Lin28B/Let-7 Regulates Expression of Oct4 and Sox2 and Reprograms Oral Squamous Cell Carcinoma Cells to a Stem-like State

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Abstract

Lin28, a key factor for cellular reprogramming and generation of induced pluripotent stem cell (iPSC), makes a critical contribution to tumorigenicity by suppressing Let-7. However, it is unclear whether Lin28 is involved in regulating cancer stem–like cells (CSC), including in oral squamous carcinoma cells (OSCC). In this study, we demonstrate a correlation between high levels of Lin28B, Oct4, and Sox2, and a high percentage of CD44⁺/ALDH1⁺ CSC in OSCC. Ectopic Lin28B expression in CD44⁺/ALDH1⁺ OSCC cells was sufficient to enhance Oct4/Sox2 expression and CSC properties, whereas Let7 co-overexpression effectively reversed these phenomena. We identified ARID3B and HMGA2 as downstream effectors of Lin28B/Let-7 signaling in regulating endogenous Oct4 and Sox2 expression. Let7 targeted the 3’ untranslated region of ARID3B and HMGA2 and suppressed their expression, whereas ARID3B and HMGA2 increased the transcription of Oct4 and Sox2, respectively, through promoter binding. Chromatin immunoprecipitation assays revealed a direct association between ARID3B and a specific ARID3B-binding sequence in the Oct4 promoter. Notably, by modulating Oct4/Sox2 expression, the Lin28B–Let7 pathway not only regulated stemness properties in OSCC but also determined the efficiency by which normal human oral keratinocytes could be reprogrammed to iPSC. Clinically, a Lin28B(high)/Let-7(low) expression pattern was highly correlated with high levels of ARID3B, HMGA2, OCT4, and SOX2 expression in OSCC specimens. Taken together, our results show how Lin28B/Let-7 regulates key cancer stem–like properties in oral squamous cancers.

Introduction

Head and neck squamous cell carcinoma (HNSCC), including oral squamous cell carcinoma (OSCC), is the sixth most prevalent malignancy worldwide (1, 2). Most patients relapse within months after current therapeutic treatments and around 50% of patients die of this disease or die from complications within 5 years under current therapies. Embryonic stem cell (ESC) signatures, including Oct4, Sox2, Nanog, Klf4, and c-Myc, have been reported to maintain the self-renewal property and drive cellular reprogramming into pluripotent state in normal somatic cell as well as in malignant transformed cells. Exogenous induction of ESC-stemness gene has been shown to promote dysplastic growth in adult epithelial tissues (3, 4). A recent study indicated that premature termination of reprogramming may produce cells that are equipped with several stemness properties but fail to become successfully induced pluripotent stem cell (iPSC), and finally generate neoplasia resembling Wilms tumor (5). These interesting findings suggested a possible link between ESC/stemness signature-mediated reprogramming and tumor transformation.

Lin28 has been shown to be a key RNA-binding protein and plays a critical role in regulating the balance between stemness and differentiation in ESCs (6). Lin28 is highly expressed during embryogenesis and has emerged as a factor that defines stemness state in several tissue lineages (7). Lin28 was found to be a master regulator of developmentally timed changes in hematopoietic stem cells (HSC) programs with the high-mobility group AT-hook 2 (HMGA2) serving as its specific downstream modulator of HSC self-renewal potential (8). Overexpression of Lin28 was detected in various cancers and involved in the oncogenesis of human malignancies (9–12). Moreover, through inhibiting Let7 biogenesis, Lin28 influences mRNA translation, regulates the self-renewal of ESCs, and promotes the malignant state in several cancer tissues. 

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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transformation of cancers (8, 13–15). Recent reports showed that the Lin28–Let7 pathway plays a critical role in regulating the stemness, self-renewal, tumor initiation, and epithelial–mesenchymal transition–derived metastasis in malignant tumors and cancer stem–like cells (CSC; refs. 8, 9, 12, 13, 15, 16). However, the detailed molecular mechanism involved in the Lin28/Let7–driven stemness regulation and cancer reprogramming is still an open question.

Oct4 is a key reprogramming factor and balances the pluripotent and differentiated states in stem cell and cancer development (4, 17, 18). Sox2 is critical in maintaining self-renewal of ESC (17), and Sox2-mediated pathway was also shown to regulate the acquisition of CSC-like and radiochemoresistant properties in HNSCC (19). A genomic copy number gain of the Sox2 locus was reported in OSCC, which resulted in an increase of Sox2 transcriptional activity and might be critically involved in OSCC initiation and progression (20). Although Oct4 and Sox2 are seemingly involved in the induction of CSC properties, the regulatory mechanisms of ESC/stemness signature–dependent modulation of CSC properties in OSCC are still blurred. Here, we reported that the Lin28B–Let7 pathway positively regulates Oct4 and Sox2 expression, inducing a reprogramming–like phenomenon, switching non–CSCs to CSCs with tumor-initiating and self-renewal properties in oral CSC. We identified the AT-rich interaction domain molecule 3B (ARID3B) and HMGA2 as direct targets of Let7, mediating Oct4 and Sox2 transcriptional activity and might be critically involved in the acquisition of CSC properties in OSCC, which resulted in an increase of Sox2 transcriptional activity and might be critically involved in OSCC initiation and progression (20). Although Oct4 and Sox2 are seemingly involved in the induction of CSC properties, the regulation of ESC/stemness signature–dependent modulation of CSC properties in OSCC are still blurred.

Materials and Methods

Cell culture, CD44 “ALDH1+” OSCC cells isolation, and tumor sphere enrichment

All procedures of tissues acquirements have followed the tenets of the Declaration of Helsinki and are approved by the Institutional Ethics Committee/Institutional Review Board of Taipei Veterans General Hospital and Chiang Sang Hospital (Taipei, Taiwan). All samples were obtained after patients provided informed consent. All culture protocols and information of OSCC cell isolated from patients were obtained from Accutase (STEMCELL Technologies) followed by cell sorting analysis (FACS; refs. 21, 22). For enrichment of spheres, 10^3 cells were plated in low-attachment 6-well plate and cultured at a concentration of 1 × 10^3/mL in 37°C DMEM with 2% FCS. To identify CD44-positive and ALDH1+ positive cells, we stained cells with CD44 antibody (phycoerythrin-conjugated; BioLegend) and applied Aldefluor assay (STEMCELL Technologies) followed by fluorescence-activated cell sorting analysis (FACS; refs. 21, 22). For enrichment of spheres, 10^3 cells were plated in low-attachment 6-well plate (Corning Inc.) with tumor sphere medium consisting of serum-free DMEM/F12 medium (GIBCO), N2 supplement (GIBCO), 10 ng/mL human recombinant basic fibroblast growth factor (bFGF) and 10 ng/mL epidermal growth factor (EGF; R&D Systems). For serial passage of spheroid cells, single cells were passaged onto mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers, and cultured using human ESC medium [DMEM/F12 (Gibco) supplemented with 20% knockout serum replacer (KSR; Invitrogen), 0.1 mmol/L nonessential amino acids (Invitrogen), 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 10 ng/mL recombinant human bFGF, and antibiotics (Gibco)]. SB431542 (2 μmol/L; Stemgent), PD0325901 (0.5 μmol/L; Stemgent), and thiazovivin (0.5 μmol/L) were added to the culture medium to aid colony formation and this medium was refreshed daily until iPSC colonies appeared (25). Undifferentiated iPSCs were maintained on mitotically inactivated MEFs (50,000 cells/cm^2) in human ESC medium. To prevent MEF contamination, human iPSCs were transferred to a feeder-free/serum-free CSTI-8 medium (Cell Science & Technology Institute Inc.) without KSR supplementation. The detailed information for generating and long-term maintaining iPSCs has been described in our previous report (23, 24).

SPONGE constructions

Let7–SPONGE, scramble, and antisense Let7 and microRNA were constructed using a pcDNA 6.2-GW/EmGFP-miR plasmid (Invitrogen; ref. 26). microRNA SPONGE sequence design was based on Ebert and colleagues (27), and the sequence of Let7–SPONGE was listed in Supplementary Table S3.

Bisulfite pyrosequencing

Bisulfite treatment and DNA cleanup was performed by using the EpiTect 96 Bisulfite Kit according to the previous protocol (23, 26). In brief, 500 ng of genomic DNAs were reacted with freshly prepared bisulfite/hydroquinone solutions to convert unmethylated cytosine to uracil, and methylated cytosine was protected from the chemical reaction. The bisulfite reaction was stopped by neutralization using sodium acetate, and the genomic DNAs were precipitated with glycygen by ethanol DNA precipitation method. PCR amplification primers and sequencing primers were designed by PyroMark Assay Design Software 2.0. PCR amplification of target region was performed by using the PyroMark PCR Kit (Qiagen). Details are described in the Supplementary Data.
Let7 angomiR and liposome-mediated delivery

Let7 RNA oligos were synthesized as identical sequences of Let7 with modifications. The phosphodiester backbones were modified by phosphothiolate backbones and the ribose was replaced by lock-nucleic-acid (LNA) ribose. The delivery of the Let7 angomiR was mediated by liposome-based nucleic acid delivery method. In brief, Let7 was dissolved in D5W (5% dextrose water) and packaged with specialized lipid components into liposomes. The packaged liposomes (final concentration of Let7 angomiR is 10 ng/μL) were delivered into the same locus of intracranially xenotransplanted tumor-initiation cells.

Statistical analysis

Data are presented as mean ± SD. A Student t test or analysis of variance (ANOVA) test was used to compare the continuous variables between groups, as appropriate. The \( \chi^2 \) test or Fisher exact test was used to compare the categorical variables. \( P < 0.05 \) was considered statistically significant.

Results

Elevated Lin28B in high-grade and CSC-like OSCC cells were correlated with increased Oct4, Sox2, and sphere formation

Lin28 is recently a focus in the field of regenerative researches as its role in RNA regulation has been linked to the modification of miRNA landscape during ESCs differentiation as well as somatic cell reprogramming. Although stemness factors, such as Oct4 and Sox2, have been implicated in malignant development and stemness property acquisition of cancers, the role of Lin28 in cancer stemness, however, is still unclear. We first analyzed and compared the expression levels of Lin28B in nine pairs of tumorous and nontumorous tissues of oral cancer patients. Immunohistochemistry (Fig. 1A) and quantitative real-time PCR (qRT-PCR; Fig. 1B) showed that Lin28B was highly expressed, both in protein and mRNA levels, in oral cancer specimens, compared with the nontumorous counterparts. Further analysis of Lin28B levels in different stages of OSCC specimens revealed a correlation between high Lin28B levels and advanced stage of OSCC (Fig. 1C).

OSCC cells that express CD44 and ALDH1, and are able to form spheres in suspension culture, were proposed to be CSCs with tumor-initiating potential (28). These markers, therefore, were used to identify OSCC-CSCs. Using flow cytometry, we isolated CD44+ and ALDH1+ cells from 3 patients’ tumorous specimens (Pt1-3) and found that cells expressing these CSC markers had high mRNA expression levels of Lin28B (Fig. 1D). Serial transfection of the isolated cells in NOD/SCID mice showed significant higher tumor-initiating potential of CD44+/ALDH1+ cells than CD44−/ALDH1− cells (Supplementary Fig. S1E). We then exogenously overexpressed Lin28B in three patient-derived CD44+/ALDH1+ cells (CD44+/ALDH1+/pLV-Lin28B; Fig. 1H; Supplementary Fig. S1A) and showed that overexpressed Lin28B increased the percentage of CD44+/ALDH1+ cells (Fig. 1E) as well as the sphere-forming ability (Supplementary Fig. S1B). Moreover, cells expressing CD44 and ALDH1, individually or simultaneously, also presented high level of Oct4 and Sox2, compared with their nonexpressing counterparts (Fig. 1F; Supplementary Fig. S1C and S1D). Similarly, in the CD44+/ALDH1+/pLV-Lin28B cells, Oct4 and Sox2 were increased in mRNA and protein levels, compared with the parental cells and empty vector-transfected controls (Fig. 1G and H; Supplementary Fig. S1A).

Functional analysis showed that CD44+/ALDH1+/pLV-Lin28B cells presented enhanced sphere formation capability in comparison with control cells; whereas knockdown of Lin28B in CD44+/ALDH1+ cells (CD44+/ALDH1+/shLin28B) suppressed the formation of suspended spheres (Supplementary Fig. S2A) and soft agar colonies (Supplementary Fig. S2B). Co-knockdown of Oct4 and Sox2 by short hairpin RNA (shRNA) in CD44+/ALDH1+/pLV-Lin28B cells (Fig. 1H) decreased the sphere number (Fig. 1I), indicating a role of Lin28B in regulating self-renewal and tumorigenesis in OSCC cells. In addition, CD98 was recently shown as a marker to enrich a subpopulation of HNSCC cells with stem cell properties (29). However, our flow cytometry data showed no significant difference of CD98 level between the functionally proved CSC-like CD44+/ALDH1+/shCtrl and non-CSC-like CD44+/ALDH1+/shLin28B cells (data not shown). Finally, transplantation of these stable cells in the neck region of immunocompromised mice showed that overexpression of Lin28B increased tumor growth in CD44+/ALDH1− cells, whereas co-knockdown of Oct4 and/or Sox2 (CD44+/ALDH1+/pLV-Lin28B/shOct4+shSox2) decreased it (Fig. 1I). Notably, the Oct4 and Sox2 seemed to have an additive effect in this assay, as knockdown of both presented a more suppressive effect on tumor growth than knockdown of each (Fig. 1I). Consistently, subcutaneous transplantation of these stable cell lines in the neck region of immunocompromised mice with different cell numbers indicated that Lin28B overexpressed cells were able to induce tumor formation with as less as 1,000 cells, whereas the control cell line could not generate in vivo tumors even with a cell number up to 100,000 (Supplementary Fig. S1E). Co-knockdown of Oct4 and Sox2 in the CD44+/ALDH1+/pLV-Lin28B cells hampered the tumor-initiation ability in vivo (Supplementary Fig. S1E). These data indicated that Lin28B induced stemness genes expression such as Oct4 and Sox2, CSC markers expression such as CD44 and ALDH1, self-renewal potential, and tumor growth and initiation. A novel Lin28B-Oct4–Sox2 pathway was identified in regulating stemness property of OSCC.

Let7 is a negative downstream effector of Lin28B in the regulation of Oct4/Sox2 and tumorigenesis in OSCC

Let7 is a negative downstream molecule of Lin28 (8, 14, 15); Lin28-mediated inhibition of Let7 resulted in upregulated self-renewal and epithelial–mesenchymal transition in HSCs and cancers (8, 14, 15). We, therefore, investigate whether Let7 is one of the major downstream effectors of Lin28B in regulating CSC-like and reprogramming property in OSCC. We first stably suppressed the level of Let7 in patient-derived CD44+ALDH1+ cells by knockdown of Let7 expression using the SPONGE system. SPONGE is a competitive inhibitor of miRNA through strong binding with the miRNA of interest by its multiple binding sites, relieving the targets of the miRNA from being inhibited (27). We established Let7 SPONGE, a competitive inhibitor containing a transcript expressing multiple tandem binding sites to Let7. When vectors encoding Let7 SPONGE are expressed in cells, the SPONGE strongly competes with the targets of Let7, “absorbs” Let7 in cells, and relieves the targets of Let7 from being suppressed by Let7. In this study, we used Let7 SPONGE (Spg-Let7) as a knockdown
method of Let7, and validate the hypothesis of Let7 as a key mediator linking stemness genes with Lin28. In line with our hypothesis, suppressing Let7 expression in CD44+ ALDH1+ cells, which express higher level of Let7 than CD44- ALDH1+ cells, resulted in increased Oct4 and Sox2 protein levels (Fig. 2A). We then overexpressed Let7 in CD44+ ALDH1+/pLV-Lin28B cells to generate a stable cell line (CD44+ ALDH1+/pLV-Lin28B+Let7). An empty vector-transfected control cell line was also established simultaneously (CD44+ ALDH1+/pLV-Lin28B+Vec). We have shown that the Lin28-elevated protein levels of Oct4 and Sox2 were decreased upon co-overexpression of Let7 (Fig. 2B). Delivery of Let7-mimic had the same effect (Supplementary Fig. S2C and S2D). Sphere formation (Fig. 2C, left) and soft agar (Fig. 2C, right) assays indicated that delivery of Let7-mimic reversed the Lin28B-increased sphere and colony numbers, resulting in a decrease of sphere formation ability and anchorage independence down to a level similar to CD44+ ALDH1+/pLV-Ctrl cells.

To assess the effects of the Lin28B–Let7 pathway in tumor initiation and growth, we established a panel of CD44+ ALDH1+ stable cell lines with Lin28B and Let7 being knocked down by shRNA and Sponge (26), respectively (Supplementary Fig. S2E).

Figure 1.
Elevated Lin28B in high-grade and CSC-like oral cancer cells was correlated with increased Oct4, Sox2, and sphere formation. A and B, nine pairs of tumor and nontumor specimens were derived from OSCC patients and subjected to IHC and qPCR analysis to assess the Lin28B protein and mRNA levels. One representative IHC stain of Lin28B is shown in A. C, samples from patients with different stages (stage I to IV) of OSCC were collected and subjected to Western blot analysis for the protein expression levels of Lin28, Oct4, and Sox2. D, primary culture cells derived from patients were subjected to sort out CD44+ALDH+ cells. Relative Lin28B expression level is shown in the quantitative chart on the right. E, CD44+ALDH+ cells were transfected with pLV-Lin28B or empty vector (pLV-Ctrl) using lentiviral system. Both cells were subjected to flow cytometry analysis for their CD44 expression and ALDH activity. F, CD44+ ALDH1+ and CD44+ ALDH1- cells were subjected to qPCR analysis to assess the Oct4 and Sox2 mRNA expression. G, pLV-Ctrl and pLV-Lin28B-transfected cells were analyzed by qPCR for the Oct4 and Sox2 expression. H, patient-derived CD44+ ALDH1+ cells with stably transfected plasmid constructs as indicated were subjected to Western blot analysis for the protein expression levels of Lin28, Oct4, and Sox2. I, pLV-Lin28B cells with or without double knockdown of Oct4 and Sox2 were subjected to a sphere formation assay. The results of tumorsphere formation by phase contrast microscopy are presented at the top and the quantified sphere numbers is presented in the graph at the bottom. This experiment was repeated in three patient-derived CD44+ ALDH1+ cells. *, P < 0.05 versus control. Scale bar, 100 μm. J, stable cell lines as indicated were transplanted in the neck subcutaneous region of immunocompromised mice (n = 3; each mouse was injected by 106 cells). Tumor volumes (mean tumor volume cm3 ± SEM) were measured by a caliper and monitored up to 6 weeks.
Consistent with previous results, shLin28B suppressed the sphere formation (Fig. 2D) and Oct4 and Sox2 expression (Fig. 2E; Supplementary Fig. S2E), whereas Sponge-Let7 (Spg-Let7) reversed these effects (Fig. 2D and E). We then transplanted these stable cells lines in the neck subcutaneous region of immunocompromised mice with the indicated number of cells. In CD44+ ALDH1+ cells, shLin28B suppressed the tumor growth and initiation, both of which were rescued by Spg-Let7 (Fig. 2F and G). These results demonstrated that Let7 is the key downstream effectors of Lin28B, negatively mediating Lin28B-dependent regulation of tumorigenesis and stemness-derived reprogramming properties in OSCC.

**Figure 2.** Let7 is a negative downstream effector of Lin28B in the regulation of Oct4/Sox2 and tumorigenesis in OSCC. A, Oct4 and Sox2 expression in CD44+ ALDH1+ cells transfected with or without Sponge-Let7 (Spg-Let7) and Sponge-control (Spg-Ctrl) was assessed by Western blot analysis. B, pLV-Ctrl, pLV-Lin28B, and pLV-Let7-transfected CD44+ ALDH1+ stable cells were subjected to Western blot analysis for Lin28B, Oct4, and Sox2 expression (top) and qPCR for Let7 levels (bottom). C, CD44+ ALDH1+ cells stably transfected with pLV-Ctrl, pLV-Lin28B, scrambled miR-mimic control, and Let7-mimic were subjected to sphere formation (left) and soft-agar (right) assay. The numbers of sphere and colony were calculated and are presented in the graphs. *, \( P < 0.05 \) versus control; \#, \( P < 0.005 \) versus Spg-Ctrl. D, CD44+ ALDH1+ cells with or without knockdown of Lin28B (shLin28B) and Let7 (Spg-Let7) were subjected to a sphere formation assay for up to 21 days. The size of sphere from each stable cell line is presented by phase contrast microscopy on the left, and the quantitative sphere number is presented in the graphs on the right. Only spheres with a diameter more than 50 μm were counted. E, CD44+ ALDH1+ cells with or without knockdown of Lin28B and Let7 were subjected to qPCR to assess the expression level of Oct4 and Sox2. F, CD44+ ALDH1+ stable cell lines were transplanted in immunocompromised mice and the xenograft tumor volume was monitored up to 6 weeks. G, CD44+ ALDH1+ stable cell lines as indicated were subcutaneously transplanted in the neck region of immunocompromised mice with different cell number from 1,000 to 100,000 cells per transplantation. Each cell line was repeated in three mice, and the tumor formation was monitored for at least 3 months. The number of mice that presented visualized tumor mass is listed in the table.

ARID3B and HMGA2 are targets of Lin28B/Let7 signaling to regulate Oct4 and Sox2. Following the finding of the Lin28B–Let7–Oct4/Sox2 pathway, we then analyzed the direct regulatory mechanism of how Lin28B mediates the expression of the two important stemness factors. We applied a bioinformatics analysis in three pairs of isolated OSCC cells: CD44+ ALDH1+ versus CD44+ ALDH1+ Oct4–/C0 versus CD44+ ALDH1+ /Oct4–/C0, and primary culture of NHOK versus NHOK-iPSC, and compared their gene expression profiles. We identified ARID3B and HMGA2 as potential regulators (Fig. 3A). ARID3B is a DNA-binding protein belonging to the AT-rich interactive domain (ARID)
family of transcription factors that are involved in diverse biological processes (30), and HMGA2 is a member of the high-mobility group AT-hook protein family and has been reported to be a Let7 target (8) and directly binds to Sox2 promoter in glioblastomas to induce Sox2 expression (26). To validate the bioinformatics data, we established a panel of patient sample-derived CD44^+/ALDH1^+ OSCC cells with different combinations of overexpressed Lin28B, Let7, and Spg-Let7. We found that overexpression of Lin28B increased the protein levels of ARID3B, HMGA2, Oct4, and Sox2, whereas overexpression of Let7 reversed this effect of Lin28B and resulted in suppressed protein levels of the four molecules down to a level similar to control cells (Fig. 3B). Spg-Let7 dramatically increased the expression of the four molecules, and this increase was even more significant with co-overexpression of Lin28B (Fig. 3B).

Moreover, we dissected the regulatory causal effects between ARID3B, HMGA2, Oct4, and Sox2 by individually overexpressing the four molecules in CD44^+/ALDH1^+ human iPSC cell line. Overexpression of ARID3B and HMGA2 enhanced the protein levels of Oct4 and Sox2, respectively, whereas overexpression of Oct4 and Sox2 did not affect the levels of ARID3B and HMGA2 (Fig. 3C and D). Knockdown of ARID3B and HMGA2 in CD44^+/ALDH1^+ cells resulted in suppressed Oct4 and Sox2, respectively, but not vice versa (Fig. 3E and F). Taken together, these results demonstrated the causal effect of Lin28B/Let7-driven ARID3B–HMGA2-Oct4–Sox2 pathway in OSCC.

Let7 regulates tumor initiation of OSCC through targeting the 3’-UTR of ARID3B and HMGA2

Using the TargetScan program, we identified potential Let7-targeting sites in the 3’-untranslated region (3’-UTR) regions of ARID3B. We constructed reporter plasmids containing either full-length, serial deletion (D1-D4, the potential Let7 targeting site was deleted), or mutated forms of the 3’-UTR region of

**Figure 3.** The Lin28B–Let7–Oct4–Sox2 pathway mediated the stemness and tumorigenesis properties in OSCC. A, three pairs of OSCC cells, CD44^+/ALDH1^+ versus CD44^−/ALDH1^−, CD44^−/ALDH1^+ /Ctrl versus CD44^−/ALDH1^+ /Lin28B, and the primary culture of NHOK versus NHOK-iPSC were subjected to microarray and bioinformatics analysis to compare their gene expression profiles. The consistently changed molecules in the three pairs of cells were selected as potential downstream factors of the Lin28B–Let7 pathway in OSCC tumorigenesis and reprogramming process. In our data, we identified ARID3B, HMGA2, Oct4, and Sox2 as subjects for further investigations. B, CD44^+^/ALDH1^+^ OSCC cells with different combinations of overexpressed Lin28B, Let7, and Spg-Let7 were analyzed by Western blot analysis for the protein expression levels of ARID3B, HMGA2, Oct4, and Sox2. The human ES cells (HESC-S6, human ESC line) and human iPSC were the positive control for the four molecules in the blot. C and D, CD44^+^/ALDH1^+^ /shLin28B cells were transfected with or without ARID3B, HMGA2, Oct4, and Sox2 before being subjected to Western blot analysis. E and F, CD44^+^/ALDH1^+^ /Spg-Let7 cells with or without knockdown of ARID3B, HMGA2, Oct4, and Sox2 were analyzed by Western blot analysis.
ARID3B (Fig. 4A and B). Luciferase reporter assays demonstrated that Let7 reduced the luciferase activity of reporter plasmids containing full-length ARID3B 3′-UTR (Fig. 4C). However, when the potential Let7-targeting site was deleted or mutated, Let7 no longer affected the luciferase activity (Fig. 4C). Consistently, the protein and mRNA levels of ARID3B were decreased in the presence of Let7 (Fig. 4D) and increased in the absence of Let7 (Fig. 4E). These results identified a crucial Let7-binding site on the 3′-UTR of ARID3B for Let7 to suppress its expression. To further link this regulatory mechanism to tumorigenesis of OSCC, we subcutaneously transplanted parental CD44−ALDH1+ /Spg-Ctrl, CD44−ALDH1+ /Spg-Let7, and CD44−ALDH1+ /Spg-Let7 + shARID3B cells in the neck region of immunocompromised mice and monitored the tumor growth up to 6 weeks. Spg-Let7 enhanced the tumor growth, while shARID3B inhibited the Spg-Let7−induced tumor growth (Fig. 4F). Previous studies demonstrated that the 3′-UTR of HMGA2 contains seven sequences complementary to the Let7 family of miRNAs (31), allowing Let7 to negatively regulate HMGA2 mRNA and protein expression. In our OSCC model, we also found that Let7 suppressed HMGA2 expression through binding on its 3′-UTR (Supplementary Figs. S2F and S2G and S3).

ARID3B and HMGA2 regulate the promoter activities of Oct4 and Sox2, respectively, through direct promoter binding To investigate whether ARID3B directly regulates Oct4 expression, we searched for potential AT-rich regions in the proximal promoter of Oct4 and found several potential binding sites of ARID3B (Fig. 5A). We then constructed a series of Oct4 promoter-driven luciferase reporter plasmids containing either full-length promoter, promoter with different length of deletions (D1-3), or promoter with mutations in the potential binding sites
Reporter assays showed that ARID3B induced the luciferase activity of full-length, D1, and D2 Oct4 promoter reporters, but not others (Fig. 5A). These data indicated that ARID3B positively regulated the promoter activity of Oct4 and that this regulation depended on a specific sequence within the Oct4 promoter. A chromatin immunoprecipitation (ChIP) assay showed that Oct4 promoters were coprecipitated with ARID3B. However, when the ARID3B-binding sequences were mutated or removed from Oct4 promoter, the coprecipitation was no longer observed (Fig. 5B). The association between ARID3B and Oct4 promoter was further confirmed by a qPCR of the ChIP assay (Fig. 5C). Moreover, an electrophoretic mobility shift assay (EMSA) further supported the binding of ARID3B to the specific regions of Oct4 promoters (Fig. 5D).

We previously reported that HMGA2 regulates the expression of Sox2 through a direct promoter binding, leading to enhanced CSC properties in glioblastoma cells (26). This mechanism has not been shown in OSCC cells, however. Using the same approaches, we confirmed that HMGA2 bound to and increased the activity of Sox2 promoter (Supplementary Fig. S4). Taken together, our results demonstrated a novel ARID3B-mediated Lin28B/Let7 effect on the regulation of Oct4 expression through a direct binding between ARID3B and Oct4 promoter.

The Lin28B–Let7 pathway regulates the stemness-driven reprogramming in normal oral keratinocytes and OSCC cells

Reprogramming process has become a method to evaluate the strength of subject factors in regulating ESC-stemness and self-renewing property (31). Yu and colleagues (32) reported that using a combination of Oct4, Sox2, Nanog, and Lin28 (OSNL) is able to reprogram human somatic cells into iPSC. Stem cells and cancer cells share similar properties such as self-

Figure 5. ARID3B regulated the promoter activity of Oct4 through direct promoter binding. A, schematic presentation of the constructed full-length, deleted, and mutated Oct4 promoter-driven reporter plasmids. The full-length Oct4 promoter contains three AT-hook regions. The reporter plasmids were cotransfected with Let7 in CD44++ ALDH1++ OSCC cells and the luciferase activity was assessed and is presented in the graph. B, ChIP analysis of Arid3B at Oct4 promoter region. Empty vector or ARID3B-overexpressing plasmid was cotransfected with Oct4 promoter constructs in CD44++ ALDH1++ OSCC cells and subjected to a ChIP assay using anti-ARID3B antibody. IgG antibody was used as a background control. The Northern blot of the ChIP assay is presented in the figure. C, the qPCR analysis of ChIP assay with four different amplification regions (NC and ARID3B RE1-3) is presented. Input, 2% of total lysate. D, EMSA and competition assays were performed using Arid3B, biotin-labeled, or unlabeled probes containing the Arid3B binding sequence of Oct4 promoter. Probes containing mutated Arid3B-binding site were used as negative control. Unlabeled probe were added at concentrations 10-fold (lane 3) or 50-fold (lane 4) greater than biotin-labeled probe. *, P < 0.05 versus full length.
Lin28/Let-7 Reprogramming OSCC into Stem-like State

renewal and the expression of stemness signature (33). Aberrant reprogramming and epigenetic alteration deregulate stemness properties and result in the induction of cancer stem-like properties and tumor development (34–36). Because Oct4 and Sox2 mediate the ESC/stemness-acquisition, we then aimed to investigate the roles of Lin28B–Let7 signaling in cellular reprogramming in NHOK primary cells (21) and OSCC cells using a reprogramming protocol as described in the Supplementary Data. In brief, iPSCs were reprogrammed from NHOK cells via the transduction of pMXs vectors encoding the transcription factors Oct4, Sox2, Klf4, and c-Myc using a virus infection system (23). On day 7 of after infection, target cells were passaged onto mitotically inactivated MEF feeder layers, and cultured using human ESC medium. The iPSC cells were passaged onto mitotically inactivated MEF feeder cells were reprogrammed from NHOK primary cells (21) and OSCC cells using a reprogramming protocol as described in the Supplementary Data. In brief, iPSCs were reprogrammed from NHOK cells via the transduction of pMXs vectors encoding the transcription factors Oct4, Sox2, Klf4, and c-Myc using a virus infection system (23). On day 7 of after infection, target cells were passaged onto mitotically inactivated MEF feeder layers, and cultured using human ESC medium. The iPSC colony formation was aided by adding SB431542 (2 μmol/L; Stemgent), PD0325901 (0.5 μmol/L; Stemgent), and thiazovivin (0.5 μmol/L; ref. 25). The iPSCs were later transferred to feeder-free/serum-free culture system (37). First, we executed a reprogramming protocol (23) on NHOK with OSN (Oct4+/Sox2+/Nanog), OSN+/Lin28B, and OSN+/Spg-Let7, based on Yu and colleagues’ protocol (32), and compared the reprogramming efficiency (Fig. 6A). As expected, the OSN+/Lin28B displayed the best reprogramming efficiency, while OSN failed to reprogram NHOK without Lin28B. To our interest, Spg-Let7 had the same effect as Lin28B in cooperating with OSN to reprogram NHOK. Co-overexpression of Let7 in all the three reprogramming experiments dramatically reduced the iPSC colonies. These results suggested that suppression of Let7 is a crucial checkpoint for NHOK reprogramming. We further examined the role of the Lin28B–Let7 pathway mediated reprogramming efficiency of NHOK cells. A. NHOK cells were subjected to reprogramming procedure using Oct4+/Sox2+/Nanog (OSN), OSN+/Lin28B, or OSN+/Spg-Let7 with or without exogenous Let7. The iPSC colonies were stained with alkaline phosphate and photographed (top). The quantification of iPSC colony number is presented at the bottom. B, a Oct4+/Sox2+/Klf4+/c-Myc (OSKM) reprogramming protocol was performed in NHOK in the presence or absence of shLin28B and shLin28B+/Spg-Let7. The iPSC colonies were stained and photographed (top). The number of colonies was calculated and is presented as relative value in the graph (bottom). C, the iPSC colonies from B were analyzed by Western blot analysis. D, bisulfite sequencing analysis of Oct4 and Nanog promoter regions in NHOK, NHOK-iPSC, CD44+/ALDH1+/Ctrl, and CD44+/ALDH1+/Lin28B cells. E, quantitative PCR analysis of Lin28B and Let7 levels in NHOK, NHOK-iPSC, CD44+/ALDH1+/Ctrl, and CD44+/ALDH1+/Lin28B cells. F, CD44+/ALDH1+/shCtrl, CD44+/ALDH1+/shLin28B, CD44+/ALDH1+/Ctrl, and CD44+/ALDH1+/Lin28B cells were subjected to a SL-PCR analysis to assess the changes of Let7 family (including Let7a, Let7b, Let7c, Let7d, Let7f, and Let7g) expression level. miR98, a miRNA with a similar sequence to Let7, was used as a control to show the distinguishability of the method.

Figure 6. The Lin28B–Let7 pathway mediated reprogramming efficiency of NHOK cells. A. NHOK cells were subjected to reprogramming procedure using Oct4+/Sox2+/Nanog (OSN), OSN+/Lin28B, or OSN+/Spg-Let7 with or without exogenous Let7. The iPSC colonies were stained with alkaline phosphate and photographed (top). The quantification of iPSC colony number is presented at the bottom. B, a Oct4+/Sox2+/Klf4+/c-Myc (OSKM) reprogramming protocol was performed in NHOK in the presence or absence of shLin28B and shLin28B+/Spg-Let7. The iPSC colonies were stained and photographed (top). The number of colonies was calculated and is presented as relative value in the graph (bottom). C, the iPSC colonies from B were analyzed by Western blot analysis. D, bisulfite sequencing analysis of Oct4 and Nanog promoter regions in NHOK, NHOK-iPSC, CD44+/ALDH1+/Ctrl, and CD44+/ALDH1+/Lin28B cells. E, quantitative PCR analysis of Lin28B and Let7 levels in NHOK, NHOK-iPSC, CD44+/ALDH1+/Ctrl, and CD44+/ALDH1+/Lin28B cells. F, CD44+/ALDH1+/shCtrl, CD44+/ALDH1+/shLin28B, CD44+/ALDH1+/Ctrl, and CD44+/ALDH1+/Lin28B cells were subjected to a SL-PCR analysis to assess the changes of Let7 family (including Let7a, Let7b, Let7c, Let7d, Let7f, and Let7g) expression level. miR98, a miRNA with a similar sequence to Let7, was used as a control to show the distinguishability of the method.
pathway in Yamanaka 4-gene (OSKM; Oct4-Sox2-Klf4-cMyc) reprogramming protocol (38) and showed that knockdown of Lin28B hampered the reprogramming efficiency as well as the protein expression levels of OSKM, while unchanged knockdown of Let7 partially recovered it (Fig. 6B and C).

Altering of DNA methylation pattern of stemness genes is an important epigenetic hallmark as well as a crucial barrier for cell reprogramming (39). We compared the bisulfite sequencing results of Oct4 and Nanog promoters in NHOK and OSNL-driven HNOK-iPSCs, and showed that the Oct4 and Nanog promoters were dramatically demethylated in NHOK-iPSC, compared with NHOK cells (Fig. 6D). Moreover, overexpression of Lin28B in CD44-ALDH1 OSCC cells also partially demethylated Oct4 promoter but had little effect on Nanog promoter, compared with nontransfected CD44-ALDH1 OSCC cells (Fig. 6D). This may indicate an incomplete reprogramming in OSCC cells, contributing the switch from non-CSC to CSC state. Moreover, the results of qPCR analysis in Fig. 6E confirmed a pattern of high expression level of Lin28B and low expression level of Let7 in the NHOK-iPSC and CD44-ALDH1/Lin28B. In addition, let7 family members have recently been shown to regulate cancer progression and self-renewal in stem cells (8, 40, 41). To further explore whether Lin28 could precisely regulate the endogenous expression of Let7 family in OSCC, the size-coded ligation-mediated PCR (SL-PCR), a method for detecting multiple miRNAs simultaneously in total RNA (42), was used in CD44-ALDH1- and CD44-ALDH1- OSCC cells. The SL-PCR data confirmed the downregulation of endogenous Let7 family upon Lin28B overexpression in CD44-ALDH1 cells, while knockdown of Lin28B in CD44-ALDH1 cells elevated the endogenous Let7 family (Fig. 6F). Taken together, these results strongly suggested that Lin28B/Let7 signaling plays a crucial role in cell reprogramming and regulates the acquisition of ESC-stemness properties both in NHOK and OSCC cells.

Clinical significance and potential application of the Lin28B-Let7-Arid3b/Hmga2-Oct4/Sox2 signaling in OSCC patients

To evaluate whether Lin28B reprograms the tumorigenesis properties in OSCC, we preinjected GFP-labeled CD44-ALDH1- /Lin28B and CD44-ALDH1- /Spp-let7 cells transfected with shArid3b or shArid3b/shHmga2 in immune-compromised mice. Through monitoring GFP signal, we found that overexpressed Lin28B or knockdown of Let7 increased the tumor volume of CD44-ALDH1 cells, whereas co-knockdown of ARID3B and HMGA2 decreased it (Fig. 7A and B). Moreover, the tumor growth of highly tumorigenic CD44-ALDH1 cells was suppressed by shLin28B but rescued by co-overexpression of ARID3B and HMGA2 (Fig. 7C, top). The overexpression ARID3B and HMGA2 plasmids used in this study does not contain 3'-UTR. Therefore, the overexpressed HMGA2 and ARID3B would not be suppressed by endogenous or exogenous Let7. Overexpressing Let7 in CD44-ALDH1 cells was able to suppress their tumor growth, but with co-overexpression of ARID3B and HMGA2, the Let7-suppressed tumor growth was recovered (Fig. 7C, bottom). These tumor growth rates were in line with the expression levels of Oct4 and Sox2 in the tumor sections (Fig. 7D). Notably, our therapeutic experiments indicated that delivery of synthetic hsa-Let7 may possess therapeutic potential in OSCC patients (Supplementary Fig. S5). These data showed that Lin28B, as well as its downstream effectors ARID3B, were able to increase the tumorigenicity in non-CSC OSCC cells, and Oct4 and Sox2 are important downstream mediators of Lin28B/Let7/ARID3B/HMGA2 in regulating OSCC tumorigenesis.

In OSCC clinical specimens, IHC analysis further showed that patient samples expressing high level of Lin28B also expressed ARID3B, HMGA2, Oct4, and Sox2 proteins (Fig. 7E) but low level of Let7 (Fig. 7F). Analysis on a cohort of clinical specimens revealed that more than 78% of Lin28B-high/Let7-low patients expressed high level of ARID3B, HMGA2, Oct4, and Sox2; while only less than 22% expressed low levels of these molecules (Fig. 7G). Collectively, these data indicate a clinical correlation between Lin28B-high, Let7-low, ARID3B-high, Oct4-high, HMGA2-high, and Sox2-high expression in OSCC patients, supporting the clinical importance of Lin28B-Let7-ARID3B/HMGA2-Oct4/Sox2 signaling in OSCC patients.

Discussion

CSCs share numerous features with normal stem cells, including hallmark properties, such as self-renewal and undifferentiated state, and CSCs were suggested to be arisen from non-stem-like cancer cells through a reprogramming mechanism very similar to the generation of iPSC (5). Both iPSC and CSC present unlimited proliferation and self-renewing activities (43), and share similar characteristics of cell metabolism. Moreover, many of the key transcription factors, such as Oct4 and Sox2, mediating the induction of iPSC and the maintenance of stemness phenotype in embryonic and somatic stem cells are also overexpressed in CSC (4, 18, 44). Recently, Ohnishi and colleagues (5) demonstrated that premature termination of the reprogramming process leads to cancer development in various tissues through altered epigenetic regulation. We believe that the premature reprogramming is highly related to the teratoma formation of iPSC, as well as the development of CSC. In this study, we found a correlation of high Lin28B level and low Let7 level in CSC-like OSCC cells. Furthermore, we demonstrated that the Lin28B-Let7 pathway regulates cancer stemness in OSCC and pluripotency in NHOK through mediating Oct4 and Sox2 expression. The Lin28B-Let7-dependent regulation of Oct4 and Sox2 provides further insights into the complex stemness regulatory networks. Further investigation may reveal its roles in regulating key stemness pathways during reprogramming, differentiation, and tumorigenesis.

We identified ARID3B as a novel downstream target of Let7. ARID3B is involved in survival of neural crest during embryogenesis and is expressed in a portion of neuroblastoma (45–47); it synergized with Mycn to control ESC proliferation (48). Recent studies indicated that ARID3B participates in tumor development and is highly expressed in differentiated layers of squamous epithelium in malignant tissues (49). We revealed a direct binding of Let7 in the 3'-UTR region of ARID3B mRNA, resulting in suppressed ARID3B expression. ARID3B directly binds to Oct4 promoter, confirmed by ChIP assay, and subsequently enhanced the mRNA and protein expression of Oct4. In response to Lin28B expression, suppression of Let7 allows ARID3B binding to a specific sequence in Oct4 promoter and promote its transcription. Our data also confirmed that Let7 directly targets the 3'-UTR of HMGA2, which upregulates Sox2 expression through promoter binding in response to Lin28B overexpression or Let7 suppression. The Let7-suppressed tumor-initiation and self-renewal...
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The pathologic reprogramming process in cancer has been shown to be critical in modulating the CSC induction and cancer recurrence (39, 50). Premature reprogramming was demonstrated to be leading to cancer development through altering epigenetic signatures (5). In our data, the Lin28B–Let7 pathway not only mediates cancer stemness, but also regulates cellular reprogramming. The Lin28B–Let7 pathway interferes with reprogramming efficiency in NHOK-derived iPSCs through regulating Oct4 and Sox2 expression. Modulating the Lin28B/Let7 signaling altered the gene expression signature and pluripotency of iPSCs, methylation patterns of Oct4 and Nanog, and shifted the gene expression pattern between the dynamic change of stem-like and non-stem-like states in both NHOK and OSCC cells. Through demethylating Oct4 and Nanog promoters, Lin28B-mediated pathway enhanced reprogramming efficiency of NHOK. It has to be noted that the effect of Lin28B on Nanog promoter demethylation seemed very little in

Figure 7.
Clinical significance of the Lin28B–Let7–ARID3B–HMGA2–Oct4–Sox2 pathway in OSCC. A and B, immunocompromised mice were transplanted in the neck subcutaneous region with 10^7 cells of GFP-labeled CD44^+ALDH1^+ cells stably transfected with Lin28B, shARID3B, shHMGA2, or Spg-Let7 as indicated. The xenograft tumors were incubated for 6 weeks and the tumor size was visualized by GFP signal and quantified by a caliper. C and D, an in vivo tumor growth assay was performed with stable CD44^+ALDH1^+ cell lines as indicated in immunocompromised mice (mean tumor volume cm^3 ± SEM). The tumor growth was monitored up to 6 weeks, and the tumor sections were analyzed by qPCR for their Oct4 and Sox2 expression. E, patient specimens expressing high level of Lin28B were analyzed by IHC staining to assess the protein expression of ARID3B, HMGA2, Oct4, Sox2, and Lin28B. One representative patient specimen is presented. F, a qPCR analysis of Let7 mRNA levels in a cohort of Lin28B-high and Lin28B-low patient samples. G, IHC analysis of 46 Lin28B-high/Let7-low and 44 Lin28B-low/Let7-high OSCC patient samples for their expression of HMGA2, ARID3B, Oct4, and Sox2. The negative and positive expression of each of the four molecules were calculated and statistically correlated in the two groups of patient samples. H, schematic presentation of the proposed Lin28B–Let7–ARID3B–HMGA2–Oct4–Sox2 pathway in the current study.

capability was rescued by overexpression of ARID3B and HMGA2, which also rescued the suppressed Oct4 and Sox2, respectively. We believe that in OSCC, Lin28B upregulates stemness transcription factors, such as Oct4 and Sox2, as well as self-renewal and tumor-initiation ability, through increased ARID3B and HMGA2. Clinical specimens revealed a significant correlation between Lin28B-high, Let7-low, ARID3B-high, Oct4-high, HMGA2-high, and Sox2-high in OSCC patients. Most importantly, therapeutic delivery of Let-7-mimics in orthotopic-transplanted NOD/SCID mice reduced the tumor growth (Supplementary Fig. S5). Collectively, these results demonstrate a Lin28B/Let7-driven ARID3B/Oct4 signaling pathway that activates the stemness-derived tumor initiation and malignance during oral cancer formation.
OSCC cells, implying a partial or premature reprogramming. The role of Lin28B/Let-7 signaling in stemness pathway and pluripotency regulation during cell reprogramming echoes with its role in cancer stemness, and it would be interesting to investigate the demethylation mechanism of stemness gene promoters in the Lin28B/Let-7-modulated reprogramming process.

Conclusively, we reported a Lin28B–Let-7 pathway in OSCC through ARID3B/HMGAA2–mediated Oct4/Sox2 signaling, this pathway enhanced the ESC-stemness properties, tumor initiation, and malignance of OSCC. This study would greatly contribute to a deeper understanding of cancer reprogramming and ESC-stemness acquisition in OSCC, and promote the development of effective therapies for OSCC eradication.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Lin28/Let-7 Reprogramming OSCC into Stem-like State

Lin28B/Let-7 Regulates Expression of Oct4 and Sox2 and Reprograms Oral Squamous Cell Carcinoma Cells to a Stem-like State

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