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MicroRNA-27b suppresses tumor progression by regulating ARFGEF1 and focal adhesion signaling

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The non-receptor tyrosine kinase c-Src is frequently activated during progression of colon cancers. In this study, we found that among the c-Src-regulated microRNAs (miRNAs), miR-27b is also repressed by activation of K-Ras/H-Ras. Inhibitor studies suggested that the phosphatidylinositol 3-kinase pathway is involved in the repression of miR-27b. MicroRNA-27b was repressed in various colon cancer cell lines and tumor tissues. Re-expression of miR-27b in human colon cancer HCT116 cells caused morphological changes and suppressed tumor growth, cell adhesion, and invasion. We also identified ARFGEF1 and paxillin as novel targets of miR-27b, and found that miR-27b-mediated regulation of ARFGEF1 is crucial for controlling anchorage-independent growth, and that of paxillin is important for controlling cell adhesion and invasion. Re-expression of miR-27b suppressed the activation of c-Src induced by integrin-mediated cell adhesion, suggesting that repression of miR-27b may contribute to c-Src activation in cancer cells. These findings show that miR-27b functions as a tumor suppressor by controlling ARFGEF1 and the paxillin/c-Src circuit at focal adhesions.

Malignant progression of colorectal tumor is induced by subsequent accumulation of genetic alterations, often including mutations in K-Ras, B-Raf, phosphatidylinositol 3-kinase (PI3K), and p53, as well as gains or losses of chromosomes.[1] In addition, the tyrosine kinase c-Src is often upregulated or activated during the progression of colon cancer.[2–5] However, despite the accumulation of genomic data on human cancers, mutations in the *SRC* gene have rarely been observed;[6] therefore, the upregulation of c-Src (and the resultant contribution to cancer progression) is thought to result from dysregulation of c-Src expression or activity.

The non-receptor tyrosine kinase c-Src serves as a molecular switch that coordinately controls various cellular functions, including cell proliferation, adhesion, migration, invasion, and metastasis.[7] In the resting state, c-Src is inactivated through phosphorylation at the negative regulatory site Tyr527 by CSK.[8] After stimulation with growth factors or ECM proteins, c-Src is activated and triggers downstream signaling pathways, including Ras/MAPK, PI3K/Akt, and STAT3. Although the underlying mechanisms remain elusive, many studies have shown that the expression levels and specific activity of c-Src are elevated during the development of various human cancers, including lung, breast, prostate, and colon cancers.[9] To elucidate the molecular mechanisms underlying c-Src-induced transformation and its role in tumor progression, we developed a model system using $Csk^{-/-}$ mouse fibroblasts, in which activated wild-type c-Src induces cell transformation.[10] Using this system, we have analyzed molecular events downstream of upregulated c-Src. Our results revealed that c-Src upregulation induces repression of a group of microRNAs (miRNAs), including miR-99a, miR-542, miR-503, miR-322 (miR-424 in human), miR-27b, miR-23b, and miR-450a.[11] Subsequent studies showed that miR-99a controls tumor growth by targeting mammalian target of rapamycin (mTOR) and fibroblast growth factor receptor (FGFR) in human lung cancer, and that miR-542-3p targets integrin-linked kinase, resulting in the downregulation of cell adhesion and invasion of human colon cancer.[12] In addition, the miR-503/-424 cluster strictly controls tumor progression by targeting Rictor, one of the components of mTORC2.[13] These findings suggest that specific miRNAs are involved in controlling tumor progression induced by c-Src upregulation.

To further extend our understanding of the role of miRNA in c-Src-mediated tumor progression, we focused on determining the function of miR-27b, which is downregulated in human cancers, including colon, lung, breast, and prostate cancer,[14,15] suggesting that it may function as a tumor suppressor.[16–18] The mechanisms underlying miR-27b downregulation, as well as the critical targets of this miRNA in human cancers, remain to be elucidated.

Here, we show that miR-27b expression is repressed not only by c-Src upregulation, but also by activation of K-Ras/H-Ras. MicroRNA-27b is also repressed in various human cancer cell lines and tumor tissues, implying that its expression is controlled downstream of a wide range of oncogenic signals. We also show that miR-27b directly targets ARFGEF1 and paxillin to suppress tumor growth and invasion in human colon cancers, and that miR-27b-mediated repression of paxillin

attenuates focal adhesion-mediated signaling. The latter finding suggests that repression of miR-27b accounts for the activation of c-Src in human cancers. Our results suggest that repression of miR-27b contributes to malignant progression of a wide range of human colon cancers, and raises the possibility that miR-27b serves as a prognostic marker in human colon cancers.

Materials and Methods

Tissue samples. Snap-frozen colon tissues were divided visually into tumor (T) and non-cancerous (N) regions that were then confirmed histologically. The research protocol for the collection of human samples was approved by the ethical review board of the Graduate School of Medicine, Osaka University (Osaka, Japan). Informed consent was obtained from all patients in writing before enrolment in the study.

***In vivo* tumorigenicity.** Cells (2×10^6 in 200 μL serum-free medium) were s.c. injected into nude mice (BALB/cAJcl-nu/nu) purchased from SLC (Hamamatsu, Japan). Tumor length (L) and width (W) were measured every 2–3 days. Tumor volume was calculated as $0.5 \times L \times W^2$. All animal experiments were carried out in accordance with the protocols approved by the Animal Research Committee of Research Institute for Microbial Diseases, Osaka University.

Other methods are described in Data S1.

Results

MicroRNA-27b downregulated by PI3K/Akt pathway in cancer cells. Previously, we showed that the expression of miR-27b was repressed by c-Src upregulation in a model system based on $Csk^{-/-}$ cells.[11] In this study, we first examined mouse embryonic fibroblasts transformed with v-Src, H-Ras, or K-Ras. Quantitative real-time PCR analysis revealed that miR-27b was significantly repressed by transformation induced by all of the oncogenes we tested, suggesting that miR-27b expression is controlled downstream of multiple oncogenic pathways involving Src and Ras (Fig. 1a).

Next, we analyzed miR-27b expression in human colon cancer cell lines (Fig. 1b). Quantitative real-time PCR analysis revealed that miR-27b levels were substantially reduced in HCT15, HCT116, and HT29 cells relative to those in normal cells (FHC), whereas SW480 and SW620 cells exhibited more moderate repression of miR-27b. We also observed repression of miR-27b in human prostate cancer cell lines (Fig. S1a). Western blot analysis showed that HCT15, HCT116, and

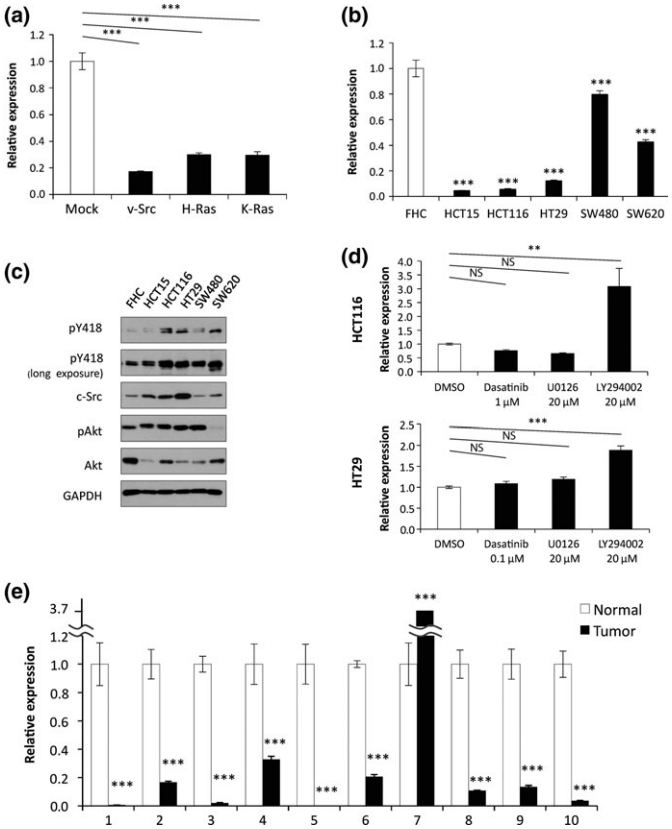

Fig. 1. MicroRNA-27b (miR-27b) is downregulated in human colon cancer. (a) Quantitative real-time PCR analysis of miR-27b expression in mouse embryonic fibroblasts transfected with v-Src, H-Ras, or K-Ras. (b) Expression levels of miR-27b in human colon cancer cells (HCT15, HCT116, HT29, SW480, and SW620) and in normal human colon epithelial cells (FHC). (c) Western blot analysis of expression and activity of c-Src and Akt. (d) Effects of the indicated inhibitors on miR-27b expression in HCT116 and HT29 cells. (e) Expression levels of miR-27b in human colon cancer tissues (black bar) and the adjacent normal tissues (white bar). $^{**}P < 0.01$, $^{***}P < 0.001$, *t*-test. NS, not significant.

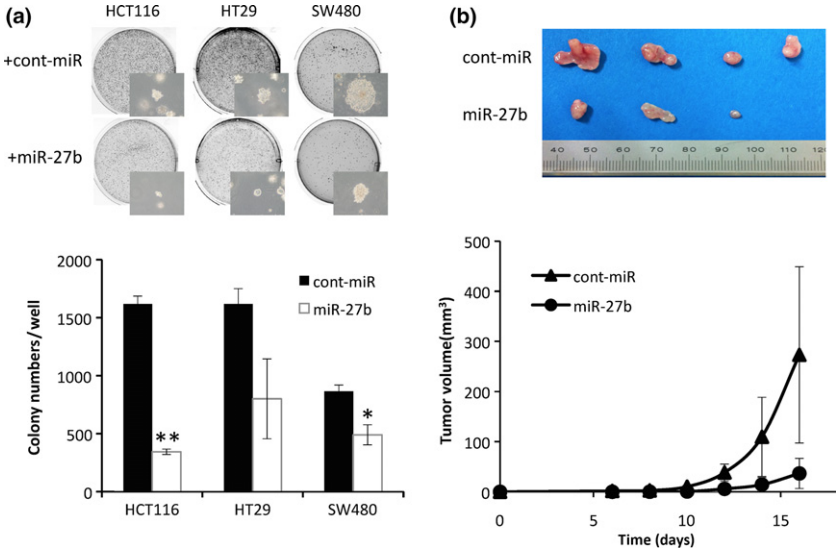

Fig. 2. MicroRNA-27b (miR-27b) suppresses tumor growth in human colon cancer cells *in vitro* and *in vivo*. (a) Colon cancer cells transfected with 30 nM cont-miR (control) or miR-27b were subjected to colony formation assay. (b) HCT116 cells transfected with 30 nM cont-miR or miR-27b were subjected to tumorigenicity assay in nude mice. $^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test.

HT29 cells had higher levels of c-Src activity and protein, as well as activated Akt (Fig. 1c). By contrast, SW480 cells had lower levels of c-Src activity and protein, and SW620 cells showed specific reduction in Akt activity. These results suggest that expression levels of miR-27b correlate with the activation status of c-Src and/or Akt.

To identify pathways leading to the repression of miR-27b, we used dasatinib, U0126, and LY294002 to inhibit Src family kinases, the MAPK pathway, and the PI3K pathway, respectively. Quantitative real-time PCR analysis of HCT116 and HT29 cells revealed that only LY294002 increased miR-27b expression (Fig. 1d), suggesting that the PI3K/Akt pathway plays a role in the repression of miR-27b. Unexpectedly, inhibition of Src kinases with dasatinib did not affect miR-27b expression. This phenomenon might be explained by the fact that HCT116 and HT29 cells have activating mutations in PI3K; consequently, even though c-Src was inhibited, the constitutively-activated PI3K/Akt pathway repressed miR-27b expression. The inefficacy of MEK inhibitor U0126 further suggested that the Ras/PI3K pathway, rather than the Ras/MAPK pathway, is the primary cause of miR-27b repression. The same analysis in SW480 cells that have wild-type PI3K showed that LY294002 had no effect on miR-27b expression (Fig. S2a). In addition, introduction of a constitutively active PI3K suppressed miR-27b expression in normal epithelial MCF10A cells (Fig. S2b). These observations suggest that the PI3K/Akt pathway, which can be activated downstream of c-Src and/or Ras, contributes significantly to repression of miR-27b in human cancers.

Because mutations in K/H-Ras and PI3K are frequently detected in human colon cancers, it is likely that miR-27b is repressed in a wide range of colon cancer tissues. We revealed that the level of miR-27b expression was markedly decreased in tumors from 9 out of 10 patients, relative to the level in adjacent normal tissues (Fig. 1e). These results suggest that expression of miR-27b is repressed in a wide range of human cancers, potentially through the Src/Ras/PI3K pathway.

MicroRNA-27b suppresses tumor growth of human colon cancer cells. To evaluate the role of miR-27b in human cancers, we investigated whether miR-27b would suppress tumor growth of human colon cancer cells. Soft agar colony formation assays showed that re-expression of miR-27b markedly decreased the number of colonies in HCT116 and HT29 cells (Fig. 2a), but was less effective in SW480 cells, which express

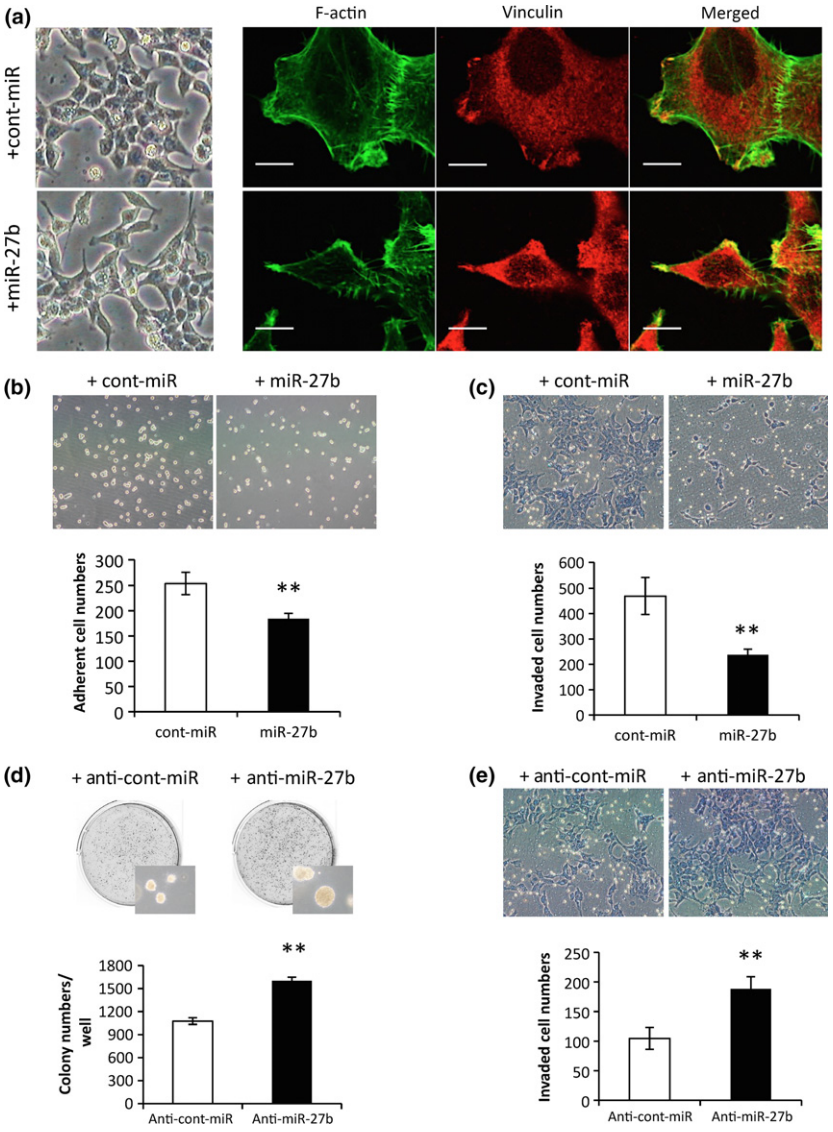

Fig. 3. MicroRNA-27b (miR-27b) suppresses colon cancer cell adhesion and invasion. (a) Phase-contrast images of HCT116 cells transfected with 30 nM cont-miR (control) or miR-27b (left panels). Cells were stained for F-actin (green) and vinculin (red) (right panels). Scale bar = 10 μm. (b) HCT116 cells were transfected with 30 nM cont-miR or miR-27b for 2 days and subjected to cell adhesion assay on fibronectin-coated dishes. (c) HCT116 cells were transfected with 30 nM cont-miR or miR-27b, and subjected to invasion assay using a Matrigel-coated invasion chamber. (d, e) HCT116 cells were transfected with 5 nM anti-cont-miR or anti-miR-27b, and subjected to colony formation assay (d) and invasion assay (e). $^{**}P < 0.01$, *t*-test.

miR-27b at higher levels than other cell types (Fig. 1b). These growth-suppressive effects were also observed in prostate cancer cells (Fig. S1b). Re-expression of miR-27b grossly suppressed tumorigenicity of HCT116 cells in nude mice (Fig. 2b). Thus, miR-27b has the potential to suppress tumor growth of human colon cancer cells both *in vitro* and *in vivo*.

MicroRNA-27b suppresses cell adhesion and invasion of human cancer. The effects of miR-27b re-expression on the cell morphology and invasiveness of HCT116 cells was examined. Transfection of miR-27b caused morphological changes to the spindle-like shape (Fig. 3a). Notably, staining for F-actin, vinculin, and paxillin revealed that miR-27b transfection suppressed the formation of focal adhesions (Figs 3a and S3a). Because focal adhesions are crucial for cell adhesion, motility, and invasion, we tested whether miR-27b would inhibit adhesion and invasion in these cells. Assays on fibronectin-coated plates revealed that miR-27b induced a significant decrease of adhered cells (Fig. 3b). In addition, Matrigel assays revealed that miR-27b significantly suppressed the invasive activity of HCT116 cells (Fig. 3c). We observed similar effects of miR-27b on focal adhesions and cell invasion in DU145 prostate cancer cells (Fig. S3b,c).

To confirm the role of miR-27b in cancer cells, we introduced anti-miR-27b into HCT116 cells, in which miR-27b is still expressed, albeit at a low level. Colony formation and Matrigel invasion assays revealed that inhibition of miR-27b significantly promoted anchorage-independent cell growth (Fig. 3d) and invasive activity (Fig. 3e). These findings suggest that miR-27b has the ability to suppress tumor growth and progression in human cancers.

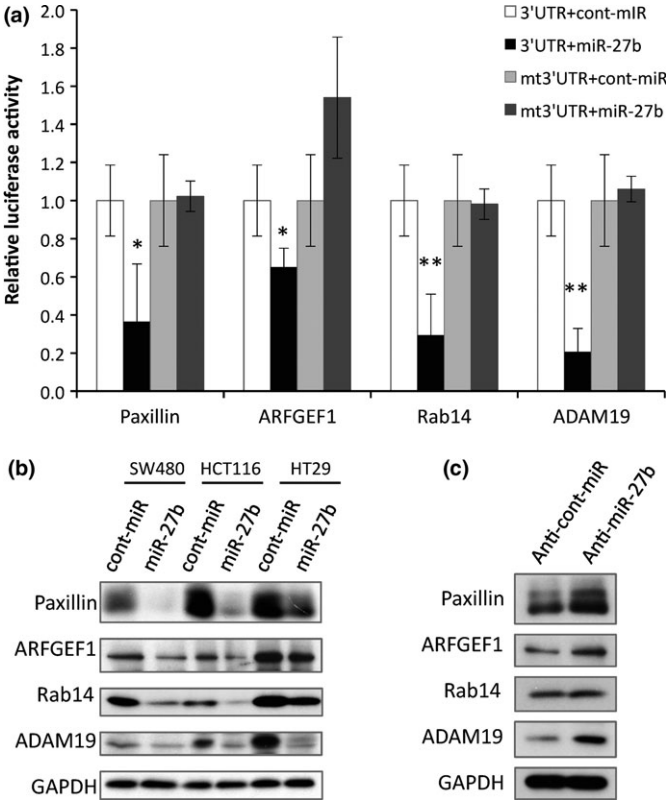

Fig. 4. MicroRNA-27b (miR-27b) directly targets paxillin, ARFGEF1, Rab14, and ADAM19. (a) $Csk^{-/-}$/c-Src cells were cotransfected with the pMIR-Luc plasmid containing the wild-type or mutated (mt) 3′-UTR of each target and 30 nM cont-miR (control) or miR-27b, and subjected to luciferase reporter assay. (b) Western blot analysis of miR-27b targets in colon cancer cells transfected with 30 nM cont-miR or miR-27b. (c) Western blot analysis of the targets in HCT116 cells transfected with anti-cont-miR or anti-miR-27b (5 nM). $*P < 0.05$, $**P < 0.01$, *t*-test.

MicroRNA-27b targets paxillin/ARFGEF1/Rab14/ADAM19. We searched potential targets of miR-27b in human genes using TargetScan. Among the predicted genes, we chose 20 related to cell adhesion and invasion, and validated our findings by luciferase reporter assay in c-Src-transformed cells. The luciferase activities of constructs containing the predicted target sites of paxillin, ARFGEF1, Rab14, and ADAM19 were significantly reduced in miR-27b transfected cells (Fig. 4a). The miR-27b-mediated reduction of luciferase activities was abolished by mutation of the recognition sites, confirming that miR-27b interacts specifically with these target sequences. Consistent with the results of the luciferase assays, Western blot analysis revealed that paxillin, ARFGEF1, Rab14, and ADAM19 protein levels were decreased by miR-27b treatment of human cancer cells (Fig. 4b). Conversely, anti-miR-27b increased the levels of these proteins (Fig. 4c). These findings suggest that miR-27b suppresses tumor growth and progression by targeting multiple genes related to cell adhesion and invasion.

MicroRNA-27b-mediated regulation of ARFGEF1 expression is crucial for tumor growth. Although these candidate targets have different functions, they are all implicated in cell dynamics. Paxillin is a component of the focal adhesion complex, which links the ECM to F-actin in cells.[19] ARFGEF1 is the guanine nucleotide exchange factor for the ADP ribosylation factors ARF1 and ARF3, which play important roles in membrane trafficking.[20,21] Rab14 is also involved in membrane trafficking between the Golgi complex and endosomes; in particular, it transports ADAM10 and FGFR, which are involved in the regulation of cell–cell junctions and embryonic development.[22–24] A member of the a disintegrin and metalloproteinase (ADAM) family of proteins, ADAM19 promotes shedding of growth factors and cytokines, such as neuregulin.[25,26]

To elucidate the roles of these targets in cancer-related phenotypes, we used shRNAs to knock down paxillin, ARFGEF1, Rab14, and ADAM19 in HCT116 cells (Fig. 5a). Soft agar colony formation was suppressed by knockdown of paxillin or ARFGEF1, but not Rab14 or ADAM19 (Fig. 5b). Notably, ARFGEF1 knockdown decreased the number of colonies significantly, to a level comparable with that in miR-27b-transfected cells (Fig. 2a). Similar results were obtained in HT29 and DU145 cells (Fig. 5c). Re-expression of ARFGEF1 rescued the colony formation of the ARFGEF1 knockdown cells, confirming that suppression of tumor growth by sh-ARFGEF1 is not due to an off-target effect (Fig. 5d). Furthermore, overexpression of ARFGEF1 restored colony formation of cells transfected by miR-27b (Fig. 5e). These results suggest that ARFGEF1 is crucial for tumor growth and that repression of ARFGEF1 largely contributes to miR-27b-mediated growth suppression.

The target of ARFGEF1, ARF1, promotes activation of Akt and ERK1/2 in breast cancer.[27] In addition, ARFGEF1 promotes activation of Akt and ERK1/2 in neuronal cells.[28] Consistent with these findings, when cells were cultured on non-adherent polyhydroxyethylmethacrylate-coated dishes, levels of the active form of Akt (pAkt) were reduced in ARFGEF1-knockdown cells, as well as in miR-27b-transfected cells (Fig. 5f,g). However, re-expression of ARFGEF1 did not attenuate the suppression of pAkt caused by miR-27b (Fig. 5h),

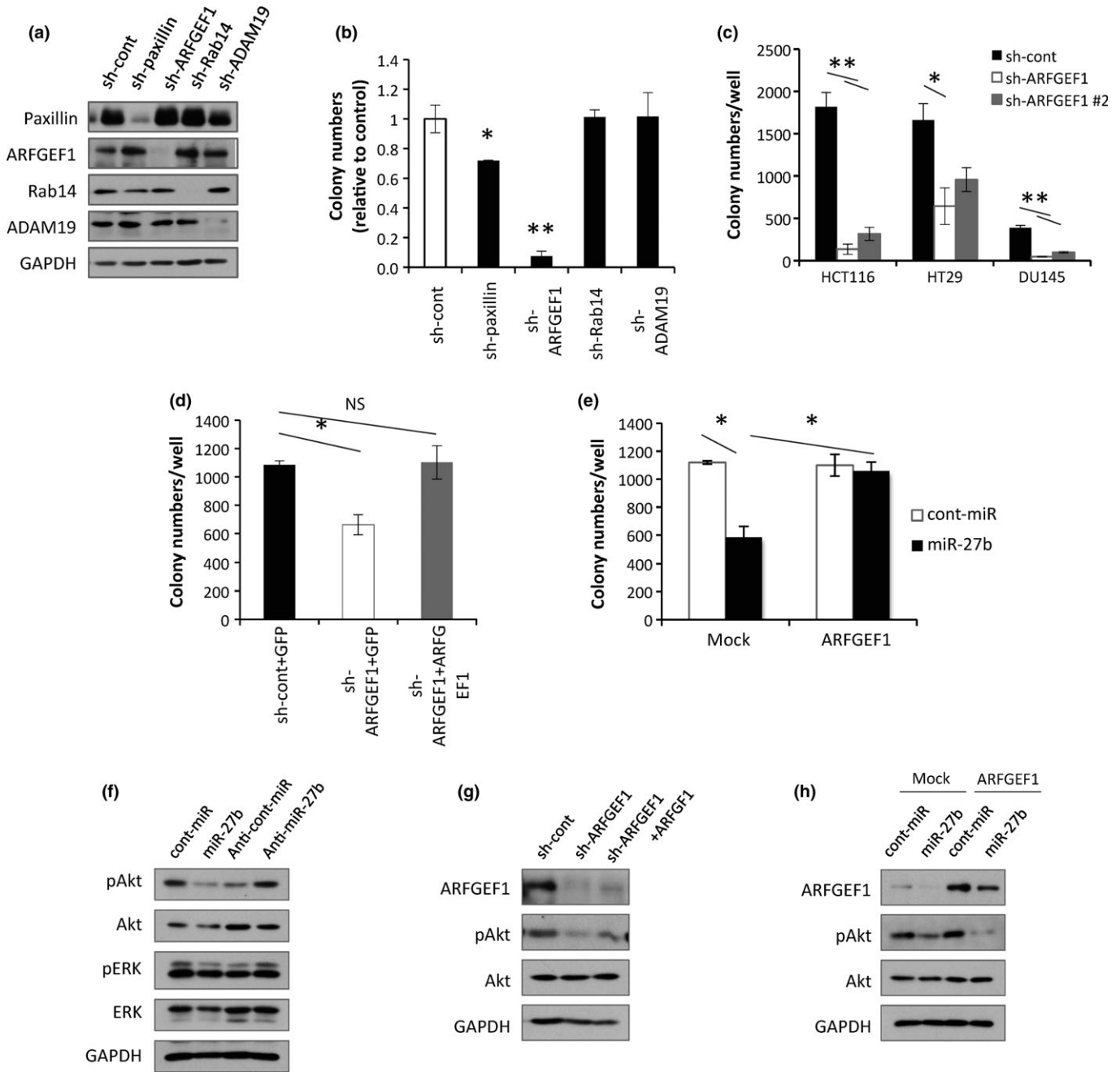

Fig. 5. ARFGEF1 is crucial for colon cancer tumor growth. (a) Western blot analysis of microRNA-27b (miR-27b) targets in HCT116 cells, in which target was knocked down by each shRNA. (b) Cells used in (a) were subjected to colony formation assay. (c) Colony formation assay of ARFGEF1-depleted cancer cells. (d) Effect of ARFGEF1 re-expression on colony formation of ARFGEF1-depleted HCT116 cells. (e) Effect of ARFGEF1 overexpression on the growth inhibition by miR-27b in HCT116 cells. (f) HCT116 cells transfected with the indicated miRNAs were cultures on polyhydroxyethylmethacrylate coated dishes, and the activity status of Akt or ERK was assessed by Western blot analysis. (g, h) Cells used in (d, e) were cultured on polyhydroxyethylmethacrylate-coated dishes, and the activity status of Akt was assessed by Western blot analysis. $*P < 0.05$, $**P < 0.01$, *t*-test. NS, not significant.

suggesting that other downstream targets of ARFGEF1, which are responsible for tumor growth, remain to be identified.

MicroRNA-27b-mediated regulation of paxillin is important for cell adhesion and invasion. We next examined contributions of paxillin, ARFGEF1, Rab14, and ADAM19 to cell adhesion and invasion. It was revealed that paxillin knockdown significantly attenuated cell adhesion and invasion by HCT116 cells (Fig. 6a,b). As mentioned above, miR-27b caused morphological changes and reduced the number of focal adhesions, concomitant with suppression of cell adhesion and invasive activity, in HCT116 cells (Fig. 3). Overexpression of paxillin in miR-27b-treated cells reversed the morphological changes (Fig. 6c,d,e) and restored cell adhesion and invasion (Fig. 6f, g). These findings suggest that paxillin is a target of miR-27b responsible for controlling cell adhesion and invasive potential in cancer cells.

MicroRNA-27b inhibits oncogenic signaling stimulated by cell adhesion. Paxillin serves as a platform on which tyrosine

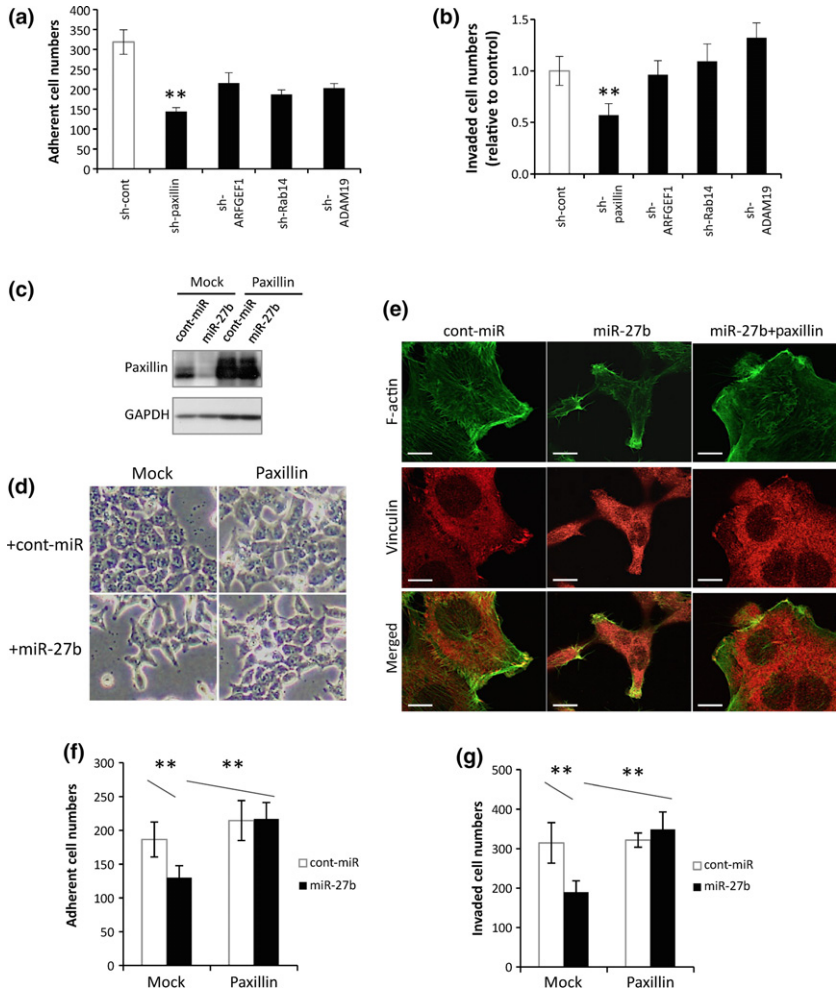

Fig. 6. Paxillin is crucial for microRNA27-b (miR-27b)-mediated regulation of colon cancer tumor progression. (a) HCT116 cells transfected with the indicated shRNA were subjected to adhesion assay on collagen-coated dishes. (b) Cells used in (a) were subjected to invasion assay. (c) HCT116 cells expressing mock or paxillin were transfected with 30 nM cont-miR (control) or miR-27b, and paxillin was detected by Western blotting. (d) Phase-contrast images of the cells used in (c). (e) HCT116 cells expressing mock or paxillin were transfected with 5 nM cont-miR or miR-27b, and subjected to cell staining for vinculin (red) and F-actin (green). Scale bar = 10 μm. (f, g) Cells used in (c) were subjected to adhesion (f) and invasion (g) assays. $^{**}P < 0.01$, *t*-test.

kinases such as c-Src and focal adhesion kinase (FAK) are activated.[29] To determine whether miR-27b regulates the activity of c-Src by regulating paxillin, we stimulated HCT116 cells by integrin-mediated cell adhesion. In cells transfected with cont-miR, c-Src was activated at sites of cell adhesion. By contrast, activation of c-Src was suppressed in miR-27b-transfected cells (Fig. 7). Furthermore, activation of ERK1/2 and FAK, a downstream signaling component, was also suppressed by miR-27b, indicating that miR-27b may negatively regulate c-Src signaling evoked by integrin-mediated cell adhesion.

Discussion

In this study, we showed that activation of the oncogenic c-Src/Ras/PI3K pathway induces repression of miR-27b, which plays a tumor-suppressive role in human colon cancer cells. MicroRNA-27b directly targets ARFGEF1 and paxillin, which are required for tumor growth and cancer cell adhesion/invasion, respectively. Therefore, we postulate that miR-27b-mediated upregulation of ARFGEF1 contributes to tumor growth by activating the ARFGEF1/Akt pathway, and that paxillin upregulation promotes formation of focal adhesions, resulting in the activation of cell motility and invasive/metastatic potential (Fig. 8).

Cancer cells accumulate mutations in proto-oncogenes and tumor suppressors, including K-Ras, TP53, and PI3K during tumor progression. We showed that miR-27b is downregulated by transformation by not only c-Src/v-Src, but also K-/H-Ras and PI3K. These results suggest that aberrant expression of miR-27b may be a relatively early event in cancer progression. Bioinformatic analysis of prostate cancer has shown that miR-27b is significantly downregulated in primary tumors, and this downregulation becomes more remarkable in metastatic tumors (Fig. S4). These observations raise the possibility that miR-27b could be useful as a prognostic marker in human cancers.

The ARFGEF1 protein localizes to the trans-Golgi network and activates the class I ADP ribosylation factors, ARF1 and ARF3, by promoting GDP–GTP exchange to initiate membrane vesicle formation. ARF1, which plays a role in vesicular trafficking,[30] is upregulated in invasive breast cancers and gastric cancers.[31,32] It also promotes the activation of the PI3K/Akt pathway and retinoblastoma protein, thereby promoting cell proliferation, migration, and invasion in breast cancer cells.[27,31] ARFGEF1 is also involved in cell adhesion and migration by regulating proper glycosylation and function of integrin β1. These facts implicate ARFGEF1/ARF1 in the progression of cancer phenotypes; to date, however, the direct link between ARFGEF1 and cancer has not been addressed. In this study, we found that depletion of ARFGEF1 significantly suppresses tumor growth of human cancer cells. This is the first evidence for a contribution of ARFGEF1 to cancer, suggesting that ARFGEF1 represents a novel therapeutic target for human cancer.

Focal adhesions connect the ECM and intercellular F-actin to control cell morphology, adhesion, and motility, which are important for cancer invasion and metastasis. Following cell

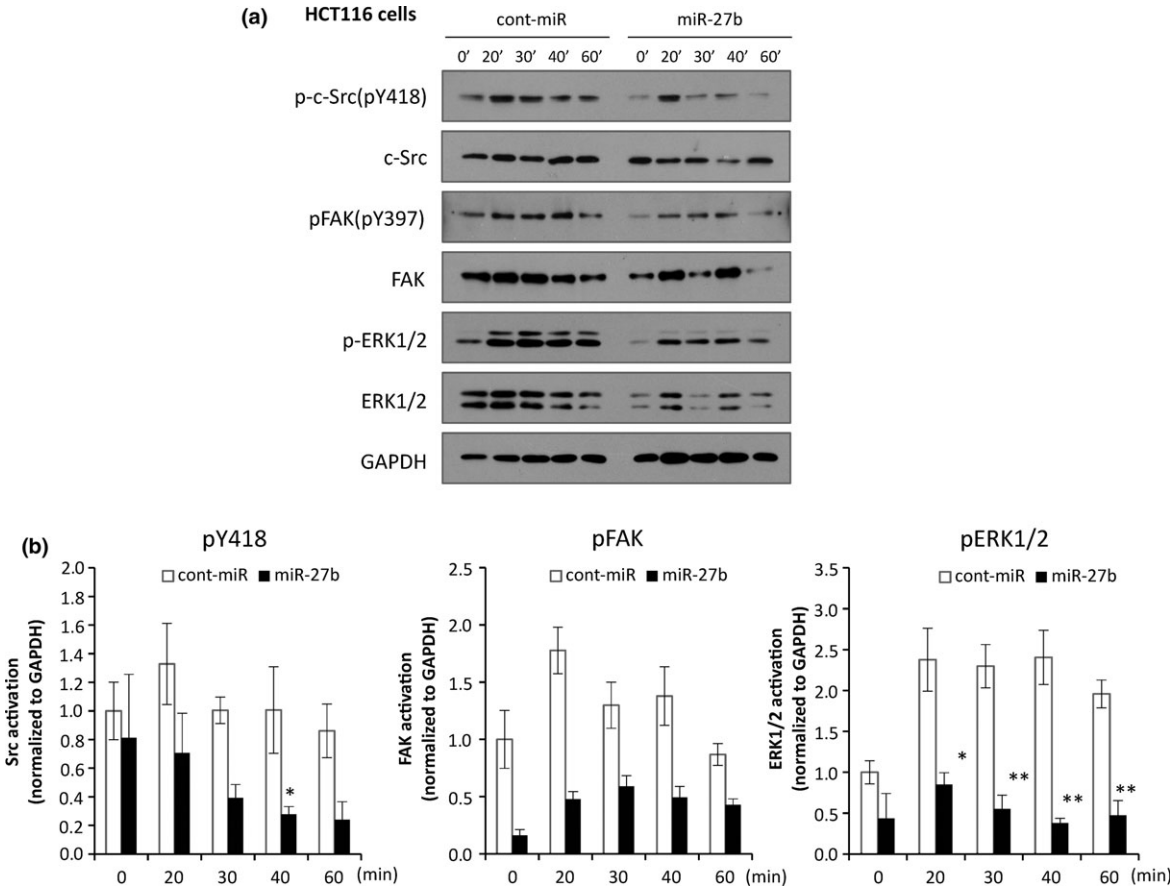

Fig. 7. MicroRNA-27b (miR-27b) suppresses c-Src activation stimulated by cell adhesion. (a) HCT116 human colon cancer cells transfected with 30 nM cont-miR (control) or miR-27b for 2 days were detached from the dishes and incubated in serum-free media at 37°C for 30 min. These cells were then plated on fibronectin-coated dishes, and the lysates of attached cells obtained at the indicated time points were analyzed for the activity status of the indicated proteins by Western blotting. (b) Quantitation of the signal intensities of c-Src (pY418), focal adhesion kinase (FAK) (pY397), and p-ERK1/2. Data are shown as means ± SD from three independent experiments. $*P < 0.05$, $**P < 0.01$, *t*-test. FAK, focal adhesion kinase.

adhesion by way of integrins, paxillin is heavily phosphorylated by c-Src and FAK, creating a platform for adaptor proteins that activate downstream intracellular signaling, thereby promoting cell growth and survival. Paxillin is upregulated in various human cancers and involved in tumor malignancies. Previously, however, the molecular connections between upregulated paxillin and downstream effectors, such as c-Src, and the mechanisms underlying paxillin upregulation remained unclear. In this study, we showed that repression of miR-27b by oncogene activation causes upregulation of paxillin. This upregulation may lead to elevated activation of c-Src signaling delivered from focal adhesions, resulting in the promotion of malignant phenotypes, that is, invasion and metastasis.

In colon cancers, c-Src activity is elevated with the progression of cancer stages, implying that the protein plays a role in malignant progression, that is, the acquisition of invasive and metastatic phenotypes. However, in contrast to other oncogenes such as *K-Ras* and *PI3K*, mutations in the *SRC* gene are rarely detected. In this study, we proposed a new mechanism for the upregulation of c-Src activity: upregulation of paxillin through downregulation of miR-27b. Recent work showed that c-Src is directly targeted by miR-23b, a tumor suppressor that is silenced by methylation in prostate cancer.[33] Both miR-23b and miR-27b are encoded by the same gene cluster, *miR-23b/27b/24-2*, located in the last intron of the aminopeptidase O gene, suggesting that expression of miR-23b and miR-27b is regulated through a common pathway. Indeed, we observed that the

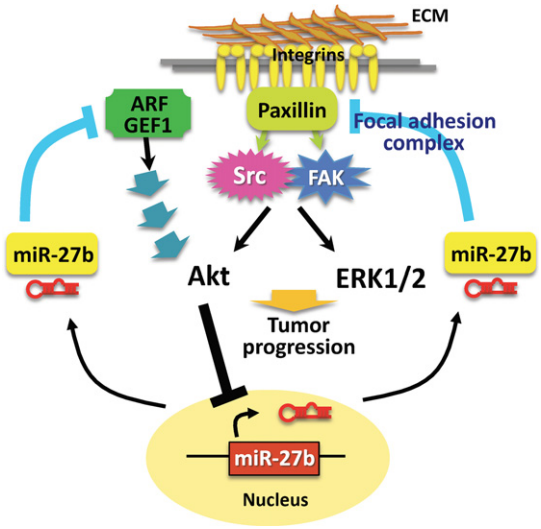

Fig. 8. Schematic model of microRNA-27b (miR-27b)-mediated regulation of colon cancer tumor progression. In normal cells expressing miR-27b, expression of paxillin and ARFGEF1 is limited to avoid unregulated cell growth and/or motility. Once miR-27b expression is downregulated due to activation of the Ras/Src/PI3K pathway, expression of paxillin and ARFGEF1 is elevated, resulting in rapid cell growth and stimulation of cell adhesion and invasion.

expression of miR-23b was repressed by c-Src-induced transformation with the same kinetics as miR-27b (Rei Matsuyama, unpublished data). If this is the case in human cancers, it is possible that the *miR-23b/27b/24-2* cluster regulates c-Src through dual mechanisms: regulation of the c-Src kinase activity by indirect upregulation of paxillin, and direct regulation of the c-Src protein level by miR-23b. Because the expression of miR-27b is regulated downstream of c-Src (Fig. 8), upregulation of c-Src may further amplify the positive-feedback loop mediated by the *miR-23b/27b/24-2* gene cluster, thereby promoting tumor malignancy mediated by c-Src activity. This model may account for the frequent upregulation of c-Src in various human cancers.

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Disclosure Statement

The authors have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials and methods.

Fig. S1. MicroRNA-27b (miR-27b) suppresses tumor cell growth in prostate cancer.

Fig. S2. PI3K/Akt pathway is responsible for microRNA-27b (miR-27b) repression.

Fig. S3. Inhibitory effect of microRNA-27b (miR-27b) on focal adhesion formation and cell invasion.

Fig. S4. Analysis of the microRNA-27b (miR-27b)/paxillin/ARFGEF1/Rab14/ADAM19 pathway in prostate cancer.