Published in final edited form as:

Oncogene. 2014 August 21; 33(34): 4296–4306. doi:10.1038/onc.2013.385.

MiR-26a enhances miRNA biogenesis by targeting Lin28B and Zcchc11 to suppress tumor growth and metastasis

Xianghui Fu¹, Zhipeng Meng^{1,2}, Wei Liang³, Yan Tian³, Xichun Wang¹, Weidong Han¹, Guiyu Lou¹, Xiaoqiong Wang¹, Fang Lou¹, Yun Yen^{2,4}, Hua Yu^{2,5}, Richard Jove^{2,3}, and Wendong Huang^{1,2,*}

¹Division of Molecular Diabetes Research, Department of Diabetes and Metabolic Diseases Research, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA

² Irell & Manella Graduate School of Biological Sciences, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA

³Department of Molecular Medicine, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA

⁴Department of Molecular Pharmacology, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA

⁵Department of Cancer Immunotherapy and Tumor Immunology, Beckman Research Institute, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA

Abstract

Human cancers often exhibit attenuated miRNA biogenesis and global underexpression of miRNAs, thus targeting the miRNA biogenesis pathway represents a novel strategy for cancer therapy. Here, we report that miR-26a enhances miRNA biogenesis, which acts as a common mechanism partially accounting for miR-26a function in diverse cancers, including melanoma, prostate and liver cancer. miR-26a was broadly reduced in multiple cancers and overexpression of miR-26a significantly suppressed tumor growth and metastasis both *in vitro* and *in vivo*, including melanoma, prostate and liver cancer. Notably, miR-26a overexpression was accompanied by global upregulation of miRNAs, especially let-7, and let-7 expression was concordant with miR-26a expression in cancer cell lines, xenograft tumors and normal human tissues, underscoring their biologic relevance. We showed that miR-26a directly targeted Lin28B and Zcchc11, two critical repressors of let-7 maturation. Furthermore, we have demonstrated that Zcchc11 promoted tumor growth and metastasis and it was prominently overexpressed in human cancers. Our findings thus provide a novel mechanism by which a miRNA acts as a modulator of miRNA biogenesis. These results also define a role of the miR-26a and Zcchc11 in tumorigenesis and metastasis, and have implications to develop new strategies for cancer therapy.

*To whom correspondence should be addressed: Wendong Huang, Department of Diabetes and Metabolic Diseases Research, Beckman Research Institute, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA. ; Email: whuang@coh.org.

Conflict of interest

The authors declare no conflict of interest.

Keywords

miR-26a; Zcchc11; let-7; miRNA biogenesis; tumorigenesis; metastasis

Introduction

microRNAs (miRNAs) are often deregulated in human malignancies and can function as either oncogenes or tumor suppressor genes;^{1,2} therefore, they are considered emerging “hallmarks” of cancer.³ Notably, human cancers often exhibit attenuated miRNA biogenesis and global underexpression of miRNAs,^{4,5} highlighting the importance of miRNA biogenesis during the development of cancer. Indeed, critical components of miRNA biogenesis, including Dicer 1, Drosha, TARBP2 and XPO5, have been identified as haploinsufficient tumor suppressors.⁶⁻¹⁰ Therefore, there is considerable interest in understanding the mechanisms of miRNA biogenesis and it is thought that targeting the miRNA biogenesis pathway may lead to the development of novel cancer therapies.¹¹⁻¹⁴

miRNA biogenesis consists of a series of biochemical steps and disruption of any step could influence miRNA abundance and contribute to tumorigenesis. The best-studied repressor of miRNA biogenesis is Lin28, an RNA-binding protein initially identified in *Caenorhabditis elegans*, which has crucial and widespread roles in development and diseases.¹⁵⁻¹⁹ These roles are enacted at least partially through blocking the maturation of let-7,²⁰⁻²² which is one of the most abundant miRNA families in mammals, and members of this family function as tumor suppressors by targeting multiple oncogenes, including RAS, HMGA2, and c-Myc.²³⁻²⁶ Mammals have two Lin28 homologs, Lin28A (also known as Lin28) and Lin28B, which have similar suppressive roles in let-7 biogenesis.²⁷⁻³⁰ Given that the importance of the Lin28/let-7 regulatory circuit in development, pluripotency, tumorigenesis, this circuit provides novel therapeutic opportunities for cancer.²² However, the mechanisms controlling the Lin28/let-7 circuit remain largely unknown.

In the studies described herein we discovered that miR-26a directly targets Zcchc11 and Lin28B, thereby disrupting the Lin28/let-7 circuit, enhancing miRNA biogenesis, and consequently inhibiting tumorigenesis and metastasis of several cancers, including melanoma, prostate and liver cancer. We also defined Zcchc11 as an oncogene for the development of these cancers and established a direct link between Zcchc11 and human liver and colorectal cancers. These findings not only provide novel insights into the mechanism by which miRNAs mediate tumor suppression, but also offer new targets for cancer treatments.

Results

Downregulation of miR-26a in multiple cancer types

Previously, we identified miR-26a, but not miR-26b, as one of several miRNAs that are highly expressed in mouse liver.³¹ Here we sought to further determine the expression of miR-26a in human tissues. QRT-PCR analysis revealed that miR-26a was universally expressed at high-levels in normal human tissues (Figure 1a). Moreover, expression of

miR-26a was dramatically decreased in human cancer cell lines when compared to the corresponding normal tissues/cell lines (Figure 1b). Then we determined miR-26a function in multiple cancer cell lines of diverse origins, including melanoma (A375, A2058, SK-MEL-5, and SK-MEL-28), prostate liver (DU145, LNCAP, and PC-3), and liver cancer (HepG2, Huh 7, and SK-HEP-1). miR-26a overexpression, driven by transfection with its miRNA precursor (pre-26a), modestly, but significantly inhibited cell proliferation, whereas knockdown of miR-26a expression by an antisense oligonucleotide (anti-26a) enhanced cell proliferation (Figure 1c and 1d; Supplementary Figure 1). These modest effects on tumor cell growth may result from the short-term treatment of miR-26a overexpression/knockdown.

As another approach, we introduced miR-26a to three cancer cell lines—A375, DU145 and SK-HEP-1—through a retroviral vector MDH-PGK-eGFP 2.0 (henceforth referred to as MPG) with an insertion of a human miR-26a precursor sequence.³² This approach led to 4-6 fold increase of miR-26a in these three cell lines (Figure 1e). We found that this moderate upregulation of miR-26a was sufficient to suppress tumor cell proliferation (Figure 1f) and to decrease the efficiency of colony formation in all three cell lines (Figure 1g). Taken together, these results suggest that miR-26a has a broad role in suppressing the growth and transformation of multiple cancers, namely melanoma, prostate and liver cancer.

miR-26a inhibits the growth and metastasis of human tumor xenografts

To determine the potential roles of miR-26a in tumor growth and metastasis simultaneously *in vivo*, we used a modified mouse xenograft system in which fewer cancer cells than are used in the classic xenograft system were injected subcutaneously into NOD-SCID mice and sufficient time allowed for spontaneous metastases to grow.³³ We injected NOD-SCID mice with tumor cells (5×10^5) of diverse origins (melanoma [A375], prostate [DU145] and liver cancers [SK-HEP-1]) transduced with either the MPG control vector or MPG-miR-26a expression vector. Tumor growth was monitored regularly and spontaneous metastases in liver and lung were examined when mice were euthanized. miR-26a-overexpressing tumors were smaller and weighed less than control tumors (Figure 2a-f). A375 tumor growth was very rapid (Figure 2a) and no metastasis could be observed in either livers or lungs of xenograft mice (Supplementary Figure S2a-b). In contrast, DU145 tumor growth was slowly (Figure 2c), and a few tiny metastases were emerged in lung of 50% control mice, but not in mice injected with miR-26a-overexpressing cells, as evidenced by H&E staining (Supplementary Figure S2c-f), suggesting a role for miR-26a in tumor metastasis.

The probable function of miR-26a in tumor growth and metastasis was further supported by analysis of SK-HEP-1-derived tumors. miR-26a overexpression not only significantly suppressed the growth of SK-HEP-1-derived tumors (Figure 2e and 2f), but also almost completely blocked spontaneous metastasis in both the liver and lung (Figure 2g-f; Supplementary Figure S1g-j). All control mice had many visible metastases in their livers and lungs (Figure 2g and 2h). However, only 17% of mice injected with miR-26a-overexpressing cells displayed metastases, and these metastases were less frequent and much smaller (Figure 2h). In line with pulmonary metastatic burden, mice injected with miR-26a-overexpressing cells maintained an almost normal lung:body weight ratio, compared to a

two-fold increase of the ratio in control mice (Figure 2h). Concordantly, cell proliferation, as measured by staining for proliferating cell nuclear antigen (PCNA), was much greater in the livers and lungs of control mice than in mice injected with miR-26a-overexpressing SK-HEP-1 cells (Figure 2i and 2j).

The above-described xenograft system was unable to generate spontaneous metastases using A375 melanoma cells because of their extremely rapid growth, we therefore determined the effects of miR-26a in melanoma metastasis using an experimental metastasis model. We injected NOD-SCID mice, via the tail vein, with A375 cells (1×10^6) transduced with either MPG-miR-26a expression or MPG control vector and evaluated metastasis 3 weeks after the injection. Melanomatic metastatic lesions, which consisted of solid white masses, were detected in the livers and lungs of all mice. However, control mice had much more lung and liver metastases than mice that received miR-26a-overexpressing cells (Figure 2k and 2l). Collectively, these results demonstrate that miR-26a represses the growth and metastasis of multiple cancers, including melanoma, liver and prostate cancer *in vivo*.

miR-26a significantly upregulates let-7 expression in human tumor xenografts

Strikingly, expression of many miRNAs, especially members of the let-7 family, was significantly greater in miR-26a-overexpressing A375 tumors than in control tumors (Figure 3a and Supplementary Figure S3a). This led us to profile the expression of various miRNAs in three melanoma cell lines (A2058, A375, and SK-MEL-5) transduced with either the MPG control vector or MPG-miR-26a expression vector. We found that the expression of these miRNAs was also increased in miR-26a-overexpressing melanoma cells (Figure 3b and Supplementary Figure S3b). As global underexpression of miRNAs is a common feature of human cancers, we hypothesized that the global increase in miRNA expression induced by miR-26a overexpression may be a common mechanism that partially accounts for the effects of miR-26a in the development of tumors of diverse origins.

Given that let-7 is a potent tumor suppressor in various cancers and its expression was consistently upregulated by miR-26a in melanoma tumors and cell lines, we focused our attention on this family and found that let-7 was also upregulated in both DU145 and SK-HEP-1 miR-26a-overexpressing tumors relative to control tumors (Figure 3c and 3d). We next correlated the let-7 level with the miR-26a level by using a panel of 20 diverse normal human tissues. There was a strong positive correlation between let-7d and miR-26a levels (Figure 3e, left panel, $R^2 = 0.5372$, $p = 0.0002$), as well as let-7f and miR-26a levels (Figure 3e, right panel, $R^2 = 0.3795$, $p = 0.0038$), further underscoring their biologic relevance.

Next, we examined the contribution of let-7 in miR-26a-mediated inhibition on tumor growth *in vitro*. To this end, we transfected miR-26a overexpressing A375, DU145 and SK-HEP-1 cells by the let-7 family inhibitor (designated as let-7 FI), which can inhibit the expression of all let-7 family members. We found that inhibition of let-7 in these cells reversed the decreased growth caused by miR-26a overexpression (Figure 3f and 3g), indicating that the tumor suppressor effects of miR-26a are mediated, at least partially, through regulation of let-7.

Let-7 expression can be regulated at either the transcriptional or posttranscriptional levels. To clarify this, we assessed the expression of pri-let-7 in xenograft tumors. The expression of pri-let-7-a/f/d and pri-let-7g was unchanged obviously between miR-26a-overexpressing tumors and control tumors (Figure 3h), supporting a posttranscriptional regulation. As a control, the levels of mature miR-16a and pri-miR-16a exhibited no significant changes (Figure 3a-d and 3h). Collectively, these data indicate that miR-26a overexpression leads to the upregulation of let-7, which may function as a common mechanism underlying the inhibitory effect of miR-26a in tumorigenesis and metastasis.

Lin28B and Zcchc11 are directly targeted by miR-26a

Let-7 biogenesis could be posttranscriptionally regulated by Lin28 and Zcchc11 (zinc finger CCHC domain-containing 11, also known as TUT4),^{27,30} we thus pursued the potential relationship between Lin28/Zcchc11 and miR-26a. Interestingly, both human Lin28B and Zcchc11 are predicted as targets of miR-26a by public algorithms (TargetScan)³⁴ (Supplementary Figure S3). To test it directly, we cloned the 3'UTRs of Lin28B and Zcchc11 into the psiCheck2.2 luciferase expression vector and examined luciferase activity upon miR-26a overexpression. Ectopic expression of miR-26a significantly suppressed the luciferase activity of wild-type Lin28B and Zcchc11 3' UTR, but not their mutant 3' UTR, indicating that miR-26a suppress Lin28B and Zcchc11 expression through direct binding to their 3' UTR (Figure 4a). miR-26a overexpression inhibited the mRNA expression of both Lin28B and Zcchc11 in HEK293 cells (Figure 4b). Moreover, miR-26a overexpression inhibited the endogenous expression of both Lin28B and Zcchc11 protein in cancer cell lines (Figure 4c) and xenograft tumors (Figure 4d). Collectively, these data demonstrate that Lin28B and Zcchc11 are direct targets of miR-26a.

Zcchc11 promotes tumorigenesis and metastasis

The importance of Lin28B in tumorigenesis has been extensively investigated, but the role of Zcchc11 in cancer development remains poorly understood.^{35,36} Moreover, direct evidence linking Zcchc11 to human cancers has not yet been provided. We therefore focused on the function of Zcchc11 in tumorigenesis.

Initially, we measured the expression of Zcchc11 in various cancer cell lines. QRT-PCR and immunoblot analysis showed that Zcchc11 was broadly expressed (Figure 4e and 4f). Then we introduced specific siRNA against Zcchc11 into three cancer cell lines—A375, DU145 and SK-HEP-1 and found that Zcchc11 depletion impaired tumor cell growth (Figure 5a and Supplementary Figure S5a). As another approach, we designed 4 short hairpin RNAs (shRNAs) against the human Zcchc11 gene and initially determined their efficiency in HEK293T cells (Supplementary Figure S5b). We then established A375, DU145 and SK-HEP-1 cell lines with stable Zcchc11 depletion by using two of these shRNAs, sh-Zcc3 and sh-Zcc4 (Supplementary Figure S5c), and evaluated common aspects of tumorigenesis and transformation in these cells. Zcchc11 depletion repressed proliferation (Figure 5b) and colony formation (Figure 5c-5e) of A375, DU145 and SK-HEP1 cells. Transwell invasion assays demonstrated that Zcchc11 depletion and miR-26a overexpression strongly suppressed the invasive capacity of SK-HEP-1 cells (Figure 5f and Supplementary Figure

S5d). Taken together, these data suggest that Zcchc11 promotes tumorigenesis and transformation *in vitro*.

Knockdown of Zcchc11 represses the growth and metastasis of human liver tumor xenograft

As shown above, the SK-HEP-1 xenograft model is excellent for examining tumor growth and metastasis simultaneously. Therefore, we assessed the effect of Zcchc11 downregulation in this model and found that mice bearing Zcchc11-depleting SK-HEP-1 xenografts had smaller tumors that weighed less than those of control mice (Figure 5g). Notably, all control mice had many visible metastases in the livers and lungs, whereas only 25% and 37.5% mice injected with Zcchc11-depleted cells had liver and lung metastases, respectively, and these metastases were less frequent and much smaller (Figure 5h and 5i; Supplementary Figure S5e and 5f). In line with pulmonary metastatic burden, mice injected with Zcchc11-depleted cells exhibited significant decrease of lung:body weight ratio compared to control mice (Figure 5i). Furthermore, tumors of control mice had a high-level of cellular proliferation, as measured by PCNA staining, in livers and lungs relative to mice injected with Zcchc11-depleted cells (Figure 5j and 5k). Taken together, these data demonstrate that Zcchc11 depletion has similar effects as miR-26a overexpression and suggest that Zcchc11 promotes tumorigenesis and metastasis *in vivo*.

Overexpression of miR-26a inhibits Lin28B and Zcchc11 in human tumor xenografts

As shown in Figure 4, miR-26a directly targets Lin28B and Zcchc11. To further confirm this regulation *in vivo*, we assessed the expression of Lin28B and Zcchc11 by immunohistochemistry (IHC) in A375, DU145 and SK-HEP-1 tumor xenografts. This analysis revealed decreased Lin28B and Zcchc11 staining in miR-26a-overexpressing tumors as compared with control tumors (Figure 6a-c), consistent with an inhibitory role of miR-26a on their expression. Of note, IHC analysis also showed that Lin28B was primarily localized in the nucleus, whereas Zcchc11 was predominantly localized in the cytoplasm. These results demonstrate that miR-26a represses Lin28B and Zcchc11 in the development of cancers *in vivo*.

We also performed IHC analysis on Zcchc11-depleting SK-HEP-1 tumor xenografts. As expected, Zcchc11-depleting tumors had much lower Zcchc11 staining than those of control mice (Figure 6d). In line with reduced Zcchc11 staining, Zcchc11-depleting tumors exhibited decreased Lin28B staining (Figure 6d), probably resulting from an indirect effect of Zcchc11 inhibition. For example, reduced Zcchc11 leads to increased activities of let-7 and miR-26a, thereby decreasing Lin28B expression.

The effects of miR-26a and Zcchc11 on tumor cell proliferation and apoptosis

To explore whether the slowing of tumor growth induced by miR-26a overexpression was due to impaired cell proliferation, increased apoptotic cell death, or both, we used immunohistochemical analysis to evaluate tumor cell proliferation and apoptosis in xenografts (A375, DU145, and SK-HEP-1). For all xenografts, the percentage of PCNA-positive cells was significantly lower in miR-26a-overexpressing tumors compared with control tumors (Figure 6a-c). In contrast, miR-26a-overexpressing tumors exhibited

considerably more apoptotic bodies, as determined by TUNEL assay, than did control tumors (Figure 6a-c). As a direct target of miR-26a, Zcchc11 was speculated to have opposite effects on tumor cell proliferation and apoptosis. Consistent with this idea, tumors derived from Zcchc11-depleted SK-HEP-1 cells exhibited reduced tumor cell proliferation and increased apoptosis compared with control tumors (Figure 6d). These results demonstrate the opposing effects of miR-26a and Zcchc11 on tumorigenesis and indicate that this effect is associated with tumor cell proliferation and apoptosis.

Upregulation of Zcchc11 in human liver and colorectal cancers

To determine the relevance of our experimental findings on Zcchc11 to human cancer patients, we examined Zcchc11 expression in primary human cancers. QRT-PCR analysis revealed that the expression of Zcchc11 was increased at least 1.5 fold in 65% liver tumors (6 out of 9 paired hepatocellular carcinoma [HCC] samples) (Figure 7a). We further assessed the expression of Zcchc11 by IHC in another liver cancer cohort consisting of 9 paired samples of liver cancers with normal tissue. This analysis revealed elevated Zcchc11 staining in 7 out of 9 tumor tissues as compared with adjacent non-tumor tissues (Figure 7b). We also evaluated Zcchc11 expression in 10 paired samples of colorectal cancers with normal tissue through IHC, and found that Zcchc11 was significantly upregulated in 8 out of 10 tumor tissues compared with adjacent non-tumor colon tissues (Figure 7c). Notably, IHC analysis also showed that Zcchc11 was predominantly localized in the cytoplasm (Figure 7b and 7c), consistent with our IHC analysis on xenografts and previous reports.^{27,35} Together, these data indicate Zcchc11 has an oncogenic role in human liver and colorectal cancer, consistent with the results obtained from *in vitro* experiments.

Discussion

miRNAs have been identified as key regulators in tumorigenesis and several stages of metastasis.^{14,37} Previous studies have demonstrated the suppressive role of miR-26a in tumor growth.³⁸⁻⁴¹ Our study has expanded these findings by revealing a specific function of miR-26a in metastasis. That is, miR-26a not only inhibits tumorigenesis, but also strongly prevents spontaneous and distant metastasis, indicating that miR-26a is involved in the development of both primary tumors and metastatic disease, at least including melanoma, prostate and liver cancer. This expansion of miR-26a function to metastasis is of clinical importance, as metastasis is responsible for more than 90% of cancer-associated mortality. Of note, miR-26a is universally expressed at high-level in human tissues. Taken together, these data suggests that miR-26a represents a potential therapeutic target to treat both tumor growth and metastasis.

Here we show that overexpression of miR-26a results in increased expression of let-7 and knockdown of let-7 reverses the inhibitory effects of miR-26a overexpression in tumor cell growth. Moreover, let-7 expression is concordant with miR-26a expression in normal human tissues, cancer cell lines, and human xenograft tumors, underscoring coordinated regulation of these two miRNAs. Let-7 has been regarded as a bone fide tumor suppressor and accumulating evidence demonstrates its crucial roles in the development of cancer. For example, let-7 targets multiple oncogenes, including RAS, HMGA2, and c-Myc. Recently,

let-7 has been shown to act in a metastasis-signaling cascade involving the RAF kinase inhibitory protein.^{42,43} Thus, miR-26a-induced let-7 provides a conserved mechanism for the suppressive role of miR-26a during tumorigenesis and metastasis of diverse cancers. However, it is likely that miR-26a also directly represses distinct oncogenes within different cancer context,^{38,41} which contributes to its function.

Mechanistically, miR-26a directly targets Lin28B and Zcchc11, which are critical repressors of the maturation of miRNAs, particularly let-7.^{21,22} Previous studies have defined a regulatory loop consisting of Lin28 and let-7, in which Lin28A/Lin28B suppress let-7 maturation, while let-7 directly targets them. Recently, it has shown that Lin28A is predominantly cytoplasmic and recruits Zcchc11 to pre-let-7 to induce 3'-uridylation, thereby blocking Dicer cleavage and let-7 maturation. Unlike Lin28A, Lin28B localizes primarily to the nucleus and blocks let-7 maturation by binding to pri-let-7 and interfering with Drosha processing through a Zcchc11-dependent mechanism. Our finding that miR-26a targets both Lin28B and Zcchc11 suggests that miR-26a may modulate Lin28/let-7 regulatory loop through either Zcchc11-dependent (Lin28A) or Zcchc11-independent (Lin28B) mechanism.

Compared to Lin28B, much less is known about the function of Zcchc11 in cancer development^{35,36}. Piskounova et al. suggested that Zcchc11 enhances the tumorigenicity and invasiveness of Lin28A-expressing cancer cells, but has no effect on these properties in Lin28B-expressing cancer cell lines,³⁵ indicating that the function of Zcchc11 in tumorigenesis depends on its uridylyltransferase activity for let-7 maturation. In contrast, Blahna et al. showed that Zcchc11 promotes proliferation of lung cancer cells independent of its uridylyltransferase activity.³⁶ Here we provide several lines of evidence to demonstrate the oncogenic function of Zcchc11 in melanoma, prostate and liver cancer *in vitro*, and in liver xenograft mouse models. Overall, Zcchc11 displays an opposite function to that of miR-26a in tumor development and metastasis, suggesting reciprocal regulation of these two genes. Furthermore, we reveal that Zcchc11 expression is consistently elevated in human liver and colorectal cancers, which establishes the first clinical link between Zcchc11 and human cancers. It will be great interest for future investigations to explore the underlying mechanism for Zcchc11 function in tumorigenesis.

In summary, we find that miR-26a directly targets Lin28B and Zcchc11 and define the suppressive function of miR-26a and Zcchc11 in tumor growth and metastasis. These findings reveal new insights into an miRNA-mediated tumor suppression through enhancing miRNA biogenesis, which may provide novel approaches for cancer treatments.

Materials and methods

Cell Culture

Melanoma (A2058, A375, SK-MEL-5, and SK-MEL-28), hepatoma (HepG2, Huh7, and SK-HEP-1), prostate cell lines (DU145, LNCAP, and PC3), colon [HCT116(p53⁺) and HCT116 (p53⁻)], kidney (786-O), lung (A549), breast (MB-MDA-231), and acute myelocytic leukemia (K562) cell lines were obtained from American Type Culture

Collection (ATCC) and cultured according to instructions. NHDFs (normal human dermis fibroblasts) were kindly provided by Dr. J. Wu (City of Hope).

RNA Extraction and Real-Time PCR

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) following the supplier's instructions. miR-26a and let-7 expression in human tissues was measured with First-Choice Human Total RNA Survey Panel (Applied Biosystems, Foster City, CA). For mRNA analysis, cDNA was synthesized using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). For miRNA analysis, cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) as described previously.⁴⁴ Both mRNA and miRNA quantitative real-time PCR analyses were performed with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Expression of mRNA and miRNA was normalized to actin and 5S RNA, respectively. Sequences of the QRT-PCR primers are provided in Supplementary Information Table 1.

miRNA and siRNA Transfection

miRNA mimic, miRCURY LNATM miR-26a inhibitor and let-7 family inhibitor was purchased from Ambion (Austin, TX) and Exiqon (Vedbaek, Denmark), respectively. esiRNA human ZCCHC11 was purchased from Sigma (St. Louis, MO). Transfections of miRNA or siRNAs were performed using HiPerfect (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Retroviral and lentiviral Transduction

The human miR-26a-2 gene was amplified by PCR and directionally inserted into the MDH1-PGK-GFP 2.0 retrovirus vector (Addgene, cat no. 11375). Retrovirus was generated and infected cancer cells as described previously.³² A multiplicity of infection of 3 to 6 was used to generate miR-26a-overexpressing stable cell lines. For Zcchc11 knockdown, the pLKO.1 lentivirus vector (Addgene, cat no. 8453) was used. Transductions with lentiviral particles were performed using 2 mg/ml of polybrene (Sigma, St. Louis, MO) and positive cells were selected with puromycin (Sigma, St. Louis, MO). A list of Zcchc11 shRNA sequences is provided in the Supplementary Information Table 2.

Cell Proliferation

Cells (5000 cells/well) were seeded in a 96-well plate and transfected with miR-26a precursors/inhibitors. 48 hours post transfection, rates of cell proliferation were determined using CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI).

Colony formation Assay

miR-26a-overexpressing, zcchc11-depleted stable cell lines and their corresponding controls were used for colony formation assay. Briefly, cells were seeded at low density and allowed to grow for 14 days. Colonies were photographed and counted.

Invasion Assay

Transwell invasion assays performed according to the manufacturer's protocol (BD Biosciences, San Jose, CA). Briefly, cells (5×10^4) were added to the upper chamber of transwell inserts (BD Biocoat Matrigel 24-well invasion chamber) in serum-free medium containing 0.1% BSA, and the number of cells that invaded into the lower chamber were stained with Diff-QuikTM stain and counted after 24 hours of incubation at 37°C with 5% CO₂. The cell nucleus stained purple and the cytoplasm stained pink. Each experimental group had two replicates, and three fields in each replicate were randomly chosen for quantification of invasive cells.

Immunoblotting

Total cell lysis and western blot analysis were performed as described previously.⁴⁵ Western blot analysis used the following antibodies: anti-Zcchc11 (1:1000 dilution), anti-Lin28B (1:1000 dilution) (Abcam, Cambridge, UK), and anti-β-actin (1:5000 dilution) (Sigma, St. Louis, MO).

Luciferase Reporter Assays

The Luciferase-Zcchc11-3'UTR and Luciferase-Lin28B-3'UTR constructs were based on psiCHECK-2 vector (Promega, Madison, WI). The predicted miR-26a binding site was mutated using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HeLa cells were plated in a 96-well plate and cotransfected with the luciferase reporter vector and 20 nM miR-26 mimic (Ambion, Austin, TX) by using HiPerfect (Qiagen, Valencia, CA). Firefly and renilla luciferase activities were analyzed by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) 24 hours after transfection.

Mouse xenograft experiments

To study the effect of miR-26a overexpression in tumorigenesis *in vivo*, A375, DU145, and SK-HEP-1 cells (5×10^5) transduced with either the miR-26a expression vector or the control vector were trypsinized, resuspended in sterile PBS, and injected subcutaneously into the flank of female NOD-SCID mice. To study the effect of Zcchc11 depletion in tumorigenesis *in vivo*, SK-HEP-1 cells (5×10^5) transduced with either the Zcchc11-depleting vector or the control vector were used as described above. Tumor size was measured with Vernier calipers and tumor volumes were calculated according to the following formula: $L \times W^2/2$, where L is the length and W is the width of the tumor. After mice were euthanized, liver and lung metastases were examined and tumor samples were collected for RNA and protein extraction.

***In vivo* metastasis assay**

miR-26a-overexpressing stable A375 cells generated by retrovirus transduction were used. Briefly, 1×10^6 A375 cells were trypsinized, resuspended in sterile PBS, and injected into female NOD-SCID mice through the tail vein. Three weeks after injection, mice were euthanized. Livers and lungs were then harvested and tumor nodules were counted.

Human liver and colorectal samples

Total RNAs of 10 liver tumors and their adjacent normal livers were obtained from City of Hope National Medical Center (Duarte, CA). Formalin-fixed, paraffin-embedded liver cancer sections and colorectal cancer sections were obtained from Third Military Medical University (Chongqing, China) and Zhejiang University (Hangzhou, China), respectively.

Histology and immunostaining

Samples were formalin-fixed, paraffin-embedded, and sectioned. Hemotaxyl-eosin (H&E) was performed according to standard histopathological procedures. The number of proliferating cells was determined by staining paraffin sections with the PCNA staining kit (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. Apoptosis was evaluated by staining paraffin sections with In situ cell death detection kit, TMR red terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) system (Roche, Indianapolis, IN) according to the manufacture's protocol. Slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Immunohistochemical staining was performed by City of Hope Pathology Core according to the manufacture's protocol using anti-Zcchc11 antibody (1:50 dilution, Sigma) and anti-Lin28B antibody (1:50 dilution, Abcam).

Statistical analysis

Results of experiments are displayed as mean $\pm$ SEM in the Figures. Two-tailed Anova assay and Student's *t* test were used to determine differences between data groups (two-tailed; $P < 0.05$ was considered statistically significant) unless otherwise noted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Drs. Arthur Riggs and Rama Natarajan for reviewing the manuscript, Dr. Kyle Sousa and other members of the Huang lab for helpful discussions, and Dr. Keely Walker for editing manuscript. This work was supported by the National Cancer Institute (P30 CA033572) and the American Cancer Society (RSG-11-132-01-CCE).

References

1. Farazi TA, Spitzer JI, Morozov P, Tuschl T. miRNAs in human cancer. *J Pathol.* 2011; 223:102–115. [PubMed: 21125669]
2. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med.* 2012; 4:143–159. [PubMed: 22351564]
3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144:646–674. [PubMed: 21376230]
4. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A.* 2006; 103:2257–2261. [PubMed: 16461460]
5. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature.* 2005; 435:834–838. [PubMed: 15944708]
6. Kumar MS, Pester RE, Chen CY, Lane K, Chin C, Lu J, et al. Dicer1 functions as a haploinsufficient tumor suppressor. *Genes Dev.* 2009; 23:2700–2704. [PubMed: 19903759]

7. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet.* 2007; 39:673–677. [PubMed: 17401365]
8. Melo SA, Moutinho C, Ropero S, Calin GA, Rossi S, Spizzo R, et al. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell.* 2010; 18:303–315. [PubMed: 20951941]
9. Melo SA, Ropero S, Moutinho C, Aaltonen LA, Yamamoto H, Calin GA, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet.* 2009; 41:365–370. [PubMed: 19219043]
10. Ravi A, Gurtan AM, Kumar MS, Bhutkar A, Chin C, Lu V, et al. Proliferation and tumorigenesis of a murine sarcoma cell line in the absence of DICER1. *Cancer Cell.* 2012; 21:848–855. [PubMed: 22698408]
11. Newman MA, Hammond SM. Emerging paradigms of regulated microRNA processing. *Genes Dev.* 2010; 24:1086–1092. [PubMed: 20516194]
12. van Kouwenhove M, Kedde M, Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. *Nat Rev Cancer.* 2011; 11:644–656. [PubMed: 21822212]
13. Kasinski AL, Slack FJ. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev Cancer.* 2011; 11:849–864. [PubMed: 22113163]
14. Lujambio A, Lowe SW. The microcosmos of cancer. *Nature.* 2012; 482:347–355. [PubMed: 22337054]
15. Yuan J, Nguyen CK, Liu X, Kanellopoulou C, Muljo SA. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science.* 2012; 335:1195–1200. [PubMed: 22345399]
16. Viswanathan SR, Powers JT, Einhorn W, Hoshida Y, Ng TL, Toffanin S, et al. Lin28 promotes transformation and is associated with advanced human malignancies. *Nat Genet.* 2009; 41:843–848. [PubMed: 19483683]
17. Zhu H, Shyh-Chang N, Segre AV, Shinoda G, Shah SP, Einhorn WS, et al. The Lin28/let-7 axis regulates glucose metabolism. *Cell.* 2011; 147:81–94. [PubMed: 21962509]
18. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell.* 2009; 139:693–706. [PubMed: 19878981]
19. Jiang X, Huang H, Li Z, Li Y, Wang X, Gurbuxani S, et al. Blockade of miR-150 Maturation by MLL-Fusion/MYC/LIN-28 Is Required for MLL-Associated Leukemia. *Cancer Cell.* 2012; 22:524–535. [PubMed: 23079661]
20. Newman MA, Hammond SM. Lin-28: an early embryonic sentinel that blocks Let-7 biogenesis. *Int J Biochem Cell Biol.* 2010; 42:1330–1333. [PubMed: 20619222]
21. Viswanathan SR, Daley GQ. Lin28: A microRNA regulator with a macro role. *Cell.* 2010; 140:445–449. [PubMed: 20178735]
22. Thornton JE, Gregory RI. How does Lin28 let-7 control development and disease? *Trends Cell Biol.* 2012; 22:474–482. [PubMed: 22784697]
23. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. *Cell.* 2005; 120:635–647. [PubMed: 15766527]
24. Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science.* 2007; 315:1576–1579. [PubMed: 17322030]
25. Sampson VB, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P, et al. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res.* 2007; 67:9762–9770. [PubMed: 17942906]
26. Hammond SM, Sharpless NE. HMGA2, microRNAs, and stem cell aging. *Cell.* 2008; 135:1013–1016. [PubMed: 19070572]
27. Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, et al. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell.* 2009; 138:696–708. [PubMed: 19703396]
28. Heo I, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell.* 2008; 32:276–284. [PubMed: 18951094]

29. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. *Science*. 2008; 320:97–100. [PubMed: 18292307]
30. Hagan JP, Piskounova E, Gregory RI. Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat Struct Mol Biol*. 2009; 16:1021–1025. [PubMed: 19713958]
31. Meng Z, Fu X, Chen X, Zeng S, Tian Y, Jove R, et al. miR-194 is a marker of hepatic epithelial cells and suppresses metastasis of liver cancer cells in mice. *Hepatology*. 2010; 52:2148–2157. [PubMed: 20979124]
32. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004; 303:83–86. [PubMed: 14657504]
33. Talmadge JE. Models of metastasis in drug discovery. *Methods Mol Biol*. 2010; 602:215–233. [PubMed: 20012401]
34. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120:15–20. [PubMed: 15652477]
35. Piskounova E, Polyarchou C, Thornton JE, LaPierre RJ, Pothoulakis C, Hagan JP, et al. Lin28A and Lin28B inhibit let-7 microRNA biogenesis by distinct mechanisms. *Cell*. 2011; 147:1066–1079. [PubMed: 22118463]
36. Blahna MT, Jones MR, Quinton LJ, Matsuura KY, Mizgerd JP. Terminal uridylyltransferase enzyme Zcchc11 promotes cell proliferation independent of its uridylyltransferase activity. *J Biol Chem*. 2011; 286:42381–42389. [PubMed: 22006926]
37. King CE, Wang L, Winograd R, Madison BB, Mongroo PS, Johnstone CN, et al. LIN28B fosters colon cancer migration, invasion and transformation through let-7-dependent and -independent mechanisms. *Oncogene*. 2011; 30:4185–4193. [PubMed: 21625210]
38. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell*. 2009; 137:1005–1017. [PubMed: 19524505]
39. Ji J, Shi J, Budhu A, Yu Z, Forgues M, Roessler S, et al. MicroRNA expression, survival, and response to interferon in liver cancer. *N Engl J Med*. 2009; 361:1437–1447. [PubMed: 19812400]
40. Mavrikis KJ, Van Der Meulen J, Wolfe AL, Liu X, Mets E, Taghon T, et al. A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL). *Nat Genet*. 2011; 43:673–678. [PubMed: 21642990]
41. Sander S, Bullinger L, Klapproth K, Fiedler K, Kestler HA, Barth TF, et al. MYC stimulates EZH2 expression by repression of its negative regulator miR-26a. *Blood*. 2008; 112:4202–4212. [PubMed: 18713946]
42. Dangi-Garimella S, Yun J, Eves EM, Newman M, Erkeland SJ, Hammond SM, et al. Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7. *EMBO J*. 2009; 28:347–358. [PubMed: 19153603]
43. Yun J, Frankenberger CA, Kuo WL, Boelens MC, Eves EM, Cheng N, et al. Signalling pathway for RKIP and Let-7 regulates and predicts metastatic breast cancer. *EMBO J*. 2011; 30:4500–4514. [PubMed: 21873975]
44. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA*. 2005; 11:1737–1744. [PubMed: 16244135]
45. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. *Cancer Res*. 2007; 67:863–867. [PubMed: 17283114]

Figure 1. miR-26a inhibits tumor cell growth

(a, b) QRT-PCR analysis of miR-26a expression in the indicated normal human tissues (a), and cell lines derived from liver, prostate and skin cancers and corresponding normal tissues/cell lines (b).

(c, d) Indicated cells were transfected with miR-26a precursor (pre-26a), inhibitor (anti-26a), or negative controls (N.C.). 48 hours post transfection, miR-26a expression (determined by QRT-PCR) (c) and cell proliferation (determined by MTS analysis) (d) were measured.

(e-g) Indicated cells were transduced with either MPG-control (MPG-C) or MPG-26a overexpression (MPG-26a) retroviral vector. A multiplicity of infection of 3 to 6 was used to generate miR-26a-overexpressing stable cell lines. (e) QRT-PCR analysis of miR-26a expression (f) cells (5000 cells/well) were plated in a 96-well plate. After 48 hours culture, cell proliferation was determined by MTS analysis. (g) Colony formation assay.

Data are shown as means $\pm$ SEM. Statistical significance, determined by two-tailed Student's t test (b, d, f, g), is indicated by *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$.

after the injection. **(k)** Number of total tumors. **(l)** Representative pictures of livers and lungs with tumors. Arrows indicate metastases.

Data are shown as means $\pm$ SEM. Statistical significance, determined by two-tailed ANOVA (**a, c, e**) and two-tailed Student's t test (**b, d, f, h, k**), is indicated by *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$.

Figure 3. let-7 is upregulated in miR-26a-overexpressing tumors

(a) Relative expression of indicated miRNAs in miR-26a-overexpressing A375 tumors and control tumors (n = 5 tumors/group).

(b) Fold changes in the expression of indicated miRNAs of miR-26a-overexpressing versus control melanoma cell lines.

(c, d) Relative expression of indicated miRNAs in miR-26a-overexpressing DU145 (c) and SK-HEP-1 (d) and control tumors.

(e) Correlation of miR-26a and let-7 expression within normal human tissues, as determined by linear regression.

(f, g) Indicated cells were transfected with miR-26a precursor (pre-26a), miR-26a precursor plus let-7 family inhibitor (pre-26a + let-7 FI), or negative controls (N.C.). 48 hours post transfection, let-7 expression (determined by QRT-PCR) (f) and cell proliferation (determined by MTS analysis) (g) were measured.

(h) Relative expression of indicated pri-miRNAs in miR-26a-overexpressing and control tumors.

Data are shown as means $\pm$ SEM. Statistical significance, determined by two-tailed Student's t test (a, c, d, f-h), is indicated by *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$.

Figure 4. miR-26a directly targets Zcchc11 and Lin28B

(a) Relative luciferase activity in HeLa cells transfected with reporter constructs containing the 3' UTR of Zcchc11, Lin28B or control vector (psiCheck) and cotransfected with an miR-26a precursor (pre-26a) or negative control (N.C.). The relative luciferase activity of psiCheck (pre-26a/N.C.) is set as 1. Mut: mutant.

(b) Expression of Lin28B and Zcchc11 mRNA in HEK293 cells transfected with negative control (N.C.), pre-26a or let-7d precursor (pre-let7d) for indicated times.

(c) Immunoblot analysis of Zcchc11 and Lin28B expression in indicated cell lines transfected with pre-26a or N.C. for 48 hours. Quantification of Zcchc11 and Lin28B is shown (right panel).

(d) Immunoblot analysis of Zcchc11 and Lin28B in A375, DU145 and SK-HEP-1 xenograft tumors. Quantification of Zcchc11 and Lin28B is shown (right panel).

(e, f) Expression of Zcchc11 mRNA (e) and protein (f) in indicated cancer cell lines. Data are shown as means ± SEM. Statistical significance, determined by two-tailed Student's t test (a-d), is indicated by *, p < 0.05; **, p < 0.01; and ***, p < 0.005.

Figure 5. Zcchc11 represses the tumorigenesis and metastasis of cancer cells

(a) Cell proliferation (determined by MTS assay) of indicated cell lines transfected with control siRNA (siEGFP) or siRNA specific for Zcchc11 (siZcc).

(b) Cell proliferation (determined by MTS assay) of cell lines transduced with one of two specific shRNAs for Zcchc11 (shZcc-3 or shZcc-4) or scrambled control shRNA (SCR).

(c-e) Colony formation assay of A375 (c), DU145 (d), and SK-HEP-1 (e) transduced with one of two specific shRNAs for Zcchc11 (siZcc-3 or siZcc-4) or SCR. Representative images are shown in the right panel.

(f) Invasion assay of SK-HEP-1 cells transduced with one of two specific shRNAs for Zcchc11 (shZcc-3 or shZcc-4), pre-26a, or SCR.

(g-k) Stable SK-HEP-1 cells expressing either SCR or shZcc-3 were injected subcutaneously into NOD-SCID mice and tumor volume was monitored over time (n = 6 mice/group). (g) Tumor growth (left panel) and weight (right panel). (h) Representative pictures of livers, lungs and tumors. Arrows indicate metastases. (i) Percentage of mice with metastases, number of metastases, size of metastases, and organ:body weight ratio. (j,k) Representative H&E and PCNA staining of lung (j) and liver (k) tissues. White cycles indicate metastases. Scale bars, 100 μm.

Data are shown as means $\pm$ SEM. Statistical significance, determined by two-tailed ANOVA (**g**, left panel) and two-tailed Student's t test (**a-f, g**, right panel; **i**), is indicated by *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. The effects of miR-26a and Zcchc11 in tumor cell proliferation and apoptosis
H&E, Lin28B, Zcchc11, PCNA, and TUNEL staining performed in A375 (a), DU145 (b), and SK-HEP-1 (c) miR-26a-overexpressing (MPG-26a) xenograft or control (MPG-C) tumors, and SK-HEP-1 Zcchc11-depleted (d) xenograft (sh-Zcc3) or control tumors (SCR). Scale bars, 100 μ M.

Figure 7. Zcchc11 is upregulated in human cancers

(a) Relative expression of Zcchc11 in 9 samples of human liver cancer with paired normal tissues.

(b, c) Representative Zcchc11 IHC staining in human liver cancers (b) and colorectal cancers (c) with paired normal tissues. Scale bars, 100 μ M.