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Short Communication

MiRNA-3163 limits ovarian cancer stem-like cells via targeting SOX-2 transcription factor

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ABSTRACT

Cancer stem cells (CSCs) are pivotal in both cancer progression and the acquisition of drug resistance. MicroRNAs (miRNAs) play a crucial role in modulating CSC properties and are being explored as potential targets for therapeutic interventions. MiR-3163 is primarily known for its tumor suppressive properties in various human malignancies, with lower expression reported across different cancer types. However, its role in regulating the ovarian CSC phenotype and the underlying mechanism remain largely unknown. Here, we report a remarkable downregulation of miR-3163 in ovarian cancer stem-like cells (CSLCs). Enforced expression of miR-3163 in ovarian adherent and CSLCs, significantly disrupts the stemness phenotype. Moreover, downregulation of miR-3163 expression in ovarian cancer cells (OV2008 and OVCAR-3) inhibits the stem-like cells characterized by CD44+CD117+ expression. Sphere formation assay results reveal that overexpression of miR-3163 in ovarian cancer cells significantly inhibits spheroid formation ability, confirming the regulatory properties of miR-3163 on ovarian CSLCs. Mechanistic investigation reveals that miR-3163 depletes ovarian CSLCs via targeting SOX-2. Furthermore, we establish SOX-2 as a direct target of miR-3163 through dual-luciferase assay. Taken together, our study demonstrates that overexpression of miR-3163 could be a promising strategy for efficiently eradicating the CSC population to prevent chemoresistance and tumor relapse in ovarian cancer patients.

1. Introduction

The cancer stem cells (CSCs) usually play a pivotal role from the inception to progression of malignancies across different cancer types based on the histological origin. They consist of minute population of cells capable of self-renewal, differentiation and,potent tumor-initiating capability. With the progression of the disease the bulk tumor usually becomes more heterogenous in nature which acts as an efficient fuel for therapy resistance [1]. According to the hierarchical model of tumor heterogeneity, it arises from phenotypically and functionally distinct group of cells with dysregulated self-renewal capacity [2]. Over the years substantial amount of research indicated that CSC population is intricately involved in metastasis, therapy resistance, immune evasion strategies, and often in molding of the tumor microenvironment (TME) towards pro-tumoral niche [3]. Contributing to its pluripotency, major transcription factors like NANOG, SOX2, OCT4, c-Myc, KLF4 are elevated in stem cells, with some of the prominent cell surface markers such as CD44, CD133 and CD73 [4]. This concept of cancer stemness holds so much importance in today's research for ultimate development of therapeutic interventions.

In this scenario, among all the gynecological cancers, it was observed that ovarian cancer causes the maximum cancer-related deaths world-wide with a poor 5-year survival rate [5]. Several efforts were made to understand the TME in the context of stemness properties and the associated pro-tumoral phenotype of the non-cancerous cells. These scientific testimonies highlighted the definite and multilayered role of tumor immune landscape, soluble mediators and non-coding RNAs like miRNAs, lncRNAs, cirRNAs etc. For a long time, these non-coding RNAs were classified as 'junk RNAs', but it was gradually established that they are involved in epigenetic, and post-translational regulation of gene expression in different pathophysiological conditions. MiRNAs, described as 19–25 nucleotide long single stranded RNAs, are capable of

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binding to specific 3′ UTR region of their target mRNAs, thus interfering with its translational process [6]. They are synthesized as primary miRNAs, which eventually get processed into mature miRNAs and even are known to shuttle between subcellular compartments [7]. The dysregulated miRNAs are involved in stem cell signaling pathways that give rise to CSC subpopulation.Cumulative research has highlighted that stem cell related pathways like the Wnt, Notch, JAK-STAT, Hedgehog signaling serve as efficient targets for specific miRNAs [8]. Given that miRNAs expression and regulation of biological functions are tissue-specific, one miRNA can act as oncogene in one cell type whereas it can act as tumor suppressor in the other [9,10]. Notably, aberrant expression of certain miRNAs are involved in pathogenesis and regulation of cancer stemness features [11]. But in case of ovarian cancer, only a few miRNAs have been reported to modulate the CSC phenotype till date.

Exclusive clinical database mining revealed the negative correlation of miR-3163 expression with cancer progression. Various cancer models have highlighted the multifaceted role of miR-3163 in countering cancer growth, proliferation and chemoresistance. Sponging of miR-3163 in colorectal and cervical cancer through FAM201A and lncRNA lead to invasion, metastasis, and enrichment of stemness properties. Infact, aggressive malignancies like nasopharyngeal, NSCLC, breast, and cervical cancers manifest downregulated expression of miR-3163 [12–15]. Therefore, the diversified role of miRNAs usually serves as a potential biomarker, predictor of treatment success and promising tool for precision treatment in cancers [16]. Herein, we determined the miR-3163 levels in ovarian CSLCs isolated from cell lines. Moreover, we identified SOX2, a stem cell marker, as a direct target of miR-3163. Enforced expression of miR-3163 reduces the CSCs population via targeting SOX2.

2. Materials and methods

2.1. Cell culture

OV2008 and OVCAR3 ovarian cancer cell lines were gifted by Prof. Qi-En Wang from The Ohio State University, USA. Cells were cultured in RPMI 1640 medium (Gibco, cat. no-11875-093) supplemented with 10 % FBS (Gibco) and 1% Penicillin-Streptomycin antibiotic (Gibco, cat.no-10378-016) solution. Cells were maintained in 5 % CO2 humidified incubator at 37 °C. For CSCs enriched spheroids generation and culture, cancer cells were seeded and cultured in CSC specific media which comprises of DMEM/F12 + GlutaMAX™ (1X) medium (Gibco, cat. no-10565018) supplemented with bFGF (20 ng/ml) (Gibco, USA), EGF (10 ng/ml) (Gibco, USA) and 20 % Knockout ™ SR (Gibco, USA).

2.2. Transfection of miRNA mimic and inhibitor

For transfection experiment, miRNA-3163 mimic and inhibitor were purchased from Eurogentec (Cat.No.-SR-HP001-004) and Invitrogen (Cat.No.-4464084), respectively. Both cell lines were transfected using Lipofectamine 2000 reagent (Invitrogen, cat no.-11668-019) and opti-MEM media (Gibco, USA) and the transfection efficiency were determined after 48 h of incubation.

2.3. Molecular cloning

The psicheck-2 vector (Promega) was used to successfully insert the sequence containing the binding site for miR-3163 in the 3′ UTR region of SOX2 mRNA. Dual luciferase reporter assay was used to determine the binding potential of miR-3163 with 3′-UTR region of SOX2 mRNA.

2.4. Western blot

Cells transfected with miRControl, miR-3163 mimic or inhibitor were trypsinized and lysed using RIPA lysis buffer (Sigma-Aldrich) followed by the quantification of the protein level using the Bicinchoninic acid method (Pierce BCA protein assay kit, Thermo Scientific, USA). Proteins were transferred from the electrophoresis gel onto PVDF membrane, which was then blocked with 5 % BSA solution. Primary antibodies were incubated overnight at 4 °C, followed by 2 h of incubation with secondary antibodies. Protein bands were visualized using chemiluminescence (ECL) and images were acquired with a Bio-rad chemidoc instrument. Primary antibodies used were SOX2 (CST) and GAPDH(CST) along with Horseradish Peroxidase (HRP)-anti-rabbit-IgG secondary antibody(abcam) used to detect the protein levels.

2.5. Real time PCR

Total RNA was isolated from the cells using RNAiso Plus (Takara cat. no. - 9109), quantified and the cDNA was prepared with high efficiency cDNA kit (Invitrogen cat.no- 4368814). The primer sequences used for amplification were as follows- GAPDH forward primer-GACTAACCCTGCGCTCCTG; GAPDH reverse primer-GAAGATGGTGATGGGATTTC; SOX2 forward primer-ACCGTGATGCCGACTAGAAA; SOX2 reverse primer- GCGCCTAACG-TACCACTAGAA. RT-PCR was performed in RT-qPCR apparatus (Bio-Rad, USA). For miR-3163 assay, mature miRNA assay kit, assay ID - 244181_mat, assay name - hsa-miR-3163, (Cat.No. – 442797) was used. Quantification was done through $2^{-\Delta\Delta Ct}$ method for relative gene expression change. RNU6B (Invitrogen, PN 4427975) and GAPDH were used as endogenous controls for miRNA and mRNA, respectively.

2.6. Flow cytometry

2 x 10^5 cells were seeded in 60 mm dish. Cells were transfected with either miRcontrol, mimic or inhibitor.h. Flow cytometry analysis of CD44(MACS Miltenyi Biotec), CD117(MACS Miltenyi Biotec) double positive cells were carried out after 48 h of transfection using BD LSRFortessa™ flow cytometer. All the data were re-analyzed using FCS express flow cytometry software.

2.7. Spheroid formation assay

After transfection with miRControl, mimic or inhibitor, around 1000 cells/well were seeded in a six well ultra-low attachment plate (Corning, USA) with CSC media. The spheroids were allowed to form for 7 days in humidified incubator with 5 % CO2 at 37 °C. Afterward, the spheroid forming ability was determined by the number of spheres under a brightfield microscope along with average spheroids diameter. Spheroids were stained with CFDA-SE (Invitrogen,cat. no. -V12883A) dye for 20mins(25μM) at 37 °C in the dark and subsequently washed with PBS. CSC culture media was added to each group, and images were taken in fluorescent cell imager (Bio-Rad, Hercules, CA, USA).

2.8. Wound healing assay

Around 2 x 10^5 cells were seeded in a 12-well plate and maintained until a confluent cell monolayer was formed. A scratch was introduced using the 20μl tip and wound closure images were taken at 0^{th} and 24th hour with inverted light microscope. The relative level of wound closure was quantified in ImageJ software.

2.9. Dual luciferase assay

Cells were transfected with the vector construct (psiCHECK-2- 3′UTR SOX2) in the first group and co-transfected with vector construct plus miRNA mimic (psiCHECK-2- 3′UTR SOX2 + miR-3163) in the second group for 48 h.Thereafter relative luciferase activity was estimated using dual luciferase reporter assay kit (Promega, Cat. no.- E1910, USA).

2.10. Statistical analysis

All the statistical analysis and graph generation were performed in GraphPad Prism software 8(GraphPad Software). All the experiments were executed in triplicates and the data were shown as mean ± SD. Unpaired Student's t and one-way ANOVA were performed to analyze all the experimental results, where, * = P < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

3. Results –

3.1. Poor prognosis is associated with SOX2 expression in ovarian cancer

Besides its definitive role in embryogenesis, SOX2 is strongly associated with neoplastic differentiation and solicit cancer cell migration, metastasis, proliferation, and invasion [17]. Quiescent tumor cells express elevated levels of SOX2, contributing to chemoresistance and tumor recurrence [18]. Analysis of patient data suggests that SOX2 plays a stronger role in tumor relapse and exhibit greater tumor initiating potential compared to OCT4, NANOG, and other stemness markers[19]. Moreover,SOX2 functioning as a transcription factor is considered as undruggable target [20]. Expression levels of SOX2 in tumor samples compared to adjacent normal tissue, extracted from the TNMplot database, indicate elevated expression in tumor samples (Fig. 1A) [21]. Several databases were explored to identify SOX2 expression correlation with cancer stages along with gene amplification and mutation. Slight upregulation of SOX2 gene expression (transcripts per million) was noticed in advanced stages (stage 3 and 4) compared to early stages (stage 1 and 2) of ovarian cancer patients (cBioportal) (Fig. 1B) [22]. Although the stage wise variation of SOX2 is not that significant but the consistant upregulated expression of the gene in ovarian cancer compared to non-cancerous ovarian tissue is evident. In addition to that, SOX2 gene is emerging as a promising predictor of relapse and death in advanced high grade ovarian serous carcinoma [23]. In fact, the relationship between putative copy number variations such as deletions, alterations, gene amplification and mutation counts as well as mRNA expression data across tumor samples was extracted from GISTIC module on cBioportal (Fig. 1. C, D). Moreover, the gene effect score of SOX2 in various ovarian cancer cell lines was extracted from DepMap portal to highlight the negative effect of SOX2 knockdown on cancer cell proliferation, progression and metastasis (Fig. 1E) [24]. Bioinformatics data extracted from these databases related to SOX2 gene expression establish the association of SOX2 expression with poor prognosis among ovarian cancer patients.

3.2. MiR-3163 as a potential regulator of SOX2

Ovarian cancer is among the few cancer types which gets usually detected at later stages of the disease. Clinical data from the Cancer-MIRNome database on 489 patients, revealed insights into ovarian cancer stages, survival probabilities over time, and patient mortality (Supplementary Fig. 1. A, B, C) [25]. It also highlighted the age wise diagnosis of ovarian cancer (Supplementary Fig. 1. D). So, in our study, we wanted to explore the specific role of miR-3163 which has well

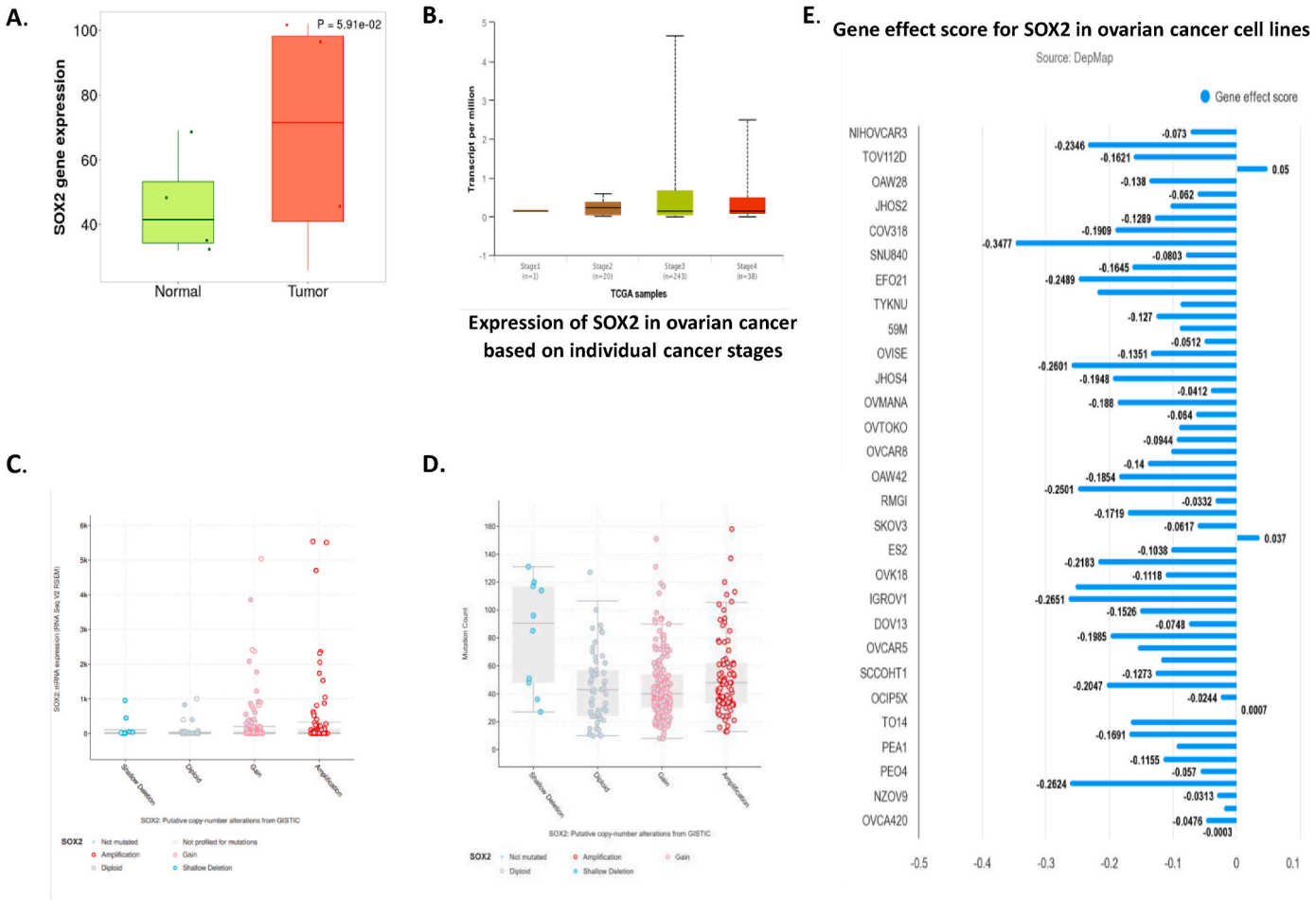

Fig. 1. Clinical significance of SOX2 expression in ovarian cancer patients (**A.**) The relative SOX2 expression level in normal vs tumor tissue (TNMplot). (**B.**) The relative transcripts per million of SOX2 among different cancer stages in ovarian cancer. (**C. D.**) The mutation count, and SOX2 mRNA expression level with the copy number variation data were extracted from GISTIC. (**E.**) Gene effect score for SOX2 in different ovarian cancer cell lines (DepMAp online portal).

known anti-tumorigenic activities in curbing the stemness property in the ovarian cancer patients [12–15,26–28]. Futhermore, pan cancer analysis from the dbDEMC database underscored miR-3163's prevalent downregulation in normal vs tumor tissues (Supplementary Fig. 2 A, B) [29]. By traversing through the miRNA target predicting databases like miRbase (https://www.mirbase.org), targetscan (https://www.targetscan.org/vert_80/), miRDB (https://mirdb.org/) and ENCORI (https://rnasysu.com/encori/), SOX2 was identified as a potential target for miR-3163. Additionally, the pan cancer kaplan meier survival analysis for the miRNA level from TCGA dataset was extracted from cancerMIRnome database (Fig. 2A). The expression level of extracellular miR3163 was retrieved from circulating miRNome dataset (Fig. 2B). All these cumulative data indicate a consistantly low average expression of miR-3163 across cancers, as reflected in low hazard ratio for survival analysis.

3.3. Downregulated expression of miR-3163 in ovarian CSLCs

Lower expression of miR-3163 has been noticed in ovarian CSLCs as compared to corresponding adherent cells. Subsequently, we demonstrated a higher expression of SOX-2 in ovarian CSLCs, reflecting an inverse correlation between miR-3163 and SOX-2 (Fig. 2 C). Similar trend was observed in our database analysis of ovarian cancer patients.

3.4. MiR-3163 negatively regulates the SOX2 level in ovarian cancer

Based on our in-silico analysis, we speculated that miR-3163 targets SOX-2 by binding to its 3′ UTR region. To validate this, the 3′ UTR region of the SOX-2 gene was cloned into the psiCHECK-2 vector. To check the binding of the miR-3163 with its target sequence, we performed the dual luciferase reporter assay by transfecting the psiCHECK-2 vector containing the 3′ UTR region of SOX-2 or psiCHECK2-3′ UTR region along with miR-3163 mimic in both cell lines (OV2008 & OVCAR-3). As shown in (Fig. 3 A), luciferase activity got significantly declined in the psiCHECK-2- 3′UTR SOX2+miR-3163 transfected group compared to the psiCHECK-2- 3′UTR SOX2 transfected alone group. This observation strongly indicates the binding of miR-3163 with its target sequence in 3′ UTR region of SOX2 mRNA. Moreover, Western blot analysis and real time quantitative PCR results also showed downregulated expression of SOX2 in the miR-3163 mimic transfected cells at the protein and mRNA levels, respectively (Fig. 3 B, C). Similarly, ovarian CSLCs, showed decreased mRNA levels of SOX2 upon miRNA mimic transfection compared to controls (Fig. 3 D). All these results suggest a negative correlation between miR-3163 and SOX-2.

3.5. MiR-3163 decreases the stemness property of CSLCs

In order to ascertain the functional role of miR-3163 targeting the SOX2 transcription factor, we performed flow cytometry analysis to

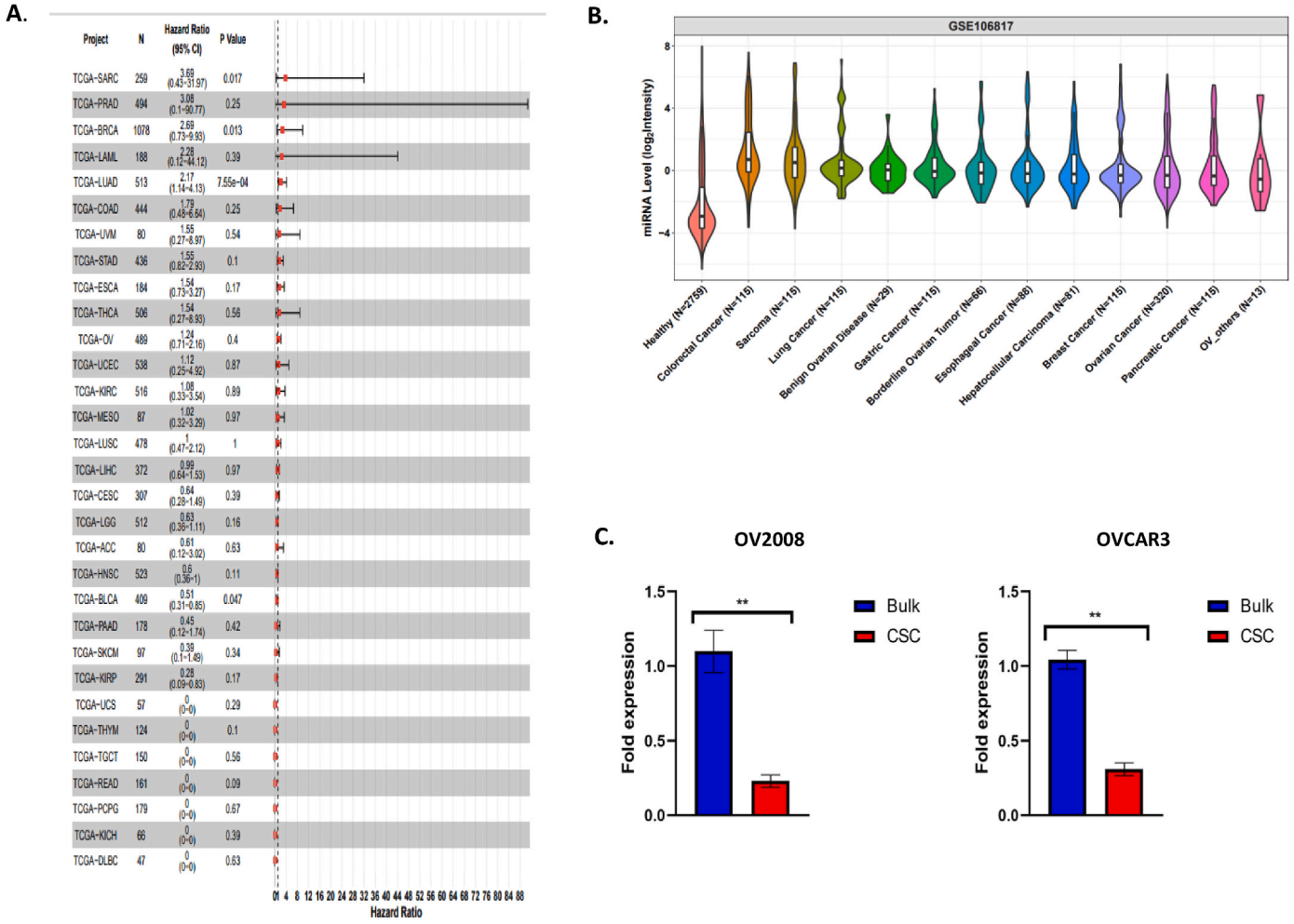

Fig. 2. Pan cancer and cell line-based analysis of miR-3163 levels. (**A.**) Pan cancer kaplan meier survival analysis for the miR-3163 levels from TCGA database (CancerMIRNome database). (**B.**) Extracellular miR-3163 levels from selected circulating miRNome dataset. (**C.**) The expression analysis of miR-3163 in bulk and CSCs of OV2008 and OVCAR-3 cell lines using real-time PCR

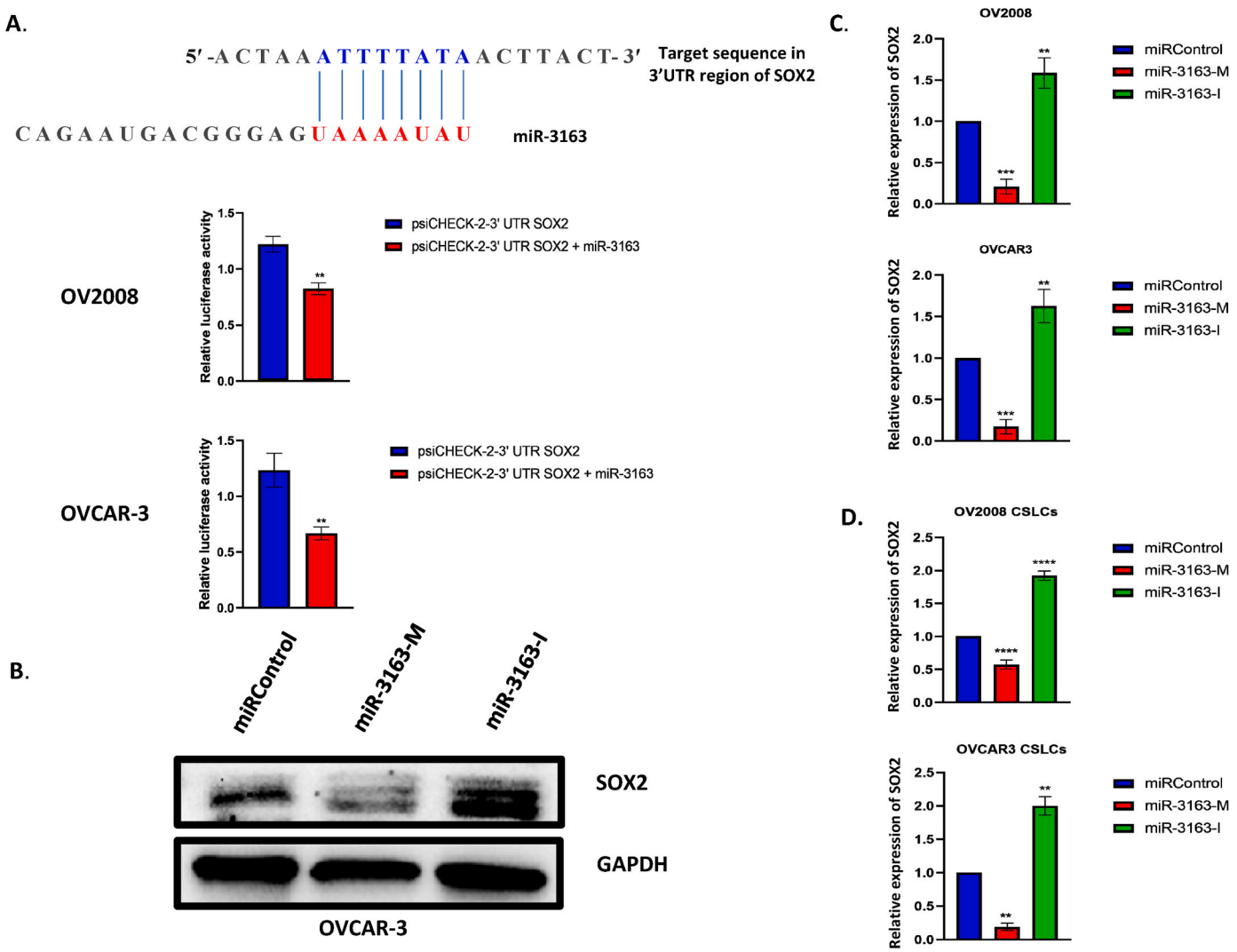

Fig. 3. MiRNA-3163 negatively regulates SOX2 expression in ovarian cancer (A.) OV2008 and OVCAR3 cells were used for luciferase reporter assay. Luciferase activity was detected 2 days after transfection. **(B, C.)** The relative expression levels of SOX2 at the protein and mRNA levels in control, mimic and inhibitor transfected groups were determined by immunoblotting and RT-PCR, respectively. **(D.)** The relative mRNA level of SOX2 in miR-control, mimic and inhibitor transfected CSLCs was determined by RT-PCR, where, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

examine the stem cell markers. Ovarian cancer cells were transfected with miRcontrol, miR-3163 mimic, and inhibitor for 48 h. As shown in Fig. 4A, overexpression of miR-3163 significantly reduced the ovarian CSLCs population characterized by CD44+CD117+ compared to the control group. Similarly, transfection with the miR-3163 inhibitor significantly enhanced the ovarian CSLCs population in both cell lines (Fig. 4A), indicating a clear reduction in the stemness properties of the cancer cells with miR-3163 transfection. Eventually, the spheroid formation assay echoed the same result, showing the reduced sphere-forming ability in the miR-3163 mimic-transfected cells along with a remarkable reduction in the average spheroid diameter (Fig. 4B and C). Another set of spheroids was live-stained with CFDA-SE dye, and average spheroid diameters were observed in the similar manner (Supplementary Fig. 3). Additionally, wound healing assay was performed to analyze the effect of SOX2 downregulation with miR-3163 on the migratory property of the cells (Supplementary Fig. 4). We observed a definite reduction in the migratory and wound healing potential of the mimic-transfected group compared to control group. Thus, miRNA-based approaches are well-documented and applied for better prognosis. Here, we have identified diminished expression of miR-3163 as important for the maintenance of ovarian CSLCs. Therefore, designing therapeutic agents that overexpress miR-3163 in CSLCs represents a novel strategy for the eradication of these cells.

4. Discussion

Cancer stemness remains one of the most controversial and multi-dimensional concepts yet to be fully explained with utmost clarity. With pan cancer studies, there is a constant search for clinically relevant stemness markers at different stages of cancer progression. SOX2, OCT4 and NANOG seems to be universal transcription factors regulating multiple genes at the transcriptional and post-translational levels which are involved in the induction and maintenance of stemness properties of cancer cells. Among them SOX2 belongs to the HMG box (SOX) family of transcription factors and is the most studied member in the context of cancer progression and metastasis [30]. Over the years, numerous research articles have stressed the difficulty in diagnosing ovarian cancer at early stages and highlighted the dynamic role of cancer stem cells in it. Even the therapy resistant, highly aggressive ovarian cancer cells express elevated levels of SOX2 [31]. Although, stage wise variation of the SOX2 expression level was not that prominent but experimental evidences validated the consistent upregulation of SOX2 irrespective of the ovarian cancer stage. In fact, the upregulated expression is directly been correlated with poor prognosis, tumor relapse and death in ovarian

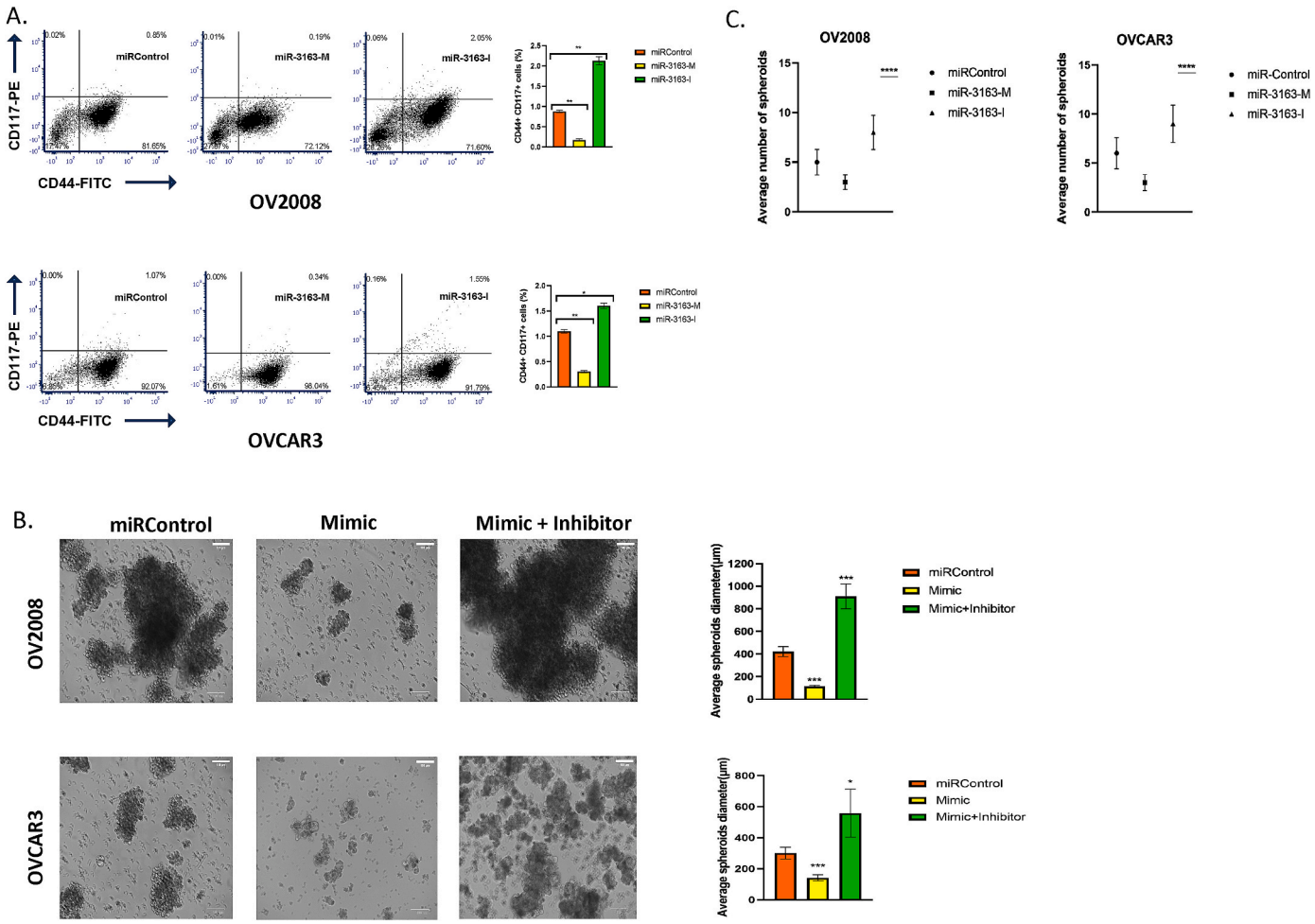

Fig. 4. MiRNA-3163 attenuates the stemness property of the ovarian cancer cells. (**A.**) Flow cytometry analysis showing percentage of CD44+CD117+ cells in OV2008 and OVCAR3 cell lines following transfection with miRcontrol, mimic and inhibitor. (**B.**) Spheriod assay and bar diagram showing average spheroids diameter formed in different groups,scale bar=100μm. (**C.**) Average number of spheroids formed in each microscopic fields with miRcontrol, mimic and inhibitor transfected cells. where, * = P < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

serous carcinoma [23]. Contributing to cancer chemoresistance, SOX2 actively participates as a master transcription regulator in regulating ABC drug transporters, pro- and anti-apoptotic proteins, immune evasion and lineage plasticity [32]. Here, we have tried to establish miR-3163 as a potential candidate for targeting the master regulator to counter the initiation and maintenance of cancer stemness. The mi-3163 could potentially be used as adjuvant therapy for 'hard to treat' ovarian cancer patients at advance stages to combat the tumor growth and EMT process with elevated SOX2 expression. As far as the predictive information from different bioinformatics database is concerned, SOX2 is predicted to be a target of miR-3163. Here we have identified miR-3163 to be potentially targeting SOX2 mRNA expression which was confirmed with western blot and quantitative real time PCR analysis. In fact, the diminished expression of SOX2 in the miR-3163 mimic transfected group impacted the stemness and invasive properties of the cancer cells. In order to investigate downstream consequences, we observed a reduced CSLCs population in mimic transfected group as compared to the control, confirming the CSCs depleting ability of miR-3163. Similarly, metastatic and wound healing capacity were also hampered in mimic transfected group. Similarly, spheroids generated from the diffrent groups even showed considerable differences in diameter. The miR-3163 mimic transfected group exhibited diminished potential in spheroid forming ability as compared to miRcontrol.

Moreover, we confirmed the SOX-2 as a direct target of miR-3163 through dual luciferase assay, confirming that miR-3163 exert its therapeutic efficacy via targeting SOX-2. The significance of our result depends on the fact that downregulation of SOX2 could counter the enrichment of stemness properties of the cancer cells. Thus SOX2 inhibitors can be used as a part of adjuvant therapy in conjunction with existing therapeutic intervention. Targeting SOX2 via miR-3163 overexpression may potentiate the response to chemotherapeutic drugs and possibly increase the 5-year survival rate of the patients with aggressive ovarian cancer.

5. Conclusion

The results of this particular study reveals that miR-3163 is remarkably downregulated in ovarian cancer stem like cells compared to adherent cells. Expression level data in the ovarian cancer patients extracted from online databases also demonstrated diminished expression of the miR-3163 across various malignancies. In fact, we demonstrated that miR-3163 could negatively regulate the cancer stemness properties of ovarian CSLCs by targeting the expression level of SOX2 transcription factor. MiR-3163 could modulate the cancer cell spheroid forming ability, proliferation, metastasis and more. Since the ovarian cancer usually gets detected in later stages of cancer progression, reduced expression of miR-3163 could serve as a prognostic indicator. At the same time, miR-3163 overexpression strategies can be used as adjuvant therapy to counter the expansion of cancer stem cell- like population and prevent tumor relapse. Further in-depth investigation

would certainly lead to a better understanding of the cancer stemness property and designing of therapeutic strategies based on miRNAs for patients with aggressive ovarian cancer.

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CRediT authorship contribution statement

Bilash Chatterjee: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Subhankar Bose:** Methodology. **Richa Singh:** Writing – review & editing, Validation. **Amit Kumar Dixit:** Formal analysis, Validation, Writing – review & editing. **Lalrin Puia:** Formal analysis, Validation, Writing – review & editing. **Amit Kumar Srivastava:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.06.012.

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