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Pancreatic cancer-secreted miR-155 implicates in the conversion from normal fibroblasts to cancer-associated fibroblasts

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Key words

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Abstract

Cancer-associated fibroblasts (CAF) are a major constituent of the pancreatic cancer microenvironment and that the meaning is as intended. Pancreatic cancer cells can induce normal fibroblasts to convert into CAF and, reciprocally, CAF promote tumor invasions and proliferations. The mechanism of the conversion from normal fibroblasts (NF) to CAF remains unclear. MicroRNA are short non-coding RNA involved in the post-transcription gene regulation, which have been defined as an imperative controller in tumor invasions, proliferations and colony formations. Microvesicles (MV) have been proved to be an important mediator of intercellular communication and can selectively transport secreted microRNA from a donor cell into a recipient cell. In this study, we isolated primary pancreatic fibroblasts from wild type C57 mice and co-cultured them with pancreatic cancer cell lines, BxPC-3 and SW1990, and observed the conversion from NF to CAF, or at least CAF-like cells. This phenomenon could also be replicated in primary fibroblasts treated with MV separated from a cancer cell media. We identified that miR-155 was upregulated in PaC-derived MV and we confirmed that normal fibroblasts could convert into CAF after MV containing miR-155 had been taken up. TP53INP1 is a target of miR-155 in fibroblasts and a downregulation of TP53INP1 protein levels could contribute to the fibroblasts' activation. These results indicated that pancreatic cancer cells might reprogram normal adjacent fibroblasts into CAF by means of secreted MV containing miR-155. Targeting the circulating microRNA might be a potential therapy for malignant tumors.

Pancreatic cancer (PaC) is a highly malignant digestive carcinoma, and is the fourth leading cause of cancer-associated death in the USA.[1] The average survival time for patients of advanced PaC receiving recommended first-line chemotherapeutics (i.e. single-agent gemcitabine chemotherapy) is approximately 6 months.[2] The overall 5-year survival rate of PaC is less than 5% and this has changed little in the past 20 years.[3] New therapeutic strategies are being explored.

The microenvironment of PaC, characterized by an extensive desmoplastic reaction, has been proved to have significant effects in the progression of carcinomas. The interactions by cell signaling between pancreatic epithelium and stroma can largely determine tumor behavior by influencing tumor migration, angiogenesis, proliferation and immunological recognition.[4] Among stromal cells, fibroblasts are the most abundant and normal fibroblasts (NF) have been shown to prevent tumor growth and invasiveness, while cancer-associated fibroblasts (CAF) have been reported to play a key role in malignant progression.[5] In PaC, the overexpression of Smo in CAF activates the Hedgehog signal pathway and promotes the PaC cell growth.[6] The mechanism of the interrelationship between PaC and CAF, however, remains unclear.

In PaC cells, miR-155 plays an important role in the regulation of cancer cell invasion and migration by modulating the STAT3 signaling pathway and reducing the SOCS1 expression.[7] In addition, the downregulation of tumor protein 53 induced nuclear protein 1 (TP53INP1), a p53-target molecular that induces cell growth arrest and apoptosis by modulating p53 transcriptional activity, is considered to be associated with the escalation of oncogenic miR-155 in PaC.[8] Recently, microvesicles (MV) were defined as vectors that can mediate intercellular information.[9] The studies by Valadi, Skog and Zhang[10–12] demonstrate that MV can transport secreted miRNA into target cells, and can modulate biological functions by downregulating the expression of the target genes. This

kind of intercellular connection achieves signal transmission between different cells, tissues and organs.

We hypothesize that PaC cells can secrete MV containing microRNA to impact tumor-adjacent NF to convert them into CAF. In this study, we have revealed that MV, containing miR-155, might be released by PaC cells and incorporated by co-cultivated NF. Furthermore, TP53INP1 might be a target gene of miR-155 in fibroblasts, which may mediate the proliferation and the activation of normal fibroblasts, and manifest the characteristics of CAF.

Materials and Method

Isolation and culture of pancreatic primary fibroblasts. Primary pancreatic NF were isolated from the mouse pancreas tissues of wild C57 mice. Briefly, after washing with sterile D-hanks, several 2–3 cm^3 pieces of pancreas were incubated with collagenase V (Sigma-Aldrich, Missouri, C9263) at 37°C for 20 min. The solution containing the cells in suspension was centrifuged at 200 g for 5 min and the pellet was plated with DMEM containing 15% FBS (Gibco, Carlsbad, CA). To isolate the fibroblasts, the primary cultures were incubated at 37°C and the medium was exchanged after 24 h for the first time and every third day thereafter. All primary fibroblasts used for this study were between passages 2 and 5.[13,14]

Pancreatic cell lines and culture conditions. Human PaC cell lines BxPC-3 and SW1990 were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS. Both of the cell lines were grown in a humidified 5% CO_2 with a temperature of 37°C.

Indirect co-culture. For indirect co-cultures, transwell plates with two compartments separated by a polycarbonate membrane with 0.4-μm pores were used. Fibroblasts were seeded in the lower compartment (0.4×10^5cells per well) and the PaC cells in the upper compartment (1.2×10^5cells per well). These cells were cultured for 4–5 days together. Fibroblasts alone were used as controls (0.4×10^5 cell per well).

Microvesicle isolation. The MV were isolated from the cell culture media by differential centrifugation according to previous publications.[10,11] After removing the cells and other debris by centrifugation at 300 *g*, 1200 *g* and 10 000 *g*, the supernatant was centrifuged at 110 000 × *g* for 2 h (all steps were performed at 4°C). The MV were collected from the pellet and resuspended in an FBS-free medium, and the BCA method was employed to quantify the total protein content in the MV. The fluorescence labeling of the MV was performed as previously described.[12]

Immunofluorescence assay. The fibroblasts were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized with 0.5% Triton X for 30 min, and then blocked with 5% BSA (diluted in PBS) for 20 min at room temperature. The fibroblasts were further incubated with antibodies as blow: α-smooth muscle actin (α-SMA; Abcam, ab5694, 1:200), FAP (Abcam, ab28244, 1:50), vimentin (Santa Cruz, sc-7557, 1:100) and TP53INP1 (Santa Cruz, sc-68919, 1:30), at 4°C overnight. After that, the cells were incubated with Cy3-labeled IgG (Beyotime, China, 1:2000) for 2 h at 37°C. Finally, DAPI (Beyotime, 1:3000) was added for 10 min at room temperature before the fibroblasts were viewed under a fluorescence microscope.

Cell migration assay. The migration ability of the PaC cells was tested in a transwell boyden chamber with an 8-mm pore size of the polycarbonate membranes. Primary fibroblasts, with or without 4 days co-culture with SW1990 or BxPC-3 cells, were resuspended with 10% FBS at a concentration of 3×10^4 cells/mL and then added to the lower compartment (0.5 mL/well). Simultaneously, corresponding PaC cells were suspended in a serum-free DMEM culture medium at a concentration of 1×10^5 cells/mL and then added to the upper chamber (100 μL/well). The transwell-containing-plates were incubated for 12 h. Cell migration was quantified by the blind counting of migrated cells on the lower surface of the membrane, with three fields per chamber.

RNA isolation and quantitative RT-PCR of mature miRNA. The total RNA of the NF and the MV derived from 10^8 cells were extracted using a TRIzol Reagent (Invitrogen, Carlsbad, CA). qRT-PCR was performed using TaqMan miRNA probes (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The miRNA expression was normalized to U6 snRNA.

To quantify TP53INP1, pre-miR-155 and β-actin mRNA, a real-time PCR was performed using forward and reverse primers. The sequences of the primers were as follows: TP53INP1(F): 5′-GCACCCTTCAGTCTTTTCCTGTT-3′; TP53INP1(R):5′-GGAGAAAGCAGGAATCACTTGTATC-3′; pre-miR-155(F):GTTAAT GCTAATTGTGATA; pre-miR-155(R):TAATGCTAACAGGTAGGAG; β-actin (F):5′-AGGGAAATCGTGCGTGAC-3′; and β-actin(R):5′-CGCTCATTGCCGATAGTG-3′.The relative amount of TP53INP1 and pre-miR-155 mRNA was normalized to β-actin.

Cell Transfection with ncRNA, anti-miR-155 or pre-miR-155, and cell transfection with GFP-labeled miR-155 overexpression lentivirus. PaC cells were seeded on 60-mm dishes and were transfected the following day using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To knock down miR-155, 200 pmol of anti-miR-155 or scrambled negative control anti-miRNA (anti-ncRNA; GenePharma, China) was employed. To overexpress miR-155, 100 pmol of pre-miR-155 or scrambled negative control pre-miRNA (pre-ncRNA) was used. Cells were harvested 24 h after transfection, and supernatants were used for miR-155-deficient MV isolation as mentioned above.

A green fluorescence protein (GFP)-labeled miR-155 overexpression lentivirus was purchased (GeneChem, China) and transfected into PaC cells according to the manufacturer's instructions. The GFP-labeled miR-155-containing MV was isolated as mentioned above.

Protein isolation and western blotting. Protein levels were quantified by western blot analysis of the whole-cell extracts using antibodies as below: FAP of Abcam (ab28244), GAPDH of Santa Cruz (sc-32233), α-SMA, vimentin and TP53INP1 as mentioned above.

EdU proliferation assay. Cell proliferation was detected with an EdU Cell Proliferation Assay Kit (Ribobio, China). The cell nuclei were stained with DAPI. The proportion of the cells incorporated by EdU was determined with fluorescence microscopy.

Statistical analysis. All of the images are representative of at least three independent experiments. The results are presented as the mean ± SEM of at least three independent experiments. The differences were considered statistically significant at $P < 0.05$ using Student's *t*-test.

Results

Conversion from normal fibroblasts to cancer-associated fibroblast-like cells after co-cultivation with pancreatic cancer cells. The microphotographs of the isolated NF from C57 are shown in Figure S1a, and the immunostaining of vimentin was

performed (Fig. S1b) for further validation. To explore the interaction between the tumor cells and the adjacent fibroblasts, we co-cultivated NF with PaC cells (BxPC-3 and SW1990) on transwell plates. Primary normal pancreatic fibroblasts were cultured alone under the same conditions and served as negative control. After 96 h of cultivation, the biomarkers of the CAF, the α-SMA and the fibroblast activation protein (FAP),[15,16] were assessed, to investigate whether the primary fibroblasts had converted to CAF. As shown in Figure 1a and b, the fibroblasts in the co-culture system expressed a higher protein level of CAF characteristic markers. The proliferation activities of the fibroblasts were also validated by an EdU assay. The results showed that after being co-cultivated with BxPC-3 and SW1990, a higher proliferative ability of the fibroblasts was induced (Fig. 1c,d). In the meantime, the BXPC-3 and SW1900 cells presented an increased migration ability in the treated-NF surroundings than did the NF (Fig. 1f,g), which is a feature of a CAF microenvironment. Thus, we propose that after co-cultivation with PaC, NF might be converted to CAF, or at least CAF-like cells.

In addition, the microRNA expression levels were evaluated between the NF and the treated NF. We picked a series of

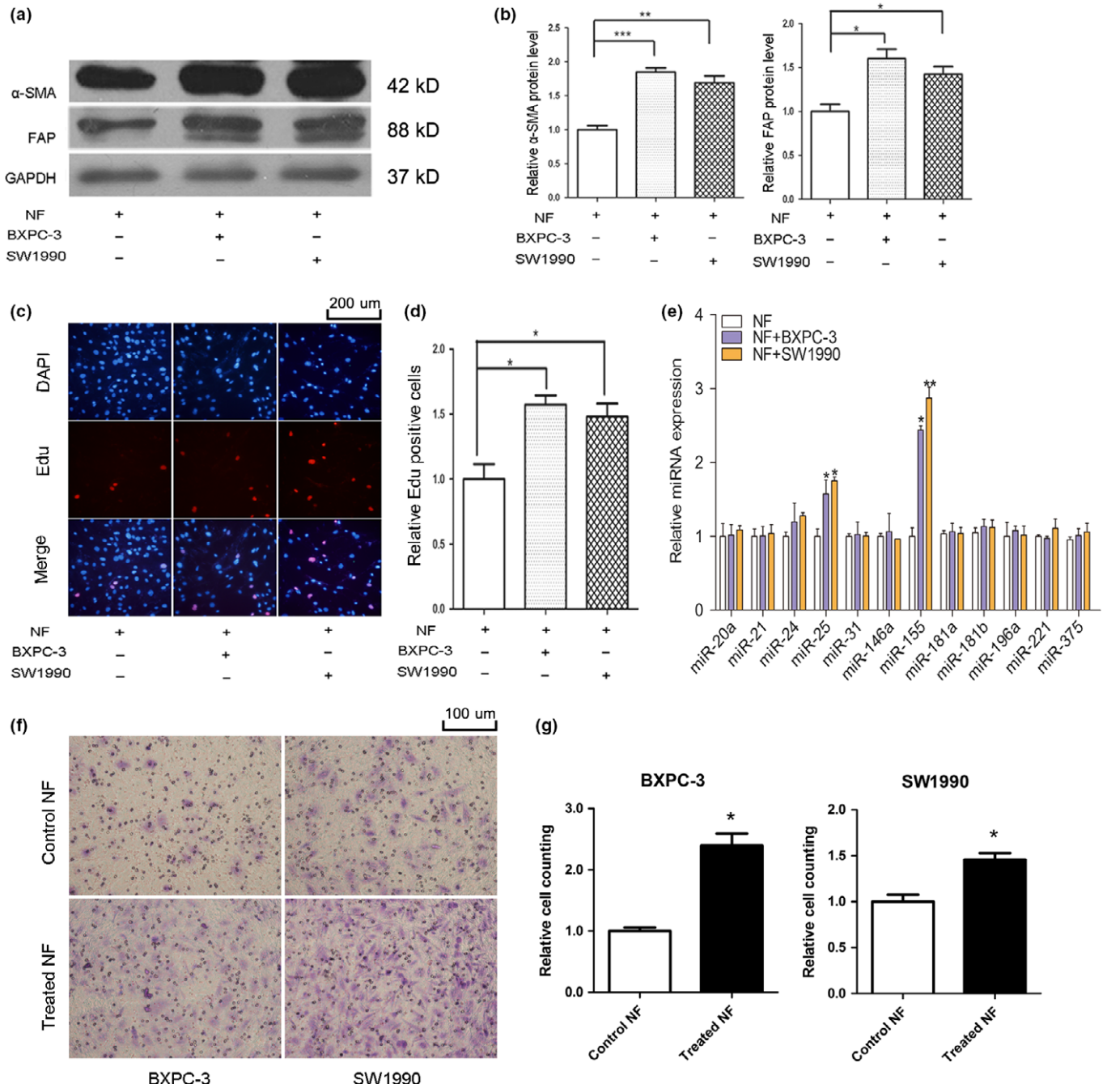

Fig. 1. Normal fibroblasts (NF) converted into cancer-associated fibroblasts (CAF) after being co-cultivated with pancreatic cancer (PaC) cells. (a, b) Western blot analysis of α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) protein levels in NF co-cultivated with PaC cells. (c, d) Edu proliferation assay analysis in NF and NF co-cultivated with PaC cells. (e) Quantitative RT-PCR analysis of miRNA expression levels in NF and NF co-cultivated with PaC cells. (f, g) Cell migration analysis in NF and NF co-cultivated with PaC cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

miRNA which were generally considered to be dysregulation in the tissues or in the sera of PaC patients.[17–21] A relatively higher expression level of miR-25 and miR-155 in the CAF when compared to the NF was confirmed. This suggested that these two miRNA might function as signal molecules in this procedure of the modification of cellular features (Fig. 1e).

Conversion from normal fibroblasts to cancer-associated fibroblast-like cells after being treated with pancreatic cancer cell-derived microvesicles. PaC cell media was collected after 2 days of cultivation with BxPC-3 and SW1990, and the MV were separated from the media for further investigation. We found that after separating the PaC cell medium into two parts by sequential centrifugation (the MV and MV-free fractions), a profile of miR-21, miR-25 and miR-155 was primarily localized in the MV fraction, while miR-24, miR-196a and miR-221, were localized in the MV-free fraction (Fig. S1c). Interestingly, miR-155 and miR-25, but not miR-21, were also elevated in the activated fibroblasts. Meanwhile, DiI-labeled MV rapidly entered into the cultured NF after incubation (Fig. S1d). These results implied that miR-155 and miR-25 might be packed in MV and engaged by NF. Afterward, the NF treated with PaC-derived MV were verified to assess whether the features of the NF had efficiently changed. As depicted in Figure 2a and d, the upregulation of α-SMA and FAP protein levels, as well as the proliferation ability, was seen in MV-treated NF. Additionally, the CAF-like environment that was presented after co-cultivation (Fig. 2f,g), and the α-SMA immunostaining of the treated NF by the BXPC-3 and SW1990 MV, also supported this conversion (Fig. S1e). Meanwhile, a similar trend of miRNA alterations between the NF before and after the MV treatment was uncovered (Fig. 2e), which indicated that the PaC-derived MV could induce the formation of CAF-like cells, and that the molecules packed in the MV might be mediators. Furthermore, to exclude the MV contamination from cell culture medium or FBS, we prepared MV-free 10% FBS DMEM by ultracentrifugation, in which the PaC cell lines were cultured, and the MV of PaC cells supernatants were collected after 4 days cultivation. We re-analyzed the alpha-SMA and FAP protein levels, which showed similar results, indicating that contaminations of MV derived from bovines or from DMEM impact little on this NF–CAF conversion (Fig. S1f,g).

Role of secreted miR-155 and PaC-derived microvesicles in the conversion process. Accumulating evidence has consistently demonstrated stable miRNA in extracellular spaces, such as in various body fluids,[22] and MV are important vectors for miRNA. MiR-155 has a significantly higher level in PaC tissue and PaC patients' sera, when compared with normal adjacent pancreatic tissue or healthy controls.[19,21] In our study, we have hypothesized that under a pancreatic cancer environment, miR-155 would be secreted into circulation in a stable and cell-free form and would serve as a signaling molecule that influenced the fibroblasts. As shown in the data above, the MV derived from PaC cells containing miR-155 could significantly convert the NF to CAF-like cells. In addition, when the primary fibroblasts were treated with pre-miR-155, the protein markers of the CAF were also notably increased (Fig. S2a,b), as well as the proliferation abilities (Fig. S2c,d). In view of the MV containing various substances and being shown to target recipient cells, to exchange RNA, proteins and lipids,[23,24] we next tested whether a "neutralization" of miR-155 in the PaC-derived MV could induce the conversion. We transfected BxPC-3 and SW1990 with anti-miR-155, and the MV were isolated from the cell media after 48 h of cultivation. The PaC cell lines transfected with anti-ncRNA were served as negative controls. The decreased level of miR-155 in miR-155-deficient MV is demonstrated in Figure S2e. The medium of NF was changed with PaC cells, a MV-free-medium, and equal amounts (50ug total protein) of different MV were introduced into the NF. We found that those fibroblasts treated with these miR-155-deficient MV showed no remarkable changes in the protein markers of the CAF (Fig. 3a–d) or in the proliferation abilities (Fig. 3e–h). Furthermore, to exclude the possibility that endogenous miR-155 in NF after PaC MV stimuli affected the NF conversion, we compared the level of pre-miR-155 in NF and CAF-like cells. The result showed tiny change (Fig. S2f), suggesting that altered miR-155 levels in fibroblasts after MV stimuli are not attributed to endogenous miR-155 expression but tumor-secreted miR-155 via MV. Consequently, we concluded that miR-155 packaged in the MV derived from PaC cells were predominant and participated in the conversion to CAF-like cells.

Inverse correlation of TP53INP1 and miR-155 and contributions to the conversion. As MV deliver miRNA into recipient cells in which these exogenous miRNA can silence their target genes and trigger downstream signaling events,[12,25] we considered the TP53INP1 gene to be the target of miR-155 in the conversion from NF to CAF-like cells. It has been shown that TP53INP1 is a proapoptotic stress-induced p53 target molecular and could be directly repressed by miR-155 in a pancreatic tumor development.[8] We found that in primary pancreatic NF, the inverse correlation of TP53INP1 and miR-155 also exists (Fig. S3a–c). However, whether a reduction of the TP53INP1 protein level itself contributes to this conversion has not been explored. In a subsequent assay, we constructed TP53INP1 siRNA (siRNA validation data shown in Fig. S3d–f) and the transfection with siRNA could augment the protein levels of α-SMA and FAP in fibroblasts (Fig. S3 g,h), and together, could appreciably intensify cell proliferation ability (Fig. S3i,j). In addition, the TP53INP1 protein levels were significantly declined in CAF-like cells, whether they were induced by co-cultivation with PaC cells (Fig. 4a,b) or by treatment with cancer cell MV (Fig. 4c,d). In addition, decrement of TP53INP1 immunostaining was seen in these CAF-like cells (Fig. S4a). However, after the "neutralization" of miR-155 in the PaC-derived MV with anti-miR-155, the decrement of TP53INP1 protein levels vanished (Fig. 4e–h) in accordance with the NF-CAF-like cell conversion. Thus, we concluded that the expression level of the TP53INP1 protein might play an influential role in NF-CAF conversion in a PaC microenvironment and it could be regulated by exogenous miR-155 from the adjacent PaC cells.

Discussion

Emerging evidence has shown that miRNA could be a potent remedy in various diseases,[26] and the use of exosomal miRNA was attempted previously.[27] Although CAF constitute tumorigenic microenvironments, the exact mechanism of how fibroblasts in a quiescent stage convert into CAF is still under research.[28] We report that the general features of CAF could be found in mouse pancreatic fibroblasts, co-cultured with PaC cells, and that PaC-derived MV could produce the same results, which prompted that the effective agents converting NF to CAF might be packed in the MV.

Accumulating evidence has consistently demonstrated that circulating miRNA can be stable in extracellular spaces and are tightly correlated with various pathological conditions.[29–31]

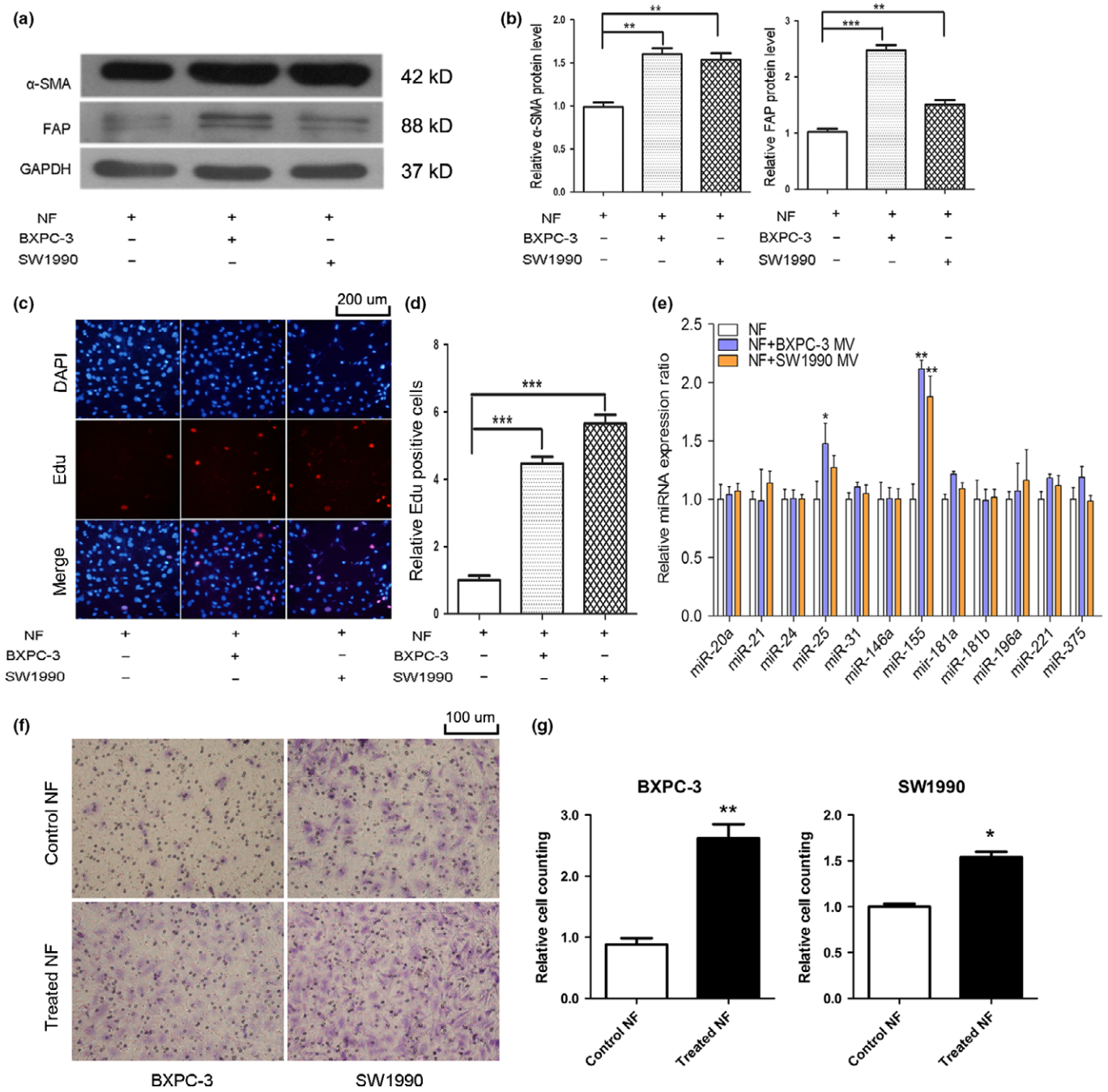

Fig. 2. Normal fibroblasts (NF) converted into cancer-associated fibroblasts (CAF) after being treated with pancreatic cancer (PaC)-derived microvesicles (MV). (a, b) Western blot analysis of α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) protein levels in NF treated with PaC-derived MV. (c, d) Edu proliferation assay analysis in NF and NF treated with PaC-derived MV. (e) Quantitative RT-PCR analysis of miRNA expression levels in NF and NF treated with PaC-derived MV. (f, g) Cell migration analysis in NF and NF treated with PaC-derived MV. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Specific miRNA populations may be preferentially sorted into MV under certain physiological or pathophysiological conditions, and MV-enclosed miRNA are thought to be taken up when the MV are internalized by endocytosis, phagocytosis, or by direct fusion with the plasma membrane.[12,22] Our data has illustrated that the NF activation could be induced by PaC-derived MV bearing miR-155. Using PaC-derived GFP-labeled-miR-155 containing MV, we also demonstrated the translocation of miR-155 from PaC cells to fibroblasts via MV (Fig. S4b). We then used miR-155-deficient MV from PaC cells to confirm that the major effect of MV on NF was due to containing miR-155 rather than the many other constitutions of the MV, basing on the fact that endogenous miRNA do not substantially alter after the induction of exogenous MV and that negligible alterations of protein/mRNA/miRNA levels in miRNA antisence transfected MV could be found, shown by Yin et al [32]. Thus, our study has revealed at least one of the important approaches of how PaC cells could influence periph-

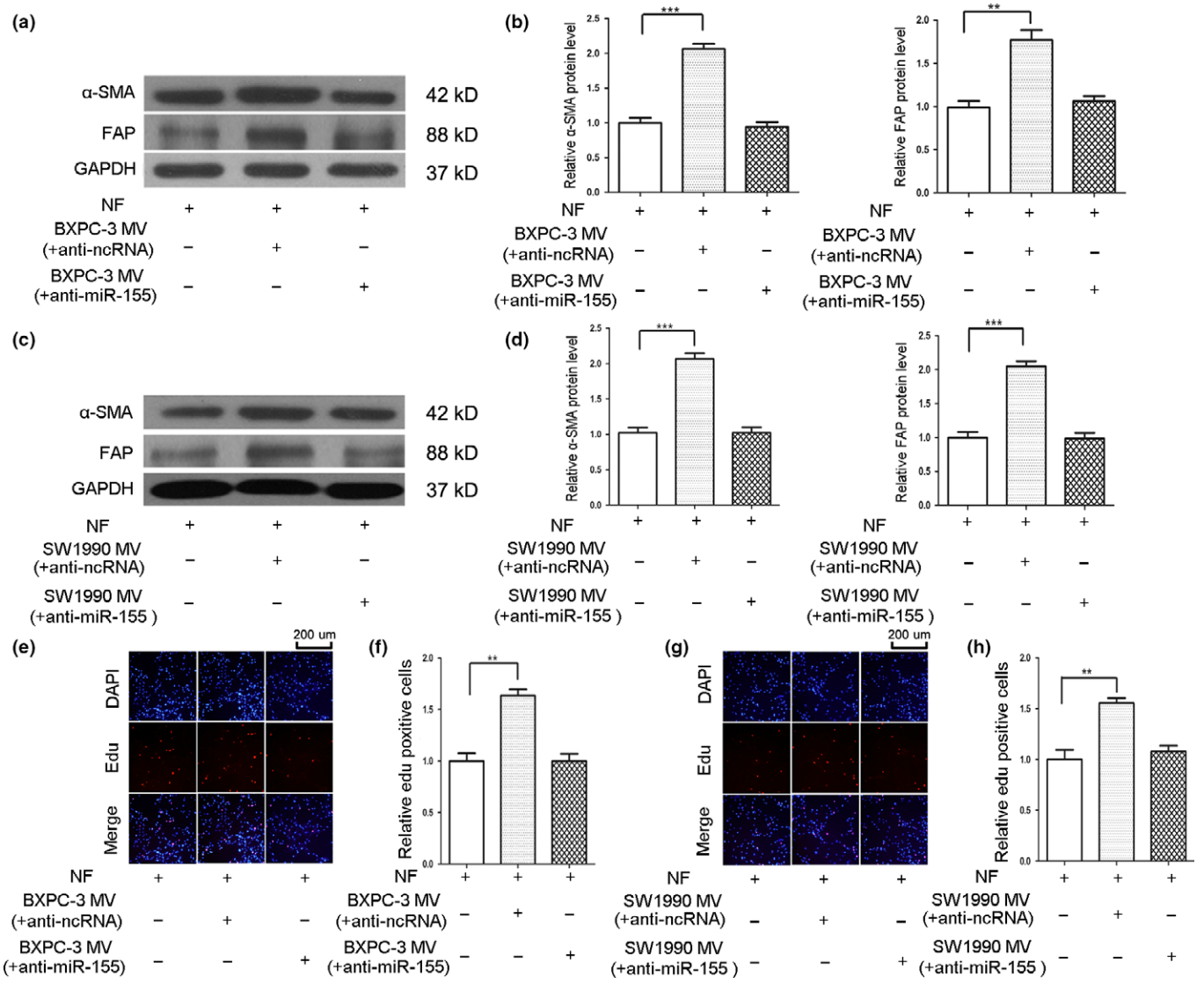

Fig. 3. Unapparent conversion from normal fibroblasts (NF) to cancer-associated fibroblasts (CAF) if treated with miR-155-deficient microvesicles (MV). (a, b) Western blot analysis of α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) protein levels in NF and NF treated with miR-155-deficient BxPC-3-derived MV and controls. (c, d) Western blot analysis of α-SMA and FAP protein levels in NF treated with miR-155-deficient SW1990-derived MV and controls. (e, f) Edu proliferation assay analysis of NF and NF treated with miR-155-deficient BxPC-3-derived MV and controls. (g, h) Edu proliferation assay analysis of NF and NF treated with miR-155-deficient SW1990-derived MV and controls. $^{**}P < 0.01$; $^{***}P < 0.001$.

eral fibroblasts. Furthermore, miR-155 was also deemed to induce reprogramming of NF into CAF in ovarian cancer,[33] which could support our findings.

TP53INP1, a key element in p53 function, induces growth inhibition and autophagic cell death, represses tumor cell migration, and leads to cell growth arrest and apoptosis upon DNA damage stress.[34] TP53INP1 expression is repressed by miR-155 in PaC, and also in breast cancer, and its restoration inhibits pancreatic tumor development.[8,35]. Recent reports indicate that inactivation of the p53 gene is detected in the stroma of human cancers, and mouse models have validated the tumor-promoting effects of deleting p53 in fibroblasts that convert them from NF to CAF.[36] We noticed that over a 160-fold increase of the miR-155 level showed a slight decrease of TP53INP1. One of the explanations for this discrepancy is that the transient transfection of miRNA mimics gives rise to supra-physiological miRNA levels in cells (as found when the miRNA level is assessed by qRT-PCR). The supraphysiological levels of transfected miRNA do not represent the functional levels, because the majority of transfected miRNA is not accessible for loading into Argonaute as functionally active miRNA, but is actually in non-functional locations such as in lysosomes.[37]

There are some limitations in our study. Because there are no stable human fibroblast cell lines, we used mice pancreatic NF as our research model, and co-cultured these NF with human PaC cell lines, based on the fact that the nucleotides in the seed recognition sites of TP53INP1 3′UTR and miR-155 are highly conserved across species, including humans and mice (Fig. S4c). This makes the regulation of the mouse gene by miRNA from human cell lines plausible. In contrast, previous *in vivo* studies have shown an angiogenetic effect in mice of human cell-derived MV,[38] as well as a reduction in the growth of *Plasmodium falciparum* induced by human

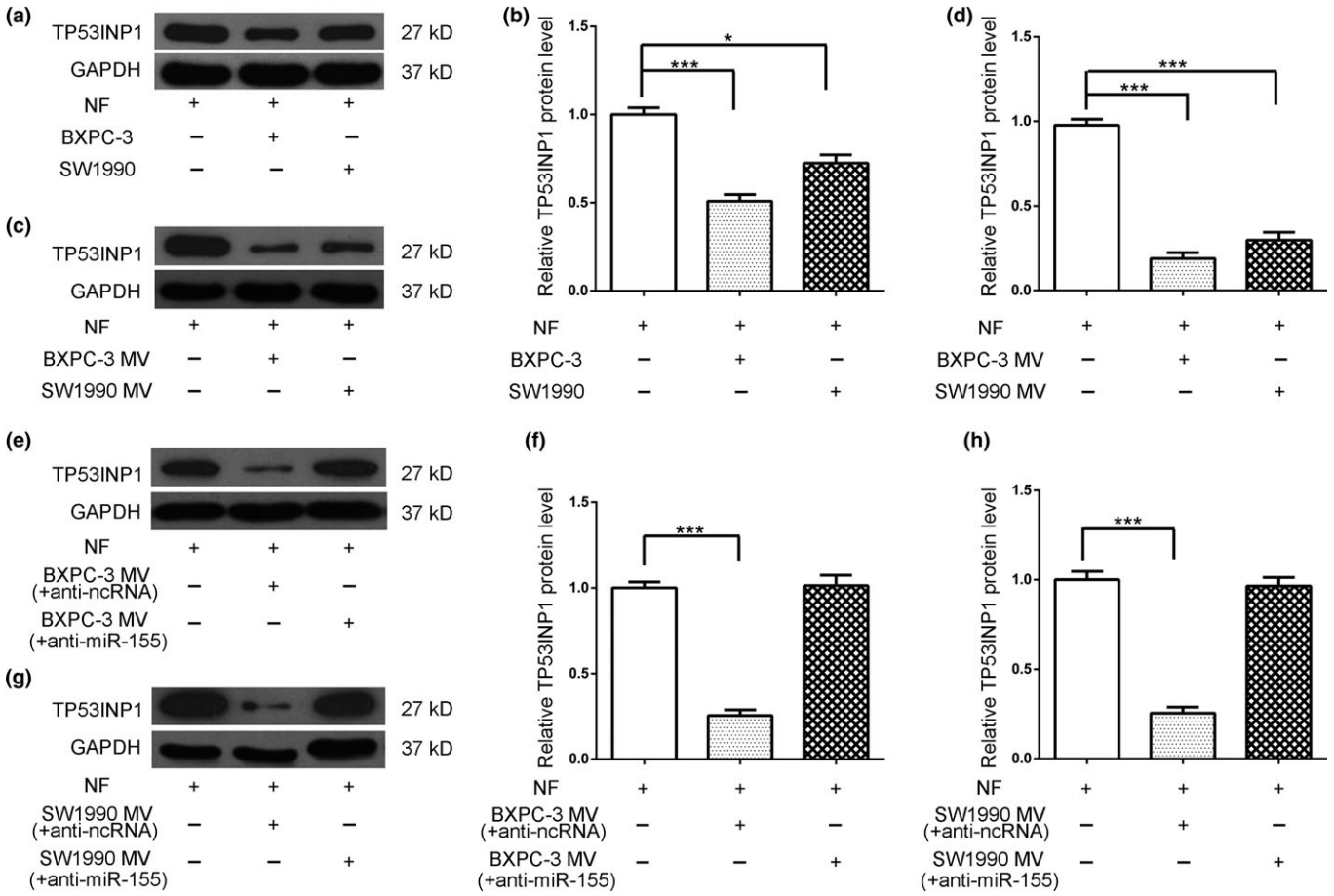

Fig. 4. Alteration of TP53INP1 protein levels in normal fibroblasts (NF) to cancer-associated fibroblast (CAF) conversion. (a, b) Western blot analysis of TP53INP1 protein levels in NF co-cultivated with pancreatic cancer (PaC) cells. (a, b) Western blot analysis of α-smooth muscle actin (α-SMA) and fibroblast cells. (c, d) Western blot analysis of TP53INP1 protein levels in NF treated with PaC-derived microvesicles (MV). (e, f) Western blot analysis of TP53INP1 protein levels in NF treated with miR-155-deficient BxPC-3-derived MV and controls. (g, h) Western blot analysis of TP53INP1 protein levels in NF treated with miR-155-deficient SW1990-derived MV and controls. $*P < 0.05$; $***P < 0.001$.

miRNA,[39] confirming the roles of miRNA in cross-species communication. In addition, the NF-CAF conversion is a complex disorder and the downregulation of TP53INP1 induced by MV-enclosed miR-155 might be an important pathogenesis, while other mechanisms involved remain to be investigated. Besides, given that PaC-derived MV dramatically decreased TP53INP1 expression but miR-155 increased only twofold, we cannot totally rule out the possibility that TP53INP1 expression is suppressed by factors other than miR-155. Therefore, at this stage, our investigation is simply describing a new critical pathway, and one of the major pathways, in NF activation.

In conclusion, our study has demonstrated a conversion from NF to CAF, or at least CAF-like cells, *in vitro*, through the mediation of MV from PaC cells, and that the secreted miR-155 might play a leading role. Our research has identified a candidate mediator, and has proposed the possibility of a new strategy of treatment, targeting the tumor stroma with miRNA inhibitors.

Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10–29.
2 Lee KH, Kim MK, Kim YH, *et al.* Gemcitabine and oxaliplatin combination as first-line treatment for advanced pancreatic cancer: a multicenter phase II study. *Cancer Chemother Pharmacol* 2009; **64**: 317–25.
3 Hidalgo M. Pancreatic cancer. *N Engl J Med* 2010; **362**: 1605–17.
4 Kleeff J, Beckhove P, Esposito I, *et al.* Pancreatic cancer microenvironment. *Int J Cancer* 2007; **121**: 699–705.
5 Bremnes RM, Donnem T, Al-Saad S, *et al.* The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. *J Thorac Oncol* 2011; **6**: 209–17.
6 Walter K, Omura N, Hong SM, *et al.* Overexpression of smoothened activates the sonic hedgehog signaling pathway in pancreatic cancer-associated fibroblasts. *Clin Cancer Res* 2010; **16**: 1781–9.
7 Huang C, Li H, Wu W, Jiang T, Qiu Z. Regulation of miR-155 affects pancreatic cancer cell invasiveness and migration by modulating the STAT3 signaling pathway through SOCS1. *Oncol Rep* 2013; **30**: 1223–30.
8 Gironella M, Seux M, Xie MJ, *et al.* Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A* 2007; **104**: 16170–5.
9 Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes /microvesicles as a mechanism of cell-to-cell communication. *Kidney Int* 2010; **78**: 838–48.

10 Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007; **9**: 654–9.
11 Skog J, Wurdinger T, van Rijn S, *et al.* Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008; **10**: 1470–6.
12 Zhang Y, Liu D, Chen X, *et al.* Secreted monocytic miR-150 enhances targeted endothelial cell migration. *Mol Cell* 2010; **39**: 133–44.
13 Cai J, Tang H, Xu L, *et al.* Fibroblasts in omentum activated by tumor cells promote ovarian cancer growth, adhesion and invasiveness. *Carcinogenesis* 2012; **33**: 20–9.
14 Witowski J, Jorres A. Peritoneal cell culture: fibroblasts. *Perit Dial Int* 2006; **26**: 292–9.
15 Xouri G, Christian S. Origin and function of tumor stroma fibroblasts. *Semin Cell Dev Biol* 2010; **21**: 40–6.
16 Rasanen K, Vaheri A. Activation of fibroblasts in cancer stroma. *Exp Cell Res* 2010; **316**: 2713–22.
17 Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J Gastrointest Surg* 2008; **12**: 2171–6.
18 Szafranska AE, Davison TS, *et al.* MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* 2007; **26**: 4442–52.
19 Bloomston M, Frankel WL, Petrocca F, *et al.* MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* 2007; **297**: 1901–8.
20 Liu R, Chen X, Du Y, *et al.* Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. *Clin Chem* 2012; **58**: 610–8.
21 Liu J, Gao J, Du Y, *et al.* Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. *Int J Cancer* 2012; **131**: 683–91.
22 Liang H, Gong F, Zhang S, Zhang CY, Zen K, Chen X. The origin, function, and diagnostic potential of extracellular microRNAs in human body fluids. *Wiley Interdiscip Rev RNA* 2014; **5**: 285–300.
23 Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2002; **2**: 569–79.
24 Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics* 2010; **73**: 1907–20.
25 Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem* 2010; **285**: 17442–52.
26 Urbich C, Kuehbacher A, Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 2008; **79**: 581–8.
27 Iguchi H, Kosaka N, Ochiya T. Secretory microRNAs as a versatile communication tool. *Commun Integr Biol* 2010; **3**: 478–81.
28 Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012; **21**: 309–22.
29 Park NJ, Zhou H, Elashoff D, *et al.* Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clin Cancer Res* 2009; **15**: 5473–7.
30 Hanke M, Hoefig K, Merz H, *et al.* A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. *Urol Oncol* 2010; **28**: 655–61.
31 Chen X, Ba Y, Ma L, *et al.* Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008; **18**: 997–1006.
32 Yin Y, Cai X, Chen X, *et al.* Tumor-secreted miR-214 induces regulatory T cells: a major link between immune evasion and tumor growth. *Cell Res* 2014; **24**: 1164–80.
33 Mitra AK, Zillhardt M, Hua Y, *et al.* MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer. *Cancer Discov* 2012; **2**: 1100–8.
34 Shahbazi J, Lock R, Liu T. Tumor Protein 53-Induced Nuclear Protein 1 Enhances p53 Function and Represses Tumorigenesis. *Front Genet* 2013; **4**: 80.
35 Zhang CM, Zhao J, Deng HY. MiR-155 promotes proliferation of human breast cancer MCF-7 cells through targeting tumor protein 53-induced nuclear protein 1. *J Biomed Sci* 2013; **20**: 79.
36 Drake LE, Macleod KF. Tumour suppressor gene function in carcinoma-associated fibroblasts: from tumour cells via EMT and back again? *J Pathol* 2014; **232**: 283–8.
37 Thomson DW, Bracken CP, Szubert JM, Goodall GJ. On measuring miRNAs after transient transfection of mimics or antisense inhibitors. *PLoS ONE* 2013; **8**: e55214.
38 Li J, Zhang Y, Liu Y, *et al.* Microvesicle-mediated transfer of microRNA-150 from monocytes to endothelial cells promotes angiogenesis. *J Biol Chem* 2013; **288**: 23586–96.
39 LaMonte G, Philip N, Reardon J, *et al.* Translocation of sickle cell erythrocyte microRNAs into Plasmodium falciparum inhibits parasite translation and contributes to malaria resistance. *Cell Host Microbe* 2012; **12**: 187–99.

Supporting Information

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

Fig. S1. (a) Microphotographs of isolated fibroplasts. Magnification at 100× and 200×, respectively. (b) Representative images of the immunostaining of vimentin in isolated NFs. (c) Quantitive RT-PCR analysis of miRNA expression levels in the MV and MV-free medium of PaC cells. (d) Incorporation of human MVs by mice NFs. (e) Representative images of the immumostaining of alpha-SMA in NFs treated with MVs from PaC cells. (f, g) Western blot analysis of α-SMA and FAP protein levels in NFs treated with PaC-derived MVs (PaC cell lines cultured in MV-free 10% DMEM medium).

Fig. S2. (a, b) Western blot analysis of α-SMA and FAP protein levels in NFs transfected by pre-miR-155. (c, d) Edu proliferation assay analysis in NFs and NFs transfected by pre-miR-155. (e) Decrement of the miR-expression in fibroblasts treated with miR-155-deficient MVs from PaC cells. (f) Quantitive RT-PCR analysis of pre-miR-155 expression levels in NFs and NFs treated with PaC-derived MVs.

Fig. S3. (a, b) TP53INP1 protein levels in NFs tranfected by pre-miR-155. (c) Quantitive RT-PCR analysis of miRNA expression levels in NFs and NFs transfected by pre-miR-155. (d) Quantitive RT-PCR analysis of TP53INP1 miRNA levels NFs and NFs transfected by TP53INP1-siRNA. (e, f) Downregulation of TP53INP1 protein levels in NFs and NFs transfected by TP53INP1-siRNA. (e, f) Upregulation of α-SMA and FAP protein levels in NFs and NFs transfected by TP53INP1-siRNA. (i, j) Edu proliferation assay analysis in NFs and NFs transfected with TP53INP1-siRNA.

Fig. S4. (a) Representative images of the immunostaining of TP53INP1 in NFs treated with Mvs from PaC cells. (b) Representative images of the translocation of fluorescence-labelled miR-155 from the PaC cells to fibroblasts via MV. (c) Schematic description of the hypothetical duplexes formed by the interations between the binding sites of TP53INP1 3′UTR and miR-155. The seed recognition sites are denoted and all nucleotides in these regions are highly conserved across species, including human and mouse seed recognition sites.