The miR-30 Family Inhibits Pulmonary Vascular Hyperpermeability in the Premetastatic Phase by Direct Targeting of Skp2

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Abstract

Purpose: Before metastasis, primary tumor can create a premetastatic niche in distant organ to facilitate the dissemination of tumor cells. In the premetastatic phase, the permeability of pulmonary vasculatures is increased to accelerate the extravasation of circulating tumor cells. However, it is not clear whether local miRNAs contribute to the vascular hyperpermeability of the premetastatic niche.

Experimental Design: The expression of total miRNAs was determined using microarray in series of premetastatic lungs from tumor-bearing mice. Significantly differentially expressed miRNAs were identified and validated with qRT-PCR. Vascular permeability assays, vascular mimic systems, and orthotopic tumor models were used to investigate roles of selected miRNAs and target genes in premetastatic hyperpermeability.

Results: We identified a miRNA signature in premetastatic lungs. Among these miRNAs, miR-30a, b, c, d, and e were significantly attenuated. Subsequent investigations elucidated that lung fibroblast-derived miR-30s stabilized pulmonary vessels. Overexpression of miR-30s in lungs postponed metastasis and extended overall survival of B16 tumor-bearing mice. Following studies uncovered that Skp2 was directly targeted by miR-30s. Overexpression of Skp2 could disrupt pulmonary vessels, promote lung metastasis, and decrease overall survival of B16 tumor-bearing mice.

Conclusions: These findings illuminate a novel mechanism for the modulation of premetastatic niches by miR-30s, which suggest that miR-30s represent not only promising targets for antimetastasis therapy but also indicators for metastasis.

Introduction

Distant metastasis is the primary cause of mortality in patients with cancer. Metastasis is a multistep process that requires tumor cells to leave the primary tumor through intravasation, survival in the circulation, and extravasation into distant organs that provide appropriate microenvironments. Among the multiple factors underlying metastasis, it is well-recognized that a process called premetastatic niche formation (1–3) is crucial for the development of metastasis. Previous studies have described 2 primary premetastatic events in the lung: bone marrow–derived cell (BMDC) mobilization (2, 4) and vascular hyperpermeability (5). Induction of vascular destabilization is crucial for the extravasation of tumor cells and subsequent colonization. We and others have reported that certain factors, such as MMPs, angiopoietin2, CCL2, S100A8 (6), and angiopoietin-like 4, can disrupt pulmonary vascular endothelial tight junctions and break the integrity of capillary walls to facilitate metastasis (6–10). microRNAs are a class of small noncoding RNAs (19–24 nt) which mediate mRNA destabilization or translational repression typically by base pairing to the 3′-untranslated region (UTR) of a target mRNA (11). It is well-recognized that miRNAs play important roles in multiple aspects of the tumor progression. Interestingly, miRNAs are involved in the regulation of the vascular integrity in both autocrine and paracrine manner to promote tumor metastasis. Recent studies have discovered primary tumor-derived exosomal miRNAs and suggested their roles in defining the metastatic potential of cancer cells (12, 13). However, the role of host-derived miRNAs in premetastatic niches has not yet been examined.

Here we identify distant lung-derived miR-30s as novel factors that inhibit premetastatic niche formation. miR-30s are downregulated in fibroblasts of premetastatic lungs, and overexpression of miR-30s disrupts the development of premetastatic niches. Moreover, we define a set of premetastatic-associated miR-30 targets, including Skp2, and reveal the molecular mechanism of the miR-30 machinery in lung metastasis.

Materials and Methods

Cells and reagents

B16, 4T1, 3T3, and HEK293T cells obtained from the ATCC were cultured according to the manufacturer’s instructions. Cells were grown in DMEM supplemented with 10% FBS and 1%
Metastasis is the primary cause of mortality in patients with cancer. Here, we show that many distant organ-derived miRNAs have altered expression in the premetastatic niche and may play key roles in tumor cell dissemination, presenting an evidence for Paget's theory of metastatic spread. One challenge of the delivery of antimiR treatment is the heterogeneity of spreading tumor cells. Therefore, the host-derived miR-30 family and its target gene Skp2 appear promising targets to bypass the diversity of tumor cell for antimetastasis treatment and indicators of metastasis.

**Translational Relevance**

Antimetastasis is central for managing cancer patients due to its high mortality. Here, we show that many distant organ-derived miRNAs have altered expression in the premetastatic niche and may play key roles in tumor cell dissemination. One challenge of the delivery of antimiR treatment is the heterogeneity of spreading tumor cells. Therefore, the host-derived miR-30 family and its target gene Skp2 appear promising targets to bypass the diversity of tumor cell for antimetastasis treatment and indicators of metastasis.
injected intrapleurally weekly for 2 weeks to stably overexpress or knockdown the target genes in the lung tissue. Then B16 cell conditioned media (BCM), which was from serum-free medium cultured B16 cells for 24 hours, were intravenously injected to establish the premetastatic lung. After 72 hours, Evans blue dye (EB, 100 μL of 20 mg/mL; Sigma-Aldrich) was intravenously injected. Lungs were perfused with PBS to remove blood 2 hours after injection and then removed and trimmed of extraneous tissues. After grinded into powder in liquid nitrogen, EB exuded from lungs was extracted by incubating the powder of one lung in 1-mL formamide for 16 hours at 60°C, and tissue EB content (μg EB/g lung) was calculated by correcting $A_{520}$ for the presence of heme pigments: $A_{520}$ (corrected) = $A_{520}$ – (1.426 × $A_{340}$ + 0.030) and comparing this value with a standard curve EB in formamide/PBS.

Cloning of 3'UTR reporter constructs and reporter assays

The 3'-UTRs of Klip, Nedd4L, Rab38, Skp2, and Ugt8a were amplified by PCR from mouse lung fibroblast and cloned into pCMV-reporter vector between HindIII and MluI sites. Mutations of 3'-UTRs were generated using overlap PCR and were also cloned to pCMV-reporter vector. 3T3 cells were plated in 12-well plates at 1 × 10⁵ cells per well and cotransfected with pCMV-reporter vectors along with miRNA mimics. Forty-eight hours after transfection, whole-cell lysates were prepared using cell lysis buffer, and then soluble fraction was used for reporter assay. Luciferase activities were measured in triplicate using Bright-Glo luciferase assay system (Promega). All experiments were performed in triplicate with data pooled from at least 2 independent experiments.

In situ hybridization

miR-30 in situ hybridization was performed on 10-μm cryosections of mouse lung tissue as described previously with slight modifications (15). The 5′-biotin-labeled probes (Ribobio Co.) against miR-30s were listed in Supplementary Table S3. Briefly, the cryosections were fixed with 4% paraformaldehyde. After wash steps, slides were acetylated for 5 minutes in the acetylation buffer. For hybridization, 100 pmol of 5′ biotin-labeled probe diluted in 100 μL hybridization buffer (50% formamide, 5 × SSC, 1 × Denhardt solution, 500 μg/mL yeast tRNA) were applied per slide. After hybridization and washing steps, the slides were incubated for 30 minutes at room temperature in blocking buffer (0.5% blocking reagent, 0.5% BSA) and then incubated in FITC-streptavidin (1:100, Abcam) for 30 minutes at room temperature. Finally, the slides were mounted using mounting medium with 4′,6-diamidino-2-phenylindole (DAPI).

In vivo lectin/dextran vascular permeability assay

The assay was performed as previous described with slight modifications (5). After lentivirus and BCM injection, mice were intravenously injected with rhodamine-dextran (Sigma) at 50 mg/kg. Then, mice were intravenously injected with FITC-lectin (Sigma) at 5 mg/kg after 3 hours. Ten minutes later, mice were anesthetized and perfused with PBS and 4% paraformaldehyde. Then, tissues were cryosectioned at 30 nm and examined.

Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committee of Tsinghua University (Beijing, China). For miRNA microarray analysis, B16 cells (5 × 10⁶) were mixed with GFR-Matrigel (BD Pharmingen) and intradermally injected into the flank of female C57Bl/6 mice (8-week-old). At days 0, 4, 8, 12, and 16 after B16 tumor implantation, lung tissues were perfused and subjected to miRNA microarray. For survival and metastasis analysis, 100 μL lentiviruses (5 × 10⁵ TU/mL) were injected intrapleurally weekly for 2 weeks. The mice inoculated with B16 cells (5 × 10⁵) for indicated days and then were killed, and lungs were dissected and applied to hematosylin and eosin (H&E) staining. For lung retention assay, 100 μL lentiviruses (5 × 10⁵ TU/mL) were injected intrapleurally weekly for 2 weeks. Then BCM were intravenously injection to establish the premetastatic lung. GFP-B16 cells (1 × 10⁵ per mouse) were intravenously injected into the mice 72 hours later. Lungs were obtained to detect tumor cell retention in the lungs 48 hours later.

Statistical analysis

Statistically significant differences between groups were determined using a 2-tailed unpaired Student’s t test or ANOVA, where P < 0.05 is considered significant.

Results

The miRNA signature in premetastatic lungs

Lung is one of the most preferentially metastatic organs for numerous types of tumor. To explore the function of miRNA in the premetastatic niche, we focused on the lungs before the formation of metastasis. Lungs of B16 tumor–bearing mice were collected at days 0 to 16, by which time lung metastasis does not happen and is considered as the premetastatic phase (2, 4, 5). We systematically examined the miRNA expression by miRNA chip and identified distinct miRNA expression signatures through SAM (16). Twenty-five miRNAs were significantly downregulated in premetastatic lungs (Fig. 1A). Intriguingly, miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e, all of which belonged to the miR-30 family, had at least 50% decreases at day 16. To validate the miRNA chip result, we examined levels of these miRNAs in premetastatic lungs by qRT-PCR with Bulge-Loop miRNA Primer which can detect mature miRNA with high sensitivity and specificity. There were 21 miRNAs confirmed to be significantly suppressed by both qRT-PCR and the chip through Pearson correlation analysis (Supplementary Table S4). Interestingly, miR-30s were proved to be downregulated through days 4 to 16 (Fig. 1B). Then, in situ hybridization was performed to detect the localization of miR-30s in the lung. The results show that the positive staining was present in the cytoplasm for all miR-30s probes compared with control probe (Supplementary Fig. S1). Furthermore, miR-30s were localized at the mesenchyme of the lung tissue, which was mainly composed of fibroblasts. Consistent with the ChIP and qRT-PCR results, miR-30s were significantly decreased in premetastatic lung tissues compared with normal one. In addition, we randomly selected miR-181b as a negative control, which was not significantly changed in the microarray. In premetastatic lungs analyzed by qRT-PCR, miR-181b was not altered either (Supplementary Fig. S2A).

To further investigate the involvement of miR-30s in distant lungs, we determined their expression in lungs challenged with BCM (2). After 48 hours, miR-30s were attenuated by more than 50% (Fig. 1C). Following studies revealed that miR-30s were only repressed in fibroblasts isolated from lungs of BCM challenged mice (Fig. 1D), but not in cells other than fibroblasts (Supplementary Fig. S2B). In addition, miR-30s were remarkably
suppressed in cultured lung fibroblasts treated with BCM (Supplementary Fig. S2C), but not in endothelial cells (Supplementary Fig. S2D).

Taken together, these findings demonstrate that miR-30s are reduced in premetastatic lung fibroblasts, indicating potential inhibitory roles of miR-30s in premetastatic niche formation.

**miR-30s stabilize pulmonary vessels in premetastatic lungs**

MMPs played crucial roles in the disruption of endothelial monolayer and interruption of vessel integrity (5). To test roles of miR-30s in vascular permeability, we detected effects of miR-30s on MMPs by qRT-PCR. Overexpression of miR-30s in fibroblasts decreased mRNA levels of MMP2, MMP3, MMP9, MMP10, and MMP14. Particularly, MMP9 was remarkably suppressed by miR-30s (Fig. 2A). Thus, we detected effects of miR-30s on the proteolytic activity of MMP9 by gelatin zymography. Overexpression of miR-30s in primary lung fibroblasts significantly inhibited proteolytic activity of MMP9, but not MMP2 (Fig. 2B), suggesting potential involvement of miR-30s in vascular remodeling. In addition, in murine endothelial cells treated by the conditioned media from miR-30s–transfected lung fibroblasts, levels of MMP2, MMP3, MMP9, MMP10, and MMP14 were not significantly changed compared with miR-NC group (Supplementary Fig. S2E). These results suggest that miR-30s modulate premetastatic lung vessels mainly through MMPs derived from fibroblasts other than endothelial cells.

To assess the function of miR-30s in vascular destabilization, an early event in premetastatic niche formation (2, 4, 5), we first evaluated endothelial leakage by vascular mimic system in which endothelial cell monolayer with basement membrane was induced by the conditioned media of primary fibroblasts. Enhanced expression of miR-30s in fibroblasts remarkably attenuated the leakage of endothelial cell monolayer (Fig. 2C). Therefore, we compared levels of miR-30s between hyperpermeable and poorly permeable regions in lungs of BCM challenged mice. miR-30s exhibited about 2.5-fold increase in poorly permeable regions compared with that of hyper permeable regions (Fig. 2D and E), suggesting their protective effects on vessel integrity. To specifically determine the function of miR-30s in the premetastatic niche, we intrapleurally injected mouse with lentivirus encoding pri–miR-30s for 2 weeks according to previous description (5), followed by treatment of BCM. The efficiency of lentivirus was evaluated (Supplementary Fig. S2F). Then mice were applied for vascular permeability assay. The results showed that ectopic expression of miR-30s dramatically inhibited vascular hyperpermeability in premetastatic lungs compared with controls (Fig. 2F and G), demonstrating the protective effects of miR-30s on premetastatic vascular hyperpermeability.

**miR-30s inhibit metastasis and improve overall survival of tumor-bearing mice**

To address whether miR-30s could block lung colonization of tumor cells, mice were preintraperitoneally injected with miR-30s and applied for lung retention assay. Overexpression of miR-30s remarkably attenuated the number of GFP-B16 cells compared with controls (Fig. 3A and B). We next investigated whether miR-30s could block the metastasis of well-established tumor models. After preintraperitoneal injection of lentiviruses, primary tumor growth was unaffected in neither B16 nor 4T1 tumors (Supplementary Fig. S3A and S3B). On the other hand, lung metastasis was dramatically blocked by miR-30s in both B16 (Fig. 3C and D) and 4T1 (Supplementary Fig. S3C) tumor-bearing mice compared with vector-injected mice. To confirm the overexpression efficiency of lentivirus, we detected miR-30s levels in both bone marrow and B16 cells. As expected, miR-30s in lung fibroblasts of miR-30–treated group exhibited more than 3-fold increase compared with vector group (Supplementary Fig. S3D). On the other hand, the level of miR-30s in tumor cells was unchanged (Supplementary Fig. S3E). Then, the median survival of mice preintraperitoneally injected with vectors was 21 days, whereas mice received treatments of miR-30b, miR-30c, miR-30d, and miR-30e significantly extended their median survival to at least 31 days (Fig. 3E). Most...
miR-30–treated mice died of the outgrowth of primary tumor, without severe lung metastatic burden, whereas vector-treated mice died earlier accompanied with substantial macrometastases in lungs.

We further analyzed antimetastasis effects of miR-30s by systemic administration of miR-30s. Lentiviruses encoding pri–miR-30s or vectors were intravenously injected into mice twice a week for 2 weeks. In the following, mice were intrapleurally transfected with miR-30s weekly for 2 weeks and applied for lung vascular permeability assay (n = 7). Error bars, min to max. Data were representative of ≥3 independent experiments (Student t test; * and ***, P < 0.01; **** and ***, P < 0.001).

Collectively, these results suggest that either intrapleurally or intravenously administration of miR-30s restrains metastatic spread to the lung, therefore prolongs overall survival of B16 tumor-bearing mice.

**Klf9, Nedd4l, Rab38, Skp2, and Ugt8a are direct targets of miR-30s**

In the following, we screened for specific miR-30s’ targets with potential relevance in the regulation of premetastatic niche formation. According to 4 miRNA target prediction algorithms, a set of 158 genes was predicted to be targeted by miR-30s. Subsequently, a previously reported cohort of premetastatic lung-associated genes (5) was probed with these 158 genes. There were 14 genes shared in both (Fig. 4A). We next examined whether these 14 genes could be influenced by overexpression of miR-30s. Fibroblasts were transfected with miR-30s and collected for qRT-PCR. Nine of 14 genes were suppressed by overexpression of miR-30s (Fig. 4B), whereas the other 5 genes were unaffected (Supplementary Fig. S4A). In premetastatic lungs, 8 of 9 genes were increased (Fig. 4C and Supplementary Fig. S4B). In BCM challenged lung fibroblasts, only Klf9, Nedd4l, Rab38, Skp2, and Ugt8a (Fig. 4D and Supplementary Fig. S4C) were increased. Pearson correlation analysis between the left 5 putative targets and miR-30s in premetastatic lungs (Supplementary Table S5) revealed that these 5 genes were negatively associated with miR-30s. Taken together, these analyses narrowed the list down to 5 genes that were potentially influenced by miR-30s in premetastatic models.

To prove that effects of miR-30s on Klf9, Nedd4l, Rab38, Skp2, and Ugt8a were dependent on putative binding sites, we generated reporter constructs in corresponding 3’-UTR where miR-30s are predicted to bind (Supplementary Fig. S4D). Overexpression of miR-30s conferred repression to wild-type 3’-UTR but not to mutants (Fig. 4E). Also, protein levels of 5 targets were suppressed by miR-30s (Supplementary Fig. S4E). Although all targets were
downregulated by miR-30s, there was certain heterogeneity among these results. For example, miR-30a and b downregulated the expression of Nedd4l, Skp2, and Ugt8a to more than 50%. miR-30d attenuated protein level of Rab38 and Ugt8a to most extent than others. miR-30e had strong inhibitory effects on Klf9, Skp2, Rab38, and Ugt8a. These results were mostly consistent with the reporter assay (Fig. 4E). These results were mostly consistent with the reporter assay (Fig. 4E). These results demonstrate that miR-30s directly target Klf9, Nedd4l, Rab38, Skp2, and Ugt8a through specific interactions with corresponding 3'UTRs. Furthermore, these 5 genes exhibit strong negative correlation with miR-30s throughout in vivo and in vitro premetastatic models.

Besides, we noticed that several genes, such as Bcl2 (17), Bcl2l11 (18), Ccne2 (19), Gab1 (20), Ganti2 (21), Snai1 (22), Socs1 (23), and Ube2i (24), were previously reported to be targets of miR-30s in mouse. To evaluate whether these genes were involved in premetastatic niche formation, we detected their expression levels in premetastatic lungs. Snai1, Socs1, and Ube2i were induced during premetastatic phase (Supplementary Fig. S5A). However, only Snai1 and Socs1 were increased in BCM-treated lung fibroblasts (Supplementary Fig. S5B). Therefore, we suspected that Snai1 and Socs1 could also take part in premetastatic niche formation. Additional studies revealed that these targets were only elevated in fibroblasts isolated from lungs of BCM challenged mice, but not in cells other than fibroblasts (Supplementary Fig. S5C and S5D).

Taken together, these results illustrate that Klf9, Nedd4l, Rab38, Skp2, Sna1, Socs1, and Ugt8a are direct targets of miR-30s, and they have potential effects on premetastatic niche formation.

Skp2 facilitates pulmonary vascular destabilization in premetastatic lungs, promotes metastasis, and reduces overall survival of tumor-bearing mice

Given that miR-30s could interfere with premetastatic niche formation through protecting vessel integrity, we next compared effects of Klf9, Nedd4l, Rab38, Skp2, Sna1, Socs1, and Ugt8a on endothelial cell permeability. In vascular mimic system, Skp2-transfected fibroblast induced a more than 4-fold increase in the leakage of endothelial monolayer compared with control (Fig. 5A), whereas Klf9 and Ugt8a increased the leakage for about 2-fold. Following experiments showed that knockdown of Skp2 or overexpression of miR-30s protected endothelial integrity and additional overexpression of Skp2 reversed the inhibitory effect of miR-30s (Fig. 5B), demonstrating that miR-30s inhibited vascular destabilization via suppression of Skp2. Because miR-30s protected pulmonary vascular integrity through modulation of MMP9, we detected the effect of Skp2 on the proteolytic activity of MMP9. As a result, the proteolytic activity of MMP9 was significantly elevated by Skp2 in primary lung fibroblasts (Fig. 5C), suggesting that Skp2 disrupted vascular integrity through activating MMP9.
We next compared levels of Klf9, Skp2, and Ugt8a between hyperpermeable and poorly permeable regions in premetastatic lungs. All of them were increased in hyperpermeable regions compared with poorly permeable regions (Fig. 5D), indicating their roles in modulating vessel integrity in premetastatic niches. Then, overexpression of Klf9, Skp2, and Ugt8a remarkably increased premetastatic lung vascular leakage to at least 2-fold (Fig. 5E and F). Consistently, Skp2 displayed the strongest effect. We further assessed functions of Klf9, Skp2, and Ugt8a individually. Preintrapleurally injected Skp2 induced a more than 7-fold leakage in premetastatic lungs, whereas knockdown of Skp2 or overexpression of miR-30s inhibited vascular destabilization (Fig. 5G and H). Additional overexpression of Skp2 reversed the inhibitory effect of miR-30s, indicating that miR-30s inhibited vascular destabilization through Skp2. Klf9 and Ugt8a showed similar effects but to much less extent (Supplementary Fig. S6A–S6D).

We confirmed the effect of Skp2 on pulmonary vascular permeability through in vivo lectin–dextran permeability assay. As shown in Fig. 6A and B, large amounts of rhodamine-dextran were leaked from blood vessels in Skp2 group (12-fold increase), indicating that Skp2 significantly enhanced the pulmonary vascular permeability. Knockdown of Skp2 or overexpression of miR-30s inhibited vascular destabilization. Additional overexpression of Skp2 reversed the inhibitory effect of miR-30s. These results were consistent with our findings by Evans blue assays.

To address whether miR-30 targets could facilitate lung metastasis, mice were preintrapleurally injected with lentivirus encoding Skp2 and examined for lung metastasis. Primary tumor growth was unaffected (Supplementary Fig. S7). Numbers of metastatic nodules in lungs were significantly induced by overexpression of Skp2 compared with vector-injected mice (Fig. 6C and D). Furthermore, the median survival of mice preintrapleurally injected with vectors was 36 days, whereas median survival of mice received treatments of Skp2 was significantly reduced to 22 days (Fig. 6E).

Therefore, the above results prove that Skp2, as a direct target of miR-30s, disrupts vascular integrity in premetastatic lungs and decreases overall survival of tumor-bearing mice by accelerating tumor metastasis.
DNMT1 (29), and TGF

have been reported to be suppressed by BMP-2 (27), c-Myc (28),

blasts mediates the downregulation of miR-30s. Besides, miR-30s

explore whether primary tumor

ability to promote lung metastasis (5, 8). It will be interesting to

cated as a tumor-secreted inducer of distant organ hyperperme-

bination between tumor cells and lung

modulating the premetastatic niche (26), it is possible that

models. Because tumor-derived exosomes play crucial roles in

vascular permeability in premetastatic lungs through Evans blue assay. Mice were

preintrapleurally transfected with Klf9, Skp2, or Ugt8a (n = 7). F, quantification of E. Error bars, min to

max. G, analysis of vascular permeability in premetastatic lungs through Evans blue assay. Mice were

preintrapleurally transfected with indicated lentiviruses (n = 7). H, quantification of G. Error bars, min to

max. P values, Student t test. Data were representative of ≥3 independent experiments. Error

bars, SD; *P < 0.05; ** and

***, P < 0.01; ****, P < 0.001.

Figure 5. Skp2 induced pulmonary vascular hyperpermeability. A, quantified results of vascular mimic system

induced by conditioned media from fibroblasts transfected with indicated miR-30 targets. Error bars, SD. B, quantified results of vascular mimic system induced by conditioned media from fibroblasts transfected with indicated reagents. Error bars, SD. C, gelatin zymography analysis of effects of Skp2 on the proteolytic activity of MMP2 and MMP9. D, qRT-PCR analysis of Klf9, Skp2, and Ugt8a in hyperpermeable and poorly

permeable regions of mouse lungs. Error bars, SD. E, analysis of vascular permeability in premetastatic lungs through Evans blue assay. Mice were

preintrapleurally transfected with Klf9, Skp2, or Ugt8a (n = 7). F, quantification of E. Error bars, min to

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max. P values, Student t test. Data were representative of ≥3 independent experiments. Error

bars, SD; *, P < 0.05; ** and

***, P < 0.01; ****, P < 0.001.

Discussion

The precise mechanisms that dictate metastasis of a primary tumor to a predetermined metastatic location are unclear. Specific unknown stimuli provided by fibroblasts create a niche that promotes the metastatic spread of tumor cells in distant organs (25). Here, we present direct evidence that host-derived miRNAs play important roles in the formation of permissive niches in distant organs for metastatic tumor cells. Within days following tumor implantation, miR-30s become downregulated in resident fibroblasts within lungs which are conventional sites of metastasis for B16 melanoma tumor. Subsequently, Skp2, a direct target of miR-30s, is upregulated in lung fibroblasts to facilitate the extravasation of tumor cells by permeabilizing lung endothelium. These studies suggest multiple possibilities for clinical translation of the findings.

Our results illustrate that B16 cells repress miR-30s independent of cell-to-cell contact interactions throughout in vitro and in vivo models. Because tumor-derived exosomes play crucial roles in modulating the premetastatic niche (26), it is possible that exosomal communication between tumor cells and lung fibroblasts mediates the downregulation of miR-30s. Besides, miR-30s have been reported to be suppressed by BMP-2 (27), c-Myc (28), DNMT1 (29), and TGFβ (22). Intriguingly, TGFβ has been implicated as a tumor-secreted inducer of distant organ hyperpermeability to promote lung metastasis (5, 8). It will be interesting to explore whether primary tumor–derived TGFβ along with other factors, which facilitate the formation of premetastatic niche, can inhibit expression of miR-30s in lung fibroblasts.

Although miR-30s share high sequence homology, the expression of this family is differentially regulated. In mice, the miR-30 family is expressed in different clusters: miR-30b and miR-30d are in one cluster; miR-30c-1 and miR-30e are in another cluster. Some of our data show differences between each miR-30s, which is possible to be partially dependent on miRNA cluster expression. Tumor-induced adaptation of endothelial cells occurs during early premetastatic niche formation (3). Enhanced vascular permeability could then facilitate tumor cell dissemination through multiple means (13). We and others have previously described that certain factors can disrupt endothelial tight junctions and break the integrity of capillary walls to facilitate extravasation (5–8). Here, our investigation into the functional target genes of miR-30s reveals direct targeting of a number of novel premetastatic genes, including Klf9, Nedd4l, Rab38, Skp2, Snai1, Soc31, and Ugt8a. Skp2 is an F-box protein that forms the SCF complex to constitute an E3 ligase for ubiquitylation. Previous observations demonstrate that Skp2 overexpression contributes to tumorigenesis in various types of cancer (30, 31). Moreover, inactivation of Skp2 represents a promising approach for cancer prevention and treatment (32, 33). Together with our findings, systematic inhibition of Skp2 may exert an overall arrest for tumor progression by targeting both primary tumor and metastatic niche. Understanding mechanisms of Skp2-induced vascular destabilization, which is an ongoing
direction in our laboratory, may reveal additional strategies for premetastatic intervention. Besides Skp2, other target genes of miR-30s possibly will regulate the formation of premetastatic niche through mechanisms yet to be unraveled.

It is likely that additional pathways regulated by miR-30s also contribute to the antitumor effects. Although overexpression of miR-30s in distant organs has no effect on primary tumor growth, interestingly, miR-30s have been reported as tumor suppressors that inhibit proliferation in pancreatic cancer (34), hepatocellular carcinoma (35), breast cancer (36), myeloma (37), and colorectal cancer (17). The miR-30 family is also implicated to inhibit epithelial–mesenchymal transition in prostate cancer cells (38), pancreatic cancer cells (39), and hepatocytes (22). In addition, miR-30s are crucial in the inhibition of breast tumor initiation (24). Low serum level of miR-30d is associated with improved prognosis and overall survival in NSCLC (40). The upregulation of miR-30 plays biologic roles in cellular senescence (41) and trastuzumab-induced cell growth arrest (19). In together with our findings, these may suggest combined effects of miR-30s on both primary tumors and distant organs, resulting in the inhibition of tumor proliferation, intravasation, and extravasation. Understanding these mechanisms and their relevance to cancer progression will provide further rationales for using miR-30s as a treatment for metastatic tumors.

Our findings indicate several avenues for potential translational applications in the clinical management of metastasis. Overexpression of miR-30s significantly reduces metastases in vivo. Consistently, treatment of mice with Skp2 is capable of facilitating metastasis. Therefore, miR-30s and Skp2 have the potential for targeted antimetastasis therapeutics.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: F. Qi, T. He, N. Song, Y. Luo
Development of methodology: F. Qi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Qi, L. Jia, L. Guo, X. Ma, C. Wang, M. Xu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. He
Writing, review, and/or revision of the manuscript: F. Qi, T. He, L. Li, Y. Luo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Fu
Study supervision: T. He, Y. Fu

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