Oncogene (2013) **32,** 2747–2755

www.nature.com/onc

ORIGINAL ARTICLE

Leukemia cell to endothelial cell communication via exosomal miRNAs

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Recent findings indicate that specific microRNAs (miRNAs), such as those of the miR-17-92 cluster, may be responsible for regulating endothelial gene expression during tumor angiogenesis. Secreted miRNAs enclosed in exosomes also have an important role in cell–cell communication. To elucidate whether miRNAs secreted from neoplastic cells transfer into endothelial cells and are functionally active in the recipient cells, we investigated the effect of exosomal miRNAs derived from leukemia cells (K562) on human umbilical vein endothelial cells (HUVECs). As K562 cells released the miR-17-92 cluster, especially miR-92a, into the extracellular environment, K562 cells, transfected with Cy3-labeled pre-miR-92a, were co-cultured with HUVECs. Cy3-miR-92a derived from K562 cells was detected in the cytoplasm of HUVECs, and the Cy3-miR-92a co-localized with the signals of an exosomal marker, CD63. The expression of integrin α5, a target gene for miR-92a, was significantly reduced in HUVECs by exosomal miR-92a, indicating that exogenous miRNA via exosomal transport can function like endogenous miRNA in HUVECs. The most salient feature of this study is the exosome, derived from K562 cells with enforced miR-92a expression, did not affect the growth of HUVECs but did enhance endothelial cell migration and tube formation. Our results support the idea that exosomal miRNAs have an important role in neoplasia-to-endothelial cell communication.

Oncogene (2013) **32,** 2747–2755; doi:10.1038/onc.2012.295; published online 16 July 2012

Keywords: exosomal miRNAs; cell-to-cell communication; endothelial migration; tube formation

INTRODUCTION

Pathological angiogenesis is a hallmark of cancer. Concentrated research efforts are leading to the discovery of a growing number of pro- and anti-angiogenic molecules, some of which are already in clinical trials.[1] The complex interactions among these molecules and how they affect vascular structure have not been fully elucidated. MicroRNAs (miRNAs), small (18–22 nucleotides) non-coding RNAs, suppress the translation of target mRNAs by binding to their 3′-untranslated region.[2] Evidence suggests that miRNAs are key regulators of several cellular processes, including angiogenesis. miRNAs, such as the miR-17-92 cluster, miR-126 and miR-296, have been shown to have an important role in angiogenesis.[3] Among the miRNAs, the miR-17-92 cluster is one of the most well-characterized, consisting of six miRNAs: miR-17, -18a, -19a, -20a, -19b and -92a. Recent reports have demonstrated that the miR-17-92 cluster, especially miR-92a, has potent angiogenesis-regulating activity in addition to oncogenic activity,[4] but the contribution to angiogenesis may differ among individual miRNAs within this cluster.[5]

Emerging evidence suggests that exosomes, small membrane vesicles of endocytic origin secreted by most cell types, contain miRNA, and they are thought to serve an important purpose in intercellular communications. A recent report by Kosaka *et al.*[6] demonstrated that extracellular miRNAs are actively controlled through ceramide-dependent machinery associated with exosome secretion. Zhang *et al.*[7] also showed that THP-1 cells selectively package miRNAs (miR-150) into multivesicular bodies and actively secrete them into the extracellular environment. Extracellular miR-150 can then enter endothelial cells and enhance endothelial migration via repression of *c-Myb*, the target gene of miR-150.

These findings led us to consider the possibility that miRNA transferred from cancer cells into neighboring endothelial cells can be one of the extracellular signaling molecules and can affect the biological properties of endothelial cells. We therefore set out to determine the interaction of a human leukemia cell line, K562, and human umbilical vein endothelial cells (HUVECs), using a non-contact co-culture system. We found that Cy3-labeled miR-92a derived from K562 cells was transferred to HUVECs via exosomes. Here, we show a possible association between secreted exosomal miRNAs and endothelial migration, as well as tube formation.

RESULTS

Screening of extracellular and intracellular miRNAs in K562 cells co-cultured with HUVECs by TaqMan low-density array

We first performed miRNA expression profiling of donor and recipient cells, using a TaqMan low-density array for human miRNAs (NCBI, Gene expression omnibus, GEO, GSE31114). We used K562 cells, which constitutively secrete miRNAs,[8] as donor cells and HUVECs as recipient cells. The expression of the miR-17-92 cluster was remarkably and highly expressed in K562 cells, whereas its expression was extremely low in HUVECs, indicating that high expression of the miR-17-92 cluster is a distinct feature of the miRNA profile of K562 cells. In contrast, high expression of miR-24 and miR-222 was found in HUVECs; these miRNAs had low expression in K562 cells.

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Received 5 September 2011; revised 25 May 2012; accepted 10 June 2012; published online 16 July 2012

Table 1. Top 20 downregulated miRNAs in the presence of HUVECs

Rank	*miRNA*	*Supernatant of K562 cells* $2^{-\Delta CT}$	*Supernatant of K562 cells with HUVECs* $2^{-\Delta CT}$
1[a]	hsa-miR-19b	36.8622	15.8308
2[a]	hsa-miR-17	29.7929	6.5075
3	hsa-miR-106a	29.6192	6.0042
4[a]	hsa-miR-20a	29.28	8.0605
5	hsa-miR-223	7.998	1.0083
6[a]	hsa-miR-92a	6.541	1.0761
7	hsa-miR-146b-5p	3.7037	0.4304
8[a]	hsa-miR-19a	3.6347	1.5142
9	hsa-miR-186	0.9347	0.367
10	hsa-miR-20b	0.9215	0.2645
11	hsa-miR-224	0.9191	0.3235
12	hsa-miR-191	0.9085	0.1256
13	hsa-miR-376a	0.8954	0.2698
14	hsa-miR-93	0.4635	0.2923
15	hsa-miR-376c	0.4572	0.1285
16	hsa-miR-30c	0.4061	0.064
17	hsa-miR-342-3p	0.2765	0.0622
18	hsa-miR-197	0.2628	0.0627
19[a]	hsa-miR-18a	0.2321	0.0265
20	hsa-miR-127-3p	0.2266	0.0417

Ranking: K562 miRNAs, which were downregulated less than twofold in the presence of HUVECs, are ranked by the expression level. [a]miR-17-92 cluster.

Table 2. Top 20 upregulated miRNAs in the presence of HUVECs

Rank	*miRNA*	*Supernatant of K562 cells* $2^{-\Delta CT}$	*Supernatant of K562 cells with HUVECs* $2^{-\Delta CT}$
1	hsa-miR-126	7.333	15.4229
2	hsa-miR-222	0.0037	5.3569
3	hsa-miR-24	0.9054	4.8537
4	hsa-miR-21	0.968	2.0449
5	hsa-miR-99a	0.0035	0.7921
6	hsa-miR-345	0.0576	0.2329
7	hsa-miR-29a	0.0586	0.1281
8	hsa-miR-195	0.057	0.0939
9	hsa-miR-27a	0.0323	0.0648
10	hsa-miR-139-5p	0.0141	0.0634
11	hsa-miR-365	0.0071	0.0383
12	hsa-miR-152	0.0036	0.0256
13	hsa-miR-361-5p	0.0071	0.0221
14	hsa-miR-642	0.0561	0.0208
15	hsa-miR-29c	0.0071	0.0185
16	hsa-miR-324-3p	0.007	0.0174
17	hsa-miR-324-5p	0.007	0.0161
18	hsa-miR-27b	0.0094	0.0158
19	hsa-miR-337-5p	0.0087	0.0158
20	hsa-miR-140-3p	0.007	0.0042

Ranking: K562 miRNAs, which were upregulated greater than twofold in the presence of HUVECs, are ranked by the expression level.

To clarify the dynamics of extracellular miRNA derived from donor cells, K562 cells and HUVECs were non-contact co-cultured using Transwell (Corning Inc. Life Sciences, Lowell, MA, USA, pore size: 0.45 μm) for 24 h; we compared the expression of miRNAs in supernatants between mono-culture medium (supernatant of K562 cells only) and co-culture medium (supernatant of K562 cells with HUVECs). Notably, subsets of miRNAs were decreased in the presence of HUVECs, indicating that extracellular miRNA, derived from K562 cells, may transfer into HUVECs. The rank order of the $2^{(-\Delta Ct)}$ values is shown in Table 1. We found that all miRNAs from the miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, miR-92a) were present in the top 20 downregulated miRNAs (Table 1). In the presence of HUVECs, miR-222 and miR-24, possibly derived from the HUVECs, were upregulated (Table 2).

Intracellular and extracellular miRNA expression of K562 cells with or without HUVECs

To confirm the results obtained from the TaqMan low-density array, we quantified the expression of individual miRNAs (miR-17, miR-20a, miR-92a, miR-223, miR-24) by real-time quantitative reverse-transcription (QRT)–PCR. In the supernatant, the miRNAs (miR-17, miR-20a, miR-92a, miR-223) that were preferentially expressed in K562 cells, were significantly reduced in the presence of HUVECs (Figure 1a). In contrast, the expression level of miR-24 was significantly upregulated in the supernatant of K562 cells co-cultured with HUVECs (Figure 1a, right). However, the expression of intracellular miRNAs in K562 cells did not differ with or without co-culture with HUVECs (Figure 1b), indicating that intracellular miRNAs in K562 cells were not influenced by the presence of HUVECs.

miRNA expression of HUVECs with or without K562 cells

To prove the uptake of miRNAs in recipient cells, we analyzed the expression levels of miRNAs in HUVECs. We found significant increases in miRNAs, except for miR-24, in HUVECs (Figure 1c). The results in Figures 1a and c suggest that the extracellular miRNAs derived from K562 cells may transfer into HUVECs rather than decreased miRNA secretion from K562 cells.

We subsequently concluded that there might be at least two types of miRNA; one preferentially expressed in K562 cells (for example, miR-17-92 cluster, miR-223) and the other preferentially expressed in HUVECs (for example, miR-24). The miRNA profiling, using the co-culture with HUVECs, revealed a subset of extracellular miRNAs, including miR-92a, decreased in the presence of HUVECs (Supplementary Figure 1). On the basis of the results obtained from the ranking order of the expression levels and the miR-17-92 cluster being essential in angiogenesis, we focused in particular on miR-92a for further study.

Transfer of extracellular miR-92a from the donor cells to the endothelial cells

To visualize the transport of extracellular miRNA derived from K562 cells into HUVECs, K562 cells were transfected with Cy3-labeled pre-miR-92a and co-cultured with HUVECs, using Transwell. K562 cells and HUVECs were pre-cultured in serum-free AIM V medium. The next day, K562 cells were seeded onto the inside of an insert, and co-culture was started by setting the insert on HUVECs. Twenty-four hours after initiation of co-culture, Cy3-miR-92a signal was detected in the cytoplasm of HUVECs (Figure 2a). The Cy3-miR-92a co-localized with the signals of an exosomal marker, CD63. It should be noted that the structures observed by anti-CD63 immunofluorescence were much larger than individual exosomes (Figures 2b and d); thus, it is not possible to determine whether they represent uptake of larger material through pinocytosis, endocytosis, or other mechanisms. Indeed, the mechanism of exosome uptake remains unclear.

To confirm whether the transferred miR-92a was derived from exosomal miRNA, we extracted exosomal fractions by Exoquick (System Biosciences, Mountain View, CA, USA) or ultracentrifugation from the culture medium of K562 cells transfected with Cy3-labeled pre-miR-92a (K562/Cy3-miR-92a cells). K562/Cy3-miR-92a cells were cultured in serum-free AIM V medium for 24 h, and the culture medium was then collected and used for exosome preparation. To observe and compare in detail the structure of exosomes separated by two different methods (Exoquick and ultracentrifugation), we used transmission electron microscopy.

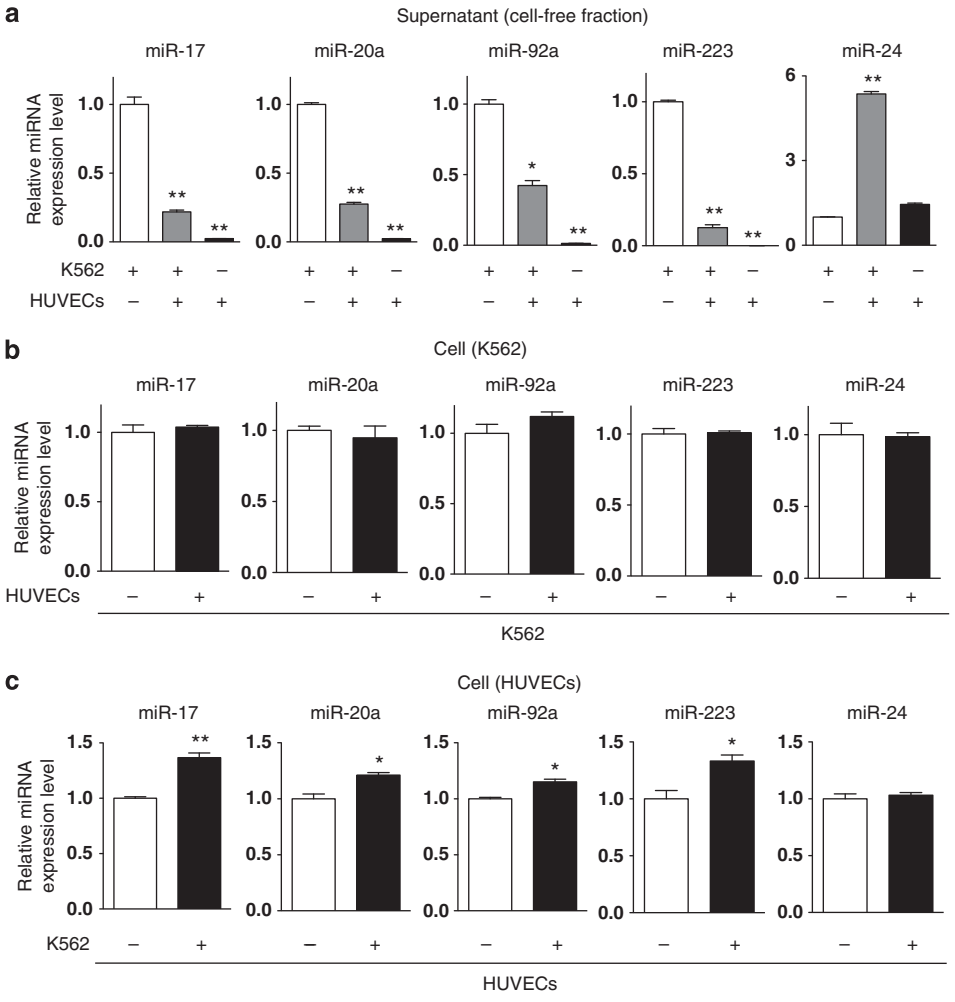

Figure 1. Quantification of individual miRNAs in the supernatant and cells. The *y* axis represents relative miRNA expression level. Results are presented as means ± s.d. of three independent experiments, and ath-miR-159 (for supernatant) and RNU6B (for cell) were used as an invariant control. (**a**) Comparison of extracellular miRNA expression in the supernatant. The mir-17, miR-20a, miR-92a and miR-223 were preferentially expressed in the supernatant of K562 cells, whereas they were hardly detectable in that of HUVECs. The expression was significantly downregulated in the supernatant of K562 cells co-cultured with HUVECs (*$P<0.05$; **$P<0.005$). (**b**) miRNA expression in K562 cells with or without HUVECs. We could not find any difference between K562 cells co-cultured with HUVECs and K562 cells (mono-culture). (**c**) miRNA expression in HUVECs with or without K562 cells. When HUVECs were co-cultured with K562 cells, a modest, but statistically significant, increase of miRNA expression was seen for miR-17 (**$P<0.005$), miR-20a (*$P<0.05$), miR-92a (*$P<0.05$), and miR-223 (*$P<0.05$).

The exosomes fractionated by Exoquick appeared as cluster of vesicles of 40–100 nm in diameter (Figure 3a), and each of those vesicles showed the classical cup-shaped appearance (Figure 3b). The configuration of exosomes fractionated by ultracentrifugation was similar to those fractionated by Exoquick (Figure 3c). When we added the exosomes isolated by Exoquick to HUVEC mono-culture, we found the signals of Cy3-miR-92a in accordance with CD63 in HUVECs (Figure 3e). The same experiment was performed using exosomes isolated by ultracentrifugation, which also revealed the signal of Cy3-miR-92a in the cytoplasm of HUVECs (Supplementary Figure S2). Moreover, we used miRNA microarrays to compare the two methods (Exoquick and ultracentrifugation, NCBI, Gene expression omnibus, GEO, GSE35256), and Supplementary Figure S3 shows that there were no differences in exosomal miRNA populations isolated by the two different collection methods. Therefore, we used the exosome fraction isolated by Exoquick in this study.

Additionally, we treated K562/Cy3-miR-92a with GW4869, a specific neutral sphingomyelinase (nSMase) inhibitor, and obtained exosome fraction from the culture medium. The exosome fraction was then added to HUVEC mono-culture. Kosaka *et al.*[6,9] showed that GW4869 suppressed the secretion of exosomal miRNAs via inhibiting ceramide biosynthesis. As a result of GW4869 treatment, it became impossible to observe the Cy3 signals in HUVECs (Figure 3f). The Cy3-miR-92a was detected when HUVECs were cultured with the exosome of K562/Cy3-miR-92a cells (73%), whereas the GW4869 treatment suppressed the incorporation of Cy3-miR-92a in HUVECs (22.1%) ($P<0.005$) (Figure 3g). This indicates that exosomal miR-92a was indeed transferred into HUVECs. Time-lapse imaging for the transfer of extracellular miRNA (see movies in Supplementary Material) also supported the idea of cell–cell communication via exosomal miRNA.

Exosomal miR-92a derived from K562 directly regulates the target gene in HUVECs

To test whether exosomal miR-92a regulates the target gene directly, we performed a luciferase reporter assay. Our luciferase reporter vector for assessing miR-92a–specific activity expressed firefly luciferase containing complementary miR-92a sequences in its 3′-untranslated region. The luciferase reporter vector and β-gal

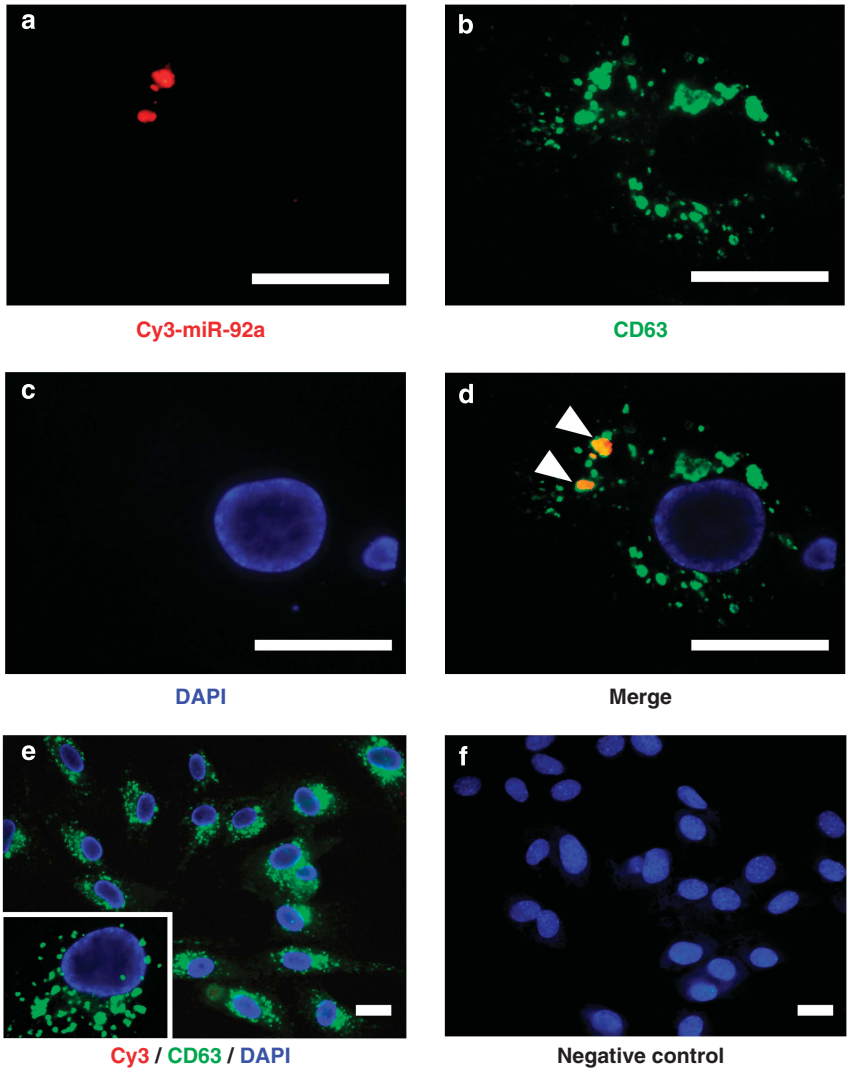

Figure 2. Localization of extracellular miRNA transferred into HUVECs. HUVECs were non-contact co-cultured with K562/Cy3-miR-92a cells (K562 cells transfected with Cy3-labeled pre-miR-92a) using Transwell. K562 cells and HUVECs were pre-cultured in serum-free AIM V medium (Invitrogen, Carlsbad, CA, USA). The next day, K562 cells were seeded onto the membrane of a Transwell cell culture insert, and co-culture was started by putting the insert into the HUVEC cell culture dish. Twenty-four hours after co-culture, HUVECs were fixed in 2% paraformaldehyde, and analyzed by using a fluorescent microscope. (**a**) Red shows Cy3-miR-92a expression. (**b**) Green shows CD63 expression. (**c**) Nuclear counterstaining was performed using DAPI (blue). (**d**) The Cy3-miR-92a signals co-localized with CD63 (arrowheads). (**e**) As a negative control for detection of the Cy3 signals, HUVECs were co-cultured with K562/non-labeled miR-92a. (**f**) As a negative control for anti-CD63 immunostaining, normal mouse IgG fraction was used as the primary antibody. The size bar indicates 10 μm.

control vector allowed simultaneous monitoring of miR-92a activity and transfection efficiency, respectively. When HUVECs transduced with the reporter and control plasmids were incubated with exosomes derived from K562/Cy3-miR-92a cells, the firefly luciferase activity was drastically reduced as compared with exosomes derived from K562/negative control miR cells (K562 cells transfected with non-targeting negative control miRNA) ($P<0.05$; Figure 4a). In contrast, the exosomes derived from K562/Cy3-miR-92a did not reduce the luciferase activity when we used a mutated vector of the miR-92a (Figure 4b).

One of the target genes, integrin α5, was significantly repressed in the treatment with exosomal miR-92a (Figure 4c), indicating that exogenous miRNA via exosomal transport might function like endogenous miRNA in HUVECs. We also investigated the possible association between exogenous miRNA and cell growth of HUVECs; however, neither exosomes derived from K562/Cy3-miR-92a cells nor directly transfected pre-miR-92a affected cell growth of HUVECs (data not shown). This further indicates the possibility that incorporation of miR-92a might be related to certain endothelial cell functions, with the exception of cell proliferation.

Exosome derived from K562/Cy3-miR-92a cells enhances cell migration and tube formation in HUVECs

To investigate whether HUVECs were affected by exogenous miR-92a via exosomes, we performed a migration assay using an 8-μm pore Transwell. HUVECs were cultured with either exosomes derived from K562/Cy3-miR-92a cells or K562/Cy3-negative control miR cells (K562 cells transfected with Cy3-labeled negative control miRNA). After pre-culture for 24 h, HUVECs were washed and added to the upper chamber of the fibronectin-coated Transwell at a density of 5×10^5 cells/well. Two hours later, migrating cells on the bottom side of the chamber were manually counted in five random microscopic fields using a fluorescence microscope. We found migrated cells containing Cy3-miR-92a on

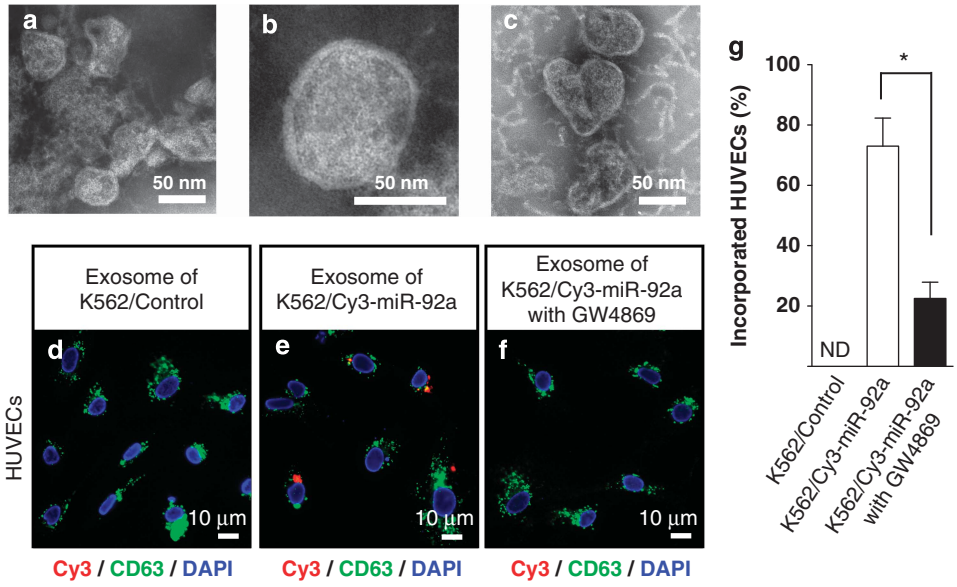

Figure 3. Ultrastructure and localization of exosomal miRNAs transferred into HUVECs. (**a**, **b**) Photomicrographs of exosomes fractionated by Exoquick. (**c**) Photomicrographs of exosomes fractionated by ultracentrifugation. (**d**) The exosomes from K562/negative control miR cells (K562 cells transfected with negative control miRNA) were added to HUVEC mono-culture. (**e**) The exosomes from K562/Cy3-miR-92a cells were added to HUVEC mono-culture. Cy3-miR-92a signals (red) were co-localized with CD63 (green) in HUVECs. (**f**) K562/Cy3-miR-92a cells were treated with 10 μM GW4869 for 24 h. The exosomes from GW4869-treated K562/Cy3-miR-92a cells were added to HUVEC mono-culture. Nuclear counterstaining was performed using DAPI (blue). (**g**) Quantification of the HUVECs that had incorporated Cy3-miR-92a. The cells were manually counted in each of five random microscopic fields ($n = 3$, $^*P < 0.005$). Values are mean ± s.d.

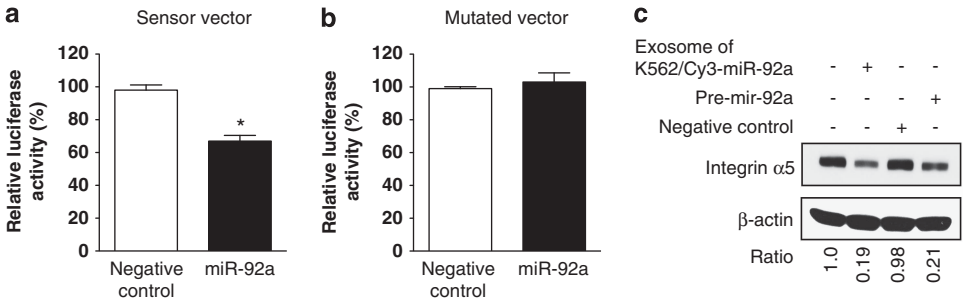

Figure 4. Functional analysis of exosomal miRNAs transferred into HUVECs. (**a**, **b**) Luciferase assay. HUVECs were transfected with the reporter and control plasmid for the luciferase assay. Luciferase activity was normalized with β-gal activity as shown. Mean ± s.d. of triplicates. Relative luciferase activity compared with control (HUVECs were cultured with the exosomes from K562 cells transfected with negative control miRNA) is shown. (**a**) Sensor vector: luciferase activity of HUVECs culturing with exosomes from K562/Cy3-miR-92a cells was significantly reduced when compared with HUVECs culturing with exosomes from K562/Cy-3 negative control ($n = 3$; $^*P < 0.05$). (**b**) Mutated vector: there was no difference in luciferase activity between exosomes derived from K562/Cy3-miR-92a cells and those derived from K562/Cy-3 negative control cells. Mean ± s.d. of replicates. (**c**) Protein expression in HUVECs transfected with pre-miR-92a or co-cultured with the exosomes from K562/Cy3-miR-92a cells. Expression level of integrin α5 was expressed as a ratio of integrin α5 signal/β-actin signal. Integrin α5 expression was significantly repressed in HUVECs cultured with the exosome from K562/Cy3-miR-92a cells as compared with HUVECs cultured with the exosomes from K562/control cells. Decrease of integrin α5 expression was also found in HUVECs transfected with pre-miR-92a as compared with those transfected with negative control miRNA. Bands are quantified by the ImageJ program.

the bottom side of the chamber for HUVECs cultured with exosomes derived from K562/Cy3-miR-92a cells (Figures 5a and b). Similarly, migrated cells (measured as the ratio of migrated cells in the bottom side to the non-migrated cells in upper side) were significantly elevated for HUVECs cultured with exosomes derived from K562/Cy3-miR-92a cells ($P < 0.05$, Figure 5e). These findings indicate that exosome, derived from K562 cells with enforced miR-92a expression, enhanced HUVEC migration.

We tested further effects of exogenous miR-92a via exosomes using an *in vitro* model of angiogenesis. When HUVECs were cultured on Matrigel with exosomes derived from K562/Cy3-miR-92a cells, we found that the tube-like structures were enhanced (Figures 5g and h). Collectively, the exogenous miR-92a via exosome derived from K562 cells enhanced endothelial migration and tube formation.

DISCUSSION

This is the first report to circumstantiate leukemia cell to endothelial cell communication via exosomal miRNA, which may be, in part, associated with angiogenic activity in endothelial cells. First, we visualized the transport of K562-derived miRNAs to HUVECs. Second, we proved that exogenous miRNA via exosomal transport could function like endogenous miRNA in endothelial

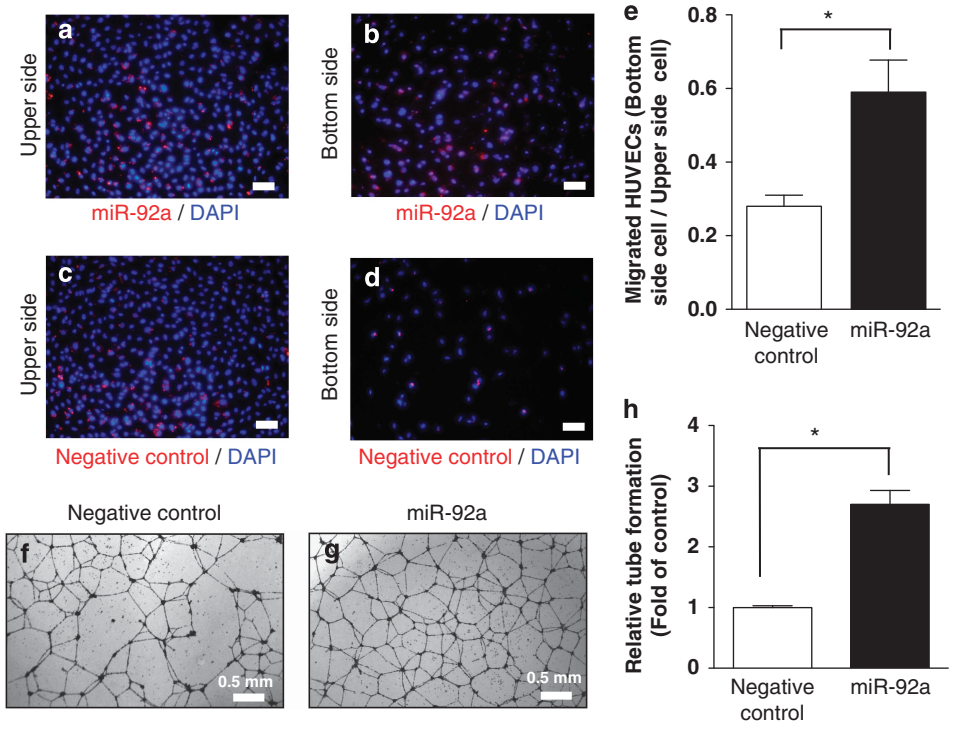

Figure 5. Exosome derived from K562/Cy3-miR-92a cells enhanced endothelial migration and tube formation in HUVECs. (**a**, **b**) HUVECs were treated with the exosomes derived from K562/Cy3-miR-92a cells for 24 h. HUVECs were then washed and added to the upper chamber of the Transwell at a density of 5×10^5 cells/well. HUVECs containing miR-92a signals (red) migrated from the upper side (**a**) of membrane to the bottom side (**b**). (**c**, **d**) HUVECs were treated with the exosomes derived from K562 cells transfected with Cy3-negative control miRNA as the control. Migration was not seen in the bottom side of membrane. The size bar indicates 100 μm. (**e**) Migrating cells on the bottom side of the chamber were counted manually in five random microscopic fields. The *y* axis represents the percentage of migrated cells. Results are presented as means ± s.d. (*$P < 0.05$). Migrated cells were significantly increased when HUVECs were pre-treated with the exosomes derived from K562/Cy3-miR-92a cells. (**f**, **g**) Tube formation assay: the formation of tube-like structures was observed under bright field. (**h**) The tube-like structures determined by pixel density are significantly enhanced by the addition of exosome derived from K562/Cy3-miR-92a cells (*$P < 0.05$). Values are mean ± s.d.

cells. Finally, we noted that cell-to-cell communication via exosomes of K562 cells, transfected with miR-92a, affected endothelial migration and tube formation rather than cell growth. This suggests the possibility that miRNAs originating from other cells (that is, cancer cells) can regulate the angiogenic phenotype of endothelial cells.

Tumor angiogenesis is a complex multistep process that requires endothelial cells carry out specific activities. Recent findings indicate that specific miRNAs might also be required to regulate endothelial gene expression during angiogenesis.[1,3] The miR-17-92a cluster is known to be a regulator of angiogenesis. The pro-angiogenic functions of miR-17-92 have been ascribed to direct repression of the secreted, anti-angiogenic molecules thrombospondin-1 and connective tissue growth factor within tumor cells, thereby promoting angiogenesis in the adjacent tumor endothelium by a paracrine mechanism.[10] Conversely, this cluster has a dual function of pro- or anti-angiogenesis by the cooperation of individual miRNAs in endothelial cells. For instance, miR-17 promotes the regulation of endothelial cell migration and proliferation by targeting tissue inhibitor of metalloproteinase 1,[11] and miR-18a and miR-19a have been shown to target protein containing thrombospondin type 1 repeats, which often have anti-angiogenic activity.[5] In contrast, miR-17, miR-20, and miR-92a do not affect the expression of thrombospondin type 1 repeat protein[5,12] and thereby exhibit anti-angiogenic activity in the endothelial cells. Moreover, Bonauer *et al.*[13] reported that overexpression of miR-92a in endothelial cells represses angiogenesis by direct repression of the pro-angiogenesis protein integrin α5, whereas antagomir-based inhibition of miR-92a in ischemic mice induces neovascularization and functional recovery after ischemia. Bonauer *et al.*[13] demonstrated miR-92a dynamics in endothelial cells using an ischemic mouse model. Arterial ligation may induce hypoxic conditions in endothelial cells; therefore, miR-92a dynamics were likely to be different from those in the normoxic conditions used in our experiments. Indeed, we used HUVECs in normoxic condition and assessed their dynamics by exogenous excess miRNAs. Our *in vitro* model aimed to study the effect of leukemia-derived miRNAs to endothelial cells, while Bonauer *et al.*[13] focused on angiogenesis from the viewpoint of cardiovascular research, rather than tumor angiogenesis.

The question has thus arisen why exogenous miR-92a does not affect endothelial cell growth. A recent study by Kuehbacher *et al.*[14] clearly demonstrated that nonspecific knockdown of miRNAs by dicer siRNA suppressed angiogenic activity, including cell migration, sprouting and tube formation, suggesting the complexity of miRNA-dependent regulation of angiogenesis. We, therefore, conclude that exosome-mediated miR-92a may have an important role in a part of angiogenesis as a result of increased migration and tube formation. It is, however, insufficient in orchestration of tumor angiogenesis because of microvesicles, including exosomes, contain cellular components other than miRNAs.

Recently, use of exosomal miRNAs for cancer therapy was attempted.[15] Oncogenic miRNAs, or tumor suppressor miRNAs in exosomes, can be transferred to cancer cells and are functional in them, and they are considered to be promising drugs to induce apoptosis and/or cell cycle arrest in cancer cells that depend on miRNA dysregulation for growth and survival. Thus, there is

growing evidence that miRNA therapy could be a potent means to curtail tumor growth. It might be possible to induce anti-angiogenesis by using exosomal miRNAs to block tumor angiogenesis as well (for example, miR-221 and miR-222).[16]

One of the most important issues we should address is that exogenous miR-92a, which is secreted from leukemia cells, works as one of the mediators of endothelial migration and tube formation. Uptake of exosomal miRNA into endothelial cells provides further evidence that the extracellular miRNAs can be transferred from one cell to another as signaling molecules mediating cell–cell communication. It has been shown that there are several mechanisms of cell–cell communications, including direct contact mediated by integral membrane proteins, as seen in lateral inhibition mediated by Notch.[17,18] Indirect contact via the extracellular matrix[19] and diffusible factors, such as sonic hedgehog and bone morphogenic protein, also have a role in cell–cell communication.[20–23] In addition to these classical mechanisms of cell–cell communication, we particularly highlighted the circulating miRNA via exosomes in cell–cell communication in a microenvironment. Recent reports indicate that most of the extracellular miRNA in plasma and cell culture media are independent not only of exosomes, but also binding to Ago2 protein, a part of the RNA-induced silencing complex.[24,25] We investigated only the exosomal fractions, and there is a possibility that other extracellular miRNAs like the Ago2-binding miRNA also influence cell–cell communication.

In this study, we demonstrated that exosomal miRNAs can be transported from leukemia cells to endothelial cells and that certain exogenous miRNAs modulate endothelial migration and tube formation. Our study provides a therapeutic possibility of antago-miR-92a in combination with currently available vascular endothelial growth factor inhibitors. This study may provide an opportunity to open the door to neoplasia-oriented intervention.

MATERIALS AND METHODS

Cell culture and preparation of culture medium

To observe the neoplasia–endothelial cells communication via extracellular miRNAs, we used a non-contact co-culture system with HUVECs and the human leukemia cell line K562. As K562 cells secrete a large amount of miR-17-92 cluster into culture medium,[8] we used them as the donor cells. The K562 cells were cultured in RPMI 1640 supplemented with penicillin (Sigma Aldrich, Milan, Italy, 100 U/ml), streptomycin (Sigma Aldrich, 100 mg/ml), and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Pooled HUVECs were purchased from Lonza Inc. (Allendale, NJ, USA) and cultured in endothelial basal medium (EBM; Lonza Inc.) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor, gentamycin and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, until the third passage.

K562 cells and HUVECs were co-cultured separately using a Transwell filter (polycarbonate membrane insert, 0.45-μm pore; Corning Inc.). Before co-culture, HUVECs were plated onto the bottom of a 12-well plate at a density of 5×10^5 cells/well, and K562 cells and HUVECs were pre-cultured in serum-free AIM V medium (Invitrogen, Carlsbad, CA, USA). The next day, K562 cells were seeded onto the inside of an insert above the membrane at a density of 5×10^5 cells/ml, and co-culture was started by setting the insert on the 12-well plate. After 24-h co-culture, the culture medium was collected and centrifuged at 2000 *g* for 15 min. The supernatant was filtered through a 0.22-μm polyvinylidene difluoride (PVDF) filter (Millipore, Billerica, MA, USA) to further eliminate cellular debris.

RNA isolation

Isolation of extracellular and intracellular miRNAs was performed using the mirVana PARIS kit (Ambion, Austin, TX, USA). Five hundred microliters of culture medium (after filtration) or cell lysate was diluted with 500 μl of binding solution. After a 5-min incubation, 1 μl of 1 nM ath-miR-159 (Hokkaido System Science, Hokkaido, Japan) was added to each aliquot as a spike control for losses in preparation, followed by vortexing for 30 s and incubation on ice for 10 min. Subsequent phenol extraction and cartridge filtration were carried out according to the manufacturer's instructions.

MiRNA expression profile and QRT–PCR

To assess levels of specific miRNAs in cell and culture medium samples, a fixed volume of 3 μl of RNA solution from 50 μl of the eluate was used as input in each RT reaction. An RT reaction and pre-amplification step were set up according to manufacturers' recommendations. miRNAs were reverse-transcribed using the Megaplex Primer Pools (Human Pools A v2.1) from Applied Biosystems (Bedford, MA, USA). RT reaction products from the culture medium sample were further amplified using the Megaplex PreAmp Primers (Primers A v2.1). The expression profile of miRNAs was determined using the Human Taqman miRNA Arrays A (Applied Biosystems). RNU6B and a spike control (ath-miR159) were used as an invariant control for the cell and culture medium, respectively. QRT–PCR was carried out on an Applied Biosystems 7900HT thermal cycler using the manufacturer's recommended program. Finally, all the raw data from each array were retrieved from the 7900HT and run on Data Assist Software ver. 3.1 (Applied Biosystems).

Real-time quantitative reverse-transcription PCR (QRT–PCR)

To determine the amount of the individual miRNAs, we measured their levels by TaqMan miRNA assays, using real-time PCR. All of TaqMan miRNA assays (hsa-miR-17: 002308, hsa-miR-20a: 000580, hsa-miR-92a: 000431, has-miR-223: 002295, hsa-miR-24: 000402, RNU6B: 001093, hsa-miR159: 000338) were purchased from Applied Biosystems. The reactions were run in duplicate. The relative quantities of intracellular miRNA were calculated using the comparative Ct methods after normalization to RNU6B. The expression of extracellular miRNAs derived from a fixed volume (500 μl) of culture medium was calculated based on their Ct values normalized by ath-miR159, which was spiked in each aliquot of QRT–PCR.

Transfection of K562 cells with Cy3-labeled pre-miR miRNA precursor

Pre-miR miRNA precursor (has-miR-92a; Ambion) was labeled with Label IT siRNA Tracker Cy3 Kit, according to the manufacturer's instructions (Mirus, Madison, WI, USA). K562 cells (1×10^5) were transfected with 10 nM of Cy3-labeled pre-miR miRNA precursor using HiPerFect (Qiagen, Düsseldorf, Germany) (K562/Cy3-miR-92). The day after transfection, cells were washed three times with PBS, and the medium was switched to fresh serum-free AIM V medium (Invitrogen). After incubation for a day, the culture medium was collected and used for exosome preparation.

Preparation of exosomal fraction

The culture medium was collected and centrifuged at 3000 *g* for 15 min. The supernatant was filtered through a 0.22-μm PVDF filter (Millipore). The appropriate volume of Exoquick Exosome Precipitation Solution (System Biosciences) was added to the filtered culture medium and mixed well by inverting. After refrigeration for 12 h, the mixture was centrifuged at 1500 *g* for 30 min and all supernatant was removed by aspiration. Exosome pellets were resuspended with 500 μl of the serum-free AIM V medium (Invitrogen).

Inhibiton of exosome release

To validate that miRNAs were transferred via exosomes, exosome release was blocked by using a specific inhibitor for neutral sphingomyelinase 2 (nSMase2). K562 cells were transfected with Cy3-labeled pre-miR-92a in a 12-well plate. The cells were reseeded and cultured in a six-well plate for 24 h with 10 μM GW4869 (Calbiochem, Darmstadt, Germany). After the incubation, the culture medium was collected and used for exosome preparation. The exosomes from GW4869-treated K562 cells were added to HUVEC mono-culture for 24 h.

Luciferase assay

Synthetic oligonucleotides bearing the miR-92a-binding sequence (5′-ACAGGCCGGGACAAGTGCAATA-3′), containing *Hin*dIII and *Spe*I restriction sites, were cloned into the firefly luciferase reporter plasmid pMIR-Report (Ambion), according to the manufacturer's protocol. We also generated the mutation of the seed sequence (G**C**G**T**AATA) of miR-92a by using a QuickChange site-specific mutagenesis kit (Stratagene, La Jolla, CA,

USA). For measuring luciferase activity, HUVECs were grown in 24-well plates until 50%–60% confluence. The luciferase plasmid (0.01 ng) was co-transfected with 0.01 ng of pMIR-Report β-gal control plasmid (Ambion) as control for the transfection efficiency and 10 pmol of pre-miR-92a (Ambion) or Negative Control pre-miR (Ambion) using HiPerFect Transfection Reagent (Qiagen), according to the manufacturer's protocol. The activity of luciferase and β-gal was assessed after 48 h with the Dual-Light System (Applied Biosystems).

Immunocytochemistry

HUVECs were seeded on glass coverslips (Matsunami Glass, Osaka, Japan) and co-cultured with K562 cells using a Transwell filter. HUVECs were fixed in 2% paraformaldehyde for 15 min and washed twice with cold PBS. Nonspecific binding of antibodies was blocked by incubation with 2% bovine serum albumin (Sigma-Aldrich) for 30 min. Monoclonal anti-CD63 antibody (Progen Biotechnik GmbH, Heidelberg, Germany) was used for the detection of exosomes. Alexa Fluor 488-conjugated anti-mouse IgG goat serum (Cell Signaling Technology, Danvers, MA, USA) was used as a secondary antibody. 4′,6-Diamidino-phenylidole (DAPI; Abbott, Abbott Park, IL, USA) was used for nuclear staining. Analyses were performed with a fluorescent microscope (Biozero BZ-8000, Keyence, Osaka, Japan). As a negative control, normal mouse IgG fraction (Vector Laboratories, Burlingame, CA, USA) was used as the primary antibody.

Western blot analysis

For western blot analysis, HUVECs were lysed in RIPA lysis buffer (Sigma) containing protease and/or phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min at room temperature. After centrifugation for 15 min at 14 000 *g* (room temperature), the protein content of the samples was determined using the Bradford method. Equal amounts of protein were separated on SDS–polyacrylamide gels, blotted onto PVDF membranes, and blocked in 5% nonfat dry milk or bovine serum albumin in TBS, with 0.1% Tween-20 (TBS-T). Western blots were probed with antibodies directed against integrin α5 (rabbit polyclonal anti-integrin α5; 1:1,000, Cell Signaling Technology) or tubulin (mouse monoclonal anti-tubulin β, 1:2,500, Chemicon International, Temecula, CA, USA). Secondary antibodies were purchased from GE healthcare (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA).

Migration assay

To determine migration of endothelial cells, HUVECs were detached with trypsin, harvested by centrifugation, resuspended in EBM, counted, and placed in the upper chamber of a Transwell (5×10^4 cells per chamber, pore size 8 μm, Corning) coated with 1 μg/ml human fibronectin (Nitta Zeratin, Osaka, Japan). The chamber was placed in a 24-well culture dish containing EBM. After incubation for 4 h at 37°C, the cells on the upper and lower side were fixed with 4% formaldehyde. For quantification, cell nuclei were stained with DAPI. Migrating cells on the bottom side of the chamber were counted manually in five random microscopic fields using a computer-assisted fluorescence microscope (BZ-8000, Keyence)

Tube formation assay

The formation of capillary-like structures was assessed in a 24-well plate using growth factor–reduced Matrigel (BD Biosciences, San Jose, CA, USA). For this procedure, HUVECs (4×10^4 cells/well) were plated on top of Matrigel (280 μl/well) and treated with the exosome derived from K562/Cy3-miR-92a cells. After 24 h, cells were stained by CellTracker Green (Invitrogen) and visualized under the fluorescent microscope (BZ-8000, Keyence). The total tube area was quantified as mean pixel density obtained from image analysis of five random microscopic fields using ImageJ software (http://rsb.info.nih.gov/nih-image/).

Transmission electron microscopy

For electron microscopy analysis, exosomes were prepared, fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3. The samples were adsorbed to carbon-coated nickel grids and negatively stained for 10 min with 2% methylamine tungstate. The samples were observed in a JEM-1200EX electron microscope (Nihon Denshi, Tokyo, Japan) at 80 kV.

Statistical analysis

Data are expressed as means ± s.d. Two treatment groups were compared by Mann–Whitney *U*-test or Student's *t*-test. Multiple group comparisons were done by one-way ANOVA. For statistical analysis, GraphPad Prism version 5c for Macintosh (GraphPad Inc., La Jolla, CA, USA) was used. Results were considered statistically significant when $P < 0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Shinobu Ueda for processing of miRNA microarray analysis.

This work was supported by the Private University Strategic Research-Based Support Project: Epigenetics Research Project Aimed at General Cancer Cure Using Epigenetic Targets from MEXT (Ministry of Education, Culture, Sports, Science and Technology), Tokyo, Japan.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)