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KMT2C modulates migration and invasion processes in osteosarcoma cell lines

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ABSTRACT

In this study we investigated the role of KMT2C (a chromatin-modifying and remodelling protein) in osteosarcoma progression through cell migration and invasion assays in osteosarcoma primary and metastatic cell lines. Wound healing and transwell assays were used to detect changes of cell migration and matrigel assay was used to evaluate changes of cell invasion in primary and metastatic osteosarcoma cell lines after *KMT2C* siRNA transfection. We found that primary osteosarcoma cell lines showed the highest capacity of migration before mRNA *KMT2C* silencing and the highest capacity of invasion after mRNA *KMT2C* silencing; on the contrary, osteosarcoma metastatic cell line showed the highest capacity of migration after mRNA *KMT2C* silencing and the highest capacity of invasion before mRNA *KMT2C* silencing.

Our study supports data in favour of selective enhancer changes, KMT2C-mediated, in metastatic osteosarcoma probably due to the different microenvironment between primary and metastatic sites.

1. Introduction

Osteosarcoma is a bone malignancy that frequently metastasizes especially to the lung and the 5-year survival rate for patients with metastases at the time of diagnosis is only 20–28% [1,2]. Metastasis is a complex process that involves the detachment of osteosarcoma cells from the primary tumor, adhesion to extracellular matrix (ECM), local migration and distant invasion to colonize distant sites [3]. In every single step, tumour cells acquire specific behaviours that allow to distinguish them from the primary tumour [4]. In our previous study of Whole Exome Sequencing (WES) analysis, we found that the *KMT2C* gene, a key component of histone H3 lysine 4 methyltransferase complexes, showed the highest number of variations in most of the samples being analysed [5]. Previously, it has been shown that highly conserved epigenetic regulators are frequently mutated in cancer and some studies suggest that variations in coding sequences of regulating elements, which act on enhancers to recognize specific transcriptional factors, may be the cause of tumour development [6]. Moreover, in our study of gene expression analysis, we observed that some genes were upregulated only in metastatic cell line and that these values were modulated after *KMT2C* mRNA silencing performed on osteosarcoma cell lines [7]. According to our data, a recent study supports an enhancer dysregulation that determines the acquisition of metastatic behaviour and the epigenomic profiling reveals differential enhancer activity in primary versus metastatic tumours [8]. The goal will be to understand which are the targets with an altered enhancer activity; indeed, a therapy preventing metastasis development could represent a significant improvement in outcome for osteosarcoma patients since the therapeutic protocols include a conventional, nonspecific and toxic chemotherapy that has never changed since the 1960s [9]. In our study, we hypothesized that KMT2C affecting negatively or positively the enhancer activity of genes involved in osteosarcoma progression, could play a role of oncogene or oncosuppressor, particularly in the degradation and attachment of tumour cells to ECM [7]. The aim of this study is to better understand the role of KMT2C in osteosarcoma progression through its evaluation in cell migration and invasion.

Abbreviations: KMT2C, lysine (K)-specific Methyltransferase 2C; ECM, extracellular matrix; WES, whole exome sequencing; ATCC, American Type Culture Collection; FBS, fetal bovine serum; siRNA, small interfering RNA; cDNA, complementary DNA; PBS, phosphate-buffered saline; FAM, 6-carboxyfluorescein; PCR, polymerase chain reaction; Ct, cycle threshold; SPSS, statistical package for social science; H3K4, histone 3 lysine 4; CXCL12, C-X-C motif chemokine Ligand 12; CXCR4, C-X-C motif chemokine Receptor 4

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2. Materials and methods

2.1. Cell culture

143B, Saos-2, and Hs888 human osteosarcoma cells lines obtained from American Type Culture Collection (ATCC, Manassas, VT, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% to 15% fetal bovine serum (FBS), 1 Mm sodium piruvate, 100 U/mL of penicillin, and 100 mg/mL of streptomycin, at 37 °C and 5% CO_2, according to ATCC recommendations. 143B, and Saos-2 were osteosarcoma primary cell lines while Hs888 was an osteosarcoma metastatic cell line (lung). All cell lines derived from young adults with osteosarcoma diagnosis, one male (Hs888) and two females (143B and Saos-2).

2.2. KMT2C siRNA transfection

siRNA sequences targeting *KMT2C* and a negative control siRNA were designed and synthesized by Invitrogen (Life Technologies, Foster City, CA, USA). The siRNAs were designed according to the *KMT2C* complementary DNA (cDNA) sequence (Gene ID: 58,508). 143B, Saos-2, and Hs888 cells were seeded without antibiotics at $1/10^5$ per well in 6-well plates 2 days before transfection. When cells were 90% confluent, the cells were washed with PBS and siRNAs were transfected into 143B, Saos-2 and Hs888 cells using Lipofectamine RNAiMAX (Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions. The cells were harvested before and 24 h after transfection with *KMT2C* siRNA; then the cells were subjected to RealTime PCR to determine the mRNA expression of *KMT2C*. Total RNA was extracted with a SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's instructions. We confirmed the purity and quantity of RNA by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reverse transcribed into cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR products for human *KMT2C* and *β-ACTIN*, used as an internal standard, were detected using gene-specific primers and probes labelled with reporter dye FAM, which yielded a predicted amplicon of 58 and 171 bp, respectively. TaqMan RealTime quantitative PCR for *KMT2C* and *β-ACTIN* mRNA was performed on an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). PCR reaction was carried out in triplicate on 96-well plate with 10 u L per well using 1 × TaqMan Master Mix. After an incubation for 2 min at 50 °C and 10 min at 95 °C, the reactions continue for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. At the end of the reaction, the results were evaluated using the ABI PRISM 7500 software (Applied Biosystem, Foster City, CA, USA). The Ct values for each set of 3 reactions were averaged for all subsequent calculations. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression.

2.3. Wound healing assay

A wound-healing assay was used to evaluate 143B, Saos-2 and Hs888 cell motility before and after *KMT2C* siRNA transfection. Osteosarcoma cells (1×10^6/well) were seeded in six-well plates and cultured overnight. Then, confluent osteosarcoma cells were scratched carefully using 200 u L sterile pipette tips, and cell debris was discarded washing with culture medium twice and cultured again for up to 24 h with serum-reduced medium containing 1% FBS. Images of the plates were taken under a microscope at 0 and the gap closure was measured at 24 h and analysed using Image J software (Rawak Software, Inc. Germany).

2.4. Transwell assays

We used a Transwell insert (24-well insert, pore size 8 lm; Corning, Inc., Corning, NY) to determine the effect of *KMT2C* siRNA transfection on cell migration in vitro. The transfected and non-transfected cells were first starved in serum-free medium overnight, and 3×10^4 cells were re-suspended in serum-free medium and placed in the top chambers in triplicate. The lower chamber was filled with 10% FBS as the chemoattractant and incubated for 48 h. At the end of the experiments, the cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed and stained with 0.1% crystal violet. Five visual fields of each insert were randomly chosen under a light microscope and migrated cell population was evaluated by Image J software (Rawak Software, Inc. Germany).

2.5. Invasion assay

We used the Cell Invasion Assay Kit (Chemicon International, Temecula, CA, USA) to determine the effect of *KMT2C* siRNA transfection on cell invasion in vitro. It was performed in an Invasion Chamber, a 24-well tissue culture plate with 12 cell culture inserts. The inserts contain an 8 µm pore size polycarbonate membrane, over which a thin layer of ECMatrixTM is dried. Frist, 300 µl of warm serum free media was added to the interior of the inserts allowing to rehydrate the ECM layer for 1–2 hours at room temperature. Then, 500 µl of media containing 10% FBS was added to the lower chamber and a cell suspension containing 0.5×10^6 cells/ml in serum free media was added to each insert and incubated for 48 h. Invasive cells on lower surface of the membrane was stained by dipping inserts in the staining solution for 20 min and then washed two times. Five visual fields of each insert were randomly chosen under a light microscope and invasion cell population was evaluated by Image J software (Rawak Software, Inc. Germany).

2.6. Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). Two group comparisons were performed using two-tailed Student's t-test. P value < 0.05 was considered statistically significant.

3. Results

3.1. Effective targeting of KMT2C with RNA interference

A duration of 24 h after transfection with *KMT2C* siRNA, quantitative real-time PCR assay was performed to analyse the expression of *KMT2C* mRNA. The expression of *KMT2C* mRNA was suppressed in the groups transfected with *KMT2C* siRNA compared with the group without transfection. 143B and Saos-2 primary osteosarcoma cell lines showed an expression decrease of 95% and 97% respectively whereas the metastatic cell line Hs888 showed an expression decrease of 92%. The expression of *β-actin* mRNA showed no change as an internal control.

3.2. Cell migration

We investigated the effect of *KMT2C* mRNA silencing on the chemotactic motility of 143B, Saos-2 and metastatic Hs888 osteosarcoma cell lines using a wound-healing assay. Confluent osteosarcoma cells were scratched, and a continuous cell-free region was observed (T0). After 24 h the wound-healing assay showed that the motility capacity of primary osteosarcoma cells to move into the 'wound' region was significantly reduced in the group transfected with *KMT2C* siRNA compared with the group without transfection ($p < 0.0005$, $p < 0.0006$; Fig. 1a-b). In contrast, Hs888 metastatic cells' motile abilities were enhanced by *KMT2C* mRNA silencing compared with its control (Fig. 1c).

In the same way, the results of the transwell assay showed that the

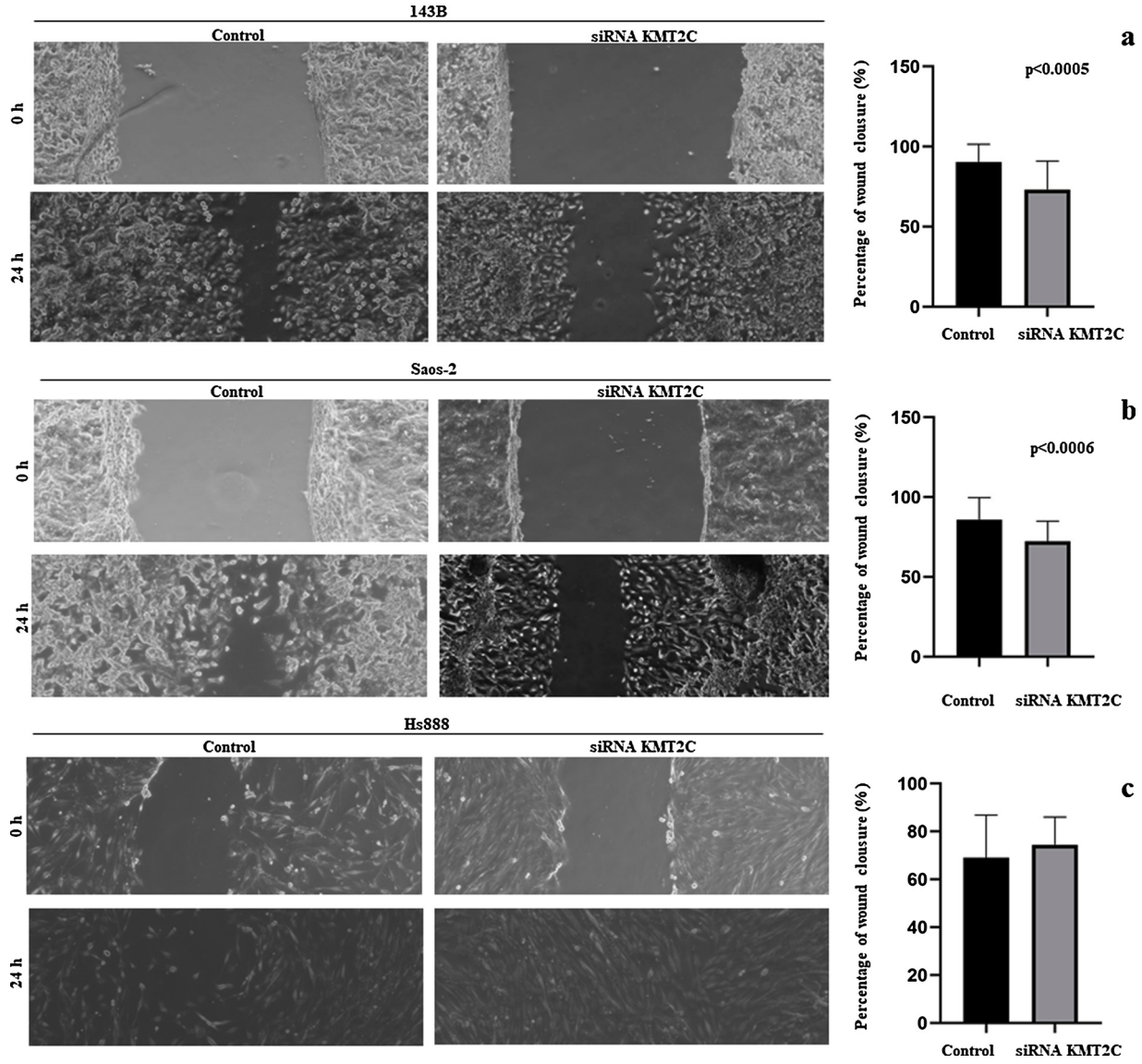

Fig. 1. Effect of *KMT2C* siRNA transfection on the migration capability of 143B, saos-2 and Hs888 osteosarcoma cell lines. Representative wound-healing images at 0 and 24 h (magnification: X100). The experiment was repeated at least three times independently. a) 143B primary osteosarcoma cell line; b) Saos-2 primary osteosarcoma cell line; c) Hs888 metastatic osteosarcoma cell line.

migrated primary osteosarcoma cells were more likely concentrated in the control group (without transfection), while a much lower number of primary osteosarcoma cells were found in the *KMT2C*-silenced group ($p < 0.0001$, $p < 0.0001$; Fig. 2a-b). Additionally, the migration ability of Hs888 metastatic cell line was enhanced in the group transfected with *KMT2C* siRNA compared to its control ($p < 0.0001$; Fig. 2c).

3.3. Cell invasion

To examine the effect of *KMT2C* mRNA silencing on tumour invasion of 143B, Saos-2 and Hs888 osteosarcoma cell lines, we performed an invasion assay and we obtained completely opposite data compared to cell migration. Indeed, primary osteosarcoma cells were able to move more easily through the ECM matrix when they were transfected with *KMT2C* siRNA compared to control ($p < 0.0242$; Fig. 3a-b); on the contrary, the metastatic cell line Hs888 showed a better ability to invade the ECM matrix when not transfected with *KMT2C* siRNA ($p < 0.0008$; Fig. 3c).

4. Discussion

Metastasis is the main problem of osteosarcoma patients affecting their prognosis and survival and the mechanisms underlying this process remain largely unclear [10]; for these reasons it is necessary to identify new targets to develop new therapies that could prevent metastasis development in osteosarcoma. We obtained valuable information about KMT2C in osteosarcoma that allowed us to hypothesize that KMT2C could affect negatively or positively the enhancer activity of genes involved in osteosarcoma progression [7]. Accumulated evidence indicates that the mono-methylation of H3K4 occurs more frequently on enhancer regions; it is therefore possible that mutations in proteins like KMT2C could inactivate tumour suppressors or activate oncogenes [11]. Also, we have previously shown that *KMT2C* is frequently mutated in osteosarcoma [5] and some studies suggest that variations in the coding sequences of regulatory elements could act on enhancers to recognize specific transcription factors and modify their expression [12]. Moreover, our previous data showed that KMT2C differentially regulated the expression of some genes between primary and metastatic cell lines [6]. Coherent with our data, a recent study based on

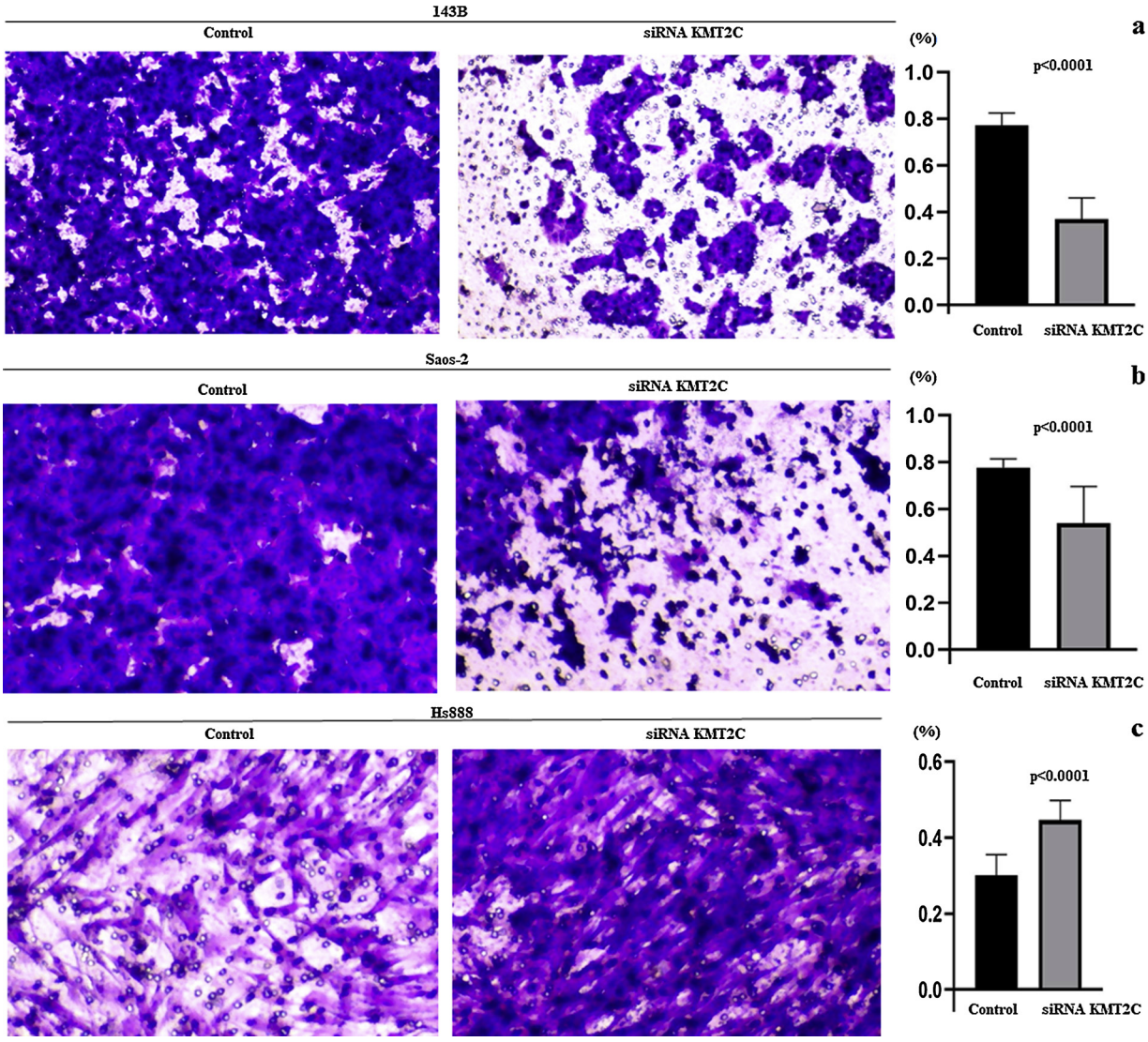

Fig. 2. Effect of *KMT2C* siRNA transfection on the migration capability of 143B, saos-2 and Hs888 osteosarcoma cell lines. Representative transwell images before and after transfection. Cells were stained with crystal violet (magnification: ×100). The experiment was repeated at least three times independently. a) 143B primary osteosarcoma cell line; b) Saos-2 primary osteosarcoma cell line; c) Hs888 metastatic osteosarcoma cell line.

epigenomic profile, reveals a differential enhancer activity in primary versus metastatic osteosarcoma so that an altered enhancer activity would allow to promote osteosarcoma metastasis [8]. Indeed, it was shown how the enhancer epigenome changes in metastatic progression as well as in physiological process [8]. In this study, we evaluated the role of KMT2C in migration and invasion of osteosarcoma primary and metastatic cell lines after its mRNA silencing. We showed how KMT2C plays a key role in the modulation of migration and invasion in osteosarcoma cell lines and how these processes are differently modulated by KMT2C in primary and metastatic cell lines. Indeed, our data showed that KMT2C mainly acts on migration in primary osteosarcoma cell lines and on invasion in metastatic cell line probably through the regulation of selected genes thus strengthening the hypothesis of differential, albeit non-random, enhancer activity in primary versus metastatic osteosarcoma [8]. This difference could likely be due to the lung microenvironment that could stimulate osteosarcoma cells to change their epigenomic profile. Understanding which genes are modulated due to an altered activity of KMT2C in osteosarcoma metastatic cells could suggest potential targets for antimetastatic therapies.

Moreover, in our previous study we noted that the expression of one gene, involved in migration and invasion in osteosarcoma (*CXCL12*), was strongly downregulated following mRNA *KMT2C* silencing in osteosarcoma metastatic cell line [7]. Recently, the chemokine CXCL12/CXCR4 signalling has been extensively investigated in osteosarcoma due to the relevance in metastasis progression and poor patient outcome [12]. Indeed, this signalling drives the tumour cells homing to distant sites (e.g. lung). Moreover, being CXCL12 also involved in T-cell homing, it has been shown that in osteosarcoma CXCL12 expression is associated with the number of intra-tumoral cytotoxic T-cells and it has been hypothesized that alteration of this signalling could be the cause of the failure of the use of checkpoint inhibitors in osteosarcoma immunotherapy [13–15]. Our hypothesis is that the epigenetic alteration of CXCL12/CXCR4 axis expression could affect the ability of tumour cells to metastasize and prevent the T-cell homing to the tumour site, and that the failure of osteosarcoma immunotherapy could be also due to a lack of patient's stratification based on CXCL12/CXCR4 expression.

5. Conclusions

In conclusion, our study shows how KMT2C modulates differently the migration and invasion processes in osteosarcoma metastatic cell line. KMT2C, as a chromatin remodelling protein, could act on different

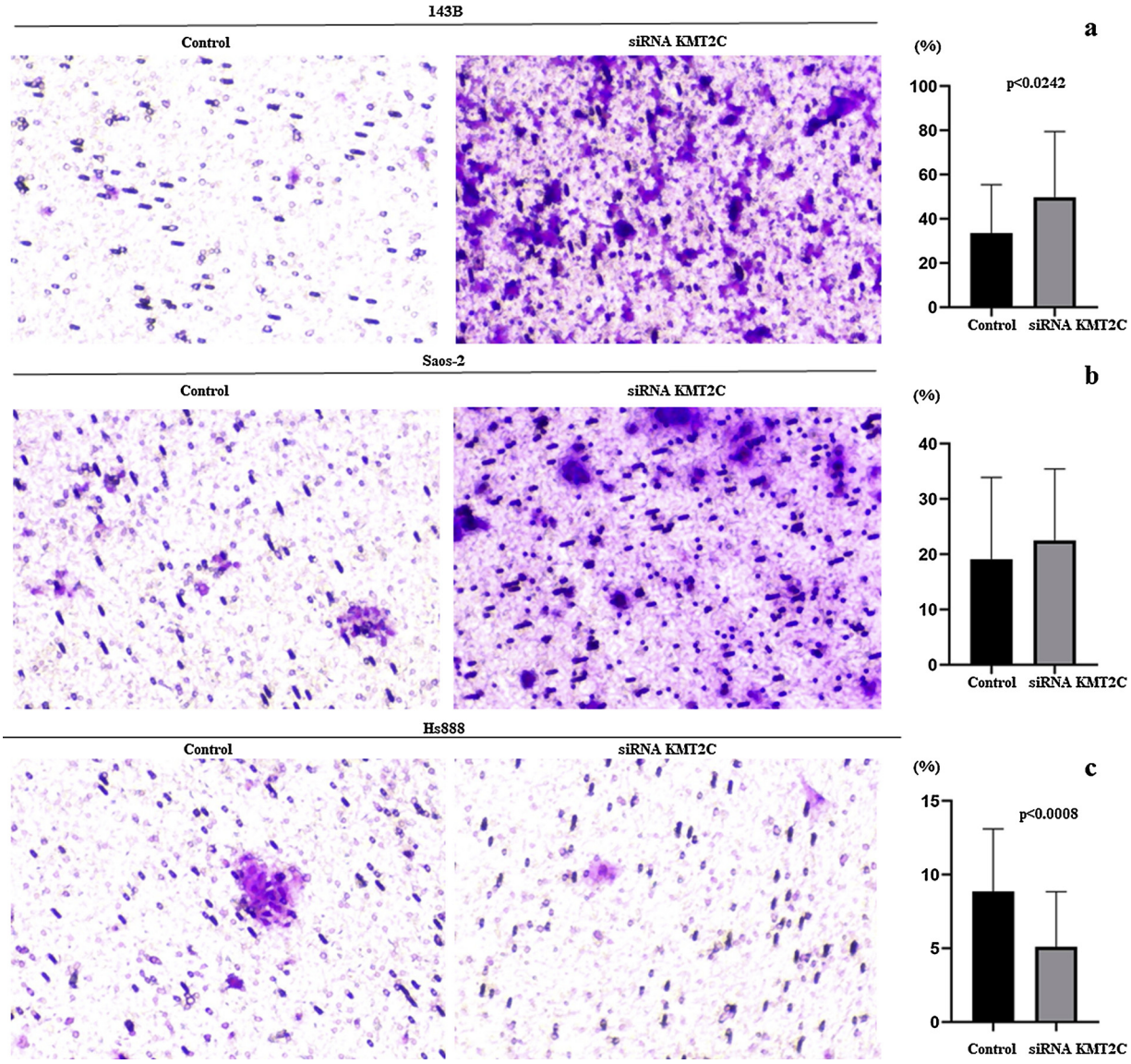

Fig. 3. Effect of *KMT2C* siRNA transfection on the invasion capability of 143B, saos-2 and Hs888 osteosarcoma cell lines. Representative invasion images before and after transfection. Cells were stained with crystal violet (magnification: ×100). The experiment was repeated at least three times independently. a) 143B primary osteosarcoma cell line; b) Saos-2 primary osteosarcoma cell line; c) Hs888 metastatic osteosarcoma cell line.

targets that differ from the primary tumour probably due to the different microenvironment. So, identifying the KMT2C targets could be important to obtain an antimetastatic therapy for osteosarcoma treatment. Moreover, in the field of immunotherapy, it would be interesting to better understand the role of KMT2C in the modulation of CXCL12-CXCR4 axis in osteosarcoma metastatic progression to provide a tailored combined immunotherapy for each patient based on their CXCL12-CXCR4 profile.

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