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Research Paper

Let-7a suppresses Ewing sarcoma CSCs' malignant phenotype via forming a positive feedback circuit with STAT3 and lin28

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ABSTRACT

Cancer stem cells (CSCs) have been documented to be closely related with tumor metastasis and recurrence, and the same important role were identified in Ewing Sarcoma (ES). In our previous study, we found that let-7a expression was repressed in ES. Herein, we further identified its putative effects in the CSCs of ES (ES-CSCs). The expression of let-7a was consistently suppressed in the separated side population (SP) cells, which were identified to contain the characteristics of the stem cells. Then, we increased the expression of let-7a in ES-CSCs, and found that the ability of colony formation and invasion of ES-CSCs were suppressed *in vitro*. The same results were found in the tumor growth of ES-CSCs' xenograft mice *in vivo*. To further explore the putative mechanism involved, we also explored whether signal transducer and activator of transcription 3 (STAT3) was involved in the suppressive effects. As expected, excessive expression of let-7a could suppress the expression STAT3 in the ES-CSCs, and repressed the expression of STAT3 imitated the suppressive effects of let-7a on ES-CSCs, suppressing the ability of colony formation and invasion of ES-CSCs. Furthermore, we found lin28 was involved in the relative impacts of let-7a, as well as STAT3. Let-7a, STAT3 and lin28 might form a positive feedback circuit, which serve a pivotal role in the carcinogensis of ES-CSCs. These findings maybe provide assistance for patients with ES in the future, especially those with metastasis and recurrence, and new directions for their treatment.

1. Introduction

Ewing sarcoma is a cancer of the soft tissue and bone. Among them, the Ewing sarcoma of bone is a kind of greatly malignant primary bone tumor, which is common in children and adolescents, which progresses rapidly [1]. For patients whose lesion is localized, drug chemotherapy combined with radiotherapy or surgery can remarkably improve the survival rate to 70%, but for patients whose lesion is metastatic, the survival rate is only 30% [2]. Thus, understanding the mechanism involved in tumor metastasis and recurrence may help us enhance the survival rate of individuals with metastatic.

CSCs are produced by dysregulation of proliferation, as well as differentiation of normal stem cells, with stem-like characteristics and potential for proliferation, as well as self-renewal. CSCs is the origin of tumor occurrence, development and metastasis, although it's a small part of tumors [3]. CSCs have the characteristics of drug resistance due to high expression of ATP-binding cassette transporter G 2 (ABCG2), which can be used to the isolation of CSCs. Suva et al. successfully isolated and identified CSCs in ES [4], they confirmed that miR-145 could down modulate the expression of OCT4, NANOG and SOX2[5]. Meanwhile, when comparing the miRNA expression profiles of CSCs, mesenchymal stem cells (MSCs) and human induced pluripotent stem cells (iPSCs) in ES, they found that the precursors of miR-125b, miR-145, miR-30a, miR-7, let-7a, as well as miR-143 in ES-CSCs, which are remarkably higher than the others. Overexpressing of miR-143 and miR-145 in ES-CSCs inhibited the ability of proliferation and the growth of

Abbreviations: CSCs, Cancer stem cells; ES, Ewing Sarcoma; ES-CSCs, CSCs of ES; SP, side populationl; STAT3, signal transducer and activator of transcription 3; ABCG2, ATP-binding cassette transporter G 2; MSCs, mesenchymal stem cells; iPSCs, human induced pluripotent stem cells; ATCC, American Type Culture Collection; FBS, fatal bovine serum; PBS, phosphate buffer saline; PI, propidium iodide; ORF, open reading frame; MMP2, Matrix Metallopeptidase 2.

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xenograft tumor in nude mice. The results indicated that miRNAs have an inhibitory effect on ES-CSCs [6], Therefore, it would make sense to explore the mechanism of miRNA in ES-CSCs.

In our previously reported research, we found that let-7a function as a tumor repressor in ES cell lines [7], and STAT3 is the target gene of let-7a [8], which is published associated with CSCs[9,10]. As a member of let-7 miRNA family [11], let-7a has been documented to be involved in functional modulation of breast cancer stem cells [12,13]. However, the putative effect of let-7a on the ES-CSCs remains unknown. In this study, we confirmed that let-7a represses the malignant phenotype of ES-CSCs, and this function may be achieved by forming a positive feedback circuit with STAT3 and lin28.

2. Materials and methods

2.1. Cell line and culture conditions

Human ES cell lines A673, and SK-ES-1 were acquired from the American Type Culture Collection(ATCC) and were grown in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) added fetal bovine serum (10%) (FBS; PAA, Linz, Austria), penicillin (100 mg/ml), as well as streptomycin (100 mg/ml) (Invitrogen, USA). Incubation of all the cells was conducted in an incubator (5% CO_2 at 37℃).

2.2. Isolation of ES SP cells

CSCs can exported the Hoechst 33,342 fluorescent dye due to drug resistance, which is attributed to the high expression of ABCG2, therefore these cells can be isolated by flow cytometry. As documented by Goodell et al. isolation of SP cells was carried out as per the side populations protocol [14]. The suspension of cells was done in RPMI 1640 medium(1×10^6 cells/ml) enriched with 10% fatal bovine serum (FBS) (PAA, Pasching, Austria), 100 mg/ml penicillin (Sigma-Aldrich, California, USA) and 100 ug/ml streptomycin (Sigma-Aldrich, California, USA), as well as 5 μg/mL Hoechst 33,342 (Sigma-Aldrich, California, USA), with or without ABCG2 repressor, verapamil (150 μmol/L)[15] (Sigma-Aldrich, California, USA), were incubated in 37 °C incubator for 90 min. Then, incubation of the cells were conducted for 30 min in a shaking water bath, followed by placing the cells on ice to terminate dye efflux. After that, rinsing of the cells was carried out in 2 ml cold phosphate buffer saline (PBS) enriched with FBS (5%) (PAA, Pasching, Austria). The dead cells were labeled with 2 ug/ml propidium iodide (PI) (Sigma-Aldrich, California, USA), and the proportions of SP cells were analyzed by flow cytometry.

Signals were collected under Linear mode, and PI fluorescence was detected by a 630/30 BP filter, which was used to separate dead cells from Hoechst staining cells alive. PI-negative cells were collected by FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The divergence of the Hoechst33342 dye were measured by two detectors :Hoechst Blue and Hoechst Red. The 405/20 BP filter detected the Blue scattered light (Hoechst Blue) and the 660 nm long pass filter detected the Red scattered light (Hoechst Red),

Two-dimensional scatter plots of fluorescence were made with Hoechst Red as X-axis and Hoechst Blue as Y-axis respectively. The areas with low Hoechst Red and low Hoechst Blue, and the missing part of cells treated with verapamil were set as the gate of SP cells, and the percentage was calculated by analysis software. SP cells and non-SP cells were isolated and collected.

2.3. Oligonucleotide transfection

Let-7a mimic, siRNAs (distinct for STAT3, and lin28) scrambled mimic, as well as siRNA control oligonucleotides were bought from Dharmacon (Austin, TX, USA). Transfection of all the oligonucleotides into cells (50 nM) was carried out using Dharmafect 1 (Dharmacon, Austin, TX, USA) as outlined in the manufacturer provided protocol. The medium of the culture was refreshed via changing 6 h after transfection. The cells were grown another 48 h, then harvested for evaluation.

2.4. RNA extraction and RT-qPCR

For *in vitro* quantitation of the let-7a expression, the TRIzol reagent (Life Technologies, Darmstadt, Germany) was employed in isolating total RNA as per the manufacturer provided protocol. After that, gel electrophoresis was done to verify the integrity of the RNA. Then, cDNA was generated via reverse transcription of the tRNA with the First- Strand cDNA Synthesis kit (Life Technologies, Darmstadt, Germany) using gene-specific primers coupled to a TaqMan probe. Small nuclear U6 served as the endogenous control gene, and the non-SP cells or ES-CSCs transfection with scramble mimic served as control. The Quanti-Tect SYBR Green PCR mixture was employed in carrying out quantitative PC, which was run on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, USA). The sequences of the specific primers for reverse transcription, as well as the RT-PCR reaction are indicated in the Supplementary Table S1. The resulting data were standardized using the internal standard, and evaluation of the relative expression levels done via the $2^{-\Delta\Delta Ct}$ approach. All experiments were replicated thrice.

2.5. Cell colony formation assay

Following 48 h of infection, the cells were collected, followed by planting of 200 cells in a 6-well plate and allowed to grow for 14 days. The medium was changed after one week, then the medium was replaced twice a week. At the end of 14 days, rinsing of the cells was done twice in PBS. Thereafter, fixing of the cells for 20 min was done using methanol, then 20 min staining in 0.1% crystal violet (Beyotime Biotechnology, Shanghai, China) conducted. Lastly, cells were counted and photographed.

2.6. Cell invasion assays

All the tests were conducted in modified Boyden chambers (BD Biosciences, San Jose, CA, USA) with 8-μm pore filter inserts in 24-well plates. We coated the Transwell chambers with Matrigel (BD Biosciences, San Jose, CA, USA) and solidification done for 3 h at 37 °C. 24 h after transfection, introduction of the 2×10^5 ES-CSCs inserted with let-7a or scramble mimic via transfection and suspended in serum-free medium was done in the upper chamber. Furthermore, the medium enriched with 20% FBS was introduced to the lower chamber to act as a chemoattractant. At the end of 24 h, cells retained in the upper chambers were gently removed with a cotton swab. Staining of the cells in the lower chambers was done using crystal violet, then air dried, and images acquired. All experiments were independently replicated thrice. Imaging of 6 fields from each chamber was done at 100 × using on an Olympus IX81 microscope with CCD camera.

2.7. Plasmid construction

Amplification of the complete open reading frame (ORF) of STAT3, as well as lin28 was conducted, followed by cloning into the pcDNA3.1 vector to create constructs of pcDNA3.1-STAT3, as

well as pcDNA3.1-lin28, respectively. Sequencing of the complete STAT3, as well as lin28 genes was done at Beijing Tianyi Huiyuan Bioscience & Technology Inc., Beijing, China for verification of the genes. An empty construct of pcDNA3.1 served as a negative control. Regarding the rescue tests, ES-CSCs were first inserted with constructs of pcDNA3.1-STAT3 or pcDNA3.1 (2.0 μg) via transfection in 6-well culture dishes. 48 h following transfection, we harvested the cells at intervals and assayed.

2.8. Immunoblot analysis

After 48 h of transfection, we harvested the ES-CSCs in ice-cold PBS. Thereafter, lysing of the cells was done on ice using cold-modified radioimmunoprecipitation buffer enriched with protease repressors. The BCA Protein Assay Kit (Bio-Rad, Italy) was employed to determined the protein concentrations. After that, fractionation of the proteins was carried out on SDS-PAGE gel, then transfer-embedded onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Blocking of the membranes was done for 2 h using non-fat milk powder (5%) enriched with 0.1% Tween-20, followed by overnight-incubation with primary antibodies incubated at 4 °C and then conjugation with the peroxidase-labeled secondary antibodies performed via another incubation. Visualization of the protein bands was done on an enhanced chemiluminescence system (Millipore, Billerica, MA, USA). The primary antibodies used herein included anti-STAT3, anti-c-Myc, anti-p-STAT3, anti-MMP2 (Matrix Metallopeptidase 2), as well as anti-lin28 (all from Cell Signaling Technology, Danvers, MA, USA). GAPDH served as the internal standard (Zhong-Shan JinQiao, Beijing, China). All the experiments were replicated thrice. The concentrations of primary antibody were all 1:500, and that of secondary antibody is 1:5000.

2.9. Xenograft model assays in vivo

In the xenograft assays, subcutaneous administration of 1×10^5 ES-CSCs was done into the posterior flanks of 12 six-week-old female nude mice. When tumors attained a size of 200 mm^3, we selected 8 mice with almost equivalent volumes of tumors for subsequent experiments. These mice were randomly grouped into two groups (n = 4). A suspension of Let-7a or scramble mimic (5 μg) in 100 μl of Dharmafect 1 solution was administered into each tumor, with the injections done every 3 days for up to 7 times. After 7 days of ES-CSCs injection, measurement of the tumor diameter was done, and then after every successive 3 days. After 28 days following the last administration, we sacrificed all the mice, excised the tumors, and then weighed them. At the same time, the following formula was employed to determine the tumor volume; length $\times$ width^2 $\times$ 1/2. The mice xenograft experiments were conducted as per the institute guidelines.

2.10. Immunohistochemistry of xenograft tumor tissues

The tumor tissues of xenograft were processed, paraffin embedded and sectioned. The sections were incubated at 60℃ for 4 h, followed be deparaffinized in xylene and anhydrous ethanol. TBST containing 5% serum were be used to block the sections for 1 h. Sections incubated with primary antibody (anti-STAT3, anti-lin28, the concentration were 1:200; (Cell Signaling Technology, Danvers, MA, USA)) at 4℃ overnight. Normal immunoglobulin G were incubated with control section. Then, the secondary antibody (1:200; Cell Signaling Technology, Danvers, MA, USA) incubated the sections at room temperature for 30 min in the next day to staining. Finally, liquid DAB (DakoCytomation, Carpinteria, CA, USA) were used to counterstained, then sections be observed and photographed under the microscope.

2.11. Statistical analyses

Data are indicated as mean and standard deviation. The SPSS 15.0 software was employed in computing the statistical analyses. Comparisons between 2 groups was done using the student's *t*-test, and analysis of variance was employed for comparison among 3 groups. Moreover, the chi-squared test was utilized for occurrence analysis. $P \leq 0.05$ signified statistical significance.

3. Result

3.1. Isolation and identification of ES-CSCs

Since SP cells were recognized as cancer stem-like cell populations, we focused to utilize this sub clone to inspect the putative influences of let-7a on the malignant phenotype of ES-CSC cells. Firstly, we extracted the SP cells via staining the grown A673, as well as SK-ES-1 cells with the fluorescent DNA-binding dye Hoechst33342 and then FACS sorting. Consequently, the flow cytometric assay of Hoechst-labeled A673 and SK-ES-1 cells for blue, as well as red fluorescence disclosed a small population of cells with low fluorescence, suggesting active extrusion of the dye (Fig. 1A and 1B). The side population features of these cells with low fluorescence were validated via "normal fluorescence" (Fig. 1A and 1B) after administration with inhibitors of ATP-binding cassette transporters, verapamil. Utilizing verapamil for characterization, 1.3% ± 0.2% of the total cells in A673 culture, and 1.5% ± 0.2% of the total cells in SK-ES-1 growth demonstrated side population characteristics, respectively.

Furthermore, to identify whether the sorted cells were malignant stem-like cells, the expression of CD133 and Sox2 and the ability of colony formation, as well as invasion was inspected. Obviously, the expression of CD133 and Sox2 (Fig. 1C) and the ability of colony formation (Fig. 1D), as well as invasion (Fig. 1E) were both increased in SP cell population of A673 and SK-ES-1 cells , suggesting the stem-like characteristics of ES-SP cells.

3.2. Let-7a performed an suppressor in ES-CSCs

Our previous research has uncovered the let-7a repressive effects in ES cells[7]. Although the repressive influences of let-7a in the CSCs of other kinds of cancers have been widely reported, its role in the ES-CSCs has not been identified. We inspected the expression of let-7a in extracted ES-SP cells, as well as ES-non-SP cells via RT-qPCR inspection. The results showed that the expression of let-7a was remarkably diminished in the ES-SP cells compared with the ES-non-SP cells (Fig. 2A). Then we changed the let-7a expression in ES cells through transfected the let-7a or scramble mimic into A673, as well as SK-ES-1 cells, respectively (Fig. 2B). Then, its effects on the ratio of SP cells were performed. Interestingly, overexpression of let-7a could reduce the percentage of SP cells in ES cells (Fig. 2C), suggesting the putative suppressive effects of let-7a in ES-CSCs.

Then, we further inspected the influence of let-7a on the ES-SP cells malignant phenotypes. The sorted ES-SP cells were separated and cultured respectively, named as A673-SP cells, as well as SK-ES-1-SP cells. We transfected the A673-SP, as well as SK-ES-1-SP cells with let-7a mimic or scramble mimic. Evidently the expression of let-7a was markedly increased in ES-SP cells while treated with let-7a (Fig. 2D). The effects of let-7a on the ability of colony formation and invasion of ES-SP cells were tested by colony formation assays and Matrigel invasion assays. As shown in the figures, comparing with the control group, ES-SP cells treated with scramble mimic, the colony formation ability and invasive ability of ES-SP cells treated with let-7a were all decreased. These experiments

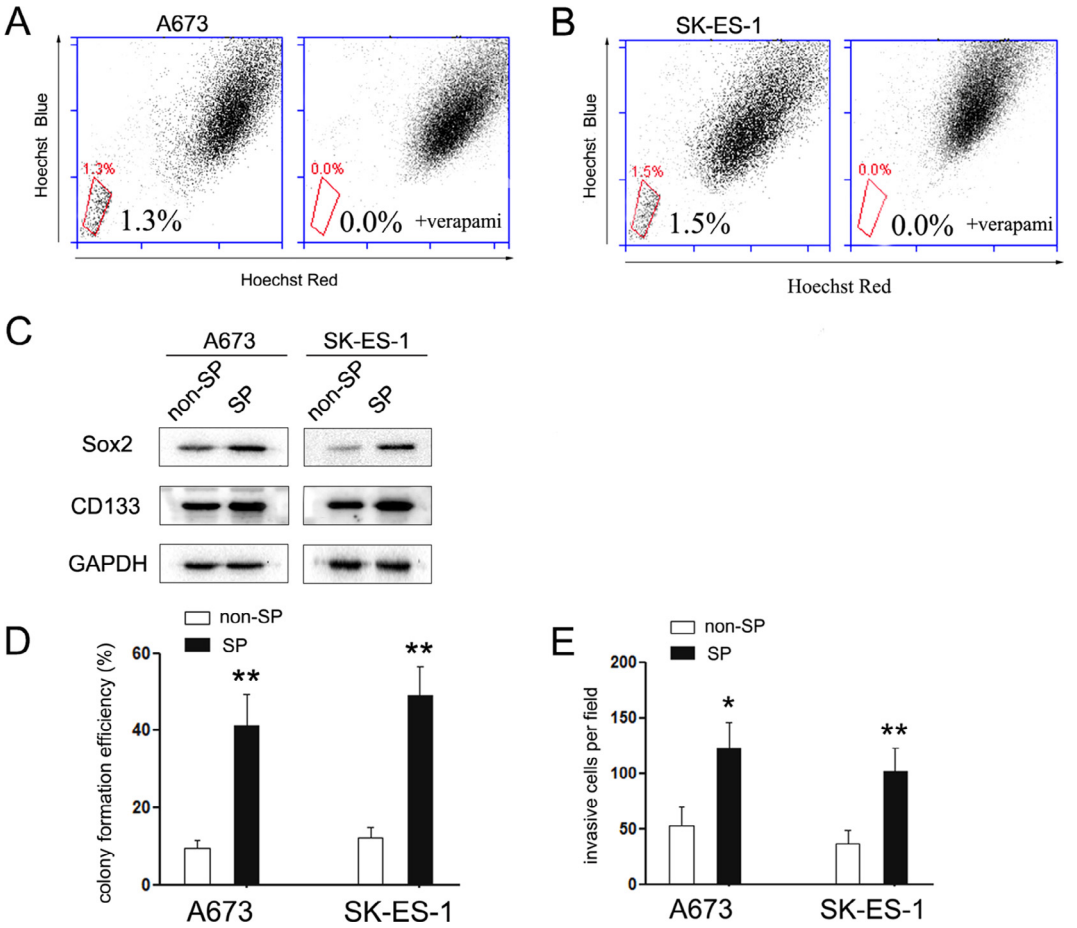

Fig. 1. Isolation and identification of ES-CSCs. Flow cytometric analysis of Hoechst-labeled A673 (A), as well as SK-ES-1 (B) cells. Upon treated with verapamil, a part of cells with low fluorescence were sorted. C. Western blot evaluation were employed to identify the expression of Sox2 and CD133 in SP cells. The expression of Sox2 and CD133 were increased in SP cells. D. The colony formation assays were employed to tested the colony formation capability in sorted cells, and the results showed that the colony formation capability of SP cells were more capable compared with non-SP cells. E. The Matrigel invasion assays were employed to tested the invasive capability in sorted cells, and the results showed that the invasive capability of SP cells were more capable compared with non-SP cells.$^{*}P < 0.05$, $^{**}P < 0.01$.

indicated that let-7a could suppress the malignant phenotype of ES-CSCs.

3.3. STAT3 pathway might participate in the let-7a-mediated effects in ES-CSCs

STAT3 pathway is a crucial signal pathway, which has been reported to be activated in many cancers, especially in the CSCs formation [9,10] . We found that STAT3 is one of direct target genes of let-7a through miRanda and PicTar (Fig. 3A), which were two different kinds of miRNA target genes biology information website, and the results showed that we got 15 target genes. STAT3 was the only one which have been reported associated with CSCs[16]. The repressive influences on malignant phenotype in ES-CSCs had been confirmed, and then we explored whether STAT3 is involved in the influences furtherly in ES-CSCs. To begin with, we explored the expression of STAT3 and its subsequent genes phopho-STAT3 (p-STAT3), MMP2 and c-Myc in A673-SP and SK-ES-1-SP cells (Fig. 3B). Like other kinds of CSC cells, the STAT3 signaling pathway was activated in SP cells comparing with the non-SP cells. Then, we monitored their expression while silencing the STAT3 expression in ES-SP cells, and found that the expression of STAT3 signaling pathway and its downstream targeted gene were also suppressed (Fig. 3C).

The results of colony formation assays showed that ES-SP cells treated with si-STAT3 were remarkably repressed on cells colony formation relative to the control group (Fig. 3D). The invasive ability showed the same results (Fig. 3E). As well as transfection with let-7a, decreased the expression of STAT3 suppressed the malignant phenotype of ES-CSCs, too. Which means the influences of silencing the expression of STAT3 imitated the increased let-7a expression on ES-CSCs. Since let-7a could directly target the 3′-UTR of STAT3 mRNA, we suspected STAT3 might also participate in the repressive effect of let-7a on ES-CSCs. Lastly, we explored the expression of STAT3 and its downstream genes p-STAT3, MMP2, C-Myc, after transfection with let-7a in ES-CSCs. The expression of STAT3 and its subsequent genes were consistently decreased upon transfection with let-7a (Fig. 3F). Collectively, these data further confirmed our hypothesis that the STAT3 signaling pathway might participate in the repressive effects of let-7a on ES-CSCs.

3.4. Upregulation STAT3 mediates let-7a repression and lin28 might participate in the modulation

STAT3 classically behaves as an activator protein. Interestingly, in our previous paper, we found the negative relationship between let-7a and STAT3 expression, especially, we found the STAT3 expression repressed the expression of let-7a in ES cells[8]. Thus, we also detected the expression of let-7a after suppressing the expression of STAT3 in ES-SP cells. Obviously, the expression of let-7a in ES-SP cells were consistently decreased upon transfection

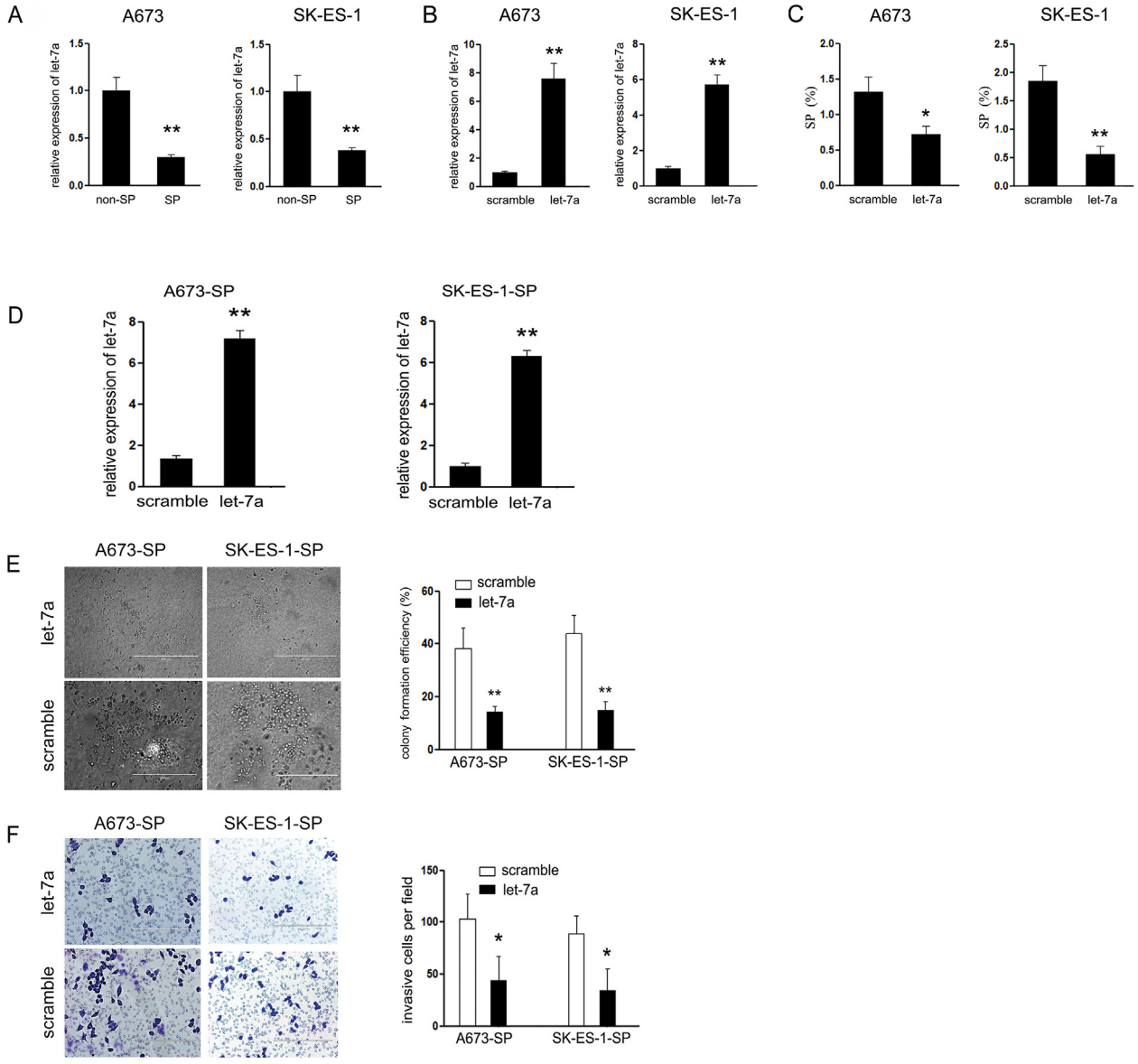

Fig. 2. Let-7a performed as a tumor repressor in the SP cells. A. RT-qPCR inspection were conducted to explore the expression of let-7a in the SP and non-SP cells. B. The expression of let-7a were increased in ES cells upon transfection with let-7a. C. The ratios of ES cells were decreased upon transfection with let-7a. D. RT-qPCR inspection were conducted to demonstrated that the overexpression of let-7a in A673-SP cells and SK-ES-1-SP cells upon transfection with let-7a. E. The inhibiting effects of let-7a on the colony formation capability of A673-SP cells and SK-ES-1-SP cells were tested by colony formation assays. F. The inhibiting effects of let-7a on the invasive capability of A673-SP cells and SK-ES-1-SP cells were tested by Matrigel invasion assays. Small nuclear U6 served as the endogenous control gene and evaluation of the relative expression levels done via the $2^{-\Delta\Delta Ct}$ approach in the RT-qPCR inspection. $^{*}P < 0.05$, $^{**}P < 0.01$.

with pcDNA-STAT3 (Fig. 4A). While, the expression of let-7a were increased when the expression of STAT3 were decreased in ES-SP cells (Fig. 4B). Then, we further explored what mediates the suppression of let-7a by STAT3. Except for NF-κB as previously reported, whether there were other genes involved[8].

Lin28 is an important transcription factor that also serves a pivotal role in iPSCs [17]. It is not only performed as an important transcription factor through blocking the cracking of pri-let-7a and pre-let-7a thus inhibit the production of mature let-7a[18], but also contain two binding sites of STAT3 on its 5′-UTR [19]. Overexpression of STAT3 remarkably up modulated the expression of lin28 in ES-CSCs (Fig. 4C), and the contrary results were found when the expression of STAT3 was suppressed (Fig. 4D). These results suggested a putative feedback circuit in ES-CSCs, which is let-7a targeted suppresses the STAT3 expression, and STAT3 in turn suppresses the expression of let-7a through lin28.

To identify the putative mechanism, we further explored the effects of lin28 on the expression let-7a and STAT3 in ES-CSCs. Lin28 could block the crack of pre-let-7a and pri-let-7a and thus suppress the expression of mature let-7a. We established that overexpression of lin28 actually repressed the expression of let-7a in ES-SP cells (Fig. 4E); conversely, knockdown its expression increased the expression of let-7a (Fig. 4F). On the other side, the expression of STAT3 increased correspondingly when transfected with pcDNA-lin28 (Fig. 4G), and the expression was suppressed upon transfection with si-lin28 (Fig. 4H). These data further suggest that the feedback circuit containing let-7a, STAT3 and lin28 might perform important roles in ES-CSCs.

A

Gene Symbol	Description
RPUSD3	RNA pseudouridylate synthase domain containing 3
FNDC3B	fibronectin type III domain containing 3B
CDC34	Ubiquitin carrier protein
ZZZ3	zinc finger, ZZ domain containing 3
EDEM3	ER degradation-enhancing alpha-mannosidase-like 3
APPBP2	Amyloid protein-binding protein 2
POGZ	Pogo transposable element with ZNF domain
CDH22	Cadherin-22 precursor
ZNF710	Zinc finger protein 710
WAPAL	Wings apart-like protein homolog
RFXDC1	Regulatory factor X domain containing 1
GDF6	Growth/differentiation factor 6 precursor
EPHA3	Ephrin type-A receptor 3 precursor
SURF4	Surfeit locus protein 4
STAT3	Signal transducer and activator of transcription 3

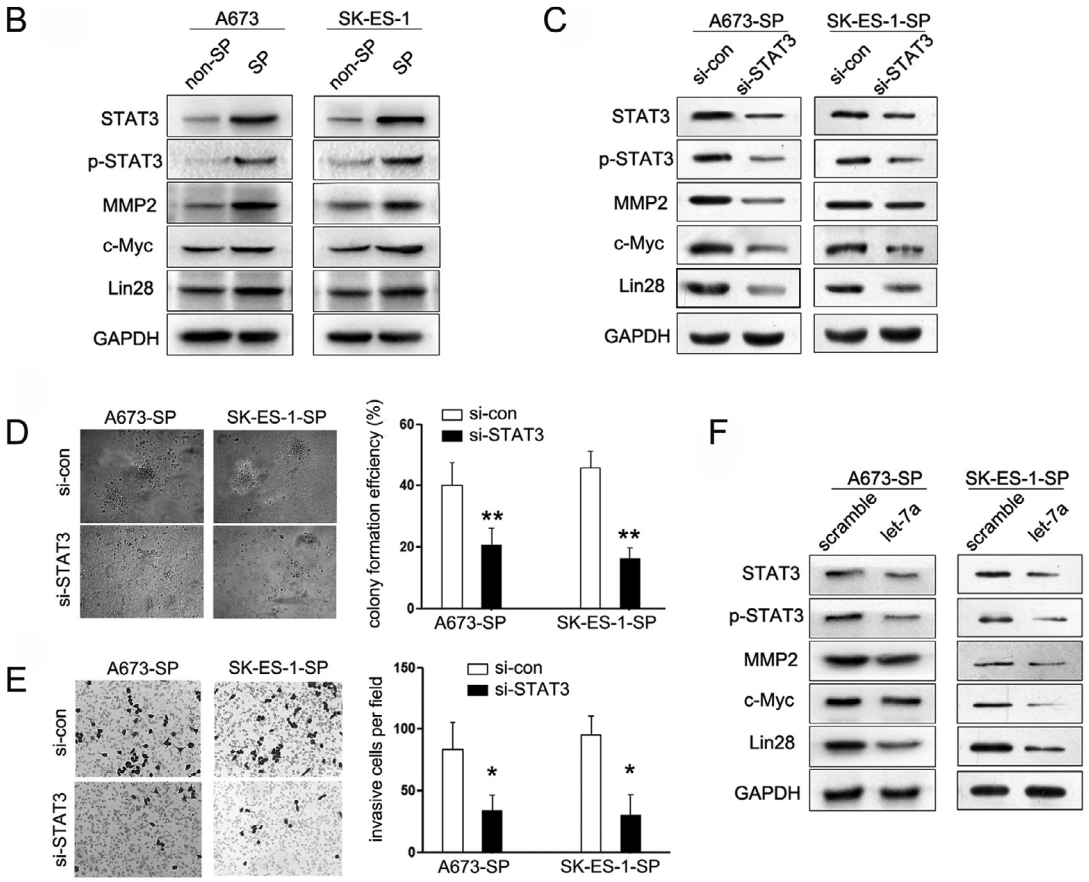

Fig. 3. STAT3 might participate in let-7a-mediated repressive effects on SP cells. A. The two different miRNA target gene biological information websites (miRanda and PicTar) were used to predict the possible target gene of let-7a, and these 15 gene could be predicted by both of them. B. Western blot evaluation were carried out to inspect the expression of STAT3 and its down-stream genes p-STAT3, MMP2, c-Myc and lin28 in SP cells, as well as non-SP cells. C. The expression of STAT3 and its downstream genes were decreased through transfection with si-STAT3 construct. D. The inhibiting effects of si-STAT3 on the colony formation capability of ES-SP cells were tested by colony formation assays. E. The inhibiting effects of si-STAT3 on the invasive capability of ES-SP cells were tested by Matrigel invasion assays. F. Overexpression of let-7a in ES-SP Cells, the expression of STAT3 and its down-stream genes were decreased. $^{*}P < 0.05$, $^{**}P < 0.01$.

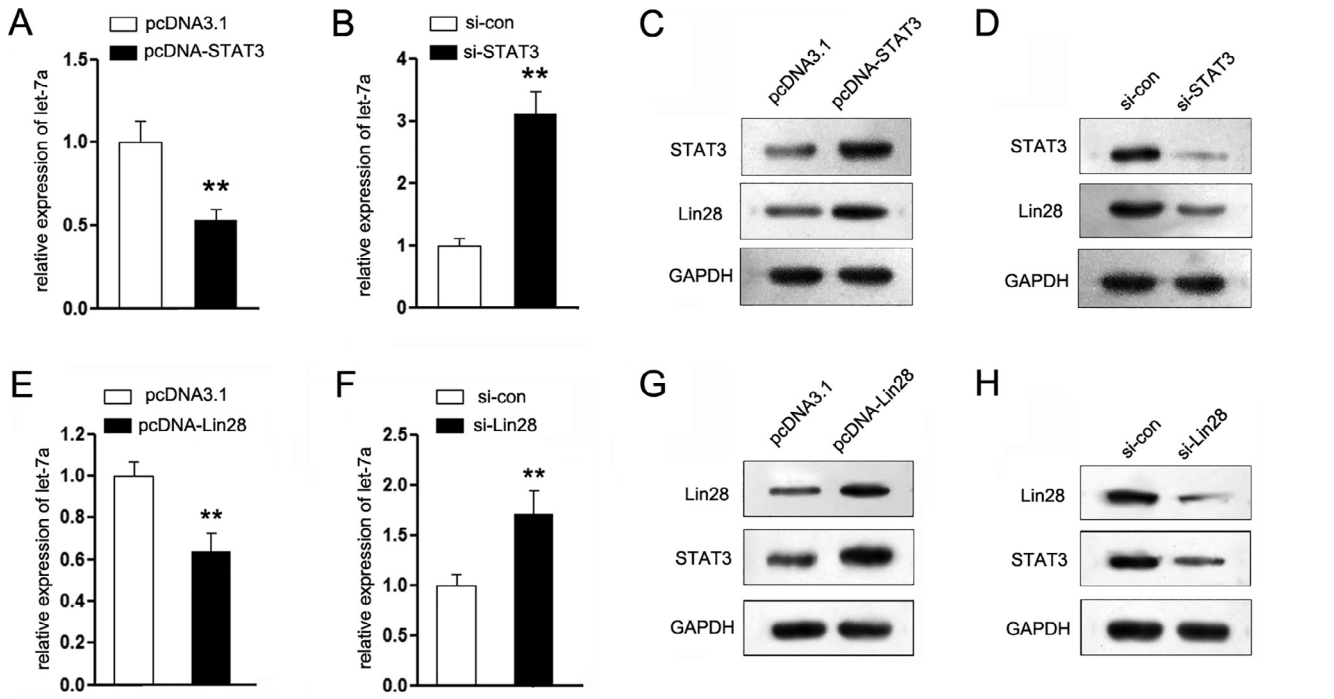

Fig. 4. STAT3 could modulate the expression of let-7a through lin28 in ES-SP cells. A. RT-qPCR inspection showed the expression of let-7a in A673-SP cells were decreased upon transfection with pcDNA-STAT3. B. The expression of let-7a in A673-SP cells were increased upon transfection with si-STAT3. C. Western blot evaluation showed the expression of lin-28 in A673-SP cells were increased upon transfection with pcDNA-STAT3. D. The expression of lin-28 in A673-SP cells were suppressed upon transfection with si-STAT3. E. RT-qPCR inspection demonstrated the expression of let-7a in A673-SP cells were suppressed upon transfection with pcDNA-lin28. F. The expression of let-7a in A673-SP cells were increased upon transfection with si-lin28. G. Western blot evaluation indicated the expression of STAT3 in A673-SP cells were increased upon transfection with pcDNA-lin28. H. The expression of STAT3 in A673-SP cells were decreased upon transfection with si-lin28. Small nuclear U6 served as the endogenous control gene and evaluation of the relative expression levels done via the $2^{-\Delta\Delta Ct}$ approach in the RT-qPCR inspection. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.5. Restoration of let-7a represses ES tumor growth in vivo

In order to verify the role of the positive feedback circuit *in vivo*, 1×10^5 ES-CSCs were implanted into the posterior flanks of immunocompromised nude mice, with the let-7a or scramble mimic was directly administered into the resultant tumors after its formation (Fig. 5A). We observed that the volume of subcutaneous tumor injected with let-7a mimic was distinctly smaller than those injected with scramble mimic (Fig. 5B), and congruent with the tumor volume (Fig. 5C), the average tumor weight was also remarkably reduced (Fig. 5D). Finally, we also inspected the expression of let-7a, STAT3, and lin28 in xenografts. Immunohistochemical analysis of STAT3 and lin28 protein contents in tumor tissue evaluated the expressions of STAT3 and lin28 in the xenografts. Congruent with our results *in vitro*, STAT3 and lin28 expression were decreased (Fig. 5F) when let-7a expression was increased (Fig. 5E) in tumors. Therefore, the let-7a/STAT3/lin28 circuit may mediated the growth of ES tumor through serve as a key role in ES-CSCs.

4. Discussion

CSCs have been documented to serve a crucial role in tumor recurrence and metastasis [20]. As to ES, the problem about recurrence and metastasis restrict the prognosis of patients for a long time [2]. Therefore, CSCs research has increasingly become an important entry point to improve the efficacy of patients with ES. Suva et al. successfully isolated and identified CSCs in ES cells previously [4] and confirmed the modulatory role of miRNA in ES-CSCs. Meanwhile, when comparing the expression profiles of miRNA in ES-CSCs and non-CSCs, they found that the expression levels of multiple microRNA precursors, including let-7a, were remarkably increased[5,6].

As a let-7 miRNA family member, let-7a is composed of 10 distinct members (let--7f-1, 7a-1, −7a-3, −7e, −7g, −7b, −7f-2, −7a-2, −7i, as well as mir-98)[11]. In previous researches, we have confirmed that let-7a is under-expressed in ES tissues relative to the normal vicinal tissues[8], and it functions in ES as a tumor repressor gene[7]. In recent years, mounting studies have shown that let-7a has an suppressive effect on CSCs[13,21–24]. Therefore, we speculate that let-7a may also play the same role in ES CSCs, thus affecting its malignant phenotype.

Tumors are composed of variety of heterogeneous cells, and CSCs as an important part of them. There were some scholars came up with an idea that CSCs are the only cells who can initiate and promote tumor growth even though they only account for a less proportion of tumors[25]. Currently, the isolation of CSCs mainly rely on cell surface specific antigen. However, as a tumor come from mesenchymal tissue, there is no recognized specific surface marker to ES. Therefore, we isolated ES-CSCs by a method named side population cell sorting, and found the expression of let-7a were lower-expressed in ES-CSCs. At present, the function of let-7a in ES-CSCs and its detailed mechanism are still unclear. In our research, we established that overexpression of let-7a in ES cells lines can led to the decrease of the proportion of CSCs. In addition, overexpression of let-7a in ES CSCs, both the potential to form tumors in nude mice *in vivo* and the capacity of colony formation and invasion *in vitro* are all suppressed. These data implied that let-7a can suppress the malignant phenotype of ES-CSCs.

To further explore the detailed mechanism of the let-7a in ES-CSCs, we found the predict target genes to let-7a through two different kinds of miRNA target genes biology information website (miRanda and PicTar), the results showed that we got 15 target

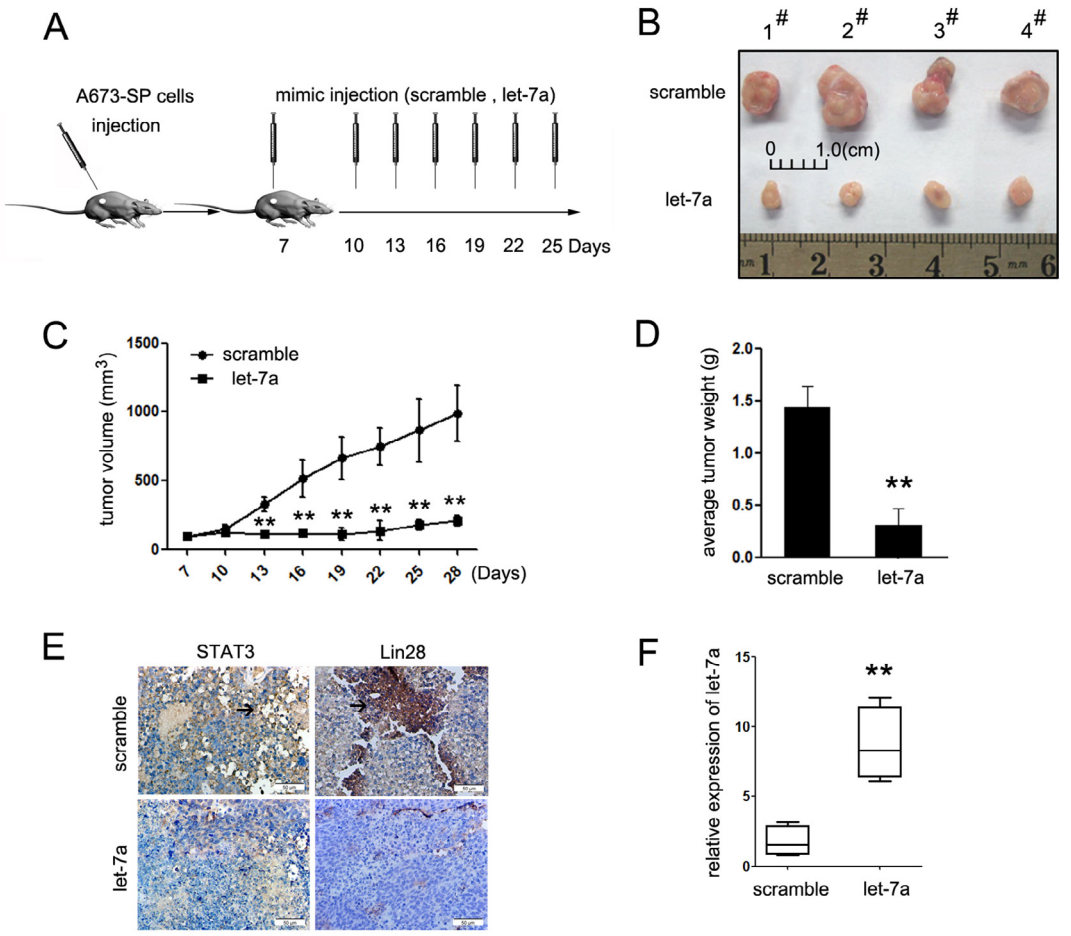

Fig. 5. Let-7a suppressed the tumor growth of SP cells *in vivo*. A. Diagram indicating the experimental procedure employed in the mouse xenograft experiments. B. Illustrative images of xenograft tumors at 28 days following the injection when all the mice be sacrificed and tumors be excised. Graph illustrating the average tumor volume(C), as well as the weight (D) for each mouse groups at the end of the experiment. E. Immunohistochemistry analysis of lin28 and STAT3 in tumors from xenograft mice (200 ×). F. The expression of let-7a in xenografts from mice injected with A673-SP cells transfected with or without let-7a mimic. Small nuclear U6 served as the endogenous control gene and evaluation of the relative expression levels done via the $2^{-\Delta\Delta Ct}$ approach in the RT-qPCR inspection. $^{*}P < 0.05$, $^{**}P < 0.01$.

genes, the only one which have been reported associated with CSCs is STAT3[9,10]. STAT3 is an important transcriptional activator in the JAK/STAT signaling pathway, which is related to multiple biological behaviors, e.g., cell proliferation and differentiation, gene expression, inflammatory response, and immune escape, and is also a important pathway connecting the signal transduction of cells inside and outside[26]. Some research reported that STAT3 serves a vital role in the proliferation and differentiation of CSCs of neuroglioma and breast cancer[16,27,28]. In the research we had published[8], we had confirmed that STAT3 is the target gene of let-7a in ES, and in ES-CSCs, we found that excessive expression of let-7a could also have repressed the expression of STAT3 pathway. These findings provide a potential mechanism to explain the suppressive effect of let-7a on ES-CSCs. In order to verify our hypothesis, we silenced the expression of STAT3 in ES-CSCs to detect its colony formation ability and invasion ability. The results showed that STAT3 activation had a certain influence on the ES-CSC malignant phenotype. In addition, we also found that with the expression of STAT3 changed, not only the expression of let-7a, but also lin28 would make a difference.

As the one of the 4 transcription factors, lin28 is employed to iPSCs currently[17]. There are two binding sites of STAT3 on its upstream of 5′-UTR, and the results of immunoprecipitation show that STAT3 can bind to the above two sites simultaneously to up modulate the expression of lin28[19]. Meanwhile, lin28 can bind to the let-7a precursors like pre-let-7a or pri-let-7a, blocking its cleavage and then suppresses the production of mature let-7a [18]. In addition, several researches had reported that STAT3 and lin28 are consistently highly expressed in various tumor pathological tissues[29,30]. Therefore, we put forward an assumption that maybe the lower expression of mature let-7a launched the high expression of STAT3 and lin28 in ES CSCs. Moreover, after overexpression of lin28, it will bind to the precursors of let-7a like pri-let-7a or pre-let-7a, suppress its cleavage and then reduce the production of the mature let-7a. However, the low expression of mature let-7a would leads to the overexpression of STAT3 and lin28, forming a positive feedback circuit, leading to the continuous activation of let-7a/STAT3/lin28 circuit, which makes the abnormal differentiation on normal stem cells and form the ES-CSCs with infinite proliferation ability, and then bring about the occurrence, metastasis and recurrence on ES. To verify our assumption, we altered the expression of lin28 in ES-CSCs and detected the expression of let-7a, STAT3 and lin28 respectively. As predicted, when the expression of lin28 changed, the expression of STAT3 and let-7a showed an opposite trend.

To sum up, we find an positive feedback circuit with a modulatory effect on malignant phenotype of ES-CSCs. Because of the high rate of relapse and metastasis seriously restricts the prognosis of patients with ES, meanwhile ES-CSCs as an crucial role in them, our research may provide a new train of thought and scheme for

the treatment of ES, like target any gene in the circuit for targeted regulation to inhibit the carcinogenesis of ES-CSCs and thus suppress the malignant biological behavior of Ewing sarcoma, or to carry out greater clinical impact assessment on the expression of key markers in patient tissues. It will provide a far-reaching influence on the prognosis of patients.

5. Conclusion

In conclusion, the deficiency of let-7a promoted the upregulation of STAT3, a upstream of lin28, increased the expression of lin28, and then lin28 block the release of mature let-7a furtherly. A positive circuit might formed among them which serve a pivotal role in the carcinogensis of ES-CSCs. These findings maybe provide assistance for patients with ES in the future, especially those with metastasis and recurrence, and new directions for their treatment.

6. Author Statement

6.1. Ethics approval and consent to participate

All animal experiments were approved by the First Affiliated Hospital of Nanchang University Medical Research Ethics Committee

6.2. Consent for publication

Not applicable.

6.3. Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Appendix A. Supplementary data

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