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LncRNA CBR3-AS1 promotes osteosarcoma progression through the network of miR-140-5p/DDX54-NUCKS1-mTOR signaling pathway

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Long noncoding RNA (lncRNA) CBR3-AS1 (termed as CBR3-AS1) has been reported to be upregulated in several cancers including osteosarcoma. Its positive impact on the proliferation, migration, and invasion of osteosarcoma cells has been unveiled; nevertheless, whether it also affects the stemness and epithelial-mesenchymal transition (EMT) of osteosarcoma cells is unclear. The purpose for this study was to explore the effects of CBR3-AS1 on the stemness and EMT of osteosarcoma cells as well as its underlying mechanism. qRT-PCR and western blot were applied to detect target gene expression. Function assays were conducted to evaluate the effect of genes on the stemness and EMT of osteosarcoma cells. Mechanism assays were done to verify the association among different genes. *In vivo* assays were also performed. The obtained data showed that CBR3-AS1 demonstrated a high expression in osteosarcoma cells. CBR3-AS1 could promote stemness and EMT of osteosarcoma cells as well as osteosarcoma tumor growth. Mechanically, CBR3-AS1 sponged miR-140-5p and recruited DDX54 to upregulate NUCKS1, thus activating the mTOR signaling pathway. Furthermore, NUCKS1 could facilitate stemness and EMT of osteosarcoma cells. In summary, this study reveals that CBR3-AS1 exerts an oncogenic role in osteosarcoma through modulating the network of the miR-140-5p/DDX54-NUCKS1-mTOR signaling pathway.

INTRODUCTION

Osteosarcoma is a kind of malignant tumor that damages the bones, especially the long bones.[1] It is the top primary bone tumor besetting children and adolescents.[2] According to a study, in the US, there are 5 osteosarcoma patients among 1,000,000 children aged at or under 19 years.[3] Osteosarcoma is a rare cancer diagnosed at any age,[4] and it presents a high tendency to metastasize to the lung[5] and cause recurrence after therapy.[6] Although the improvement in treatment methods, including surgery and multidrug chemotherapy, has brought benefits for osteosarcoma patients, the overall survival rate and prognosis are still not optimistic due to its metastasis.[2,7] Therefore, it is essential to explore the pathogenesis as well as the underlying molecular mechanisms of osteosarcoma so as to achieve new breakthrough in the therapy of osteosarcoma.

Long noncoding RNAs (lncRNAs) are a category of RNAs characterized by non-protein encoding ability and are over 200 nucleotides in length.[8] Considerable studies have revealed their participation in the development of cancers including osteosarcoma. For instance, lncRNA LET has been reported to be involved in osteosarcoma cell proliferation and invasion.[9] LncRNA GAS5 could suppress cell growth and metastasis of osteosarcoma.[10,11] Moreover, it has been reported that lncRNA CBR3-AS1 could induce the proliferation, migration, and invasion, while repressing the apoptosis of osteosarcoma cells.[12] However, the impact of CBR3-AS1 on the stemness and epithelial-mesenchymal transition (EMT) of osteosarcoma cells remains unclear. Hence, this study aimed to explore the impact of CBR3-AS1 on stemness and EMT of osteosarcoma cells.

According to current evidence, the competing endogenous RNA (ceRNA) network, that lncRNA modulates messenger RNA (mRNA) expression by competitively binding to microRNA (miRNA), has been reported as a common mechanism for RNAs to participate in the initiation and development of cancers.[11] Based on the previous studies, lncRNA SNHG3 could enhance osteosarcoma cell invasion and migration via modulating the miRNA-151a-3p/RAB22A axis.[13] LncRNA MEG3 modifies negatively the proliferation, migration, and invasion of osteosarcoma cells through absorbing miR-361-5p to upregulate FoxM1.[14] In addition, CBR3-AS1 functions as the sponge of miR-509-3p to upregulate HDAC9, consequently pushing the progression of non-small cell lung cancer (NSCLC).[15] Hence, in this research, we tried to figure out if CBR3-AS1 could work through a ceRNA network to exert influence on the stemness and EMT of osteosarcoma cells.

Emerging lncRNAs have been revealed to be crucial regulators in cellular activities whose functions significantly lie on their protein interactors, including traditional RNA-binding proteins (RBPs).[16] Moreover, various proteins, such as PTBP1 and DEAD-box helicase

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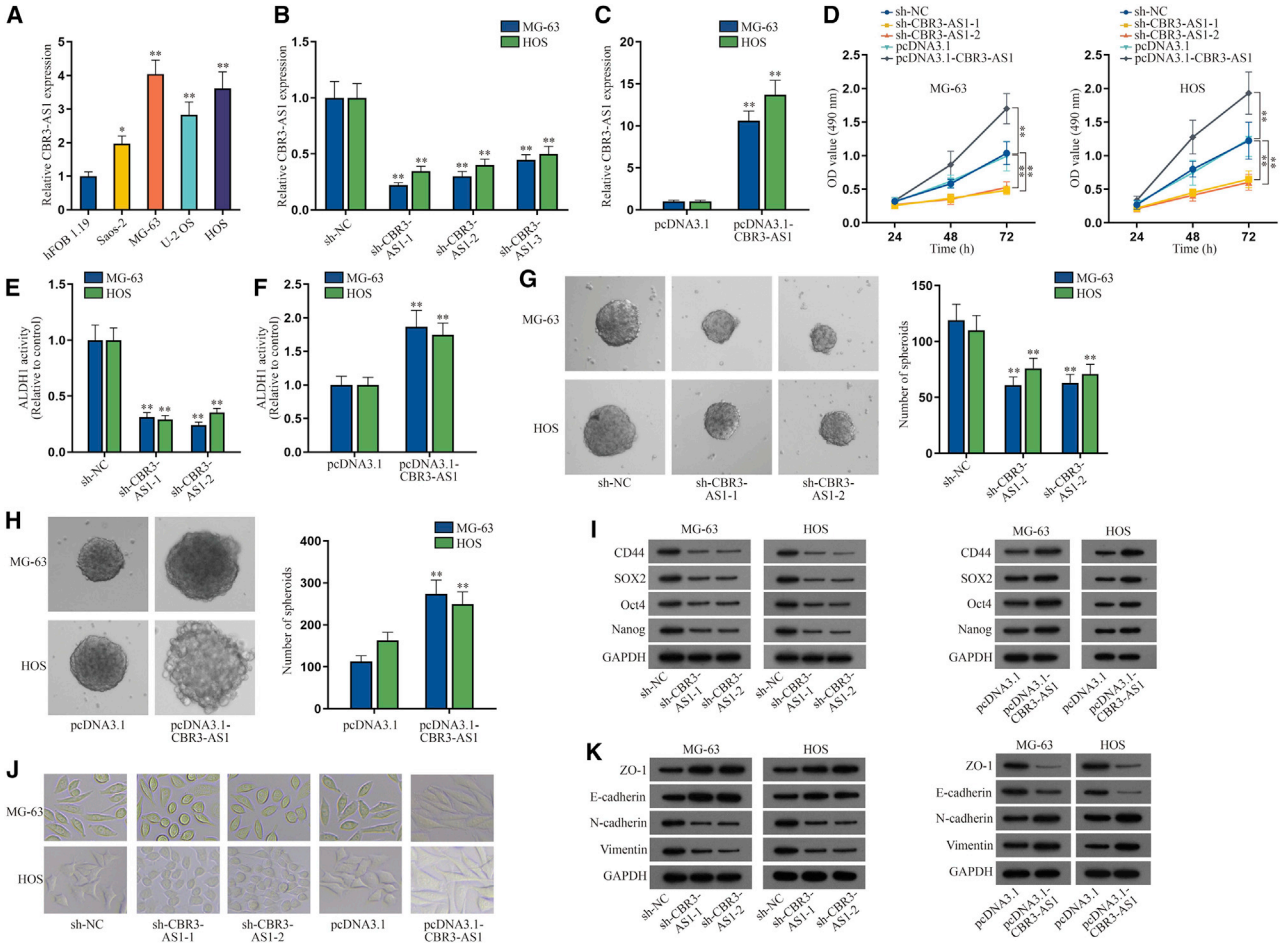


Figure 1. CBR3-AS1 promotes the stemness and EMT of osteosarcoma cells

(A) The expression of CBR3-AS1 in human osteosarcoma cell lines and the normal osteoblast cell line was detected via qRT-PCR. (B and C) The efficiency of sh-CBR3-AS1-1/2/3 and pcDNA3.1-CBR3-AS1 in MG-63 and HOS cells was tested by qRT-PCR. (D) MG-63 and HOS cell proliferation ability under the condition of ectopic expression (knockdown or overexpression) of CBR3-AS1 was evaluated by CCK-8 assay. (E) ALDH1 activity in MG-63 and HOS cells under the condition of CBR3-AS1 knockdown was determined via ALDH1 activity assay. (F) ALDH1 activity in MG-63 and HOS cells was measured by ALDH1 activity assay after CBR3-AS1 overexpression. (G and H) Number of spheroids was counted in sphere-formation assay when CBR3-AS1 was depleted or elevated in MG-63 and HOS cells. Scale bar = 8 00 μm. (I) The expression levels of stemness-associated proteins (CD44, SOX2, Oct4, and Nanog) were detected via western blot when CBR3-AS1 was downregulated or upregulated in MG-63 and HOS cells. (J) EMT of MG-63 and HOS cells was observed through the microscope when CBR3-AS1 was knocked down or overexpressed. Scale bar = 800 μm. (K) The expression levels of EMT-related proteins (ZO-1, E-cadherin, N-cadherin, and Vimentin) were detected via western blot after CBR3-AS1 reduction or elevation. Error bars indicate SD. *p < 0.05, **p < 0.01.

54 (DDX54), have been identified as RBPs to be implicated in cancer progression through binding to lncRNAs.[17] For example, lncRNA HOTTIP has been revealed to facilitate cell proliferation, invasion, and migration in osteosarcoma through interacting with PTBP1 to elevate KHSRP level.[18] In addition, lncRNA SNHG10 has been confirmed to maintain the stability of PBX3 mRNA by recruiting DDX54, thus promoting gastric cancer cell growth.[19] Accordingly, the interaction between CBR3-AS1 and certain RBP is of great value in this study.

Taken together, we focus on the exploration of how CBR3-AS1 affects the stemness and EMT of osteosarcoma cells via ceRNA regulatory mechanism as well as RBP recruitment in this study, which might provide new evidence for developing novel potential biomarkers in osteosarcoma.

RESULTS

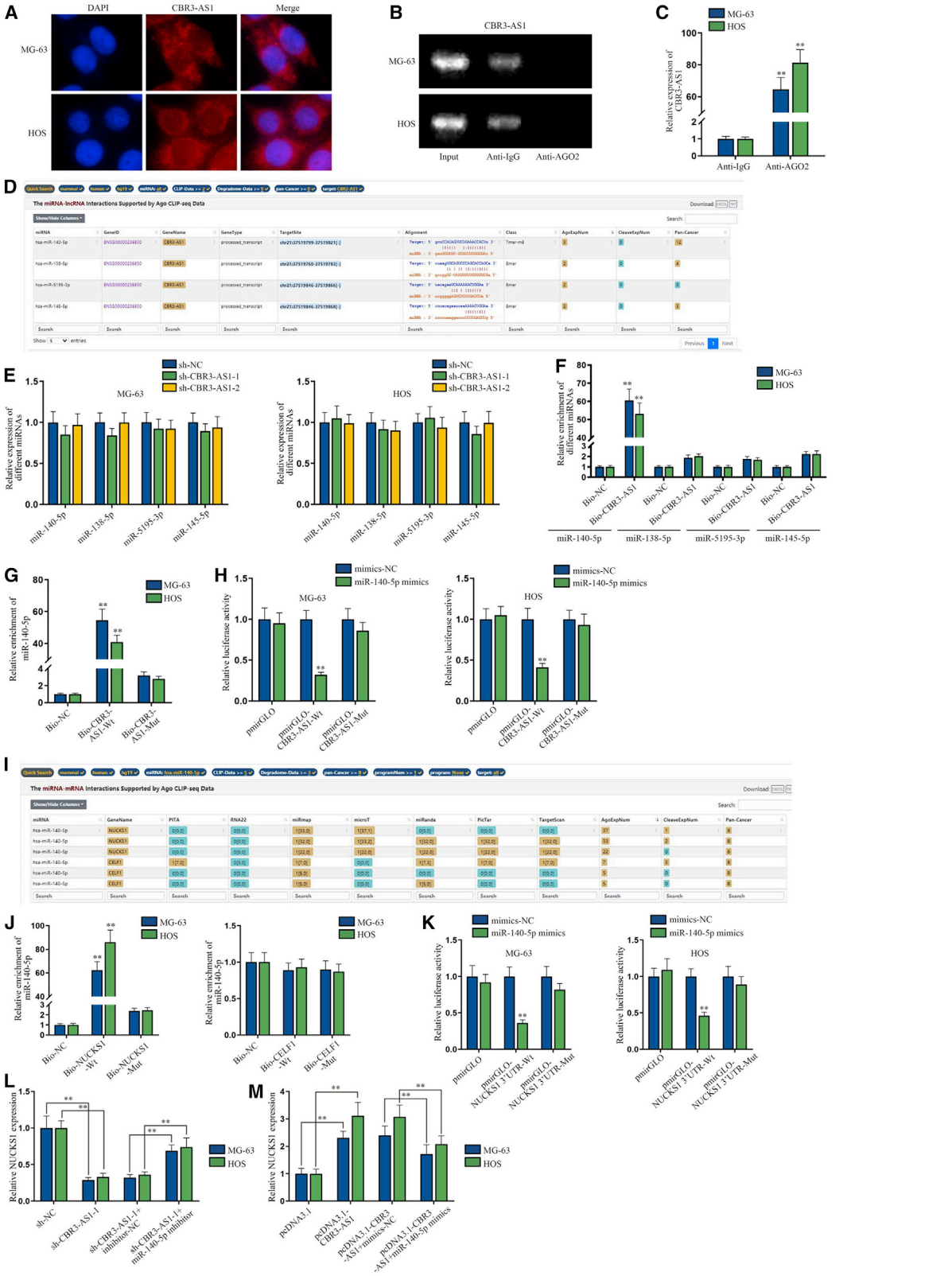
CBR3-AS1 promotes stemness and EMT of osteosarcoma cells

Previous studies have revealed that lncRNAs exert influences on malignant phenotypes of cancer cells, including osteosarcoma cells.[11,20] Thereof, we conducted a line of experiments to understand the functions of CBR3-AS1 in osteosarcoma cells. At the very beginning, we compared the expression of CBR3-AS1 in normal human osteoblasts hFOB 1.19 and four human osteosarcoma cell lines

(Saos-2, MG-63, U-2 OS, and HOS). Based on the results of quantitative reverse transcription polymerase chain reaction (qRT-PCR), CBR3-AS1 displayed a higher expression in osteosarcoma cells used in this study than in normal human osteoblasts, especially in MG-63 and HOS cell lines (Figure 1A). Accordingly, MG-63 and HOS cells were involved in the following experiments. Via qRT-PCR, the efficiency of CBR3-AS1 knockdown was quantified, and the collected data indicated that short hairpin (sh)-CBR3-AS1-1 and sh-CBR3-AS1-2 were more efficient than sh-CBR3-AS1-3 in MG-63 and HOS cells (Figure 1B). Thus, the former two were selected for the subsequent assays. Moreover, the efficiency of CBR3-AS1 overexpression was also measured via qRT-PCR and shown in Figure 1C. Subsequently, to probe into the influences of CBR3-AS1 on the phenotypes of osteosarcoma cells, cell counting kit-8 (CCK-8) assays were performed in MG-63 and HOS cells. The results showed that cell proliferation capability was highly repressed due to knockdown of CBR3-AS1, whereas it could be apparently enhanced because of the overexpression of CBR3-AS1 (Figure 1D). Reviewing existing research work, aldehyde dehydrogenase 1 (ALDH1) has been regarded as a marker of stem cells in multiple cancers.[21] In this study, we detected ALDH1 activity under different conditions, finding that ALDH1 activity declined as CBR3-AS1 was downregulated (Figure 1E) and it inversely increased when CBR3-AS1 expression was upregulated (Figure 1F). The results shown in Figures 1E and 1F reflected that CBR3-AS1 could facilitate the stemness of osteosarcoma cells. After sphere-formation assay, we found that the sphere-forming capabilities of MG-63 and HOS cells were inhibited as the result of CBR3-AS1 knockdown (Figure 1G). When CBR3-AS1 was overexpressed, sphere-forming capabilities of MG-63 and HOS cells were enhanced (Figure 1H). Recent evidence has suggested that CD44, SOX2, Oct4, and Nanog are allowed to serve as the markers of cancer stem cells.[22–24] Therefore, to further explore the influences of CBR3-AS1 on the stemness of osteosarcoma cells, the protein levels of CD44, SOX2, Oct4, and Nanog were detected via western blot. Based on the results of western blot, CBR3-AS1 downregulation could hamper osteosarcoma cell stemness while CBR3-AS1 upregulation could stimulate the stemness of osteosarcoma cells (Figure 1I). In addition, the impacts of CBR3-AS1 on EMT of osteosarcoma cells were examined. Under different conditions, the shapes of MG-63 and HOS cells were captured. As shown in Figure 1J, the shape of MG-63 and HOS cells got more rounded due to CBR3-AS1 knockdown while becoming further elongated after CBR3-AS1 elevation. This finding indicated that knockdown of CBR3-AS1 hindered the EMT of osteosarcoma cells, whereas overexpression of CBR3-AS1 promoted the EMT of these cells. Western blot was also conducted to determine the changes of EMT-related protein levels before and after knockdown or overexpression of CBR3-AS1. In Figure 1K, we observed the gain of ZO-1 and E-cadherin expression and the loss of N-cadherin and Vimentin expression as CBR3-AS1 was knocked down. The opposite findings were obtained when CBR3-AS1 was overexpressed. Meanwhile, we also conducted *in vivo* assays. The results displayed in Figures S1A–S1C show that CBR3-AS1 knockdown restricted xenograft tumor growth as the tumor volume and weight in sh-CBR3-AS1-1 groups were smaller than the control groups. To summarize, CBR3-AS1 could promote the features of stemness and EMT of osteosarcoma cells.

CBR3-AS1 can regulate NUCKS1 expression by sponging miR-140-5p

Recently, mounting evidence has proven that lncRNA is able to function as ceRNA at the post-transcriptional level via competitively binding to miRNA.[24] To explore the underlying mechanism of CBR3-AS1 in osteosarcoma cells, we firstly conducted fluorescent *in situ* hybridization (FISH) assay to determine the distribution of CBR3-AS1 in MG-63 and HOS cells. As shown in Figure 2A, CBR3-AS1 was mainly located in cytoplasm of MG-63 and HOS cells. Accordingly, we speculated that CBR3-AS1 might serve as a ceRNA to regulate the expression of downstream genes. Subsequently, an RNA-binding protein immunoprecipitation (RIP) assay was performed to confirm that CBR3-AS1 could be enriched in anti-Argonaute 2 (AGO2). The results of agarose gel electrophoresis (AGE) and qRT-PCR after RIP assays verified that CBR3-AS1 existed in anti-AGO2 (Figures 2B and 2C). As CBR3-AS1 could not combine with anti-AGO2 antibodies directly, it was reasonable to postulate that CBR3-AS1 might exert its function in a ceRNA-related manner. We searched on starBase (http://starbase.sysu.edu.cn/) under the condition of CLIP-Data >=2, sorting out four potential candidate miRNAs, namely miR-140-5p, miR-138-5p, miR-5195-3p, and miR-145-5p (Figure 2D. After knockdown of CBR3-AS1 in MG-63 and HOS cells, the expressions of these candidate miRNAs had almost no variation (Figure 2E). Through RNA pull-down assay, we found that miR-140-5p enrichment in Bio-CBR3-AS1 was the highest among all potential candidate miRNAs (Figure 2F). Hence, miR-140-5p was determined to be involved in the following investigation and the rest was excluded from the research. Then, we used biotinylated (Bio) wide-type (WT), and mutant (Mut) CBR3-AS1 probes and, based on the enrichment of miR-140-5p in different groups, the binding relationship between CBR3-AS1 and miR-140-5p was further confirmed (Figure 2G). In addition, luciferase reporter assays were implemented, and the results shown in Figure 2H indicated that CBR3-AS1 could bind to miR-140-5p through their binding sites as the luciferase activity of pmirGLO-CBR3-AS1-WT was weakened while that of pmirGLO-CBR3-AS1-Mut had no obvious change. To complete the ceRNA network in this study, we then searched on starBase under multiple conditions (CLIP-Data >= 5, Degradome-Data >= 3, pan-Cancer >= 8, and programNum >= 1) to find the downstream gene of miR-140-5p. The search results presented that simply NUCKS1 and CELF1 were screened out (Figure 2I). After RNA pull-down assay, the enrichment of miR-140-5p under different conditions were quantified via qRT-PCR, and the outcomes demonstrated that miR-140-5p was more inclined to combine with NUCKS1 rather than CELF1 (Figure 2J). Thereby, CELF1 was excluded. According to the changed luciferase activities of pmirGLO-NUCKS1 3′ UTR-WT after miR-140-5p augment, we double checked the binding relationship between miR-140-5p and NUCKS1 3′ UTR in MG-63 and HOS cells (Figure 2K). To understand the regulatory mechanism of CBR3-AS1-miR-140-5p-NUCKS1, rescue experiments were performed as follows. The corresponding results suggested that downregulating



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miR-140-5p could largely counteract the inhibitory effect of CBR3-AS1 knockdown on NUCKS1 expression (Figure 2L). Meanwhile, upregulating miR-140-5p could greatly offset the promoting influence of CBR3-AS1 overexpression on NUCKS1 expression (Figure 2M). However, miR-140-5p could only largely but not fully countervail the effect of CBR3-AS1 on NUCKS1 expression. Thereby, we considered that there might be other approaches for CBR3-AS1 to regulate NUCKS1 expression. In conclusion, CBR3-AS1 could regulate NUCKS1 expression by sponging miR-140-5p in osteosarcoma cells.

NUCKS1 facilitates the stemness and EMT of osteosarcoma cells

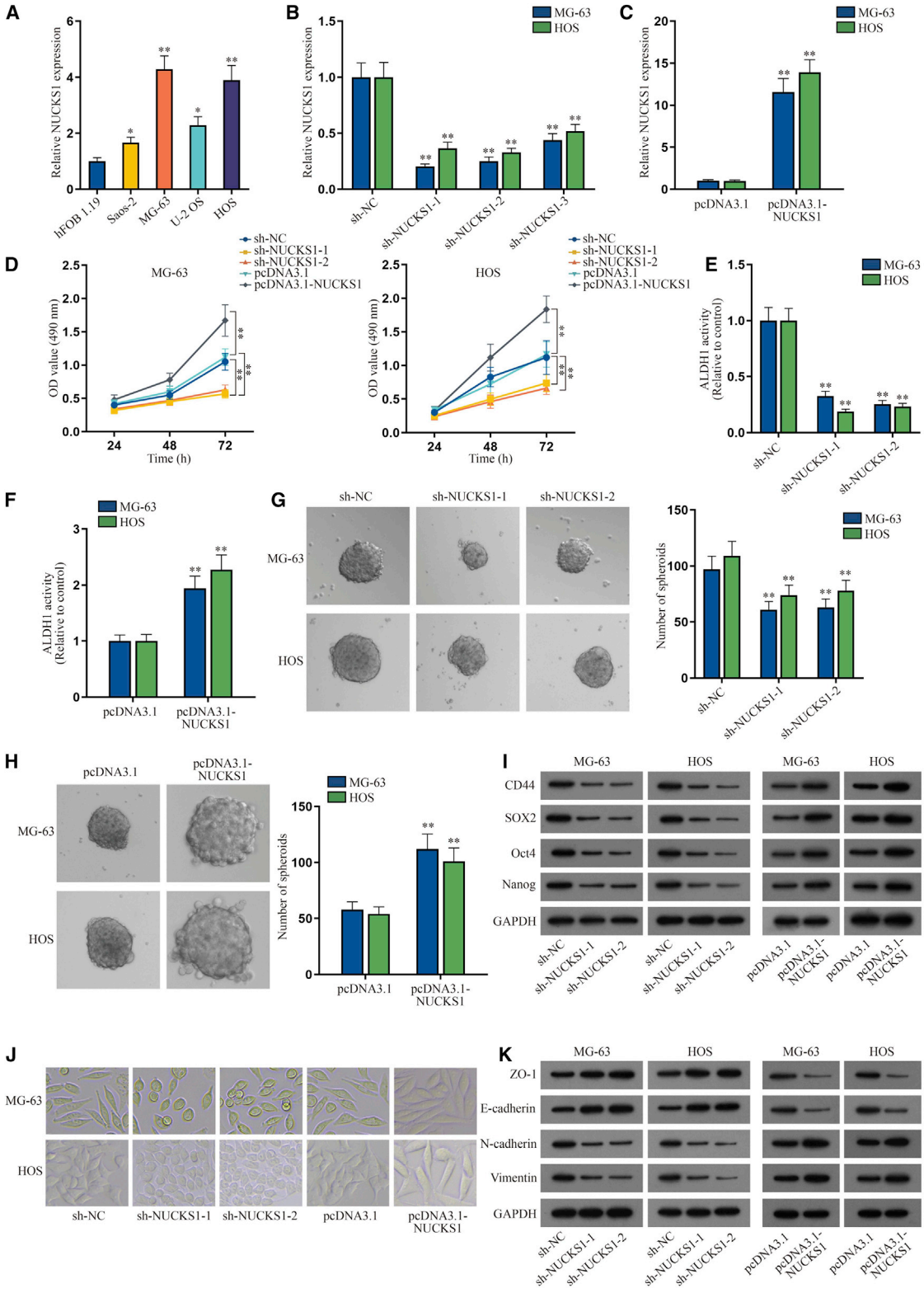
NUCKS1 has been reported to facilitate proliferation and invasion of gastric cancer cells.[25] Our research also tried to fathom out the influences of NUCKS1 on malignant phenotypes of osteosarcoma cells. Thus, we quantified the expression of NUCKS1 in normal human osteoblast hFOB 1.19 and human osteosarcoma cell lines via qRT-PCR. The results indicated that NUCKS1 was highly expressed in osteosarcoma cells, particularly in MG-63 and HOS cells (Figure 3A). Prior to functional assays, the efficiency of NUCKS1 knockdown and overexpression in MG-63 and HOS cells was confirmed to be high via qRT-PCR (Figures 3B and 3C). And sh-NUCKS1-1/2 was involved in the following study for its higher efficiency. To explore the influences of NUCKS1 on the proliferation of osteosarcoma cells, CCK-8 assays were done in MG-63 and HOS cells. As shown in Figure 3D, the cell viability was greatly restricted due to NUCKS1 knockdown, and it was significantly promoted because of NUCKS1 overexpression. Afterward, ALDH1 activities in MG-63 and HOS cells were tested before and after NUCKS1 knockdown. Since ALDH1 activities markedly reduced due to NUCKS1 knockdown, we could determine that NUCKS1 downregulation would hamper the stemness of osteosarcoma cells (Figure 3E). On the contrary, upregulating NUCKS1 would promote the stemness of osteosarcoma cells based on the results of ALDH1 activity assay (Figure 3F). Subsequently, sphere-formation assays were implemented to double check the stemness of osteosarcoma cells under different conditions. The results shown in Figures 3G and 3H further verified the above findings as NUCKS1 downregulation restricted the sphere-forming capability of MG-63 and HOS cells, while NUCKS1 upregulation resulted in the opposite outcomes. Similarly, we detected the expressions of stemness-related proteins (CD44, SOX2, Oct4, and Nanog) in these two osteosarcoma cells. The outcomes of western blot showed that knockdown of NUCKS1 would lead to the decline of these protein levels, which meant the stemness of MG-63 and HOS cells was inhibited. The overexpression of NUCKS1 brought about the increase of these protein levels, showing that the stemness of MG-63 and HOS cells was promoted (Figure 3I). Aside from that, we also observed the shape of MG-63 and HOS cells when NUCKS1 was knocked down or overexpressed (Figure 3J). The images shown in Figure 3J reflected that the EMT of these osteosarcoma cells was hindered by NUCKS1 knockdown as there were more rounded cells and, on the contrary, the EMT was stimulated due to NUCKS1 overexpression since fusiform cells increased. The expressions of EMT-related proteins, ZO-1, E-cadherin, N-cadherin, and Vimentin, were measured before and after NUCKS1 was knocked down or overexpressed. Based on the data of western blot, the expression of ZO-1, E-cadherin was enhanced while that of N-cadherin and Vimentin was reduced after NUCKS1 knockdown, and NUCKS1 overexpression gave rise to the contrary results, which indicated NUCKS1 could promote osteosarcoma cell EMT (Figure 3K). In summary, NUCKS1 could promote the stemness and EMT of osteosarcoma cells.

DDX54 can be recruited by CBR3-AS1 to stabilize NUCKS1 mRNA

According to the above-mentioned results of rescue experiments (Figures 2L and 2M), we noticed that miR-140-5p could not completely recover the influences of CBR3-AS1 on NUCKS1 expression. Naturally, we inferred that other approaches existed for CBR3-AS1 to modulate the expression of NUCKS1. With the help of starBase, the potential RBPs likely to combine with CBR3-AS1 and NUCKS1 were selected out under the condition of CLIP-Data >= 3 or CLIP-Data >= 5. DDX54, FUS, DGCRB, HNRNPC, and U2AF2 were RPBs shared by CBR3-AS1 and NUCKS1 (Figures 4A, 4B). Reviewing previous studies, these RBPs have been discussed in cancer research. U2AF2 has been found to facilitate tumorigenesis of lung cancer.[26,27] HNRNPC can induce breast cancer.[28] FUS promotes the incidence of prostatic cancer [29] and DDX54 is able to regulate gastric cancer.[19] However, the influences of U2AF2, HNRNPC, FUS, DGCRB, and DDX54 have not been well explored in osteosarcoma yet. Through qRT-PCR and western blot analysis, we could find that the expressions and protein levels of these genes had merely slight change after CBR3-AS1 knockdown (Figures 4C and 4D). Afterward, RNA pull-down assays were performed and only DDX54 was pulled down by Bio-CBR3-AS1 in MG-63 and HOS cells (Figure 4E). To further confirm the

Figure 2. CBR3-AS1 can regulate NUCKS1 expression by sponging miR-140-5p

(A) The distribution of CBR3-AS1 in MG-63 and HOS cells was determined by FISH assay. Scale bar = 800 μm. (B and C) The CBR3-AS1 enrichment in anti-AGO2 and anti-IgG was evaluated by AGE and qRT-PCR separately after RIP assay. (D) Potential miRNAs for CBR3-AS1 were screened out from starBase under the condition of CLIP-Data >= 2. (E) The expression of potential miRNAs in MG-63 and HOS cells was detected via qRT-PCR under the condition of CBR3-AS1 knockdown. (F) The enrichment of potential miRNAs in Bio-NC and Bio-CBR3-AS1 was detected via RNA pull-down assay. (G) The binding affinity between Bio-NC/Bio-CBR3-AS1-WT/Bio-CBR3-AS1-Mut and miR-140-5p was identified by RNA pull-down assay. (H) Luciferase reporter assays were implemented to detect the luciferase activity of pmirGLO-CBR3-AS1-WT/Mut in MG-63 and HOS cells with miR-140-5p augment. (I) Potential mRNAs for miR-140-5p were screened out from starBase with specific conditions (CLIP-Data >= 5, Degradome-Data >= 3, pan-Cancer >= 8, and programNum >= 1). (J) The binding of miR-140-5p to potential mRNAs (NUCKS1 and CELF1) was evaluated via RNA pull-down assay. (K) The binding of miR-140-5p and NUCKS1 was evaluated via luciferase reporter assay. (L) Rescue assays were conducted to detect the expression of NUCKS1 in MG-63 and HOS cells separately transfected with different plasmids: sh-NC, sh-CBR3-AS1, sh-CBR3-AS1+inhibitor-NC, and sh-CBR3-AS1+miR-140-5p inhibitor. (M) The expression of NUCKS1 was examined via qRT-PCR after MG-63 and HOS cells were transfected with pcDNA3.1, pcDNA3.1-CBR3-AS1, pcDNA3.1-CBR3-AS1+mimics-NC, or pcDNA3.1-CBR3-AS1+miR-140-5p mimics. Error bars indicate SD. **$p < 0.01$.



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binding relationship between CBR3-AS1 and DDX54, RIP assays were performed and CBR3-AS1 was largely enriched by anti-DDX54 antibody (Figure 4F). Subsequently, qRT-PCR was performed to evaluate the efficiency of DDX54 knockdown and overexpression in MG-63 and HOS cells (Figures 4G and 4H). The results demonstrated that DDX54 was conspicuously downregulated by sh-DDX54, particularly by sh-DDX54-1 and sh-DDX54-2, and overtly upregulated by pcDNA3.1-DDX54. Furthermore, the expressions and protein levels of NUCKS1 were detected before and after DDX54 knockdown or overexpression through qRT-PCR and western blot. As shown in Figures 4I–4K, DDX54 downregulation would reduce NUCKS1 expression, and DDX54 upregulation could increase NUCKS1 expression in MG-63 and HOS cells. Thus, we conjectured DDX54 might interact with NUCKS1 to regulate NUCKS1 expression. Co-immunoprecipitation (co-IP) assays were first conducted, which helped us verify that DDX54 could not interact with NUCKS1 protein (Figure 4L). Next, luciferase reporter assays were conducted in MG-63 and HOS cells. The luciferase activity of the pGL3-NUCKS1 promoter was hardly changed, showing that DDX54 could not bind to NUCKS1 promoter (Figure 4M). Nevertheless, as shown in Figure 4N, DDX54 was allowed to bind to NUCKS1 3′ UTR as the luciferase activity of pmirGLO-NUCKS1 3′ UTR declined. After MG-63 and HOS cells were treated with α-amanitin, qRT-PCR was performed to test the expression of NUCKS1. The results in Figures 4O and 4P suggested that the stability of NUCKS1 mRNA weakened owing to DDX54 downregulation in MG-63 and HOS cells. In short, DDX54, an RBP recruited by CBR3-AS1, could stabilize NUCKS1 mRNA.

CBR3-AS1 activates mTOR signaling pathway in osteosarcoma cells

Emerging studies have shown that the mTOR signaling pathway plays a role in osteosarcoma cells.[30] Accordingly, we speculated that CBR3-AS1 could stimulate the mTOR signaling pathway to further regulate the stemness and EMT of osteosarcoma cells. To test this speculation, we measured the expressions of key proteins correlated with the mTOR signaling pathway, mTOR, p-mTOR, SREBP-1c, and Cyclin D1, respectively, in MG-63 and HOS cells with CBR3-AS1 depletion. The corresponding western blot results indicated that the expressions of p-mTOR, SREBP-1c, and Cyclin would decline due to CBR3-AS1 knockdown, and overexpressing CBR3-AS1 led to contrary results (Figures 5A and 5B), indicating CBR3-AS1 could activate the mTOR signaling pathway. It has also been reported that NUCKS1 could activate the mTOR signaling pathway to regulate the proliferation of mammary epithelial cells.[31] This study revealed that CBR3-AS1 could upregulate NUCKS1 by modulating miR-140-5p or DDX54 in MG-63 and HOS cells. Hence, we further explored how NUCKS1 engaged in activating the mTOR signaling pathway. As shown in Figures 5C and 5D, the decreased expression of p-mTOR, SREBP-1c, Cyclin D1, and NUCKS1 caused by miR-140-5p augment could be partially recovered by CBR3-AS1 overexpression. Moreover, the suppressive effect of DDX54 knockdown on the four proteins could be partly offset by NUCKS1 overexpression (Figures 5E and 5F). To summarize, CBR3-AS1 could stimulate the mTOR signaling pathway in osteosarcoma cells by regulating miR-140-5p/NUCKS1/DDX54.

DISCUSSION

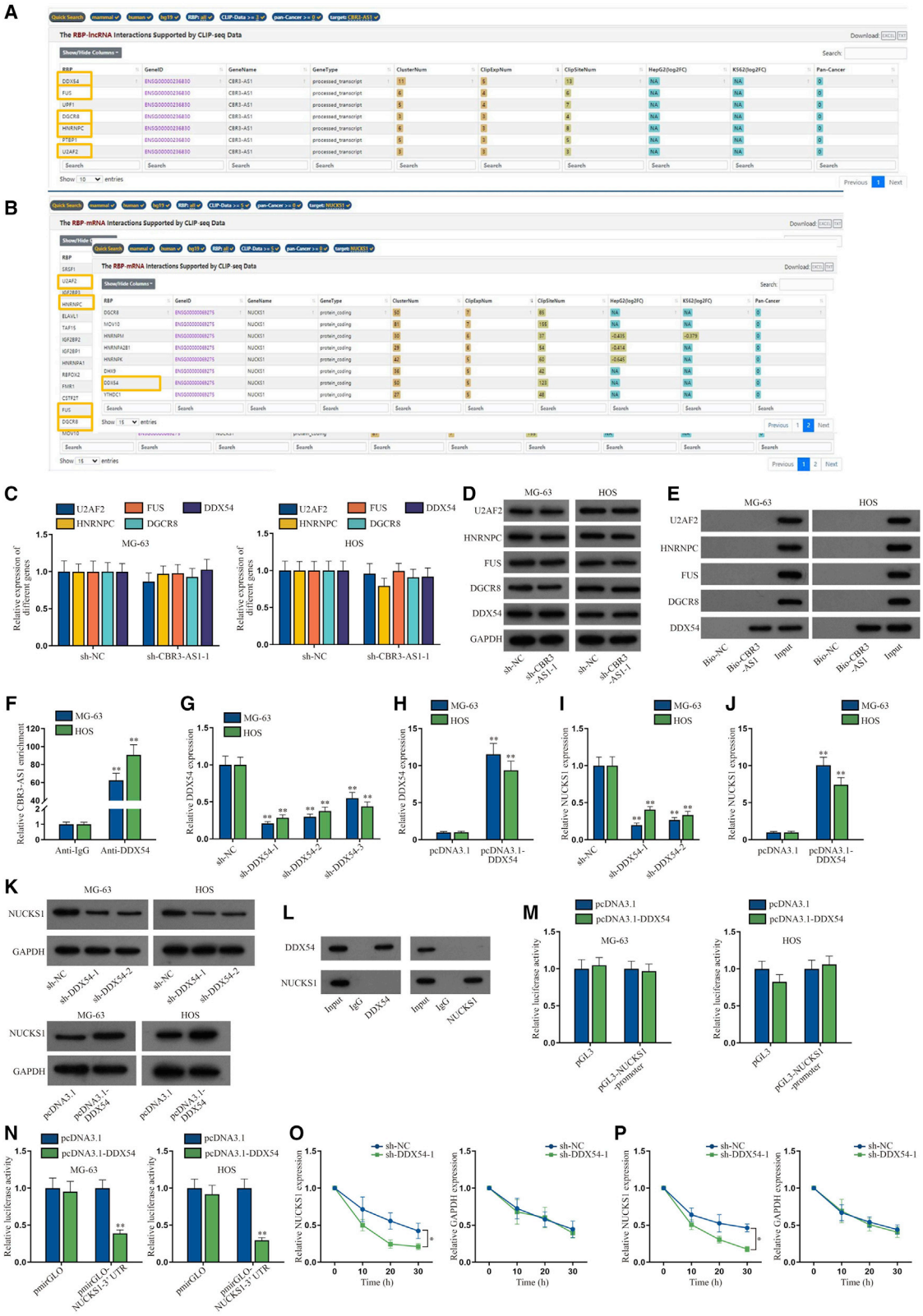
Although bone tumors represent rare malignancies, they still cause high mortality over the world.[32] Osteosarcoma is known to be the most common malignant bone tumor,[33] and some research work has reported that lncRNAs play pivotal roles in the tumorigenesis and progression of multiple cancers, including osteosarcoma.[7,20,34] Specifically speaking, CBR3-AS1 has been found to be highly expressed in osteosarcoma tissues and functions as an oncogene to promote proliferation, migration, and invasion of osteosarcoma cells, and inhibits cell apoptosis. However, the molecular mechanism of CBR3-AS1 has not been well explored in previous research, and the influences of CBR3-AS1 on the stemness and EMT of osteosarcoma cells have not be discussed either. In this study, we firstly showed the functional influence of CBR3-AS1 and the stemness and EMT of osteosarcoma cells as well as the regulatory mechanism of CBR3-AS1.

Consistent with the previous findings,[12] we also confirmed that CBR3-AS1 was upregulated in osteosarcoma cells. The following functional analyses and *in vivo* assays indicated that downregulation of CBR3-AS1 restrained the stemness and EMT of osteosarcoma cells as well as osteosarcoma tumor growth. On the contrary, the stemness and EMT of osteosarcoma cells was promoted as CBR3-AS1 was overexpressed. In summary, CBR3-AS1 was certified to serve as an oncogene in osteosarcoma, which also backed up the above-mentioned finding of this gene in previous research work.

In terms of regulatory mechanisms concerning CBR3-AS1, we determined that CBR3-AS1 was mainly located in cytoplasm. Considering the hypothesis of the ceRNA network, we surmised that CBR3-AS1 probably acted as a ceRNA, competitively binding to a certain miRNA and ultimately regulating the expression of the target mRNA. Indeed, the subsequent mechanism analysis demonstrated our speculation, showing that CBR3-AS1 acted as the sponge of miR-140-5p. After

Figure 3. NUCKS1 facilitates the stemness and epithelial-mesenchymal transition of osteosarcoma cells

(A) The expressions of NUCKS1 in human osteosarcoma cell lines and normal osteoblast cell line were detected via qRT-PCR. (B and C) The efficiency of sh-NUCKS1 and pcDNA3.1-NUCKS1 in MG-63 and HOS cells was tested by qRT-PCR. (D) MG-63 and HOS cell proliferation ability was evaluated by CCK-8 assay under the condition of ectopic expression (knockdown or overexpression) of NUCKS1. (E and F) ALDH1 activity in MG-63 and HOS cells under the condition of NUCKS1 decrease or increase was determined via ALDH1 activity assay. (G and H) Number of spheroids was counted in sphere-formation assay when NUCKS1 was downregulated or upregulated in MG-63 and HOS cells. Scale bar = 800 μm.(I) The expression levels of stemness-associated proteins were detected via western blot when NUCKS1 was diminished or overexpressed in MG-63 and HOS cells. (J) EMT phenotype of MG-63 and HOS cells was observed through the microscope after NUCKS1 knockdown or augment. Scale bar = 800 μm. (K) The expression levels of EMT-related proteins were detected via western blot when MG-63 and HOS cells were transfected with sh-NUCKS1 or pcDNA3.1-NUCKS1.Error bars indicate SD. *$p < 0.05$, **$p < 0.01$.



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bioinformatics perdition and mechanism assays, we verified the ceRNA network of CBR3-AS1/miR-140-5p/NUCKS1, which was different from the discoveries by Zhang et al.[12] Moreover, by rescue experiments, we also noticed that miR-140-5p could only partially counteract the effects of CBR3-AS1 knockdown or augment NUCKS1 expression, and there might be other ways to regulate the expression of NUCKS1.

Based on recent research work, NUCKS1 has been reported to propel an invasive phenotype of gastric cancer cells.[25] However, the roles of NUCKS1 in osteosarcoma cells remain unclear. In this section of the study, we firstly validated that NUCKS1 was highly expressed in MG-63 and HOS cells. Furthermore, the results of loss-/gain-of-function assays and western blot suggested that NUCKS1 exerted an oncogenic effect to promote the stemness and EMT process of osteosarcoma cells, which demonstrated the same role in NSCLC cells.[35]

As miR-140-5p could not completely recover the influences of regulating CBR3-AS1 expression, we probed into other regulatory mechanisms regarding CBR3-AS1 and NUCKS1. According to current understanding of molecular mechanisms, RBP is commonly required to interact with lncRNA to direct RNA function.[36] Based on the prediction from starBase and verification of RNA pull-down, as well as RIP assays, DDX54 was found to serve as an RBP combining with CBR3-AS1 and NUCKS1. The following co-IP experiments reflected that DDX54 could not interact with NUCKS1, but was recruited by CBR3-AS1 to stabilize NUCKS1 mRNA. Aside from that, the rescue study revealed that CBR3-AS1 could activate the mTOR signaling pathway via regulating the miR-140-5p/NUCKS1 axis or the DDX54/NUCKS1 axis.

To summarize, this study confirmed that CBR3-AS1 played an oncogenic role in the progression of osteosarcoma, and it could promote stemness and EMT of osteosarcoma cells. In terms of molecular mechanism, CBR3-AS1 could regulate the expression of NUCKS1 by competitively binding to miR-140-5p or recruiting DDX54, which further switched on the mTOR signaling pathway. As to the limitation of this study, we did not involve clinical samples. Hopefully, this work might provide more evidence for understanding the molecular mechanism of osteosarcoma and developing potential therapeutic targets.

MATERIALS AND METHODS

Cell culture

Normal human hFOB 1.19 osteoblasts and four human osteosarcoma cell lines, namely Saos-2, MG-63, U-2 OS, and HOS, were all procured from the American Type Culture Collection. The cells involved in this study were cultured in DMEM medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco) and penicillin-streptomycin solution at 37°C. When the cells incubated reached 80% convergence, 0.25% trypsin solution was utilized for subculture.

qRT-PCR

According to the manufacturer's guidelines, total RNAs were extracted by TRIzol (Takara, Japan). A RevertAid First Strand cDNA Synthesis Kit and a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) were separately applied to reversely transcribe lncRNAs/mRNAs and miRNAs into their cDNAs. Subsequent qPCR analyses were carried out using a qRT-PCR kit (QR0100-1KT, Sigma-Aldrich, USA). GAPDH served as internal reference for lncRNA and mRNA, while U6 was used for miRNA. All qRT-PCR outcomes were calculated and presented as $2^{-\Delta\Delta Ct}$.

Plasmid construction and cell transfection

sh-RNAs targeting CBR3-AS1, NUCKS1, and DDX54, as well as their corresponding negative control (sh-NC), were all purchased from RiboBio (Nanjing, China). Plasmids pcDNA3.1-CBR3-AS1, pcDNA3.1-NUCKS1, and pcDNA3.1-DDX54, along with the empty vector pcDNA3.1, were also synthesized. The constructed plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfected cells were used in the following experiments after 48-h transient transfection, and qRT-PCR was performed to test the knockdown and overexpression efficiency. miR-140-5p mimics, miR-140-5p inhibitors, mimic-NC, and inhibitor-NC were also purchased from RiboBio.

CCK-8 assay

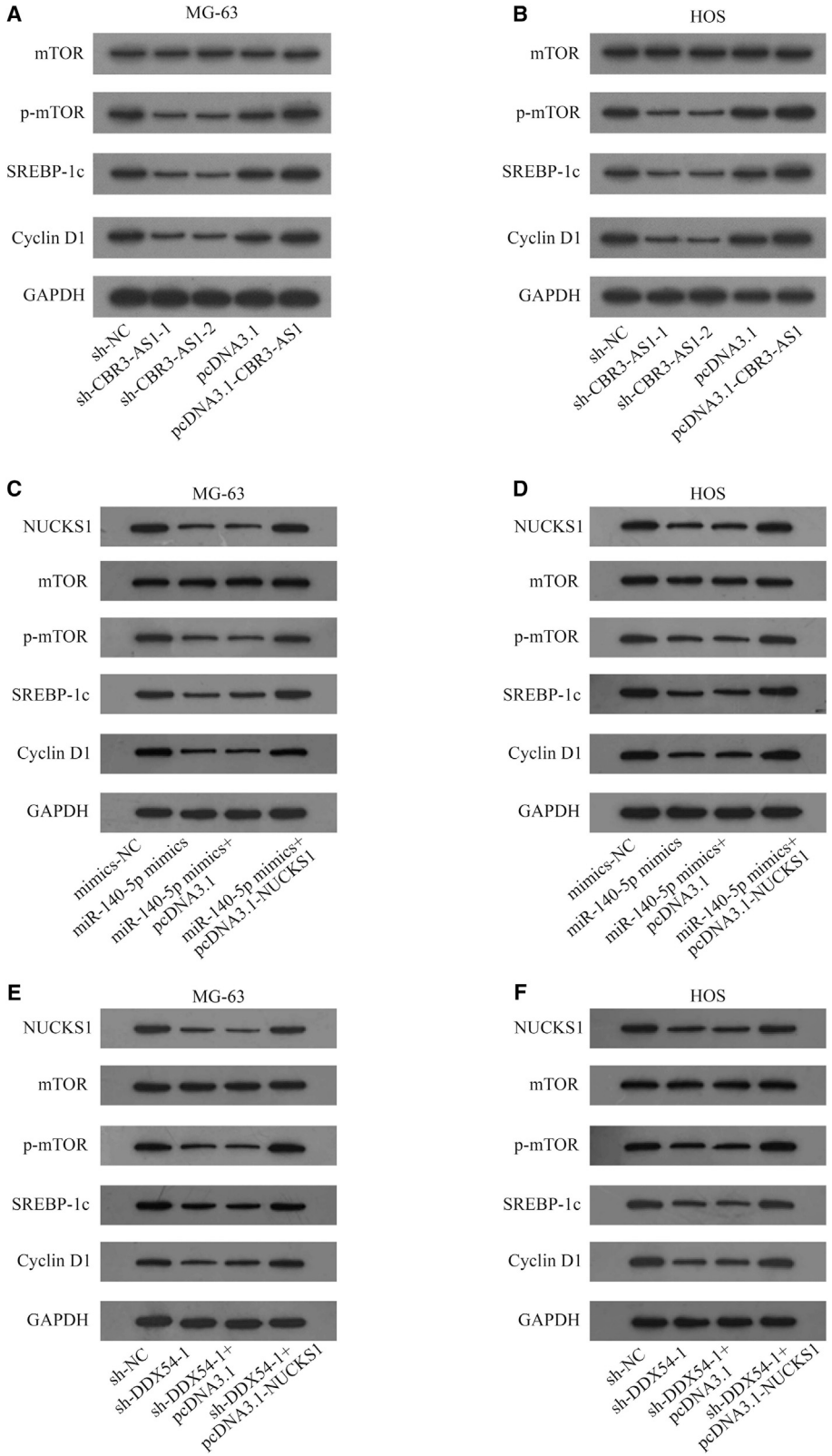
Transfected cells were planted into 96-well plates, and CCK-8 Kit (ab228554, Abcam, Shanghai, China) was applied to test cell viability. The manufacturer's instructions were strictly followed. The optical density values were detected at a wavelength of 490 nm using a microplate reader (SuPerMax-3100, Shanghai Flash Spectrum Biotechnology).

Sphere-formation assay

MG-63 and HOS cells were cultured and re-suspended as single cells in serum-free DMEM medium. Then, the cells were planted into 96-well plates and the medium was refreshed every 2 days. The images of randomly selected fields were shot using an MShot Image Analysis System.

Figure 4. DDX54 can be recruited by CBR3-AS1 to stabilize NUCKS1 mRNA

(A and B) Potential RBPs for CBR3-AS1 and NUCKS1 were screened out in starBase database. (C) The expressions of potential RBPs were analyzed by qRT-PCR when CBR3-AS1 expression was cut down. (D) The protein levels of potential RBPs were analyzed by western blot after CBR3-AS1 knockdown. (E) The binding of CBR3-AS1 to potential RBPs was determined by performing RNA pull-down assay. (F) The binding of CBR3-AS1 to DDX54 was evaluated via RIP assay. (G and H) The efficiency of sh-DDX54-1/2/3 and pcDNA3.1-DDX54 in MG-63 and HOS cells was tested by qRT-PCR. (I and J) The expression of NUCKS1 was evaluated by qRT-PCR under the condition of DDX54 depletion or elevation. (K) The protein level of NUCKS1 was evaluated by western blot under the condition of DDX54 downregulation or upregulation. (L) The interaction between DDX54 and NUCKS1 was verified via co-IP assay. (M and N) The binding between DDX54 and NUCKS1 promoter/NUCKS1-3′ UTR was evaluated by means of luciferase reporter assay. (O and P) The impact of DDX54 knockdown on NUCKS1 mRNA stability was analyzed via qRT-PCR. **Error bars indicate SD**$^{*}p < 0.05$, $^{**}p < 0.01$.



(legend on next page)

Western blot

Total proteins were acquired from the transfected cells with protein lysis buffer (KeyGen, Nanjing, China) and extracted by Total Protein Extraction Kit (PROTTOT-1KT, Sigma-Aldrich). The protein extractions were treated using an SDS Quick Match Gel Kit (P0670, 250 mL, Beyotime Biotechnology, Shanghai, China) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Vicmed, China). The PVDF membranes were subsequently blocked with 5% skimmed milk and incubated with anti-CD44, anti-SOX2, anti-Oct4, anti-Nanog, anti-ZO-1, anti-E-cadherin, anti-N-cadherin, anti-Vimentin, anti-U2AF2, anti-HNRNPC, anti-FUS, anti-DBCRB, anti-DDX54 (ab76947, Abcam), anti-NUCKS1 (ab80425, Abcam), anti-mTOR, anti-SREBP-1c, anti-Cyclin D1, or anti-GAPDH (KC Bio, China) antibodies, respectively, at 4°C overnight. Afterward, their corresponding secondary antibodies were incubated for another hour at room temperature. GAPDH acted as the internal reference.

FISH

MG-63 and HOS cells plated on the slides were washed with PBS (Vicmed) and fixed with 4% paraformaldehyde for 30 min. After digestion of the contaminating DNAs with Dnase at 37°C, FISH probes targeting CBR3-AS1 were added into the pre-warmed hybridization buffer and hybridized at 65°C overnight. Then, DAPI solution was used for staining nuclei, and the fluorescent signal was captured using a Zeiss confocal microscope (Smart zoom 5).

RIP

A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA) was utilized based on the manufacturer's instructions. The cells were subjected to lysis buffer, and the obtained cell lysates were incubated with magnetic bead-conjugated anti-immunoglobulin G (IgG) or anti-AGO2 antibody at 4°C overnight. After purification, the levels of immunoprecipitated RNAs were further analyzed by AGE or qRT-PCR.

RNA pull-down assay

Pierce Streptavidin Magnetic Beads were used in this assay. Structure buffer with 15 μL streptavidin beads was added into 1 μL Bio-NC, Bio-CBR3-AS1-WT, or Bio-CBR3-AS1-Mut for 2 h incubation at 4°C. Similarly, Bio-NUCKS1-WT, Bio-NUCKS1-Mut, Bio-CELF1-WT, and Bio-CELF1-Mut were cultured. Afterward, the biotinylated RNA-bead complexes were mixed with cell lysates at 4°C overnight. Finally, purified RNAs or proteins were subjected to qRT-PCR or western blot.

Luciferase reporter assay

The wide-type or mutant sequences of CBR3-AS1 were, respectively, cloned into pmirGLO vectors to construct pmirGLO-CBR3-AS1-WT or pmirGLO-CBR3-AS1-Mut. PmirGLO, PmirGLO-CBR3-AS1-WT, or pmirGLO-CBR3-AS1-Mut were co-transfected with mimics-NC or miR-140-5p mimics into the cells used. Likewise, pmirGLO-NUCKS1 3′ UTR-WT and pmirGLO-NUCKS1 3′ UTR-Mut were obtained by sub-cloning the corresponding sequences into pmirGLO vectors. The sequence of NUCKS1-promoter was amplified and sub-cloned into pGL3 vectors to gain the pGL3-NUCKS1 promoter. After co-transfection for 24 h, the Luciferase Reporter Assay Substrate Kit (Abcam) was applied and, using a GloMax20/20 Luminometer (Progema), the luciferase activities were detected according to the manufacturer's instructions.

Co-IP

Cells were first treated with IP lysis buffer and then the obtained cell lysates were incubated with anti-IgG, anti-DDX54, or anti-NUCKS1 antibodies, as well as streptavidin-coated magnetic beads, for 4 h. The immunocomplexes were analyzed by western blot.

Xenograft assay

BALB/c nude mice (6–8 weeks old) were procured from the Institute of Model Animal, Wuhan University. *In vivo* experiments were approved by Affiliated Cancer Hospital of Zhengzhou University (Henan Cancer Hospital). The mice were casually divided into two groups. MG-63 cells were separately transfected with sh-NC or sh-CBR3-AS1-1 plasmids and then cultivated for 48 h. Subsequently, MG-63 cells transfected with different plasmids were subcutaneously injected into each group of mice. The tumor volume was monitored every 3 days after the 7 day (formula: length × width^2/2). After 28 days, the xenograft tumors were excised from the sacrificed mice and the weights measured.

Statistical analysis

The SPSS statistical software package was utilized for statistical analysis. Each experiment was conducted at least three times and all data collected are shown as mean ± standard deviation (SD). Student's t test, one-way analysis of variance (ANOVA), or two-way ANOVA was used for difference analysis and Dunnett's test or Tukey test for back testing. $p < 0.05$ was viewed as statistically significant in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2022.03.001.

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Figure 5. CBR3-AS1 activates mTOR signaling pathway in osteosarcoma cells

(A and B) The protein levels of key proteins in the mTOR pathway were detected via western blot when CBR3-AS1 expression was reduced or heightened. (C and D) The expression levels of mTOR pathway-related proteins were evaluated in MG-63 and HOS cells with miR-140-5p augment or NUCKS1 overexpression via western blot. (E and F) Western blot assays were conducted to examine the levels of mTOR pathway-related proteins in osteosarcoma cells under different treatments (DDX54 knockdown or NUCKS1 elevation). Error bars indicate SD.

AUTHOR CONTRIBUTIONS

W.Y. and C.W. revised and improved the study in depth, and revised the manuscript. J.H. and G.L. made statistical analysis. F.W. and Q.Y. conducted functional assays. L.G. co-designed the study. All authors performed constructive discussions in this study.

DECLARATION OF INTERESTS

The authors declare competing interests.

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