

# Lin28A Promotes the Amplification and Cancer Stemness of Lung Cancer Cells Via Activating MAPK Pathway Dependent on Let-7c Functions

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## Primary research

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**Lin28A promotes the amplification and cancer stemness of lung cancer cells via activating MAPK pathway dependent on let-7c functions**

**Running title:** Lin28A/let-7c facilitates lung cancer stem cells

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## **Abstract**

Background: Metastasis and relapse of lung cancer are the main cause of disease-related deaths. It's reported that tumor metastasis and relapse originated from cancer stem cells (CSCs) which possess more potential in proliferation and invasion. In our previous studies, we established a conditional BME-based three-dimensional culture (3D culture) system to mimic the growth environment *in vivo* and further amplified lung cancer stem cells (LCSCs) in our system. However, the molecular mechanisms of the amplification and development of LCSCs in our 3D culture system are still not very clear.

Methods: We tested the expression of Lin28 and let7 by western blot and qPCR, and constructed A549 cells either knockdown of Lin28 or overexpression of let7, followed by investigating the expression of stemness markers by flow cytometry and qPCR, and stem cell like phenotypes including cell proliferation, colony formation, mammosphere culture, cell apoptosis, migration, invasion and drug resistance *in vitro*, as well as tumorigenicity *in vivo*.

Results: Here we observed Lin28A/let-7c was dysregulated in LCSCs both from the 3D culture system and from lung cancer tissues. Further, the abnormal expression of Lin28A/let-7c was correlated with poor survival outcomes. We found over-expression let-7c inhibited the maintenance of LCSC properties, while the results for knockdown of Lin28A showed Lin28A was critical for the enrichment and amplification of LCSCs via MAPK signaling pathway. Importantly, we found that either knockdown of Lin28A or over-expression of let-7c inhibited carcinogenesis and disrupted LCSC expansion *in vivo*.

Conclusions: Our study uncovered the functions and mechanisms of the "Lin28A/let-7c/MAPK" signaling pathway in promoting the amplification and cancer stemness of LCSCs, which might be a potential therapeutic target for lung cancer therapy by reducing and even eliminating LCSCs in the future.

**Keywords:** Lin28A; cancer stemness; lung cancer; MAPK pathway; let-7c

## Introduction

Lung cancer is one of the most common malignant tumors with high morbidity and mortality worldwide [1, 2]. Although the molecular classification and targeted therapy has achieved significant progress in recent years, patients have limited benefit from regular therapies and the 5-year survival rate of lung cancer has not been effectively improved [3, 4]. Metastasis and recurrence are the leading cause for disease-related deaths of lung cancer [2, 5]. Recently, the roles of cancer stem cells (CSCs) in the occurrence and development of tumors have received widely attention [6-8]. Many studies have shown that lung cancer stem cells (LCSCs) play an important role in the process of occurrence, metastasis, recurrence and drug resistance, on account that the stem cells possess the characteristics of high proliferation and invasion, multi-directional differentiation, and long-life span [9]. However, there is currently no large-scale clinical treatment which target LCSCs, mainly caused by the problems in the isolation and purification of LCSCs. Furthermore, the molecular mechanisms in regulating LCSCs are still not clear. Therefore, the establishment of a culture method for LCSC enrichment and further illumination of the molecular mechanisms during the occurrence and development of LCSCs are of great significance for lung cancer clinical research to screen more effective drugs by targeting LCSCs.

The traditional two-dimensional culture (2D culture) method *in vitro* is difficult to simulate the local microenvironment, therefore, the 2D culture is not suitable for the mechanism research and clinical transformation of LCSCs [10, 11]. In our previous study, we established a BME-based three-dimensional culture (3D culture) system with the addition of FGF1 and IGF1, which could provide the cultured cells with vital growth factors [12]. Furthermore, the 3D structure facilitated the formation of tissue-specific structures to effectively mimic a local microenvironment *in vivo*. The 3D culture system we established could effectively achieve the enrichment and amplification of LCSCs. We observed that FGF1 and IGF1 promoted LCSC amplification and cancer stemness by activating MAPK signaling pathway in our 3D

culture system. But the molecular mechanisms of the amplification and development of LCSCs in our 3D culture system are still not very clear.

MiRNAs are the small non-coding nucleic acids that negatively regulate gene expression post-transcriptionally [13-15]. An increasing number of studies have shown that miRNAs play critical roles in regulating the stemness of cancer cells by targeting different target genes [16-18]. In lung cancer, miRNAs have been shown to be the central regulatory molecules for the maintenance of properties of LCSCs associated with metastasis, drug resistance, and tumor self-renewal by effectively targeting the vital genes involved in various signaling pathways [19, 20]. And a number of translational studies have implied that the combined potential of miRNA and anticancer agents showed a positive outcome at the pre-clinical level [21-24]. Hence, targeting of miRNA might be a promising method for the elimination of CSCs.

In this study, in order to investigate the most critical miRNA(s) in regulating the stemness of cancer cells in our 3D culture system, we searched on two popular miRNA databases and filtered out 25 candidate miRNAs which might have abnormal expression levels in lung cancer, followed by examining the levels of them in different lung cancer cell lines cultured in our 3D system. Here we detected that the levels of let-7 miRNAs, especially let-7c were significantly down-regulated in LCSCs amplified in our 3D culture system. Reduced let-7c expression occurred most frequently in cancer and typically correlated with poor prognosis in previous studies [25-27]. Forced let-7 expression inhibited sphere formation and carcinogenesis of CSCs, and reduced the proportion of undifferentiated cells; similarly, antagonizing let-7 miRNAs enhanced self-renewal and prompted aggressiveness [28-32]. However, some studies revealed that let-7c could also enhance the invasion and tumor growth via the DVL3/ $\beta$ -catenin axis [33]. Considering that let-7c played an important dual role in regulating tumorigenic and metastatic abilities of cancer, and even the role of let-7c in LCSC development is still not very clear, we explored the functions of let-7c during the occurrence and development of LCSCs in the study.

By searching let-7c target genes on Targetscan and further validation, we observed the level of Lin28A, but not Lin28B, was significantly up-regulated in LCSCs amplified in our 3D culture system. Lin28A is one of the reprogramming factors to induce pluripotency in adult human fibroblast cells [34], which has been reported to be over-expressed in advanced human malignancies and play an important role in the maintenance of CSCs [35, 36]. Previous studies showed that let-7 family binds to the 3' UTR of Lin28A mRNA to regulate negatively Lin28A expression, while Lin28A itself blocks let-7 expression, thereby establishing a double negative feedback loop [36]. However, none study focusing on the expression and roles of Lin28A, as well as the relationship between Lin28A and let-7c in LCSCs has ever been reported.

Here we elucidated that Lin28A/let-7c was dysregulated both in LCSCs from BME-based 3D culture system and from lung cancer tissues. And the abnormal expression of Lin28A/let-7c was correlated with poor survival outcomes of patients from our cohort and TCGA database. Then we forced expression of let-7c in A549 cells, and investigated that let-7c could inhibit the maintenance of LCSC properties via disrupting MAPK signaling pathway. By knockdown of Lin28A, we also uncovered Lin28A was indispensable for tumorigenesis and promoting LCSC expansion. Further, either knockdown of Lin28A or over-expression of let-7c inhibited carcinogenesis and disrupted LCSC expansion *in vivo*. Therefore, our study demonstrated the vital role of the "Lin28A/let-7c/MAPK" cascade signaling pathway in promoting the amplification and cancer stemness of LCSCs *in vitro and in vivo*, which might be a potential therapeutic target for lung cancer therapy by reducing LCSC expansion.

## **Materials and Methods**

### **Patient information**

This study recruited 125 cases of LUAD patients treated with partial lung resection surgery at the Department of Lung Cancer of Tianjin Medical University Cancer Institute and Hospital. And 4 cases of LUAD tumor tissues were collected for isolation of CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells by flow sorting. No prior treatments, including chemotherapy or radiotherapy, were conducted before lung resection surgery was performed. This project was approved by the Ethics Committee of Tianjin Medical University. All experiments were performed in accordance with the principles of the Declaration of Helsinki. Written consents were obtained from the patients.

### **Cell lines and cell culture**

Human LUAD cell lines A549, H1299, LUSC cell lines NCI-H520 and SK-MES-1, were obtained from Chinese Academy of Medical Sciences tumor cell libraries, validated by short tandem repeat DNA profiling and mycoplasma tested. For cells in 2D culture system, A549, H1299 and NCI-H520 were cultured in RPMI-1640 (Gibco BRL, USA) containing 10% FBS (Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL) at 37°C, under 5% CO<sub>2</sub>. SK-MES-1 was cultured in MEM (Gibco BRL) containing 10% FBS, 1% non-essential amino acids, 1mM sodium pyruvate and 1% penicillin/streptomycin. For cells in BME-based 3D culture system, we first added 50 µl/well BME in the 96 well-plate, warmed at 37°C for 1h, and formed a reconstituted basement membrane with specialized thickness and stiffness. Then cells suspension (1×10<sup>4</sup> cell/200 µl/well) were seeded on top of the plate coated with BME and inspected every 3 days. The seeded A549 cells could migrate into the inner layer of BME and generate cell aggregate. On the third day of culture, 20 ng/ml FGF1 and 50 ng/ml IGF1 were added simultaneously into complete medium as described previously [12]. For all experiments, three parallel wells were set up in each group.

### **Mice**

Female NOD-SCID mice, which were 7-week old and weighed about 17~18 g, were obtained from Beijing SPF Biotechnology Co., Ltd. (China). The mice were maintained in the SPF animal laboratory of Tianjin Medical University Cancer Hospital and Institute. All procedures involving mice were approved by the Ethics Committee for Animal Experiments at the Tianjin Medical University Cancer Hospital and Institute and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

### **Flow cytometry analysis**

FITC anti-human CD326, APC anti-human CD44, and PE anti-human CD24 antibodies (BD Biosciences, USA) were used to label human LCSCs. Isotype-matched IgG1 antibodies (BD Biosciences, USA) were used as the negative control. The flow cytometry analysis was performed using a BD FACS Canto™ II flow cytometer (BD Biosciences). The gating strategy was described in our previous work [12].

### **Statistical analysis**

Statistical analysis was performed using SPSS 20.0 and GraphPad Prism 5.0 software. The measured data are presented as the mean  $\pm$  SD. Spearman correlation analysis was used to analyze the correlation between two data sets. One-way ANOVA and Two-way ANOVA were performed followed by Bonferroni post hoc analysis to compare two groups after ANOVA in multiple comparison. Cumulative survival was determined via Kaplan–Meier method. All the data meet the normal distribution. The level of statistical significance was set to  $P < 0.05$ .

**More detailed methods were described in supplemental data.**

## Results

### Lin28A/let-7c was dysregulated in LCSCs from BME-based 3D culture system

In our previous studies, we applied FGF1 and IGF1 to establish the conditional BME-based three-dimensional culture system with growth factors (3D+F) to amplify LCSCs in human lung adenocarcinoma (LUAD) cell line A549 cells (A549-3D+F) *in vitro* [12]. In this study, in order to investigate the most critical miRNA(s) in regulating the stemness of cancer cells in A549-3D+F, we firstly filtered the candidate miRNAs based on online databases. We searched on HMDD database v3.2 (the Human miRNA Disease Database version 3.2) [37], which is curated by experiment-supported evidence for human miRNA and disease associations. We found out 305 miRNAs dysregulated in lung cancer, among which there are 109 miRNAs that have direct causality with lung cancer. To further screen the miRNAs involved in lung cancer development, we also searched miRNAs on OncomiR database [38], and it showed 379 miRNAs associated with tumor development of lung cancer, as well as 109 miRNAs associated with survival outcome of lung cancer. We obtained the intersection of 25 miRNAs screened out from the two databases (**Figure S1**), and then we collected the cell pellets of A549 cells in 2D culture system (A549-2D) and A549-3D+F, and performed quantitative-polymerase chain reaction (qPCR) to compare the RNA levels of the 25 differentially expressed miRNAs we screened out. Compared to A549-2D, some miRNAs dramatically increased in A549-3D+F, including mir-873, mir-31, mir-582, mir-196b, and mir-212, while some miRNAs significantly decreased in A549-3D+F, including mir-99a, mir-145, mir-181c, mir-30a, mir-29b, let-7g, let-7f, mir-490, and let-7c. Among them, let-7c, let-7f, and let-7g are belonged to the same miRNA family, which have the lowest expression levels in A549-3D+F (**Figure 1A**).

In order to investigate which biological processes are let-7 miRNAs involved in, we searched them on DIANA database-miRPath v2.0 [39], another popular database which can predict the pathways that the miRNA targets are enriched in, provided by

the DIANA-microT-CDS algorithm and/or experimentally validated miRNA interactions derived from DIANA-TarBase v6.0. The analysis results showed that no matter based on microT-CDS or TarBase algorithm, the let-7 miRNA target genes are enriched in “signaling pathways regulating pluripotency of stem cells” (**Figure 1B**), indicating that let-7 miRNAs are involved in regulating cancer stemness.

Then we focused on let-7c functions in consideration of its lowest expression in A549-3D+F. We searched let-7c target genes on Targetscan [40], and found 1207 transcripts those are probably targeted by let-7c. Among the top 10 target genes, we noticed Lin28A/B (**Figure 1C**), which is one of the reprogramming factors, to induce pluripotency in adult human fibroblast cells [41]. The results of the dual-luciferase reporter assay showed that the relative luciferase activity of Lin28A 3’UTR, rather than the 3’UTR-mutant of Lin28A, 3’UTR of Lin28B, and 3’UTR-mutant of Lin28B, was significantly decreased by let-7c mimic transfection in HEK-293T cells (**Figure 1D**). Then we compared the transcription levels of Lin28A/B between A549-2D and A549-3D+F. Consistent with the results of the luciferase reporter assay, although the transcription level of Lin28A was significantly up-regulated in A549-3D+F, the transcription of Lin28B has no difference between the two groups (**Figure 1E**). All the results above indicated that Lin28A/let-7c was dysregulated in LCSCs from BME-based 3D culture system, suggesting Lin28A/let-7c might play roles in the development of LCSCs.

### **Lin28A/let-7c expressed abnormally in LCSCs from other lung cancer cell lines cultured in 3D system**

We then further attempted to establish 3D+F culture to amplify LCSCs in H1299 cells, another LUAD cell line. As before, we applied the co-stimulation of FGF1 and IGF1 in BME-based 3D system to establish our special 3D+F culture. The results of spheroid formation assay showed that although it could hardly form spheroid in H1299 cultured in 2D system (H1299-2D) (**Figure 2A**, left panel), it could successfully form several big spheroids in H1299 cultured in 3D+F system

(H1299-3D+F) (**Figure 2A**, right panel). The migration ability of H1299-2D and H1299-3D+F was evaluated via wound healing test. The wound closure rates (WCRs) of H1299-3D+F were significantly higher than H1299-2D after 48h (**Figure 2B**).

Furthermore, we detected the levels of let-7 miRNAs and Lin28A in H1299-2D and H1299-3D+F. Consistent with the results in A549, it showed that let-7c, let-7f, and let-7g were significantly down-regulated in H1299-3D+F compared to H1299-2D (**Figure 2C**), while the protein expression of Lin28A was significantly up-regulated in H1299-3D+F (**Figure 2D**), indicating the expression of let-7c/Lin28A loop was widely dysregulated in LUAD CSCs.

Then we also established the 3D+F culture systems to amplify LCSCs in two lung squamous cell carcinoma (LUSC) cell lines, H520 and SK-MES-1 cells. The sphere formation and wound healing assays also supported that the cells cultured in 3D+F system performed more stemness phenotypes (**Figure 2E-F, 2I-J**), here implying we successfully enriched and enhanced stemness of LCSCs both in LUAD and LUSC cell lines efficiently in our 3D+F rather than regular 2D system. Furthermore, as expected, let-7 miRNAs showed down-regulation in cells cultured in 3D+F system compared to in 2D system (**Figure 2G, 2K**), while the protein expression levels of Lin28A were significantly up-regulated in cells cultured in 3D+F system (**Figure 2H, 2L**). All the results above indicated that the abnormal expression of let-7c/Lin28A loop was a common phenomenon in different subtypes of LCSCs.

### **Lin28A/let-7c was dysregulated in LCSCs from lung cancer tissues and its abnormal expression was correlated with poor survival outcomes**

As CD326<sup>+</sup>, CD44<sup>+</sup>, and CD24<sup>-</sup> have been reported as the characteristic phenotypes of CSCs in multiple kinds of tumors [42, 43], we used CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells as phenotype of LCSCs in our study. We collected 4 cases of clinical LUAD tumor samples, followed by isolated CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells (**Figure 3A**) from fresh tissues by flow sorting. Then we examined the RNA levels of let-7c, let-7f, and let-7g individually. Consistent with the results in LCSCs from 3D

culture system, the levels of let-7c were significantly decreased in CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> group compared with non CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> group from lung cancer tissues (**Figure 3B**). Furthermore, the expression levels of Lin28A were significantly up-regulated in CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells, while the expression levels of Lin28B have no difference between the two groups (**Figure 3C**).

We then further collected 125 cases of LUAD tumors from Tianjin Medical University Cancer Institute and Hospital and performed qPCR to explore the association between let-7c/Lin28A and the patients' overall survival (OS). The patients were stratified into two groups by the transcription levels by qPCR individually and the survival analysis with Kaplan-Meier method revealed that low expression of let-7c, as well as high expression of Lin28A, correlated with poor survival outcomes (**Figure 3D-E**). Furthermore, Spearman's rank correlation showed a significant negative correlation between let-7c and Lin28A expression in the clinical LUAD tumor samples (**Figure 3F**).

To further explore the effects of Lin28A in LUAD occurrence and development in more samples, we analyzed the data from The Cancer Genome Atlas (TCGA). We found that the expression of Lin28A was significantly higher in LUAD primary tissues than in the matched adjacent normal tissues (**Figure 3G**) [44]. And the analysis based on Lin28A expression among individual cancer types showed the expression of Lin28A was significantly higher in Stage 4 tissues than in Stage 1-3 tissues (**Figure 3H**) [44]. Further, the survival analysis consistently showed high expression of Lin28A was correlated with poor survival outcomes of lung cancer primary tissues from TCGA (**Figure 3I**) [45]. Collectively, these results indicated that Lin28A/let-7c was dysregulated in LCSCs from lung cancer tissues and its abnormal expression was correlated with poor survival outcomes of LUAD patients.

### **Let-7c inhibited the maintenance of LCSC properties via disrupting MAPK signaling pathway**

In order to investigate the underlying biological roles of let-7c in LCSC

enrichment and amplification, we over-expressed let-7c in A549 cells, followed by cultured them in 3D+F system (A549<sup>OV-let-7c</sup>-3D+F). We examined the ratio of CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells in A549-2D, A549-3D, A549-3D+F, and A549<sup>OV-let-7c</sup>-3D+F using flow cytometry analysis. Consistent with our previous findings, compared to A549-2D, an increase of the expression levels of CD326 was observed in A549-3D, furthermore, there was a more obviously dramatic increase in A549-3D+F (**Figure 4A**). However, less CD326<sup>+</sup> cells were observed in A549<sup>OV-let-7c</sup>-3D+F compared to A549-3D+F. Next, by gating the CD326<sup>+</sup> cells and analyzing the proportion of CD44<sup>+</sup>CD24<sup>-</sup> cells in CD326<sup>+</sup> population, we found that the percentage of CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells increased in A549-3D+F compared to that in A549-2D and in A549-3D (**Figure 4A**), respectively. While less CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells were even observed in A549<sup>OV-let-7c</sup>-3D+F compared to A549-3D+F, indicating that let-7c inhibited the expression levels of phenotypic markers of LCSCs.

In order to evaluate the effect of let-7c on LCSC stemness in A549, we further compared the spheroid formation capacities in A549-2D, A549-3D, A549-3D+F, and A549<sup>OV-let-7c</sup>-3D+F. A549-3D+F formed more and bigger cell colonies than A549-2D and A549-3D, but less and smaller cell colonies were detected in A549<sup>OV-let-7c</sup>-3D+F compared to A549-3D+F (**Figure 4B**). We further detected the mRNA expression of classical stemness genes by RT-qPCR, including Oct4, Sox2, Nanog, and Aldh1. The mRNA levels of the above genes were significantly up-regulated in A549-3D+F compared to A549-2D and A549-3D. While the mRNA levels of them were significantly down-regulated in A549<sup>OV-let-7c</sup>-3D+F compared to A549-3D+F (**Figure 4C**). These results indicated that A549<sup>OV-let-7c</sup>-3D+F displayed significantly lower levels of phenotypic and genetic markers of LCSCs which implied that let-7c might inhibit the enrichment and amplification of LCSCs.

Next, the migration and invasion ability of A549-2D, A549-3D, A549-3D+F, and A549<sup>OV-let-7c</sup>-3D+F was evaluated via wound healing test and trans-well invasion assay. The WCRs of A549-3D+F were significantly higher than those of A549-2D

and A549-3D after 48h (**Figure 4D**). Furthermore, more A549-3D+F migrated across the Matrigel layer than A549-2D and A549-3D after 48 h (**Figure 4F**). But we detected much less cells migrated in A549<sup>OV-let-7c</sup>-3D+F in wound healing test and trans-well invasion assay (**Figure 4D, 4F**). Since epithelial-mesenchymal transition (EMT) is the most important cellular events of cancer invasion and metastasis [46], the enriched transcription of EMT-related biomarker was detected using qPCR assay. The conventionally used epithelium cell biomarker of E-cadherin was reduced, but other well-described mesenchymal cell biomarkers of N-cadherin and  $\beta$ -catenin and EMT-related transcription factors (snail, slug, zeb1, and Twist) were increased in A549-3D+F compared with those in A549-2D and A549-3D. While the expression levels of these mesenchymal cell biomarkers in A549<sup>OV-let-7c</sup>-3D+F decreased significantly (**Figure 4E**). All the results suggested that the elevated migration and invasion ability of A549-3D+F was reversed by over-expressing let-7c.

Since our previous observations indicated that our 3D+F culture promoted the amplification and stemness of LCSCs dependent on MAPK signaling pathway [12], then we collected the cell supernatants of A549-2D, A549-3D, A549-3D+F, and A549<sup>OV-let-7c</sup>-3D+F, followed by examined the levels of phosphorylated p38, JNK and ERK via western blot. Although the phosphorylation levels of these 3 main subunits of MAPK protein family were up-regulated both in A549-3D and A549-3D+F compared to A549-2D, their phosphorylation levels were obviously down-regulated in A549<sup>OV-let-7c</sup>-3D+F (**Figure 4G**), which implied that let-7c inhibited the maintenance of LCSC properties via disrupting MAPK signaling pathway.

### **Lin28A was indispensable for tumorigenesis and promoting LCSC expansion via MAPK signaling pathway**

Next, we constructed the A549 cells transfected with Lin28A shRNA and cultured them in 3D+F system (A549<sup>sh-Lin28A</sup>-3D+F) to investigate the function of Lin28A in LCSC enrichment and amplification. By flow cytometry analysis, both less CD326<sup>+</sup> and less CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells were observed in A549<sup>sh-Lin28A</sup>-3D+F

compared to A549-3D+F (**Figure 5A**), indicating Lin28A was critical for the expression levels of phenotypic markers of LCSCs.

We then further investigated the spheroid formation capacities and less and smaller cell colonies were detected in A549<sup>sh-Lin28A</sup>-3D+F compared to A549-3D+F (**Figure 5B**). Furthermore, the mRNA levels of Oct4, Sox2, Nanog, and Aldh1 were significantly down-regulated in A549<sup>sh-Lin28A</sup>-3D+F compared to A549-3D+F (**Figure 5C**). These results indicated that A549<sup>sh-Lin28A</sup>-3D+F displayed significantly lower levels of phenotypic and genetic markers of LCSCs, which implied that Lin28A was indispensable for the enrichment and amplification of LCSCs.

The results of wound healing test showed the WCRs of A549<sup>sh-Lin28A</sup>-3D+F were significantly lower than those of A549-3D+F after 48h (**Figure 5D**). And the results of trans-well invasion assay indicated less A549<sup>sh-Lin28A</sup>-3D+F migrated across the Matrigel layer than A549-3D+F (**Figure 5F**). Furthermore, the results of qPCR assay showed E-cadherin was increased, but N-cadherin,  $\beta$ -catenin, snail, slug, zeb1, and Twist were all decreased in A549<sup>sh-Lin28A</sup>-3D+F compared with those in A549-3D+F (**Figure 5E**). These results suggested that the elevated migration and invasion ability of A549-3D+F was also dependent on Lin28A functions.

Then we examined the levels of phosphorylated p38, JNK and ERK from the cell supernatants of A549-2D, A549-3D, A549-3D+F, and A549<sup>sh-Lin28A</sup>-3D+F, and observed that their phosphorylation levels were obviously down-regulated in A549<sup>sh-Lin28A</sup>-3D+F (**Figure 5G**). All the results above implied that Lin28A was indispensable for tumorigenesis and promoting LCSC expansion via MAPK signaling pathway.

### **Either knockdown of Lin28A or over-expression of let-7c inhibited carcinogenesis and disrupted LCSC expansion *in vivo***

Next, we tested if Lin28A would affect carcinogenesis *in vivo*. Cells transduced with the A549<sup>sh-Lin28A</sup> lentiviral constructs and their control cells were injected into

NOD-SCID mice subcutaneously as xenografts. After 24 days, the average volumes of tumors generated by engrafted tumor cells from the A549<sup>sh-Lin28A</sup> groups were at least two fold greater volumes when compared to the control groups (**Figure 6A**). Then we also investigated the roles of let-7c in tumorigenesis via injecting cells transduced with the A549<sup>OV-let-7c</sup> lentiviral constructs in the similar way. Consistently, the growth rates of tumors in A549<sup>OV-let-7c</sup> mice were much greater than those in H520<sup>OV-CTRL</sup> mice (**Figure 6B**).

Furthermore, RNA levels of let-7c and protein levels of Lin28A in xenografts were examined. As expected, the qPCR analysis demonstrated that the transcription of let-7c was elevated both in A549<sup>sh-Lin28A</sup> and A549<sup>OV-let-7c</sup> groups compared with their control mice respectively (**Figure 6C**). While the qPCR and western blot results showed down-regulation of Lin28A in A549<sup>sh-Lin28A</sup> and A549<sup>OV-let-7c</sup> groups (**Figure 6D-E**). And it also showed the mRNA levels of Oct4, Sox2, Nanog, and Aldh1 were reduced in A549<sup>sh-Lin28A</sup> and A549<sup>OV-let-7c</sup> groups (**Figure 6F**). Similarly, the mRNA levels of E-cadherin were reduced, while N-cadherin,  $\beta$ -catenin, snail, slug, zeb1, and Twist1 were increased (**Figure 6G**).

We further isolated the tumor tissues from the mice and analyzed the proportions of CSCs by flow cytometry. Both less CD326<sup>+</sup> and less CD44<sup>+</sup> cells were obviously observed in A549<sup>sh-Lin28A</sup> and A549<sup>OV-let-7c</sup> mice (**Figure 6H**). All these results implied that either knockdown of Lin28A or over-expression of let-7c could inhibit carcinogenesis and disrupt LCSC expansion *in vivo*.

## Discussion

The dysregulation of miRNA expression is related to and functionally important for many diseases, including cancer [14, 19]. Among all tumor suppressor miRNAs, the decrease in let-7 expression is the most common in cancer and is usually associated with poor prognosis [36, 47, 48]. Functionally, the reduction of let-7 miRNAs will lead to over-expression of their carcinogenic targets, such as RAS, MYC, HMGA2, etc [49-51]. Furthermore, in many cancers and embryonic stem cells, although the primary transcripts of let-7 are still actively transcribed, the mature let-7 is not produced as expected, which is due to the two highly conserved RNA-binding proteins, Lin28A/B inhibits the biogenesis of mature let-7 miRNAs through direct binding to either pre-let-7 and/or pri-let-7[36]. Specifically, Lin28A/B binds to the terminal loop of the precursor let-7 and recruits Terminal Uridylyl Transferase (TUTase) ZCCHC11, which polyuridylates pre-let-7, thereby blocking miRNA biogenesis and tumor suppressor functions[36]. Lin28A/B blocks the expression of let-7, and let-7 itself binds to the 3'UTR of Lin28A/B mRNA to negatively regulate the expression of Lin28A/B, thereby establishing a dual negative feedback loop[52].

Lin28A has been shown as a stem cell pluripotency factor [41]. When treating with Oct4, Sox2, Nanog and Lin28A, adult fibroblasts were successfully reprogrammed into induced pluripotent stem cells (PSCs) [53]. The up-regulation and activation of Lin28A/B is a hallmark of many human cancers, such as glioblastoma, ovarian, stomach, prostate and breast cancer [34, 35]. Although the functions and mechanisms of the double-negative feedback loop of Lin28/let-7 in CSCs have been elucidated in many cancers, the relationship and role of Lin28/let-7 in LCSCs are still not very clear.

In this study, we detected that the levels of let-7 miRNAs, especially let-7c was significantly down-regulated in LCSCs amplified in our 3D culture system. Let-7c is a member of let-7 family, which is commonly known as a putative tumor suppressor in several cancer cell lines [25, 26, 28], but was also reported to play an important

dual role in regulating tumorigenic and metastatic abilities of cancer [33]. Further, let-7c could target several genes, such as HOXA1, ITGB3, MAP4K3, TRIB2, to inhibit NSCLC cell proliferation, migration, and tumorigenesis [54-56]. While considering its roles in regulating stem cells, it was identified that let-7c significantly abolished symmetric division of stem cells by inhibiting Wnt signaling [57].

However, there is no direct evidence of the relationship between let-7c and Lin28A/B in lung cancer. By searching let-7c target genes on Targetscan, we noticed Lin28B among the top 10 genes. Unexpectedly, the transcription levels and protein levels of Lin28B have no difference between LCSCs and non-LCSCs, while the expression of Lin28A was significantly up-regulated in LCSCs amplified in our 3D culture system. The results indicated that although both Lin28A and Lin28B have been reported to play roles in the progression of lung cancer, Lin28A seemed to be more important in enrichment and amplification of LCSCs.

The abnormal expression of Lin28A/let-7c was not only detected in LCSCs from A549 cells, but also in LCSCs from other lung cancer cell lines, including LUAD and LUSC cell lines. We also uncovered that Lin28A/let-7c was dysregulated in LCSCs from lung cancer tissues. Furthermore, the abnormal expression of Lin28A/let-7c was correlated with poor survival outcomes of patients from our cohort and TCGA database. And even forced let-7 expression or inhibition of Lin28A suppressed self-renewal and carcinogenesis of stem cells, and reduced the proportion of undifferentiated cells. All these data implied that Lin28A and let-7c, but not Lin28B or other let-7 miRNAs, might play vital roles in the development of LCSCs, in different subtypes of lung cancer.

Hedgehog, Wnt, and Notch are the canonical signaling pathways in mediating various stem cell characteristics, such as self-renewal, cell fate decisions, survival, proliferation and differentiation [58, 59]. However, in our previous studies, we demonstrated that the growth factors promoted the amplification and stemness of LCSCs mainly dependent on MAPK signaling pathway [12]. Here we also show

Lin28A/let-7c regulated the properties of LCSCs via MAPK signaling pathway. Ras genes are regulated by let-7 miRNA family by virtue of the possession by these genes of let-7 complementary sites in their 3'-UTRs [49]. It has been reported that deletion of let-7c promoted the activation of the JNK and P38 MAPK pathways in mesenchymal stem cells (MSCs) [29]. Kaloyan, etc. found that Lin28A was phosphorylated by MAPK/ERK in PSCs, which increased its levels via post-translational stabilization [60]. In this study, our findings inversely indicated that MAPK signaling was the downstream of Lin28A.

In this study, we established an efficient 3D culture method for the amplification of different subtypes of LCSCs, intending to overcome the challenges during purification and amplification of LCSCs which limited the large-scale clinical treatment targeting LCSCs. We also illuminated the molecular mechanisms of "Lin28A/let-7c/MAPK" signaling pathway during the amplification and development of LCSCs, even defining an axis that connects gene, miRNA, signaling pathway, and stem cell properties. It might be a potential therapeutic target for lung cancer therapy by reducing and even eliminating LCSCs in clinical research.

## **Conclusions**

Our study uncovered the functions and mechanisms of the "Lin28A/let-7c/MAPK" signaling pathway in promoting the amplification and cancer stemness of LCSCs, which might be a potential therapeutic target for lung cancer therapy by reducing and even eliminating LCSCs in the future.

## **Declarations**

### **Ethics approval and consent to participate**

This study was approved by the Ethics Committee of Tianjin Medical University. All experiments were performed in accordance with the principles of the Declaration of Helsinki and written consent was obtained from all patients. All procedures involving animals were approved by the Ethics Committee for Animal Experiments at the Tianjin Medical University Cancer Hospital and Institute and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

### **Consent for publication**

Consent for publication was obtained from all authors and all patients contained in our manuscript. The work described was an original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part.

### **Availability of data and materials**

All data used in this study is available to the scientific community upon request.

### **Competing interests**

No conflict of interest exists in the submission of this manuscript, and manuscript has been approved by all authors for publication.

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**Authors' contributions**

Rui Zhang performed data analyses, and wrote the manuscript. Rui Zhang, Pengpeng Liu, and Xiao Zhang performed the research jointly. Yingnan Ye contributed clinical information and samples for the study. Jinpu Yu designed the study and commented on manuscript.

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Table 1. The basic clinical-pathological information of patients.

Clinical-pathological parameters	Number of patients
<b>Total</b>	125
<b>Gender</b>	
Male	63
Female	62
<b>Age</b>	
<60 years	61
≥60 years	64
<b>Stage</b>	
I	38
II	42
II-III	19
III	24
IV	2
<b>T stage</b>	
1	14
2	75
3	30
4	6
<b>N stage</b>	
0	77
1	37
2	11
<b>M stage</b>	
0	123
1	2

## Figure legends

Figure 1. Lin28A/let-7c was dysregulated in LCSCs from BME-based 3D culture system. (A) The RT-qPCR results of 25 candidate differentially expressed miRNAs. All the results shown for A549-3D+F were relative expression values compared to A549-2D. (B) The biological processes those let-7 miRNAs are enriched indicated on DIANA database. (C) Let-7c targets Lin28A and Lin28B indicated on Targetscan. (E) Dual-luciferase reporter assay using HEK-293T cells co-transfected with psiCheck2.0-Lin28A/B vector/psiCheck2.0-Lin28A/B-mutant vector and let-7c mimic/the negative control of let-7c mimic. (F) The RT-qPCR results of Lin28A and Lin28B in A549-2D and A549-3D+F. The data are shown as mean  $\pm$  SD. \* and \*\* indicate  $p < 0.05$  and  $p < 0.01$  between the groups as indicated.

Figure 2. Lin28A/let-7c expressed abnormally in LCSCs from other lung cancer cell lines cultured in 3D system. (A) Representative images of H1299-2D and H1299-3D+F in sphere formation assays. (B) Representative images of H1299-2D and H1299-3D+F in wound healing assays. (C) The RT-qPCR results of let-7c, let-7f and let-7g in H1299-2D and H1299-3D+F. All the results shown for H1299-3D+F were relative expression values compared to H1299-2D. (D) The western blot results of Lin28A and Lin28B in H1299-2D and H1299-3D+F. (E) Representative images of H520-2D and H520-3D+F in sphere formation assays. (F) Representative images of H520-2D and H520-3D+F in wound healing assays. (G) The RT-qPCR results of let-7 in H520-2D and H520-3D+F. (H) The western blot results of Lin28A and Lin28B in H520-2D and H520-3D+F. (I) Representative images of SK-MES-1-2D and SK-MES-1-3D+F in sphere formation assays. (J) Representative images of SK-MES-1-2D and SK-MES-1-3D+F in wound healing assays. (K) The RT-qPCR results of let-7 in SK-MES-1-2D and SK-MES-1-3D+F. (L) The western blot results of Lin28A and Lin28B in SK-MES-1-2D and SK-MES-1-3D+F.

Figure 3. Lin28A/let-7c was dysregulated in LCSCs from lung cancer tissues and its abnormal expression was correlated with poor survival outcomes. (A) Representative images of the flow cytometry analysis. We isolated CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells from LUAD tissues by flow sorting. (B) The RT-qPCR results of let-7c, let-7f and let-7g in CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells and non CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells from lung cancer samples. (C) The western blot results of Lin28A and Lin28B in two groups. (D) Identify let-7c<sup>-</sup> and let-7c<sup>+</sup> patients with different OS by Kaplan-Meier analysis based on the OS related genes expression value. (E) Identify Lin28A<sup>-</sup> and Lin28A<sup>+</sup> patients with different OS by Kaplan-Meier analysis based on the OS related genes expression value. (F) Spearman's rank correlation result showed the negative correlation between let-7c and Lin28A levels in 125 cases of clinical LUAD samples. (G) Lin28A

expressed differently between normal tissues and LUAD tumors from TCGA data. (H) Lin28A expressed differently among different stages from TCGA LUAD tissues. (I) Kaplan-Meier analysis for Lin28A<sup>-</sup> and Lin28A<sup>+</sup> patients from TCGA cancer tissues.

Figure 4. Let-7c inhibited the maintenance of LCSC properties via disrupting MAPK signaling pathway. (A) The flow cytometry analysis of CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells in A549-2D, A549-3D, A549-3D+F, and A549-3D+F<sup>OV-let7c</sup>. After gating the CD326<sup>+</sup> cells, we further analyzed the CD44<sup>+</sup>CD24<sup>-</sup> cells in CD326<sup>+</sup> group. The statistical results were shown on the right. (B) Representative images of A549 from four groups in sphere formation assays. The statistical results were shown on the right. (C) The RT-qPCR results of stemness gene markers. All the results shown for A549-3D, A549-3D+F, and A549-3D+F<sup>OV-let7c</sup> were relative expression values compared to A549-2D. (D) Representative images of A549 from four groups in wound healing assays. The statistical results were shown on the right. (E) The RT-qPCR results of EMT marker genes were shown as relative expression values. (F) Representative images of A549 from four groups in trans-well invasion assays. The statistical results were shown on the right. (G) Western blot results to detect phosphorylation of the pivotal molecules in MAPK signaling in A549 from four groups.

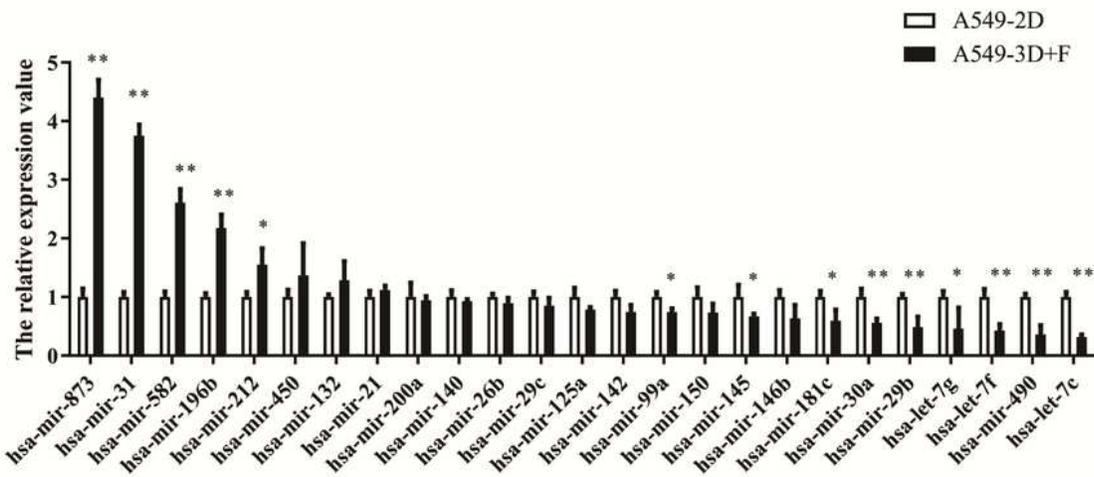
Figure 5. Lin28A was indispensable for tumorigenesis and promoting LCSC expansion via MAPK signaling pathway. (A) The flow cytometry analysis of CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells in A549-2D, A549-3D, A549-3D+F, and A549-3D+F<sup>sh-Lin28A</sup>. The statistical results were shown on the right. (B) Representative images of A549 from four groups in sphere formation assays. The statistical results were shown on the right. (C) The RT-qPCR results of stemness gene markers. All the results shown for A549-3D, A549-3D+F, and A549-3D+F<sup>sh-Lin28A</sup> were relative expression values compared to A549-2D. (D) Representative images of A549 from four groups in wound healing assays. The statistical results were shown on the right. (E) The RT-qPCR results of EMT marker genes were shown as relative expression values. (F) Representative images of A549 from four groups in trans-well invasion assays. The statistical results were shown on the right. (G) Western blot results to detect phosphorylation of the pivotal molecules in MAPK signaling in A549 from four groups.

Figure 6. Either knockdown of Lin28A or over-expression of let-7c inhibited carcinogenesis and disrupted LCSC expansion *in vivo*. (A) A549-3D+F and A549-3D+F<sup>sh-Lin28A</sup> were injected into NOD-SCID mice subcutaneously. The size of the forming tumors was shown at various time points upon injection. (B) A549-3D+F and A549-3D+F<sup>OV-let7c</sup> were injected into NOD-SCID mice subcutaneously. The size of the forming tumors was shown at various time points upon injection. (C) The

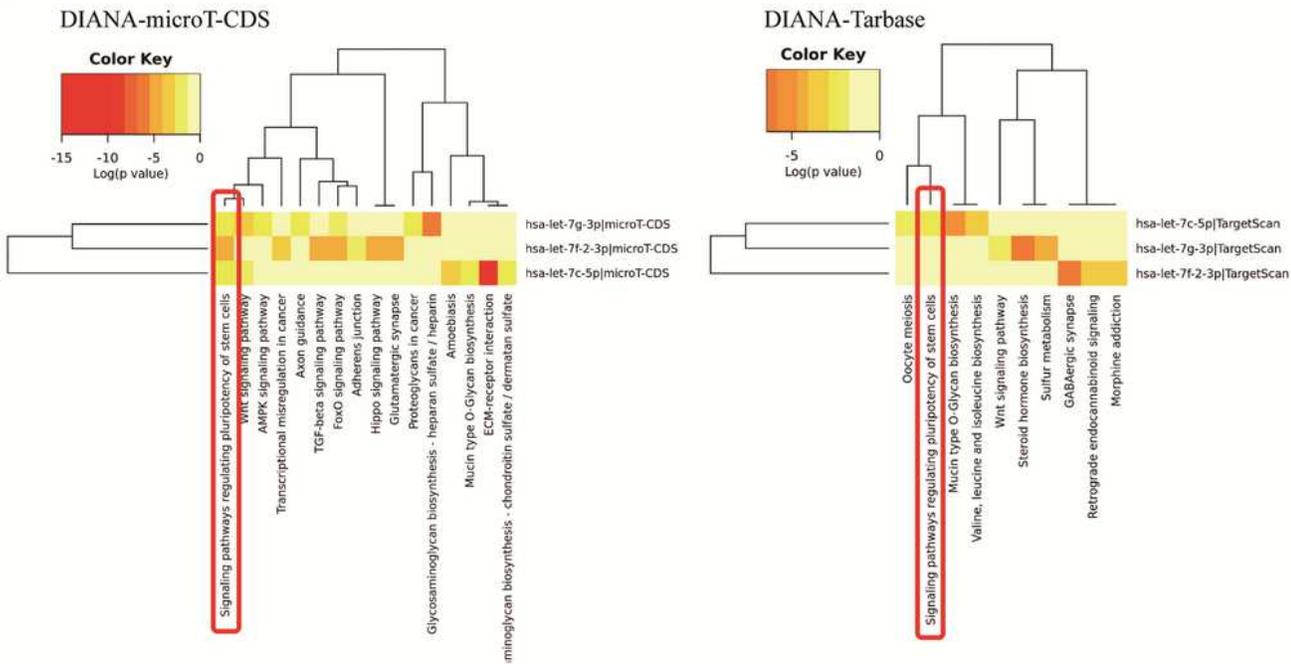
RT-qPCR results of let-7c levels in A549-3D+F, A549-3D+F<sup>sh-Lin28A</sup> and A549-3D+F<sup>OV-let7c</sup> mice. All the results shown for A549-3D+F<sup>sh-Lin28A</sup> and A549-3D+F<sup>OV-let7c</sup> were relative expression values compared to A549-3D+F group. (D) The RT-qPCR results of Lin28A expression in three groups. (E) Western blot results to detect Lin28A expression in three groups. (F) The RT-qPCR results of stemness gene markers in three groups. (G) The RT-qPCR results of EMT marker genes were shown as relative expression values. (H) The flow cytometry analysis of CD326<sup>+</sup> and CD44<sup>+</sup> cells from three groups. The statistical results were shown on the right.

# Figures

A



B

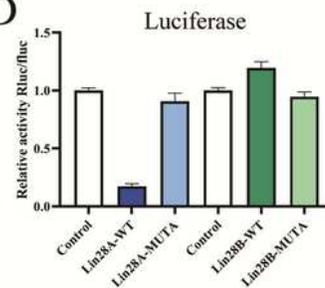


C

TargetScan

Position 905-912 of Lin28A 3'UTR 5'...GCACAGCCUAUUGAACUACCUCA...  
 hsa-let-7c-5p 3' UUGGUAUGUUGGAUGAUGGAGU  
 Position 44-51 of Lin28B 3'UTR 5'...ACCCGGUUGCAAAGU-CUACCUCA...  
 hsa-let-7c-5p 3' UUGGUAUGUUGGAUGAUGGAGU

D



E

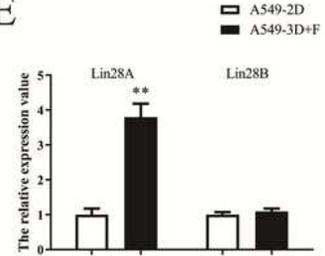
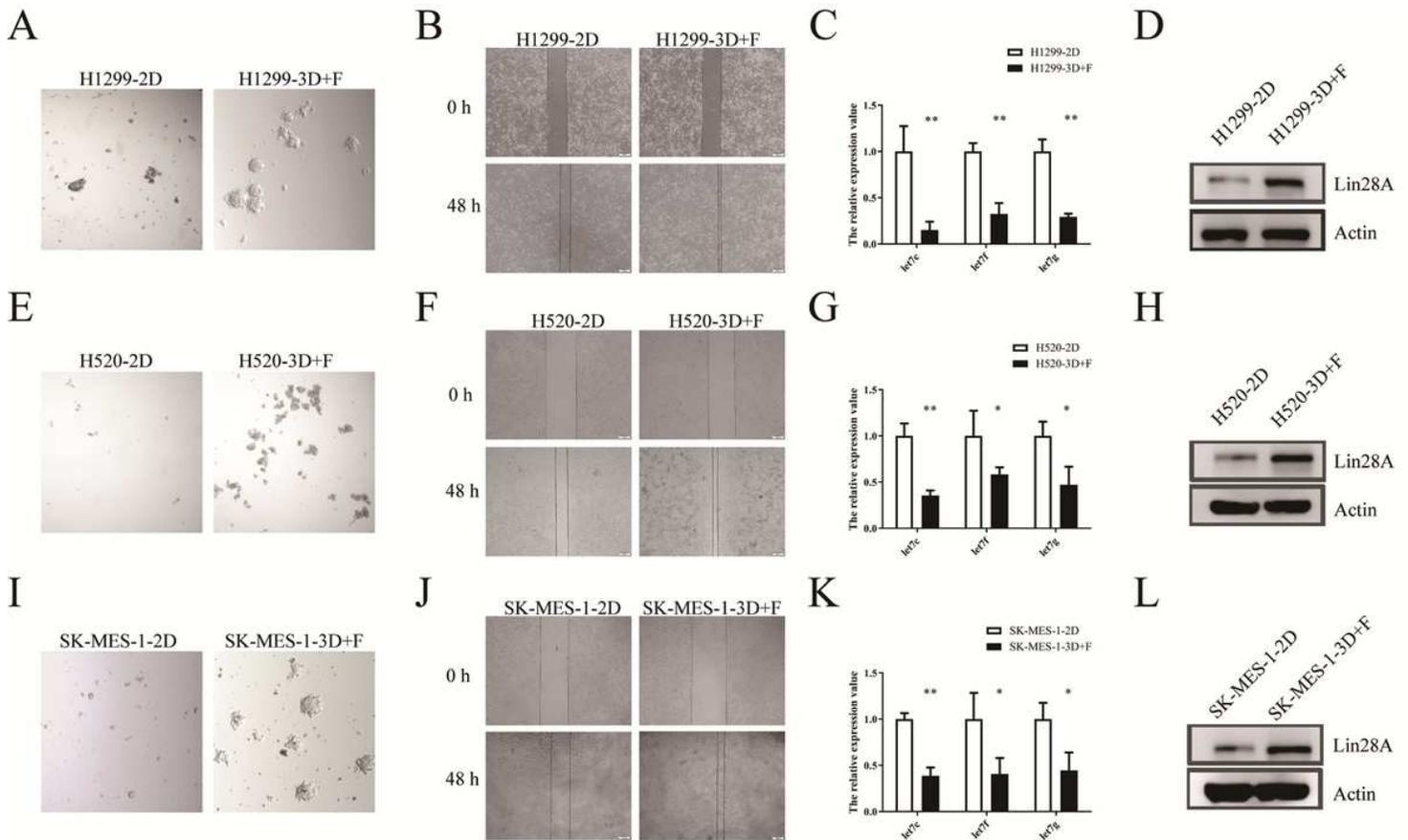


Figure 1

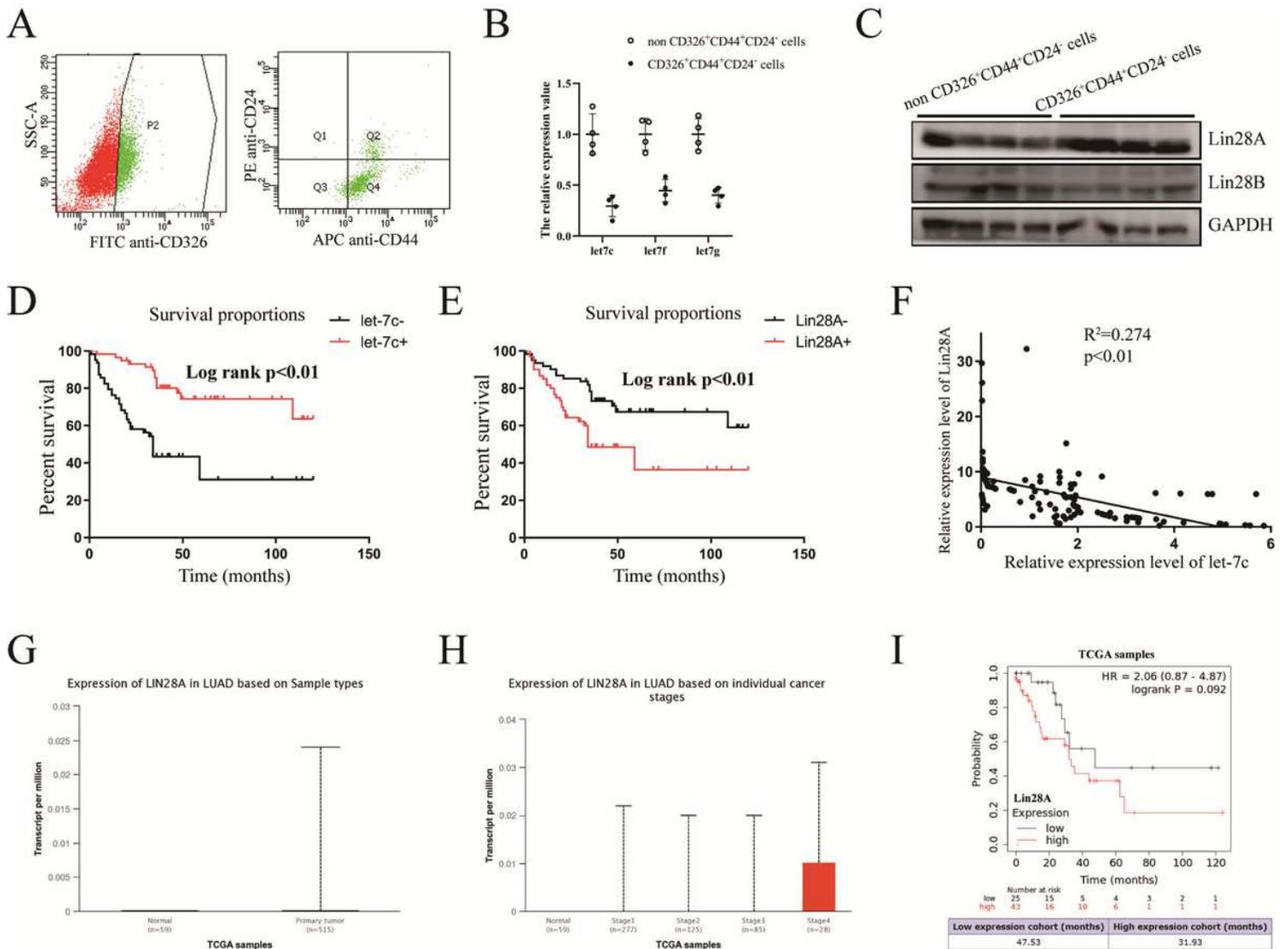
Lin28A/let-7c was dysregulated in LCSCs from BME-based 3D culture system. (A) The RT-qPCR results of 25 candidate differentially expressed miRNAs. All the results shown for A549-3D+F were relative expression values compared to A549-2D. (B) The biological processes those let-7 miRNAs are enriched

indicated on DIANA database. (C) Let-7c targets Lin28A and Lin28B indicated on Targetscan. (E) Dual-luciferase reporter assay using HEK-293T cells co-transfected with psiCheck2.0-Lin28A/B vector/psiCheck2.0-Lin28A/B-mutant vector and let-7c mimic/the negative control of let-7c mimic. (F) The RT-qPCR results of Lin28A and Lin28B in A549-2D and A549-3D+F. The data are shown as mean  $\pm$  SD. \* and \*\* indicate  $p < 0.05$  and  $p < 0.01$  between the groups as indicated.



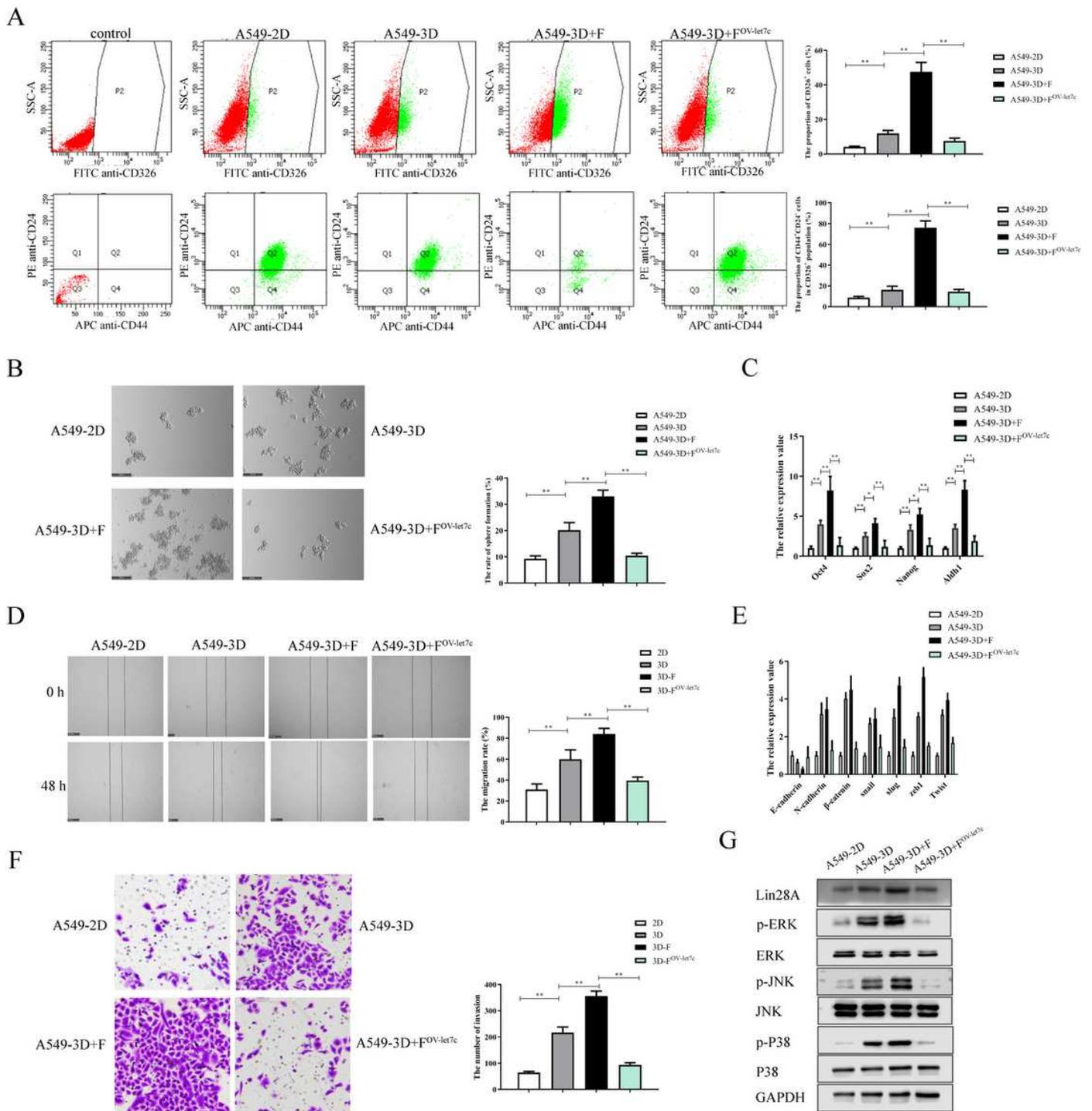
**Figure 2**

Lin28A/let-7c expressed abnormally in LCSCs from other lung cancer cell lines cultured in 3D system. (A) Representative images of H1299-2D and H1299-3D+F in sphere formation assays. (B) Representative images of H1299-2D and H1299-3D+F in wound healing assays. (C) The RT-qPCR results of let-7c, let-7f and let-7g in H1299-2D and H1299-3D+F. All the results shown for H1299-3D+F were relative expression values compared to H1299-2D. (D) The western blot results of Lin28A and Lin28B in H1299-2D and H1299-3D+F. (E) Representative images of H520-2D and H520-3D+F in sphere formation assays. (F) Representative images of H520-2D and H520-3D+F in wound healing assays. (G) The RT-qPCR results of let-7 in H520-2D and H520-3D+F. (H) The western blot results of Lin28A and Lin28B in H520-2D and H520-3D+F. (I) Representative images of SK-MES-1-2D and SK-MES-1-3D+F in sphere formation assays. (J) Representative images of SK-MES-1-2D and SK-MES-1-3D+F in wound healing assays. (K) The RT-qPCR results of let-7 in SK-MES-1-2D and SK-MES-1-3D+F. (L) The western blot results of Lin28A and Lin28B in SK-MES-1-2D and SK-MES-1-3D+F.



**Figure 3**

Lin28A/let-7c was dysregulated in LCSCs from lung cancer tissues and its abnormal expression was correlated with poor survival outcomes. (A) Representative images of the flow cytometry analysis. We isolated CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells from LUAD tissues by flow sorting. (B) The RT-qPCR results of let-7c, let-7f and let-7g in CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells and non CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells from lung cancer samples. (C) The western blot results of Lin28A and Lin28B in two groups. (D) Identify let-7c<sup>-</sup> and let-7c<sup>+</sup> patients with different OS by Kaplan-Meier analysis based on the OS related genes expression value. (E) Identify Lin28A<sup>-</sup> and Lin28A<sup>+</sup> patients with different OS by Kaplan-Meier analysis based on the OS related genes expression value. (F) Spearman's rank correlation result showed the negative correlation between let-7c and Lin28A levels in 125 cases of clinical LUAD samples. (G) Lin28A expressed differently between normal tissues and LUAD tumors from TCGA data. (H) Lin28A expressed differently among different stages from TCGA LUAD tissues. (I) Kaplan-Meier analysis for Lin28A<sup>-</sup> and Lin28A<sup>+</sup> patients from TCGA cancer tissues.

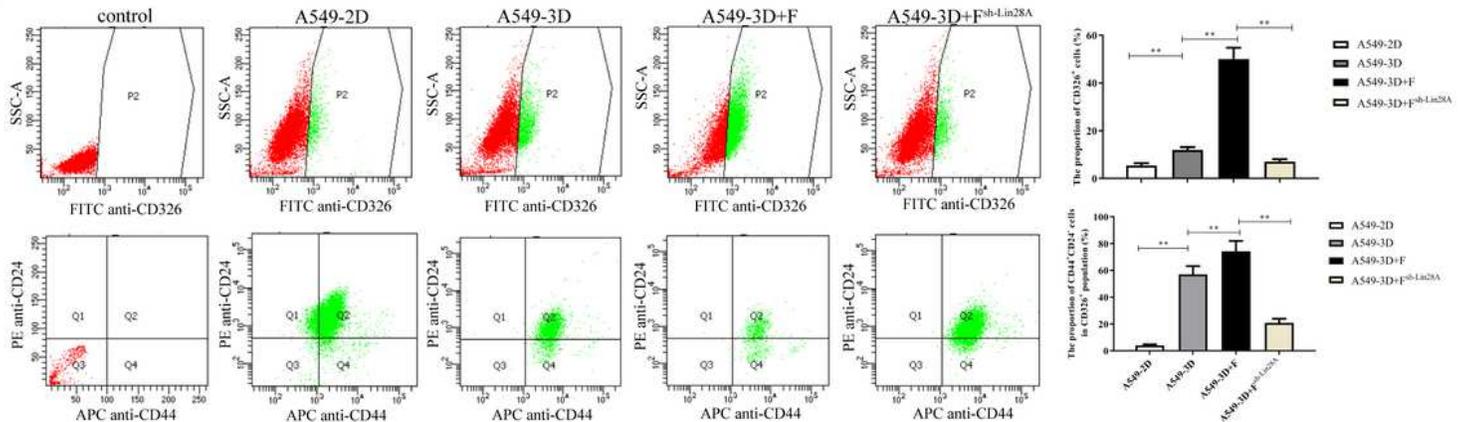


**Figure 4**

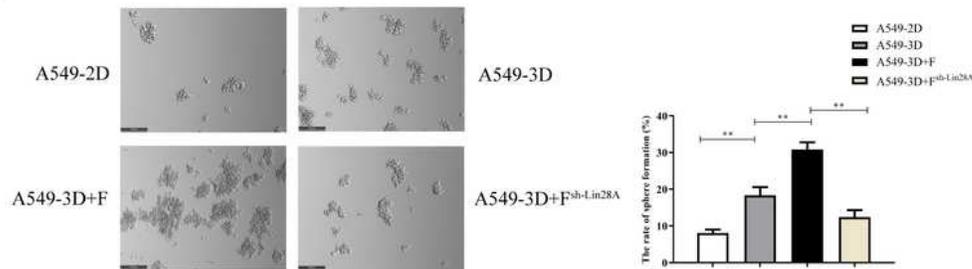
Let-7c inhibited the maintenance of LCSC properties via disrupting MAPK signaling pathway. (A) The flow cytometry analysis of CD326<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells in A549-2D, A549-3D, A549-3D+F, and A549-3D+FOV-let7c. After gating the CD326<sup>+</sup> cells, we further analyzed the CD44<sup>+</sup>CD24<sup>-</sup> cells in CD326<sup>+</sup> group. The statistical results were shown on the right. (B) Representative images of A549 from four groups in sphere formation assays. The statistical results were shown on the right. (C) The RT-qPCR results of stemness gene markers. All the results shown for A549-3D, A549-3D+F, and A549-3D+FOV-let7c were relative expression values compared to A549-2D. (D) Representative images of A549 from four groups in wound

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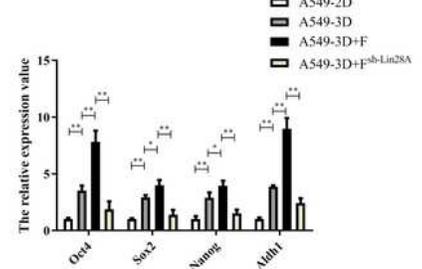
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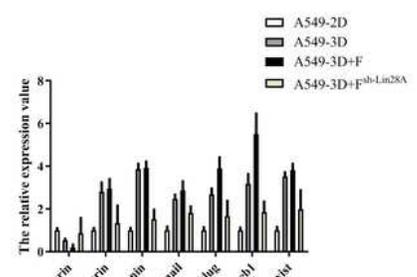
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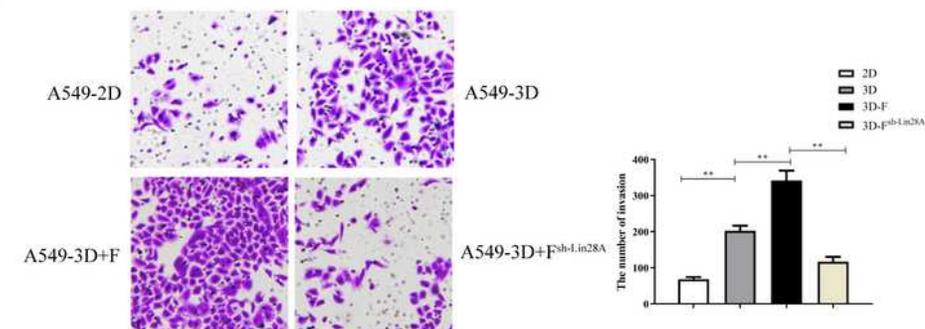
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G

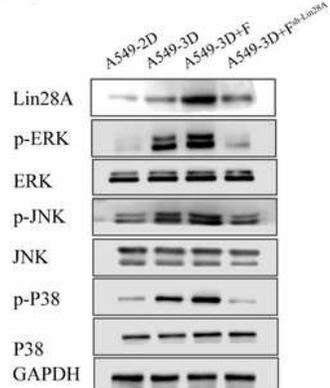


Figure 5

Lin28A was indispensable for tumorigenesis and promoting LCSC expansion via MAPK signaling pathway. (A) The flow cytometry analysis of CD326+CD44+CD24- cells in A549-2D, A549-3D, A549-3D+F, and A549-3D+Fsh-Lin28A. The statistical results were shown on the right. (B) Representative images of A549 from four groups in sphere formation assays. The statistical results were shown on the right. (C) The RT-qPCR results of stemness gene markers. All the results shown for A549-3D, A549-3D+F, and A549-3D+Fsh-Lin28A were relative expression values compared to A549-2D. (D) Representative images of A549 from four groups in wound healing assays. The statistical results were shown on the right. (E) The RT-qPCR results of EMT marker genes were shown as relative expression values. (F) Representative images of A549 from four groups in trans-well invasion assays. The statistical results were shown on the right. (G) Western blot results to detect phosphorylation of the pivotal molecules in MAPK signaling in A549 from four groups.

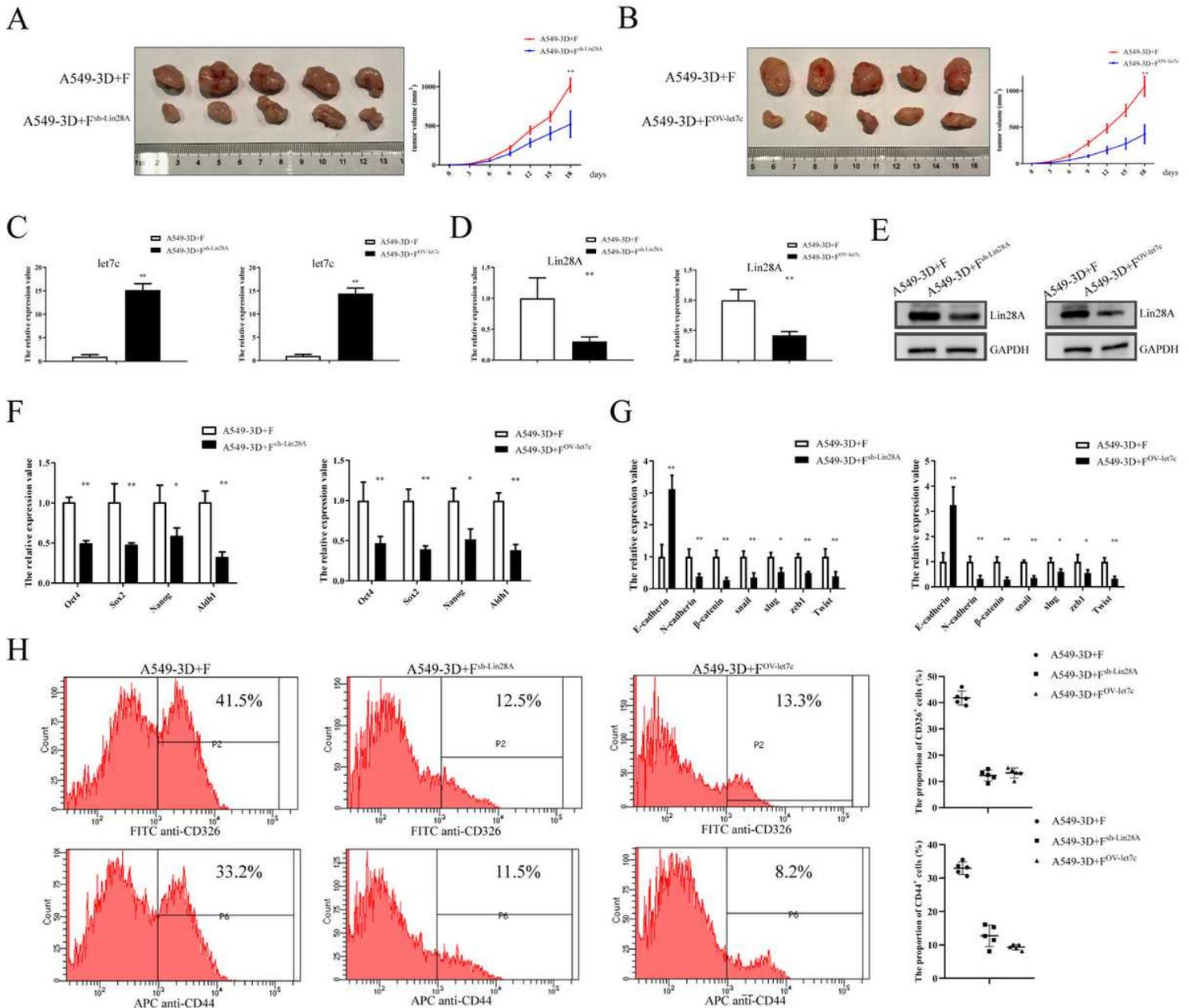


Figure 6

Either knockdown of Lin28A or over-expression of let-7c inhibited carcinogenesis and disrupted LCSC expansion in vivo. (A) A549-3D+F and A549-3D+Fsh-Lin28A were injected into NOD-SCID mice subcutaneously. The size of the forming tumors was shown at various time points upon injection. (B) A549-3D+F and A549-3D+FOV-let7c were injected into NOD-SCID mice subcutaneously. The size of the forming tumors was shown at various time points upon injection. (C) The RT-qPCR results of let-7c levels in A549-3D+F, A549-3D+Fsh-Lin28A and A549-3D+FOV-let7c mice. All the results shown for A549-3D+Fsh-Lin28A and A549-3D+FOV-let7c were relative expression values compared to A549-3D+F group. (D) The RT-qPCR results of Lin28A expression in three groups. (E) Western blot results to detect Lin28A expression in three groups. (F) The RT-qPCR results of stemness gene markers in three groups. (G) The RT-qPCR results of EMT marker genes were shown as relative expression values. (H) The flow cytometry analysis of CD326+ and CD44+ cells from three groups. The statistical results were shown on the right.

## Supplementary Files

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- [KeyMessages.pdf](#)
- [SupplementaryMaterial.pdf](#)
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