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ORIGINAL ARTICLE

MiR-26a inhibits stem cell-like phenotype and tumor growth of osteosarcoma by targeting Jagged1

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MicroRNAs (miRNAs) are important epigenetic regulators of gene expression. Although several miRNAs have been implicated in osteosarcoma, their role in regulation of osteosarcoma cancer stem cells (CSCs) remains unknown. Here we demonstrated that miR-26a is downregulated in osteosarcoma CSCs when derived by either sarcosphere generation, chemodrug or aldehyde dehydrogenase (ALDH) activity selection. Lentiviral overexpression of miR-26a in ZOS and 143B osteosarcoma cells decreases the expression of stem cell markers and suppresses sarcosphere formation, as well as ALDH activity. Moreover, miR-26a overexpression inhibits the tumor cell growth both *in vitro* and *in vivo*. We further demonstrate that miR-26a directly target Jagged1, one of the Notch ligand, and that its tumor suppressive effects are mediated through inhibition of Jagged1/Notch signaling. Importantly, reduced miR-26a expression, as determined by *in situ* hybridization in patient tumors ($n = 92$), is associated with lung metastasis and poor overall survival of osteosarcoma patients. Together, these data suggest the essential role of miR-26a/Jagged1/Notch pathway in regulating the stem cell-like traits of osteosarcoma cells and provide a potential target for osteosarcoma therapy.

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INTRODUCTION

Osteosarcoma is the most common primary malignant tumor of bone in children and adolescents.[1,2] In last two decades, the 5-year survival has remained at 60–70% in patients with localized tumor, whereas this is only 20% in patients who have lung metastasis.[3] The treatment failure may arise from an inability to eliminate the cancer stem cells (CSCs).[4,5] CSCs are a small subset of cancer cells within a tumor, which have the ability to self-renew and maintain the tumor.[6] It is believed that the persistence of CSCs is a primary cause of tumor relapse and metastasis.[7] Therefore, therapies by suppressing CSCs function may offer a promising strategy to enhance the control of osteosarcoma.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that are able to suppress gene expression by binding to the 3′-untranslated region (UTR) of target messenger RNA (mRNA).[8] Emerging literature suggests that miRNAs are involved in the regulation of stem cell function and cancer progression.[9,10] Osteosarcoma is recognized as a de-differentiation disease caused by genetic and epigenetic changes that interrupt osteoblast differentiation from mesenchymal stem cells (MSCs).[1] MSCs are also regarded as the cell of origin for osteosarcoma CSCs.[11] Therefore, abnormal expression of miRNAs involved in the osteoblastic differentiation of MSCs may be associated with the CSC regulation and osteosarcoma pathogenesis.

Notch signaling pathway has a pivotal role in the developmental processes by controlling stem cell maintenance and cell fate determination.[12] Notch signaling activation is initiated by binding of its ligands (Jagged and Delta-like) to the transmembrane Notch receptors. Upon ligand–receptor binding, the Notch receptor proteins undergo two sequential proteolytic cleavages to release Notch intracellular domain (NICD). NICD then translocates to the nucleus, interacts with RBP-Jκ transcription factors and further activate the transcription of its downstream targets, such as HES and HEY family of genes.[13] Aberrant Notch signaling activation has been found in a variety of cancers, including ovarian carcinomas, colon cancer and osteosarcoma.[14,15] Importantly, current studies indicate that the Notch signaling pathway has a vital role in maintaining CSC function.[16] However, the effect of Notch signaling on osteosarcoma CSCs and the upstream control of Notch activation in osteosarcoma currently remains unclear.

MiR-26a expression is reported to be increased during osteogenesis, and its overexpression promotes the osteoblastic differentiation of MSCs.[17] In this study, we show that miR-26a is downregulated in osteosarcoma CSCs. Overexpression of miR-26a suppresses the stem cell-like properties and induces osteosarcoma growth inhibition and chemosensitivity. Moreover, we find that miR-26a attenuates the malignancy of osteosarcoma through suppressing the Jagged1/Notch pathway. Our study provides a better understanding of miR-26a in regulating osteosarcoma CSC self-renewal and progression.

RESULTS

MiR-26a is downregulated in osteosarcoma cancer stem cells

To investigate the role of miR-26a in stem cell-like properties and progression of osteosarcoma, we first examined its expression in osteosarcoma CSCs. We previously used sarcosphere selection to enrich osteosarcoma CSCs that are more tumorigenic than their parental cells.[18] Comparison of miR-26a expression in sarcosphere versus their parental cells in six independent osteosarcoma cell

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lines using real-time PCR (qPCR) showed that miR-26a is significantly downregulated in CSC-enriched population (Figure 1a). Chemotherapeutic drugs have recently been shown to selectively enrich CSCs,[19] and we have also demonstrated that drug-resistant osteosarcoma cells possess properties of CSCs.[18] Therefore, we determined miR-26a expression levels in osteosarcoma cells treated with doxorubicin (DOX, a commonly used chemotherapeutic drug for osteosarcoma patients). We observed miR-26a to be significantly lower in survived osteosarcoma cells (except Saos-2) following 3-day DOX treatment (Figure 1b). Further, tumor cells with the increased aldehyde dehydrogenase (ALDH) activity are reported to have stem/progenitor properties and ALDH serves as a marker for CSCs.[20,21] Wang *et al.*[22] have identified a highly tumorigenic osteosarcoma cell subpopulation based on high ALDH activity. Consistent with this, we found that highly tumorigenic osteosarcoma cell lines (ZOS, ZOSM and 143B) have a higher proportion of $ALDH^{high}$ cells in comparison with poorly tumorigenic osteosarcoma cell lines (U2OS, MG63 and Saos-2; Figures 1c and d). In addition, miR-26a expression was lower in $ALDH^{high}$ than $ALDH^{low}$ population (Figure 1e). These data suggest that miR-26a downregulation may contribute to osteosarcoma CSCs.

MiR-26a attenuates stem cell-like properties of osteosarcoma cells

As miR-26a is downregulated in osteosarcoma CSCs, we determine whether miR-26a influences the stem cell-like phenotypes in osteosarcoma cells. ZOS and 143B cells were transduced with lentiviruses carrying either miR-26a or control miRNA (Figure 2a). Sarcosphere assay showed that miR-26a-overexpressing cell lines formed much smaller and significantly fewer sarcospheres (Figure 2b) and decreased the ALDH activity remarkably (Figure 2c). Next, we examined the expression of several stem cell markers—including OCT3/4, NANOG, SOX2, nucleostemin and CD133—in miR-26a and control transfectants. As shown in Figure 2d, these stem cell markers were significantly reduced in miR-26a-transduced ZOS and 143B cells. The protein expression of SOX2, known to maintain the self-renewal of osteosarcoma-initiating cells, was further confirmed to be lower by immunofluorescence (Figure 2e). In summary, miR-26 reintroduction decreases the stem cell-like population in osteosarcoma and attenuates the stemness property of osteosarcoma cells.

MiR-26 restoration inhibits tumorigenicity and chemoresistance

Next, we determined whether miR-26a impairs osteosarcoma cell survival. To this end, ZOS and 143B cells were transiently

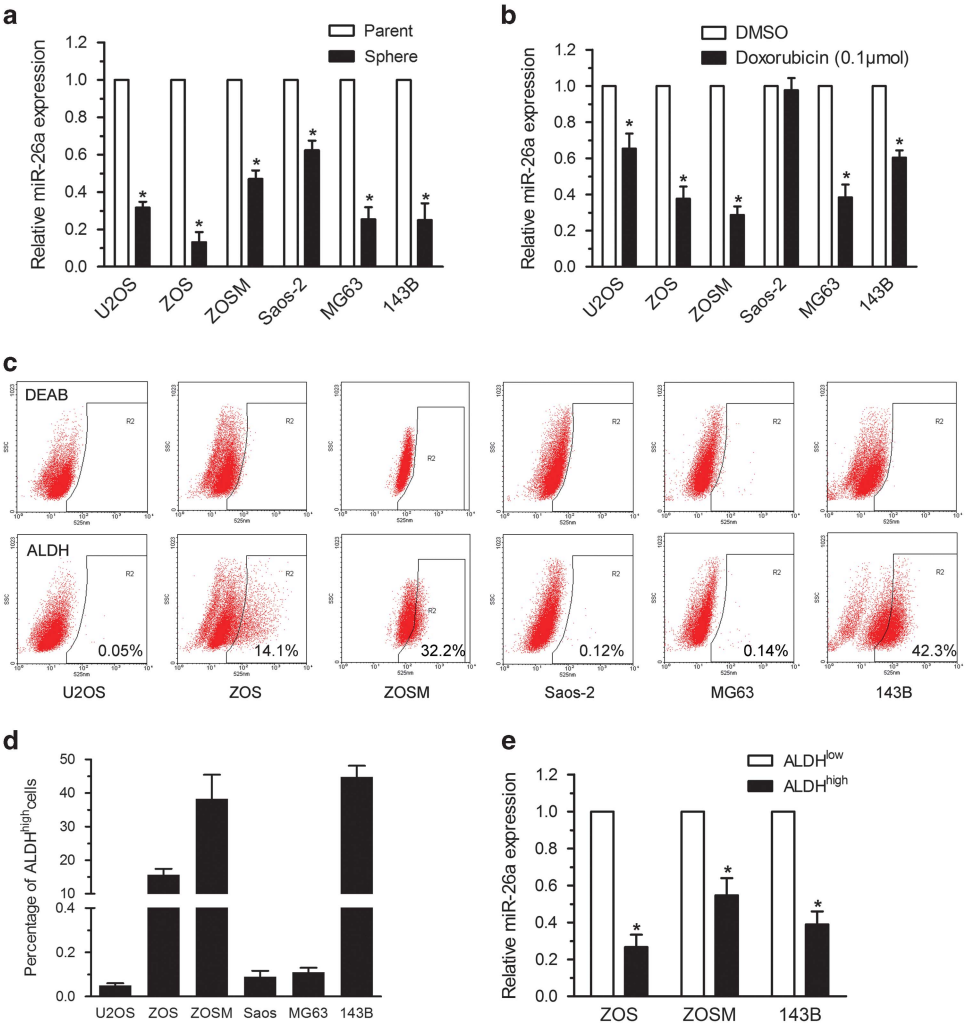

Figure 1. MiR-26a expression is downregulated in osteosarcoma cancer stem cells (CSCs). (**a**) MiR-26a expression in CSCs isolated from six osteosarcoma cell lines was determined by qPCR. (**b**) MiR-26a expression in osteosarcoma cell lines after dimethyl sulfoxide (DMSO) or DOX (0.1 μM for 3 days) treatment was analyzed by qPCR. (**c**) ALDH activity in six osteosarcoma cell lines was determined by fluorescence-activated cell sorting analysis. (**d**) The percentage of $ALDH^{high}$ cells in osteosarcoma cell lines. (**e**) MiR-26a expression in osteosarcoma cells with low or high ALDH activity was determined by qPCR. Data shown are mean ± s.d. of three independent experiments. $^*P < 0.05$ by two-tailed Student's *t*-test.

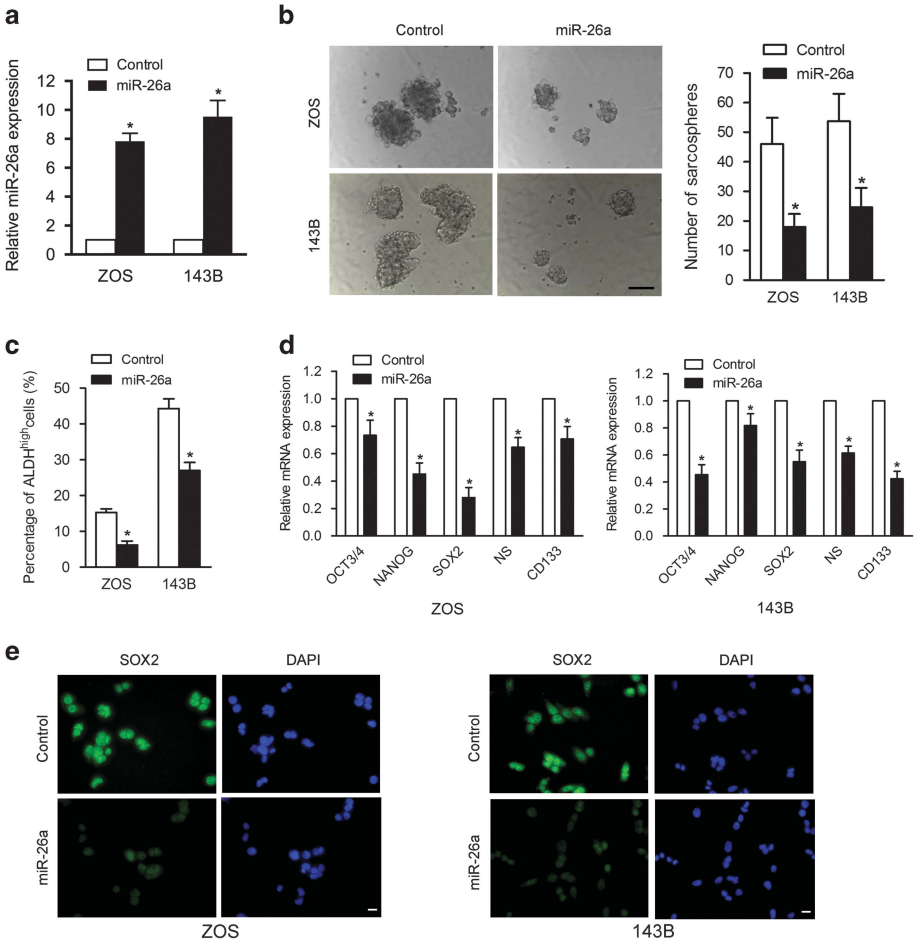

Figure 2. MiR-26a inhibits the stem cell-like properties of osteosarcoma cells. (**a**) MiR-26a expression was determined by qPCR. (**b**) Sarcosphere formation capacity of ZOS and 143B cells was analyzed after miR-26a overexpression. Scale bar, 100 μm. (**c**) The percentage of $ALDH^{high}$ cells was determined by fluorescence-activated cell sorting analysis. (**d**) The mRNA expression of stem cell markers (OCT3/4, NANOG, SOX2, nucleostemin (NS) and CD133) in control and miR-26a-overexpressing cells were examined by qPCR. (**e**) SOX2 protein expression in indicated cells was analyzed by immunofluorescence. Scale bar, 20 μm. Data shown are mean ± s.d. of three independent experiments. $^*P < 0.05$ by two-tailed Student's *t*-test.

transfected with either miR-26a mimic or inhibitor. The efficacy of miR-26a overexpression and inhibition were verified by qPCR (Supplementary Figure 1). As shown in Figure 3a, miR-26a mimic reduced, whereas the miR-26a inhibition enhanced the tumor cell proliferation. Tumor cells with miR-26a mimic also displayed increased apoptosis rate in comparison with control cells, although no significant difference in the number of apoptotic cells was observed in cells transfected with miR-26a inhibitor (Figure 3b). In clonogenic assay, miR-26a-overexpressing cells significantly repressed colony formation (Figure 3c), indicating that miR-26a suppresses the tumor-initiating potential of osteosarcoma cells. We then determined whether reintroduction of miR-26a could enhance the chemosensitivity to DOX in osteosarcoma cells, and found that miR-26a overexpression decreased the cell viability and increased cell apoptosis, following DOX treatment (Figures 3d and e). Consistently, the activity of caspase-3 was substantially higher in DOX-treated miR-26a-overexpressing cells in comparison with controls (Figure 3f).

We then evaluated the influence of miR-26a on *in vivo* tumor growth of osteosarcoma. As shown in Figure 3g, the average xenograft volume of miR-26a group at 5 weeks was remarkably decreased (>36%) than the control group. We also found that the number of Ki-67- and proliferating cell nuclear antigen- (cell proliferation markers) positive cells were substantially decreased in tumors from miR-26a-overexpressing group (Figure 3h). To further confirm whether miR-26a overexpression attenuates the tumorigenic potential of osteosarcoma cells, we performed a limiting dilution assay. The indicated cells were injected subcutaneously at decreasing numbers into nude mice; at 1×10^5 cell number, control cells formed tumors at 100% incidence (6/6), whereas only 50% of miR-26a-overexpressing cells led to tumor formation (3/6; Table 1). We noted that tumor latency was significantly longer in miR-26a group (Figure 3i). At 1×10^4 cell number, 2/6 controls developed tumors, although no tumor formed in the miR-26a group when examined at 60 days after cell injection (Table 1). These data indicate that miR-26a expression in osteosarcoma could suppress the tumor initiation and development.

To further examine the effect of miR-26a overexpression on chemotherapy sensitivity *in vivo*, mice-bearing-specific tumor xenografts were treated with DOX. As shown in Figure 3j, although miR-26a overexpression did not exhibit a greater anti-tumor effect than DOX-treated group, combination of miR-26a with DOX significantly decreased the tumor burden when compared with any single intervention. These data suggest that restoration of miR-26a enhances the sensitivity of osteosarcoma cells to chemotherapeutic drugs.

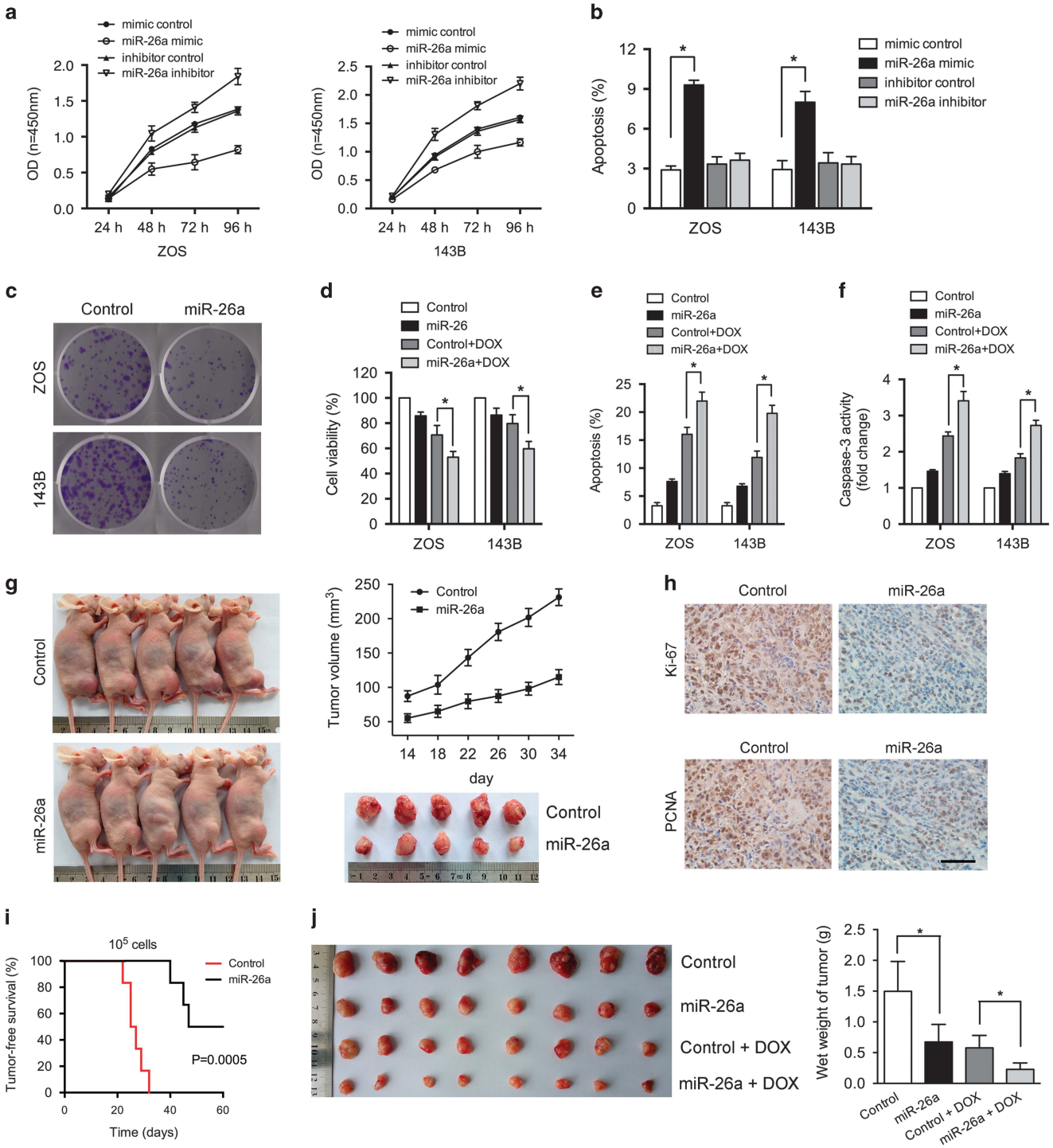

Figure 3. MiR-26a suppresses tumorigenesis and chemoresistance of osteosarcoma. (**a**) Cell proliferation of ZOS and 143B cells was determined by CCK-8 assay after transfection of control, miR-26a mimic or inhibitor. (**b**) The apoptosis rate of indicated cells were determined by fluorescence-activated cell sorting (FACS) analysis. (**c**) Clonogenic ability of ZOS and 143B cells was examined after miR-26a overexpression. (**d**) Cell viability of control and miR-26a-overexpressing cells following DOX treatment (0.1 μM for 24 h) was measured by CCK-8 assay. (**e**) The apoptosis rate was determined by FACS analysis. (**f**) Caspase-3 activity was determined by Caspase-3 Colorimetric Assay kit (Abcam). (**g**) Representative image of orthotopic tumor in the right proximal tibia of nude mice at 5 weeks after injection ($n = 10$ per group). (**h**) Immunohistochemistry analysis of Ki-67 and proliferating cell nuclear antigen in tumors from indicated 143B cells bearing mice. Scale bar, 100 μm. (**i**) Kaplan–Meier analysis of tumor-free survival for mice with 1×10^5 cells injection (log-rank test, $P = 0.0005$). (**j**) Subcutaneous tumor model was used to assess the effect of miR-26a on the chemoresistance of 143B cells *in vivo*. The tumors were collected at 4 weeks and the wet weight of tumor was measured by electronic scale. Data shown are mean ± s.d. of three independent experiments. *$P < 0.05$ by two-tailed Student's *t*-test (**b**) or one-way analysis of variance (**d**–**f** and **j**).

JAG1 is a direct target of miR-26a in osteosarcoma cells

We sought the mechanism of anti-tumor effects induced by miR-26a by screening its targets using two online prediction programs—microRNA.org and Targetscan. There were 2013 genes overlapping between the target genes predicted by these two programs (Supplementary Table 1). Here we mainly focused on the genes involved in CSC pathways[16,23] (Wnt, Notch and Hedgehog): *CCND2*, *ERBB4*, *GSK3B*, *FGF9*, *JAG1*, *SKP2*, *WNT5A*, *IL2RA*, *STK36* and *WISP1*. We then examined the expression of miR-26a and these genes of our interest in osteosarcoma cells, using qPCR. Our results showed that *JAG1* (encodes Jagged1) was significantly downregulated in miR-26a-overexpressing cells and upregulated in the cells transfected with miR-26a inhibitor (Figure 4a). Jagged1 is one of the Notch ligands and has been reported to be upregulated in osteosarcoma.[14] It is rational that downregulation of miR-26a in osteosarcoma may lead to Jagged1 overexpression. Indeed, we further observed that Jagged1 protein level were also significantly decreased after miR-26a overexpression, and conversely, increased by transfection of miR-26a inhibitor in both ZOS and 143B cell lines (Figure 4b). Moreover, a negative correlation between miR-26a and *JAG1* mRNA expression was found in human osteosarcoma tumor specimens (Figure 4c) as measured by qPCR, indicating that *JAG1* may be regulated by miR-26a. We then performed luciferase reporter assay to determine whether miR-26a could directly bind to the 3′-UTR of *JAG1* (Figure 4d). ZOS and 143B cells were then co-transfected with vectors harboring wild-type or mutant *JAG1* 3′-UTR (Figure 4e) and miR-26a mimic. Luciferase activity was markedly decreased after the transfection with wild-type vector and miR-26a mimic in ZOS cells (Figure 4f). Luciferase activity was also significantly decreased when the poorly conserved binding site 1 (6mer) was mutated, whereas mutation of conserved

Table 1. Limiting dilution assay

Cell number	*Tumor formation*	
	Control	*miR-26a*
1×10^5	6/6	3/6
1×10^4	2/6	0/6
1×10^3	0/6	0/6

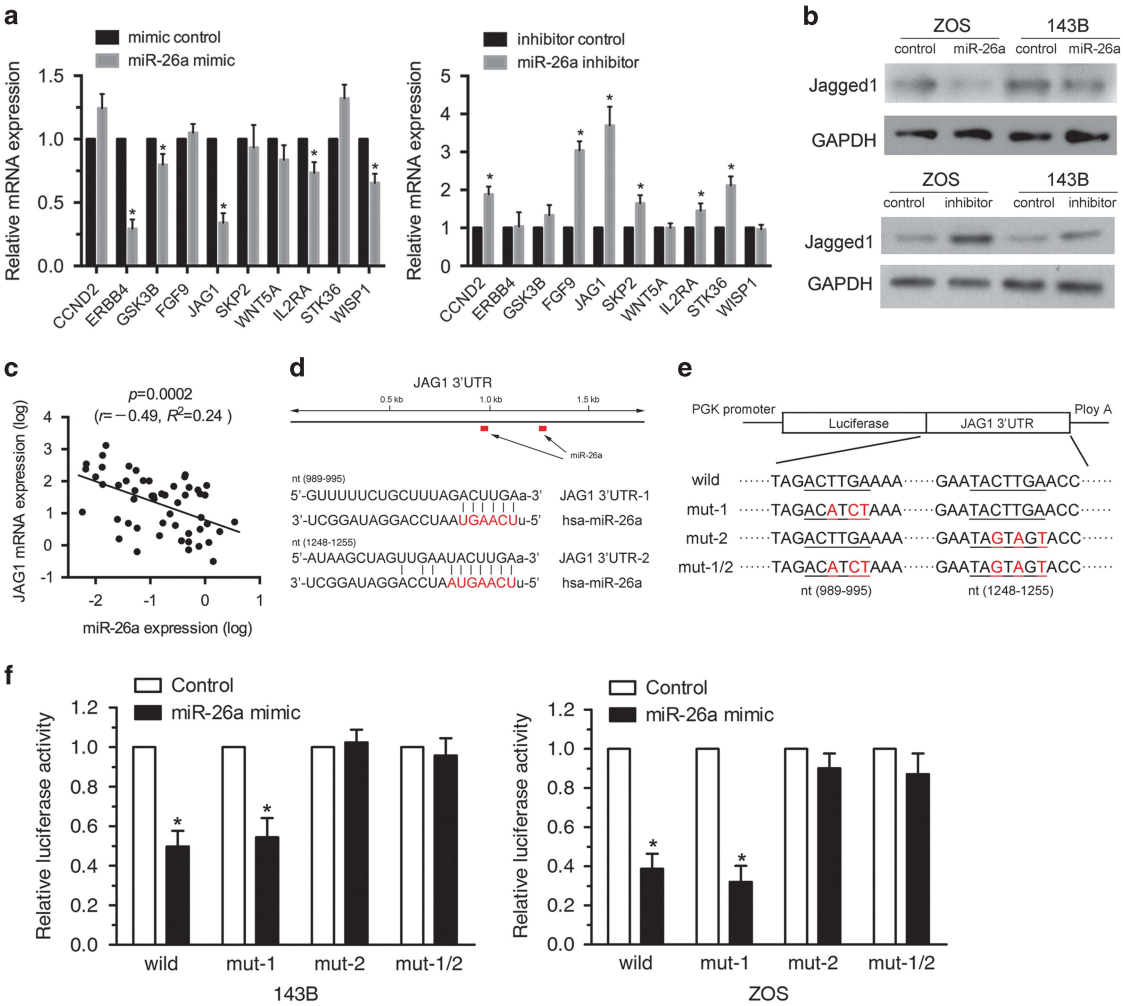

Figure 4. MiR-26a directly targets Jagged1 in osteosarcoma cells. (**a**) mRNA expression of indicated genes were determined by qPCR. (**b**) JAG1 protein levels in indicated cells were determined by western blot. (**c**) The expression of miR-26a and JAG1 in osteosarcoma tissues ($n=53$) were examined by qPCR. Pearson's correlation analysis ($r=-0.49$, $R^2=0.24$, $P=0.0002$). (**d**) Diagram of predicted binding sites of miR-26a on the 3′-UTR of JAG1 gene. (**e**) Diagram of JAG1 3′-UTR wild-type and mutant reporter constructs. (**f**) Luciferase reporter assays were performed in ZOS and 143B cells with co-transfection of indicated wild-type or mutant 3′-UTR constructs and miR-26a mimic. The data shown are representative of three independent experiments. Data shown are mean ± s.d. of three independent experiments. *$P<0.05$.

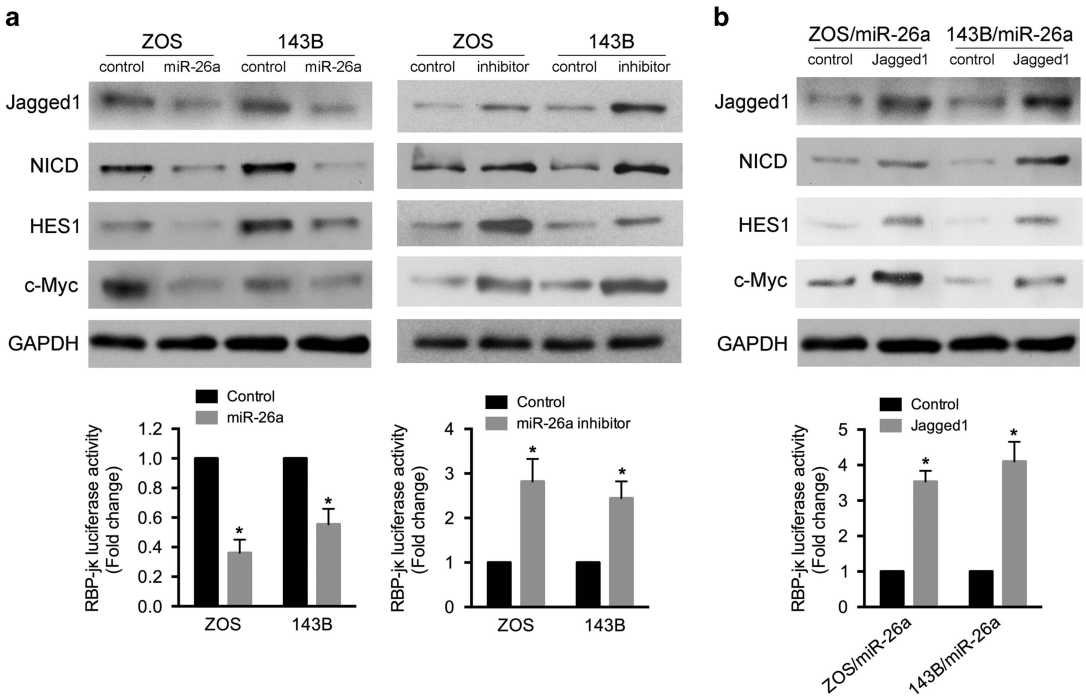

Figure 5. MiR-26a suppresses the Notch activity in osteosarcoma. (**a**) Expression of Jagged1, NCID and Notch target genes were determined by western blot. An RBP-jκ luciferase reporter assay was performed in the indicated cells. (**b**) Expression of indicated proteins and RBP-jκ luciferase reporter activity were examined in the indicated cells. The data shown are representative of three independent experiments. Data shown are mean ± s.d. of three independent experiments. $^*P < 0.05$.

binding site 2 (7mer-m8) nearly rescued the decrease. Similar results were obtained with 143B cells. These data suggest that miR-26a directly regulate *JAG1* expression through its binding to site 2 (nt1248–1255) in the 3′-UTR of *JAG1*.

MiR-26a suppressed Jagged1/Notch pathway in osteosarcoma cells

Given that Jagged1 is a ligand for Notch receptors; we next determined the influence of miR-26a over the Notch activation in osteosarcoma cells. Level of NICD was decreased after miR-26a overexpression in ZOS and 143B cells (Figure 5a). Moreover, the Notch-induced RBP-Jκ transcriptional activity and the expression of Notch target genes (HES1 and c-Myc) were consistently lower in miR-26a-overexpressing cells than control cells. In contrast, Notch activity was activated by the miR-26a inhibitor transfection (Figure 5a). The role of Jagged1 in miR-26a-induced Notch inhibition was then examined by transducing with Jagged1 or control lentivirus in miR-26a-overexpressing ZOS or 143B cells. We found that NICD levels, and HES1 and c-Myc expression was elevated upon Jagged1 overexpression in ZOS/miR-26a and 143B/miR-26a cells (Figure 5b). Luciferase activity assay also showed that Notch-induced transcriptional activity was increased in Jagged1-overexpressing cells. Together, these observations demonstrate that miR-26a impairs Notch signaling through repressing the Jagged1 expression.

MiR-26a reduces osteosarcoma malignancy via Jagged1 suppression

We next performed gain-of-function and loss-of-function analysis to determine whether the anti-tumor effect of miR-26a was mediated via repression of Jagged1 in osteosarcoma cells. First, we knocked down Jagged1 expression by lentiviral small hairpin RNA to investigate whether Jagged1 repression recapitulates the suppressive effect of miR-26a (Figure 6a). Both sarcosphere formation capacity and the proportion of $ALDH^{high}$ cells were significantly decreased after the Jagged1 repression in ZOS and 143B cells (Figures 6b and c). In the meanwhile, Jagged1 knockdown also suppressed the expression of SOX2 (Figure 6d) and led to the inhibition of tumor cell growth and colony formation similar to those seen upon miR-26a overexpression (Figures 6e and f). Second, we examined whether ectopic expression of Jagged1 can reverse miR-26a-induced tumor suppression. ZOS/miR-26a cells with Jagged1 overexpression formed sarcospheres that were comparable with those in vector controls (Figure 7a). Also, elevated expression of stem cell marker SOX2 was found in Jagged1-overexpressing ZOS/miR-26a cells (Figure 7b). Similar results were obtained using 143B cells (Supplementary Figure 2). Furthermore, the average tumor volume and tumor weight were significantly increased after Jagged1 overexpression in ZOS/miR-26a cells (Figure 7c). Taken together, our results demonstrate that miR-26a inhibits osteosarcoma malignancy by dowonregulating Jagged1.

MiR-26a is a prognostic marker for osteosarcoma patients

Finally, we tested the clinical relevance of miR-26a in human primary osteosarcoma specimens. Surgical specimens from 92 cases of osteosarcoma were examined for miR-26a expression by *in situ* hybridization (ISH). The correlations between miR-26a expression and clinical characteristics are presented in Table 2. Kaplan–Meier analysis indicated that the patients with high miR-26a expression had better overall survival than those with low expression (Figure 8a). Moreover, the risk of lung metastasis for patients in the miR-26a high-expression group was lower compared with that of low-expression group (Figure 8b). Given that miR-26a inhibits osteosarcoma malignancy by targeting Jagged1, we also examined the co-expression pattern of miR-26a and Jagged1 in these patient osteosarcoma tumor specimens. Immunohistochemical analysis for Jagged1 was performed and scored in the same cohort of 92 cases of osteosarcoma. Chi-squared analysis indicated that miR-26a expression was negatively correlated with the expression of

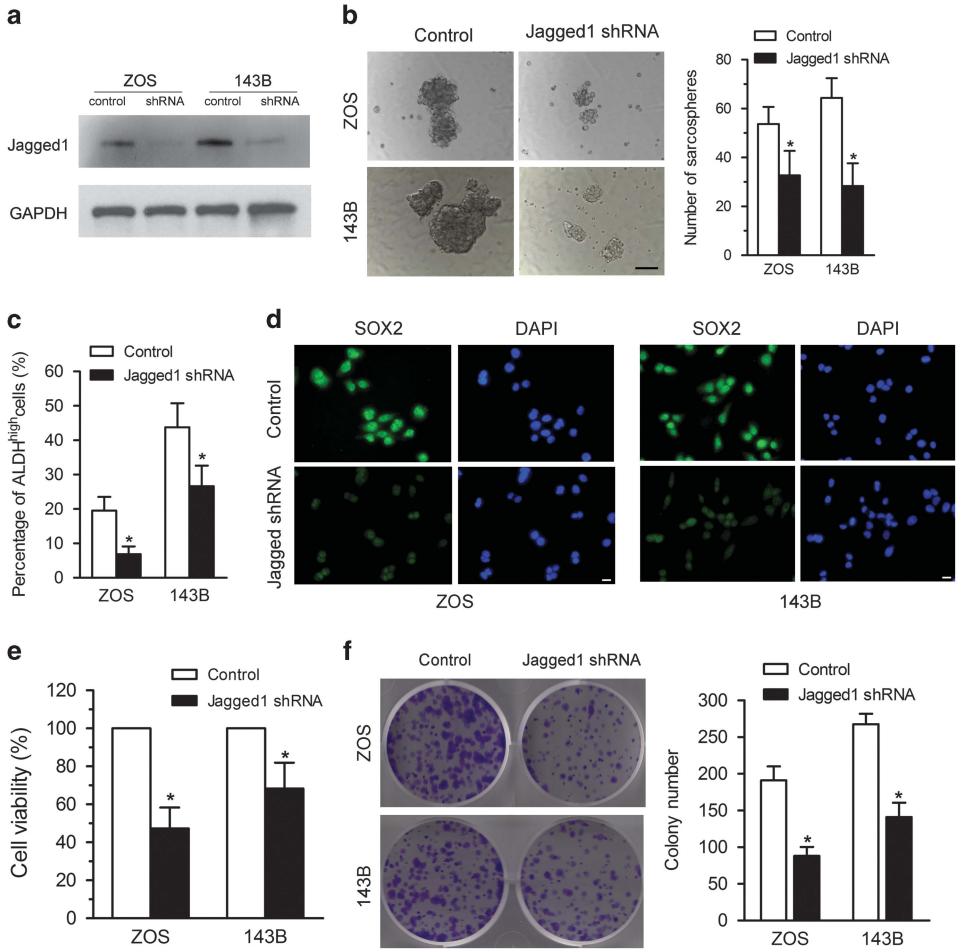

Figure 6. Jagged1 knockdown inhibits stem cell-like properties and cell growth of osteosarcoma. (**a**) Jagged1 protein expression was determined by western blot. (**b**) Sarcosphere formation capacity of ZOS and 143B cells was analyzed after Jagged1 knockdown. Scale bar, 100 μm. (**c**) The percentage of $ALDH^{high}$ cells was determined by fluorescence-activated cell sorting analysis. (**d**) SOX2 protein expression in indicated cells was analyzed by immunofluorescence. Scale bar, 20 μm. (**e**) Cell viability of indicated cells was determined by CCK-8 assay. (**f**) Clonogenic ability of ZOS and 143B cells was examined after Jagged1 knockdown. Data shown are mean ± s.d. of three independent experiments. $^*P < 0.05$.

Jagged1 ($P = 0.019$; Figure 8c), which further revealed that Jagged1 suppression is tightly linked to the anti-tumor effects of miR-26a in osteosarcoma.

DISCUSSION

MiRNAs are a class of critical gene regulators at post-transcriptional level that have been shown to be involved in almost every aspect of cancer cell function.[24] MiR-26a, a member of miRNA-26 family, regulates the differentiation of several cell types, such as pancreatic cell,[25] myoblast[26] and monocytes.[27] MiR-26a is also found to be able to facilitate the osteogenic differentiation of bone marrow-derived MSCs,[17] suggesting that miR-26a might have a key role in osteoblastic lineage commitment. Osteosarcoma is a de-differentiation cancer disease caused by the interruption of osteoblast differentiation of MSCs that are regarded as one of the cell of origin for osteosarcoma CSCs.[1,11] Therefore, we hypothesized that miRNAs, such as miR-26a, involved in osteoblast differentiation are involved in modulating osteosarcoma CSCs. Indeed, some studies have demonstrated that miR-133a and miR-29b, both of which are bone-regulating miRNAs,[28] could impair the malignant phenotypes of osteosarcoma CSCs.[29,30] Consistently, we found that miR-26a is down-regulated at osteosarcoma CSCs. Overexpressing miR-26a decreased the expression of stem cell markers and sarcosphere formation capacity in two independent cell lines, ZOS and 143B. miR-26a overexpression also inhibited tumorigenesis and chemoresistance both *in vitro* and *in vivo*. These results indicate that miR-26a is a potential regulator of osteosarcoma CSCs. Importantly, we demonstrated that miR-26a downregulation is associated with poor prognosis and lung metastasis in osteosarcoma patients. Very recently, Song *et al*.[31] reported that downregulation of miR-26a promoted migration and invasion of osteosarcoma cells, which further supports our conclusions.

Identifying the critical pathways involved in stem cell-like properties is important for developing novel molecular targets for a better control of osteosarcoma. Studies showed that the pathways governing the stem cells function have essential roles in CSCs regulation.[23] For example, Wnt signaling controls the self-renewal of mammary stem cells and regulates the mammary gland development at different stages,[32] whereas the aberrant Wnt pathway activation by stromal protein POSTN allows maintenance of breast CSCs.[33] Wnt signaling is also a key regulator of osteoblast commitment and differentiation of MSCs.[34] Our previous study demonstrated that salnomycin targets osteosarcoma CSCs by suppressing the Wnt/β–catenin pathway.[18] However, additional pathways involved in osteosarcoma CSCs function remain elusive. Notch signaling is an evolutionarily

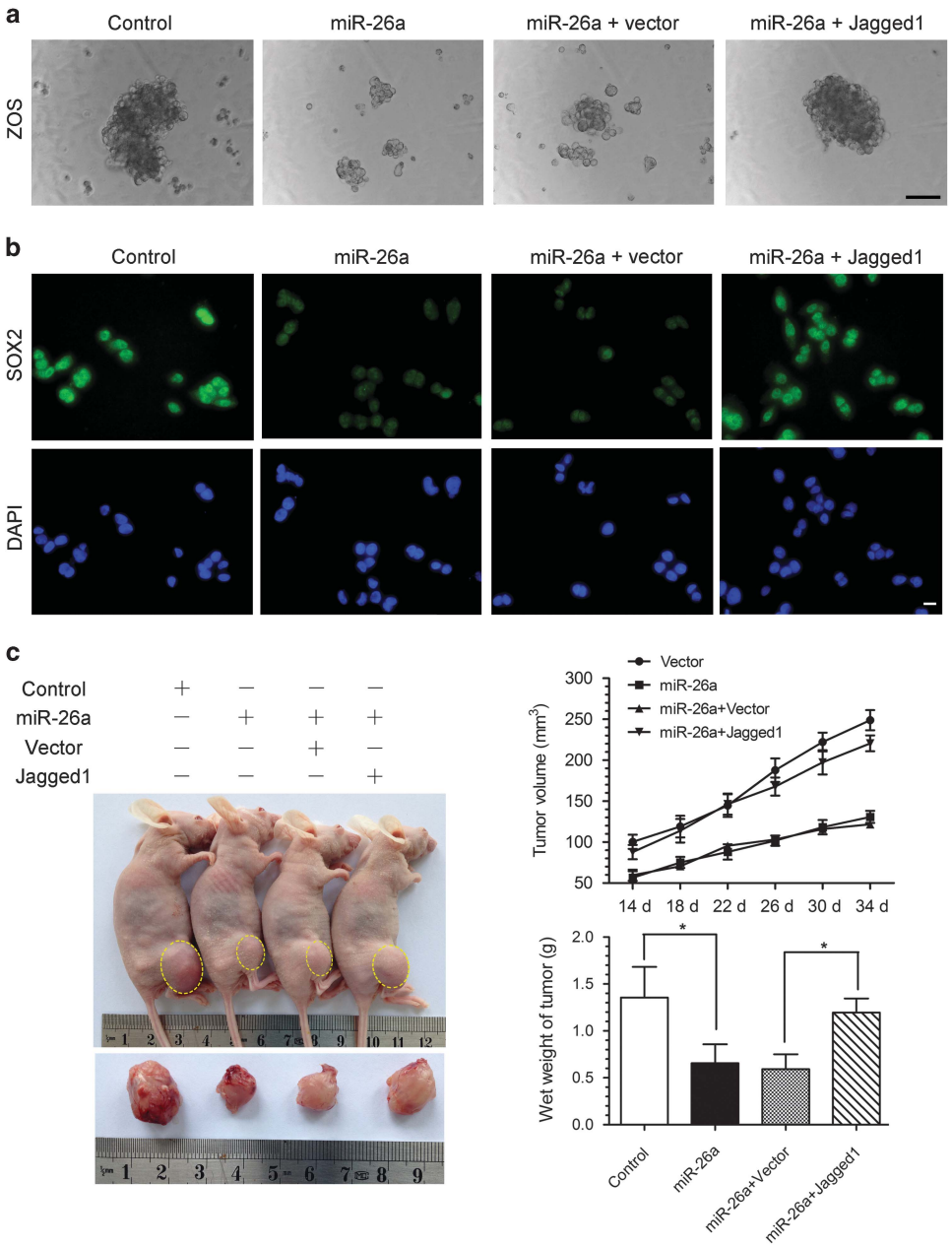

Figure 7. MiR-26a suppresses stem cell-like properties and tumor growth of osteosarcoma by targeting Jagged1. (**a**) Sarcosphere formation was performed in indicated cells. Scale bar, 100 μm. (**b**) SOX2 expression in indicated cells was determined by immunofluorescence. Scale bar, 20 μm. (**c**) The indicated cells were injected into the right proximal tibia of nude mice ($n = 10$ per group). Tumor sizes were measured every 3 days. The tumors were collected at 5 weeks and the wet weight of tumor was measured by electronic scale. $^*P < 0.05$ by one-way analysis of variance.

conserved pathway that regulates cell fate (including osteoblast) determination.[12,34] Aberrant activation of Notch has been found in osteosarcoma cell lines and tissues.[14] Although the oncogenic functions of Notch signals in osteosarcoma have been well documented,[14,35,36] its role in osteosarcoma CSCs is unclear. Our results demonstrate the Notch inactivation by either miR-26a overexpression or Jagged1 knockdown decreases stem cell markers and impairs sarcosphere formation, $ALDH^{high}$ cell numbers, chemoresistance and osteosarcoma tumor cell growth, implying Notch inhibition as an effective means of suppression for the stem cell-like properties in osteosarcoma cells. Consistently, Tao *et al*.[37] found conditional expression of the NICD in immature osteoblasts to be sufficient for driving osteosarcoma development in a mouse model. Those observations further support an essential role of Notch signaling in osteosarcoma initiation and progression. We propose that Notch is a potential therapeutic target for treating osteosarcoma.

To develop anti-CSCs therapy, it is important to specifically isolate CSCs. Recent studies have revealed several strategies to enrich for osteosarcoma CSCs that are based on: (1) cell surface markers: CD133, CD117 and Stro-1; and (2) intrinsic cellular properties: sarcosphere formation, side population, ALDH activity and chemoresistance.[38] Here we used sarcospheres, chemodrug and ALDH activity selection to enrich CSCs. We showed that all 6 osteosarcoma cell lines could form sarcosphere under suspension conditions, including two primary osteosarcoma cell lines ZOS and ZOSM. Particularly, the highly malignant 143B cells formed larger sarcospheres in a shorter time. Consistently, we found tumorigenic cells had substantially higher ALDH activity than non-tumorigenic cells, supporting higher ALDH activity as a property of

Table 2. Association of miR-26a expression and patient clinicopathological characteristics in 92 osteosarcoma tissues

	Number	*miR-26a Expression level*		P-*value*[a]
		High	*Low*	
Age				0.764
⩽20	66	30	36	
21–40	24	13	11	
>40	2	1	1	
Gender				0.401
Male	63	32	31	
Female	29	12	17	
Location				0.492
Distal femur	50	25	25	
Proximal tibia	24	10	14	
Proximal humerus	9	4	5	
Proximal femur	4	1	3	
Others	5	4	1	
Enneking				0.575
IIB	73	36	37	
III	19	8	11	
Relapse				0.784
Yes	7	3	4	
No	85	41	44	
Lung metastasis				**0.005**
Yes	30	8	22	
No	62	36	26	
Death				**0.023**
Yes	34	11	23	
No	58	33	25	

[a]Chi-square test. The bold *P*-values are statistically significant.

osteosarcoma CSCs. Although subpopulations isolated by such approaches show stem cell-like features, specific markers for osteosarcoma CSC are still missing. Further efforts are required to identify more specific markers, which will enhance our understanding of the biology of osteosarcoma CSCs.

In conclusion, we found that miR-26a is downregulated in osteosarcoma CSCs and its reintroduction suppresses the stem-like traits of osteosarcoma cells, consequently inhibiting chemoresistance and tumor growth by inactivating the Jagged1/Notch signaling. Our studies not only identify the miR-26/Jagged1/Notch axis is a critical regulator of osteosarcoma CSCs, but also provide a novel therapeutic target for osteosarcoma clinical treatment.

MATERIALS AND METHODS

Cell culture and miRNA transfection

U2OS, MG63, Saos-2 and 143B were obtained from the American Type Culture Collection (ATCC) and were cultured according to the instructions. Primary human osteosarcoma cells ZOS and ZOSM were previously described.[39] All cells were free of mycoplasma contamination and were recently authenticated by short tandem repeat profiling at China Center for Type Culture Collection (CCTCC, Wuhan, China). MiR-26a mimic, inhibitor and the negative controls were purchased from RiboBio (Guangzhou, China) and transfected into osteosarcoma cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction.

Tissue samples

The formalin-fixed, paraffin-embedded surgical specimens were collected from the tissue bank of the Department of Pathology. Patients ($n=92$) who met the following criteria were included: (1) osteosarcoma of the limb; (2) received standard neochemotherapy followed by surgical resection and postoperative chemotherapy; and (3) with available follow-up (>5 years) information. For mRNA analysis, fresh tumor specimens were snap-frozen in liquid nitrogen immediately after surgical resection and stored at −80 °C. Osteosarcoma was confirmed histopathologically, and only tumor samples ($n=53$) that were composed of >80% tumor cells were used for the study.

Sarcosphere formation assay

Sarcosphere formation assay was performed as previously described.[18] The experiments were performed in triplicate.

Real-time PCR

Total RNA was isolated from osteosarcoma cells and tissues by using RNeasy Mini Kit (Qiagen, Hilden, Germany). SYBR PrimeScript miRNA RT-PCR kit (Takara, Shiga, Japan) was used to determine the miR-26a expression. First strand complementary DNA was synthesized by First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) and real-time PCR was carried out using SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The PCR primers were listed in Supplementary Table 2.

ALDEFLUOR assay

ALDEFLUOR kit (Stem Cell Technologies, Vancouver, BC, Canada) was used to determine the ALDH activity by fluorescence-activated cell sorting according to the manufacturer's instructions. The experiments were performed in triplicate.

Lentiviral transduction studies

Precursor miR-26a clone (HmiR0044-MR03), JAG1 open reading frame expression (EX-M0722-Lv105) and small hairpin RNA (HSH004470-LVRU6P) clones were obtained from Genecopoeia. Lentivirus was produced using Lenti-Pac HIV Expression Packaging kit (Genecopoeia, Rockville, MD, USA). To generate the stable cell lines, osteosarcoma cells were transduced with the indicated lentiviruses (MOI = 10–20) and selected with 1 μg/ml puromycin (Sigma-Aldrich, St Louis, MO, USA).

Cell viability assay

Cell viability assay was performed as previously described.[40] The experiments were performed in triplicate.

Apoptosis assay

For assessment of apoptosis, indicated cells were collected and stained with the Annexin V Apoptosis Detection kit APC (eBioscience, San Diego, CA, USA). The apoptosis rate was determined by fluorescence-activated cell sorting analysis. The experiments were performed in triplicate.

Colony formations

Colony formation assay was performed as previously described. Cells were seeded in a six-well plate at a density of 500 cells per well. After incubating for 12 days, cells were fixed in methanol and stained with crystal violet. The number of colonies that contained >50 cells were counted under microscope. The experiments were performed in triplicate.

Caspase-3 activity assay

Caspase-3 activity was measured using a Caspase-3 Colorimetric Assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. The experiments were performed in triplicate.

In vivo animal study

Six-week-old female BALB/c nude mice were obtained from Guangdong Medical Laboratory Animal Center (Foshan, China). Orthotopic osteosarcoma model ($n=10$ per group) was performed as previously described.[40] For subcutaneous tumor models, 2×10^6 control ($n=16$) or miR-26a-overexpressing ($n=16$) cells were injected subcutaneously into the flanks of nude mice.[41] One week after injection, mice from control and miR-26a group were randomly separated into two groups ($n=8$ per group), respectively. DOX (6 mg/kg, Sigma-Aldrich) or DMSO (control) was administrated intraperitoneally once a week. Tumor

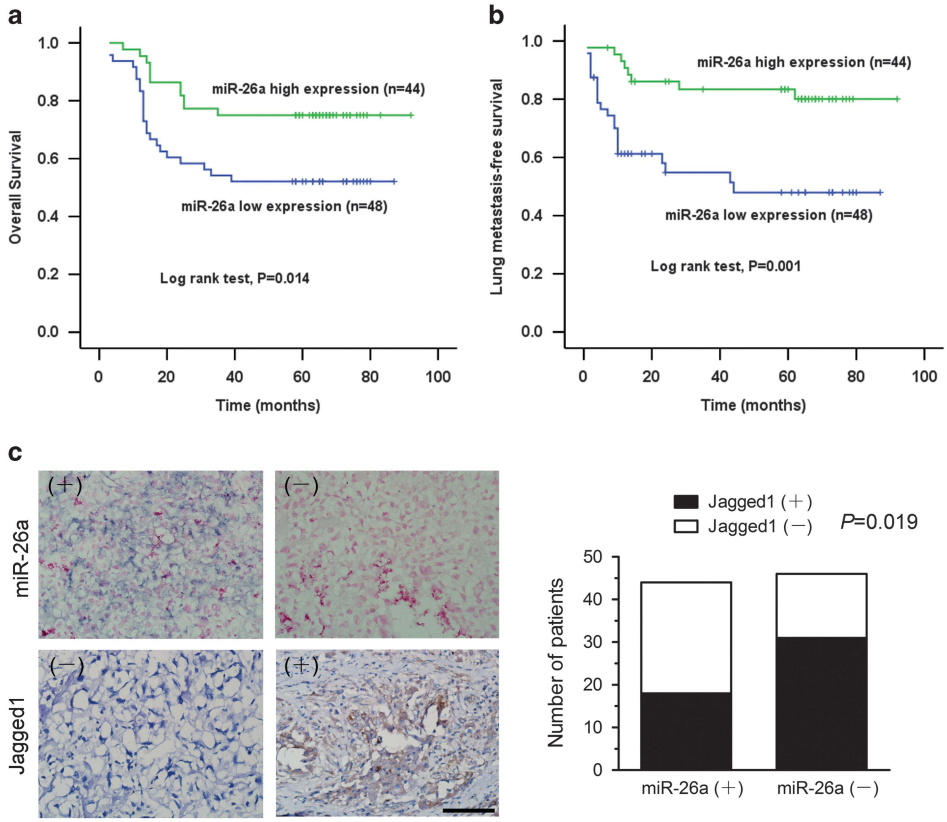

Figure 8. Reduced miR-26a expression is associated with poor prognosis of osteosarcoma patients. (**a**) Patients in high-miR-26a expression group had better overall survival (log-rank test, $P=0.014$). (**b**) The risk of lung metastasis was significantly higher in the low-miR-26a expression group (log-rank test, $P=0.001$). (**c**) Representative positive and negative staining of miR-26a and Jagged1 in human osteosarcoma tissue sections. Scale bar, 100 μm. Chi-squared analysis was performed to determine the correlation of miR-26a and Jagged1 expression in osteosarcoma sections ($P=0.019$).

volume was measured every 3 days, and tumor volume was calculated using the formula $V=1/2$ (width^2 × length). Four weeks after injection, the mice were killed and the end-point tumor mass were weighted. For the limiting dilution assay, indicated cells were mixed at 1:1 ratio with Matrigel (phenol-free growth factor-reduced, BD, San Jose, CA, USA) and injected subcutaneously into bilateral flanks of mice ($n=6$ per group).[42] Experiments were ended at 60 days or before any tumors reached 1.5 cm. Investigators who measured and analyzed the samples were blind to the group information.

Immunohistochemistry and immunofluorescence

For Immunohistochemistry, sections were incubated with antibody against Ki-67 (1:100, #ab16667, Abcam), proliferating cell nuclear antigen (1:100, #ab92552, Abcam) and Jagged1 (1:200, #ab109536, Abcam) at 4 °C overnight. Primary antibodies were detected by the Dako EnVision Kit (Dako, Glostrup, Denmark). For immunofluorescence, sections were incubated with SOX2 antibody (1:100, #ab92494, Abcam). The secondary antibody donkey anti-rabbit IgG (Alexa Fluor 488, #ab150073, Abcam) was used and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Immunofluorescence was detected by BX51WI Fixed Stage Upright Microscope (Olympus, Tokyo, Japan).

In situ hybridization

ISH was performed on 5 μm paraffin sections as previously described.[43] Digoxin-labeled miR-26a probe (miRCURY LNA Detection probe, 250 pmol, 5′-DIG labeled, Exiqon, Denmark, Germany) was used for visualization of the miR-26a.

Evaluation of immunohistochemistry and ISH staining

The immunohistochemistry and ISH staining score was determined as previously described:[40,44] 0: 0% of staining cells, 1: < 5% of staining cells, 2: 5–50% of staining cells and 3: > 50% of staining cells. Staining intensity was scored as 0: negative, 1: weak, 2: intermediate and 3: strong. Staining final score was defined as the sum of both extent and intensity score, and grouped as low (scores 0 and 2) and high (3–6) expression.

Western blot

The analysis was performed in standard procedures. Antibodies against Jagged1 (1:1000, #ab109536, Abcam), NICD (1:500, #07-1232, Millipore, Billerica, MA, USA), HES1 (1:1000, #11988, Cell Signaling Technology, Beverly, MA, USA) and c-Myc (1:10000, #ab32072, Abcam) were used. The protein bands were detected by the enhanced chemiluminescence detection system.

Luciferase reporter assay

The 823 bp human JAG1 3′-UTR containing the two predicted binding sites of miR-26a was amplified from U2OS cells using primers listed in Supplementary Table S2. The PCR products were cloned into pmirGLO vector (Promega, Madison, WI, USA) with NheI and XbaI digestion. Mutation of miR-26a-binding sites in pmirGLO-JAG1 3′-UTR was generated by QuikChange Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX, USA). Primers used for mutated vector constructs are listed in Supplementary Table S2. All mutated sequences were confirmed by DNA sequencing. Notch transcriptional activity was measured by Cignal RBP-Jk luciferase reporter kit (SABiosciences, Frederick, MD, USA). Luciferase activities were determined using a Dual-Glo luciferase assay system (Promega). The results were quantified as the ratio of firefly luciferase to Renilla luciferase activity. The experiments were performed in triplicate.

Statistics

All statistical analyses were performed with SPSS17.0 software (IBM Corporation, Somer, NY, USA). Measurements were analyzed by two-tailed Student's *t*-test or one-way analysis of variance, whereas categorical data were analyzed by the χ^2- or Fisher exact tests. Correlations between

miR-26a and JAG1 expression were analyzed with Pearson's correlation method. Kaplan–Meier method was used to calculate the overall survival, and differences were determined by the log-rank test. $P < 0.05$ was considered statistically significant. Data are shown as mean ± s.d.

Study approval

This study was approved by the Ethics committee of Sun Yat-Sen University, and written informed consent was obtained from the patients or their guardians before sample collection. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-Sen University.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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