

Double-Negative Feedback Loop between Reprogramming Factor LIN28 and microRNA *let-7* Regulates Aldehyde Dehydrogenase 1–Positive Cancer Stem Cells

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Abstract

A relatively rare aldehyde dehydrogenase 1 (ALDH1)–positive “stem cell–like” subpopulation of tumor cells has the unique ability to initiate and perpetuate tumor growth; moreover, it is highly resistant to chemotherapy and significantly associated with poor clinical outcomes. The development of more effective therapies for cancer requires targeting of this cell population. Using cDNA microarray analysis, we identified that the expression of the *Caenorhabditis elegans* lin-28 homologue (LIN28) was positively correlated with the percentage of ALDH1⁺ tumor cells; this was further validated in an independent set of tissue arrays ($n = 197$). Both loss-of-function and gain-of-function studies showed that LIN28 plays a critical role in the maintenance of ALDH1⁺ tumor cells. In addition, we found that there is a double-negative feedback loop between LIN28 and *let-7* in tumor cells, and that *let-7* negatively regulates ALDH1⁺ tumor cells. Finally, we report that a LIN28/*let-7* loop modulates self-renewal and differentiation of mammary gland epithelial progenitor cells. Our data provide evidence that cancer stem cells may arise through a “reprogramming–like” mechanism. A rebalancing of the LIN28/*let-7* regulatory loop could be a novel therapeutic strategy to target ALDH1⁺ cancer stem cells. *Cancer Res*; 70(22); 9463–72. ©2010 AACR.

Introduction

Aldehyde dehydrogenase 1 (ALDH1) catalyzes the irreversible oxidation of a range of aliphatic and aromatic aldehydes to their corresponding carboxylic acids. High endogenous ALDH1 activity has been detected in normal stem and progenitor cells of various lineages including hematopoietic, mesenchymal, neural, mammary, and prostate (1–3). Recently, ALDH1 has been successfully used to identify a unique “stem cell–like” subpopulation of cells from tumors, which shares novel characteristics with normal embryonic and somatic stem/progenitor cells: self-renewal and multipotent differenti-

ation (1). Preclinical studies have further shown that this subpopulation of cells is highly tumorigenic and resistant to chemotherapy (4–7). Moreover, these cells promote and mediate tumor metastasis *in vivo* (8–11). Importantly, a high percentage of ALDH1⁺ cells in most types of epithelial tumors, such as breast (1, 10, 12, 13), lung (14), pancreatic (15), bladder (16), ovarian (7), and prostate (17), is associated with a poorer clinical outcome for these patients. This provides robust clinical evidence that the ALDH1⁺ tumor cells play a critical role in cancer initiation and progression. Therefore, more effective cancer therapies may be developed by targeting this cell population. However, our knowledge of the cellular and molecular regulation of ALDH1⁺ cancer stem cells of human tumors is limited. In this study, an important ALDH1⁺ tumor cell regulatory loop between reprogramming factor LIN28 and miRNA *let-7* was identified by high-throughput profiling and was then further characterized in human breast and ovarian tumors.

Materials and Methods

Patients and specimens

The ovarian cancer specimens for microarray analysis ($n = 26$; Supplementary Table S1) were collected at the University of Turin (Turin, Italy). Detailed information is provided in Supplementary Materials and Methods.

Cell lines and cell culture

T47D and MCF7 cells were purchased from the American Type Culture Collection (ATCC), A2780 and 2008 cells were

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obtained from the Ovarian Cancer Tissue and Cell Bank, and HeLa tet-on cells were purchased from Clontech.

RNA isolation and cDNA microarray analysis

Total RNA was isolated with Trizol reagent (Invitrogen). cDNA microarrays were performed on the human U133+ 2.0 GeneChip (Affymetrix). Detailed information is provided in Supplementary Materials and Methods.

Tissue microarray

The tissue microarray was constructed at the University of Helsinki. Detailed information is provided in Supplementary Materials and Methods.

Immunohistochemistry and image analysis

Immunohistochemistry was performed using the Vectastain ABC kit as described by the manufacturer (Vector). The following primary antibodies were used in this study: mouse anti-human ALDH1 (clone: 44/ALDH, 1:250; BD Pharmingen) and rabbit anti-human LIN28 (1:4,000; Abcam). Antibodies were incubated overnight at 4°C, and the immunoreaction was visualized using 3,3'-diaminobenzidine. The image was analyzed using Image-Pro Plus 4.1 software (Media Cybernetics).

Lentiviral transduction and generation of stable cell lines

Two individual lentiviral short hairpin RNA (shRNA) clones targeting LIN28 were purchased from Open Biosystems. eGFP shRNA and nontarget shRNA were used as controls. The pSin-EF2-LIN28 lentiviral expression vector was purchased from Addgene. Lentiviral vector and packing vectors were transfected into the packaging cell line 293T (ATCC) using FuGENE 6 Transfection Reagent (Roche). The medium was changed 8 hours after transfection, and the lentivirus-containing medium was collected 48 hours later.

Protein isolation and Western blots

Cells were lysed in 200 μ L mammalian protein extraction reagent (Pierce). Then, 15 μ g of total protein were separated by 10% SDS-PAGE under denaturing conditions and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% nonfat milk (Bio-Rad) and then incubated with an anti-LIN28 primary antibody (1:10,000; Abcam), followed by incubation in anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP; 1:10,000; Amersham Biosciences) together with an HRP-conjugated primary antibody to β -actin (1:10,000; Sigma). Immunoreactive proteins were visualized using the LumiGLO chemiluminescent substrate (Cell Signaling).

ALDEFLUOR assay and fluorescence-activated cell sorting analysis

ALDH1 activity was detected using the ALDEFLUOR assay kit (StemCell Technologies) as described by Ginestier and colleagues (1).

Mammosphere culture

Mammosphere cultures were performed as described by Dontu and colleagues (18). Detailed information is provided in Supplementary Materials and Methods.

RNA immunoprecipitation

Detailed information is provided in Supplementary Materials and Methods.

Quantitative real-time reverse transcription-PCR and Taqman miRNA assay

Detailed information is provided in Supplementary Materials and Methods.

let-7-responsive sensor construction and transfection

The *let-7* sensor was constructed by introducing two copies of *let-7b* perfect complement sequences into the 3' untranslated region (UTR) of the Renilla luciferase gene in the psiCHECK-2 vector (Promega).

miRNA *in situ* hybridization and image analysis

In situ detection of miRNA expression was performed on tissue microarray sections by locked nucleic acid (LNA) probes (Exiqon). Detailed information is provided in Supplementary Materials and Methods.

Retroviral transduction and stable cell line generation

The retroviral human miRNA expression vector (Supplementary Fig. S1A) was purchased from GeneService. Detailed information is provided in Supplementary Materials and Methods.

Tet-on-inducible cell lines

Stable cell lines inducibly expressing *let-7b* were generated using the retrovirus-based RevTet-On system (Clontech). The human genomic sequence of *let-7b* and an upstream reporter gene (DsRed) were cloned into the pRevTRE response vector downstream of the tetracycline-responsive element (TRE; Supplementary Fig. S1B), and then pRevTet-On and pRevTRE-DsRed-*let-7b* were separately introduced into HeLa cells by retroviral gene transfer. The reporter gene expression was monitored by fluorescent microscopy (Supplementary Fig. S1C) and fluorescence-activated cell sorting (FACS) analysis (Supplementary Fig. S1D), and *let-7b* expression was measured using real-time reverse transcription-PCR (RT-PCR; Applied Biosystems).

Transfection of *let-7* mimic and inhibitor oligonucleotides

Pre-miR miRNA precursor and control oligos were purchased from Ambion, and miRCURY LNA miRNA inhibitors and control oligos were purchased from Exiqon. Transfections were performed using the Lipofectamine RNAiMAX transfection reagent (Invitrogen), and then cells were incubated in the medium containing the transfection mixture for 72 hours.

3'UTR reporter construct and assay

The full-length sequence of human LIN28 3'UTR was cloned from human genomic DNA. The PCR products were ligated to

the PCR2.1 TOPO cloning vector (Invitrogen) and subcloned into psiCHECK-2 reporter vector (Promega). Mutagenesis of miRNA binding sites on reporter vectors was performed by the approach of overlap extension by PCR. Detailed information is provided in Supplementary Materials and Methods.

Mammary gland epithelial cell isolation, infection, and colony-forming assay

Detailed information is provided in Supplementary Materials and Methods.

Statistics

Statistical analysis was performed using the SPSS statistics software package. All results were expressed as mean \pm SD, with significance at $P < 0.05$.

Results

LIN28 expression is positively correlated with a higher percentage of ALDH1⁺ tumor cells

To explore the molecular mechanisms regulating ALDH1⁺ tumor cells, we chose 26 human ovarian tumor specimens (Supplementary Table S1), in which the ALDH1 expression was characterized by immunohistochemistry, and the percentage of ALDH1⁺ tumor cells was scored by two independent investigators. Using the median value of ALDH1⁺ cells observed in these tumors (7.5%) as cutoff point, the tumors were divided into two groups: ALDH1 low ($n = 13$, ALDH1⁺ = $2.6 \pm 2.3\%$) and ALDH1 high ($n = 13$, ALDH1⁺ = $48.8 \pm 37.5\%$). An Affymetrix microarray was then used to characterize the transcriptional signature of these tumors. In summary, we found that the expression of 59 genes was significantly different between the ALDH1-high and ALDH1-low groups ($P < 0.002$; Supplementary Table S2; Fig. 1A). Interestingly, we found that expression of the homologue of *Caenorhabditis ele-*

gans lin-28 (LIN28) was remarkably higher in the ALDH1-high group ($P = 0.00007$). LIN28 is an evolutionarily conserved RNA binding protein, which is highly expressed in embryonic stem (ES) cells, progenitor cells, and developing tissues but not in most adult organs (19–24). Together with OCT4, SOX2, and NANOG, LIN28 can act as a reprogramming factor, reprogramming somatic cells to induced pluripotent stem cells (iPS cells; ref. 25). LIN28/LIN28B has also been identified as an oncogene that is upregulated/reactivated in tumors, promoting transformation (26–30). These results suggest that LIN28 may be one of the genes involved in the regulation of ALDH1⁺ tumor cells. To validate this result, we analyzed LIN28 expression and the percentage of ALDH1⁺ tumor cells in an independent ovarian cancer tissue array set ($n = 197$) using immunohistochemistry (Fig. 1B and C). Consistent with the cDNA microarray result, the percentage of ALDH1⁺ tumor cells was significantly higher in LIN28⁺ tumors ($n = 19$) compared with LIN28⁻ tumors ($n = 178$; $P = 0.007$; Fig. 1D). Similar correlation was also observed in a breast cancer tissue array set ($n = 69$; $P = 0.045$). This positive correlation of LIN28 expression with ALDH1⁺ tumor cells indicates that LIN28 may play a functional role in maintenance of ALDH1⁺ cells in human tumors.

LIN28 plays a functional role in the maintenance of ALDH1⁺ tumor cells

To further examine whether LIN28 plays a role in the regulation of the ALDH1⁺ cell population, we chose two cancer cell lines (A2780 and T47D) that highly express LIN28. Using lentiviral shRNA vectors, LIN28 was specifically knocked down in these cell lines (Fig. 2A). The percentage of ALDH1⁺ cells in these two cell lines was measured by the ALDEFLUOR assay, which was originally developed to detect ALDH1 activity in hematopoietic tissues, and that has since been successfully applied to detect progenitor and cancer stem cells in nonhematopoietic tissues such as the mammary

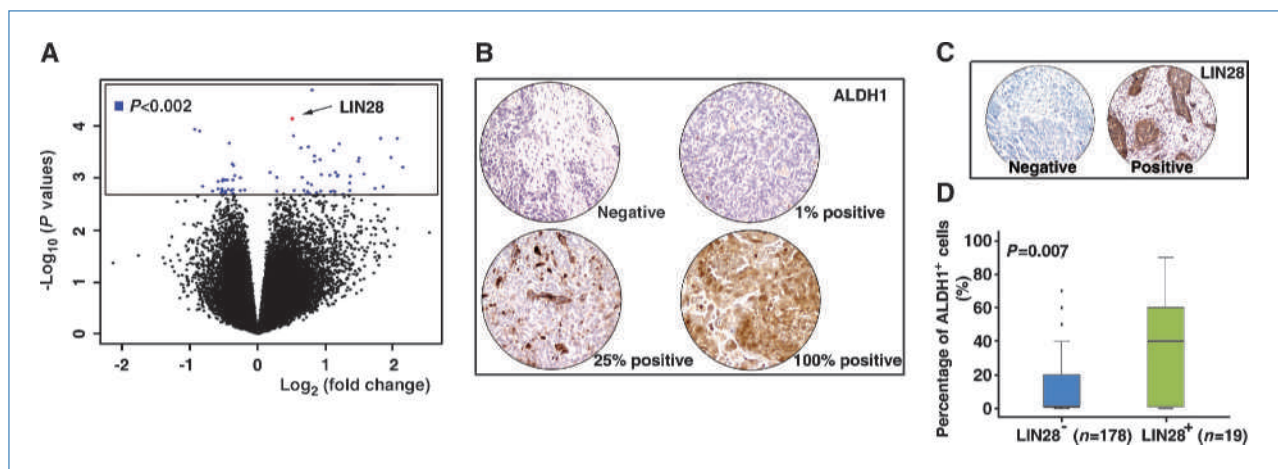


Figure 1. LIN28 expression is positively correlated with a higher percentage of ALDH1⁺ tumor cells. A, an Affymetrix cDNA microarray was used to identify the differential gene expression between ALDH1-high and ALDH1-low ovarian tumors ($n = 26$). The results indicated 59 genes (74 probes) that were significantly different between these two groups (blue spots; $P < 0.002$; Supplementary Table S2). LIN28 was positively correlated with a higher percentage of ALDH1⁺ tumor cells (red spot; $P = 0.00007$). B and C, an independent ovarian cancer tissue array was used to validate the cDNA microarray result. ALDH1 and LIN28 were detected by immunohistochemistry. D, the percentage of ALDH1⁺ tumor cells was significantly higher in the LIN28⁺ tumors compared with LIN28⁻ tumors in the validation tissue array set ($P = 0.007$; $n = 197$).

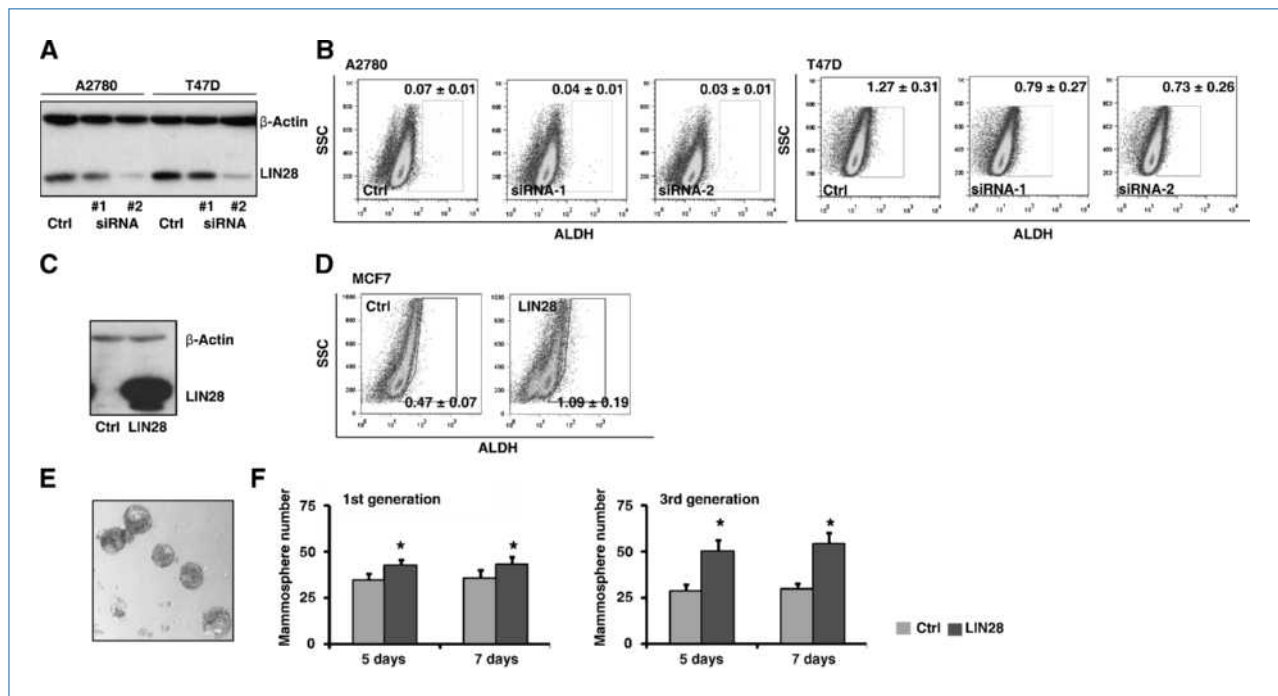


Figure 2. LIN28 plays a functional role in the maintenance of ALDH1⁺ tumor cells. A, LIN28 was knocked down by two independent lentiviral shRNA clones in two LIN28⁺ tumor cell lines (A2780 and T47D). Reduced protein expression of LIN28 was confirmed by Western blots. Full-length blots are presented in Supplementary Fig. S1. B, the percentage of ALDH1^{br} tumor cells in the above cell lines was measured using the ALDEFLUOR assay. Blocking endogenous LIN28 expression by shRNAs significantly reduced the percentage of ALDH1^{br} tumor cells. C, LIN28 was ectopically expressed in MCF7 cells by lentiviral infection. Enforced protein expression of LIN28 was confirmed by Western blots. D, the percentage of ALDH1^{br} tumor cells in the above cell lines was measured using the ALDEFLUOR assay. Enforced LIN28 expression significantly increased the percentage of ALDH1^{br} tumor cells. E, typical morphology of mammospheres in MCF7 cells. F, enforced LIN28 expression significantly increased mammosphere numbers compared with the control cells. Left, result in the first generation of mammospheres; right, result in the third generation of mammospheres. All *P* values < 0.05.

gland and breast cancer (1). We found that the knockdown of LIN28 expression in these cells significantly decreased the brightly fluorescent ALDH1 (ALDH1^{br}) cell population (Fig. 2B), and that this occurred in a dose-dependent manner; the two independent shRNA clones knocked down LIN28 expression to different levels, leading to different levels of ALDH1⁺ cells in each population. Similar observation was also found *in vivo* (Supplementary Fig. S3). To confirm this observation, we also ectopically expressed LIN28 in a LIN28⁻ cell line (MCF7; Fig. 2C). Consistent with the shRNA study, enforced expression of LIN28 remarkably increased the ALDH1^{br} population (Fig. 2D). Then, we examined the self-renewal capability of MCF7 cells using a mammosphere assay. Ectopically expressing LIN28 produced a significant increase in the number of spheres, especially in the third generation (Fig. 2E and F). Finally, other cancer stem cell markers such as CD133 and CD24/CD44 were also examined (Supplementary Fig. S4). Taken together, these results show that LIN28 plays a functional role in the maintenance of the ALDH1⁺ cancer stem cell population in tumors.

LIN28 modulates the biogenesis of miRNA *let-7* in tumor cells

Next, we examined the mechanism by which LIN28 regulates the ALDH1⁺ cell population in tumors. Results from previous

studies suggest that LIN28 maintains embryonic and somatic stem/progenitor cell pluripotency by blocking miRNA *let-7* maturation (22, 31–36). However, it is still unknown whether upregulated/reactivated LIN28 also regulates *let-7* maturation in cancer, because tumor cells may be absent in the molecular context for LIN28/*let-7* regulation. To address this question, we used RNA immunoprecipitation and real-time RT-PCR to show that LIN28 was able to specifically bind to the *let-7* precursor in A2780 cells (Fig. 3A and B). In addition, we found that knocking down LIN28 expression by shRNAs significantly increased expression of mature *let-7* in a dose-dependent manner in A2780 cells (Fig. 3C and D). Finally, using a *let-7* sensor assay, which contained a constitutively expressed reporter bearing sequences complementary to *let-7* in the downstream 3'UTR, we showed that blocking LIN28 in A2780 cells remarkably increased *let-7* activity (Fig. 3E and F). Similar observations were also found in T47D cells (Supplementary Fig. S5). Taken together, we conclude that as in embryonic and somatic stem/progenitor cells (22, 31–36), upregulated/reactivated LIN28 modulates miRNA *let-7* maturation and activity in tumor cells.

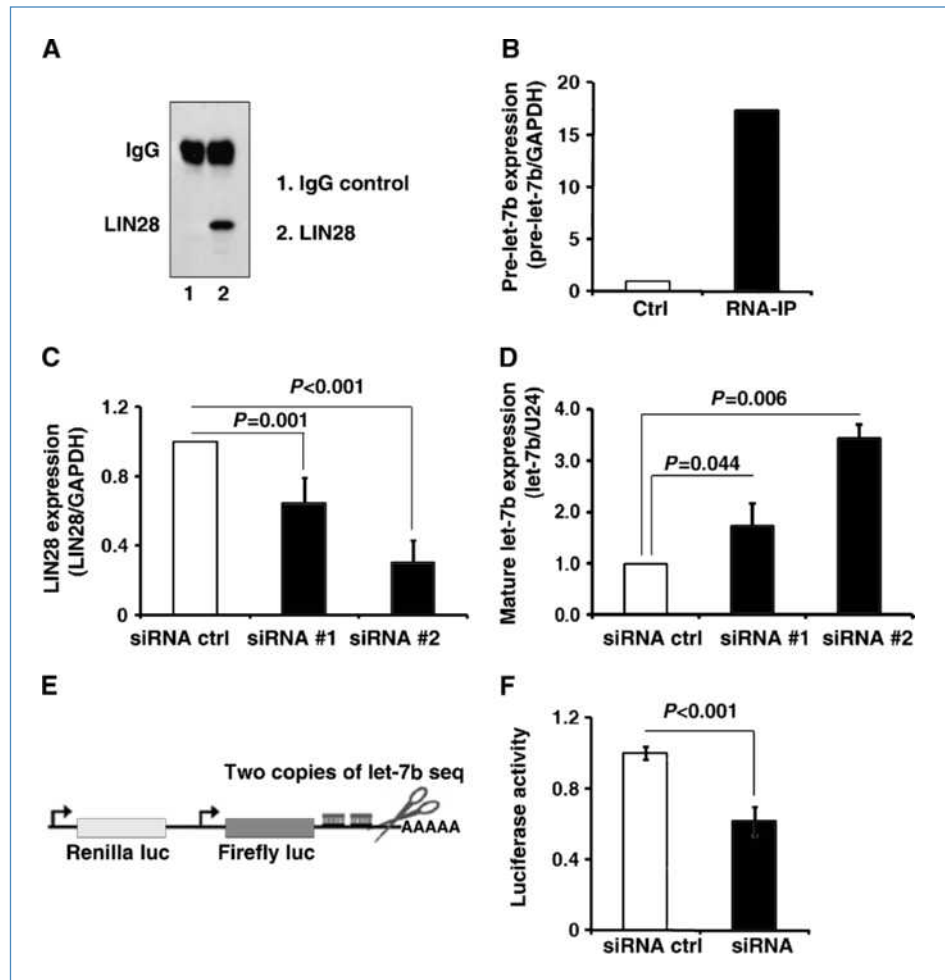
let-7 modulates the ALDH1⁺ tumor cell population in cancer

Given that *let-7* has been shown to regulate the differentiation of somatic stem/progenitor cells, as well as cancer

stem cells, into differentiated cells (37), we hypothesized that LIN28 may maintain ALDH1⁺ cell populations by modulating miRNA *let-7* maturation. To test this hypothesis, we first asked whether there was a negative correlation between *let-7* expression and ALDH1⁺ cell numbers in tumors. Using the same tissue array set as above (Fig. 1B and C), *let-7* expression was detected by *in situ* hybridization (Fig. 4A and B), and the hybridization signals were classified into two groups: no staining/weak cytoplasmic signals (*let-7* low) or moderate/strong cytoplasmic signals (*let-7* high). We found that, in contrast to LIN28, the percentage of ALDH1⁺ tumor cells in the *let-7* low group ($n = 141$) was significantly higher than the *let-7* high group ($n = 56$; $P = 0.018$; Fig. 4C). Similar correlation was also observed in a breast cancer tissue array set ($n = 69$; $P = 0.016$). These observations indicate that *let-7* may be involved in the differentiation of ALDH1⁺ tumor cells in cancer. To further functionally test our hypothesis, we stably overexpressed *let-7* in tumor cell lines using retroviral vectors (Supplementary Fig. S3A). As expected, increased *let-7* expression dramatically reduced the number of ALDH1^{br} cells (Fig. 5A). To confirm this observation, we generated an inducible cell line in which *let-7* expression was controlled by the

administration of doxycycline (Supplementary Fig. S1B–E). We found that the ALDH1^{br} cell population was negatively associated with increased *let-7* expression in a dose-dependent manner (Fig. 5B). Importantly, in our polyclonal inducible cells, only ~30% of the cells responded to doxycycline treatment, as detected by the reporter gene DsRed (Supplementary Fig. S1D; Fig. 4C). This allowed us to separate the doxycycline-treated cells into two subpopulations: DsRed⁻/*let-7*^{low} and DsRed⁺/*let-7*^{high} (Fig. 5C). We found that the percentage of ALDH1^{br} cells was >6-fold lower in the *let-7*-induced cells (DsRed⁺/*let-7*^{high}) compared with the noninduced *let-7* cells (DsRed⁻/*let-7*^{low}; Fig. 5C). Additionally, using a *let-7* inhibitor, we blocked endogenous *let-7* expression in the above cell lines and found that downregulation of *let-7* significantly increased the number of ALDH1^{br} cells (Fig. 5D). Finally, we examined the effect of *let-7* on the self-renewal ability of MCF7 cells using the mammosphere assay. In contrast to LIN28, overexpression of *let-7* led to a decrease in the number of mammospheres, whereas a *let-7* inhibitor led to an increase in the number of mammospheres in MCF7 cells (all $P < 0.05$; Fig. 5D–F). Taken together, these results show that the expression of mature *let-7* modulates the ALDH1⁺ tumor cell population in cancer.

Figure 3. LIN28 modulates the biogenesis of *let-7* in tumor cells. A, lane 2, LIN28 binding RNAs in A2780 cells were precipitated by RNA immunoprecipitation using a LIN28 antibody. Lane 1, rabbit IgG was used for the control immunoprecipitation. B, the amount of *let-7b* precursor (pre-*let-7b*) in the precipitated RNAs was detected by real-time RT-PCR. Pre-*let-7b* was remarkably enriched in the LIN28-RNA-IP compared with the control IgG-RNA-IP (17.3-fold). C, LIN28 was knocked down by two independent lentiviral shRNA clones in A2780 cells. Reduced mRNA expression of LIN28 was confirmed by real-time RT-PCR. D, mature *let-7b* expression in the shRNA and control cells was measured by stem-loop RT-PCR. Blocking endogenous LIN28 expression significantly increased mature *let-7b* expression in A2780 cells. E, a luciferase *let-7b* sensor assay, which contained a constitutively expressed firefly luciferase reporter bearing sequences complementary to *let-7b* in the downstream 3'UTR, was used to monitor the *let-7b* activity in A2780 cells. F, blocking endogenous LIN28 expression significantly reduced luciferase activity of the *let-7b* sensor, indicating that *let-7b* function was remarkably increased in the LIN28 knockdown cells.



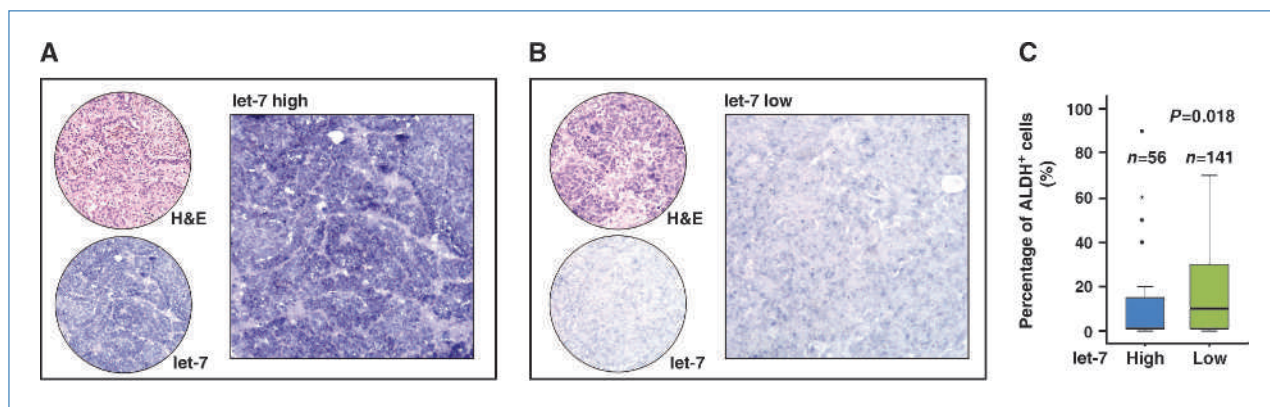


Figure 4. *let-7* expression is negatively correlated with a higher percentage of ALDH1⁺ tumor cells. A and B, mature *let-7* was detected by *in situ* hybridization using a LNA probe in the ovarian cancer tissue array. C, summary of the negative correlation of *let-7* expression and percentage of ALDH1⁺ tumor cells in the tissue array ($n = 197$; $P = 0.018$).

***let-7* targets LIN28 expression in tumor cells**

Interestingly, when we treated LIN28⁺ tumor cells (A2780 and T47D) with a *let-7* mimic, we found that the expression of both LIN28 protein and mRNA was significantly decreased (Fig. 6B and C), suggesting that *let-7* was able to regulate LIN28 via a feedback loop in tumor cells. We predicted that there was a conserved *let-7* binding site in the LIN28 3'UTR by TargetScan (Fig. 6A); this was then confirmed experimentally. Using a LIN28 3'UTR reporter assay, we showed that transfection of a *let-7* mimic was able to significantly reduce luciferase activity in the wild-type but not the *let-7* binding site mutant LIN28 3'UTR reporters (Fig. 6E). The above results show the existence of a feedback regulatory loop between *let-7* and LIN28 in tumor cells.

The LIN28/*let-7* loop controls mammary gland progenitor cell differentiation

Our studies show that the LIN28/*let-7* loop regulates the ALDH1⁺ cancer stem cell population in tumors, suggesting that this regulatory loop may also play an important role in maintenance of stem/progenitor cells under normal physiologic conditions. To test this hypothesis, we used a mouse mammary gland epithelial cell model. A standard protocol was used to isolate the heterogeneous mouse mammary gland epithelial cells containing epithelial progenitor cells. Then, LIN28 was introduced into the mammary gland epithelial cells by lentiviral infection (Fig. 7A). Protein expression was confirmed by Western blot analysis (data not shown). After short-term (48–72 hours) cultures, the percentage of the ALDH^{br} population was analyzed by FACS. Enforced LIN28 expression significantly increased the percentage of ALDH^{br} cells in the population (Fig. 7B). Another mammary gland progenitor cell marker, CD24/CD49f, was also examined (Supplementary Fig. S6). To further confirm that LIN28 indeed led to an increase in the number of mammary gland progenitor cells, we quantified the number of progenitors (colony-forming cells) using a mammary colony-forming assay in which the number of clones reflects the self-renewal capacity, whereas the type of the clones indicates the bipo-

tent differentiation ability (Fig. 7C). In summary, LIN28 significantly increased the numbers of all three types of colonies (Fig. 7D), suggesting that LIN28 was able to increase the self-renewal capacity of the mammary gland epithelial progenitor cells. In addition, we found that the enforced expression of *let-7* led to the differentiation of progenitor cells and significantly decreased total colony numbers (Fig. 7E). Interestingly, *let-7* selectively decreased progenitor cells, which had the ability to form the luminal or mixture type of colonies. Taken together, these results show that a LIN28/*let-7* regulatory loop plays a functional role in the maintenance of mammary gland epithelial progenitor cells.

Discussion

Recent research on iPS cells supports the hypothesis that cancer stem cells may arise through a “reprogramming-like” mechanism (38). By enforcing the expression of a set of genes, the so-called reprogramming factors, differentiated somatic cells can be converted to iPS cells, which have the same capabilities as ES cells to give rise to all tissue types of the body. Interestingly, most of these reprogramming factors are overexpressed or upregulated in certain types of human tumors, and at least some of them (e.g., c-MYC, KLF4, SOX2, and LIN28) are established or putative oncogenes (38). Moreover, five independent studies have shown that disabling p53, an essential tumor-suppressor gene, remarkably improves the efficiency of iPS cell production (38). Therefore, there may be overlapping mechanisms that control the functions and maintenance of iPS cells and cancer stem cells (38). LIN28, one of reprogramming factors discussed above, is very restricted in its expression; it is found only in ES cells, developing tissues, and tumors (19–24, 26–30). In human tumors, LIN28/LIN28B is upregulated and functions as an oncogene promoting malignant transformation and tumor progression (26–30). We have shown that LIN28 is positively correlated with the percentage of ALDH1⁺ tumor cells in cancer, suggesting that LIN28 may play a role in regulation of ALDH1⁺ tumor cells. In further functional studies, we have shown that LIN28 also functionally maintains this

cell population. Our data provided evidence that cancer stem cells may arise through a reprogramming-like mechanism; reactivated reprogramming factors such as LIN28 may promote the conversion of epithelial cells to a more undifferentiated stage and furthermore maintain a small subpopulation of tumor cells in this stem-like stage. In agreement with our findings, Peng and colleagues (30) recently reported that LIN28 together with OCT4 identified a subpopulation of stem cell-like cells in ovarian carcinoma. However, several critical questions need to be addressed further, including the mechanisms by which these reprogramming factors are reactivated in tumors (because most of them are completely silenced after development), the interactions between these reprogramming factors during the “reprogramming” process, and cellular safeguard mechanisms such as p53 that respond to these reprogramming events.

The present study also suggests that the maintenance of the ALDH1⁺ tumor cell population by LIN28 is mediated by the regulation of *let-7*, and that a LIN28/*let-7* regulatory loop controls ALDH1⁺ cancer stem cells. The tumor suppressor role of *let-7* in cancer was first shown by the Slack laboratory (39). They found that the *let-7* family negatively regulates let-60/RAS in *C. elegans* by binding to multiple *let-7* complementary sites in its 3'UTR (39). Moreover, having found that *let-7* expression is lower in lung tumors than in normal lung tissue, whereas RAS protein is significantly higher in lung tumors, *let-7* was proposed as a tumor suppressor gene (39–41). Increasing evidence indicates that *let-7* plays a functional role in normal and cancer stem cell differentiation. First, in *C. elegans*, *let-7* times seam cell, the stem cells that divide asymmetrically during each larval stage, terminal differentiation, possibly by acting as a

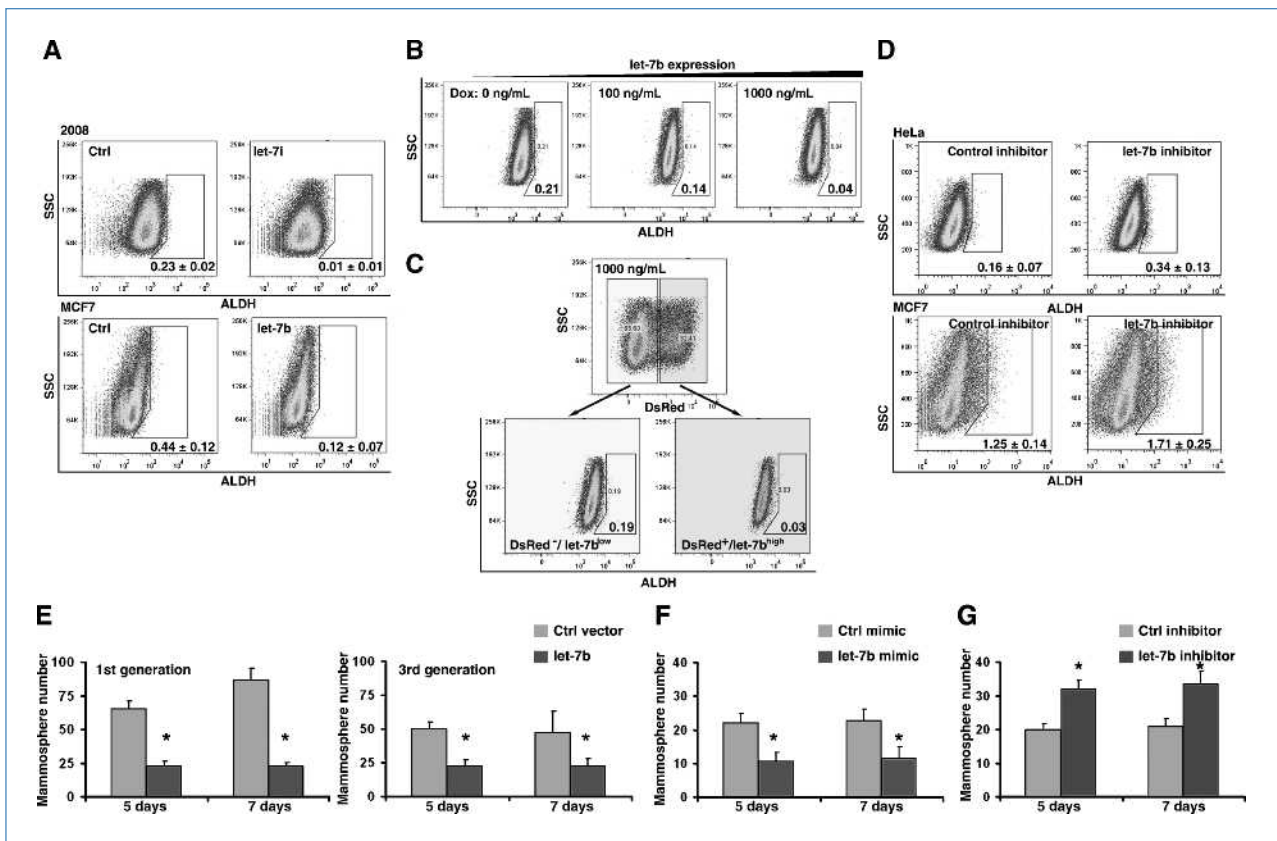


Figure 5. *let-7* modulates the ALDH1⁺ tumor cell population in cancer. A, stably enforced expression of *let-7* significantly decreased the ALDH1^{br} cell population in 2008 and MCF7 cells. Overexpression of *let-7* by retroviral miRNA expression vectors (Supplementary Fig. S3A). B, induced *let-7* expression remarkably decreased the ALDH1^{br} cell population in HeLa cells in a dose-dependent manner. The doxycycline-controlled *let-7*-inducible HeLa cells were generated by a retrovirus-based RevTet-On system (Supplementary Fig. S3B). The expression of *let-7* and reporter gene (DsRed) was induced by doxycycline treatment (Supplementary Fig. S3C–E). C, after treatment with 1,000 ng/mL doxycycline, the reporter gene DsRed (DsRed/*let-7*) was detectable in ~30% of polyclonal HeLa cells (Supplementary Fig. S3C and D). Taking this advantage, the DsRed⁺/*let-7*^{low} and DsRed⁺/*let-7*^{high} cell populations were gated, and the percentage of ALDH1^{br} cells was compared between these two groups. The percentage of ALDH1^{br} cells was significantly lower in the DsRed⁺/*let-7*^{high} population. D, LNA *let-7* inhibitor was used to block *let-7* expression in HeLa and MCF7 cells. Blocking endogenous *let-7* significantly increased the ALDH1^{br} cell population. E, enforced *let-7* expression significantly decreased the number of mammospheres compared with the control cells. Left, result in the first generation of mammospheres; right, result in the third generation of mammospheres. F, to avoid artifacts induced by retroviral infection, *let-7* was transiently overexpressed via the transfection of a *let-7* mimic. Enforced *let-7* expression significantly decreased the number of mammospheres compared with the control cells. G, endogenous *let-7* was transiently blocked via the transfection of a *let-7* inhibitor. Blocking *let-7* expression significantly increased the number of mammospheres compared with the control cells. All *P* values < 0.05.

regulator of multiple genes required for cell cycle and proliferation (42–45). Second, in ES cells, mature *let-7* is poorly expressed, although its precursor transcripts are readily detected (46). This may oppose the actions of a family of cell cycle-regulating miRNAs that maintain self-renewal in ES cells (47). Third, in mammalian embryonic and somatic stem cells, *let-7* interacts with two iPS genes, MYC and LIN28, and these autoregulatory loops [MYC/*let-7* (27) and LIN28/*let-7* (22, 31–36)] may control stem cell self-renewal and differentiation (37). Forth, Nishino and colleagues have shown that during aging, elevated levels of *let-7b* block HMGA2 and contribute to declining neural stem cell function; in contrast, HMGA2 maintains neural stem cell function in young mice through repression of the Ink4a/Arf locus (48). Fifth, Ibarra and colleagues have found that *let-7* is depleted in a population of self-renewing mammary

epithelial progenitor cells that can reconstitute the mammary gland. Enforced expression of *let-7* leads to a loss of these self-renewing cells from mixed cultures, suggesting that *let-7* plays a role in the regulation of progenitor cell maintenance (3). Finally, by comparing miRNA expression in self-renewing and differentiated breast tumor cells, Yu and colleagues (49) found that *let-7* was markedly reduced in breast cancer stem cells but increased on differentiation. These findings show that *let-7* plays a functional role in normal and cancer stem cells. In support of these notions, we have shown that *let-7* plays an opposing function to LIN28 in regulating the ALDH1⁺ tumor cell population. Importantly, we reported a double-negative feedback regulating loop of LIN28 and *let-7* in tumor cells. It has been widely reported that the *let-7* family of genes is globally downregulated in cancer (37, 39, 46), which may be one

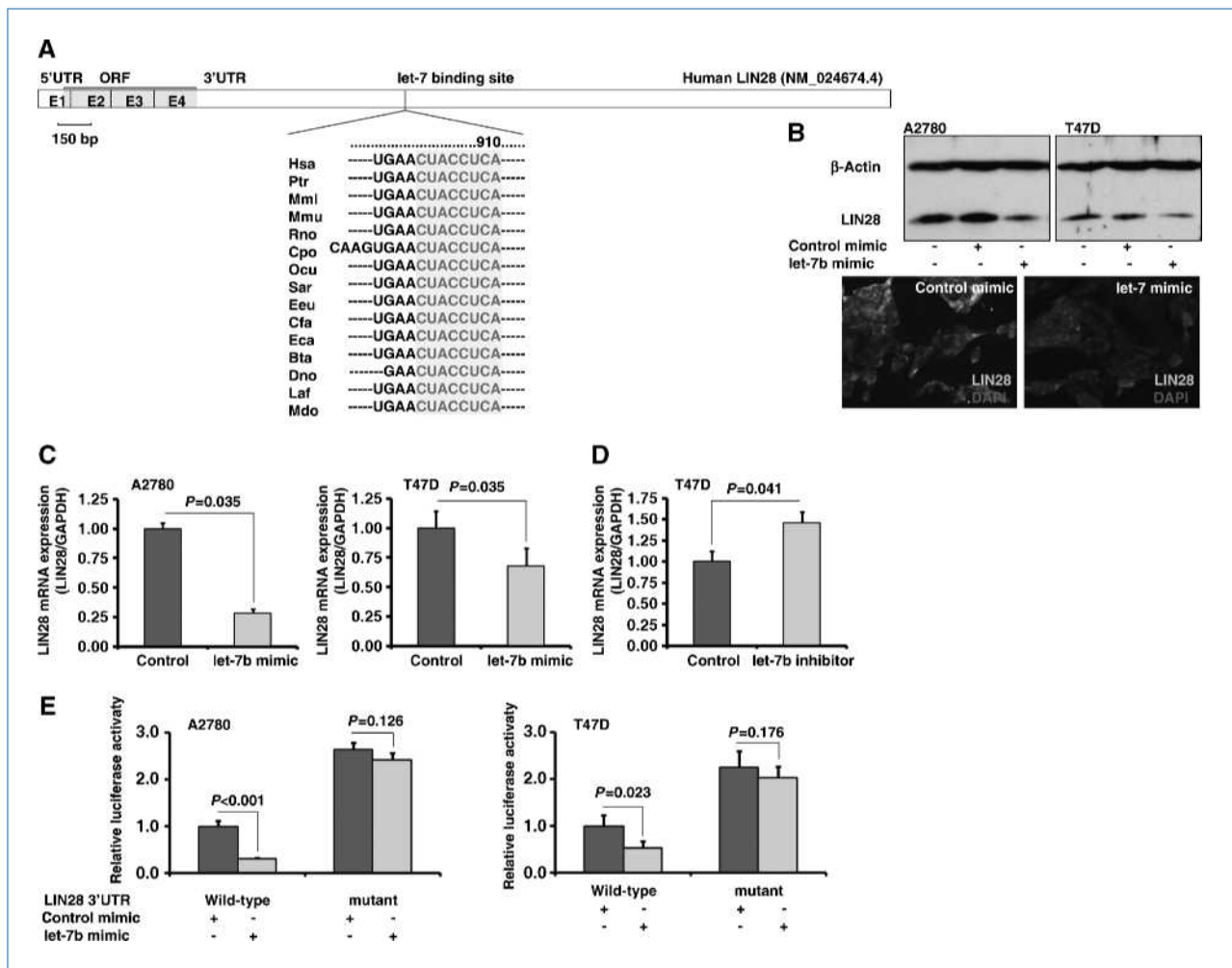


Figure 6. *let-7* targets LIN28 expression in tumor cells. A, a conserved *let-7* binding site in the human LIN28 3'UTR was predicted by TargetScan. B, transfection of a *let-7* mimic remarkably decreased LIN28 protein expression in A2780 and T47D cells. C, transfection with a *let-7* mimic significantly decreased LIN28 mRNA expression in A2780 and T47D cells. D, transfection with a *let-7* LNA inhibitor significantly increased LIN28 mRNA expression in T47D cells. E, the LIN28 3'UTR reporter assay showed that transfection of a *let-7* mimic significantly reduced the luciferase activity in the wild-type but not the *let-7* binding site mutant LIN28 3'UTR reporters.

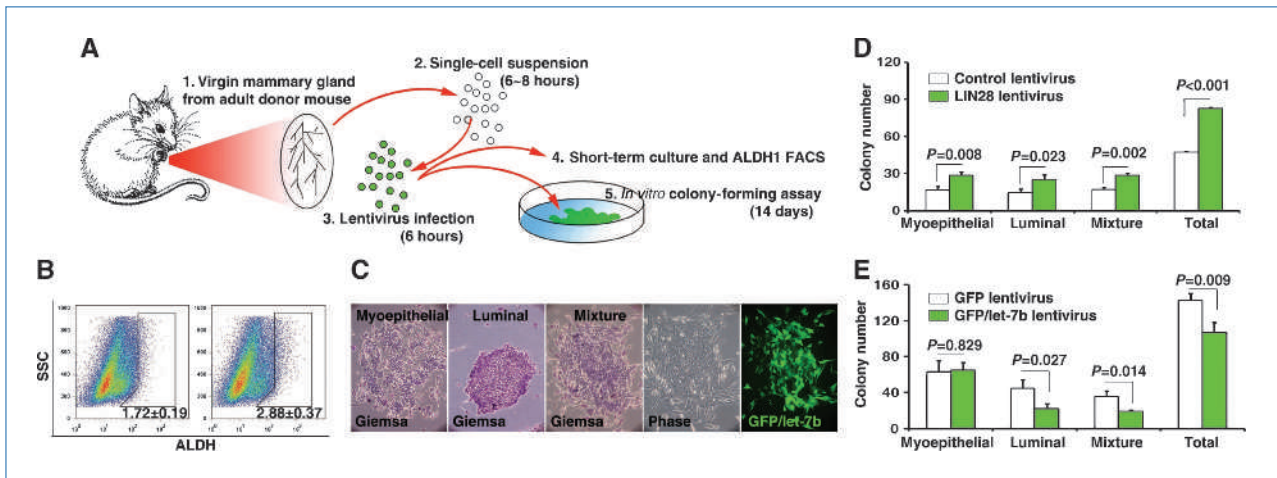


Figure 7. A LIN28/*let-7* loop controls mammary gland progenitor cell differentiation. A, protocol of mammary gland epithelial cell isolation and lentiviral infection. B, enforced expression of LIN28 significantly increased the ALDH1^{br} cell population in mammary gland epithelial cells. C, three types of colonies were identified by Giemsa staining. D, enforced LIN28 expression led to a significant increase in the number of colonies. E, enforced expression of *let-7* led to a significant decrease in the luminal and mixture types of colonies.

of mechanisms of LIN28 reactivation in tumors. Finally, LIN28 may regulate the ALDH1⁺ cell population through a *let-7*-independent pathway (20). For example, LIN28 may directly regulate other reprogramming factors in cancer stem cells, such as OCT4 (30, 50).

In summary, we have identified a novel mechanism regulating ALDH1⁺ cancer stem cells, which could lead to new therapeutic strategies for targeting the ALDH1⁺ tumor cell population. For example, recent studies indicate that LIN28 may cooperate with a terminal uridylyltransferase (TUTase) to regulate *let-7* maturation (23, 36). Given that polymerases are facile targets for pharmacologic inhibition by small chemical compounds, TUT4 may prove to be a novel target for manipulating the LIN28/*let-7* regulation loop (23). Additionally, nanoparticle-delivered LIN28 siRNA or *let-7* mimics may also be an attractive therapeutic

strategy to target ALDH1⁺ cancer stem cell population in tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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