cells and canine OS tissues in comparison with canine primary osteoblasts and adjacent tissues (Fig. 4C).

The effect of GNA on KLF5 expression in McKinley was subsequently explored. The mRNA and protein expression levels of KLF5 were both reduced in the OSc-GNA(M) group in comparison with the OSc group (Fig. 4D-E), and the nuclear translocation level and the nuclear protein expression levels of KLF5 were decreased in the OSc-GNA(M) group (Fig. 4F-G). These results suggest that KLF5 may play a key role in the development of canine OS and in GNA resistance to canine OS.

KLF5 partially rescued GNA to inhibit the proliferation, migration, and invasion of canine OS cells: The KLF5 overexpression model was established (Fig. 5A-B). Compared with the OSc^{NC} group, the apoptosis rates in the OSc^{KLF5} group were decreased, and the proliferation, migration, and invasion were increased. Compared with the OSc^{NC}-GNA(M) group, the apoptosis rates in the OSc^{KLF5}-GNA(M) group were decreased, and the proliferation, migration, and invasion were increased (Fig. 5C-G). The above results indicated that KLF5 promoted the proliferation, migration, and invasion of canine OS cells, and the overexpression of KLF5 partially rescued GNA to inhibit the proliferation, migration, and invasion of canine OS cells.

KLF5 reversed GNA-mediated inhibition of cell sphere formation and maintenance of stemness in canine OS cells: For cell sphere formation, the OSc^{KLF5} group exhibited regular sphere morphology and an increasing number of spheres compared with the OSc^{NC} group; sphere formation was enhanced in the OSc^{KLF5}-GNA(M) group relative to the OSc^{NC}-GNA(M) group (Fig. 6A). Results of stemness maintenance factors demonstrated elevated mRNA and protein expression in the OSc^{KLF5}-stem group in comparison with the OSc^{NC}-stem group. Furthermore, the mRNA and protein expression of NANOG, Oct-4, and SOX2 were markedly increased in the OSc^{KLF5}-stem-GNA(M) group relative to the OSc^{NC}-stem-GNA(M) group (Fig. 6B-C). Taken together, KLF5 promotes stemness maintenance and enhances the self-renewal ability of canine OS cells. Moreover, KLF5 overexpression reversed the inhibitory effect of GNA on the expression of stemness-related factors.

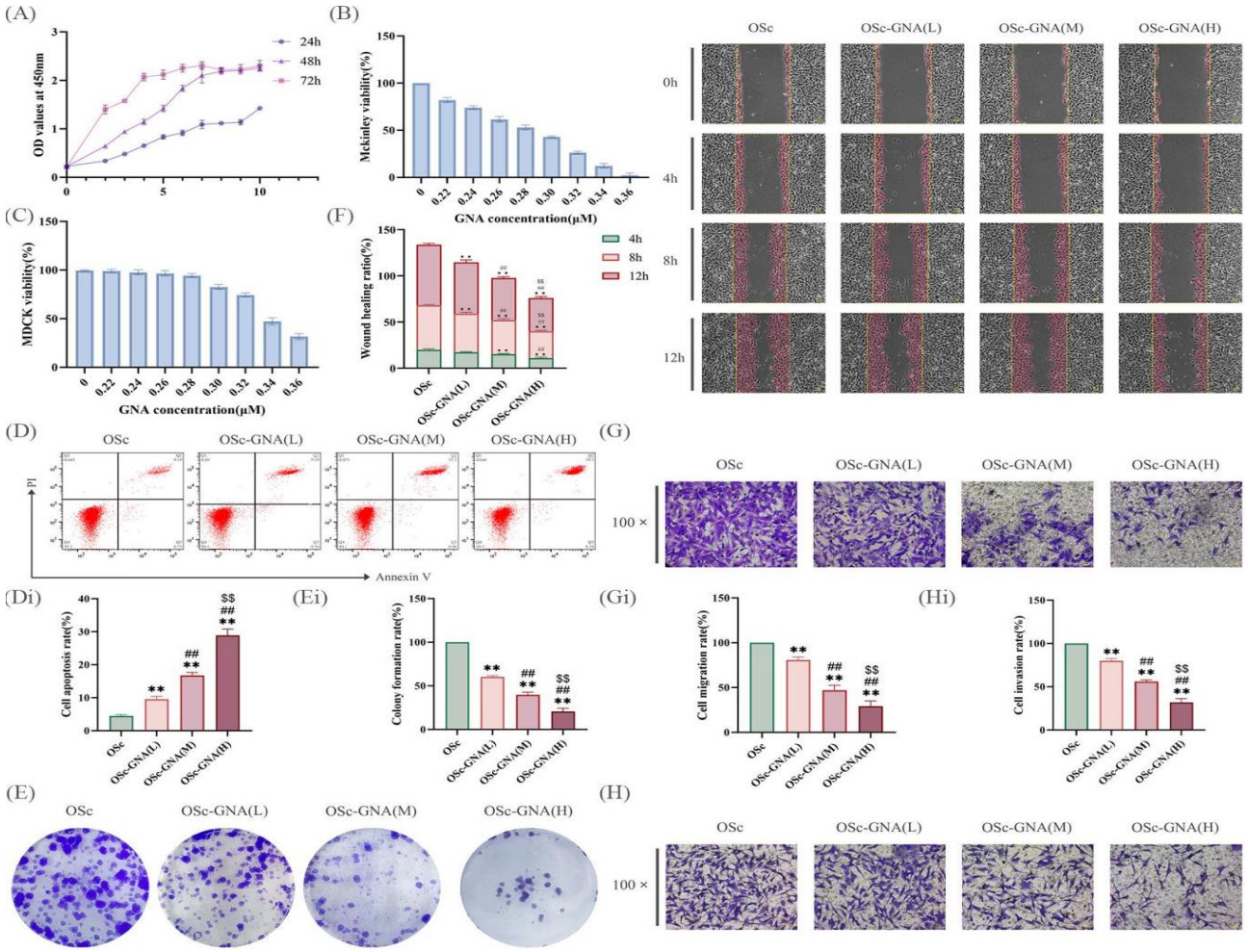

Fig. 1: Effect of GNA on proliferation, migration, and invasive ability of canine OS cells. (A) CCK-8 assay for optimal growth of McKinley cells. (B-C) CCK-8 assay for cell viability of McKinley cells and MDCK cells in the presence of different concentrations of GNA. (D) Apoptosis rate was detected by flow cytometry. (E) Representative pictures of plate clone formation and clone formation rate. (F) Representative pictures of the cell scratch at different times and scratch rates. (G-H) Representative pictures of Transwell migration and invasion. ** P<0.01 VS OSc group. ## P<0.01 VS OSc-GNA(L) group. $$ P<0.01 VS OSc-GNA(M) group.

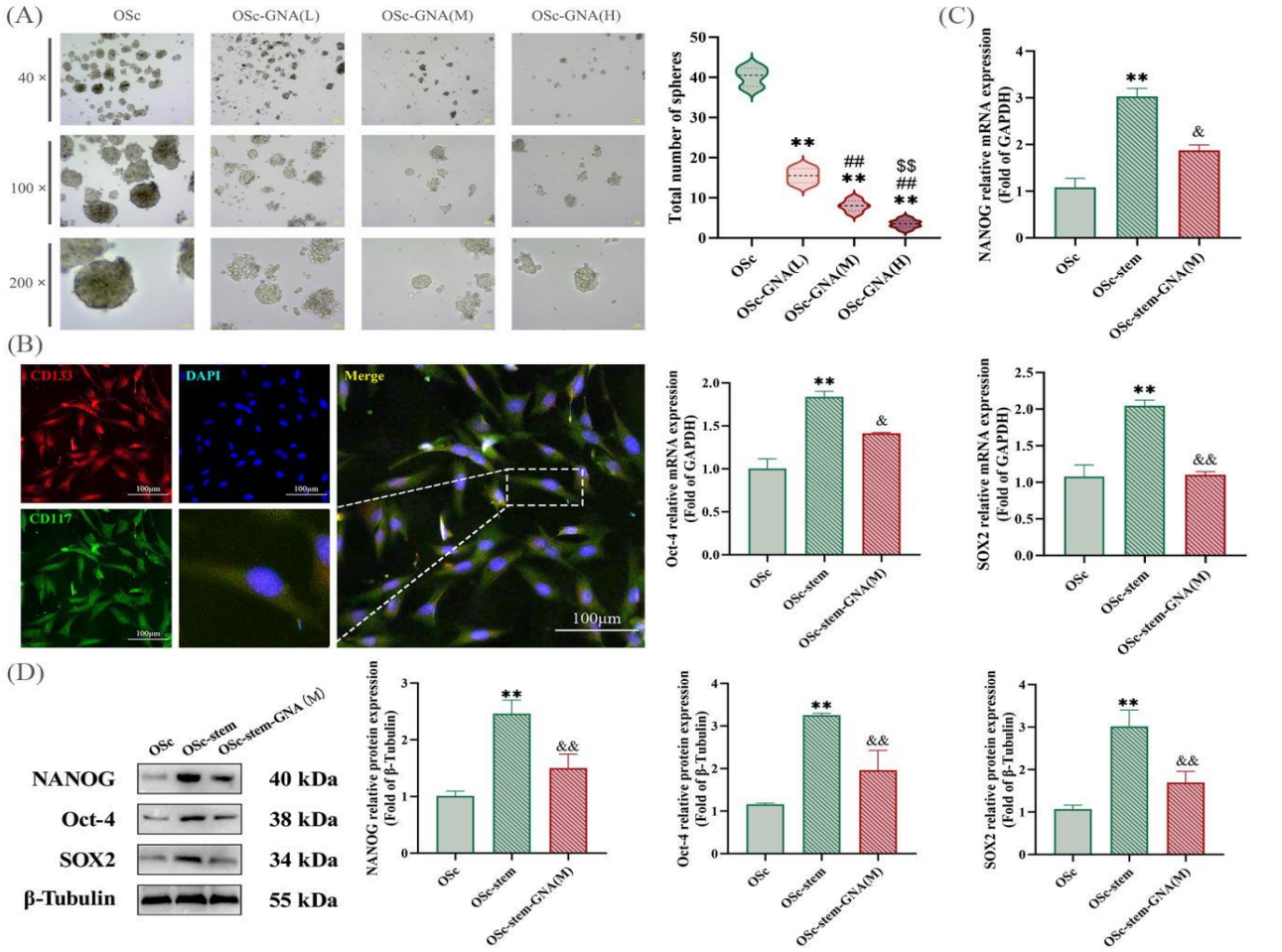

Fig. 2: Effect of GNA on sphere formation and stemness maintenance of canine OS cells. (A) Representative pictures of McKinley cell sphere formation with sphere counts. (B) Identification of stem cell sphere markers. (C) mRNA expression of stemness-related markers NANOG, Oct-4, and SOX2. (D) Protein expression of NANOG, Oct-4, and SOX2. ** P<0.01 VS OSc group. ## P<0.01 VS OSc-GNA(L) group. $$ P<0.01 VS OSc-GNA(M) group. & P<0.05 VS OSc-stem group. && P<0.01 VS OSc-stem group.

Overexpression of KLF5 interfered with GNA to promote the EMT and osteogenic differentiation in canine OS stem cells: The mRNA expressions of Slug, TWIST, and N-cadherin were significantly elevated in the OSc^{KLF5}-stem group compared with the OSc^{NC}-stem group, while E-cadherin expression was significantly downregulated. Furthermore, the mRNA expression levels of Slug, TWIST, and N-cadherin were significantly upregulated in the OSc^{KLF5}-stem-GNA(M) group in comparison with the OSc^{NC}-stem-GNA(M) group, whereas E-cadherin expression was opposite (Fig. 7A). The protein expressions and E-Cadherin and N-Cadherin expressions on the cell membrane were consistent with the mRNA expression results (Fig. 7B-C).

ALP and ARS staining demonstrated that ALP and ARS contents have a less content in the OSc^{KLF5}-stem group in comparison with the OSc^{NC}-stem group. Furthermore, ALP and ARS contents were significantly reduced in the OSc^{KLF5}-stem-GNA(M) group relative to the OSc^{NC}-stem-GNA(M) group (Fig. 8A). Analysis of osteogenic differentiation-associated factors revealed that the protein expression was significantly decreased in the OSc^{KLF5}-stem group compared with the OSc^{NC}-stem group. The protein expression levels of RUNX2, osteocalcin, and OPN were significantly reduced in the OSc^{KLF5}-stem-GNA(M) group in comparison with the OSc^{NC}-stem-GNA(M) group (Fig. 8B). In summary, KLF5 was positively correlated with EMT and negatively correlated with the differentiation ability of canine OS stem cells. Overexpression of KLF5 interfered with the tendency of GNA to promote EMT and osteoblastic differentiation in canine OS stem cells.

DISCUSSION

The potential CSC subpopulation in canine OS is the source of distal metastasis, resistance to radiation and chemotherapy, and recurrence. Furthermore, the mutational dysregulation of the transcription factor KLF5 has been closely associated with the maintenance of stemness. GNA has shown significant potential in the treatment of oncological diseases; however, its role in modulating the stemness characteristics of canine OS remains uncertain. Therefore, the present study was conducted to investigate the effects of GNA on the stemness properties of canine OS cells and to identify the relevant molecular targets involved. The findings indicate that GNA effectively inhibits the stemness characteristics of canine OS cells by regulating KLF5 expression.

In this investigation, the IC_{50} of GNA against McKinley cells over 48h was established to be 0.28μM. This value is significantly lower than those of ADM and

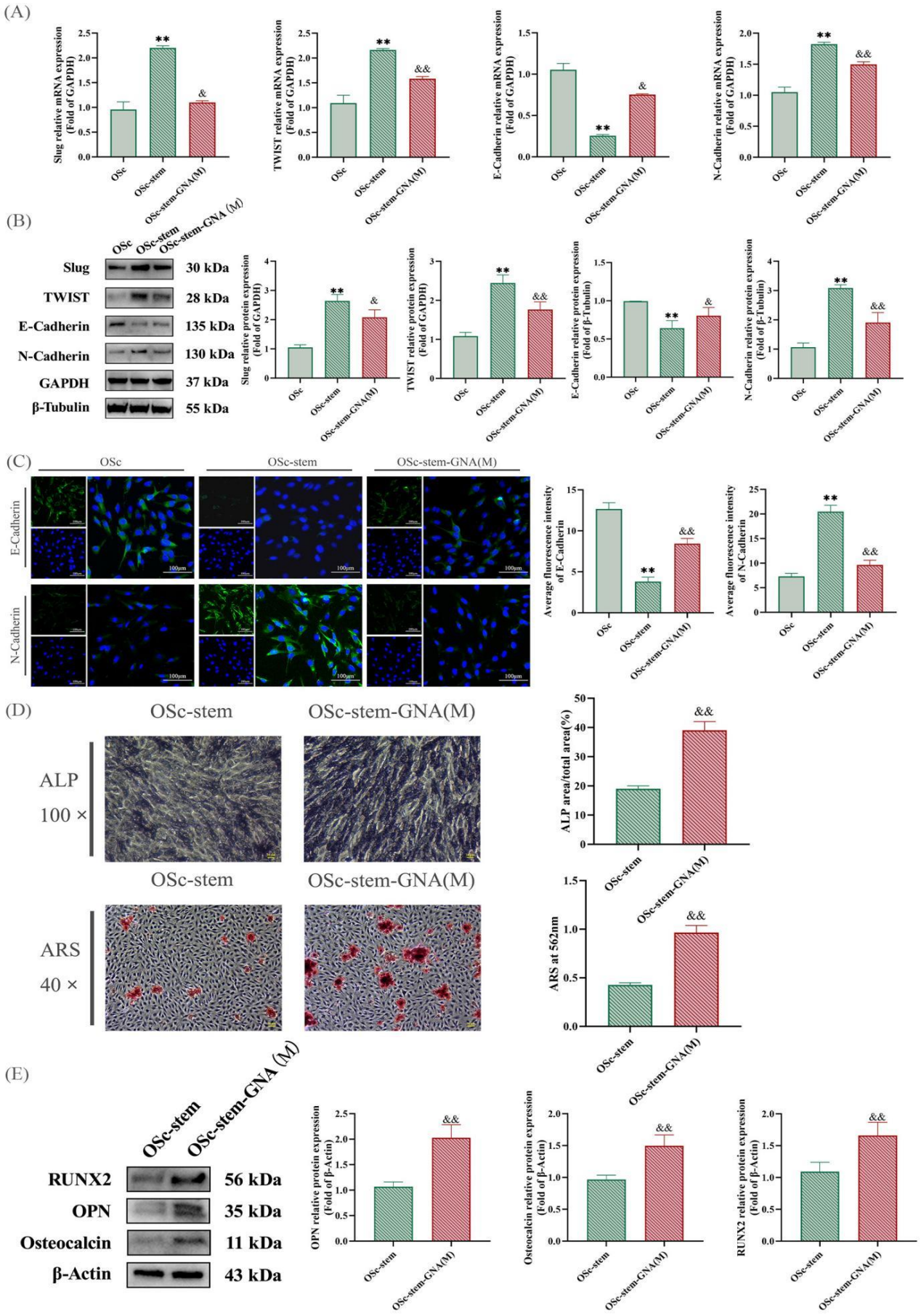

Fig. 3: Effect of GNA promotes EMT and osteogenic differentiation of canine OS stem cells. (A) mRNA expression of EMT markers Slug, TWIST, E-Cadherin, and N-Cadherin. (B) Protein expression of Slug, TWIST, E-Cadherin, and N-Cadherin. (C) Immunofluorescence detection of cell membrane E-Cadherin and N-Cadherin protein expression. (D) ALP and ARS staining. (E) Protein expression of osteogenic differentiation-related markers RUNX2, Osteocalcin, and OPN. ** P<0.01 VS OSc group. & P<0.05 VS OSc-stem group. && P<0.01 VS OSc-stem group.

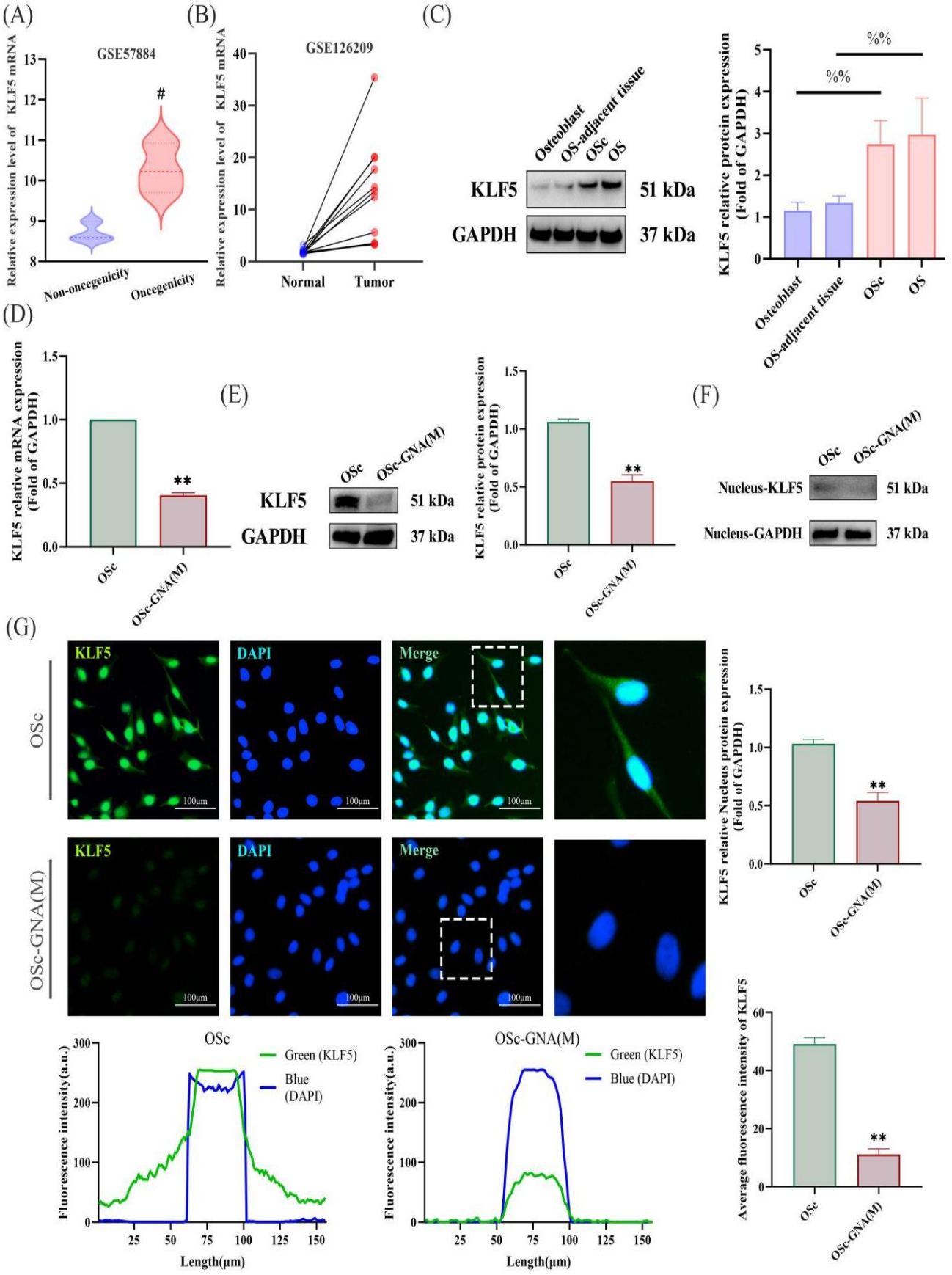

Fig. 4: Effect of GNA on KLF5 expression and nucleation. (A)KLF5 expression in GSE57884. (B) KLF5 expression in GSE126209. (C) Protein expression of KLF5 in canine primary osteoblasts, canine OS paracancerous tissues, canine OS cell lines, and canine OS tissues. (D) mRNA expression of KLF5 under GNA. (E) Protein expression of KLF5 under GNA. (F) Protein expression of KLF5 in the nucleus of canine OS cells. (G) Representative pictures and analysis of fluorescence localization of KLF5 protein into the nucleus. # P<0.05 VS Non-Oncogenicity group. %% P<0.01 indicates a comparison between the two groups. ** P<0.01 VS OSc group.

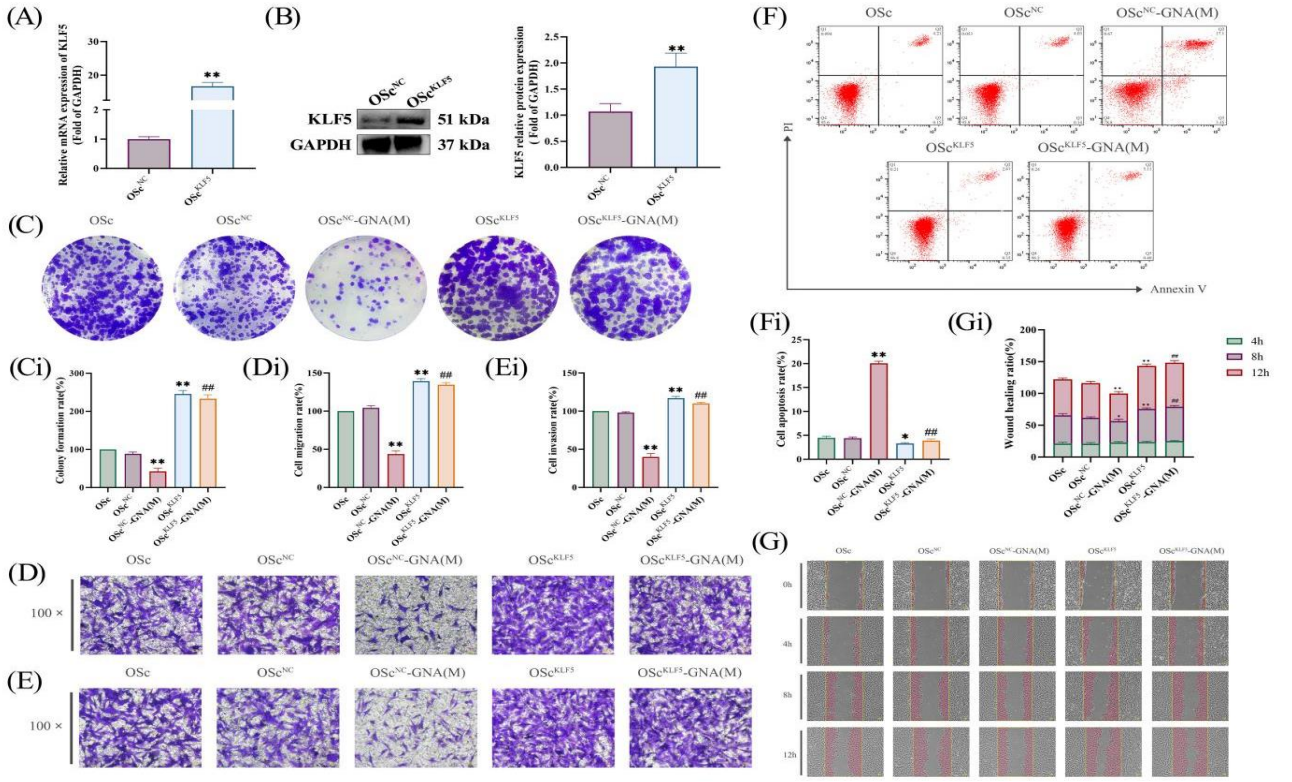

Fig. 5: Effect of KLF5 on GNA inhibition of proliferation, migration, and invasion of canine OS cells. (A-B) Validation of the KLF5 overexpression model. (C) Representative pictures of plate clone formation and clone formation rate. (D-E) Representative pictures of Transwell migration and invasion. (F) Apoptosis rate detected by flow cytometry. (G) Representative pictures of cell scratch and scratch healing rate. * P<0.05 VS OSc^{NC} group. ** P<0.01 VS OSc^{NC} group. ## P<0.01 VS OSc^{NC}-GNA(M) group.

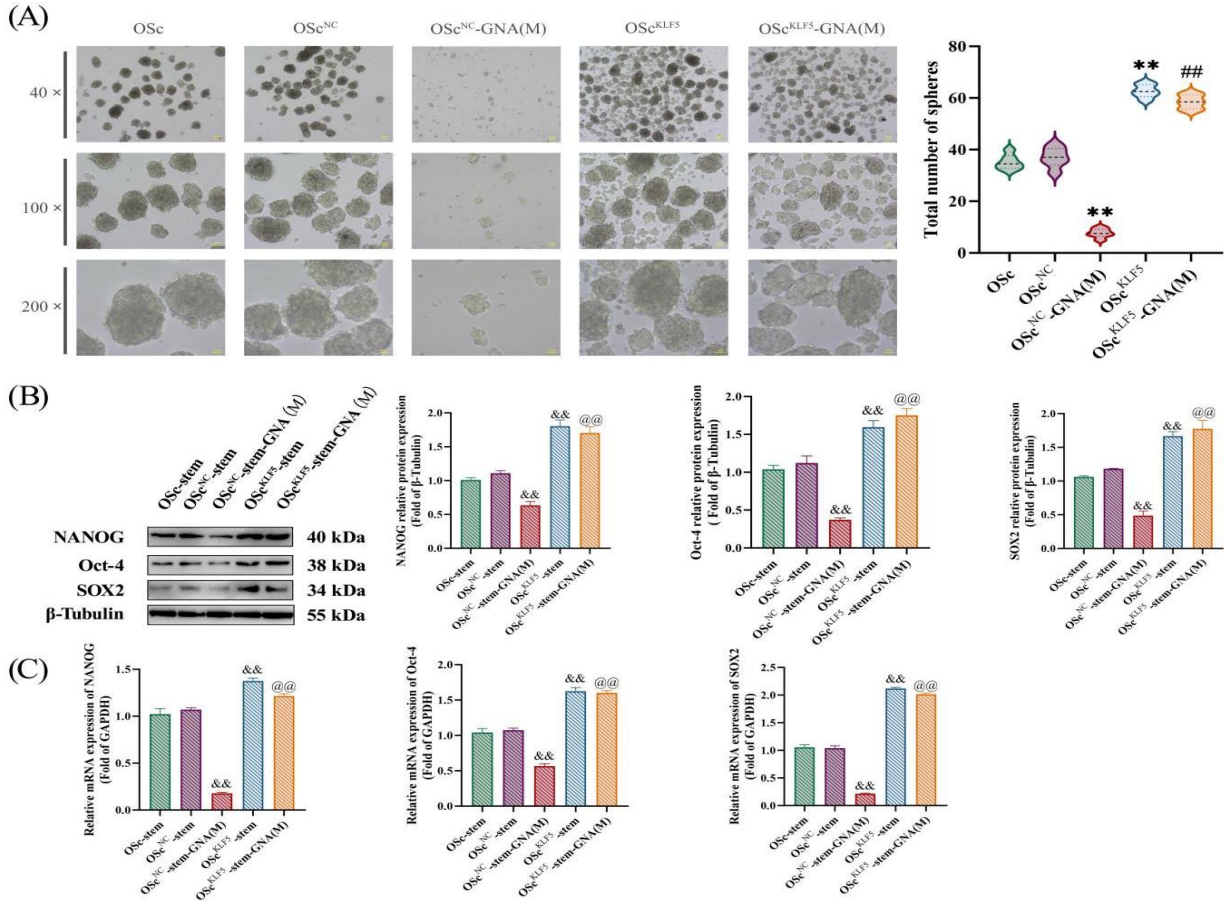

Fig. 6: Effect of KLF5 on GNA inhibition of sphere formation and stemness maintenance in McKinley cells. (A) Representative pictures of sphere formation with sphere counts. (B) mRNA expression of NANOG, Oct-4, and SOX2. (C) Protein expression of NANOG, Oct-4, and SOX2. ** P<0.01 VS OSc^{NC} group. ## P<0.01 VS OSc^{NC}-GNA(M) group. && P<0.01 VS OSc^{NC}-stem group. @@ P<0.01 VS OSc^{NC}-stem-GNA(M) group.

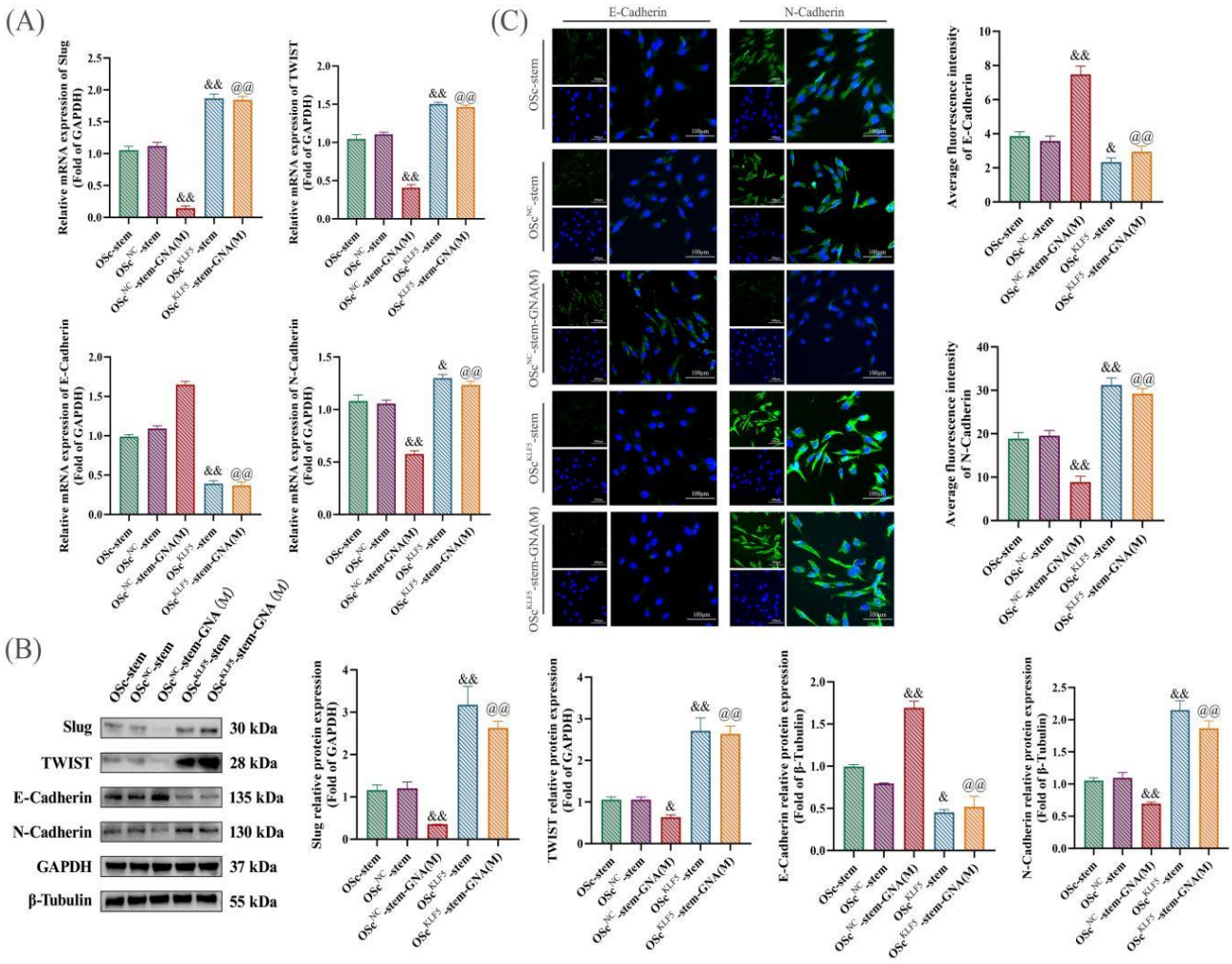

Fig. 7: Effect of KLF5 on GNA inhibition of EMT in canine OS stem cells. (A) mRNA expression of Slug, TWIST, E-Cadherin, and N-Cadherin. (B) Protein expression of Slug, TWIST, E-Cadherin, and N-Cadherin. (C) Immunofluorescence detection of cell membrane E-Cadherin and N-Cadherin protein expression. & P<0.05 VS OSc^{NC}-stem group. && P<0.01 VS OSc^{NC}-stem group. @@ P<0.01 VS OSc^{NC}-stem-GNA(M) group.

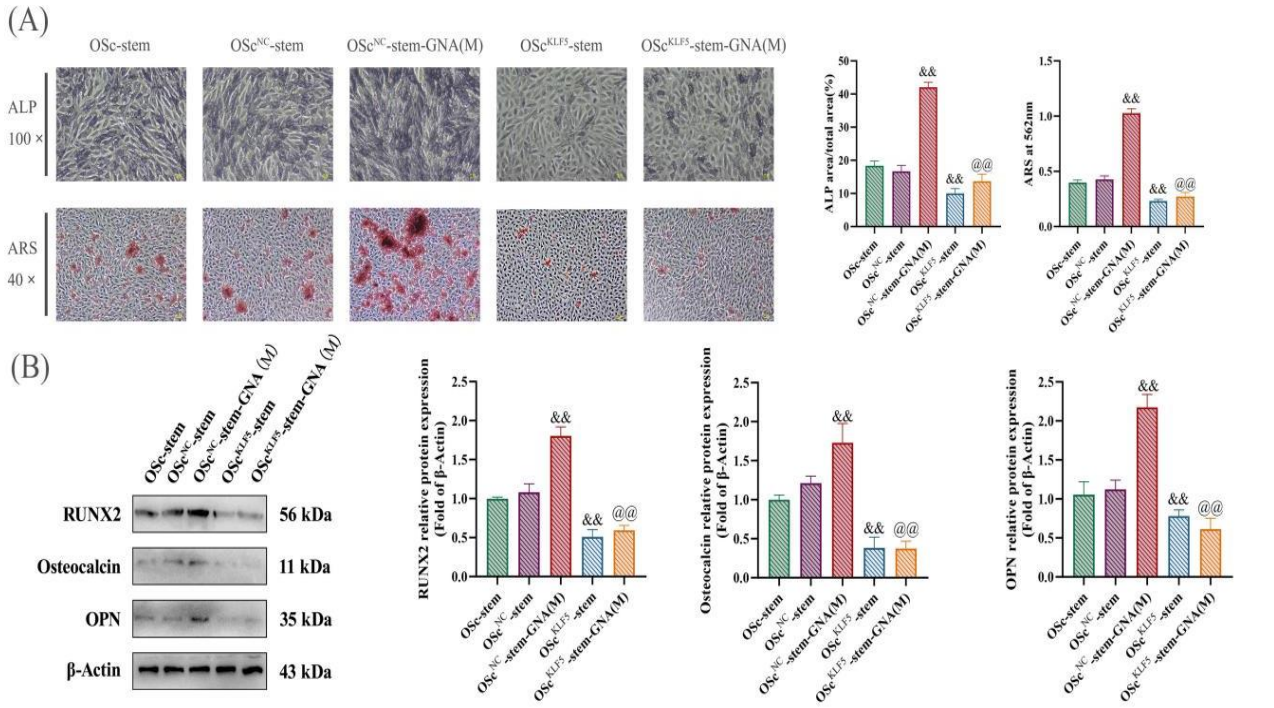

Fig. 8: Effect of KLF5 on GNA-induced differentiation of canine OS stem cells toward osteogenesis. (A) ALP and ARS staining of canine OS stem cells. (B) Protein expression of RUNX2, Osteocalcin, and OPN. && P<0.01 VS OSc^{NC}-stem group. @@ P<0.01 VS OSc^{NC}-stem-GNA(M) group.

PDD, which ranged from 0.5μM to 5μM and 10μM to 15μM, respectively, for various primary canine osteosarcoma cells (Gatti *et al*., 2018). While there exists a certain degree of variability among different canine OS cells, these findings suggest that GNA may exert substantial anticancer effects at a lower dose. Beilei W reported that GNA could significantly inhibit the growth of breast cancer in tumor-bearing mice, and the accumulation coefficient of GNA was the highest in the kidney tissue. However, GNA did not cause damage to major organs such as the kidney, liver, and heart (Wang *et al*., 2021). Therefore, the canine kidney cell line MDCK was selected to investigate the toxic effects of GNA. The current study demonstrated that cell viability in MDCK cells remained largely unaffected following exposure to 0.28μM GNA for 48 hours, indicating a favorable safety profile for the drug.

Given that sarcomas originate from mesenchymal tissue, which is typically well-vascularized, these cells are predisposed to peripheral invasion; thus, strategies aimed at preventing the proliferation and metastasis of cancer cells are crucial for inhibiting the malignant progression of OS. The results of this study revealed that GNA treatment resulted in a dose-dependent inhibition of cell motility. Similarly, the inhibitory effects of GNA on tumor cells motility have been observed in bladder cancer, where alterations in the tumor microenvironment (TME) occur due to the suppression of the RELA proto-oncogene (P65), which mediates changes in the activity of various cell adhesion factors, thereby inhibiting tumor metastasis and invasion (Schmitz and Baeuerle, 1991; Zhou *et al*., 2020).

The successful screening, enrichment, and phenotypic characterization of canine OS stem cell subpopulations are critical prerequisites for elucidating the mechanisms by which drugs affect the maintenance of cell stemness. The canine OS cell lines D-17, UWOS-1, and UWOS-2 have been shown to screen for CSCs via spheroidization (Wilson *et al*., 2008). In the present study, McKinley demonstrated that under specific culture conditions, rapid sphere formation occurred, accompanied by favorable stemness characteristics, including stable double-positive expression of CD133 and CD117, as evidenced by immunofluorescence. SOX2, NANOG, and Oct-4 proteins are essential for the stemness of normal stem cells and CSCs, and they are reflected in many types of cancer diseases as the dominant drivers of stemness. Depletion of SOX2, NANOG, or Oct-4 could lead to a systemic breakdown of the stemness regulatory network and delay tumor progression. Following the pretreatment of McKinley cells with GNA, the sphere-forming behavior of the CSC subpopulation was blocked, the stemness transformation ability was weakened, and the expression levels of the stemness regulators SOX2, NANOG, and Oct-4 were decreased. These findings align with those reported by Li *et al.* (2022). In colorectal cancer, GNA was shown to antagonize DLK1 through the activation of the Wnt signaling pathway, resulting in compromised self-renewal of colorectal CSCs and a notable decrease in the population of sphere-forming cells (Li *et al*., 2022). Effective screening, enrichment, and characterization of canine OS stem cells are the basis for subsequent related experiments to elucidate the stemness maintenance effect of GNA on CSCs and epithelial-mesenchymal transition. Because the culture of cancer stem cell spheres belongs to a three-dimensional culture model, the oxygen-rich zone outside the sphere and the hypoxic zone in the center of the sphere make a niche for CSCs at different levels, and the self-protection effect of cells in the inner sphere to drug stimulation is not the same. Therefore, studying the process of drug spheroid formation of cancer stem cells is not only to shape the survival niche of CSCs, but also to simulate the in vivo microenvironment to achieve a more accurate evaluation of drug efficacy.

The malignant feature that CSCs are involved in promoting tumor cell metastasis is beneficial for tumor cells to destroy the basement membrane and invade deeper tissues through EMT (Nguyen *et al*., 2009). EMT is increasingly considered an important feature of CSCs (Brabletz, 2012). OS stem cells induced by Melatonin were observed to regulate EMT level by suppressing SOX9-mediated signaling (Qu *et al*., 2018). The present study revealed similar results, demonstrating that canine OS stem cells exhibit typical EMT alongside a trend of increased expression of the upstream regulators TWIST and Slug. However, the expression levels of EMT-related factors were reversed in canine OS stem cells exposed to GNA. Similar pharmacological effects of GNA were also observed in melanoma cells, where TGF-β-induced melanoma cells displayed a highly active mesenchymal state. The dysregulation of cadherin and upstream transcription factor expression was reversed by GNA treatment, and cells were restored to the epithelial-like state associated with normal intercellular adhesion (Wang *et al*., 2020).

Mesenchymal stem cells (MSCs) that aggregate during the process of bone development differentiate into osteogenic precursor cells, early osteoblasts, osteoblasts, and osteocytes. The blockage of the osteogenic differentiation pathway is a critical factor in OS development. The differentiation potential is recognized as one of the stemness characteristics of CSCs. In this study, canine OS stem cells were induced to undergo osteogenic differentiation. ALP was used to detect the early process of differentiation, and ARS was utilized to evaluate the formation of calcium-phosphate deposits and calcified nodules in the middle and late stages. The results indicated that GNA significantly potentiated the sustained induction of osteogenic differentiation. Differentiation therapy has been recognized as a crucial therapeutic strategy for targeting a subset of canine OS stem cells (Gatti *et al*., 2018).To date, clinical trial drugs such as trabectedin have been conclusively demonstrated for use in OS differentiation therapy. Furthermore, natural compounds such as galangin, chrysin, and genistein have gradually emerged as viable alternatives for OS-induced differentiation therapy (Zhang *et al*., 2014; Song *et al*., 2015; Liu *et al*., 2017). In summary, GNA inhibits tumor cell stemness and EMT in canine OS, while simultaneously promoting the osteogenic differentiation of canine OS stem cells. Therefore, the present study further investigated the underlying mechanisms by which GNA affects canine OS cells.

KLF5 serves as a crucial mediator that promotes adaptive survival in response to various cellular stress stimuli encountered by cells. The present investigation

revealed that GNA treatment significantly diminished both the mRNA and protein levels of KLF5 in McKinley cells, as well as reduced its nuclear translocation. Additionally, Curcumol has also been shown to exert pharmacological effects that inhibit KLF5 expression (Gao *et al*., 2021). In this study, the overexpression of KLF5 was found to promote cell viability and positively regulate the proliferation, migration, and invasion ability of McKinley, and the same promotional effect was confirmed in previous research (He *et al*., 2018; Wu *et al*., 2023). In addition, the overexpression of KLF5 reversed the inhibitory effects of GNA on the proliferation, migration, and invasion of McKinley cells. Similar observations were observed in a study by Wei R, which indicated that KLF5 overexpression not only enhanced the motility of tumor cells but also mitigated the anticancer effects associated with a ketogenic diet (Wei *et al*., 2022).

KLF5 is also involved in regulating stemness maintenance factors, including Oct-4, NANOG, and SOX2, to regulate the stemness pathway and maintain cell stemness characteristics (Wu *et al*., 2021; Ai *et al*., 2022; Han *et al*., 2022). For instance, the overexpression of KLF5 improves the competitive binding of KLF5 to Oct-4, which is disrupted by mutations in the Oct-4 linker, ultimately restoring and maintaining the stemness characteristics of embryonic stem cells (ESCs) (Han *et al*., 2022). In this study, KLF5 overexpression was found to not only positively regulated Oct-4, NANOG, and SOX2, enhancing the self-renewal ability of canine OS stem cells, but also reverse the inhibitory effects of GNA on canine OS stem cells. At the EMT level of CSC, KLF5 overexpression enhanced the mesenchymal phenotype of dog OS stem cells, as reflected by the increased expression of N-Cadherin, TWIST and Slug, and the decreased expression of E-Cadherin. This result was consistent with the regulation pattern of KLF5 in breast cancer and lung cancer (Liu *et al*., 2022; Tung *et al*., 2022). The above studies showed that KLF5 may reverse the stemness-maintaining effect of GNA on canine OS cells, so is it possible that KLF5 is also involved in regulating the process by which GNA promotes the differentiation of canine OS stem cells towards osteogenesis? In this research, KLF5 was negatively correlated with the differentiation ability of canine OS stem cells in the present study, that is, the differentiation mechanism of canine OS stem cells overexpressing KLF5 was unbalanced, resulting in the block of osteogenic lineage induction, and the pro-differentiation effect of GNA was abolished in KLF5 over-activation. Research has demonstrated that cells derived from KLF5-deficient teratomas differentiate more into ARS-positive skeletal tissue rather than into cytosolic myogenic differentiation protein-positive skeletal muscle tissue (Aksoy *et al*., 2014). However, high expression levels of KLF5 maintain arterial intimal smooth muscle cells in a dedifferentiated state, while resveratrol restores and stabilizes the quiescent state of smooth muscle cell terminal differentiation by reducing KLF5 expression (Zhu *et al*., 2015). These studies suggest that KLF5 plays an important role in cell differentiation.

Conclusions: In summary, GNA weakened the malignant biological behavior and stemness transformation ability of canine OS cells by inhibiting both the expression and nuclear translocation of KLF5, and increasing their differentiation potential. The research mechanism roadmap is available in the graphical abstract.

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REFERENCES

Ai ZP, Xiang XY, Xiang YQ, *et al*., 2022. Krüppel-like factor 5 rewires NANOG regulatory network to activate human naive pluripotency specific LTR7Ys and promote naive pluripotency. Cell Reports 40:25.

Aksoy I, Giudice V, Delahaye E, *et al*., 2014. Klf4 and Klf5 differentially inhibit mesoderm and endoderm differentiation in embryonic stem cells. Nature Communications 5:15.

Brabletz T, 2012. EMT and MET in Metastasis: Where Are the Cancer Stem Cells? Cancer Cell 22:699-701.

Cartwright P, McLean C, Sheppard A, *et al*., 2005. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development 132:885-96.

Chen XY, Zhang XM, Cai HY, *et al*., 2020. Targeting USP9x/SOX2 axis contributes to the anti-osteosarcoma effect of neogambogic acid. Cancer Letters 469:277-86.

Gao LY, Yang X, Li Y, *et al*., 2021. Curcumol inhibits KLF5-dependent angiogenesis by blocking the ROS/ERK signaling in liver sinusoidal endothelial cells. Life science 264:12.

Gatti M, Solari A, Pattarozzi A, *et al*., 2018. In vitro and in vivo characterization of stem-like cells from canine osteosarcoma and assessment of drug sensitivity. Experimental Cell Research 363:48-64.

Gill J and Gorlick R, 2021. Advancing therapy for osteosarcoma. Nature reviews. Clinical Oncology 18:609-24.

Han D, Wu GM, Chen R, *et al*., 2022. A balanced Oct4 interactome is crucial for maintaining pluripotency. Science Advances 8:19.

He P, Yang JW, Yang VW, *et al*., 2018. Kruppel-like Factor 5, Increased in Pancreatic Ductal Adenocarcinoma, Promotes Proliferation, Acinar-to-Ductal Metaplasia, Pancreatic Intraepithelial Neoplasia, and Tumor Growth in Mice. Gastroenterology 154:1494-508.

Huang H, Han Y, Chen ZJ, *et al*., 2020. ML264 inhibits osteosarcoma growth and metastasis via inhibition of JAK2/STAT3 and WNT/β-catenin signalling pathways. Journal of Cellular and Molecular Medicine 24:5652-64.

Li Y, Lu Y, Wu M, *et al*., 2022. Neogambogic acid suppresses characteristics and growth of colorectal cancer stem cells by inhibition of DLK1 and Wnt/beta-catenin pathway. European Journal of Pharmacology 929:175112.

Liu CH, Ma MM, Zhang JD, *et al*., 2017. Galangin inhibits human osteosarcoma cells growth by inducing transforming growth factor-β1-dependent osteogenic differentiation. Biomedicine &

Pharmacotherapy 89:1415-21.

Liu P, Wang ZH, Ou XQ, *et al*., 2022. The FUS/circEZH2/KLF5/feedback loop contributes to CXCR4-induced liver metastasis of breast cancer by enhancing epithelial-mesenchymal transition. Molecular Cancer 21:19.

Liu R, Shi PG, Nie Z, *et al*., 2016. Mifepristone Suppresses Basal Triple-Negative Breast Cancer Stem Cells by Down-regulating KLF5 Expression. Theranostics 6, 533-44.

Lytle NK, Barber AG and Reya T, 2018. Stem cell fate in cancer growth, progression and therapy resistance. Nature reviews. Cancer 18:669-80.

Mason NJ, 2020. Comparative Immunology and Immunotherapy of Canine Osteosarcoma. Advances in Experimental Medicine and Biology 1258:199-221.

McConnell BB, Kim SS, Yu K,*et al*., 2011. Kruppel-Like Factor 5 Is Important for Maintenance of Crypt Architecture and Barrier Function in Mouse Intestine. Gastroenterology 141:1302-13.

Nguyen DX, Bos PD and Massague J, 2009. Metastasis: from dissemination to organ-specific colonization. Nature reviews. Cancer 9:274-84.

Niwa H, Ogawa K, Shimosato D, *et al*., 2009. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. Nature 460:118-22.

Parisi S and Russo T, 2011. Regulatory role of Klf5 in early mouse development and in embryonic stem cells. Vitamins and Hormones 381-97.

Qu H, Xue Y, Lian WW, *et al*., 2018. Melatonin inhabits osteosarcoma stem cells by suppressing SOX9-mediated signaling. Life science 207:253-64.

Radisky DC and LaBarge MA, 2008. Epithelial-mesenchymal transition and the stem cell phenotype. Cell Stem Cell 2:511-2.

Roessner A, Lohmann C and Jechorek D, 2021. Translational cell biology of highly malignant osteosarcoma. Pathology International 71:291-303.

Schmitz ML and Baeuerle PA, 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. The EMBO Journal 10:3805-17.

Shen YY, Zhu QY, Xiao MY, *et al*., 2022. Inhibitory effect of the novel tyrosine kinase inhibitor DCC-2036 on triple-negative breast cancer stem cells through AXL-KLF5 positive feedback loop. Cell Death & Disease 13:14.

Shi PG, Zhou ZM, Liu R, *et al*., 2017. Metformin suppresses triple-negative breast cancer stem cells by targeting KLF5 for degradation. Cancer Research 77:1.

Song MZ, Tian XL, Lu M, *et al*., 2015. Genistein exerts growth inhibition on human osteosarcoma MG-63 cells via PPARγ pathway. International Journal of Oncology 46:1131-40.

Tanabe A, Deguchi T, Sato T, *et al*., 2016. Radioresistance of cancer stem-like cell derived fromcanine tumours. Veterinary and Comparative Oncology 14:E93-E101.

Tung CH, Huang MF, Liang CH, *et al*., 2022. α-Catulin promotes cancer stemness by antagonizing WWP1-mediated KLF5 degradation in lung cancer. Theranostics 12:1173-86.

Wang BL, Yuan TT, Zha LQ, *et al*., 2021. Oral Delivery of Gambogenic Acid by Functional Polydopamine Nanoparticles for Targeted Tumor Therapy. Molecular Pharmaceutics 18:1470-9.

Wang M, Li SS, Wang YL, *et al*., 2020. Gambogenic acid induces ferroptosis in melanoma cells undergoing epithelial-to-mesenchymal transition. Toxicology and Applied Pharmacology 401:11.

Wei RZ, Zhou YN, Li C, *et al*., 2022. Ketogenesis Attenuates KLF5-Dependent Production of CXCL12 to Overcome the Immunosuppressive Tumor Microenvironment in Colorectal Cancer. Cancer Research 82:1575-88.

Wilson H, Huelsmeyer M, Chun R, *et al*., 2008. Isolation and characterisation of cancer stem cells from canine osteosarcoma. Veterinary Journal 175:69-75.

Wu Y, Chen SY, Shao Y, *et al*., 2023. KLF5 Promotes Tumor Progression and Parp Inhibitor Resistance in Ovarian Cancer. Advanced Science 10:19.

Wu Z, Zhou J, Zhang XY, *et al*., 2021. Reprogramming of the esophageal squamous carcinoma epigenome by SOX2 promotes ADAR1 dependence. Nature Genetics 53:881-94.

Zhang N, Ying MD, Wu YP, *et al*., 2014. Hyperoside, a Flavonoid Compound, Inhibits Proliferation and Stimulates Osteogenic Differentiation of Human Osteosarcoma Cells. Plos One 9:8.

Zhou SM, Zhao N and Wang JL, 2020. Gambogenic acid suppresses bladder cancer cells growth and metastasis by regulating NF-κB signaling. Chemical Biology & Drug Design 96:1272-9.

Zhu YC, Takayama T, Kent A, *et al*., 2015. Inhibition of restenosis by resveratrol involves down-regulation of KLF5, a marker of smooth muscle cell de-differentiation. Journal of the American College of Surgeons 221:E149.