March 2016

A Doctoral Thesis

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

(miR-27b は ARFGEF1 および focal adhesion シグナルを制御することでがん悪性化を抑制する)

Graduate School of Frontier Biosciences,
Department of Oncogene Research, Research Institute for Microbial Diseases,
Osaka University

Rei Matsuyama

Contents

Abstract

The non-receptor tyrosine kinase c-Src is frequently activated during progression of colon cancers. However, the molecular mechanisms underlying c-Src activation and c-Src–induced tumor progression remain to be elucidated. Previously, we showed that c-Src upregulation causes repression of a subset of miRNAs that target several genes implicated in tumor progression. In this study, I found that among these c-Src–regulated miRNAs, miR-27b is repressed not only by c-Src upregulation but also by activation of K-Ras/H-Ras. Inhibitor studies suggested that the PI3K pathway is involved in the repression of miR-27b, implicating miR-27b in a wider range of colon cancers. Indeed, miR-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 invasive ability. I 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. Furthermore, 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 demonstrate that miR-27b functions as a tumor suppressor by controlling ARFGEF1 and the paxillin/c-Src circuit at focal adhesions.

Key words

Src, miRNA, tumor progression, AEFGEF1, paxillin, cell adhesion

General Introduction

Cancer

Cancer is one of the leading causes of death worldwide. Cancer arises from normal cells by "the gain of function" and "the loss of function". In the gain of function, oncogenes are amplified and become constitutively active to acquire the cancer phenotypes, while in the loss of function, tumor suppressor genes are decreased or lost resulting in being unable to stop cell growth and differentiation(Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Although numerous cancer drugs have been developed, the problems such as a side effect, recurrence of cancer and metastasis remain unsolved. Since more getting older, more risks of suffering cancer people have, the number of cancer patients is expected to continue to increase in an aging society. Thus, it is required to find new remedy and therapeutic targets of human cancers.

c-Src

c-Src, the cellular form of a Rous sarcoma virus gene *v-src*, is the first discovered proto-oncogene in vertebrate (review in (Martin, 2004)), and the product of *c-Src* gene is a non-receptor tyrosine kinase that plays a pivotal role in signal transduction leading to cell proliferation, differentiation, adhesion, migration and immune function(Brown and Cooper, 1996; Thomas and Brugge, 1997)(Figure GI-1).

c-Src shares a highly conserved structure with a protein family called Src family kinases (SFKs), which includes eight member proteins: c-Yes, Fyn, c-Fgr, Lyn, Hck, Lck, Blk and c-Src itself. The structure of SFK consists of five functional domains: N-terminal domain, Src homology 3 (SH3) domain, SH2 domain, Kinase domain (or SH1), and C-terminal domain(Brown and Cooper, 1996; Thomas and Brugge, 1997). N-terminal domain contains lipid modification signal: myristylation (in all SFKs) and palmitylation (in the all

except c-Src and Blk), which are important for membrane localization. SH3 domain contains a core consensus sequence of X-P-X-X-P to bind proteins bearing proline rich region. SH2 domain also controls protein interactions by recognizing phosphotyrosine.

Tyr416 in kinase domain and Tyr527 (in chicken c-Src) in C-terminal domain play a crucial role for regulating c-Src activity(Okada, 2012). Normally, Tyr527 is phosphorylated by C-terminal Src kinase Csk(Cooper et al., 1986; Nada et al., 1991; Okada and Nakagawa, 1989) (Figure GI-1). The SH2 domain interacts with pY527 and this interaction keeps the kinase domain in a closed, inactive conformation(Xu et al., 1997; Young et al., 2001). In contrast, the dephosphorylation of pY527 by phosphatases causes activation of c-Src catalytic activity(Cartwright et al., 1987), and the following phosphorylation of auto-phosphorylation site Tyr416 allows c-Src to gain full activity. This balance between phosphorylation and dephosphorylation of c-Src is important for its function as a molecular switch.

Aside from the regulation by Csk and phosphatases, there are several mechanisms for c-Src activation. The proteins containing Src SH2 and SH3 binding domain, such as phosphorylated forms of Focal adhesion kinase (FAK) and CRK associated substrate (Cas), bind to SH2 and SH3 domains to promote activation of c-Src by abolishing intramolecular inhibitory interactions(Nasertorabi et al., 2006; Playford and Schaller, 2004; Thomas et al., 1998). In addition, growth factor signals through transmembrane tyrosine kinase receptors including platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR), and integrin-mediated cell adhesion signals also cause c-Src activation. These interactions are important for downstream signaling pathways, including MAPK, PI3K/Akt, STAT3 pathway, leading to cell proliferation and cell movements(Parsons and Parsons, 1997).

c-Src has been implicated in cancer (Figure GI-2): the expression and activity of c-Src are elevated with tumor progression in various human cancers,

including colorectal, lung, breast, pancreatic and ovarian cancers (Frame, 2002; Yeatman, 2004), and the inhibitors of c-Src activity have entered clinical trials(Aleshin and Finn). Several studies have shown that ectopic expression of v-Src into normal cells is sufficient to induce transformation(Jove and Hanafusa, 1987). In contrast, c-Src has poor transforming ability, unless c-Src is overexpressed and/or mutated(Oneyama et al., 2008). However, c-Src mutation has been rarely seen in human cancer tissues(Daigo et al., 1999; Irby et al., 1999; Laghi et al., 2001; Nilbert and Fernebro, 2000; Wang et al., 2000). Thus, the underlying mechanisms of c-Src deregulation in human cancer and how c-Src involves progression of cancer remain elusive.

miRNA

MicroRNAs (miRNA) are small non-coding, single-stranded RNAs of ~22 nucleotides found in plants, invertebrate and vertebrate animals (Figure GI-3).

miRNAs are transcribed by RNA polymerase II and are controlled by RNA pol II-associated transcription fanctors and epigenetic regulators(Lee et al., 2004). After the transcription, the primary miRNA (pri-miRNA), containing a long stem loop structure, goes thorough multi steps of maturation(Ha and Kim, 2014; Lee et al., 2002). The nuclear RNase III Drosha splices out the loop structure from pri-miRNA to release a small hairpin-shaped RNA of ~65 nucleotides called pre-miRNA(Lee et al., 2003). Pre-miRNA is exported to the cytoplasm by exportin 5 (EXP5)(Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003) and is cleaved by Dicer(Hutvágner et al., 2001). The cleaved small RNA duplex ~22 nucleotides long, named miRNA/miRNA*, is loaded on Argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC). Within the RISC, the dsRNA is unwound and the mature miRNA is selected to guide target recognition, while the complimentary strand miRNA* is subjected to degradation(Kawamata and Tomari, 2010). Finally, miRNA-RISC inhibits gene expression by translational repression, and/or mRNA degradation (Carthew and

Sontheimer, 2009). Since miRNAs recognize their targets with incomplete complementarity(Bartel, 2009; Lewis et al., 2005), miRNAs have a potential to target over one-third of human genes. Indeed, thorough the post-transcriptional repression of target genes, miRNA is involved in variety of biological functions including differentiation, development, immune system and survival(Baltimore et al., 2008; Carrington and Ambros, 2003; Kosik, 2006; Schickel et al., 2008; Wienholds and Plasterk, 2005). Conversely, deletion or incorrect expression of miRNAs leads to disease, including cancer.

Numerous reports have demonstrated that the aberrant expression of miRNAs is found in human cancers compared with normal tissues(Calin et al., 2002; Huang et al., 2010; Iorio et al., 2005; Iorio et al., 2007; Lu et al., 2005; Porkka et al., 2007), and that miRNAs play an important role in controlling tumor growth and progression by regulating the gene expression of tumor suppressor and oncoproteins(He et al., 2007; Johnson et al., 2005; Ma et al., 2010; Meng et al., 2007; Park et al., 2008; Zhang et al., 2007). For example, let-7, which is often downregulated in lung, breast and colon cancer, regulates tumor growth by targeting *RAS* oncogene(Johnson et al., 2005). Tumor suppressor *PTEN* expression is inhibited by miR-21, which is highly expressed in cancer(Meng et al., 2007). Thus, miRNAs or its antagonists have a potential to be a therapeutic target, and a prognostic and diagnostic biomarker. In addition to such benefit, analyzing the characteristic and molecular function of miRNAs contributes to clarify a new regulatory mechanism of oncogenic signaling pathways.

Introduction

Number of colorectal cancer patients and its mortality are increasing all around the world. Malignant progression of colorectal cancer is induced via subsequent accumulation of genetic alterations, often including mutations in K-Ras, B-Raf, PI3K, and p53, as well as gains or losses of chromosomes(Walther et al.). In addition, the tyrosine kinase c-Src is often upregulated or activated during the progression of colon cancer(Frame, 2002; Iravani et al., 1998; Irby and Yeatman, 2000; Talamonti et al., 1993; Walther et al.). However, despite the accumulation of genomic data on human cancers, mutations in the SRC gene have rarely been observed(Daigo et al., 1999; Irby et al., 1999; Laghi et al., 2001; Nilbert and Fernebro, 2000; Wang et al., 2000); 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.

Tyrosine kinase c-Src and cancer

c-Src serves as a molecular switch that coordinately controls various cellular functions, including cell proliferation, adhesion, migration, invasion, and metastasis(Brown and Cooper, 1996; Thomas and Brugge, 1997). In the resting state, c-Src is inactivated through phosphorylation at the negative regulatory site Tyr527 by CSK(Nada et al., 1991). Upon stimulation with growth factors or extracellular matrix proteins, c-Src is activated and triggers downstream signaling pathways, including the Ras/MAPK, PI3K/Akt, and STAT3(Thomas and Brugge, 1997). 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 cancer(Summy and Gallick, 2003). 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(Oneyama et al., 2008). Using this system, we have analyzed molecular events downstream of upregulated c-Src.

MicroRNAs are regulator of cancer

MicroRNAs (miRNAs) are endogenous small (~22 nucleotides) non-coding RNA that regulate gene expression by degrading and/or transcriptional inhibition of target mRNAs(Ambros, 2004; Bartel, 2004; Ventura and Jacks, 2009). Furthermore, miRNAs can recognize mRNAs with incomplete complementarity, increasing the set of genes that each miRNA can target(Lewis et al., 2005). Thus, miRNAs and their targets constitute a large genetic regulatory network linked to a wide range of cellular functions, including proliferation, differentiation, development, metabolism, and survival(Carrington and Ambros, 2003; Ivanovska et al., 2008; Kobayashi et al., 2008; Lee et al., 2005). Many studies have shown that miRNAs are aberrantly expressed in a substantial portion of human cancers, and that miRNAs play important roles in controlling tumor growth and metastasis through the post-transcriptional regulation of tumor suppressors and oncoproteins(Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Lu et al., 2005).

MicroRNAs involved in c-Src transformation

Our previous results revealed that c-Src upregulation in Csk-/- MEF induces repression of a group of micro RNAs (miRNAs), including miR-99a, miR-542, miR-503, miR-322 (miR-424 in human), miR-27b, miR-23b, and miR-450a(Oneyama et al., 2011). Subsequent studies showed that miR-99a controls tumor growth by targeting mTOR and 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(Oneyama et al., 2012). In addition, the miR-503/-424 cluster strictly controls tumor progression by

targeting Rictor, one of the components of mTORC2(Oneyama et al., 2013). 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, I focused on determining the function of miR-27b, which is downregulated in human cancers, including colon, lung, breast, and prostate cancer(Navon et al., 2009; Taylor et al., 2010), suggesting that it may function as a tumor suppressor(Ishihara et al., 2014; Lee et al., 2012; Wan et al., 2014). At present, the mechanisms underlying miR-27b downregulation, as well as the critical targets of this miRNA in human cancers, remain to be elucidated.

Here, I show that miR-27b expression is repressed not only by c-Src upregulation, but also by activation of K-Ras/H-Ras. miR-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. I 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. My 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.

Results

miR-27b expression is inhibited by c-Src induced transformation

As I described, miR-27b expression was downregulated by c-Src induced transformation in $Csk^{-/-}$ MEF(Oneyama et al., 2011). To further confirm c-Src-mediated miR-27b downregulation, I employed Dox-inducible system of c-Src in $Csk^{-/-}$ MEF (Figure 1a). In this system, c-Src expression and its activity increase with Dox treatment as shown in Figure 1b. Total RNAs were collected after the indicated times of Dox addition, and subjected to quantitative real-time PCR (qRT-PCR). Figure 1c shows that miR-27b expression was decreased with c-Src upregulation, suggesting that c-Src controls the expression of miR-27b for its transformation activity. To explore the role of miR-27b in c-Src-mediated transformation, I induced miR-27b expression in $Csk^{-/-}$/c-Src MEF and examined colony formation activity of the cells. Soft-agar colony-formation assay demonstrated that the number of colonies was decreased by miR-27b expression, suggesting that miR-27b suppresses c-Src-induced transformation (Figure 1d).

miR-27b is downregulated in human colon cancer

To figure out whether miR-27b is involved in transformation induced by the other oncogenes, I examined mouse embryonic fibroblasts (MEFs) transformed with v-Src, H-Ras, or K-Ras. qRT-PCR analysis revealed that miR-27b was significantly repressed by transformation induced by any of the oncogenes I tested, suggesting that miR-27b expression is controlled downstream of multiple oncogenic pathways involving Src and Ras (Figure 2a).

Next, I analyzed miR-27b expression in human colon cancer cell lines (Figure 2b). qRT-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. I also observed repression of miR-27b in human prostate cancer cell

lines (Figure 2c).

Western blot analysis demonstrated that HCT15, HCT116, and HT29 cells, had higher levels of c-Src activity and protein, as well as activated Akt (Figure 3a). By contrast, SW480 cells had lower levels of c-Src activity and protein, and SW620 cells exhibited specific reduction in Akt activity. These results suggest that expression levels of miR-27b correlate with the activation status of the c-Src and/or Akt. To identify pathways leading to the repression of miR-27b, I used dasatinib, U0126, and LY294002 to inhibit Src family kinases, the MAPK pathway, and the PI3K pathway, respectively. qRT-PCR analysis of HCT116 and HT29 cells revealed that only LY294002 increased miR-27b expression (Figure 3b), 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 on SW480 cells bearing K-Ras mutation and WT PI3K showed that LY294002 had no effect on miR-27b expression (Figure 3c). In addition, Dox-inducible constitutive active PI3K expression suppressed miR-27b in normal mammary epithelial cells, MCF10A (Figure 3d). These observations raise the possibility 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. I 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 (Figure 4). These results suggest that expression of

miR-27b is repressed in a wide range of human cancers, potentially through the Src/Ras/PI3K pathway.

miR-27b inhibited tumor growth of human colon cancer cells

To evaluate the role of miR-27b in human cancers, I 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 (Figure 5a), but was less effective in SW480 cells, which express miR-27b at higher levels than other cell types (Figure 2b). These growth-suppressive effects were also observed in prostate cancer cells (Figure 5b). Re-expression of miR-27b grossly suppressed tumorigenicity of HCT116 cells in nude mice (Figure 5c). Thus, miR-27b has the potential to suppress tumor growth of human colon cancer cells both in vitro and in vivo.

miR-27b suppresses cell adhesion and invasion of human cancer

Next, the effects of miR-27b re-expression on the cell morphology and invasiveness of HCT116 (colone) and DU145 (prostate) cells were examined. Transfection of miR-27b caused morphological changes to the spindle-like shape (Figure 6a and 6b, left). Notably, staining for F-actin and vinculin (universal focal adhesion maker) revealed that miR-27b transfection suppressed the formation of focal adhesions (Figure 6a and 6b right). It is known that c-Src localizes focal adhesions to transduce extracellular signals to its downstream substrates. Figure 7a show that the localization of pY418 to focal adhesions was reduced by miR-27b re-expression in HCT116 cells. In DU145 cells, miR-27b also interfered the localization of pY418 to invadopodia (white arrow), which is an important structure for degrading extracellular matrix and subsequent cell invasion and metastasis (Figure 7b).

Because focal adhesions are crucial for cell adhesion, motility, and

invasion, I 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 (Figure 8a). In addition, Matrigel assays revealed that miR-27b significantly suppressed the invasive activity of HCT116 cells (Figure 8b). I observed similar effects of miR-27b on cell invasion in DU145 prostate cancer cells (Figure 8c).

To confirm the role of miR-27b in cancer cells, I introduced anti-miR-27b into HCT116 cells, in which miR-27b is still expressed, albeit at a modest level. Colony formation and Matrigel invasion assays revealed that inhibition of miR-27b significantly promoted anchorage-independent cell growth (Figure 9a) and invasive activity (Figure 9b). These findings suggest that miR-27b has the ability to suppress tumor growth and progression in human cancers.

miR-27b targets paxillin/ARFGEF1/Rab14/ADAM19

To explore the mechanisms of miR-27b-mediated tumor suppression, I searched potential targets of miR-27b in human genes using the TargetScan (http://www.targetscan.org/). Since miR-27b suppressed cell adhesion and invasion, I chose 20 genes related to cell adhesion and invasion from the predicted genes. The 3'UTR fragments (~50bp) around the predicted targeting site of miR-27b were inserted to pMIR-report control vector and validated 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 (Figure 10a). 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 (Figure 10b). Conversely, anti-miR-27b increased the levels of these proteins

(Figure 10c). These findings suggest that miR-27b suppresses tumor growth and progression by targeting multiple genes related to cell adhesion and invasion.

miR-27b–mediated regulation of ARFGEF1 expression is crucial for tumor growth

Although these candidate targets appear to have different functions, they are all potentially required for the creation of dynamic cell movements. Paxillin is a component of the focal adhesion complex, which links extracellular matrix to F-actin in cells(Burridge et al., 1992). ARFGEF1 is the guanine-nucleotide exchange factor for the ADP ribosylation factors ARF1 and ARF3, which play important roles in membrane trafficking(Moss and Vaughan, 1995; Togawa et al., 1999). Rab14 is also involved in membrane trafficking, especially 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(Junutula et al., 2004; Linford et al., 2012; Ueno et al., 2011). ADAM19, a member of the A Disintegrin And Metalloproteinase (ADAM) family of proteins, promotes shedding of growth factors and cytokines, such as neuregulin(Shirakabe et al., 2001; Wei et al., 2001).

To elucidate the roles of these targets in cancer-related phenotypes, I used shRNAs to knock down paxillin, ARFGEF1, Rab14, and ADAM19 in HCT116 cells (Figure 11a). Soft-agar colony-formation activity was suppressed by knockdown of paxillin or ARFGEF1, but not Rab14 or ADAM19 (Figure 11b). Notably, ARFGEF1 knockdown decreased the number of colonies significantly, to a level comparable with that in miR-27b–transfected cells (Figure 5a). Similar results were obtained in HT29 and DU145 cells (Figure 11c). Re-expression of ARFGEF1 rescued the colony-forming activity of the ARFGEF1 knockdown cells, confirming that suppression of tumor growth by sh-ARFGEF1 is not due to an off-target effect (Figure 11d). Furthermore, overexpression of ARFGEF1 restored colony formation of cells transfected by miR-27b (Figure 11e). Taken together, these results suggest that ARFGEF1 is crucial for controlling tumor

growth and that repression of ARFGEF1 largely contributes to the miR-27b–mediated growth suppression.

ARF1, the target of ARFGEF1, promotes activation of Akt and ERK1/2 in breast cancer(Boulay et al., 2008). In addition, ARFGEF1 promotes activation of Akt and ERK1/2 in neuronal cells(Zhou et al., 2013). Consistent with these findings, when cells were cultured on non-adherent poly-HEMA coated dishes, the levels of the active form of Akt (pAkt) were reduced in ARFGEF1-knockdown cells, as well as in miR-27b–transfected cells (Figures 12a and 12b). However, re-expression of ARFGEF1 did not attenuate the suppression of pAkt caused by miR-27b (Figure 12c), suggesting that another downstream target of ARFGEF1, which is responsible for tumor growth, remains to be identified.

miR-27b–mediated regulation of paxillin is important for cell adhesion and invasion

I next examined the roles of paxillin, ARFGEF1, Rab14, and ADAM19 in determining cancer cells' capacity for cell adhesion and invasion. Knockdown studies revealed that paxillin knockdown significantly attenuated focal adhesion formation, cell adhesion and invasion in HCT116 cells (Figures 13, 14a and 14b). 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 (Figure 6 and 8). However, overexpression of paxillin in miR-27b–treated cells reversed the morphological changes (Figures 15b and 15c) and restored cell adhesion and invasion (Figures 16a and 16b). These findings suggest that paxillin is a target of miR-27b responsible for controlling cell adhesion and invasive potential in cancer cells.

miR-27b inhibits oncogenic signaling stimulated by cell adhesion

Focal adhesions are dynamic multi-protein/membrane structures that connect intracellular F-actin and extracellular matrix (ECM) through integrins. Cells attach to ECM and migrate via a cycle of formation and degradation of focal

adhesions. Paxillin is a crucial component of the focal adhesion complex, serving as a platform on which tyrosine kinases such as c-Src and FAK are activated(Schaller, 2001).

To determine whether miR-27b regulates the activity of c-Src by regulating paxillin, I stimulated HCT116 cells by integrin-mediated cell adhesion. In the 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 (Figures 17a and 17b). 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.

miR-27b hostgene APO suppresses tumor cell growth and migration

MicroRNAs are located in various genomic loci. Most mammalian miRNA genes are located in defined transcription units(Hinske et al., 2010; Kim and Nam, 2006; Rodriguez et al., 2004). The location of some intronic miRNAs is well conserved among diverse spices. For example, miR-106b~25 is located in the intron of MCM7 in both human and mice, and miR-1306 is found in the locus of DGCR8. Interestingly, some intronic miRNAs, not only are transcribed together, but also functionally interact with their host genes(Barik, 2008; Lund, 2010; Poliseno et al., 2010).

miR-27b cluster (miR-23b~27b~24b) is located in the intron of Amino-peptidase O (APO) in human, mouse and chicken. APO is a member of the M1 metalloproteinase family. APO is highly conserved, however, the function of APO in cancer and the functional association between APO and miR-27b remains to be elucidated.

To address this question, I analyzed APO expression using Csk-/- MEF in which c-Src is inducible by DOX treatment. PCR analysis demonstrated that the expression level of APO mRNA decreased by c-Src overexpression (Figure 20a),

which was almost same kinetics as miR-27b (Figure 1c), implying that APO is a candidate for a tumor suppressor. qRT-PCR analysis, however, revealed that APO mRNA is upregulated in breast cancer cell lines, T47D, MCF7 and MDA-MB-231 cells, compared to that in normal breast epithelial MCF10A cells (Figure 20b). Subsequent analysis for APO protein expression showed that APO is only expressed in MDA-MB-231 among the cell lines I tested (Figure 20c). Since MDA-MB-231 cells are classified as triple negative breast cancer cell line, whereas T47D and MCF7 are moderate luminal breast cancer cells, I hypothesized APO may promote tumor progression. To examine whether APO upregulation leads to acquisition of malignant phenotype, I tested colony-forming ability and cell migration ability of the APO overexpressed MCF7 cells. Interestingly, APO overexpression suppressed both phenotypes (Figure 20d and 20e). This result, however, is not consistent with the fact that APO was highly expressed in malignant MDA-MB-231 cells; hence further analysis using other types of cancers is required.

Discussion

In this study, I 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. miR-27b directly targets ARFGEF1 and paxillin, which are required for tumor growth and cancer cell adhesion/invasion, respectively. Therefore, I 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 (Figure 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 (Figure 18). These observations raise the possibility that miR-27b could be useful as a prognostic marker in human cancers.

ARFGEF1 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(D'Souza-Schorey and Chavrier, 2006) is upregulated in invasive breast cancers and gastric cancers(Boulay et al., 2011; Tsai et al., 2012); it promotes the activation of PI3K/Akt pathway and retinoblastoma protein, thereby promoting cell proliferation, migration, and invasion in breast cancer cells(Boulay et al., 2008; Boulay et al., 2011). 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, I 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 between the extracellular matrix and intercellular F-Actin to control cell morphology, adhesion, and motility, which are important for cancer invasion and metastasis. Upon cell adhesion via 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, I 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 cancer malignant phenotypes, i.e., 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, i.e. 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, I proposed a new mechanism for the upregulation of c-Src activity: upregulation of paxillin via 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(Majid et al., 2012). 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 both

miR-23b and miR-27b is regulated through a common pathway. Indeed, I observed that the expression of miR-23b was repressed by c-Src–induced transformation with the same kinetics as miR-27b (unpublished data). If this is the case in human cancers, it is possible that the miR-23b/27b/24-2 cluster regulates c-Src via dual mechanisms: regulation of the c-Src kinase activity via 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 (Figure 19), 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.

Conclusions

In this study, I analyzed the function of miR-27b in cancer, which expression is downregulated by c-Src-induced transformation. The results showed that miR-27b was downregulated in human colon cancer cells and tissues as well as in prostate cancer cells, and that ectopic expression of miR-27b caused morphological chages and significantly suppressed cell growth and invasion of the cancer cells. I identified novel targets of miR-27b; paxillin, ARFGEF1, Rab14 and ADAM19. Since each miRNA can target multiple genes, miRNA composes a complex signaling crosstalks and it is challenging to figure out which target is most important. The knockdown and rescue experiments of miR-27b target genes revealed that inhibition of ARFGEF1 and paxillin expression are crucial for its function as a tumor suppressor. In addition, miR-27b inhibited activation of c-Src and the subsequent downstream signaling caused by integrin-mediated cell adhesion. This finding suggests a new regulatory mechanism for the elevation of c-Src activity with cancer progression: c-Src and the related oncogenic pathway inhibit miR-27b expression, followed by the overexpression of paxillin, leading to the stabilization of focal adhesion, the enhancement and maintenance of its activity.

Materials and methods

Cell culture

All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The human colon cancer cell lines, SW480, HCT116, and HT29, were purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle's Medium (SW480 and HT29) or McCoy's 5A Modified Medium (HCT116) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin–streptomycin (Nacalai Tesque, Kyoto, Japan).

qRT-PCR analysis for miRNA

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, Japan. Informed consent was obtained from all patients in writing before enrollment in the study. Total RNA was isolated from cells and tissues using Sepasol (Nacalai Tesque). Total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Bedford, MA, USA). The resultant cDNA samples were mixed with THUNDERBIRD Probe qPCR mix (TOYOBO, Osaka, Japan), and quantitative real-time PCR analysis was performed using ABI PRISM 7900HT (Applied Biosystems). The following TaqMan MicroRNA Assays were used: hsa-miR-27b, 000409; snoRNA202, 001232; and RNU48, 001006.

miRNA transfection

miR-27b precursor (PM10750) and antisense miR-27b (AM10750) were purchased from Applied Biosystems. miRNA precursors or antisense miRNAs were transfected using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after transfection, cells were harvested using

Trypsin/EDTA and subjected to soft-agar colony-formation, adhesion, or invasion assays.

Knockdown of miR-27b target genes by shRNA

Knockdown of paxillin, ARFGEF1, Rab14, or ADAM19 was carried out using lentiviral vectors. Empty vector and vectors carrying sh-paxillin (NM_002859), sh-ARFGEF1 (NM_006421), sh-Rab14 (NM_016322), and sh-ADAM19 (NM_023038) were purchased from Sigma–Aldrich (Milan, Italy). Lentiviruses were generated from PLT cells (ectopic retrovirus packaging cell line), using MISSION Lentiviral packaging mix (Sigma–Aldrich) and FuGENE HD (Roche, Mannheim, Germany).

Retroviral and lentiviral transfection

Mouse paxillin (NM_011223.2) containing mutations in the seed region of miR-27b and mouse ARFGEF1 (NM_001102430.1) was subcloned into pCX4-puro vector. pCX4-puro-paxillin was transfected into PLT cells (ecotropic retrovirus packaging cell line) using FuGENE HD (Roche). pCX4-puro-ARFGEF1 was transiently transfected into HCT116 cells using Lipofectamine LTX.

Immunohistochemistry

Cells (5×10^4) were seeded on a 15 mm coverslip coated with 2 μg/ml fibronectin (Sigma–Aldrich) for 3 hr at room temperature. The samples were fixed in 4% paraformaldehyde (PFA, Sigma–Aldrich) at 37°C, for 10 min. After washing the samples twice with PBS for 10 min each, the cells were permeabilized with 0.03% Triton-X in PBS (T-PBS) for 20 min. The samples were treated with 1% BSA in T-PBS for 30 min to block non-specific binding of the antibodies, and then washed with T-PBS for 5 min. The diluted antibody in T-PBS was added to each well, and the samples were incubated overnight at 4°C. After the samples

were washed four times for 10 min each with T-PBS, the samples were incubated with secondary antibody in T-PBS for 2 hr at room temperature in the dark. Phalloidin (to label F-Actin) was added with the secondary antibody. After washing the samples four times with T-PBS, coverslips were mounted on glass slides using Prolong Gold (Invitrogen). The next day, the coverslips were sealed with nail polish and subjected to observation under a confocal microscope (FV1000, OLYMPUS, Tokyo, Japan).

Invasion assay

BioCoat Matrigel Invasion Chamber (BD Biosciences, San Jose, CA, USA) was used for the invasion assay. Cells (5×10^4) were seeded on an insert and moved into a chamber containing culture supernatant of NIH3T3 cells. After incubation at 37°C for 48 hr, invaded cells were fixed with 100% methanol for 10 min and then stained with 0.1% toluidine blue (Chroma-Gesellschaft Schmid GmbH & Co) for 10 min. Invaded cells were counted on micrographs; in each experiment, cells were counted on five randomly chosen fields.

Western blot

Total cell lysates were isolated with 2×SDS sample buffer and boiled for 10 min. The samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose or a PVDF membrane. The membrane was blocked with 1% BSA + 0.1% Tween 20 in TBS at room temperature for 30 min, and then incubated overnight at 4°C with the indicated primary antibodies. After two washes with T-TBS for 10 min each, the membrane was incubated with secondary antibodies in T-TBS with 1% BSA + 5% skim milk. After three washes with T-TBS for 10 min each, blots were visualized using an ECL detection kit (Immuno zeta, Wako, Osaka, Japan) on X-ray film (Fujifilm, Tokyo, Japan). The following antibodies were used for western blots: anti-paxillin (BD), anti-ARFGEF1 (Bethyl, Montgomery, TX,

USA), anti-Rab14 (Abcam), anti-ADAM19 (Abcam, Cambridge, UK), anti–c-Src (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Src pY418 (Invitrogen), anti-FAK-pY397 (Invitrogen), anti-FAK (Santa Cruz Biotechnology), anti-GAPDH (Santa Cruz Biotechnology), anti-pERK1/2 (Cell Signaling, Danvers, MA, USA), anti-ERK1/2 (Cell Signaling), anti-pAkt (Cell Signaling), and anti-Akt (Cell Signaling).

Luciferase reporter assay

Putative targets of miR-27b were predicted using TargetScan. Approximately 50 bp of 3'UTR containing the putative recognition site for miR-27b was synthesized and cloned into the pMIR-REPORT vector (Applied Biosystems), downstream of the firefly luciferase reporter gene. pRL-TK reporter vector (Applied Biosystems) was used as an internal control. These constructs and miRNA precursors were transfected into $Csk^{-/-}$/c-Src cells seeded in 24-well plates, using Lipofectamine 2000 (Invitrogen). Sixteen hours after transfection, luciferase activities were measured using the PicaGene Dual Sea Pansy Luminescence Kit (Wako).

In vivo tumorigenicit

Cells (2×10^6 in 200 μl of serum-free medium) were subcutaneously injected into nude mice (BALB/cAJcl-nu/nu) purchased from Japan SLC, Inc. Tumor length (L) and width (W) were measured every 2–3 days. Tumor volume was evaluated using the mathematical formula $V = 0.5 \times L \times W^2$. All animal experiments were conducted in accordance with the protocols approved by the Animal Research Committee of Research Institute for Microbial Diseases in Osaka University.

Acknowledgements

I would like to express my special appreciation to Prof. Masato Okada for his tremendous support, advice, discussions and encouragement. Without his support and encouragement, I couldn't have finished my Ph.D. I am deeply grateful to Dr. Chitose Oneyama for her wonderful guidance, instruction and discussions. I am very thankful to Dr. Shigeyuki Nada for his critical advices and support.

I would like to express my gratitude to Dr. Daisuke Okuzaki for his technical advice about microarray and in silico analysis, and to Dr. Takashi Akagi for the kind gift of pCX4 vector.

This work was supported by a Grant-in-Aid for JSPS Fellows.

I am deeply grateful to my family, my friends and all the lab members of Department of Oncogene Research, RIMD. I want to thank my mother for all of the sacrifices that let me keep studying. At the end, I would like to express my deepest appreciation and heartfelt thanks to my husband Yuki Matsuyama who spent the hardest time with, advised me from the different point of view and was always together.

Rferences

Aleshin, A., and Finn, R.S. SRC: a century of science brought to the clinic.

Ambros, V. (2004). The functions of animal microRNAs. Nature *431*, 350-355.

Baltimore, D., Boldin, M.P., O'Connell, R.M., Rao, D.S., and Taganov, K.D. (2008). MicroRNAs: new regulators of immune cell development and function. *9*, 839-845.

Barik, S. (2008). An intronic microRNA silences genes that are functionally antagonistic to its host gene. Nucleic Acids Research *36*, 5232-5241.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Bartel, D.P. (2009). MicroRNAs: Target Recognition and Regulatory Functions. Cell *136*, 215-233.

Bohnsack, M.T., Czaplinski, K., and GÖRlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA *10*, 185-191.

Boulay, P.L., Cotton, M., Melancon, P., and Claing, A. (2008). ADP-ribosylation factor 1 controls the activation of the phosphatidylinositol 3-kinase pathway to regulate epidermal growth factor-dependent growth and migration of breast cancer cells. The Journal of biological chemistry *283*, 36425-36434.

Boulay, P.L., Schlienger, S., Lewis-Saravalli, S., Vitale, N., Ferbeyre, G., and Claing, A. (2011). ARF1 controls proliferation of breast cancer cells by regulating the retinoblastoma protein. In Oncogene (England), pp. 3846-3861.

Brown, M.T., and Cooper, J.A. (1996). Regulation, substrates and functions of src. In Biochim Biophys Acta (Netherlands), pp. 121-149.

Burridge, K., Turner, C.E., and Romer, L.H. (1992). Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. The Journal of cell biology *119*, 893-903.

Calin, G.A., and Croce, C.M. (2006). MicroRNA signatures in human cancers. Nature Reviews Cancer *6*, 857-866.

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., *et al.* (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proceedings of the National Academy of Sciences *99*, 15524-15529.

Carrington, J.C., and Ambros, V. (2003). Role of MicroRNAs in Plant and Animal Development. Science *301*, 336-338.

Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *136*, 642-655.

Cartwright, C.A., Eckhart, W., Simon, S., and Kaplan, P.L. (1987). Cell transformation by pp60c-src mutated in the carboxy-terminal regulatory domain. *49*, 83-91.

Cooper, J.A., Gould, K.L., Cartwright, C.A., and Hunter, T. (1986). Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science *231*, 1431-1434.

D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. In Nat Rev Mol Cell Biol (England), pp. 347-358.

Daigo, Y., Furukawa, Y., Kawasoe, T., Ishiguro, H., Fujita, M., Sugai, S., Nakamori, S., Liefers, G.J., Tollenaar, R.A., van de Velde, C.J., *et al.* (1999). Absence of genetic alteration at codon 531 of the human c-src gene in 479 advanced colorectal cancers from Japanese and Caucasian patients. Cancer Res *59*, 4222-4224.

Esquela-Kerscher, A., and Slack, F.J. (2006). Oncomirs - microRNAs with a role in cancer. Nature Reviews Cancer *6*, 259-269.

Frame, M.C. (2002). Src in cancer: deregulation and consequences for cell behaviour. In Biochim Biophys Acta (Netherlands), pp. 114-130.

Ha, M., and Kim, V.N. (2014). Regulation of microRNA biogenesis. *15*, 509-524.

Hanahan, D., and Weinberg, R.A. (2000). The Hallmarks of Cancer. *100*, 57-70.

Hanahan, D., and Weinberg, Robert A. (2011). Hallmarks of Cancer: The Next Generation. *144*, 646-674.

He, L., He, X., Lim, L.P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D.*, et al.* (2007). A microRNA component of the p53 tumour suppressor network. *447*, 1130-1134.

Hinske, L.C.G., Galante, P.A.F., Kuo, W.P., and Ohno-Machado, L. (2010). A potential role for intragenic miRNAs on their hosts' interactome. BMC Genomics *11*.

Huang, Z., Huang, D., Ni, S., Peng, Z., Sheng, W., and Du, X. (2010). Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *127*, 118-126.

Hutvágner, G., McLachlan, J., Pasquinelli, A.E., Bálint, É., Tuschl, T., and Zamore, P.D. (2001). A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the let-7 Small Temporal RNA. Science *293*, 834-838.

Iorio, M.V., Ferracin, M., Liu, C.-G., Veronese, A., Spizzo, R., Sabbioni, S., Magri, E., Pedriali, M., Fabbri, M., Campiglio, M.*, et al.* (2005). MicroRNA Gene Expression Deregulation in Human Breast Cancer. Cancer Research *65*, 7065-7070.

Iorio, M.V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., Taccioli, C., Volinia, S., Liu, C.-G., Alder, H.*, et al.* (2007). MicroRNA Signatures in Human Ovarian Cancer. Cancer Research *67*, 8699-8707.

Iravani, S., Mao, W.G., Fu, L., Karl, R., Yeatman, T., Jove, R., and Coppola, D. (1998). Elevated c-Src protein expression is an early event in colonic neoplasia. Laboratory Investigation *78*, 365-371.

Irby, R.B., Mao, W., Coppola, D., Kang, J., Loubeau, J.M., Trudeau, W., Karl, R., Fujita, D.J., Jove, R., and Yeatman, T.J. (1999). Activating SRC mutation in a subset of advanced human colon cancers. Nat Genet *21*, 187-190.

Irby, R.B., and Yeatman, T.J. (2000). Role of Src expression and activation in human cancer. Oncogene *19*, 5636-5642.

Ishihara, T., Seki, N., Inoguchi, S., Yoshino, H., Tatarano, S., Yamada, Y., Itesako, T., Goto, Y., Nishikawa, R., Nakagawa, M.*, et al.* (2014). Expression of the tumor suppressive miRNA-23b/27b cluster is a good prognostic marker in clear cell renal cell carcinoma. J Urol *192*, 1822-1830.

Ivanovska, I., Ball, A.S., Diaz, R.L., Magnus, J.F., Kibukawa, M., Schelter, J.M., Kobayashi, S.V., Lim, L., Burchard, J., Jackson, A.L.*, et al.* (2008). MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Molecular and Cellular Biology *28*, 2167-2174.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D., and Slack, F.J. (2005). RAS Is Regulated by the let-7 MicroRNA Family. *120*, 635-647.

Jove, R., and Hanafusa, H. (1987). Cell transformation by the viral src oncogene. Annu Rev Cell Biol *3*, 31-56.

Junutula, J.R., De Maziere, A.M., Peden, A.A., Ervin, K.E., Advani, R.J., van Dijk, S.M., Klumperman, J., and Scheller, R.H. (2004). Rab14 is involved in membrane trafficking between the Golgi complex and endosomes. Molecular biology of the cell *15*, 2218-2229.

Kawamata, T., and Tomari, Y. (2010). Making RISC. *35*, 368-376.

Kim, V.N., and Nam, J.-W. (2006). Genomics of microRNA. *22*, 165-173.

Kobayashi, T., Lu, J., Cobb, B.S., Rodda, S.J., McMahon, A.P., Schipani, E., Merkenschlager, M., and Kronenberg, H.M. (2008). Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. Proceedings of the National Academy of Sciences of the United States of America *105*, 1949-1954.

Kosik, K.S. (2006). The neuronal microRNA system. *7*, 911-920.

Laghi, L., Bianchi, P., Orbetegli, O., Gennari, L., Roncalli, M., and Malesci, A. (2001). Lack of mutation at codon 531 of SRC in advanced colorectal cancers from Italian patients. Br J Cancer *84*, 196-198.

Lee, J.J., Drakaki, A., Iliopoulos, D., and Struhl, K. (2012). MiR-27b targets PPARgamma to inhibit growth, tumor progression and the inflammatory response in neuroblastoma cells. In Oncogene (England), pp. 3818-3825.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S.*, et al.* (2003). The nuclear RNase III Drosha initiates microRNA processing. *425*, 415-419.

Lee, Y., Jeon, K., Lee, J.T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise

processing and subcellular localization. Embo j *21*, 4663-4670.

Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. The EMBO Journal *23*, 4051-4060.

Lee, Y.S., Kim, H.K., Chung, S.M., Kim, K.S., and Dutta, A. (2005). Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the downregulation of putative targets during differentiation. Journal of Biological Chemistry *280*, 16635-16641.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15-20.

Linford, A., Yoshimura, S., Nunes Bastos, R., Langemeyer, L., Gerondopoulos, A., Rigden, D.J., and Barr, F.A. (2012). Rab14 and its exchange factor FAM116 link endocytic recycling and adherens junction stability in migrating cells. Developmental cell *22*, 952-966.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A.*, et al.* (2005). MicroRNA expression profiles classify human cancers. *435*, 834-838.

Lund, A.H. (2010). MiR-10 in development and cancer. Cell Death and Differentiation *17*, 209-214.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear Export of MicroRNA Precursors. Science *303*, 95-98.

Ma, L., Young, J., Prabhala, H., Pan, E., Mestdagh, P., Muth, D., Teruya-Feldstein, J., Reinhardt, F., Onder, T.T., Valastyan, S.*, et al.* (2010). MiR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. Nature Cell Biology *12*, 247-256.

Majid, S., Dar, A.A., Saini, S., Arora, S., Shahryari, V., Zaman, M.S., Chang, I., Yamamura, S., Tanaka, Y., Deng, G.*, et al.* (2012). miR-23b represses proto-oncogene Src kinase and functions as methylation-silenced tumor suppressor with diagnostic and prognostic significance in prostate cancer. Cancer Res *72*, 6435-6446.

Martin, G.S. (2004). The road to Src. Oncogene *23*, 7910-7917.

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T., and Patel, T. (2007).

MicroRNA-21 Regulates Expression of the PTEN Tumor Suppressor Gene in Human Hepatocellular Cancer. Gastroenterology *133*, 647-658.

Moss, J., and Vaughan, M. (1995). Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. The Journal of biological chemistry *270*, 12327-12330.

Nada, S., Okada, M., MacAuley, A., Cooper, J.A., and Nakagawa, H. (1991). Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. Nature *351*, 69-72.

Nasertorabi, F., Tars, K., Becherer, K., Kodandapani, R., Liljas, L., Vuori, K., and Ely, K.R. (2006). Molecular basis for regulation of Src by the docking protein p130Cas. Journal of Molecular Recognition *19*, 30-38.

Navon, R., Wang, H., Steinfeld, I., Tsalenko, A., Ben-Dor, A., and Yakhini, Z. (2009). Novel rank-based statistical methods reveal microRNAs with differential expression in multiple cancer types. PLoS One *4*, e8003.

Nilbert, M., and Fernebro, E. (2000). Lack of activating c-SRC mutations at codon 531 in rectal cancer. Cancer Genet Cytogenet *121*, 94-95.

Okada, M. (2012). Regulation of the SRC family kinases by Csk. Int J Biol Sci *8*, 1385-1397.

Okada, M., and Nakagawa, H. (1989). A protein tyrosine kinase involved in regulation of pp60c-src function. Journal of Biological Chemistry *264*, 20886-20893.

Oneyama, C., Hikita, T., Nada, S., and Okada, M. (2008). Functional dissection of transformation by c-Src and v-Src. In Genes Cells (England), pp. 1-12.

Oneyama, C., Ikeda, J., Okuzaki, D., Suzuki, K., Kanou, T., Shintani, Y., Morii, E., Okumura, M., Aozasa, K., and Okada, M. (2011). MicroRNA-mediated downregulation of mTOR/FGFR3 controls tumor growth induced by Src-related oncogenic pathways. In Oncogene (England), pp. 3489-3501.

Oneyama, C., Kito, Y., Asai, R., Ikeda, J., Yoshida, T., Okuzaki, D., Kokuda, R., Kakumoto, K., Takayama, K., Inoue, S.*, et al.* (2013). MiR-424/503-mediated Rictor upregulation promotes tumor progression. PLoS One *8*, e80300.

Oneyama, C., Morii, E., Okuzaki, D., Takahashi, Y., Ikeda, J., Wakabayashi, N., Akamatsu, H., Tsujimoto, M., Nishida, T., Aozasa, K.*, et al.* (2012). MicroRNA-mediated upregulation of integrin-linked kinase promotes Src-induced tumor progression. In Oncogene (England), pp. 1623-1635.

Park, S.M., Gaur, A.B., Lengyel, E., and Peter, M.E. (2008). The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes and Development *22*, 894-907.

Parsons, J.T., and Parsons, S.J. (1997). Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. *9*, 187-192.

Playford, M.P., and Schaller, M.D. (2004). The interplay between Src and integrins in normal and tumor biology. In Oncogene (England), pp. 7928-7946.

Poliseno, L., Salmena, L., Riccardi, L., Fornari, A., Song, M.S., Hobbs, R.M., Sportoletti, P., Varmeh, S., Egia, A., Fedele, G.*, et al.* (2010). Identification of the miR-106b~25 microRNA cluster as a proto-oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. Science Signaling *3*.

Porkka, K.P., Pfeiffer, M.J., Waltering, K.K., Vessella, R.L., Tammela, T.L.J., and Visakorpi, T. (2007). MicroRNA Expression Profiling in Prostate Cancer. Cancer Research *67*, 6130-6135.

Rodriguez, A., Griffiths-Jones, S., Ashurst, J.L., and Bradley, A. (2004). Identification of mammalian microRNA host genes and transcription units. Genome Research *14*, 1902-1910.

Schaller, M.D. (2001). Paxillin: a focal adhesion-associated adaptor protein. Oncogene *20*, 6459-6472.

Schickel, R., Boyerinas, B., Park, S.M., and Peter, M.E. (2008). MicroRNAs: Key players in the immune system, differentiation, tumorigenesis and cell death. Oncogene *27*, 5959-5974.

Shirakabe, K., Wakatsuki, S., Kurisaki, T., and Fujisawa-Sehara, A. (2001). Roles of Meltrin beta /ADAM19 in the processing of neuregulin. The Journal of biological chemistry *276*, 9352-9358.

Summy, J.M., and Gallick, G.E. (2003). Src family kinases in tumor progression and metastasis. Cancer and Metastasis Reviews *22*, 337-358.

Talamonti, M.S., Roh, M.S., Curley, S.A., and Gallick, G.E. (1993). INCREASE IN ACTIVITY AND LEVEL OF PP60C-SRC IN PROGRESSIVE STAGES OF HUMAN COLORECTAL-CANCER. Journal of Clinical Investigation *91*, 53-60.

Taylor, B.S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B.S., Arora, V.K., Kaushik, P., Cerami, E., Reva, B.*, et al.* (2010). Integrative genomic profiling of human prostate cancer. In Cancer Cell (United States: 2010 Elsevier Inc), pp. 11-22.

Thomas, J.W., Ellis, B., Boerner, R.J., Knight, W.B., White, G.C., and Schaller, M.D. (1998). SH2- and SH3-mediated Interactions between Focal Adhesion Kinase and Src. Journal of Biological Chemistry *273*, 577-583.

Thomas, S.M., and Brugge, J.S. (1997). Cellular functions regulated by SRC family kinases. In Annual Review of Cell and Developmental Biology, pp. 513-609.

Togawa, A., Morinaga, N., Ogasawara, M., Moss, J., and Vaughan, M. (1999). Purification and cloning of a brefeldin A-inhibited guanine nucleotide-exchange protein for ADP-ribosylation factors. The Journal of biological chemistry *274*, 12308-12315.

Tsai, M.M., Lin, P.Y., Cheng, W.L., Tsai, C.Y., Chi, H.C., Chen, C.Y., Tseng, Y.H., Cheng, Y.F., Chen, C.D., Liang, Y.*, et al.* (2012). Overexpression of ADP-ribosylation factor 1 in human gastric carcinoma and its clinicopathological significance. Cancer Sci *103*, 1136-1144.

Ueno, H., Huang, X., Tanaka, Y., and Hirokawa, N. (2011). KIF16B/Rab14 molecular motor complex is critical for early embryonic development by transporting FGF receptor. Developmental cell *20*, 60-71.

Ventura, A., and Jacks, T. (2009). MicroRNAs and Cancer: Short RNAs Go a Long Way. Cell *136*, 586-591.

Walther, A., Johnstone, E., Swanton, C., Midgley, R., Tomlinson, I., and Kerr, D. Genetic prognostic and predictive markers in colorectal cancer.

Wan, L., Zhang, L., Fan, K., and Wang, J. (2014). MiR-27b targets LIMK1 to inhibit growth and invasion of NSCLC cells. Molecular and Cellular Biochemistry *390*, 85-91.

Wang, N.M., Yeh, K.T., Tsai, C.H., Chen, S.J., and Chang, J.G. (2000). No evidence of correlation

between mutation at codon 531 of src and the risk of colon cancer in Chinese. Cancer Lett *150*, 201-204.

Wei, P., Zhao, Y.G., Zhuang, L., Ruben, S., and Sang, Q.X. (2001). Expression and enzymatic activity of human disintegrin and metalloproteinase ADAM19/meltrin beta. Biochemical and biophysical research communications *280*, 744-755.

Wienholds, E., and Plasterk, R.H.A. (2005). MicroRNA function in animal development. RNAi: Mechanisms, Biology and Applications *579*, 5911-5922.

Xu, W., Harrison, S.C., and Eck, M.J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595-602.

Yeatman, T.J. (2004). A renaissance for SRC. Nature Reviews Cancer *4*, 470-480.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes & Development *17*, 3011-3016.

Young, M.A., Gonfloni, S., Superti-Furga, G., Roux, B., and Kuriyan, J. (2001). Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. Cell *105*, 115-126.

Zhang, B., Pan, X., Cobb, G.P., and Anderson, T.A. (2007). microRNAs as oncogenes and tumor suppressors. *302*, 1-12.

Zhou, C., Li, C., Li, D., Wang, Y., Shao, W., You, Y., Peng, J., Zhang, X., Lu, L., and Shen, X. (2013). BIG1, a brefeldin A-inhibited guanine nucleotide-exchange protein regulates neurite development via PI3K-AKT and ERK signaling pathways. Neuroscience *254*, 361-368.

Achievements

科学雑誌への投稿

①Rei Matsuyama, Daisuke Okuzaki, Masato Okada, Chitose Oneyama

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

Cancer Science, Volume 107, Issue 1, pages 28–35, January 2016

②Chitose Oneyama, Yoriko Kito, Rei Asai, Jun-ichiro Ikeda, Takuya Yoshida, Daisuke Okuzaki, Rie Kokuda, Kyoko Kakumoto, Ken-ichi Takayama, Satoshi Inoue, Eiichi Morii, and Masato Okada

"MiR-424/503-Mediated Rictor Upregulation Promotes Tumor Progression."

PLoS ONE, 2013; 8(11): e80300.

国内学会・シンポジウム等における発表

●口頭発表(査読なし)

①○浅井麗伊、奥崎大介、池田純一郎、森井英一、岡田雅人、小根山千歳

「細胞接着・浸潤関連遺伝子群を標的とするマイクロ RNA による Src がん悪性化の制御機構」

第４回日本RNAi研究会（グランドプリンスホテル広島、広島県) 2012年8月30日-9月1日

②○浅井麗伊、奥崎大介、池田純一郎、森井英一、岡田雅人、小根山千歳

「細胞接着・浸潤関連遺伝子群を標的とするマイクロ RNA による Src がん悪性化の制御機構」

平成24年度がん若手研究者ワークショップ（蓼科グランドホテル滝の湯、長野県) 2012年9月5-8日

③○浅井麗伊、奥崎大介、池田純一郎、森井英一、岡田雅人、小根山千歳

"MicroRNA-mediated downregulation of ARFGEF1 controls Src-induced tumor progression"

第71回日本癌学会学術総会（ロイトン札幌、北海道) 2012年9月19-21日

④○浅井麗伊、奥崎大介、池田純一郎、森井英一、岡田雅人、小根山千歳

「miR-27bを介したSrcによるがん悪性化の制御機構」

第6回シグナルネットワーク研究会（慶應大学医学部、東京都）2014年5月10日

⑤○浅井麗伊、奥崎大介、池田純一郎、森井英一、岡田雅人、小根山千歳

「miR-27b controls c-Src-induced tumor progression by targeting Paxillin」

第73回日本癌学会学術総会（パシフィコ横浜、神奈川県）2014年9月25日-9月27日

●ポスター発表(査読なし)

①○浅井麗伊、奥崎大介、池田純一郎、森井英一、岡田雅人、小根山千歳

「ARFGEF1 および Paxillin を標的とする miRNA による Src がん形質の制御機構」

第 5 回日本 RNAi 研究会（グランドプリンスホテル広島、広島県) 2013 年 8 月 29 日-8 月 31 日

その他発表

●口頭発表(査読なし)

①○浅井麗伊

"MicroRNA-mediated downregulation of genes related to cell adhesion and invasion controls c-Src-induced tumor progression"

National Tsing Hua University-Osaka University Life Science Student Activity Fair 2013（大阪大学理学部、大阪府）2013 年 5 月 25 日

②○浅井麗伊

"miRNA controls c-Src-induced tumor progression via targeting ARFGEF1 and Paxillin"

大阪大学―ヘルシンキ大学交流会（大阪大学理学部、大阪府）2013 年 12 月 7 日

受賞歴

①優秀発表賞、National Tsing Hua University-Osaka University Life Science Student Activity Fair 2013、2013 年 5 月 25 日

②奨励賞、第 6 回シグナルネットワーク研究会、2014 年 5 月 10 日

その他特筆事項

⑴平成 26 年度日本学術振興会特別研究員(DC2)採用

②大阪大学全学教育推進機構ティーチングアシスタント

③平成 25 年度大阪大学大学院生命機能研究科リサーチアシスタント(卓越 RA)

④大阪大学大学院ヒューマンイノベーション博士課程プログラム海外インターンシップ(試行)採択, Fred Hutchinson Cancer Research Center (Seattle, WA, USA) にて研究活動に従事 (H25.9.6-H25.12.2)

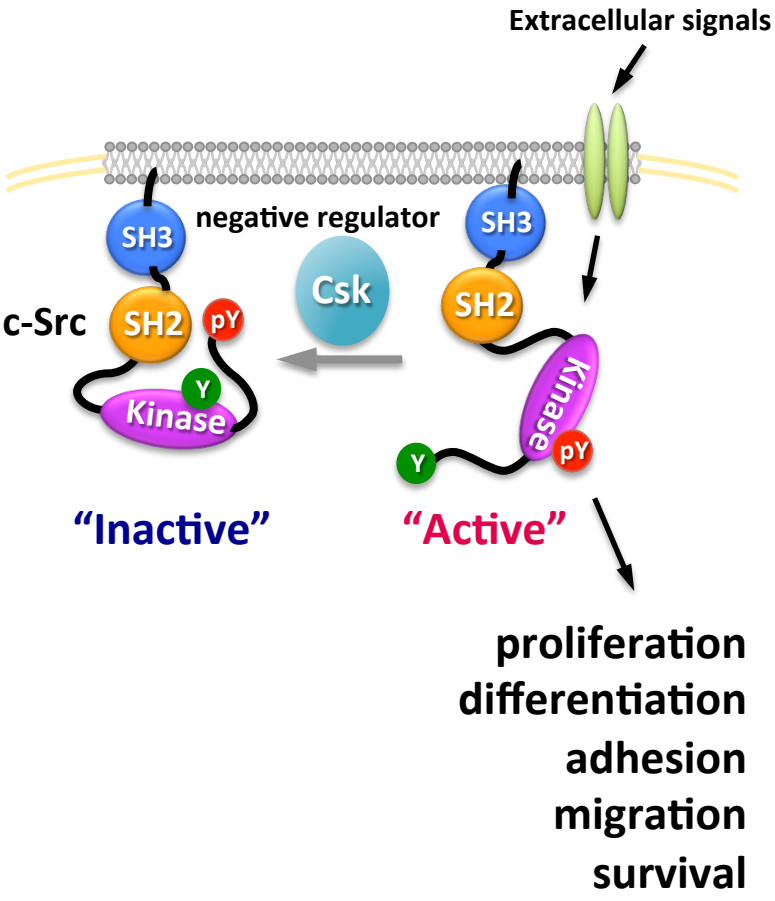

Figure GI-1. c-Src plays a critical role as a molecular switch
Normally, c-Src is inactivated thorough the phosphorylation by its negative regulator Csk. Once activated, c-Src regulates multiple signaling pathways leading to cell proliferation, differentiation, adhesion and migration.

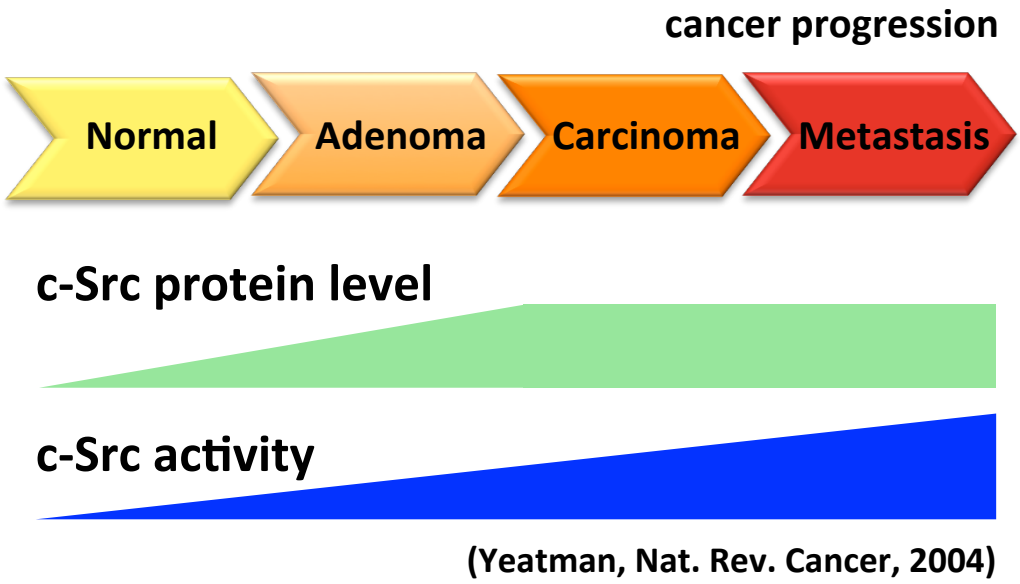

Figure GI-2. c-Src is highly expressed and activated in human cancer
Expression and activity of c-Src are associated with tumor progression, implicating that its pivotal role in cancer. However, the underlying mechanisms how c-Src is deregulated and its role in tumor progression remain unclear.

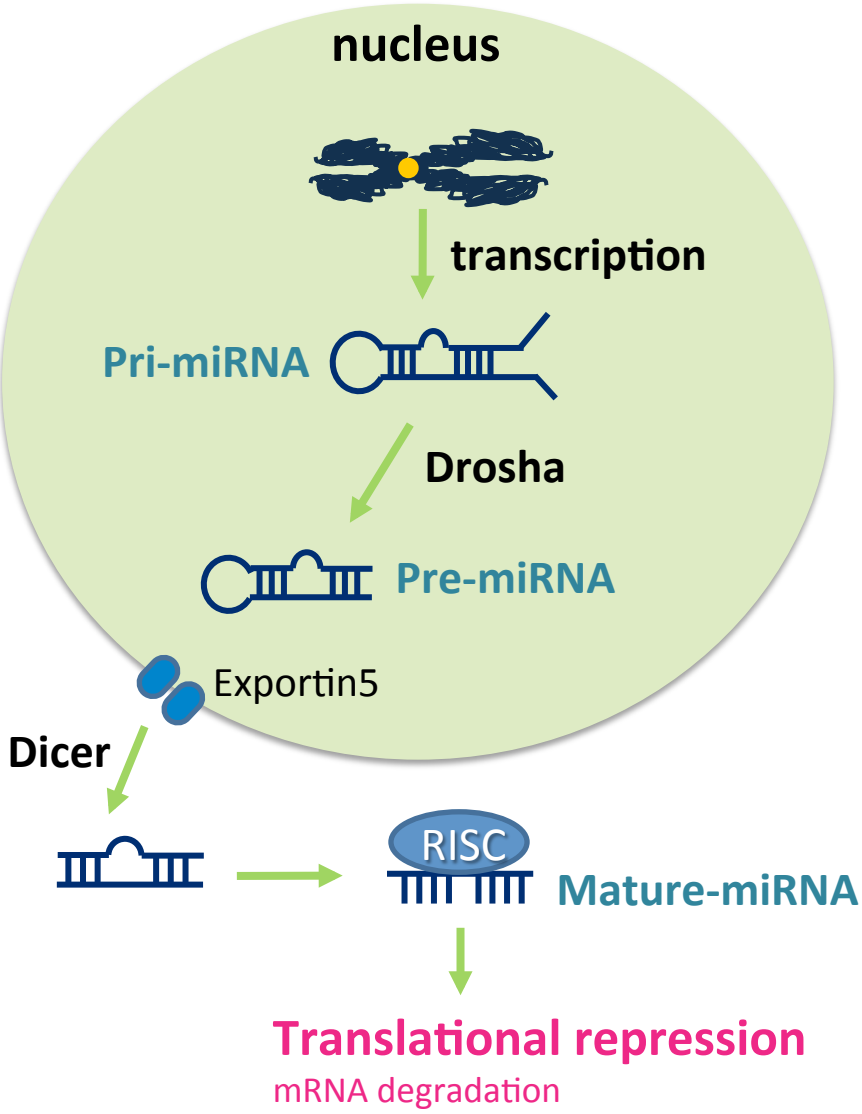

Figure GI-3. MicroRNA regulates gene expression
MicroRNAs (miRNAs) are small non-coading RNAs found in both animals and plants. miRNAs are transcribed by RNA polymerase Ⅱ, processed by Drosha and Dicer, and loaded on AGO proteins to form RISC. miRNA-RISC complex inhibits gene expression by translational repression and/or mRNA degradation.

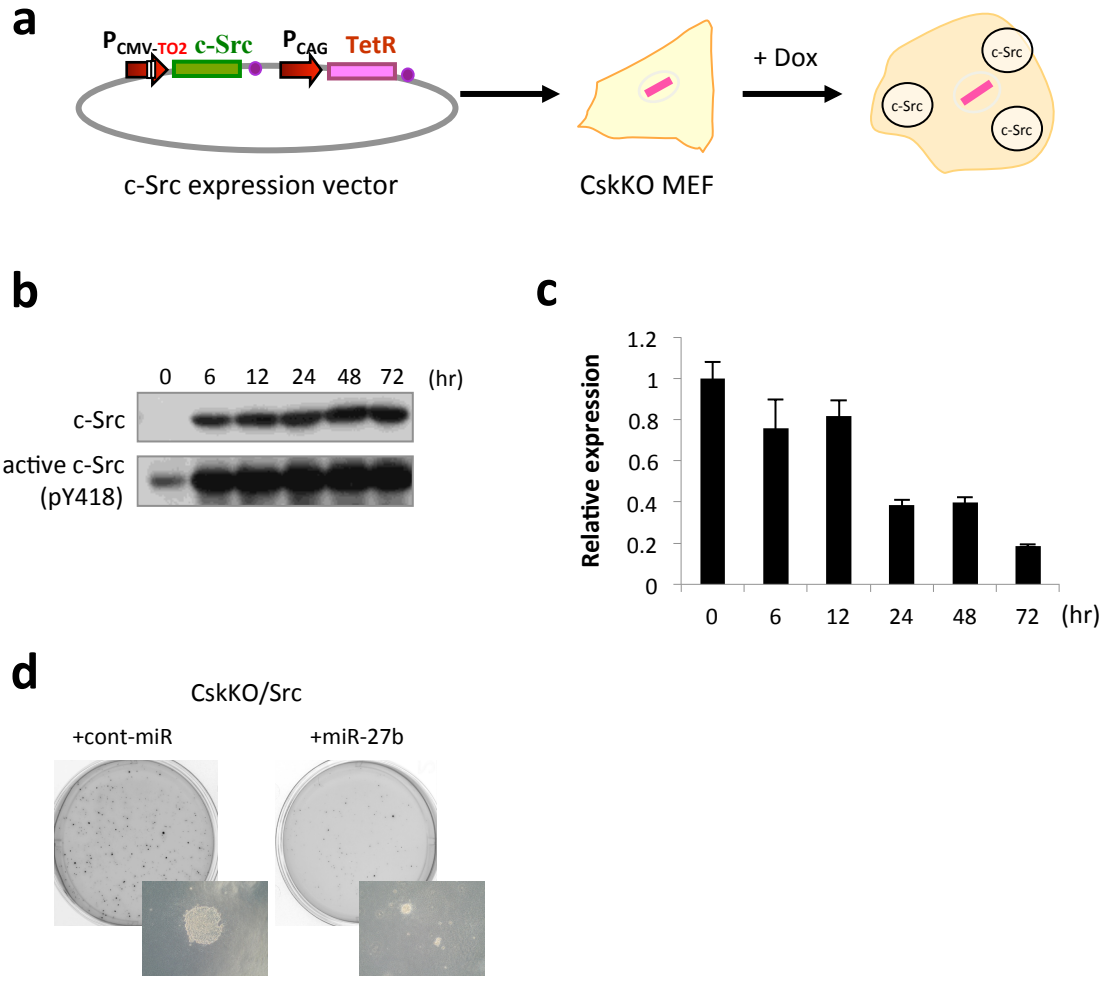

Figure 1. miR-27b expression is inhibited by c-Src induced transformation
(a) Schematic of c-Src-inducible system by DOX treatment. **(b)** Western blot for c-Src expression and its activity of the cells indicated in (a). Total cell lysates were collected at the indicated time poits after Dox treatment. **(c)** Expression levels of miR-27b in (b) Expression levels were assessed by qRT-PCR and normalized against Sno. **(d)** miR-27b inhibited colony formation of the cells transformed by c-Src ($Csk^{-/-}$/c-Src MEF). $Csk^{-/-}$/c-Src cells were transfected with 30nM of cont-miR or miR-27b. After 48 hr transfection, soft agar colony formation assay was performed.

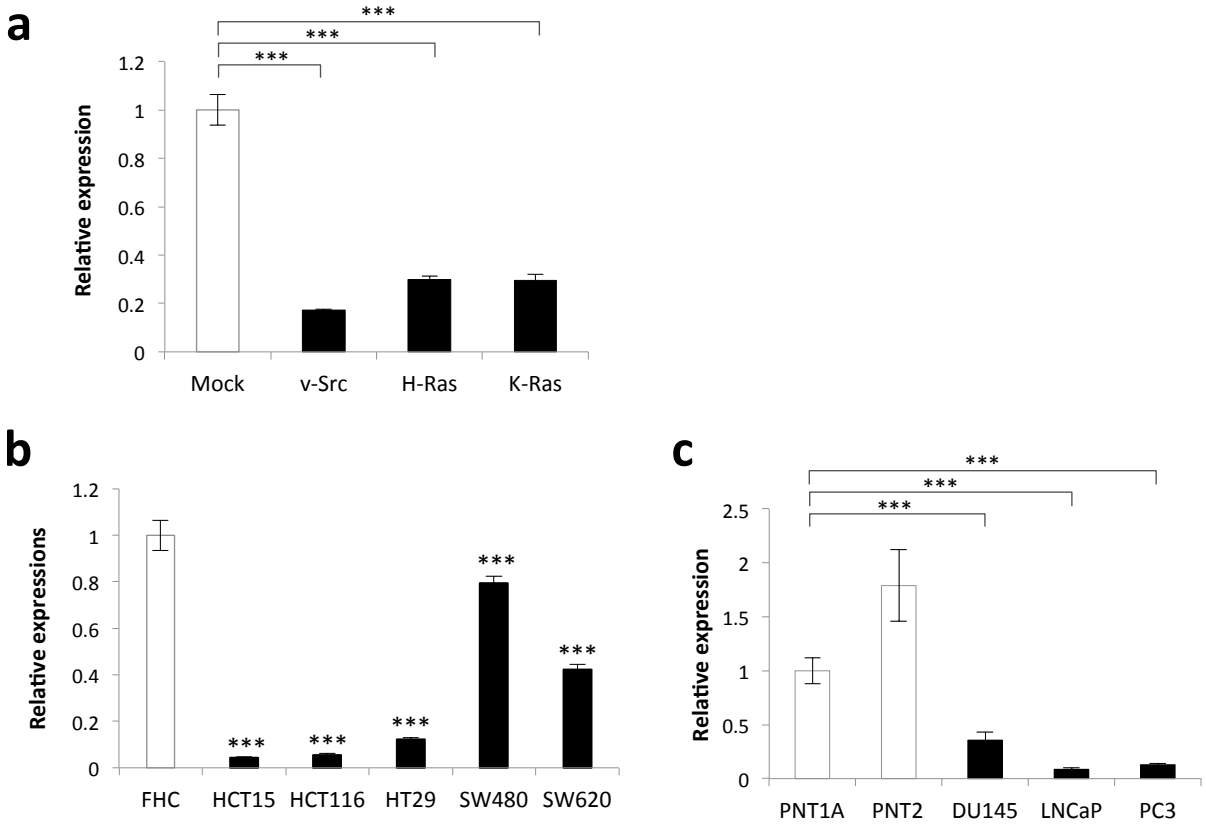

Figure 2. miR-27b expression is downregulated in human cancer

(a) miR-27b expression levels in MEFs transfected with v-Src, H-Ras, 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)** Expression levels of miR-27b in human prostate cancer cell lines (DU145, LNCaP, and PC3) were compared with those in normal human prostate epithelial cells (PNT1A and PNT2). Expression levels were assessed by qRT-PCR and normalized against RNU48. (a-c) ***P<0.001

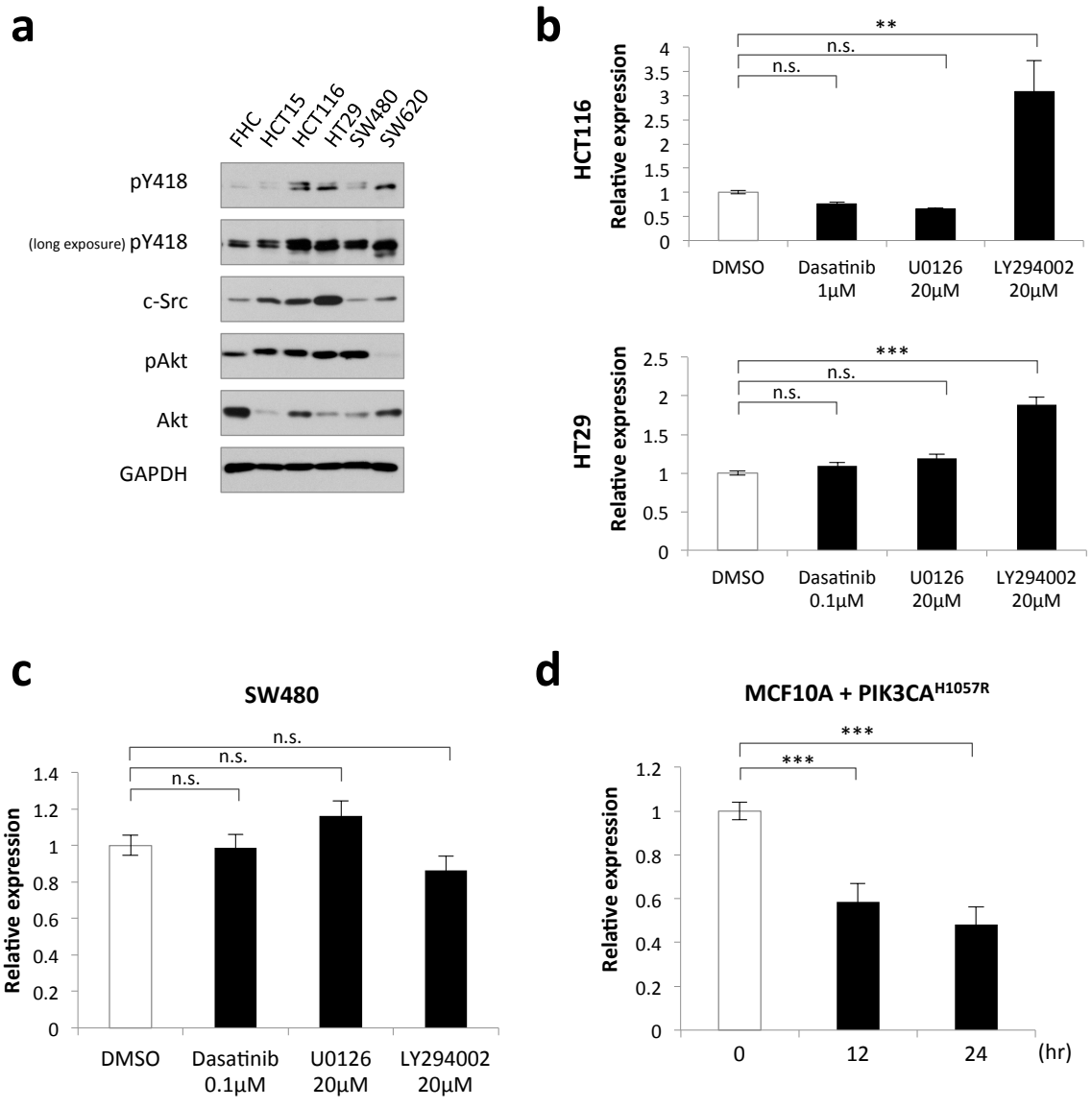

Figure 3. PI3K/Akt pathway regulates miR-27b expression

(a) Western blot analysis of expression and activity of c-Src and Akt. **(b)** Inhibition of the PI3K pathway by LY294002 upregulated miR-27b expression in HCT116 and HT29 cells. Total RNAs were isolated 48 hr after. **(c)** Inhibition of PI3K by LY294002 had no effect on miR-27b expression in SW480 cells. **(d)** qRT-PCR analysis for miR-27b in MCF10A cells expressing $PI3K^{H1047R}$ by using Retro-X Tet-On 3G Inducible Expression System. MCF10A cells stably expressing pRetroX-Tet3G vector and pRetroX-TRE3G-$PI3K^{H1047R}$ were treated with Doxycycline (1μg/ml final) and total RNAs of the cells were collected after the indicated time. (b-d) **P < 0.01, ***P<0.001

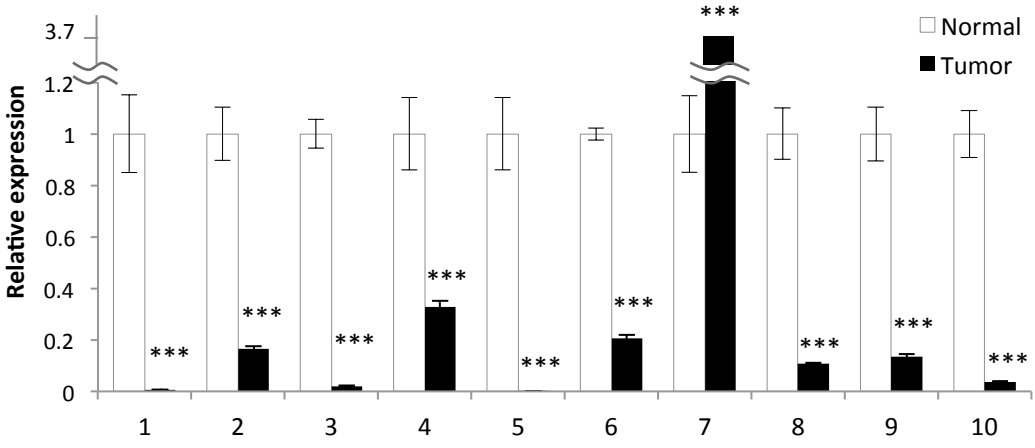

Figure 4. miR-27b is downregulated in human colon cancer tissues
Expression levels of miR-27b in human colon cancer tissues. Total RNAs were isolated from the cancer tissue (black bar) and the adjacent normal tissue (white bar). ***P<0.001

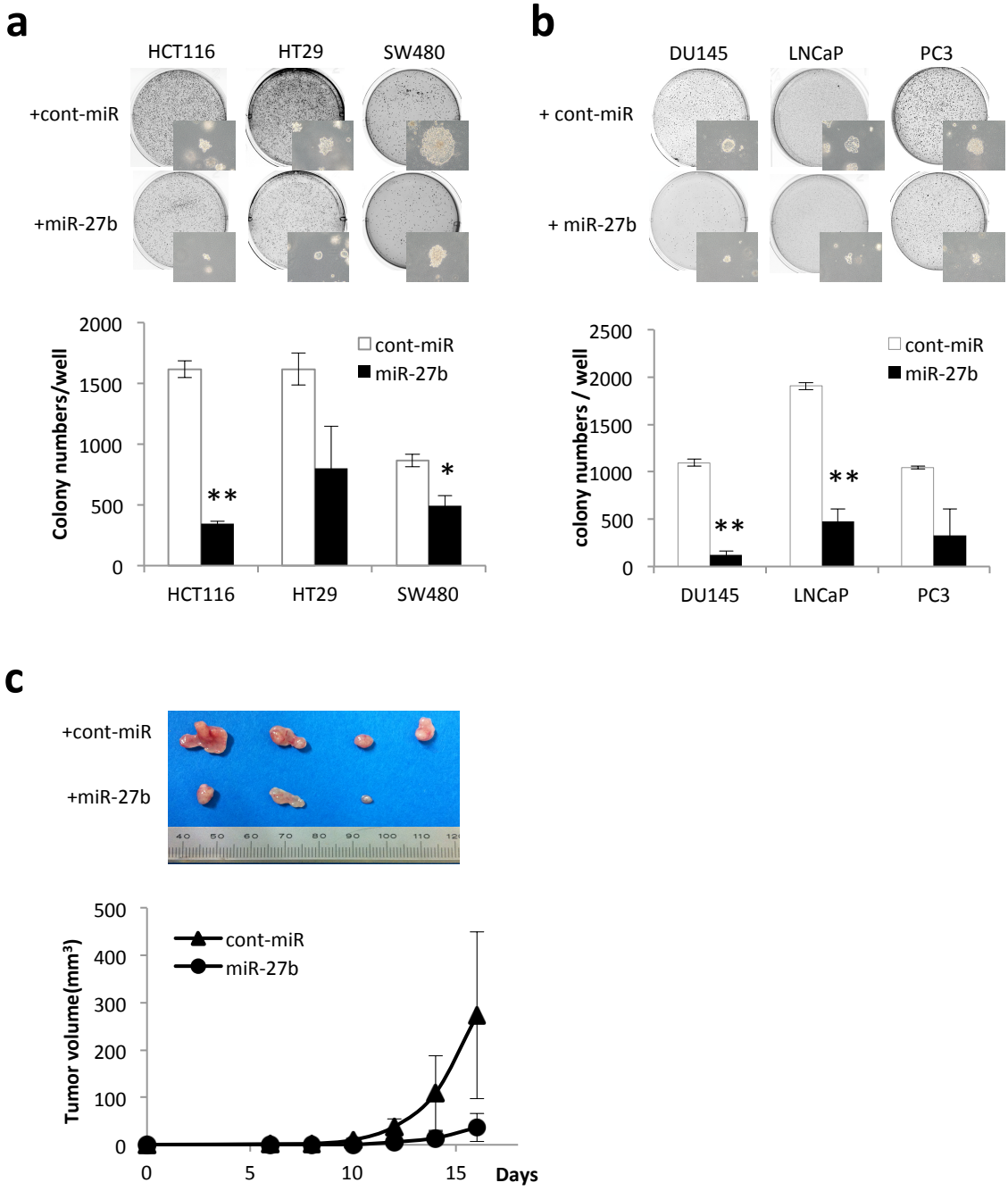

Figure 5. miR-27b suppresses tumor growth in human colon cancer cells in vitro and in vivo
Effect of miR-27b on anchorage-independent growth of colon cancer cells **(a)** and prostate cancer cells **(b)**. The cells transfected with 30 nM of cont-miR or miR-27b were subjected to colony-formation assay 2 days after. Colonies stained with MTT were counted on micrographs. *P < 0.05, **P < 0.01. **(c)** miR-27b inhibited tumorigenicity of HCT116 in nude mice. Cells transfected with 30 nM of cont-miR or miR-27b were injected into nude mice.

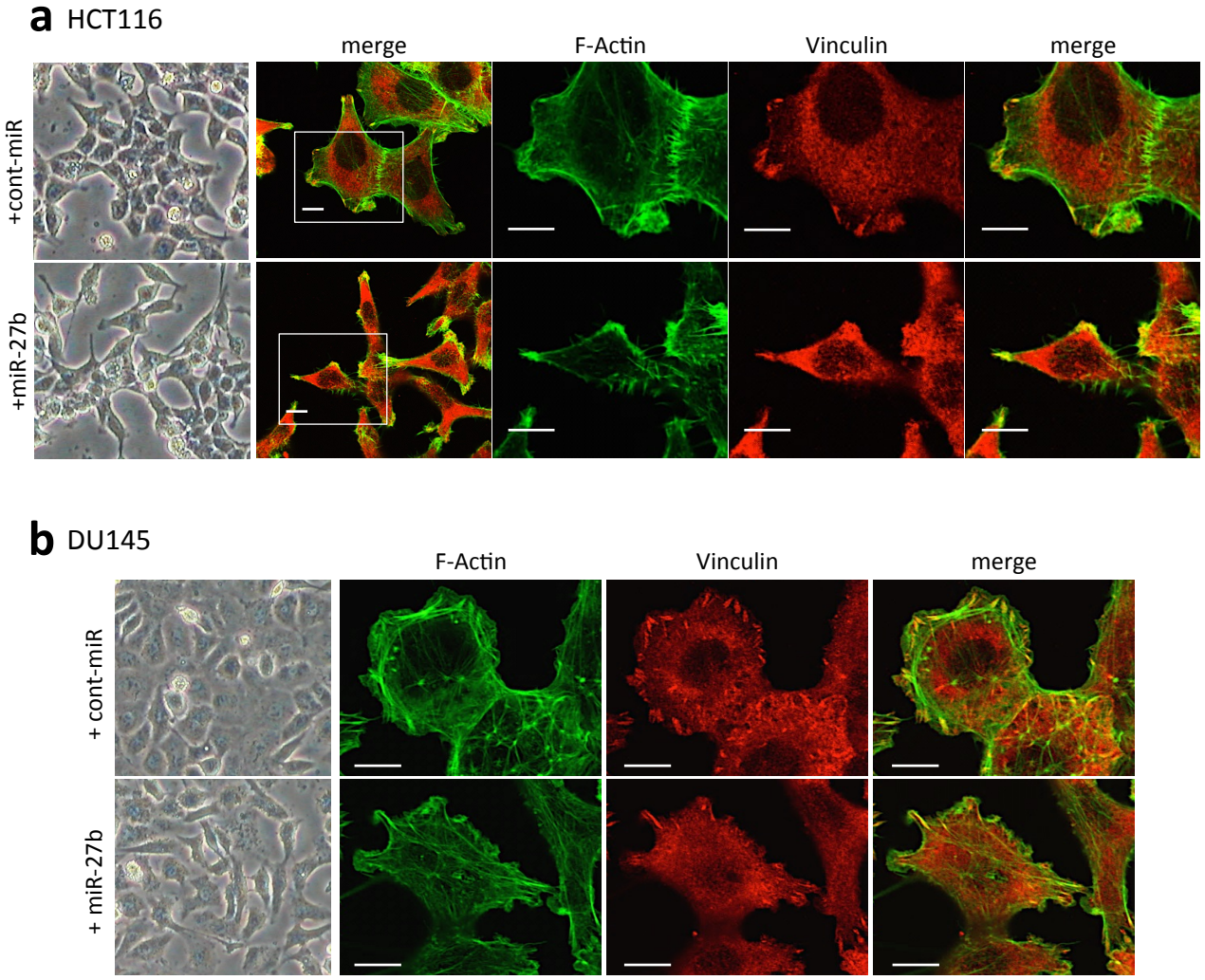

Figure 6. miR-27b causes morphological changes in human cancer.
Effect of miR-27b on morphology of HCT116 cells **(a)** and DU145 cells **(b)**. Left: optical microphotographs of cells transfected for 48 hr with 30 nM of cont-miR or miR-27b. Right: analysis of stress fibers (F-actin; green) and focal adhesions (Vinculin; red). Scale bar; 10 μm.

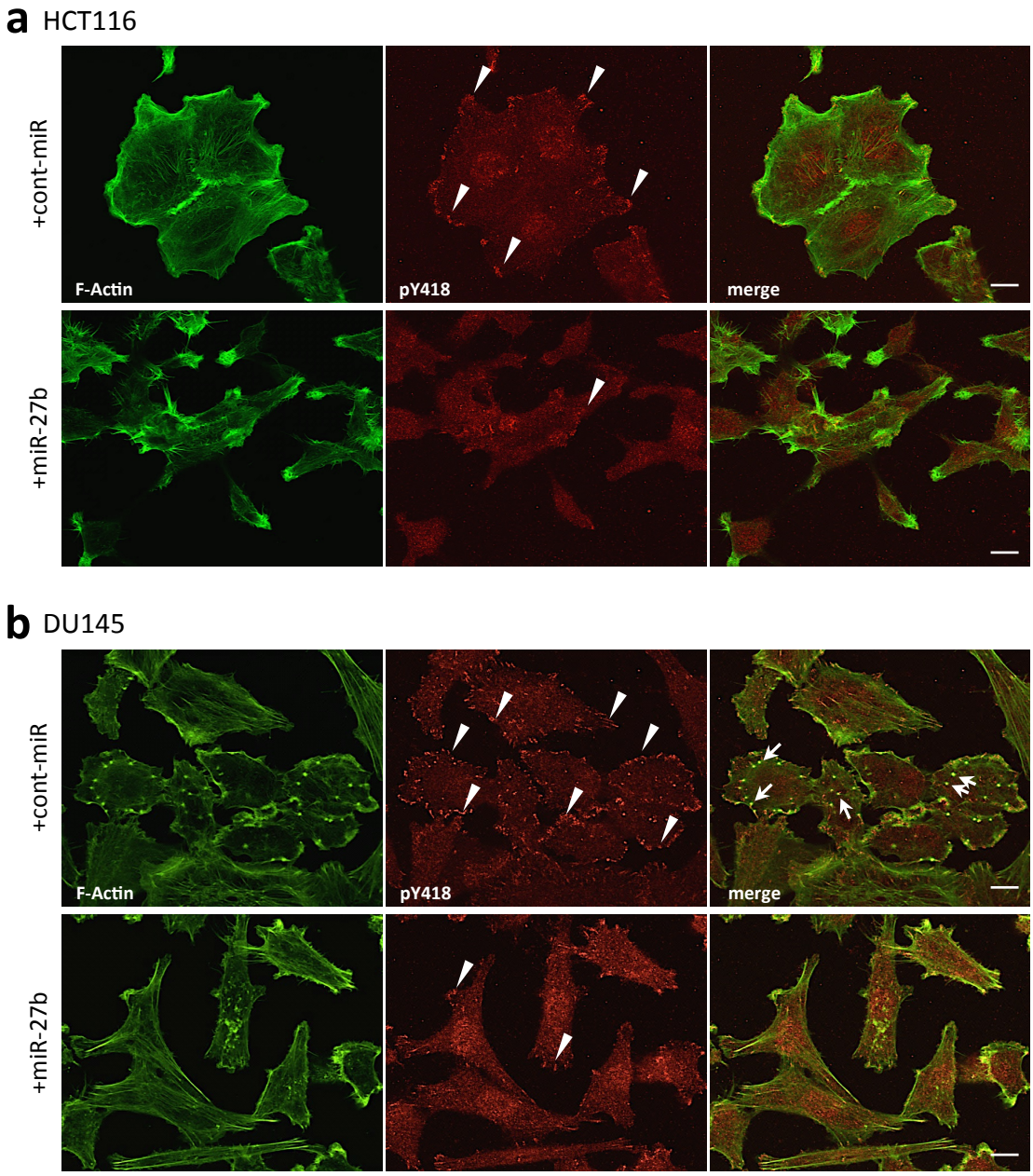

Figure 7. miR-27b suppressed c-Src localization to focal adhesions.
Effect of miR-27b on morphology of HCT116 cells **(a)** and DU145 cells **(b)**. Analysis of stress fibers (F-actin; green) and active-c-Src localization (pY418; red). Scale bar; 10 μm. Arrow heads indicate focal adhesion, white arrows indicate invadopodia.

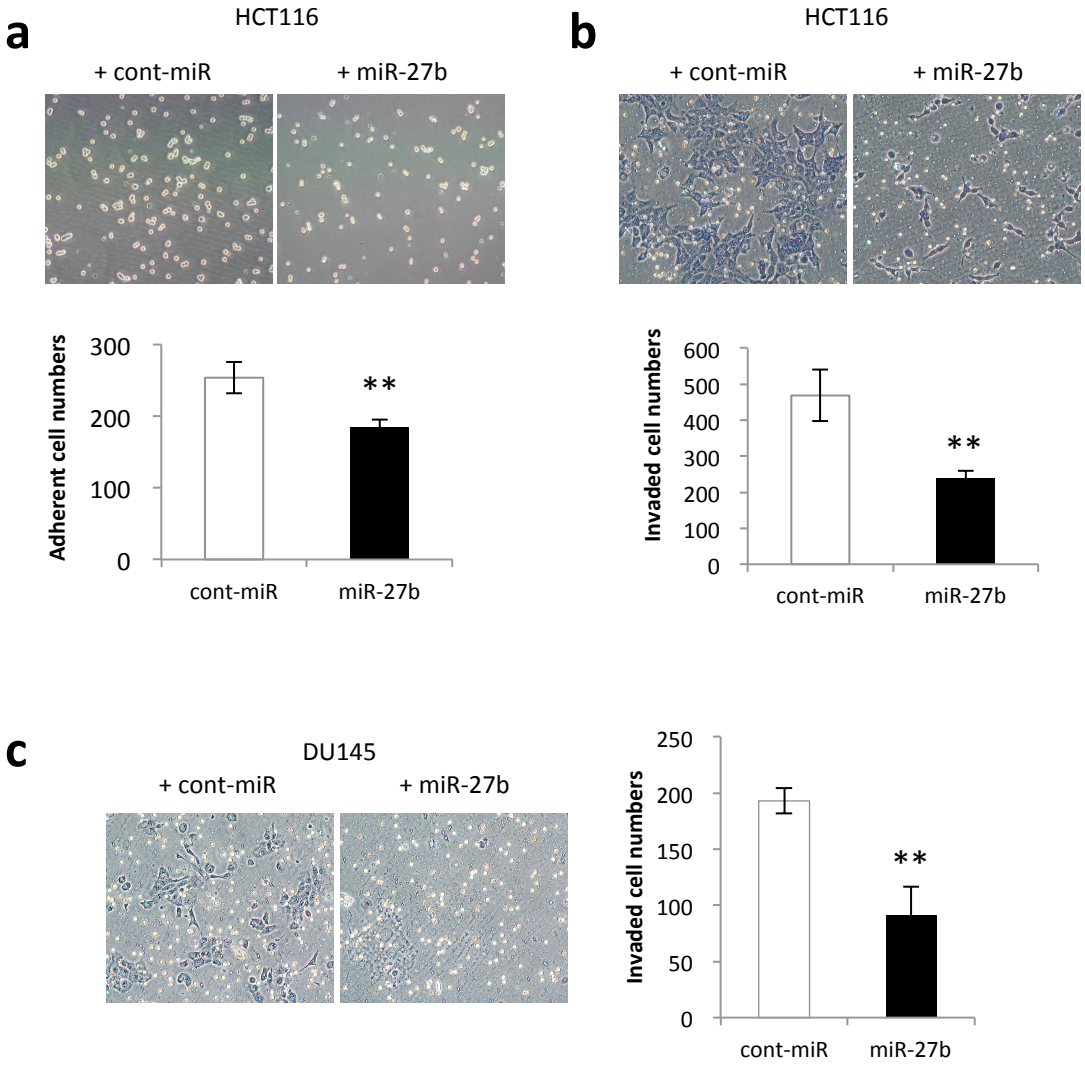

Figure 8. miR-27b suppressed cell adhesion and invasion of cancer cells.
(a) Effect of miR-27b on cell adhesion. HCT116 cells were transfected with 30 nM of cont-miR or miR-27b for 2 days. Cells were plated onto fibronectin-coated dishes and adherent cells were counted. **P < 0.01. **(b)** Effect of miR-27b on cell invasion. HCT116 cells were transfected with 30 nM of cont-miR or miR-27b for 2 days, and seeded into a Matrigel-coated invasion chamber. 48 hours later, invaded cells were counted. **(c)** Invasion assay on DU145. **P < 0.01.

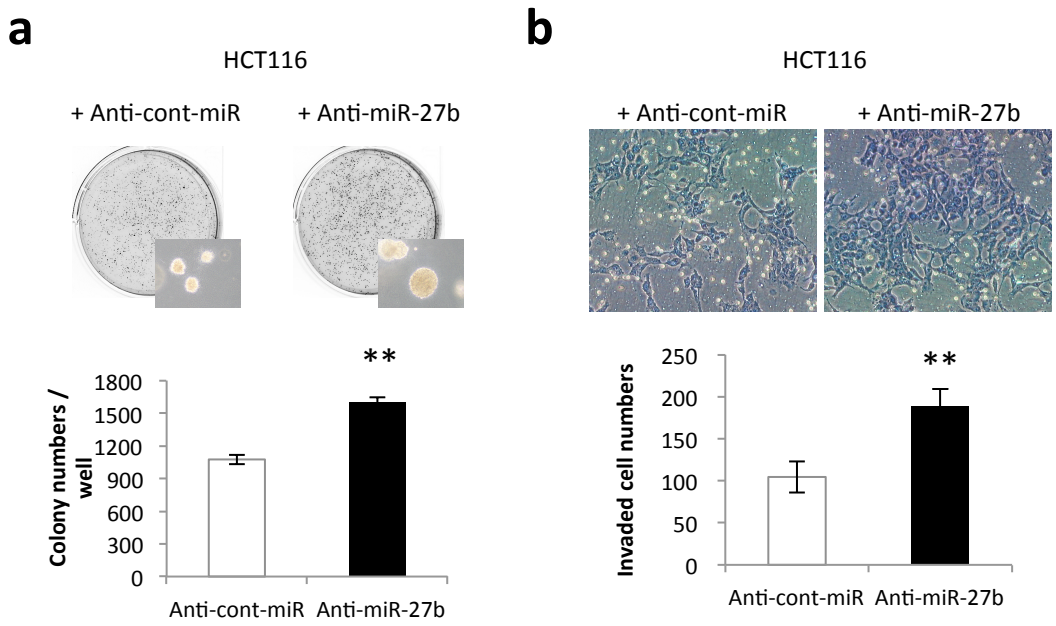

Figure 9. Inhibition of miR-27b enhances cell adhesion and invasion of cancer cells.
Functional inhibition of miR-27b. HCT116 cells were transfected with 5 nM of anti-cont-miR or anti-miR-27b for 2 days, and subjected to colony formation assay **(a)** and invasion assay **(b)**. **P < 0.01.

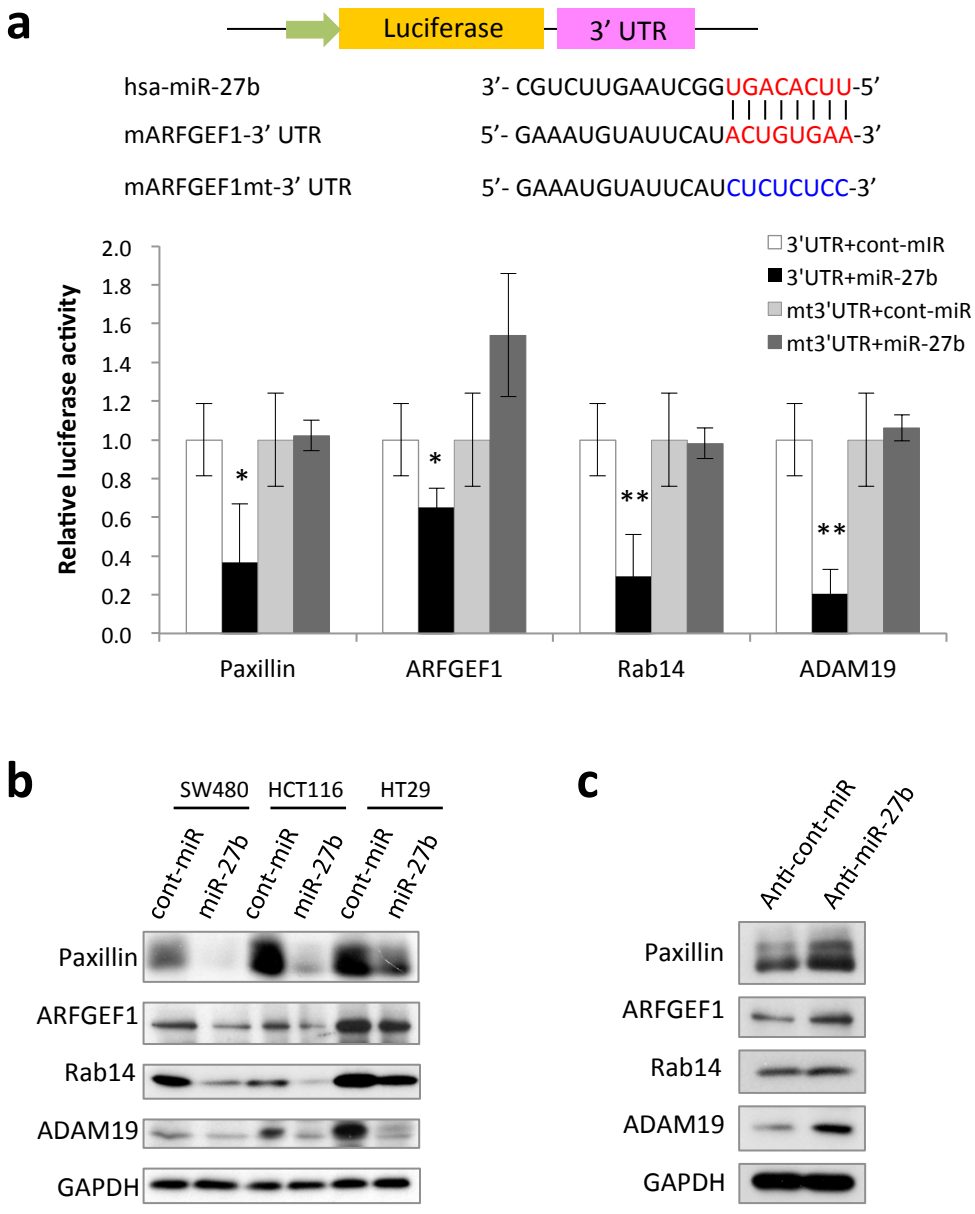

Figure 10. miR-27b directly targets paxillin, ARFGEF1, Rab14, and ADAM19.
(a) The pMIR-Luc plasmid containing the wild-type or mutated (mt) 3'-UTR of each target was co-transfected into Csk-/-/c-Src cells with 30 nM cont-miR or miR-27b. The data are shown as means ± s.d. from three independent experiments. **(b)** Western blot analysis for colon cancer cells transfected with 30 nM of cont-miR or miR-27b. **(c)** Inhibition of miR-27b increased protein levels of the targets. HCT116 cells transfected with anti-cont-miR or anti-miR-27b (5 nM) were analyzed.

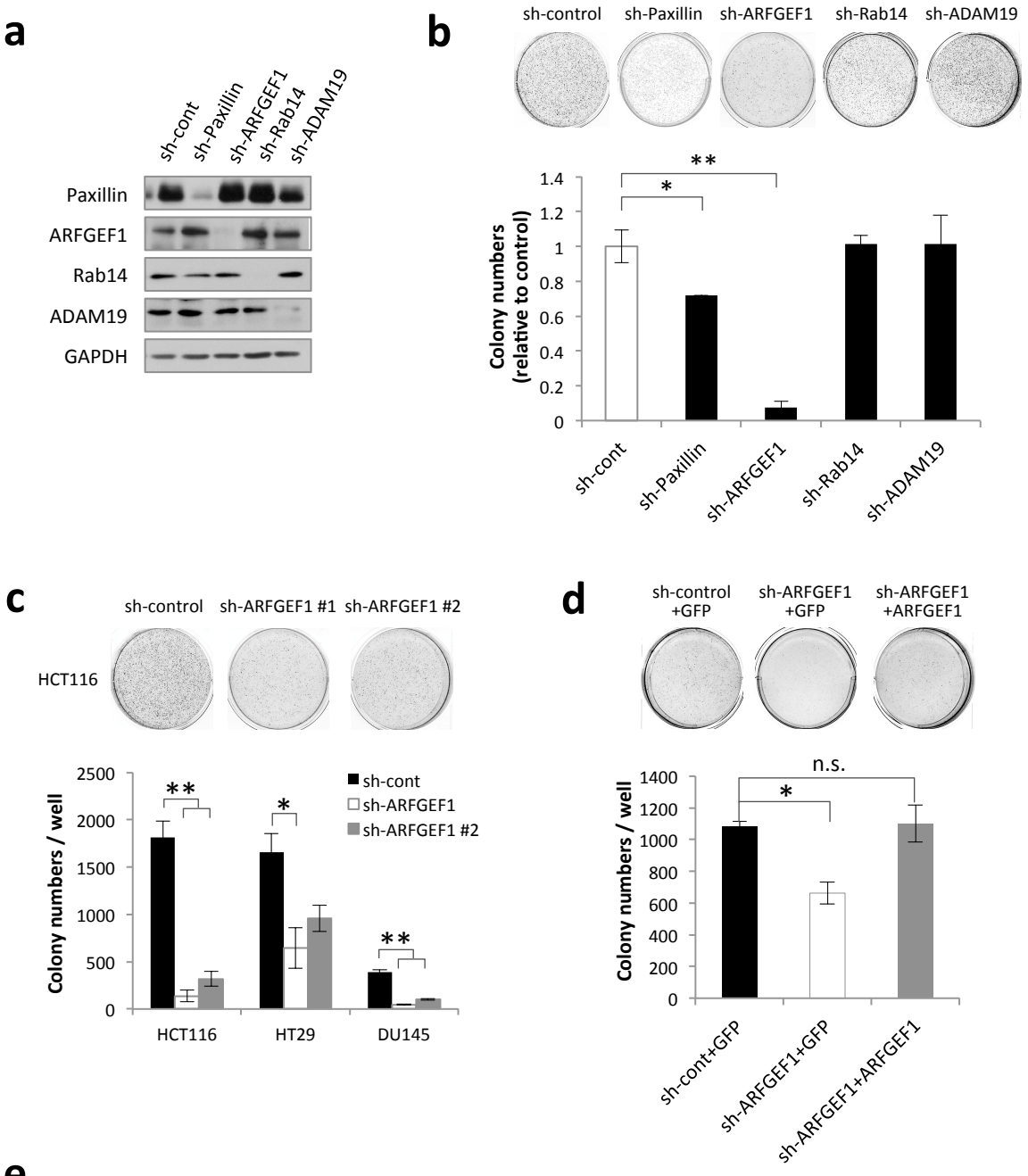
a
sh-cont
sh-Paxillin
sh-ARFGEF1
sh-Rab14
sh-ADAM19
Paxillin
ARFGEF1
Rab14
ADAM19
GAPDH
b
sh-control
sh-Paxillin
sh-ARFGEF1
sh-Rab14
sh-ADAM19
Colony numbers (relative to control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
sh-cont
sh-Paxillin
sh-ARFGEF1
sh-Rab14
sh-ADAM19
c
sh-control
sh-ARFGEF1 #1
sh-ARFGEF1 #2
HCT116
Colony numbers / well
2500
2000
1500
1000
500
0
**
*
**
sh-cont
sh-ARFGEF1
sh-ARFGEF1 #2
HCT116
HT29
DU145
d
sh-control +GFP
sh-ARFGEF1 +GFP
sh-ARFGEF1 +ARFGEF1
Colony numbers / well
1400
1200
1000
800
600
400
200
0
n.s.
*
sh-cont+GFP
sh-ARFGEF1+GFP
sh-ARFGEF1+ARFGEF1
e
Mock
ARFGEF1
+cont-miR
+miR-27b
Colony numbers / well
1400
1200
1000
800
600
400
200
0
*
*
cont-miR
miR-27b
Mock
ARFGEF1

Figure 11. ARFGEF1 is crucial for tumor growth.
(a) Knockdown of miR-27b target in HCT116 cells by each shRNA. **(b)** Effect on anchorage-independent growth of (a). *P < 0.05, **P < 0.01. **(c)** Effect of ARFGEF1 knockdown on colony formation in HT29 and DU145 cells. *P < 0.05, **P < 0.01. **(d)** Restoration of ARFGEF1 expression rescued colony formation of ARFGEF1-depleted HCT116 cells. *P < 0.05, n.s.; no significance. **(e)** Overexpression of ARFGEF1 attenuated the inhibitory effect of miR-27b on colony formation in HCT116 cells. *P < 0.05

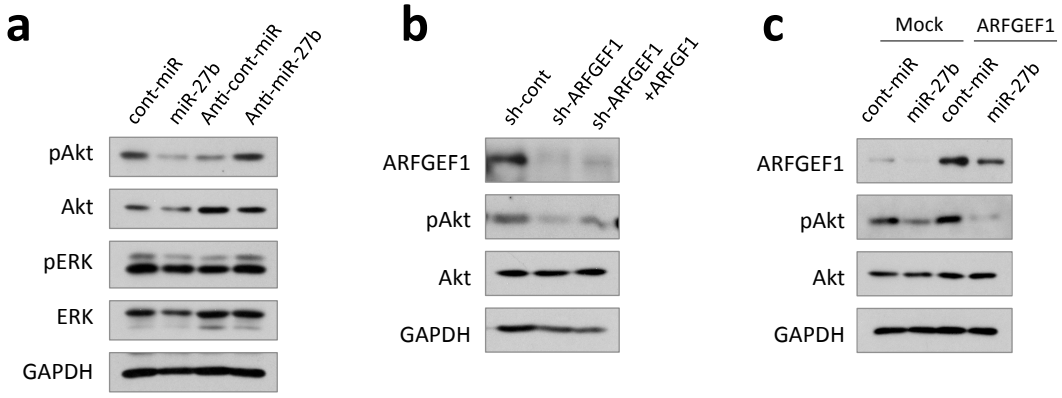

Figure 12. ARFGEF1 is involved in Akt pathway.
(a) Western blot analysis for the activation of Akt in miR-27b transfected cells. HCT116 cells transfected with 30 nM cont-miR or miR-27b for 48 hr were plated on poly-HEMA coated dishes and incubated for 12 hr. **(b, c)** Analysis of the promotion of Akt activation by ARFGEF1. The cells indicated in (Figure 11d and 11e) were plated on poly-HEMA–coated dishes and lysates were immunoblotted.

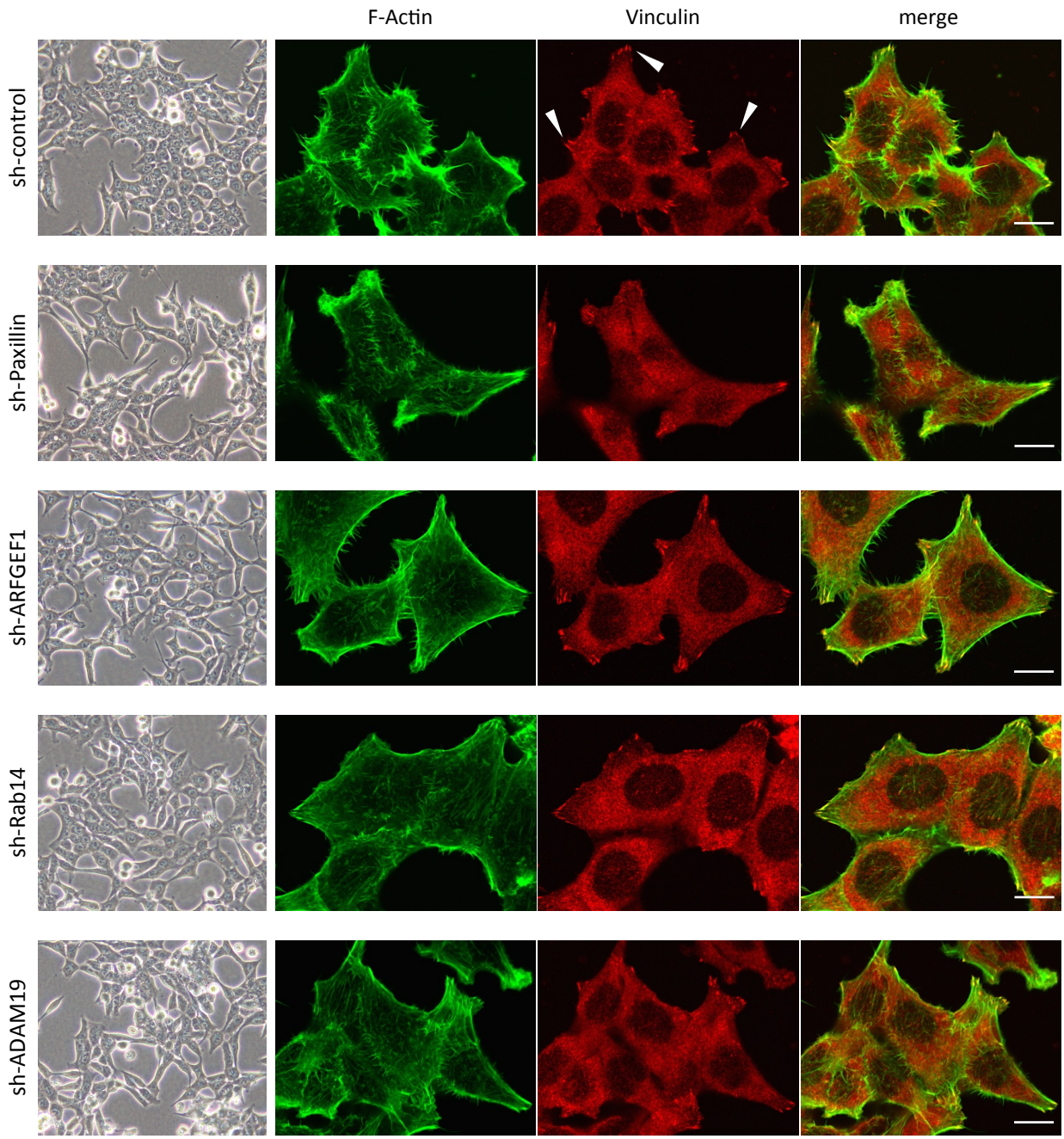

Figure 13. Paxillin promotes focal adhesion formation.
Effect of sh-RNAs on morphology of HCT116 cells.
Left: optical microphotographs of cells. Right: analysis of stress fibers (F-actin; green) and focal adhesions (Vinculin; red). Scale bar; 10 μm.

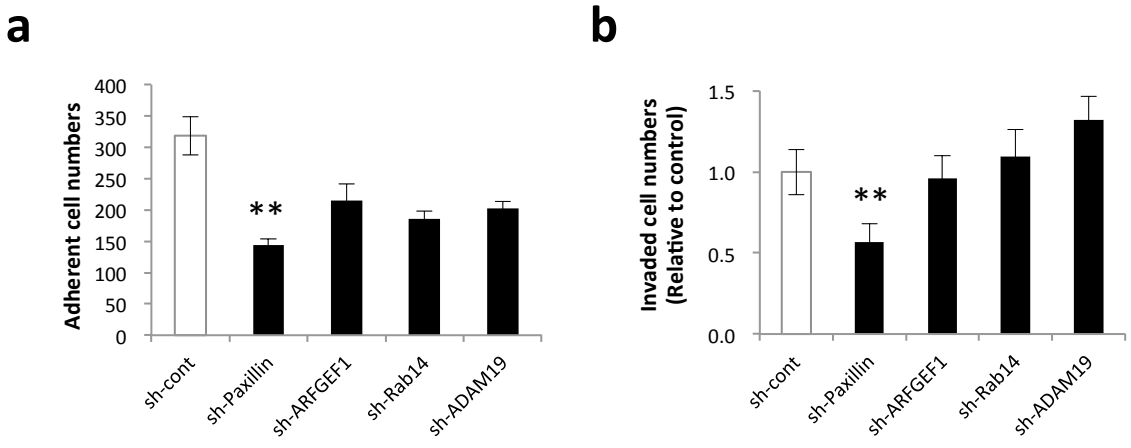

Figure 14. Paxillin is crucial for cell adhesion and invasion.
(a) Effect of knockdown of each target on cell adhesion in HCT116 cells. The indicated cells were plated on collagen-coated dishes and attached cells were counted. **P < 0.01. **(b)** Effect of knockdown of each target on cell invasion. **P < 0.01.

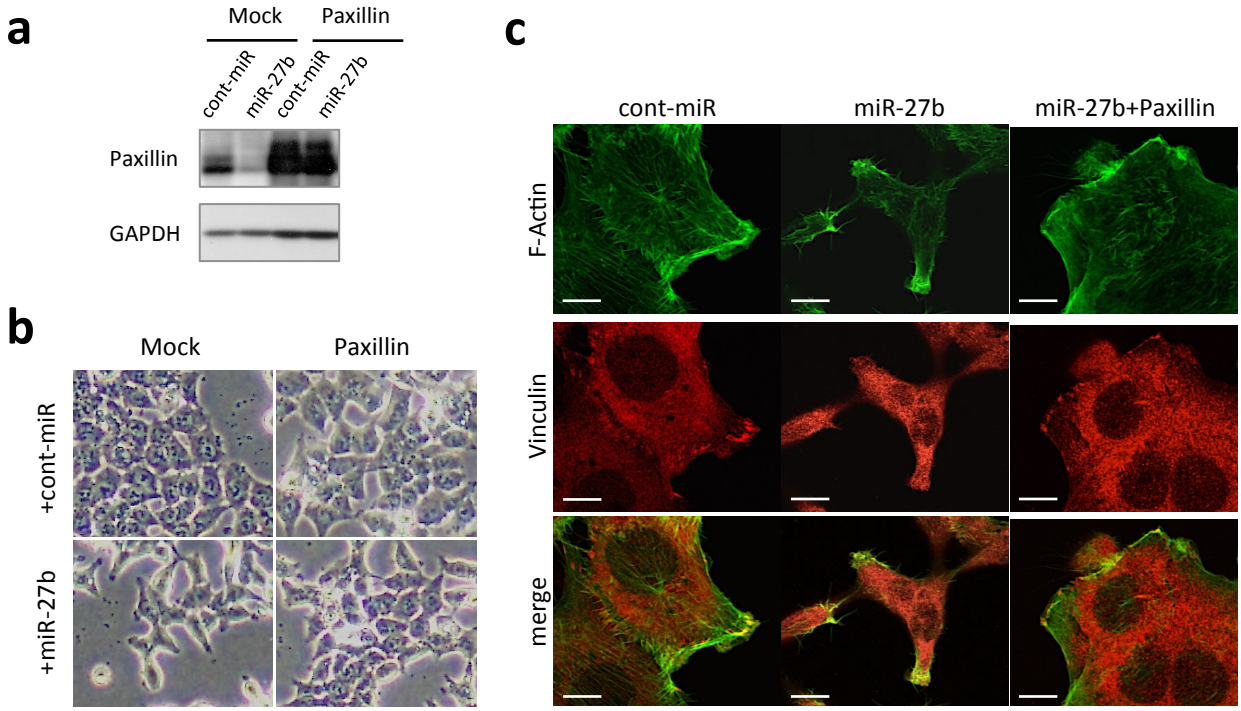

Figure 15. Overexpression of Paxillin reverses morphological changes caused by miR-27b
(a) Paxillin overexpression in HCT116 cells. Cells containing mock vector or paxillin expression vector were transfected with 30 nM cont-miR or miR-27b. **(b)** Optical micrographs of the cells indicated in (a). **(c)** HCT116 cells with mock vector or paxillin were transfected with 5 nM of cont-miR or miR-27b, and then subjected to immunostaining for focal adhesions (red) and F-Actin (green). Scale bar: 10 μm.

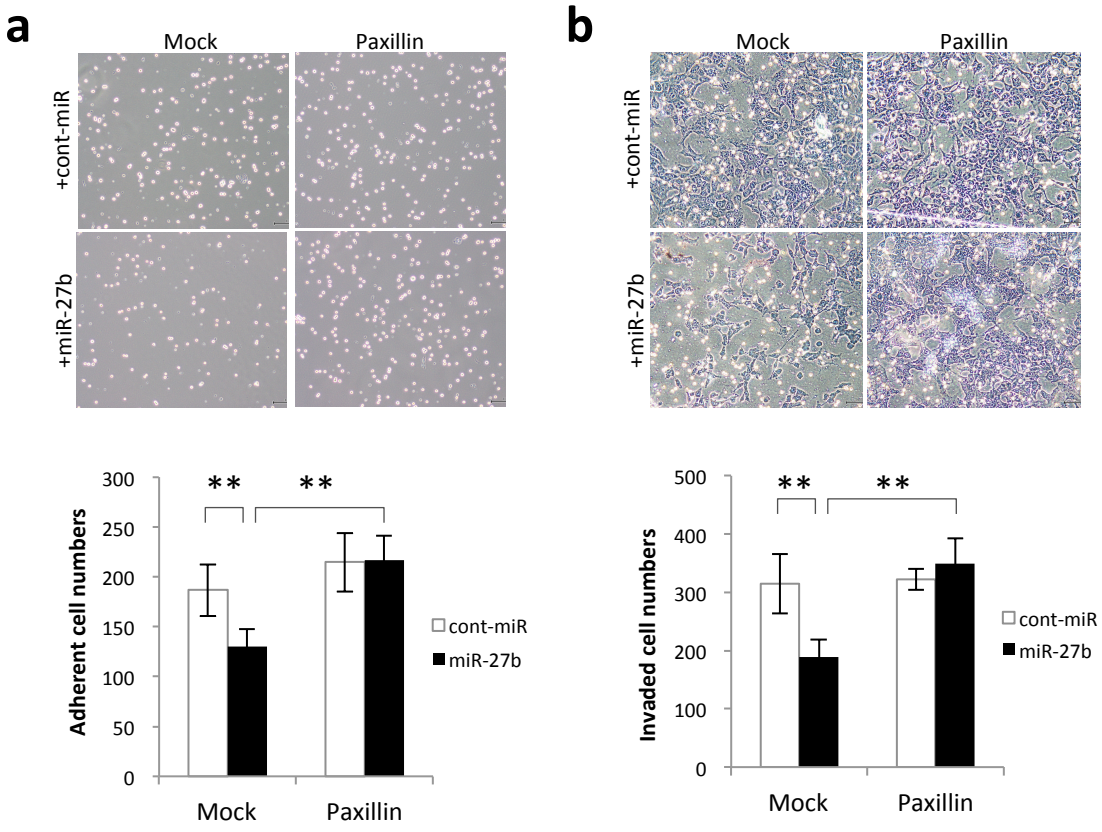

Figure 16. Paxilin expression is crucial for suppression of cell adhesion and invasion by miR-27b.
Cell adhesion **(a)** and invasion **(b)** were rescued by overexpression of paxillin. Cells indicated in (Figure 15a) were subjected to assays. **P < 0.01.

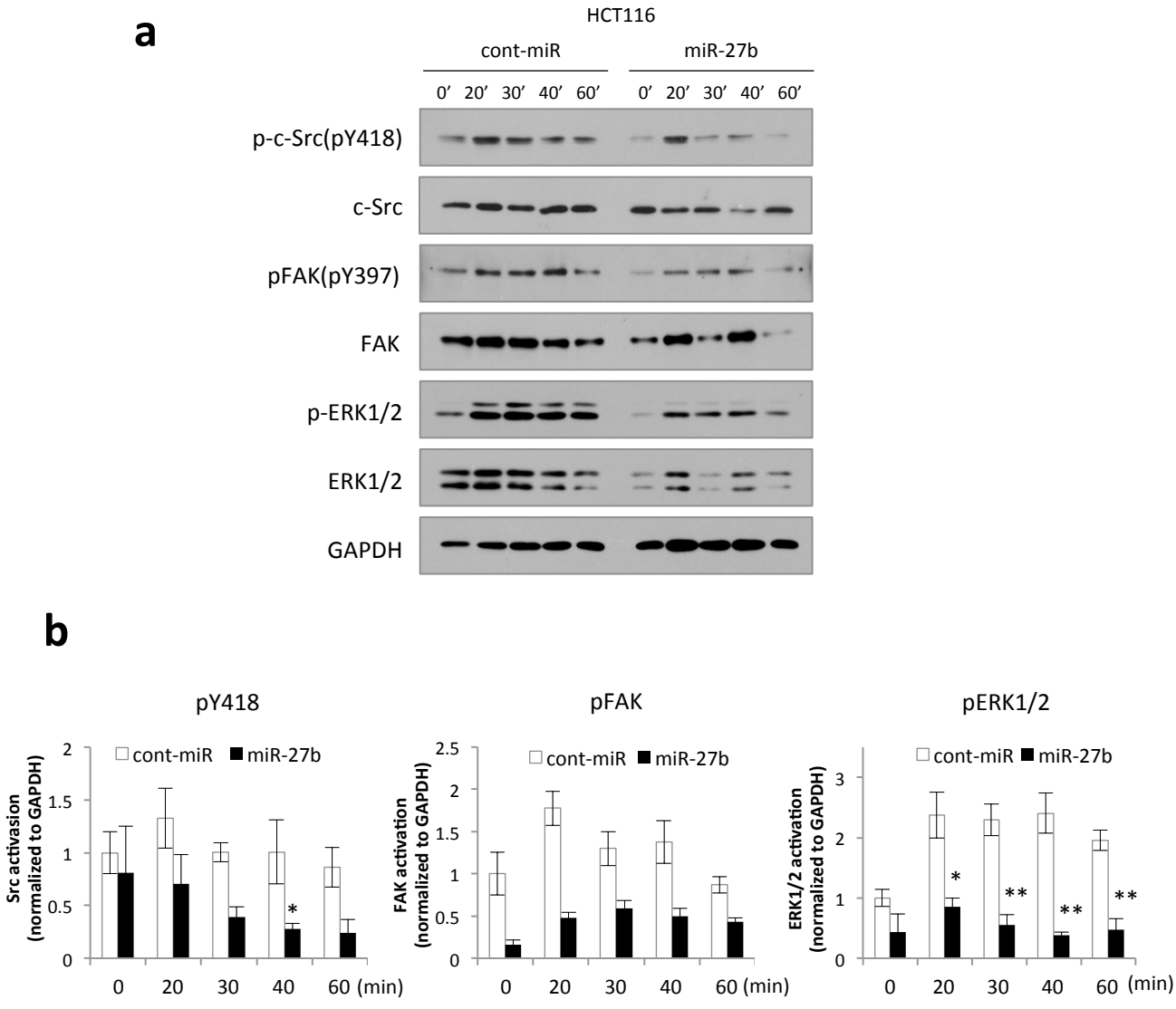

Figure 17. miR-27b suppresses c-Src activation stimulated by cell adhesion.
(a) HCT116 cells were transfected with 30 nM of cont-miR or miR-27b for 2 days, detached, and incubated in serum-free media at 37°C for 30 min. These cells were plated on fibronectin-coated dishes, and the cell lysates of attached cells obtained at the indicated time points were immunoblotted. **(b)** Quantitation of c-Src (pY418), FAK (pY397), and ERK1/2 activation. Data presented are shown as means ± s.d. from three independent experiments. *P < 0.05, **P < 0.01.

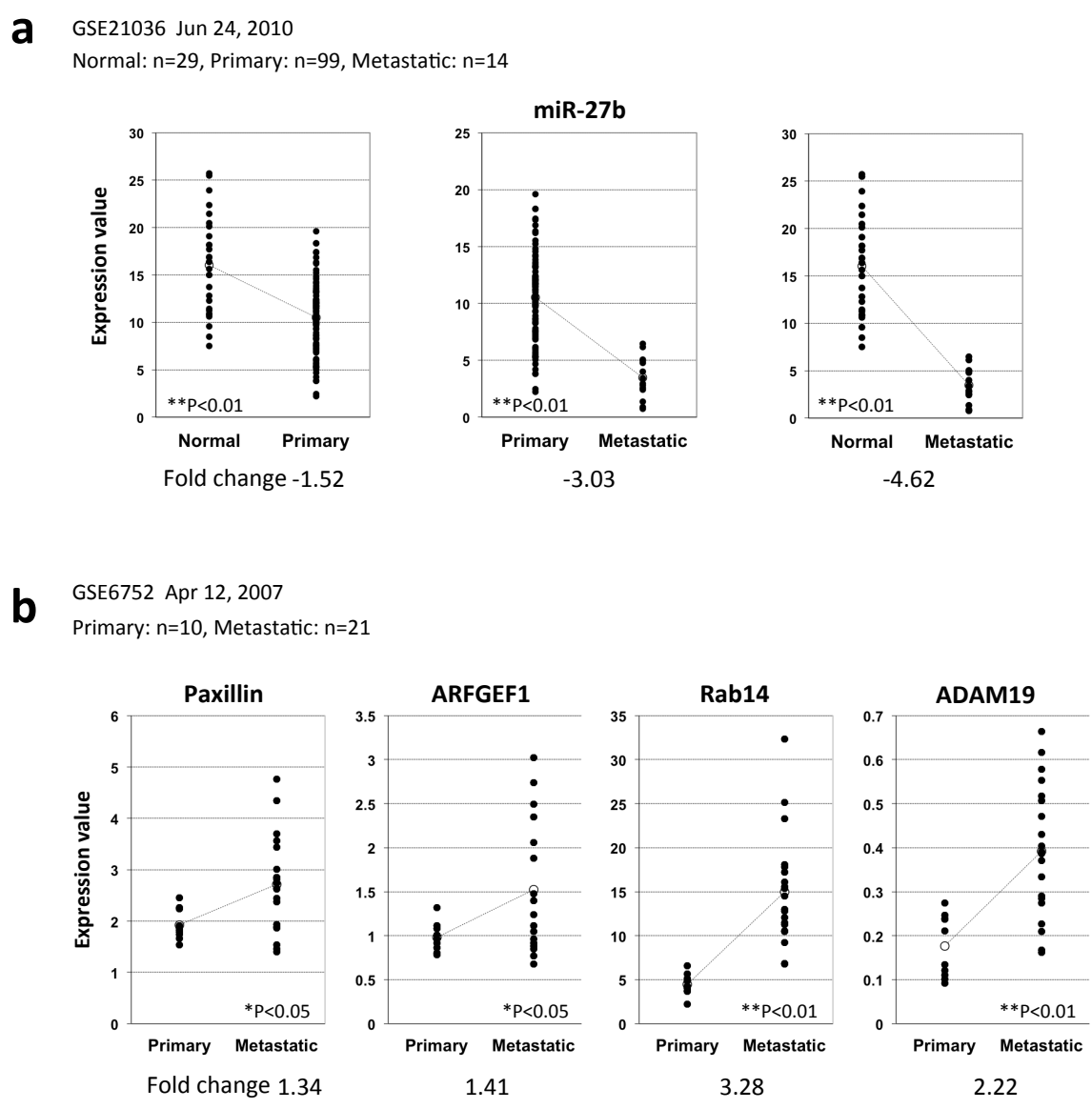

Figure 18. In silico analysis for miR-27b and its targets expression in human prostate cancer tissues.

Microarray data sets, previously published by other groups, were downloaded from the NCBI GEO Data Portal (GSE21036 and GSE6752) and reanalyzed. GSE21036 contains the microRNA expression data for human primary and metastatic prostate cancer samples and control normal adjacent benign prostate, analyzed using Agilent microRNA V2 arrays. GSE6752 contains CodeLink Expression Data from primary prostate tumor and prostate tumor metastases, analyzed using Affymetrix oligonucleotide arrays. **(a)** Expression of miR-27b in normal prostate, primary tumor and metastasized tumor. **(b)** Expression of paxillin, ARFGEF1, Rab14, and ADAM19 in primary prostate tumor and metastatic tumor.

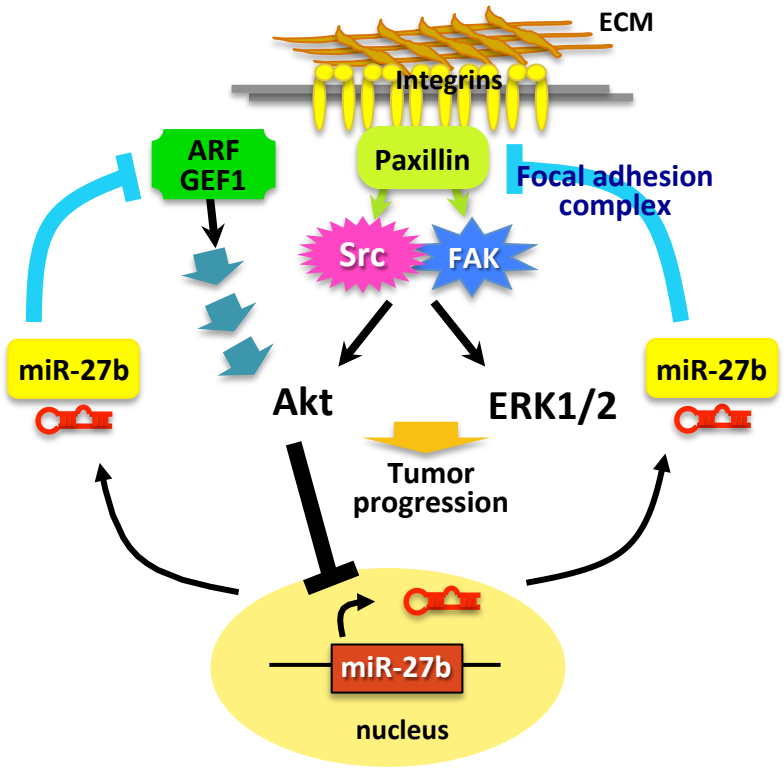

Figure 19. miR-27b mediated regulation of tumor progression
In normal cells expressing miR-27b, expression of paxillin and ARFGEF1 is limited to avoid unregulated cell growth and/or motility. Once miR-27b expression is downregulated due to activation of the Ras/Src/PI3K pathway, expression of paxillin and ARFGEF1 is elevated, resulting in rapid cell growth and stimulation of cell adhesion and invasion.

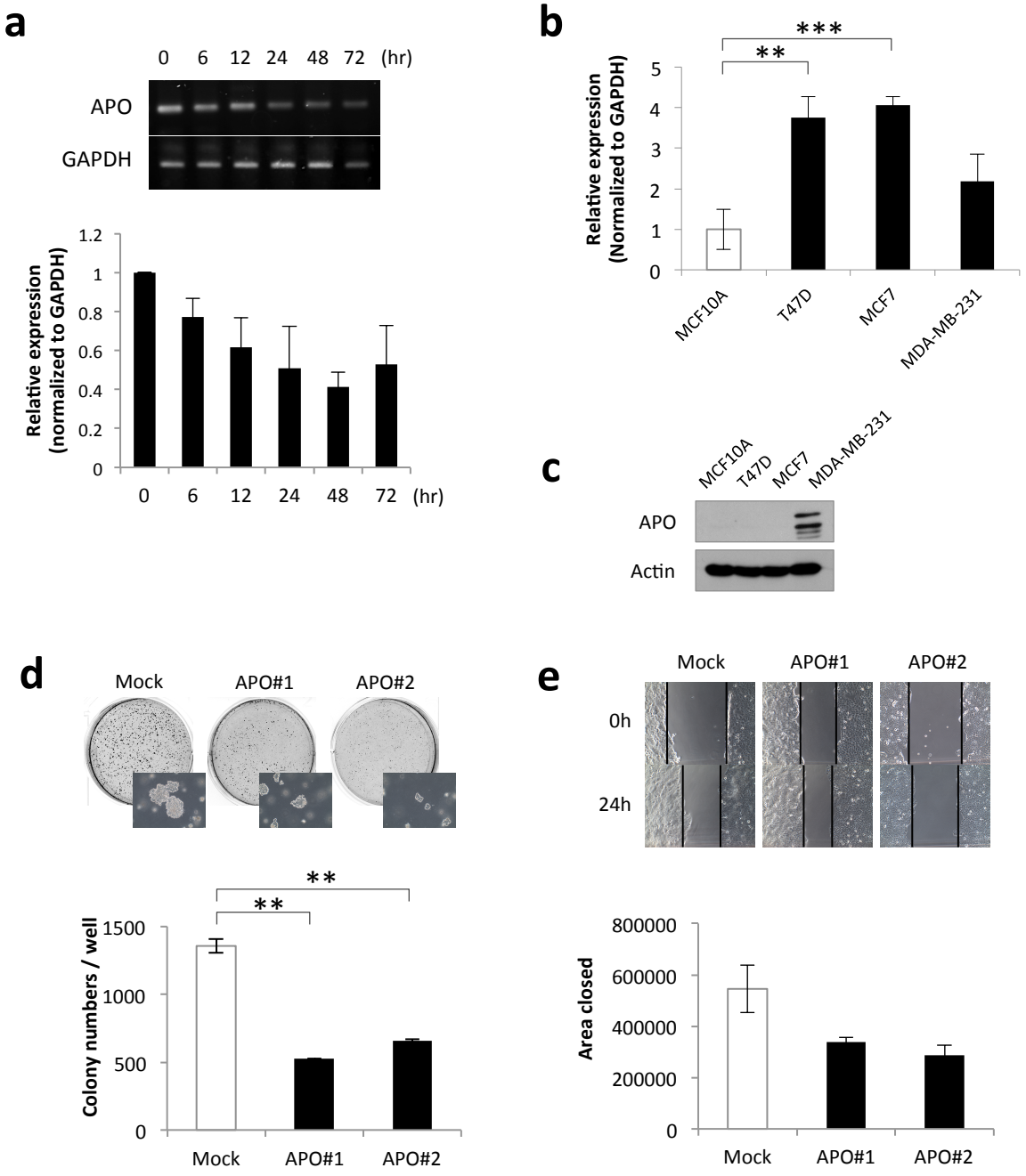

Figure 20. Analysis for miR-27b hostgene APO

(a) RT-PCR analysis for APO and GAPDH. Csk$^{-/-}$ MEFs stably expressing pRetroX-Tet3G vector and pRetroX-TRE3G-c-Src were treated with Doxycycline (1μg/ml final) and total RNAs of the cells were collected after the indicated time. The graph shows the expression levels of APO estimated from the pictures using Image J. Data presented are shown as means ± s.d. from three independent experiments. **(b)** Real time-PCR analysis for APO in breast normal (MCF10A) and cancer cells (T47D, MCF7 and MDA-MB-231). **P < 0.01, ***P<0.001. **(c)** Western blotting for APO. **(d)** Soft-agar colony-formation assay. MCF7 cells stably expressing empty vector (Mock) or pCX4-APO (APO#1, #2) were seeded in soft-agar. After a week, the number of colonies stained with MTT was counted on micrograph. **P < 0.01. **(e)** Wound healing assay. The cells indicated in (c) were plated on non-coated dishes the day before scratching. 24hrs after scratching, the closed area was quantitated from micrograph using Image J.