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Original Article

Downregulation of Kynurenine 3-Monooxygenase Inhibits cancer stemness-related genes in both human and canine breast tumor cell lines

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Abstract

Canine mammary tumors (CMT) and human breast cancer (HBC) are the most frequent cancers in both female dogs and women worldwide, and they share an urgency for the development of effective markers. Overexpression of kynurenine 3-monooxygenase (KMO), a secondary enzyme of the kynurenine pathway, has been shown to indicate poor prognosis in CMT patients. To further investigate the role of KMO in mammary tumor development, we first analyzed the significant pathways related to KMO from the Database for Annotation, Visualization and Integrated Discovery (DAVID). The results revealed that several Kyoto Encyclopedias of Genes and Genomes (KEGG) pathways might correlate with higher KMO expression, including JAK/STAT and NF-κB, two commonly-referenced cancer stem cell pathways. A canine mammary tumor cell line (CMT-1) and a triple-negative breast cancer (TNBC) cell line, MDA-MB-231, were then used to verify the correlation between KMO expression and cancer stemness. A knockdown of KMO expression using shRNA concomitantly reduced the expression of the cancer stem cell markers *POU5F1*, *SOC2*, *NANOG* and *KLF4* in both cell lines. The findings suggest that KMO plays a role in cancer stemness in both canine mammary tumors and human breast cancers and might be a potential target for controlling the development of cancer.

Keywords: comparative oncology, cancer stemness, *POU5F1*, *SOX2*, *NANOG*

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Introduction

The mammary tumor is the most common tumor occurring in intact female dogs with a high prevalence of 52% (Moe, 2001). Malignant tumors outnumber benign ones in cases of canine mammary tumors (CMTs) and CMT are the most common types of cancer affecting bitches (Varallo *et al.*, 2019). Generally speaking, the treatment option is based on tumor staging, including surgical removal, radiotherapy, chemotherapy or combinational remedies (Andrew Novosad, 2003). However, despite the combination of different strategies, the effects of treatment in CMT remain discouraging. Similar phenomena have been found in human breast cancer (HBC), the most common tumor in women worldwide, and the particularly malignant triple-negative breast cancer (TNBC) subtype, is unresponsive to current standardized treatments (Kaszak *et al.*, 2018). In addition to the shared significance mentioned, CMTs also have clinical and molecular similarities to HBCs, making this specific disease of dogs an ideal model for that in humans. In fact, in many cancer-associated gene families, dogs share closer parallels with humans than with mice. Mutation or abnormal expression of numerous HBC molecular markers, such as HER2/neu, Ki-67 and p53, also results in poor prognosis in CMTs, revealing a common pattern of gene expression and disease development in both species (Abdelmegeed and Mohammed, 2018).

Kynurenine 3-monoxygenase (KMO) is a pivotal enzyme in the kynurenine pathway, which facilitates tryptophan metabolism. It is known for its correlation with neuronal degeneration and further development into several significant neuronal diseases (Braidy *et al.*, 2009; Schwarcz *et al.*, 2012). The correlation of KMO and poor prognosis in hepatocellular carcinoma patients has also been established, revealing the distinct role of KMO in cancers (Jin *et al.*, 2015). Another study has also indicated that overexpression of NMDA receptors, whose activity can be stimulated by downstream metabolites of KMO, was found in human breast cancer cells and that inhibition of NMDA receptors could lead to impaired growth (North *et al.*, 2010). In our previous study focusing on KMO expression in canine cancers, we demonstrated that higher KMO expression is associated with higher malignancy and shorter survival time for clinical CMTs, while a knockdown of KMO expression results in lower cell viability of CMT cell lines (Chiu *et al.*, 2019).

The kynurenine pathway, which is also involved in stem cell biology, affects the characteristics of hematopoietic, mesenchymal and neuronal stem cells (Jones *et al.*, 2013). As common active signaling pathways between stem cells and cancer stem cells (CSCs) have been established, the linkage between the kynurenine pathway and CSCs should not be taken lightly (Atashzar *et al.*, 2020). Since CSCs are in the minority of cancer cells, they can initiate tumors and facilitate further progression. They are also found to maintain cell self-renewal and differentiation, generating drug resistance and promoting metastasis such that cancers are hard to destroy (Rybicka and Król, 2016). CSCs are also resistant to the chemotherapy that is inclusive of common treatments currently used in CMT and TNBC patients. Therefore, the development of a new treatment targeting CSCs should be seriously considered in cancer therapy (Timmermans-Sprang *et al.*, 2019).

Currently, no studies investigating the role of KMO in the generation or functions of CSCs in canine mammary tumors have been published. Since CMTs are regarded as a comparative model for human breast cancer, we aimed to characterize the specific gene expressions related to cancer stem cells under KMO-knockdown conditions to further elucidate the significance of KMO in cancer development.

Materials and Methods

Cell culture: The canine mammary carcinoma cell line CMT-1 (solid carcinoma) is an established primary cell line originating from a surgically resected specimen of canine mammary gland tumor (MGT). A human triple-negative breast cancer cell line, MDA-MB-231 (adenocarcinoma), was obtained from Dr. L.M. Tseng at Taipei Veterans General Hospital. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic-antimycotic containing penicillin, streptomycin and amphotericin B (AA, Simply, GeneDireX, Taipei, Taiwan) and incubated in a 5% CO_2 humidified atmosphere at 37 °C.

KMO lentiviral knockdown: CMT-1 cells were seeded in 24-well plates with 35,000 cells/well. On day 2, cells were transfected with lentivirus containing short hairpin RNAs (shRNAs), virus particles purchased from the National RNAi Core Facility, Academia Sinica, Taipei, Taiwan. Three different sets of shRNA sequences were used to target KMO, which we listed as shKMO-20 (TRCN0000064420) and shKMO-57 (TRCN0000420557). shKMO-20 was used in CMT-1, with shLuc (TRCN0000072249) as the control, at a multiplicity of infection (MOI) of 2. For MDA-MB-231, lentivirus delivering shKMO-57 was infected at a MOI of 12, while empty vector pLKO_TRC025 was used as the control. The sequences of all shRNA used are listed in Table 1. Eight μg/ml polybrene (Hexadimethrine bromide, Sigma, Germany) was added concurrently to the wells with lentivirus containing shRNA or empty plasmids. Forty-eight hours after the cells were infected, puromycin dihydrochloride (Sigma, Germany) selection at 4 μg/ml was conducted.

RNA isolation and quantitative real-time PCR (qRT-PCR): RNA was purified from each cell line using TRIzol Reagent (Invitrogen, USA) and the TriRNA Pure Kit (Geneaid Biotech Ltd., Taiwan), following the manufacturer's instructions. Deoxy HiSpec reverse transcriptase (Yeastern, Taiwan) was added according to the manufacturer's instructions after RNA extraction to 1 μg RNA to generate cDNA. All qPCR trials were performed in triplicate with the SensiFAST SYBR Lo-ROX Kit (Bioline, Germany) in the AriaMx Real-time PCR System (Agilent, USA). In the PCR protocol, activation was initiated at 95°C for 2 mins, followed by 40 cycles at 95°C for 5 secs and 60°C for 15

secs. The melt curve protocol started at 95°C for 30 secs and continued with 5 secs for each 0.5°C increment between 65°C and 95°C. Primer3 was used to design primers and the sequences are listed in Tables 2-3.

Flow cytometry: The adherent cells were detached by trypsinization and washed twice, respectively, with PBS and FACS buffer before being stained as indicated with a combination of cross-reactive fluorochrome-conjugated polyclonal antibodies as follows: mouse anti-human CD24 Alexa Fluor 647 (Clone ML5; BD Biosciences, San Jose, USA) and mouse anti-human CD44 phycoerythrin (PE) (Clone 515; BD Bioscience, San Jose, CA, USA). The cells were then left on ice for 30 mins before being washed with 1 mL FACS buffer and centrifuged for 5 mins at 300 ×g for washing. The tubes were washed three times with FACS buffer before measurements were performed with an LSR Fortessa Flow Cytometer (BD Biosciences). Data was analyzed in FlowJo Software (Tree Star).

Table 1 shRNA sequences targeting KMO for knockdown of expression.

	Sequences
shLuc	GCGGTTGCCAAGAGGTTCCAT
shKMO-57	GATAGCTCACTTCCGGAATAC
shKMO-20	GCTTGGTATTTGATGAGTTAA

Table 2 qPCR primer sequences for the detection of canine KMO and CSC genes.

	Primer sequences
Canine KMO	Forward: 5′-ATTGTAGGATGTGATGGAGCCT-3′ Reverse: 5′-TCTTGGGTGGAATCGTGAGC-3′
Canine POU5F1	Forward: 5′-CCCACATCACTCATCACTTTCTT-3′ Reverse: 5′-TAACCACAGGAGGACAAACCA-3′
Canine SOX2	Forward: 5′-ATGGGAGGAGAGTAAGAAACAGCA-3′ Reverse: 5′-CGTGAGTGTGGATGGGATTGG-3′
Canine NANOG	Forward: 5′-GGATTCTTCCACCAGTCCGC-3′ Reverse: 5′-TCCTCATCTTCTGTTTCTTGCCC-3′
Canine KLF4	Forward: 5′-GCCGTTCTCTTCCTCTCCCA-3′ Reverse: 5′-TAGTGCCTCGTCAGTTCGTCC-3′
Canine GAPDH	Forward: 5′-TGTCCCCACCCCCAATGTATC-3′ Reverse: 5′-CTCCGATGCCTGCTTCACTACCTT-3′

Table 3 qPCR primer sequences for the detection of human KMO and CSC genes.

	Primer sequences
Human KMO	Forward: 5′-GCAAGGAATGAATGCGGGCT-3′ Reverse: 5′-AGAACACAGGAAGACACAAACTAAGG-3′
Human POU5F1	Forward: 5′-ATCAGCCACATCGCCCAG-3′ Reverse: 5′-GAAAGGAGACCCAGCAGCC-3′
Human SOX2	Forward: 5′-AAACGAGGGAAATGGGAGGG-3′ Reverse: 5′-TTGCTGTGGGTGATGGGATT-3′
Human NANOG	Forward: 5′-GCAGAGAAGAGTGTCGCAAA-3′ Reverse: 5′-TGCTGGAGGCTGAGGTATTTC-3′
Human KLF4	Forward: 5′-GGAGAGAGACCGAGGAGTTC-3′ Reverse: 5′-CTGACGCTGACGAGGACAC-3′
Human β-actin	Forward: 5′-TCCTCTCCCAAGTCCACACA-3′ Reverse: 5′-GGGCACGAAGGCTCATCA-3′

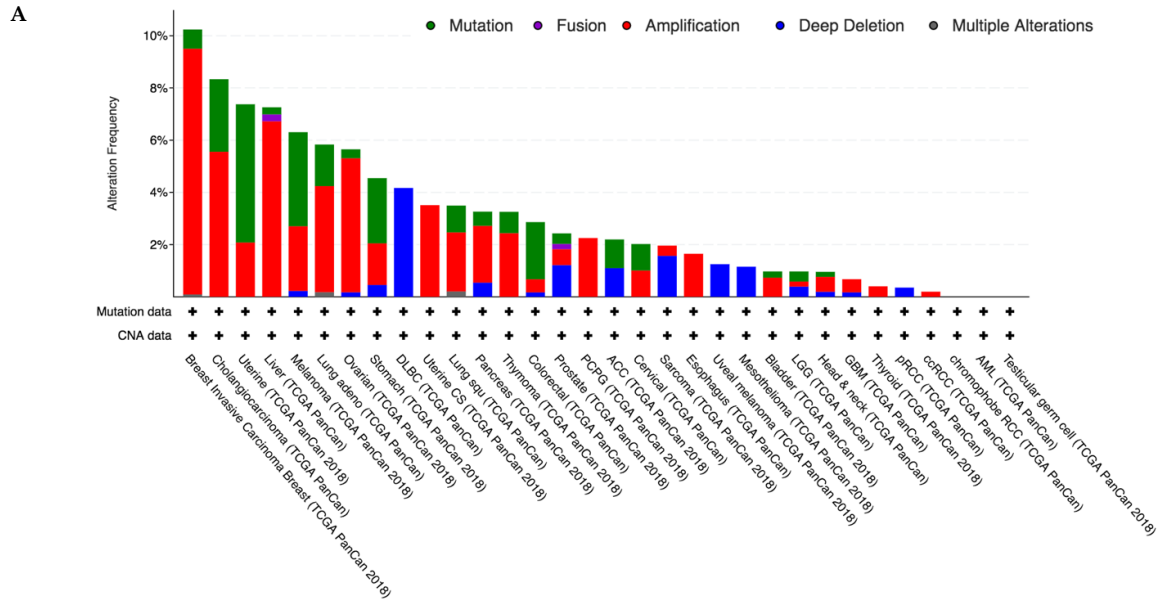
Bioinformatic analysis: Expression data of KMO and the top 500 KMO-correlated genes in breast cancer patients was retrieved from the Cancer Genome Atlas (TCGA) PanCancer Atlas Studies with cBioportal. The database for annotation, visualization and integrated discovery (DAVID) was used to carry out Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to reveal cancer stem cell-correlated pathways which might be linked to high KMO expression (Huang *et al.*, 2009).

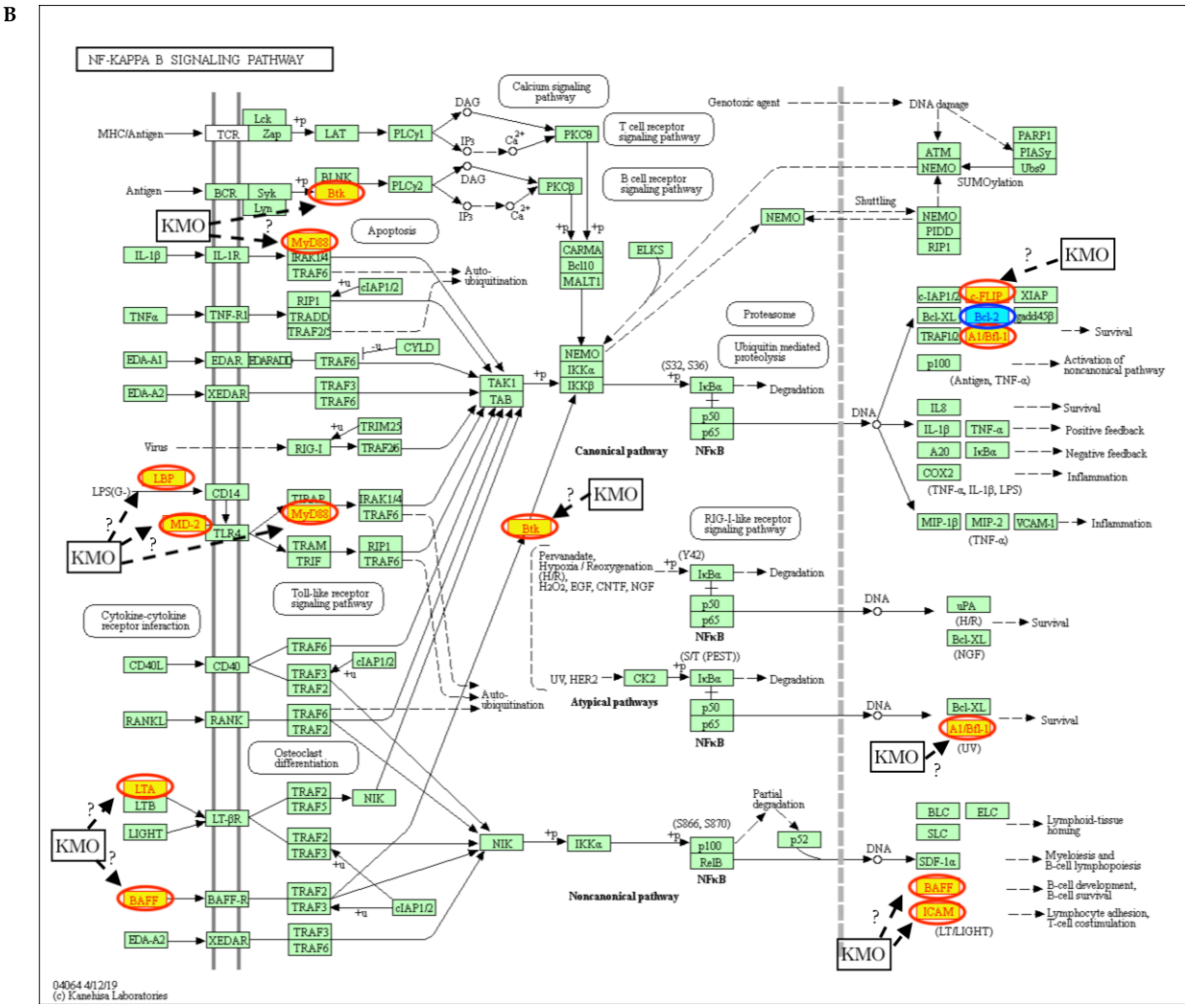
Statistical analysis: The mRNA expression data is presented as mean ± SD from 3 independent experiments. Student's *t*-test (two-tailed) was used to determine the statistical significance of differences.

Results

Bioinformatic analysis of the correlation between KMO and cancer stemness: The association between KMO and cancer stemness in human breast cancers was validated with data mining in the TCGA database by cBioportal. A higher gene alteration rate of KMO was found in patients with breast cancer than in those with other cancer types (Fig. 1A). Furthermore, the genes expressed in parallel with increased KMO were analyzed for their involvement in KEGG pathways (Fig. 1B-1C). Interestingly, the results revealed two common pathways associated with cancer stem cells, the NF-κB and JAK-STAT signaling pathways (Yang *et al.*, 2020), which were noted in the pathways which were closely related to genes whose expression correlated with KMO. Several genes, including *MYD88*, *BAFF* and other cancer-related genes, were

positively correlated with KMO in the NF-κB signaling pathway. Conversely, *BCL-2*, an apoptosis regulator gene, negatively correlated with KMO. As for the JAK-STAT signaling pathway, four and two genes involved in tumorigenesis, including *STAT*, *c-MYC* and *AKT*, positively and negatively correlated with KMO separately. The findings indicate that KMO might play a role in breast cancer stemness due to the association with the NF-κB and JAK-STAT signaling pathways.





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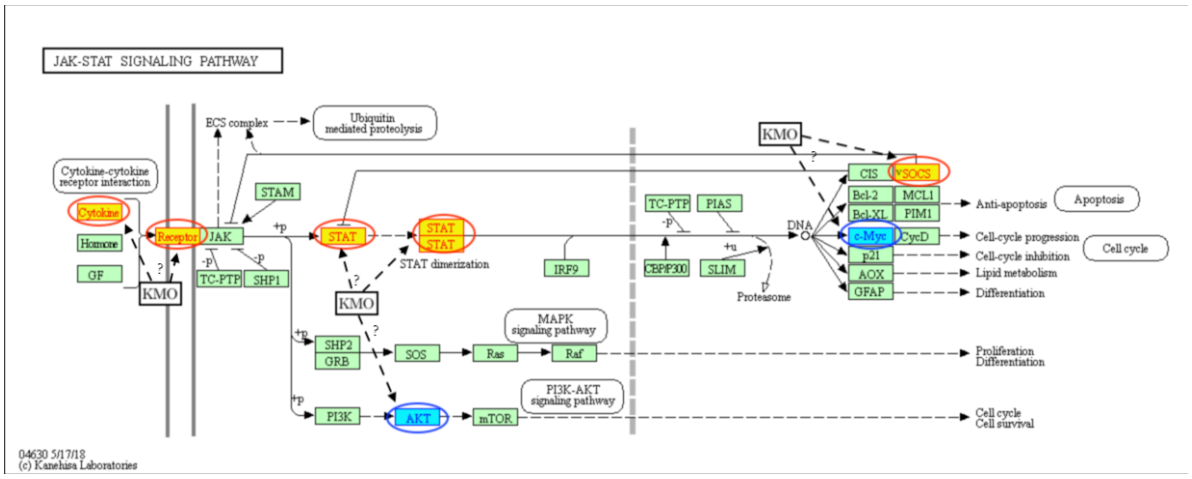
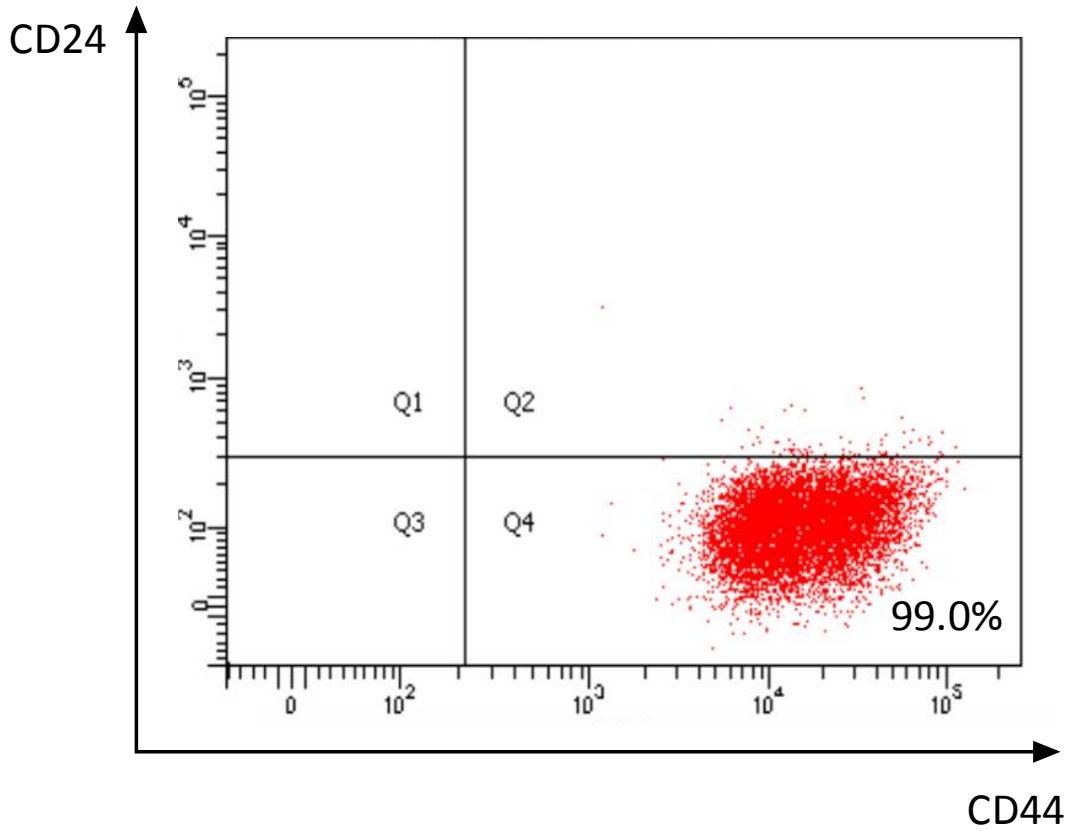


Figure 1 The correlation between KMO and cancer stemness analyzed in TCGA and KEGG pathways. (A) Alteration frequency of KMO in different cancer types. (B, C) KEGG pathways of KMO-associated genes. KMO positively-correlated genes in red circles and negatively-correlated genes in blue.

A knockdown of KMO decreased the gene expression of cancer stemness in canine mammary tumors: A canine mammary gland tumor cell line, CMT-1, was used as a model for studying the effects of KMO expression in cancer stemness. Both CD24 and CD44 are considered as potential biomarkers for cancer stem cells (Sheridan *et al.*, 2006). CMT-1 cells were identified from their stem cell-like properties as $CD44^{+}/CD24^{-}$ cells (Fig. 2A). Next, CMT-1 cells were infected with lentivirus containing shKMO-20 to silence the expression of KMO. After 48 hours of cell culture, a significant morphological change of the cells from slender to rounded shapes was observed in the KMO knockdown groups (Fig. 2B). This change in cell phenotype was not found in cells infected with shLuc-containing lentivirus. We then compared the differences in expression of a series of cancer stem cell markers, namely, *POU5F1*, *SOX2*, *NANOG*, and *KLF4*, in the knockdown and control groups by RT-qPCR. Those markers were studied in induced pluripotent stem cells (iPSCs) and were also applied in CSC studies (van Schaijik *et al.*, 2018). The results showed notably lower expression of stem cell markers in the knockdown groups (2 MOI) than in the mock control group (Fig. 2C). Therefore, the results reveal that knockdown of KMO can lead to the down expression of the genes related to cancer stemness.

A



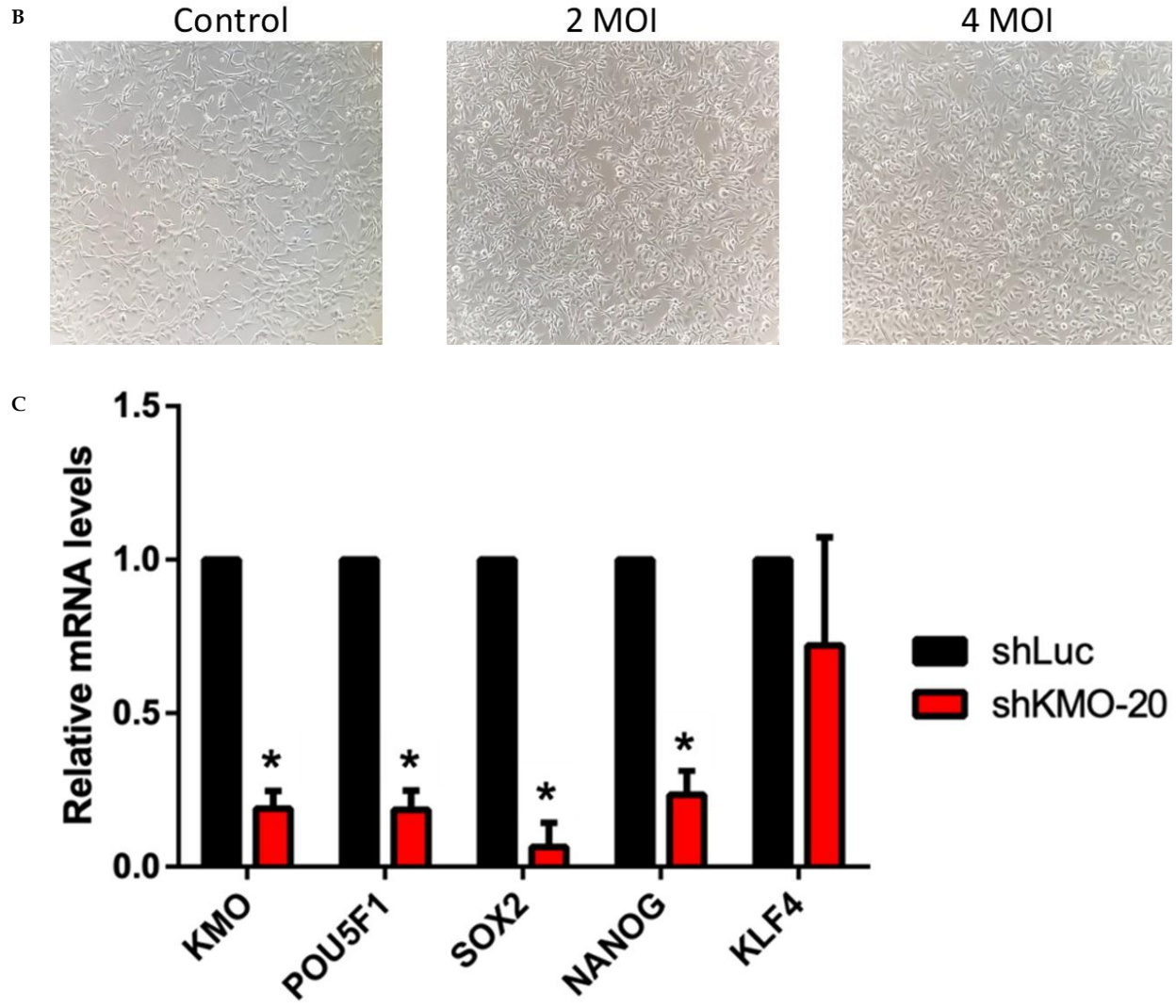


Figure 2 Expression of cancer stem cells in CMT-1. (A) Flow cytometry analysis was performed on the CMT-1 cell line to determine the expression of cancer stem cell markers. (B) Cells appeared more rounded in KMO knockdown groups. (C) Expression of a number of cancer stem cell markers, including *POU5F1*, *SOX2*, *NANOG*, *KLF4*, were evaluated with qRT-PCR. The results above showed a noticeable decreased expression of CSC markers when KMO expression is inhibited. Data was presented as mean ± SD. *: $P < 0.05$.

A knockdown of KMO decreased the gene expression of cancer stemness in human breast cancers: For human breast cancer, a triple-negative breast cancer cell line, MDA-MB-231, was used to validate the association between KMO expression and cancer stemness. This cell line expressed malignant $CD44^+/CD24^-$ markers, which were similar to those of the canine mammary gland tumor cell line, CMT-1 (Fig. 3A). Lentivirus was then used to infect cells for a knockdown of KMO expression in MDA-MB-231. RT-qPCR results also showed remarkable decreases in the stem cell markers when KMO expression was inhibited (Fig. 3B). Though no significant difference was observed after analysis with student's *t*-test, the obvious trend of decreased CSC markers cannot be ignored. The results together show that KMO expression negatively correlated with the expressions of cancer stemness genes in both canine mammary tumors and human breast cancer cell lines.

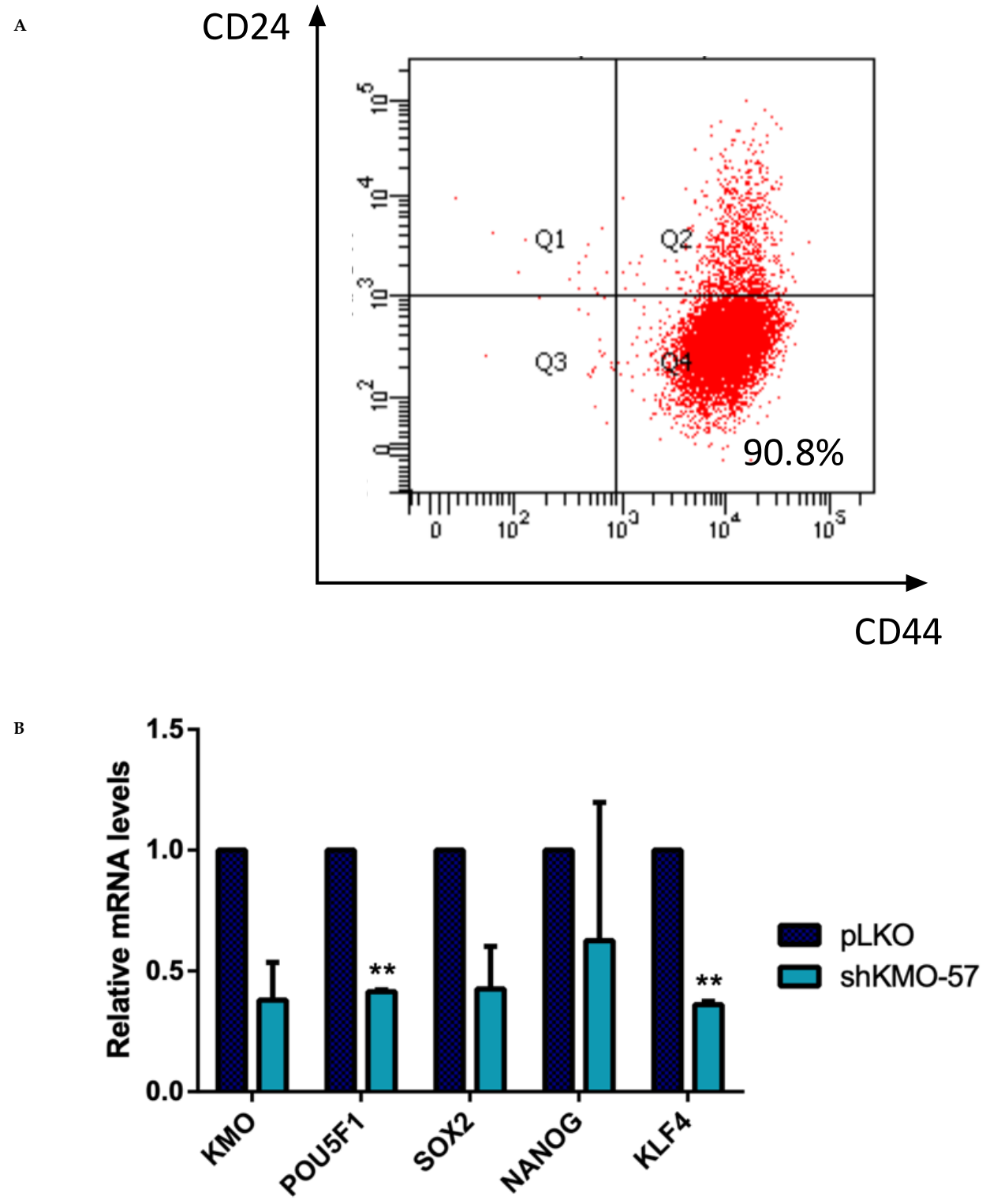


Figure 3 Correlation of KMO and CSC expression of cancer stem cells in MDA-MB-231. (A) Triple negative breast cancer cell line MDA-MB-231 also showed $CD44^{+}/CD24^{-}$ phenotype. (B) The expressions of *POU5F1*, *SOX2*, *NANOG* and *KLF4* were lower when KMO was knocked down. Data is presented as mean ± SD. **: $P < 0.01$.

Discussion

Both malignant canine mammary tumors and triple negative breast cancers show a high frequency of recurrence and metastasis, contributing to poor prognosis and lower survival rates for patients (Nguyen *et al.*, 2009; Stagg and Allard, 2013). Given that effective molecular targets cannot be defined for either, there is an urgent need to develop potential markers for both cancers. Since both molecular and clinical similarities were identified in CMT and HBC, CMT may also serve as an ideal model for the molecular characteristics of TNBC.

Epithelial–mesenchymal transition (EMT), a process which facilitates the metastasis of a tumor, is known to be coordinated by the presence of CSCs (Mani *et al.*, 2008). In fact, TNBC has been found to be composed of cells with high epithelial–mesenchymal plasticity, verifying the importance of CSC properties in this vigorous type of breast cancer (Doherty *et al.*,

2017). To support the significance of cancer stemness in CMT and TNBC, we used antibodies against CD44 and CD24, which are the established markers for CSCs (Rybicka and Król, 2016), to verify the presence of cancer stem cells in two specific cell lines, CMT-1 and MDA-MB-231. The results showed that both cell lines had the features of CSCs and therefore that further investigation regarding CSC properties could be conducted.

KMO is a unique enzyme in the kynurenine pathway, which determines the balance between 3-hydroxykynurenine and kynurenine acid, and causes the generation of several important neuronal diseases. Hence, drugs targeting KMO to treat degenerative neuronal diseases have been developed (Terness *et al*., 2002). In contrast, the correlation between KMO and cancer development is still not well recognized. Many earlier studies have instead focused on the correlation between indoleamine 2,3-dioxygenase (IDO), an upstream enzyme in the same pathway, and cancers (Macchiarulo *et al*., 2009). However, our recent study revealed that KMO over-expression is associated with increased malignancy and a lower survival rate in CMT patients, while downregulating its expression results in significant inhibition of the proliferation in CMT cell lines (Chiu *et al*., 2019). These findings suggest that KMO may play a significant role in cancer.

Since the presence of CSCs is an important property of breast cancer cells, we sought to further investigate whether KMO expression influences the characteristics of CSCs in both CMT and human TNBC cell lines. The reprogrammed transcription factors *POU5F1*, *SOX2*, *NANOG* and *KLF4* were used as CSC markers, as their expression levels have been known to correlate with poor prognosis and the malignant progression of human cancers (Li *et al*., 2011). The results of our study strongly indicated that inhibition of KMO expression in either cell line led to decreased expression of the chosen CSC markers, while a knockdown of KMO in CMT-1 gave the cells a rounded morphology, probably caused by a reversed tendency for the occurrence of EMT. Future studies will be needed to determine whether the rounding of the cell morphology is correlated with EMT or apoptosis, yet the correlation between KMO and pluripotent CSC markers was clearly observed.

Consistent with the finding of elevated KMO levels in CMT patients (Chiu *et al*., 2019), bioinformatic data retrieved from the TCGA database also revealed that the highest KMO alteration rates were those in patients with breast cancers, followed by cholangiocarcinoma and uterine tumors. Further retrieved data regarding KMO-associated signal pathways also included two CSC-related pathways, NF-κB and JAK-STAT, whose connections with CSC characteristics have been studied in various cancers (Yang *et al*., 2020). Investigation of the correlation between KMO expression and the two mentioned pathways may further establish the role of KMO in CSC characteristics, verifying the importance of KMO in CMT and TNBCs.

Genes involved in NF-κB and JAK-STAT pathways may play different roles in carcinogenesis with an increased expression of *KMO,* such as the activation of *STAT* contributing to CSC properties. (Galoczova *et al*., 2018). *c-MYC* and *AKT* facilitate tumor formation (Miller *et al*., 2012). *MYD88*, which promotes invasion and metastasis of cancers, and *BAFF*, which increases cell migration, epithelial-mesenchymal transition (EMT), and stemness in epithelial breast cancer cells (Pelekanou *et al*., 2018; Wu *et al*., 2018) correlated with higher *KMO* expression. Moreover, a downregulation of *BCL-2* was found when *KMO* was overexpressed in cancer cells while an increased level of *BCL-2* has been proven to be positively associated with a less malignant phenotype and more favorable prognosis in breast cancer (Frenzel *et al*., 2009). These findings indicate that overexpression of *STAT*, *MYD88* and *BAFF* and downregulation of *BCL-2* are involved in the development of tumorigenesis and cancer stemness when KMO is upregulated in breast cancer cell lines.

This study revealed that KMO affects the expression of CSC markers in CMT and TNBC cell lines while concurrently changing the cell morphology. We also found that cancer stemness-related genes was changed while the quantity of KMO mRNA expression declined simultaneously in HBC and CMT cell lines. Lastly, we also provided possible pathways for the further investigation of underlying mechanisms for the presented findings. Altogether, the results above strongly indicate that KMO is a potential target for TNBC and CMT treatment for inhibiting CSC expressions, which may lead to better prognosis and higher survival rates.

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