Extracellular vesicle-mediated Transfer of miRNA-1 from Primary tumor Repress Distant Metastasis Growth

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Extracellular vesicle-mediated Transfer of miRNA-1 from Primary tumor Repress Distant Metastasis Growth

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Running Title: Primary tumor represses metastases by secreting extracellular vesicles

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Abstract

Metastases originate from primary tumors and conquer distant organs. Growing evidence suggest that metastases are under control of primary tumors even out of the primary territory, however, how primary tumors remotely control metastases remains unclear. Here, we discovered a molecular mechanism by which a primary tumor suppresses the metastatic growth. Interestingly, we found that extracellular vesicles (EVs) derived from a primary tumor has an ability to inhibit the growth of metastases in vitro and in vivo. miR-1 was particularly enriched in the primary tumor-derived EVs (pTDEs) and responsible for the suppression of metastatic growth. Mechanistically, intracellular ROS and DNA damage were induced, which led to the cell cycle arrest. Collectively, our data demonstrate that the primary tumor restricts distant metastatic cancer by miR-1 in pTDEs which can potentially be used as an anticancer drug for metastasis.

Keywords: Tumor; Metastases; Communication; Extracellular vesicles; MicroRNA; miRNA-1
Introduction

Primary tumors play a dual role: they expel metastases from their origin while also maintaining control over them. Many studies have unveiled how primary tumors influence the tumor microenvironment, thereby restraining metastatic growth through various mechanisms such as interfering with angiogenesis or being eliminated by immune cells. This phenomenon was initially observed by the Ancient Roman physician Celsus, who observed the recurrence of carcinoma after excision. A century ago, Tyzzer reported that excising the primary tumors led to larger metastases. Since the year 1900 till the present, several studies have consistently demonstrated that primary tumor restrain the development of distant metastases, with sudden metastatic growth often becoming apparent after primary tumor removal. One fundamental finding is that angiostatin and endostatin, which interrupt metastatic angiogenesis and lead to nutrient starvation. Furthermore, primary tumors indirectly restrict metastases by remodeling CD8+ cytotoxic T cells and macrophages to eliminate them. Consequently, one pivotal factor contributing to the sudden increase in metastases after treating primary tumors may be the missing of the metastatic growth inhibition from the primary tumor. Since the discovery of extracellular vesicles, it has been known that cells deliver their messages by wrapping them in a lipid bilayer to communicate with distant cells. EVs are the smallest EVs (30-150 nm in diameter) containing proteins, lipids, and nucleic acids. Research on the biological function of EVs in cancer has mainly focused on their role in tumorigenesis and the formation of the tumor microenvironment, and there have been a few attempts to address the function of EVs as controllers of metastases. Despite numerous studies on tumor-derived EVs (TDEs), there is no evidence showing that pTDEs reach metastases.

In this study, we demonstrated that primary tumor uses EVs to control metastases growth. When conducting small RNA sequencing on pTDEs, miR-1 was found to be the most abundant compared to mTDEs. Additionally, we observed a reduction in the expression of miR-1 target genes in metastatic cancer cells treated with pTDEs. Moreover, exosomal miR-1 acts as a key regulator for inhibition, and engineered pTDEs for enhancing miR-1 intensify inhibition function in both metastases in cells and mouse. These findings highlight the miR-1 of primary tumor sending its messages through pTDEs in
the growth inhibition of metastases.
Materials and methods

Cell culture

Six canine mammary gland adenocarcinoma cell lines (CHMp, CHMm, CIPp, CIPm, CTBp, and CTBm) were purchased from the N. Sasaki lab (38) and grown in RPMI 1640 medium (HyClone, SH30027) containing 10% fetal bovine serum (FBS; Gibco 1600044) and 50 µg/ml gentamicin (Sigma–Aldrich, G1272). CHMp and CHMm cells were transfected with a firefly luciferase gene carrying plasmid (Addgene #18964) using Lipofectamine 3000 reagent (Invitrogen, LM3000015). Culture medium containing geneticin (G418 sulfate) was used to select stably transfected cells (Gibco, 10131035). Twenty-four hours after transfection, G418 (500 µg/ml) was supplemented and maintained for one week to eliminate untransfected cells. A single cell was isolated from stably luciferase-expressing cells to establish a stable cell line and maintained with 250 µg/ml G418 for two weeks. The expression of luciferase was confirmed with a luciferase assay. All cells were grown in a humidified incubator at 37 °C with 5% CO₂ and confirmed to be negative for mycoplasma contamination.

Mouse experiments

All mouse experiments were conducted in accordance with Seoul National University Institutional Animal Care and Use Committee (IACUC) guidelines, and the animal protocol was approved (SNU-210323-1-2). Nude mice (CrTac:NCr-Foxn1nu) were kept in pathogen-free conditions and maintained under a 12 h light/dark cycle at a controlled room temperature (22 ± 2 °C). A metastasis model was generated in 5-week-old female nude mice following orthotopic injection of a total of 5 x 10⁵ luciferase-labeled CHMp and CHMm cells into the mammary fat pad, and resection surgery was performed 21 days post implantation. Mice were anesthetized, and primary tumors were resected. Mice that underwent surgery were monitored for symptoms of pain and were sacrificed using carbon dioxide (CO₂) inhalation. IVIS bioluminescence imaging was carried out during the formation of spontaneous lung metastases. All mice were randomized before injection of EVs and blindly selected before injection. For EV injection, all treatments were administered as an intravenous injection in a final volume of 150 µl. Mice were treated with EVs six times at two-day intervals. The experimental endpoint was followed
by IACUC guidelines, and the maximal tumor volume was never exceeded.

**Extracellular vesicle isolation and labeling**

EVs were purified from cells cultured under serum-free conditions using a combined method of ultrafiltration and ultracentrifugation. Graphical method was illustrated in Supplementary figure 2a. Cells were grown to 80% confluency, washed two times with PBS and incubated in serum-free medium for 24 h. First, the cell culture supernatant was subjected to a differential centrifuge to eliminate cells, dead cells, and cell debris and filtered sequentially with 0.45 and 0.22 μm filters. The filtered supernatant was concentrated using 10K Amicon-Ultra 15 Centrifugal Filter Units (Merck, UFC903024). Filtered units were sequential centrifuged, ultracentrifuged for 80 min and washed with PBS. The EV concentration of 0.1μg/ml was applied in all of *in vitro* assays.

EVs from biological fluids were isolated using a commercial kit: ExoQuick exosome precipitation solution (System Biosciences, SBI-EXOQ5A-1). Plasma and serum samples were centrifuged at 3,000 × g for 15 min to remove cells and cell debris. ExoQuick was added to the supernatant at an appropriate volume and incubated for 30 min at 4 °C. Pelleted EVs were resuspended in Qiazol (Qiagen, 79306) for RNA isolation and Urea/SDS lysis buffer for protein isolation. Isolated EVs were stored at -80 °C for later use.

EVs were labeled with PKH67 lipophilic membrane dye (Sigma, MNI67-KIT) following the manufacturer's instructions. In brief, isolated EVs were resuspended in 1 ml of Diluent C, and 6 μl of PKH67 dye was added. The mixture was incubated for 5 min at room temperature and centrifuged at 100,000 × g for 80 min.

**Nanoparticle tracking analysis (NTA)**

NTA was used to characterize the size and concentration of EVs from the cell culture supernatant and biological fluids using the NanoSight LM10 model (Malvern). The samples were diluted with PBS (0.22 μm filtered) and injected into the laser chamber. Data were analyzed by NTA v3.2 software.
Transmission electron microscopy (TEM)

The morphology of EVs was analyzed by TEM using Talos L120C (Czech). Briefly, the samples were stained with a negative staining method using 2% uranyl acetate. One drop of diluted EVs was dropped on a glow discharged copper/carbon-coated grid. After 1 min, the grid was drained using filter paper, and one drop of 2% uranyl acetate was placed. The staining solution was drained, and the samples were observed with TEM (120 kV).

Small RNA sequencing and data analysis

The miRNA-seq library was prepared using the Small RNA Library Prep Kit (Nextflex) and sequenced as 100 bp or 150 bp paired-end reads on Illumina HiSeq 3,000 and NovaSeq 6,000 platforms. To remove adapters with low-quality reads and extract miRNA-specific sequences, cutadapt was used with options (--quality-base 33 -u 4 -m 22 -M 30 -f fastq -q 20 -O 6 -j 23 -a adapter-sequence). In this step, two different adapter sequences (TGGAATTCTCGGGTGCCAAGG and GATCGTCGGACTGTAGAAC-TCTGAAC) were used for forward and reverse reads in paired-end sequencing. Because trimmed reads are short (22–30 bp), forward and reverse reads in the same sample were merged into one fastq formatted file. Before and after the trimming step, the quality of sequenced reads was estimated using FastQC.

For known and novel miRNA analysis, the miRDeep2 package was used. Before analysis, the following two necessary files were prepared: 1) sequence files of mature and hairpin forms of dog miRNAs, which were downloaded from the miRbase database and extracted using the extract_miRNAs.pl script, and 2) indexed files from the dog reference genome (CanFam3.1) using bowtie. First, all filtered read data were merged into one file for novel miRNA analysis. The merged data were converted to a collapsed fasta-formatted file and aligned to the reference genome using the mapper.pl script with options (-e -h -j -m -p). Second, novel miRNAs were identified using the miRDeep2.pl script. The identified mature and hairpin forms of novel miRNAs were extracted and combined with known forms of miRBase prepared previously. Finally, the expression values of known and novel miRNAs were calculated using the quantifier.pl script. The CPM (counts per million), which
is counts scaled by total number of reads, was used for further analysis. MiRDeep2 analysis provides a miRNA score ranging from -10 to 10; a higher score represents genuine miRNA. We set the cutoff of 4 for strict novel miRNA identification. To estimate data reproducibility between replicate samples, Pearson correlation values were calculated and visualized using the correlation function in R. For differentially expressed miRNA analysis, fold-change in expression and significance (p value) were calculated using the EdgeR package in R. Using these calculated values, a volcano plot was visualized through the ggplot package in R.

miR-1 mimic transfection
Synthetic microRNA mimics were used to overexpress and overload microRNA into cells and EVs. CHMm cells were transfected using the lipid carrier Lipofectamine RNAiMAX (Invitrogen, 13778150) following the manufacturer's instructions. Fifty picomoles of miR-1 mimics were mixed with RNAiMAX reagent and then incubated for 15 minutes at RT. The miR-1 and RNAiMAX complexes were added to the cells and incubated for 24 h at 37 °C in a CO₂ incubator. EVs were transfected using 0.3 M CaCl₂ following the modified CaCl₂-mediated transfection method. Forty micrograms of EVs were mixed with 100 pmole of miR-1 mimics in BPS with 0.3 M CaCl₂ and incubated on ice for 30 min. Then, the mixture was heat-shocked at 42 °C for 60 sec and incubated on ice for 5 min. Transfected EVs were isolated again by ultracentrifugation and washed with PBS.

Clinical specimens
All study protocols and specimen collection were approved by the Institutional Review Board (IRB) of Seoul National University (IRB#SNU 16-10-063) and performed in accordance with guidelines. Informed consent for specimen collection was obtained by all subjects, including both humans and dog guardians, when they were enrolled.

Statistical analysis
The data represent means ± SEM. Statistical analysis was performed using Prism software (v.8.0.1, GraphPad Software). For the statistical significance of multiple groups, 2-way ANOVA combined with Tukey’s honest significant difference (HSD) test was performed, and Student’s t test was performed for analysis between CHMp and CHMm cells. Significant differences are indicated with different letters (*p<0.05, **p<0.01, and ***p<0.001) in each figure legend. The number of experimental repeats and value of n are also indicated in the figure legend.
Results

EVs from primary tumors inhibit the growth of metastases.

To investigate the role of pTDEs control the metastases, we used two types of metastatic models. The first model is spontaneous recurrence model, metastases grow after the surgical removal of primary tumor. The second model aimed to use cell lines of both primary and metastatic tumors established from the same spontaneous cancer patient. Since there is no pair of naturally occurring human breast cancer (HBC)-derived primary and metastatic tumor cell lines, we used canine mammary gland tumor (CMT), which has recently been reported to have pathological and molecular aspects similar to those of HBC\textsuperscript{24,25}. The pair of CMT cell lines (primary/metastases, CHMp/CHMm) originated from the same CMT patient. Unlike previous studies on primary tumors and metastases, we used cell lines derived from the primary tumor and metastasis of a single patient to increase the accuracy of our analysis.

Firstly, metastatic mouse model, we followed surgical methods as published by Piranlioglu et al\textsuperscript{3}. Primary tumor was surgically excised 21 days after CHMp cell injection (Supplementary Fig. 1a-c). After surgery, mice that underwent sham surgery exhibited cancer growth concentrated at the primary site rather than developing distant metastases (Supplementary Fig. 1d). In contrast, the majority of mice were confirmed to be free of residual primary tumors and injected pTDEs and control. pTDEs were isolated as described in Methods and purified EVs were cup-shaped, also positive for TSG101 and Alix (Supplementary Fig. 2a-d). PKH67-labeled pTDEs were found to be highly enriched in disseminated tumors and primary tumors (Supplementary Fig. 3a). The injection schedule is depicted in Figure 1a. Subsequently, the mice were divided into two groups: one group was regularly injected with pTDEs, and the other group with PBS as a control. The bioluminescence image showed little metastasis growth in the pTDE group compared to the control group on the 39\textsuperscript{th} day (Fig. 1b, c, Supplementary Fig. 3b). Metastases occurred in 3 out of 4 mice in control, but only very minor signals were observed in 2 out of 5 mice injected with pTDEs (Fig. 1b, c). Lungs from control mice exhibited a higher number of metastatic nodules than those from the pTDE-injected mice (Fig. 1d, e). To date, the mouse model has been shown that removing primary tumors could promote the growth of metastatic cancers\textsuperscript{26-29}, as seen in clinical findings. By comparing with negative control, we demonstrated that when pTDEs were...
injected into spontaneous metastases generated after the removal of the primary tumor, they exerted an inhibitory effect on their growth, even in metastases that were previously undetectable. Collectively, our data shows a pivotal role of pTDEs in metastases growth inhibition.

**pTDEs autonomously suppress metastasis growth in a direct and indirect manner.**

Since primary tumors affect the metastatic cancer cells both directly and indirectly through immune cells and endothelial cells that constitute the tumor mass, we next examined the impact of treating cancer cells and endothelial cells with pTDEs. We used second metastatic model, CHMm metastatic cancer cells treated with pTDE and mTDE as control to rule out any influences resulting from changes in the process of EV purification. CHMm cells treated with pTDEs showed reduced viability and proliferation, whereas mTDEs was not significantly different from PBS control (Fig. 2a, b). Moreover, pTDEs showed higher accumulation in the G2/M phase of the cell cycle compared to control (Fig. 2c). Since the accumulation of cells in the G2/M phase can be caused by cellular stress, such as cell oxidation, DNA replication, and transcription\(^{30,31}\), we investigated whether pTDEs treatment elicited stress conditions. Treatment with pTDEs caused a marked increase in intracellular ROS levels (Fig. 2d) and level of γ-H2A.X, a sensitive marker of damaged DNA (Fig. 2e, f). These results indicate that pTDEs induce an increase in intracellular ROS, genomic instability, accumulation in the G2/M cell phases and suppress growth of metastatic cells. However, this growth inhibition was not correlated with cell death and mobility (Supplementary Fig. 4a-c).

On the other hand, pTDEs had a strong effect on the tube formation of endothelial cells (Fig. 2g). Human umbilical vein endothelial cells (HUVECs) incubated with pTDEs showed a 1.5-fold decrease in the area of meshes compared to PBS, which formed regular tubes. These findings reveal that how pTDEs treated mice showed restricted metastases through directly inhibiting metastatic tumor cell growth and indirectly influence endothelial cells of tumor microenvironment by inhibiting angiogenesis.

**Primary tumor stem cell-derived EVs restrict metastases growth.**

It is speculated that cells that disseminate from primary tumors and settle in distant locations are less
likely to thrive in harsh environments if they lack stemness\textsuperscript{32-35}. Therefore, we examined the cancer stemness of primary tumors to comprehend their metastases inhibitory capability. Although many studies investigated the effect of primary cancer stem cells-derived EVs on DTCs, none have clearly addressed antitumor effects. To further investigate how primary tumor to control metastases, we \textit{compared} stemness between primary and metastatic tumors (Supplementary Fig. 5a). \textit{CD44\textsuperscript{+}/CD24\textsuperscript{−}} expression, a representative marker of breast cancer stem cells (CSCs), showed a higher CSC ratio in the primary tumor than metastases (Fig. 3a). In addition, other breast CSC characteristics, such as fast cell proliferation, high aldehyde dehydrogenase (ALDH) enzyme activity, and mammosphere formation, were substantially more enriched in CHMp than CHMm cells (Fig. 3b-d). Spheroids were fluorescently labeled with CD44 and CD24, and only CSC populations could form spheroids (Fig. 3e). We also pursued further comparisons between CHMp and CHMm cells for CD44, CD24, and ALDH1A1 protein levels and CSC-related gene expression (Supplementary Fig. 5b, c and Supplementary Table 1), including CD44, ALDH, drug resistance-related ABC transporters, stemness factors, and epithelial-mesenchymal markers. CHMp cells had higher levels of CD44, ALDH subtypes, ABCG2 and interestingly mesenchymal characteristics than in CHMm. Next, the CSC-rich CHMp cells formed a larger and faster tumor than CHMm cells and showed vast differences even the same number of cells were inoculated (Fig. 3f-h and Supplementary Fig. 5d). All these data suggest that primary tumor CHMp cells have more CSCs than CHMm cells.

We further investigated that these stemness of primary tumor produced distinct EVs which were found to exert an anti-metastatic growth effect. We further used other primary and metastatic cells, CIPp, and CIPm cells (Supplementary Fig. 5a) which exhibited fewer differences in CSC features (Fig. 3i). Notably, there was no significant difference between CIPp and CIPm cells in the amount of gene expression associated with ALDH, drug resistance, and stemness transcription factor except Nanog (Supplementary Fig. 5e). Thus, we sorted the population of \textit{CD44\textsuperscript{+}/CD24\textsuperscript{−}} cells which we named CIPp-CSCs (Fig. 3j). EVs were isolated from the maintained CIPp-CSC portion and added to CIPm cells. CIPp-CSCs inhibited the viability (Fig. 3k) and proliferation (Fig. 3l). CIPp-CSC-derived EVs significantly suppressed metastatic cell proliferation more than CIPp EVs. These data confirmed that
the growth inhibitory effect of EVs derived from primary tumor is associated with the stemness of CSCs that constitute the primary tumor. Therefore, we examined the cancer stemness of primary tumors to comprehend their metastases inhibitory capability. Although many studies investigated the effect of primary cancer stem cells-derived EVs on DTCs, none have clearly addressed antitumor effects. Analyses of cancer stemness in primary tumor cell lines CHMp and CIPp revealed that CHMp has a cancer stemness \( (CD44^+ / CD24^- / ALDH^{high} ) \) that approaches 80%, while the CIPp, although not to that extent, still has more cancer stemness compared to its metastases cell line. Moreover, EVs derived from \( CD44^+/CD24^- \) population of CIPp cell have much higher antitumor activity compared to the EVs derived from whole CIPp cell population, suggesting that this \( CD44^+/CD24^- \) population involved in the suppression of metastases.

**miR-1 is enriched in pTDEs and suppresses target gene expression in recipient metastases.**

To determine which factors, play a role in the effects of pTDEs, we performed small RNA sequencing. Procedures for RNA acquisition and QC data from small RNA sequencing are described in Supplementary Fig. 6a-e.

We identified 307 and 249 miRNAs from pTDEs and mTDEs, respectively (Fig. 4a, b). The miRNA involved in cell proliferation was the top GO term in pTDE miRNA (Supplementary Fig. 6f, top). The term ‘Regulation of stem cells’ was mainly enriched in mTDE miRNAs (Supplementary Fig. 6f, bottom). Since one miRNA silences multiple genes, we interrogated miRNA target genes using TargetScan and miRDB, which provided a reference for dogs (Supplementary Table 4). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that target genes of pTDE miRNAs were related to cellular proliferation pathways such as the cAMP, cGMP-PKG, PI3K-Akt and the TNF signaling pathway (Supplementary Fig. 6g). Based on GO and KEGG analyses, exosomal miRNA might be the cause of the reduced proliferation of metastases by pTDEs. Next, we identified for the miRNAs differentially expressed in pTDEs. Notably, cfa-mir-1-1 and -2 showed the highest expression level among the miRNAs found in pTDEs (Fig. 4c). In addition, from the miRNA-gene network analysis, miR-1 showed the highest number of nodes among miRNAs and the highest degree
of miRNA known to play a distinct role in tumor suppression (Fig. 4d).

Interestingly, the expression pattern of miR-1 between RNase-treated pTDEs and mTDEs corresponded with CHMp and CHMm cells (Fig. 4e). We integrated two databases (TargetScan and miRDB) that can retrieve the miR-1 target genes and sorted 293 common genes (Fig. 4f). KEGG and Reactome analyses of miR-1 target genes showed significant association with the cell cycle (Fig. 4g, h). Next, we selected ten miR-1 expected target genes (HACE1, C5orf51, PTPLAD1, GLCCI1, MMD, GJA1, THSB4X, SCAF11, WNK3, and SMIM14) and analyzed whether they are affected by pTDEs via qRT‒PCR. The amounts of HACE1, C5orf51, PTPLAD1, GLCCI1, and MMD were significantly decreased by pTDE treatment in CHMm cells (Fig. 4i). On the other hand, the other five genes did not show any significant changes (Supplementary Fig. 7a). Treatment with pTDEs also reduced the protein level of the HACE1 target, which then resulted in an increase in the Rac1 protein, a target of the HACE1 E3 ligase (Fig. 4j). The regulation leading to this miR-1-HACE1-Rac1 axis could help explain how pTDEs cause growth inhibition in recipient metastatic cells. Altogether, miR-1 in pTDEs can reduce the HACE1 level in recipient cells and lead to the accumulation of Rac1 that causes elevation of ROS and subsequently induces cell cycle arrest after DNA damage, which slows cell proliferation. Collectively, pTDEs from primary tumors possess miR-1 that might have a role in metastases suppression.

**pTDE-miR-1 has an anti-metastatic effect in an in vivo mouse model.**

To verify that the inhibition of metastatic growth by pTDEs is due to miR-1 present in high amounts, we treated CHMm cells with miR-1 (Supplementary Fig. 8). Treatment with miR-1 wrapped in liposomes dramatically decreased the six target genes of miR-1 (Supplementary Fig. 8a). Cell viability and growth were also significantly reduced in the group treated with miR-1 (Supplementary Fig. 8b, c). Additionally, like conventional pTDEs treatment, miR-1 also enriched the G2/M stage of the cells (Supplementary Fig. 8d). Furthermore, miR-1 increased intracellular ROS and the genomic instability, like pTDEs (Supplementary Fig. 8e, f). Lastly, miR-1 increased the levels of γ-H2A and Rac1, while decreasing the levels of HACE1 (Supplementary Fig. 8g). Collectively, miR-1 induced genomic
instability, increased G2/M phase, and reduced proliferation, similar to pTDEs.

We then engineered the pTDEs with an overload of miR-1 (pTDE-miR-1) resulting in an approximately 500-fold increase in miR-1 concentration compared to that of pTDE (Fig. 5a). The pTDE-miR-1 demonstrated significantly enhanced properties compared to pTDEs, including decreased expression of target genes, cell viability, and cell growth, as well as an increase in G2/M phase, ROS, DNA damage, and dormancy state (Fig. 5b-g, and Supplementary Fig. 8h, i). These results demonstrated that growth inhibition of metastases by primary tumors can be exerted by miR-1 present in the pTDEs.

Next, we examined the effect of pTDE-miR-1 on inhibiting metastatic growth in an animal model. As shown in Figure 1, after removing the primary tumor from the CMT mouse model, mice confirmed to have metastases of a secondary organ (lung) were grouped and treated with PBS, pTDEs, or pTDE-miR-1. The injection schedule is depicted in Figure 5h. When quantified by radiance, metastatic growth was dramatically suppressed in the pTDE group and mostly in the pTDE-miR-1 group compared to the PBS group (Fig. 5i, j). Lungs from PBS-, pTDE-, and pTDE-miR-1-injected mice showed significant differences in metastatic nodule counts (Fig. 5k). Metastases were easily found in mouse lung tissue from the PBS group, and scarcely in that of the pTDE group, and the size of metastatic tumors was smaller in the pTDE group and much smaller in the pTDE-miR-1 group compared to the PBS group (Fig. 5l). Overall, these data strongly suggest that EVs derived from primary tumors and loaded with miR-1 can inhibit the formation and growth of metastases that have already formed in the lung tissues.

**Blood exosomal miR-1 levels can be a diagnostic marker for metastases in clinical specimens.**

We then investigated whether exosomal miR-1 can be detected in the blood of CMT patients and HBC patients (Fig. 6a). Patient information is described in supplementary Table 5. EVs were isolated from plasma and serum obtained from the patients and were found to be cup-shaped and less than 150 nm in size (Fig. 6b, c). We observed that the expression of exosomal miR-1 was higher in both CMT and HBC patients compared to healthy controls, and furthermore, its levels increased gradually with the progression of breast cancer (Fig. 6d, e). The largest amount of exosomal miR-1 was detected in the sera of Stage III patients, suggesting that primary tumors in locally advanced metastatic stages of breast
cancer might generate an increased inhibitory signal of exosomal miR-1 to inhibit the growth of veiled metastatic cancer.

Next, we investigated the association between miR-1 expression and patient survival using Kaplan–Meier plotter. We found that the high miR-1 expression group had better overall survival (OS) in both lymph node-negative and -positive patients, as well as in all specimens. As high levels of miR-1 in tumor tissues may lead to high levels of exosomal miR-1 in the blood, which could result in better suppression of metastases. However, the OS patterns of HACE1 were different depending on the metastasis state, as high HACE1 expression was associated with worse OS only in the presence of metastasis (Fig. 6f). Collectively, exosomal miR-1 is significantly increased in both human breast cancer and canine mammary tumor patients. Moreover, there exists a correlation where the quantity of exosomal miR-1 increases with higher grade, indicating the potential association with the growth of metastasis.
Discussion

One of the key questions for unrevealing the metastasis is clarifying how the primary tumor controls the tumor cells that exit from their territory. During the initial stages of metastasis, disseminated tumor cells (DTCs) detach from the primary tumor, intravasate into the circulation, and eventually extravasate to colonize at distant secondary sites. The emergence of clinically significant metastatic tumors signifies a crucial juncture, where previously quiescent DTCs reactivate and regain stem cell-like properties that facilitate self-renewal and the potential for continued tumor growth. However, the underlying mechanisms governing the transition between dormant and awakened states for these cells remain incompletely understood, including the factors that determine their ultimate fate at secondary sites.

Recently, Borriello et al showed that metastasizing tumor cells were kept in a dormant state at secondary sites of the lung by primary tumor-associated macrophages. It has been reported that primary tumor can inhibit growth of metastases by inducing apoptosis in DTCs directly or regulating immune cells and endothelial cells in the tumor microenvironment. Because primary tumor inhibits DTCs, when primary tumor disappears, DTCs start to grow, regardless of where they are, due to the release from growth inhibition. This is supported by the fact that early-stage surgical removal (on day 4 after initial tumor implantation) of primary breast tumor in mice induces micro-metastases in the lymph nodes, because DTCs are present in these nodes at this stage. Conversely, late-stage surgery (on day 13) results in the formation of distant organ metastases, such as lungs, as DTCs have had sufficient time to be disseminated and colonize these organs. This is why the surgical removal of primary tumor remains a controversial topic in the field of cancer research. While it can improve patient survival and drug accessibility, it can also promote the growth of metastases. Here, we revealed that the reason why DTCs in a dormant state cannot grow is due to EVs derived from primary tumor. We also showed that the lack of EVs due to the absence of primary tumor promotes the growth of DTCs, while the primary tumor-derived EVs can inhibit the growth of DTCs, even exhibiting a therapeutic effect.

In order to demonstrate that how the primary tumors can inhibit the growth of metastases, we considered that studying the fate of DTCs after the removal of primary tumors could be an ideal model. The growth inhibition caused by pTDEs clearly suggested that the primary tumor induces antitumor
effects to prevent the development of metastasis (Fig. 1). We demonstrated that this antitumor effect of EVs was caused by the accumulation of ROS and damaged DNA. However, damaged DNA resulted in an increase in the G2/M phase of the cell cycle, leading to growth arrest. Next, we extended the role of EVs in the tumor mass, similar to Endostatin, which has been reported as an anti-angiogenic factor by Folkman. Furthermore, pTDEs limit metastatic outgrowth of DTCs by disrupting endothelial cell formation of capillary-like structures. In line with our findings, the clearance of tumor cells in EV-injected mouse model was mediated by both cellular slow-cycling tumor cells and anti-angiogenesis (Fig. 2). It was also reported that the primary tumor can induce antitumor immunity by priming T cells and macrophages. Due to immunodeficient mice that we used, we could not analyze the role of EVs in the immunity. Further investigation is warranted to determine whether pTDEs behave similarly in immunocompetent mice.

To generalize the inhibitory effect of primary tumors, we examined the difference in stemness between primary tumors and metastases. Then, we confirmed the difference in miRNA composition within EVs between the two. EVs derived from $\text{CD}44^+/\text{CD}24^-$ population of CIPp and CHMp cells have much higher antitumor activity compared to the EVs derived from whole CIPp cell population, suggesting that this $\text{CD}44^+/\text{CD}24^-$ population involved in the suppression of metastases (Fig. 3).

Analysis of exosomal miRNA revealed that miRNA-1 is abundantly present in primary tumor derived-EVs and overloading miR-1 in pTDEs significantly enhances their growth inhibitory effect (Fig. 4-5). Consistent with previous reports that miR-1 inhibits tumor growth and metastasis, miR-1 is a tumor-suppressive miRNA conserved in humans and dogs that inhibits growth and metastasis in breast cancer. Furthermore, our study showed treatment with pTDE resulted in decreased expression of HACE1, which is a target of miR-1. Decreased expression of HACE1 led the accumulation of Rac1, which in turn elevated ROS levels and induces cell cycle arrest. This is consistent with previous studies showing that HACE1 controls ROS generation by catalyzing the ubiquitination of Rac1. Taken together, these findings suggest that miR-1 is involved in the suppression of metastases.

The features of miR-1 expression in primary tumors are not confined solely to CMT, as evidenced by its detection in primary colorectal cancer obtained from human patients. In human colorectal cancer
cell lines, miR-1 expression was also higher in SW480 (primary tumor) than SW620 (metastases)\textsuperscript{39}. In this case, SW480 cells also exhibited CD133\textsuperscript{+} stem-like compared to SW620\textsuperscript{50}. Our findings in dogs provide a valuable basis for research in human patient-derived cell lines, given that miR-1 is conserved in both species. These consistent results between dogs and humans provide evidence of a relationship between primary tumors, cancer stem cells, and miR-1. However, it is of note that EVs contain not only miRNAs but also other nucleic acids, proteins, and lipids which might play roles of inhibiting metastases.

Next, we observed an intriguing potential for EVs to serve as biomarkers for both HBC and CMT, owing to their detectability in biological fluids. Notably, cancer patients with HBC and CMT exhibited increased levels of exosomal miR-1, with a progressive elevation observed in HBC patients with advancing TNM stages. We postulate that this may reflect the primary tumor's augmented release of exosomal miR-1 upon DTC dissemination and colonization of distant organs. Furthermore, in line with our clinical observations, we found that HACE1 gene expression was a predictor of inferior overall survival in patients with both primary tumors and metastases, relative to those with primary tumors only (Fig. 6).

Our study has limitations. We used a pair of cell lines for animal studies, although we have occasionally used another pair of cell lines in \textit{in vitro} studies. While we observed changes in the HACE1/Rac1 pathway due to miR-1, we did not elucidate the direct molecular pathway responsible for the observed decrease in proliferation. Additionally, we did not clarify how the abundance of CSCs in primary tumors affects growth inhibition. In future studies, we plan to address these limitations by including other primary tumor and metastatic cell lines for comparison and conducting larger and more in-depth research to elucidate the role of exosomal miR-1. Nevertheless, our research has inaugurated a new era of exploration into the metastatic inhibitory role of EVs originating from primary tumors, laying the foundation for future studies in this field.

In summary, we have provided the first evidence that EVs secreted by cancer stem cells in primary tumors can impede metastatic growth. Moreover, our investigations offer a molecular basis for the EV-mediated suppression of DTC proliferation. Specifically, pTDEs harboring miR-1 exert dual effects:
inducing ROS-mediated genomic instability and cell cycle arrest in metastatic cells while also impeding angiogenesis in nearby endothelial cells. Furthermore, our findings highlight that the most crucial factor in inhibiting metastatic growth is miR-1, which, when overloaded into pTDE, showed therapeutic potential. Additionally, the increase in exosomal miR-1 levels in the serum with the progression of breast cancer suggests its potential use as both a diagnostic marker and a therapeutic agent.
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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

Writing – original draft: HSK
Writing – review & editing: JYC and KHL
Methodology: HSK, KHL
Mouse experimental methodology: HSK and TJS
Bioinformatics methodology: KHS
Visualization: HSK and KHS
Conceptualization and study supervision: JYC

Competing interests

All other authors declare they have no competing interests.
Data and materials availability

All raw and processed exosomal miRNA-seq data for CHMp and CHMm cell lines and their biological replicates generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE213969.

Code availability

The main code scripts used for miRNA-seq processing and visualization are described in detail and available at GitHub: https://github.com/snu-cdrc/exosomal-miRNA
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Figure legends

**Figure 1. Primary tumor-derived EVs (pTDEs) restrict metastasis formation and growth. (a)** Schematic illustration of the established experimental metastasis model. Mice were inoculated with CHMp cells into the mammary fat pad to produce a primary tumor on day 0 (n = 20). On day 21, primary tumors were surgically removed. Seven days after surgery, PBS (n = 4) and pTDEs (n = 5) were administered to mice. Mice were treated with PBS or pTDEs (10µg/mouse) through the tail vein with six times every two days. **(b)** Bioluminescence images of CHMp lung metastases after surgery for confirmation of residual primary tumor mass (at D28). D33 bioluminescence images were acquired from the mice treated with PBS or pTDEs three times, and D39 images were acquired six times at two-day intervals. For each group of daily images, four mouse images were acquired at four positions (dorsal, ventral, right lateral and left lateral) to capture every possible signal from the mice. **(c)** Graph showing quantification of lung metastases treated with PBS and pTDEs in a metastasis mouse model. Unpaired Student’s t test was used to compare groups. (**p < 0.001**). **(d)** Representative pictures of lungs after PBS and pTDE treatment. Top; PBS treated, bottom; pTDE treated. Scale bar, 2 mm. **(e)** Representative H&E staining of lung tissue (4X) in the metastasis model. Left for PBS-treated mice, right for pTDE-treated mice. Magnified images: Scale bar, 1 mm. Results are presented as mean ± SD (4-5 mice were used for each group).

**Figure 2. pTDEs induce cellular ROS, DNA damage, and G2/M arrest, resulting in inhibition of proliferation in recipient cells. (a)** Recipient cell viability after tumor-derived EVs (TDEs) treatment (0.1µg/ml) was measured by MTT assay (n=8). **(b)** The proliferation rate was manually measured by cell counting in the PBS and TDEs treatment groups. pTDEs decreased cell viability and proliferation. **(c)** Cell cycle analysis of exosome-treated cells was performed using flow cytometry. pTDEs induced an increase in G2/M phase. **(d)** Representative images showing cellular ROS levels. Intracellular oxidative stress was measured with the fluorescent ROS probe H2DCFDA (2,7-dichlorodihydrofluorescein diacetate). Brighter green fluorescence indicates high ROS. EVs-treated
cells were stained with H2DCFDA to measure intracellular ROS. Scale bar, 170 μm (e) For measuring damaged DNA, γ-H2A.X (green) and DAPI (blue) were stained in EVs-treated CHMm cells. The white arrow indicates damaged DNA, which colocalized with γ-H2A.X and DAPI. Scale bar, 10 μm. (f) Western blot for intracellular γ-H2A.X. γ-H2A.X was increased with pTDEs treatment. (g) Tube formation assays of HUVECs to detect the angiogenesis potential of pTDEs. HUVECs were seeded on Matrigel, and the exosomes were treated for 24 h. Representative images showing that pTDEs inhibited tube formation compared with controls. Bottom microscopic images were analyzed by the 'ImageJ plugin Angiogenesis analyzer.' Quantification of the mean mesh size is presented as the mean ± SEM. Two-way ANOVA and Tukey’ HSD test was used to compare groups; **P < 0.01, *** p < 0.001, and ns, not significant. Results are presented as mean ± SD.

Figure 3. Cancer stemness contributes to the suppression of metastases. (a) CMT cell lines were stained with CD44 and CD24 antibodies and analyzed by flow cytometry. (b) The growth rate of CHMp and CHMm cells. Cell proliferation was measured by manual cell counting. (c) ALDH activity was examined using an ALDEFLUOR assay in CHMp and CHMm cells. Fluorescent images were examined using a 40x objective lens; the scale bar indicates 130 μm. (d) Representative confocal microscopy images of mammospheres formed by CHMp and CHMm. Scale bar, 400 μm. (e) Mammospheres were stained with antibodies against the CSC markers CD44 (green) and CD24 (red). DAPI (blue) was used as a nuclear marker. Scale bar, 100 μm. (f) Whole-body IVIS bioluminescence images on day 21. Mice were injected with equal numbers of CHMp (top) and CHMm (bottom) cells on the mammary fat pads (n = 5 per mouse group). (g) Graph showing the total flux of IVIS-imaged mice to measure their growth ability. Quantification of primary tumor size once a week. (h) Picture of surgically removed CHMp (top) and CHMm (bottom) primary tumor masses. (i) Flow cytometry analysis of CMT cell lines with CD44 and CD24 antibodies. (j) Schematic illustration of the isolation of the cancer stem cell population (CD44+/CD24-) in the CIPp cell line. (k) An MTT assay was conducted to compare CIPp exosomes and CIPp-CSC exosomes to analyze CIPm viability. (l) The proliferation rate of PBS and exosome treatment was determined by manual cell counting. CIPp-CSC exosomes decrease the proliferation rate...
of CIPm cells more than CIPp exosomes. All data are presented as the mean ± SEM. Experiments were performed in triplicate if not indicated. The statistical analysis is presented. **P < 0.01 and ***P < 0.001. ns, not significant. Results are presented as mean ± SD (5 mice were used for each group).

Figure 4. Small RNA sequencing reveals that miR-1 is enriched in pTDEs and that its network has a critical role in cell cycle regulation. (a-b) Comparison of the identified miRNAs between pTDEs and mTDEs. (c) Volcano plot showing differentially identified miRNAs between pTDEs and mTDEs. Red dots represent pTDE-enriched miRNAs, while blue dots represent mTDE-enriched miRNAs. Gray dots represent miRNAs with -Log10 (p value) and -Log2 (Fold change) values below 1.3 and 1, respectively. (d) Network analysis of miRNAs and their target genes was conducted for the top ten pTDE miRNAs. The size of colored dots indicates the number of genes regulated by the miRNA. miR-1 has the largest colored dots, which represent higher interactions than others. (e) miR-1 expression between CHMp and CHMm cells at the cellular level (left) and exosomes (right). EVs were treated with RNase for 10 min at 37 °C to degrade contamination of EV-free miRNAs. Ct (cycle of threshold) values are normalized to Uni 6 Spike-in within the same cDNA concentration. (f) Identification of target genes of miR-1. TargetScan and miRDB were used to screen miR-1 target genes using a dog database. The Venn diagram indicated that TargetScan and miRDB shared 293 genes. (g-h) KEGG and Reactome analyses of the top 100 genes shared between TargetScan and miRDB. (i-j) Expressional changes in mRNA and protein levels of miR-1 potential target genes. HACE1, C5orf51, PTPLAD1, GLCCI1, and MMD were decreased when pTDEs were administered. Ct (cycle of threshold) values are normalized to A5B gene expression within the same cDNA concentration. The protein levels of HACE1 and the HACE1 target gene Rac1 were analyzed by Western blotting. The statistical analysis is presented. Error bars represent the mean ± SEM. Two-way ANOVA was used to compare groups. *p< 0.05, ***p < 0.001.

Figure 5. pTDE-miR-1 showed therapeutic effects in mouse model. (a) Schematic illustration of miR-1 transfection into pTDEs. (b-d) Viability, proliferation and cell cycle assays were conducted in
CHMm cells treated with PBS, pTDEs, and pTDE-miR-1. Viability and proliferation were further decreased when cells were treated with pTDE-miR-1 compared with pTDEs. (D) pTDE-miR-1 further increases G2/M phases compared with pTDE treatment. (Left) Quantification of cell cycle states in CHMm cells treated with PBS, pTDEs, and pTDE-miR-1. (Right) Representative flow cytometry graph for each treated group. (e) Representative images showing cellular ROS levels; brighter green fluorescence indicates high ROS. pTDE-miR-1-treated cells produce higher ROS than pTDE-treated cells. Scale bar, 170 μm. (f) For measuring damaged DNA, γ-H2A.X (green) and DAPI (blue) stains were used. The white arrow indicates damaged DNA, which colocalized with γ-H2A.X and DAPI. Scale bar, 10 μm. (g) Western blot for H2A.X and miR-1 target genes. Downregulation of HACE1 and upregulation of Rac1 were shown by Western blot. (h) Schematic illustration of an established experimental mouse model and injection schedule. Mice were inoculated with CHMp cells into the mammary fat pad to produce a primary tumor. On day 21, primary tumors were surgically removed. Seven days after surgery, mice were treated with PBS (n=4), pTDEs (n=4) and pTDE-miR-1 (n=4). (i) Bioluminescence images of lung metastases in mice after treatment with PBS, pTDEs, and pTDE-miR-1 six times at two-day intervals. Bioluminescence images were obtained once a week. (j) Graph showing quantification of lung metastases in the mice treated with PBS, pTDEs, and pTDE-miR-1. (k) Pictures of mouse lungs after PBS and pTDEs treatment. Top; PBS treated, Middle; pTDEs treated, and Bottom; pTDE-miR-1 treated. (l) Representative H&E staining of lung tissues in the metastasis model. Left two; PBS treated, Middle two; pTDEs treated, and Right two; pTDE-miR-1 treated (4X). The statistical analysis is presented. Error bars represent the mean ± SEM. Two-way ANOVA was used to compare groups. *p < 0.05, ***p < 0.001. Results are presented as mean ± SD (4 mice were used for each group).

Figure 6. Exosomal miR-1 levels in biological fluids from dog mammary carcinoma and human breast cancer patients. (a) Schematic diagram of exosomes derived from human breast cancer patients and canine mammary carcinoma patients. (b) Nanoparticle tracking analysis (NTA) was used to analyze the size of exosomes. Size distribution of human serum-derived exosomes (left) and dog plasma-derived exosomes (right). (c) Transmission electron microscopy (TEM) showed the external appearance of
exosomes. Top; human serum-derived exosome. Bottom; dog plasma-derived exosomes. (d) The levels of miR-1 in the exosomes of canine mammary carcinoma patients (n=7) and healthy controls (n=6) were examined using qRT‒PCR and normalized to Uni 6 spike-in as a control. (e) Expression levels of miR-1 in human breast cancer patients (n=31) and healthy controls (n=9) were examined using qRT‒PCR and normalized with Uni 6 spike-in as the control. miR-1 was higher in breast cancer patients than in healthy controls, especially in late stage (TNM stage III) patients, compared to healthy controls. (stage 0 : n=9, stage I : n=9, stage II : n=9, stage III : n=4)* p< 0.05, **p < 0.01, ***p < 0.001. (f) *In silico* Kaplan‒Meier analysis of breast cancer patients (http//kmplot.com/analysis/). Overall survival (OS) curve comparing the patients with high (red) and low (black) expression of miRNA-1. miR-1 (top) and HACE1 (bottom) expression in breast cancer patients with or without lymph node metastases. Left; analyzed in all patients. Middle: analyzed for lymph node-negative patients. Right: analyzed for lymph node-positive patients.
Bioluminescence (photons s$^{-1}$ cm$^{-2}$ sr$^{-1}$ $\times 10^8$)

**PBS**

**pTDE**

***

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**Fig 1**

- PBS
- pTDE

**Fig 2**

- Histological images
- Bioluminescence images
Viability (Absorbance O.D 590)

Fig 2
**Fig 3**

(a) CD44+/CD24- cell population

(b) Cell number (1 x 10^6)

(c) ALDEFLUOR

(d) Brightfield

(e) DAPI CD44 CD24 Merged

(f) Day 21

(g) Bioluminescence (photons s⁻¹ cm⁻² sr⁻¹ × 10⁸)

(h) Cell numbers (1 x 10^4)

(i) CD44+/CD24- cell population

(j) Viability (Absorbance O.D 590)

(k) Viability

(l) Cell numbers
**Fig 4**

(a) Number of miRNAs vs. pTDE and mTDE.

(b) Annotated, Novel, Total miRNAs:
- pTDE: 49 (Annotated), 27 (Novel), 76 (Total)
- mTDE: 9 (Annotated), 9 (Novel), 18 (Total)
- Common: 198 (Annotated), 33 (Novel), 231 (Total)

(c) Log2 Fold-change vs. Log10 p-value.

(d)miR-324 and miR-328 expression.

(e) Relative miR1 expression (Normalized to Uni 6 spike-in).

(f) TargetScan and miRDB.

(g) KEGG:
- Cell cycle
- Pathways in cancer
- Pancreatic cancer
- Focal adhesion
- Chronic myeloid leukemia
- p53 signaling pathway
- Neurotrophin signaling pathway
- Renal cell carcinoma
- Regulation of actin cytoskeleton
- Protein processing in endoplasmic reticulum

(h) Reactome:
- Cell Cycle, Mitotic
- Cell Cycle
- Metabolism of proteins
- Membrane Trafficking
- Vesicle-mediated transport
- Generic Transcription Pathway
- Mitotic G2-M phases
- DNA Repair
- G2/M Transition
- Signaling by TGF-beta Receptor Complex

(i) Relative gene expression (Normalized to A5B).

(j) Relative gene expression (Normalized to A5B).
Viability (Absorbance O.D 590)

Cell numbers (1 x 10^4)

Bioluminescence (photons s–1 cm–2 sr–1 × 10^9)

Percentage of cells (%)

Fig 5
Supplementary Files

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