

Article

Not peer-reviewed version

Suppression of Metastasis of Colon Cancer to Liver by Pretreatment With Extracellular Vesicles Derived From Nanog-Overexpressing Colon Cancer Cells

Takuya Henmi , [Hideaki Matsuoka](#) , [Mikako Saito](#) *

Posted Date: 11 June 2024

doi: 10.20944/preprints202406.0614.v1

Keywords: Colon-26; Nanog; metastasis; macrophage; phagocytosis



Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Article

Suppression of Metastasis of Colon Cancer to Liver by Pretreatment with Extracellular Vesicles Derived from Nanog-Overexpressing Colon Cancer Cells

Takuya Henmi ¹, Hideaki Matsuoka ², Mikako Saito ^{1,2,*}

¹ Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan; 08henmmmm06@gmail.com

² Bioresource Laboratories, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan; mhide@cc.tuat.ac.jp

* Correspondence: mikako@cc.tuat.ac.jp

Abstract: It has been demonstrated that cancer cells that have survived cancer treatment may be more malignant than the original cancer cells. These cells are considered to be the main cause of metastasis in prognosis. A Nanog-overexpressing colon-26 (Nanog+colon26) was generated and its metastatic potential was confirmed to be enhanced, indicating higher malignancy. Extracellular vesicles (EVs) secreted from the cell line (Nanog+colon26EVs) were administered to mice at 5 µg nine times (three times per week for three weeks) via the tail vein (PBS was used as a control). Subsequently, Nanog+colon26 cells were administered to mice via the spleen, and the quantity of metastatic colonies in the liver was analyzed two weeks later. The results demonstrated that the administration of EVs suppressed metastasis. The enhanced phagocytic activity of macrophages observed in the group treated with Nanog+colon26EVs indicated the involvement of the immune system in the observed metastasis-suppressing effect. Small RNA sequencing was conducted to identify Nanog-dependent miRNAs that exhibited significant changes ($F_{\text{c}} \geq 1.5$ or $F_{\text{c}} \leq 1/1.5$; $p < 0.05$) in Nanog+colon26EVs relative to colon26EVs. Nine miRNAs (up-regulated: 4, down-regulated: 5) were identified, and 623 genes were predicted to be their target genes. Of the 623 genes identified, 17 were predicted to be relevant to immune activity, macrophage function, and phagocytosis.

Keywords: Colon-26; Nanog; metastasis; macrophage; phagocytosis

1. Introduction

While anticancer drugs and radiation treatments are effective in killing cancer cells, some cells are able to evade these treatments. It can be postulated that those cells should have acquired high resistance to drug [1–3] and radiation [4,5], and thus be regarded as malignant cancer cells. It is believed that these cells are the primary causes of recurrence and metastasis, and present a significant challenge in cancer treatment. To address this issue, vaccines utilizing extracellular vesicles (EVs) derived from cancer cells have recently garnered significant interest [6–8]. The introduction of EVs secreted from cancer cells into the body in advance as a vaccine may suppress the proliferation and metastasis of the cancer cells introduced subsequently. It has been demonstrated that dendritic cells, CD8⁺ T cells, and NK cells are activated in vitro and in vivo.

EVs comprise a multitude of components. It is observed that components with tumor-suppressive effects coexist with those with tumor-promoting effects. It is possible that some components may contribute to tumor suppression in one cancer, but that the same components may exhibit a tumor-promoting effect in another cancer. Consequently, it remains challenging to develop EV vaccines that are efficacious against a range of cancer types and at different stages of disease progression. The target is malignant cancer cells that have evaded treatment, but for which the type of cancer is known. In light of these considerations, the intention was to create highly malignant cancer cells, as they are capable of generating EVs that are effective even against such malignant cancer cells. In light of the concept of cancer stem cells [13], we postulated that a lower stage of

differentiation might result in cancer cells becoming more malignant. *Nanog* was selected as an inducer for lowering the differentiation stage.

A positive correlation was observed between the expression levels of *Nanog* and the malignancy levels in 14 types of cancer, including breast cancer, colon cancer, embryonal carcinoma, liver cancer, and skin cancer (melanoma) [14–16]. Melanoma and its metastasis to the liver were selected as the initial target cancer. A melanoma cell line, BL6-F10, was genetically modified to a more malignant cell line by overexpression of *Nanog*. The malignant cells (*Nanog*⁺F10) were used to produce an EV vaccine with metastasis-suppressing effects [18].

Colon cancer was selected as the second target cancer due to the high prevalence of metastasis to the liver, which is a critical issue in the context of colon and colorectal cancers [19]. In colon cancer, the expression of stem cell markers, including *Nanog*, was reduced by the inhibition of furin, which is involved in calcium transport [20]. Conversely, it was anticipated that an increase in the expression level of *Nanog* would enhance the stemness of cancer cells and increase their malignancy. The objective of this study is to ascertain whether extracellular vesicles (EVs) derived from malignant cancer cells have the capacity to suppress cancer metastasis.

2. Results

2.1. Properties of *Nanog*-overexpressing colon-26 (*Nanog*⁺colon26) cells

A *Nanog* overexpression vector was introduced into colon-26 cells to generate a *Nanog*⁺colon26 cell line. The *Nanog*⁺colon26 cells were tested for metastasis-related in vitro properties. As compared to colon-26, the proliferation rate was significantly higher as expected (Figure 1A). However, the migration activity was unchanged and the invasion activity was lower as determined by Transwell™ assay. In addition, metalloproteinases such as MMP2 and MMP9 were less active, suggesting low activity of invasion by degradation of cell stroma (data not shown). In contrast, metastasis to the liver was extremely prominent. White, flat colonies were formed (Figure 1B), and the area of these colonies was added up per mice to determine the quantity of metastasis (Figure 1C). The metastatic potential of *Nanog*⁺colon26 was significantly higher than that of colon-26 (Figure 1D). Therefore, *Nanog* overexpression was also effective to make colon-26 cells more malignant. However, unlike melanoma, the migration and invasion activities of colon cells seemed not to be relevant to metastasis potential.

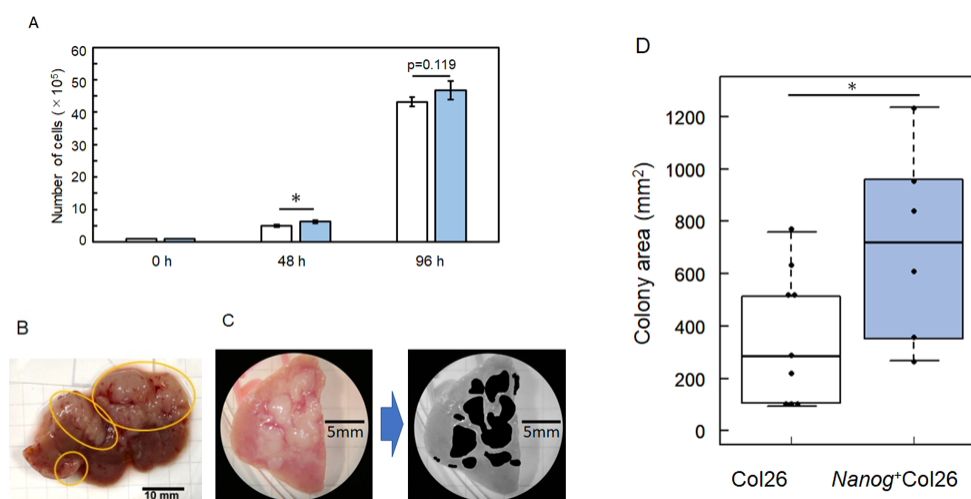
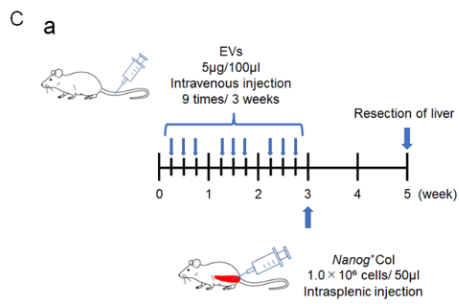
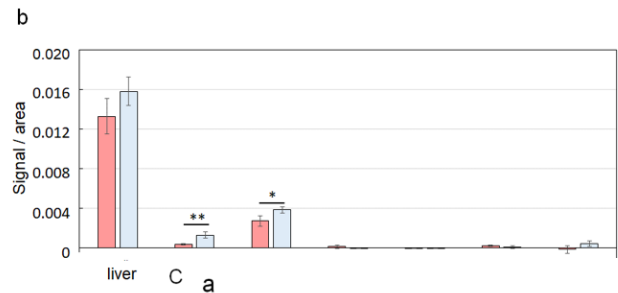
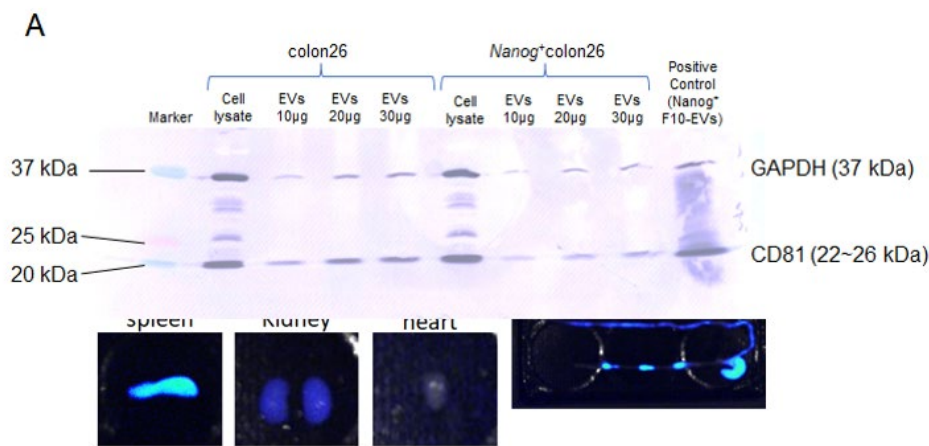


Figure 1. Properties of *Nanog*⁺colon26 cells. (A) Proliferation activity. White square: colon-26, blue square: *Nanog*⁺colon26, mean±SD for n=3. (B) Colon cancer colonies metastasizing to the liver. Colony: circled by a yellow line. (C) Quantification of metastasis by adding up the area of colonies by using ImageJ. (D) Colony area per mouse. colon-26: n=7, *Nanog*⁺colon26: n=6, *: p<0.05.

2.2. Metastasis suppression effect of *Nanog*⁺colon26EVs

EVs isolated from *Nanog*⁺colon26 cells and colon-26 cells were confirmed by Western blotting using CD81, an EVs marker (Figure 2A). Next, the circulation of EVs in the body was examined by injecting NIR815 labelled EVs (100 μ L of 50 μ g/mL) from the tail vein. Three h after the injection, organs were excised for fluorescence image analysis. Liver showed the highest uptake of EVs among seven organs (Figure 2Ba). In the lung and spleen, uptake of *Nanog*⁺colon26EVs was higher than that of colon26EVs (Figure 2Bb). In the liver, the amount of *Nanog*⁺colon26EVs taken up tended to be greater than that of colon26EVs, although the difference was not statistically significant. Then, the effect of *Nanog*⁺colon26EVs on the metastasis of colon cells to liver was investigated. *Nanog*⁺colon26EVs or colon26EVs were administered 9 times via the tail vein at 100 μ L (50 μ g/1 mL) (Figure 2Ca). *Nanog*⁺colon26 cells were then injected to the spleen, and 2 weeks later, the mice were euthanized by cervical dislocation and the livers were removed. The livers were cut into lobes and photographed. The area of tumors in the images were added up per mice. Mice treated with colon26EVs exhibited reduced metastasis compared to that treated with PBS. Mice treated with *Nanog*⁺colon26EVs demonstrated a more pronounced effect on the metastasis suppression (Figure 2Cbc). It is noteworthy that two of the mice treated with PBS died within two weeks of the injection of *Nanog*⁺colon26 cells, but all mice treated with *Nanog*⁺colon26EVs survived for two weeks.



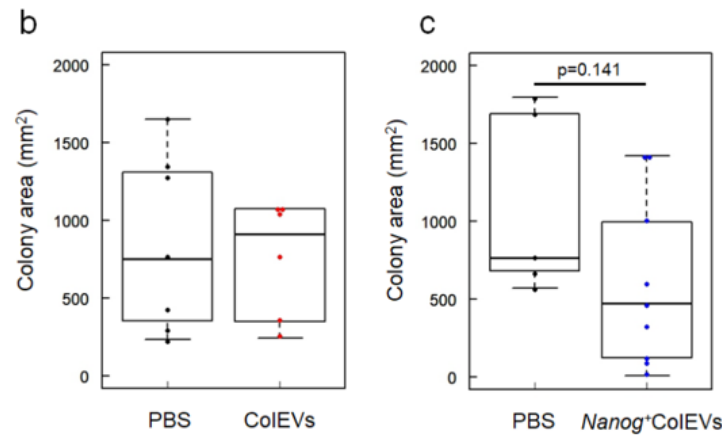


Figure 2. Effects of *Nanog*⁺colon26EVs on the liver metastasis. (A) Isolation of EVs, (B) Distribution of EVs in the body, a. Fluorescence images of NIR815-labeled *Nanog*⁺colon26EVs accumulated in each organ, b. Accumulation of *Nanog*⁺colon26EVs (■) and colon26EVs (■) in each organ, mean±SD for n=3, **p<0.01 *p < 0.05, (C) Suppressive effect of EVs on liver metastasis, a. Timeline of EVs and cell administration, b. Effect of colon26EVs, number of mice PBS: n=7, colon26EVs: n=6, c. Effect of *Nanog*⁺colon26EVs, number of mice PBS n=5, *Nanog*⁺colon26EVs: n=9.

2.3. Effects of *Nanog*⁺colon26EVs on macrophage phagocytic function

Since the metastasis-suppressive effect was obtained when EVs were used as a vaccine, it was predicted that the target cells involved were immune cells. Since the involvement of macrophages has already been reported in the suppressive effect of *Nanog*⁺F10EVs on the liver metastasis of melanoma *Nanog*⁺F10 [18], we assumed that macrophages would also be involved in the case of *Nanog*⁺colon26EVs. Specifically, we focused on the phagocytic function playing a role of elimination of unnecessary cells including cancer cells. In vitro tests were performed to examine the phagocytic function using fluorescent microbeads (MBs) as a phagocytosed model. EVs (*Nanog*⁺colon26EVs or colon26EVs) were labeled with the fluorescent dye PKH26 and used together with commercially available MBs labeled with FITC (FITC-MBs). J774.1 cells that have taken up PKH26-EVs and/or FITC-MBs were detected in the PE channel and the FITC channel, respectively, of a flow cytometer (FACS Aria II, BD).

A flowcytogram for a cell sample containing neither PKH26-EVs nor FITC-MBs was obtained as a double-negative controls (Figure 3Aa). A flowcytogram for a cell sample containing FITC-MBs alone was obtained as a PKH26-EVs negative control (Figure 3Ab). From these controls, we set the P2 and P4 gates so that the fractional number of cells within P1 and P3 gates were 0%. Figures 3Ac and 3Ad are results obtained with cell samples containing both PKH26-EVs nor FITC-MBs.

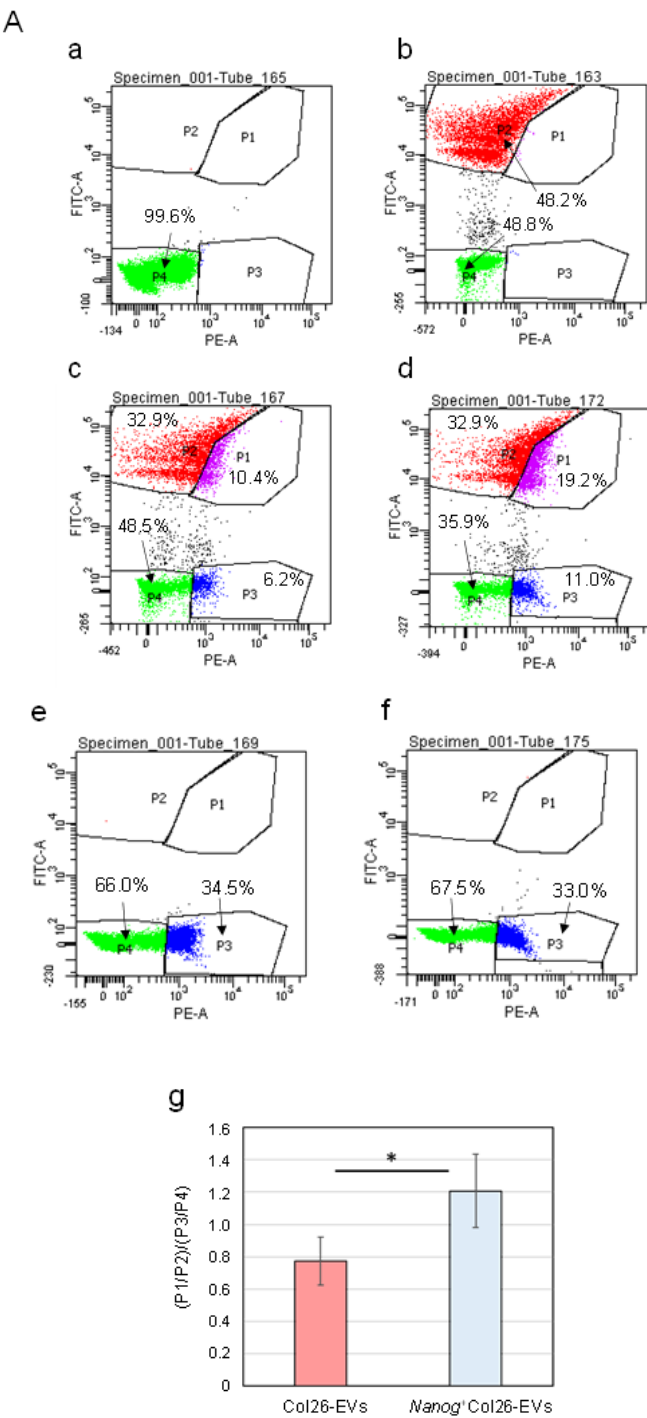
If cells that have taken up EVs have a greater tendency to take up MBs, it is expected that P1/P2 will be larger. However, a large P1/P2 ratio may also simply indicate that EVs themselves are more likely to be taken up by cells. Therefore, it is necessary to offset such an effect specific to EVs. This effect may be estimated from flowcytograms of MBs negative controls (Figure 3Ae, f). If EVs themselves are likely to be taken up by cells, it is expected that P3/P4 will be large. Therefore, we defined the effect on phagocytic activity as the net effect of EVs on the MBs uptake that may be given by $[P1/P2]_{(in\ c, d)}/[P3/P4]_{(in\ e, f)}$. If this ratio is greater than 1, the effect on phagocytic activity is promotive, whereas the ratio smaller than 1 indicates a suppressive effect. In the case of colon26EVs (Figure 3Ac and 3Ae), the ratio was 0.77 indicating suppressive effect, while in the case of *Nanog*⁺colon26EVs (Figure 3Ad and 3Af), the ratio was 1.21 indicating promotive effect. The difference between both EVs significant (p<0.05) (Figure 3Ag).

Furthermore, the number of MBs taken up by a single cell was quantified using fluorescence microscopy. After confirming the positions of J774.1 cells by a bright field image (Figure 3Ba), the numbers of MBs taken up into each cell was counted in the fluorescent images (Figure 3Ba).

Compared to the control condition where PBS was added instead of EVs, the number of MBs taken up into the cells decreased when colon26EVs were added, and increased when *Nanog*⁺colon26EVs were added (Figure 3Bc).

2.4. Effects on macrophage polarization

It was previously reported that CD80-positive M1 macrophages exhibited higher phagocytic activity compared to M2 macrophages [21]. Therefore, we investigated the effect of EVs on macrophage polarization. The results demonstrated that the expression of CD80 (a marker of M1 type) was higher in *Nanog*⁺colon26EVs than in colon26EVs, whereas the expression of CD163 (a marker of M2 type) was at the same level (Figure 3B). This suggests that *Nanog*⁺colon26EVs promoted the polarization of J774.1 cells toward M1 phenotype and consequently contributed to the enhancement of phagocytic activity.



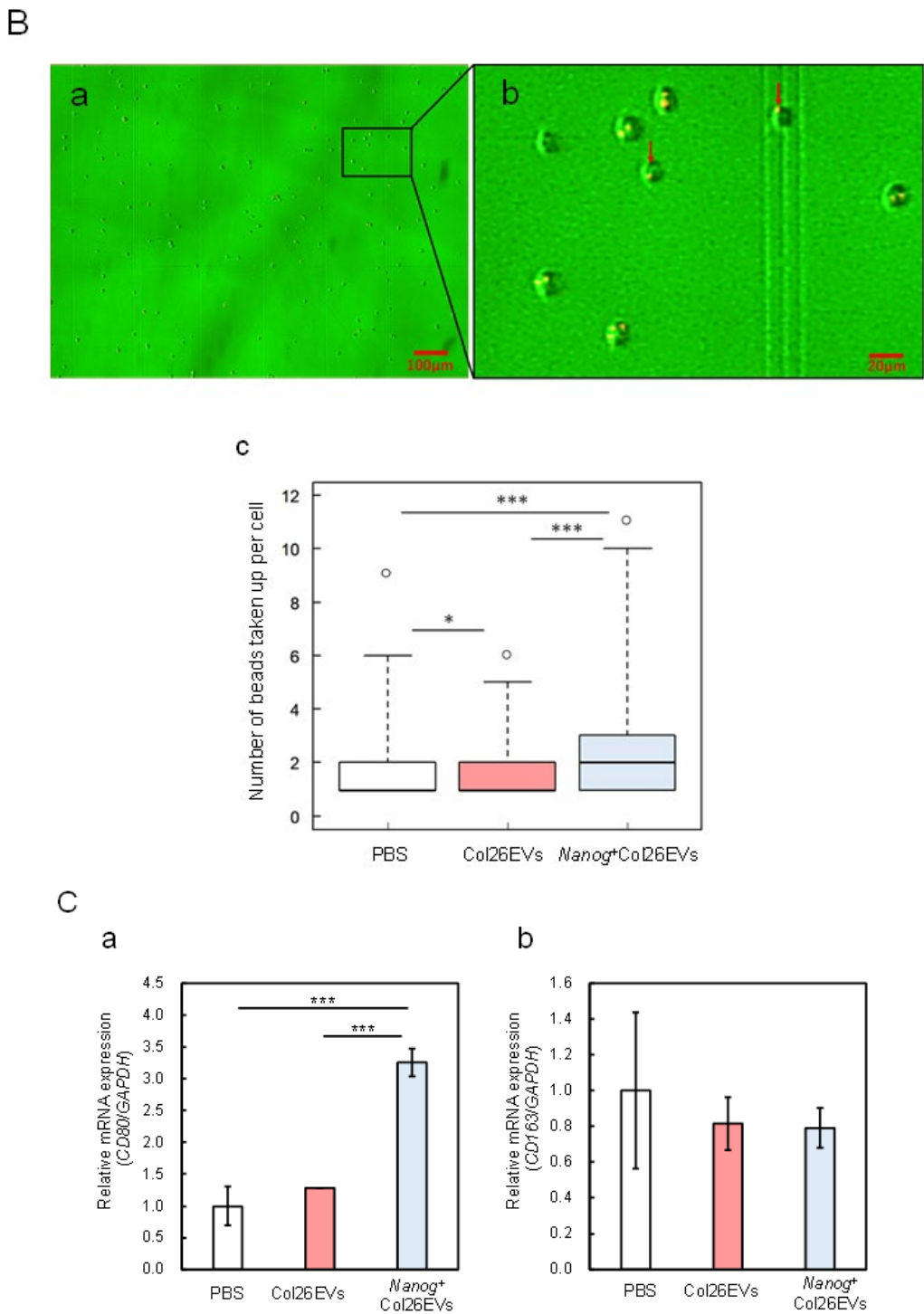


Figure 3. Effects of EVs on the phagocytic activity of J774.1 cells. (A) Flowcytograms of J774.1 cells containing PKH26-EVs and/or FITC-MBs. PKH26, Ex: 551 nm, Em: 567 nm; FITC, Ex: 441 nm, Em: 486 nm. a. EVs(-) and MBs(-), b. EVs(-) and MBs(+), c. colon26EVs (+), MBs (+), d. *Nanog*⁺colon26EVs (+), MBs (+), e. colon26EVs (+), MBs (-), f. *Nanog*⁺colon26EVs (+), MBs (-), g. Phagocytic activity, mean ± SD for n=3, *: p < 0.05. (B) Uptake of MBs by J774.1 single-cells analyzed by fluorescence microscopy. a. Brightfield image, b. Fluorescent image of an enlarged part of a, Red arrows indicate MBs. c. Number of MBs taken up per cell. °: outliers, PBS: n=377, mean=1.81, median=1. colon26EVs: n=367, mean=1.65, median=1, *Nanog*⁺colon26EVs: n=420, mean=2.19, median=2, *: p<0.05, ***: p<0.001. (C) Expression of macrophage markers determined by qPCR. a. CD80, b. CD163, mean±SD for n=3, ***: p<0.001.

2.5. Expression of miRNAs and their target genes in EVs

Small RNA sequencing was conducted to find *Nanog* dependent miRNAs that were significantly changed ($F_c \geq 1.5$ or $F_c \leq 1/1.5$; $p < 0.05$) in *Nanog*⁺colon26EVs as compared to colon26EVs. Nine miRNAs (up-regulated: 4, down-regulated: 5) were found (Figure 4) and their target genes were surveyed using TargetScanNMouse8.0. Under the condition that cumulative weighted context score was equal to or lower than -0.40, 623 genes were predicted (Table 1).

Following the prediction of 623 genes that corresponded to respective proteins, the network of those functional proteins was analyzed using STRING ver.3.10.1. and the association levels between those proteins were analyzed using cytoHubba to predict 30 genes/proteins with top highest level of association (Table 2).

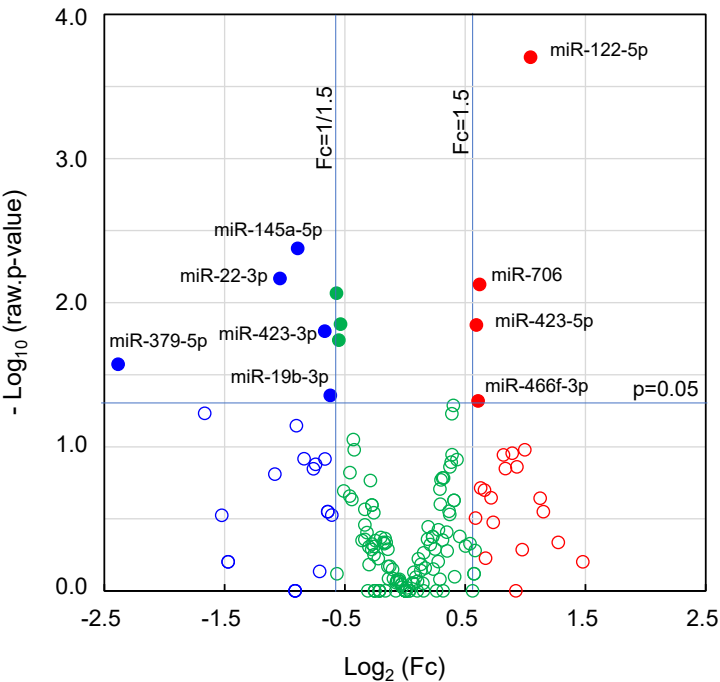


Figure 4. A volcano plot of miRNAs. Fold change (Fc) and p-value in statistical significance; $F_c \geq 1.5$ ($\text{Log}_2(F_c) \geq 0.585$) and $p < 0.05$ ($-\text{Log}_{10}(p) \geq 1.301$), or $F_c \leq 1/1.5$ ($\text{Log}_2(F_c) \leq -0.585$) and $p < 0.05$.

Table 1. Number of up-regulated and down-regulated miRNAs in *Nanog*⁺colon26EVs versus in colon26EVs.

	miRNA	$\text{Log}_2(F_c)$	Number of target genes		
			$p < 0.05$ $\text{CWCS} \leq -0.40$		Targeted by each miRNA among Top30 genes
<i>Nanog</i> ⁺ Col26EVs/ Col26EVs	miR-122-5p	1.04	29	321	2
	miR-706	0.62	62		3
	miR-466f-3p	0.61	146		6
	miR-423-5p	0.59	84		2
	miR-379-5p	-2.38	7	302	0
	miR-22-3p	-1.04	103		8
	miR-145a-5p	-0.89	48		4
	miR-423-3p	-0.67	8		1
	miR-19b-3p	-0.62	136		4
			Sum	623	30

Table 2. Top 30 genes/proteins with the highest level of association.

Rank	Gene	miRNA	Predicted effects	Rank	Gene	miRNA	Rank	Gene	miRNA
1	Actb	miR-145a-5p	cell motility and contraction→up	11	Csf1r	miR-22-3p	21	Mecp2	miR-22-3p
2	Ins1	miR-466f-3p	blood glucose decrease→down	12	Sirt1	miR-22-3p	22	Mlxipl	miR-706
3	Fgf17	miR-423-5p	normal brain development→down	13	H3f3b	miR-22-3p	23	Socs1	miR-19b-3p
4	Fgf16	miR-466f-3p	normal heart development→down	14	Angpt2	miR-145a-5p	24	Hnrnp2	miR-145a-5p
5	Grb2	miR-466f-3p	linking of cell growth factor receptor and Ras signaling→down→growth inhibition	15	Cdkn1b	miR-706	25	Pkm	miR-122-5p
6	Ngf	miR-423-5p	normal nerve growth→down	16	Ppara	miR-22-3p	26	Prkcd	miR-466f-3p
7	Erb3	miR-22-3p	development of various organs and myeloid cell differentiation→up	17	Slc2a4	miR-466f-3p	27	Arrb1	miR-22-3p
8	Fgfr2	miR-423-3p	cell proliferation, differentiation, migration, apoptosis→up	18	Mycn	miR-19b-3p	28	Sgk1	miR-19b-3p
9	Kitl	miR-466f-3p	cell survival, proliferation, stem cell maintenance→down	19	Ccnd2	miR-19b-3p	29	Hnrnpf	miR-706
10	Esr1	miR-22-3p	cellular proliferation and differentiation→up	20	G6pc3	miR-122-5p	30	Actg1	miR-145a-5p

Predicted effects of the top 10 genes are described. Generally, if miRNA is up-regulated, the target gene is down-regulated, and vice versa. Up-regulated miRNAs are shown in red and down-regulated miRNAs are shown in blue. Arrows: →up and →down indicate that functions described in Predicted effects column are up-regulated and down-regulated, respectively.

2.6. Factors involved in the activation of the immune system

Genes presumed to be closely involved in the activation of the immune system, macrophages, and phagocytic activity were searched from 623 target genes listed in Table 1. The search was conducted in three stages. The initial step involved the selection of individual genes predicted to be most affected by *Nanog* overexpression. Specifically, the top 30 genes with the highest level of association were predicted by functional association analysis using STRING, followed by association level analysis using cytoHubba. Next, enrichment analysis was conducted to investigate the possibility that multiple gene groups may be involved in a specific pathway as a cluster, and to predict dominant genes among them. The third step involved searching for genes that included the keywords "macrophage" and "phagocytosis" in their functional annotation among the 623 target genes.

Results of the first search:

The top 10 genes most highly associated with *Nanog* overexpression were examined for predicted effects listed in Table 1. The top 1 gene, *Actb*, is an actin-related gene and is associated with cell motility and contraction. The predicted effect of up-regulation of *Actb* is the increase of cell motility. Our in vitro tests of migration and invasion, however, did not seem to reflect the possible effect of *Actb*. The top 2 gene, *Ins1*, encodes insulin, a hormone that contribute to the maintenance of blood glucose level at a proper condition. Partial down-regulation of *Ins1* might cause a moderate hyperglycemia and contribute metastasis suppression as suggested by [22,23]. Top 3, 4, and 6 genes are factors promoting normal growth of brain, heart, and nerve cells, respectively. Contribution of these factors to immune system, if any, might be minimal because they are relevant to specific organs. Conversely, top 5, 7~10 genes are involved in the proliferation of various cells that may include immune system cells. Therefore, these factors are worth consideration for the regulation of immune system cells. Under the condition of Table 2, for example, top 5 (*Grb2*) and top 9 (*Kitl*) are down-regulated. From the viewpoint of an idea of direct use of target genes rather than miRNAs, however, these genes are potentially useful for growth promotion. In conclusion, the results of this search indicate that seven of the top ten genes (excluding the top 3, 4, 6) are potential candidates for the activation of immune system cells.

Results of the second search

Pathway enrichment analysis was conducted on a group of 623 target genes. Ten pathways in KEGG pathways and two pathways in WikiPathways were predicted to be enriched (Table 3). Two key words, “focal adhesion” and “PI3K-Akt(-mTOR) signaling” were found in one of KEGG pathways and one of WikiParthways in common. Especially, WP2841 was only one that contained both keywords in the title. Therefore, this pathway was thought to be most closely associated with *Nanog* dependent genes. The number of genes included in WP2841 was 316, of which 24 genes were included in the 623 target genes (Table 4). Furthermore, 11 of the 24 genes were included in the top 30 of Table 2. Seven of the top ten genes in Table 4 were identical to seven of the top ten genes in Table 2. The remaining three genes, *Csf1r*, *Angpt2*, and *Pik3cd*, were of interest. *Csf1r* is a macrophage colony-stimulating factor 1 receptor, which is upregulated and is predicted to contribute to immune system activation. *Pik3cd* is an isoform of the common keyword PI3K, which mediates immune responses. Although its target is not macrophages, *Pik3cd* is involved in the development, proliferation, and migration of B cells, and is up-regulated, contributing to their promotion. In conclusion, *Csf1r* and *Pik3cd* have been identified as key target genes for the activation of immune system cells.

Results of the third search

Nine genes were selected and listed in Table 5. *Csf1r*, which was also predicted in the second search, is involved in the proliferation and differentiation of macrophages. In contrast, *Maf1* and *Ifi204* are related to macrophage differentiation, and *Ocln* to macrophage adhesion and expansion. However, all three genes are downregulated, indicating that they are expected to show inhibitory or suppressive effects. With regard to phagocytosis, *Gulp1* is predicted to have a promoting effect due to its upregulation, whereas *Il15ra* is predicted to have an inhibitory effect due to its downregulation. It is noteworthy that *Pkm* is the only gene in Table 5 that is included in the Top 30 of Table 2. Furthermore, it was reported that *Pkm* promotes the immune checkpoint ligand PD-L1 in a STAT1 (Signal transducer and activator of transcription 1)-dependent manner in macrophages lacking circadian genes, thus acting as a brake on the immune system [24]. In our case, *Pkm* was down-regulated and PD-L1 was suppressed and, consequently, immune activity was thought to be enhanced. Based on the aforementioned evidence, it can be reasonably inferred that *Csf1r*, *Gulp1*, and *Pkm* are involved in the phagocytic function of macrophages and the subsequent activation of the immune system. It is also postulated that the immune system can be further activated if the expression of other factors is separately regulated properly.

Table 3. Pathways predicted to be enriched in 623 target genes/proteins.

	Pathway	Description	Count in network	Strength
KE	mmu04010	MAPK signaling pathway	20 of 287	0.40
	mmu04014	Ras signaling pathway	17 of 225	0.44
	mmu04015	Rap1 signaling pathway	18 of 208	0.50
	mmu04068	FoxO signaling pathway	15 of 129	0.63
	mmu04151	PI3K-Akt signaling pathway	23 of 353	0.38
	mmu04152	AMPK signaling pathway	11 of 124	0.51
	mmu04510	Focal adhesion	17of 225	0.45
	mmu04930	Type II diabetes mellitus	7 of 48	0.73
	mmu04931	Insulin resistance	10 of 109	0.52
	mmu04960	Aldosterone-regulated sodium reabsorption	6 of 38	0.76
WK	WP2841	Focal adhesion: PI3K-Akt-mTOR signaling pathway	24 of 316	0.44
	WP85	Focal adhesion	15 of 185	0.47
KE:KEGG pathways, WK: WiKiPathways				

Table 4. The target genes listed in Table 1 that are included in 316 genes constructing the pathway WP2841.

Rank	name	Score	miRNA	Rank	name	Score	miRNA	Rank	name	Score	miRNA
1	Ins1	1500	miR-466f-3p	9	Angpt2	48	miR-145a-5p	15	Rab14	2	miR-466f-3p
2	Fgf17	1490	miR-423-5p	10	Pik3cd	6	miR-22-3p	15	Efna3	2	miR-423-5p
3	Fgf16	1488	miR-466f-3p	11	Slc2a4	5	miR-466f-3p	15	Pfkfb2	2	miR-466f-3p
4	Grb2	1450	miR-466f-3p	11	Pdpk1	5	miR-466f-3p	15	Fgf12	2	miR-706
5	Kitl	1440	miR-466f-3p	13	Cdkn1b	4	miR-706	21	Gys1	1	miR-122-5p
5	Ngf	1440	miR-423-5p	13	Pdgfd	4	miR-145a-5p	21	Ppp2r5e	1	miR-19b-3p
7	Fgfr2	751	miR-423-3p	15	Ddit4	2	miR-423-5p	21	Itgb8	1	miR-145a-5p
8	Csf1r	749	miR-22-3p	15	Rab10	2	miR-706	21	Thbs3	1	miR-423-5p

Genes shown in bold are included in the top 30 (Table 2). miRNAs shown in red and blue are those that are up-regulated and down-regulated, respectively.

Table 5. Genes relevant to the functions of macrophages and phagocytosis.

Keyword	Gene	miRNA	Predicted effects
Macrophage	Maf1	miR-122-5p	Required for monocytic, macrophage, osteoclast, and islet beta cell differentiation. →down
	Pkm	miR-122-5p	Promotes in a STAT1-dependent manner, the expression of the immune checkpoint protein PD-L1 in ARNTL/BMAL1-deficient macrophages, consequently suppresses immune activity.→down
	Ocln	miR-122-5p	Regulation of the tight junction and macrophage adhesion and spreading →down
	Ifi204	miR-466f-3p	Inhibits cell growth and may be involved in macrophage differentiation.→down
	Il31	miR-22-3p	May function in skin immunity, enhances myeloid progenitor cell survival in vitro, and induces amyloid A protein expression in macrophages.→up
	Csf1r	miR-22-3p	Regulation of survival, proliferation and differentiation of macrophages and monocytes and plays an important role in innate immunity and in inflammatory processes.→up
	Pdgfd	miR-145a-5p	Plays an important role in wound healing and induces macrophage recruitment.→up
Phagocytosis	Il15ra	miR-466f-3p	Required for IL15-induced phagocytosis in neutrophils→down
	Gulp1	miR-19b-3p	Required for efficient phagocytosis of apoptotic cells.→up

miRNAs shown in red and blue are those that are up-regulated and down-regulated, respectively. Arrow notations indicate the up-regulation (→up) and down-regulation (→down) of target genes.

3. Discussion

The metastasis suppressive effect of *Nanog**colon26EVs was demonstrated and possible contribution of macrophages to this effect was investigated. It was revealed that phagocytic activity was enhanced and polarization towards the M1 type was promoted. These supported the increase in antitumor activities of macrophage. Candidates of *Nanog* dependent genes were predicted by miRNA sequencing and analysis of associated target genes. From 623 target genes predicted, 17 candidate genes were predicted (Table 6).

Table 6. Candidate genes predicted to be effective for the promotion or regulation of immune-activities.

	Effective for promoting	Potentially effective for promoting when overexpressed
Search 1	Actb, Ins1, Erbb3, Fgfr2, Esr1	Grb2, Kitl
Search 2	Csf1r, Pik3cd	—
Search 3	Csf1r, Gulp1, Pdgfd, Pkm	Maf1, Ifi204, Ocln, Il15ra

There are four research topics necessary for the development of EVs vaccines: (i) the creation of EVs with high-performance, (ii) prediction of key genes essential for high performance, (iii) development of genetic modification tools for quantitative expression control of predicted key genes,

(iv) efficient production of EVs that include expression systems for multiple key genes. Our goal is to develop vaccines that can reduce the risk of cancer recurrence and metastasis in cancer patients. Under such conditions, the type of cancer is known from the beginning but the challenge is to target the small number of highly malignant cancer cells that have evaded anti-cancer drugs and radiation therapy. Therefore, our idea was to create highly undifferentiated cancer cells in order to create a highly malignant cancer cell model. Our first answer was overexpression of Nanog gene in cancer cells and it was successful. Furthermore, unexpectedly, EVs derived from these highly malignant cancer cells showed a strong effect of suppressing metastasis, which marked an important turning point. We faced the challenge of whether the propositions "Can Nanog be used to create a malignant model of cancer cells?" and "Do EVs derived from malignant cells have metastasis suppressive effect?" can apply to all types of cancer cells, or whether they apply only to melanoma, which was the first cancer we worked on. Therefore, we worked on colon cancer as the second cancer in this study. And as expected, we were able to confirm that the two propositions apply.

A total of 17 target genes were predicted in this study. Among them, *Esr1* was common compared to the 6 genes predicted in melanoma (*Trp53*, *Hif1a*, *Esr1*, *Atm*, *Rnf11*, *Cdkn1b*) [25]. In addition, *Cdkn1b*, which was ranked in the top 13 in Table 2, was included in melanoma. On the other hand, in EVs derived from iPS cells, which have a higher level of undifferentiation, 10 genes (*Ins1*, *Kitl*, *Fgf16*, *Grb2*, *Malt1*, *Fgf1*, *Il1f6*, *Cmklr1*, *Siglec1*, *Cd28*) were predicted [25], and *Ins1*, *Grb2*, and *Kitl* were common compared to the 10 genes predicted in EVs derived from iPS cells. From a practical point of view, it is highly suggestive that *Esr1* and *Ins1* were common key genes, because they are related to hormones that can act from outside the cell. Although this study has focused on the contribution of macrophages, it is not difficult to imagine that various functions of other immune cells may contribute to metastasis suppression. In that sense, the discovery of *Pkm*'s involvement as an immune checkpoint ligand is a major discovery. At the present, it is still difficult to discuss commonalities and characteristics between types of cancer. However, the findings of this study certainly support our research in promising direction.

4. Material and Methods

4.1. Cell culture

The mouse colon-26 cell line was obtained from RIKEN BRC and cultured in RPMI-1640 (Gibco) containing 10% FBS and 1% penicillin-streptomycin at 37°C under 5% CO₂. When the cells reached 70-80% confluence, the medium was removed from the dish and the cells were washed twice with PBS. Subsequently, 400 µL of a solution containing 0.25 (w/v) % trypsin and 1 mM EDTA was added to the dish. Following incubation at 37°C under 5% CO₂ for 5 min, the cells were detached from the dish for subculture. Mouse macrophage J774.1 was obtained from RIKEN BRC. J774.1 cells were cultured in accordance with the methodology previously described for mouse melanoma cells [18].

4.2. Animals

This study was conducted in accordance with the ALLIVE guidelines. The BALB/c male mice were sourced from Kiwa Laboratory Animal Research Center (Wakayama, Japan). The mice were used in the experiments following a one-week period of acclimation. The breeding room was maintained under SPF conditions with a 12-hour light-dark cycle. The mice were provided with solid food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan).

4.3. Generation of a Nanog-overexpressing colon cancer cell line

A cell line overexpressing *Nanog*, designated *Nanog*⁺colon26, was generated by introducing the pCAG-*Nanog*-IRES-puroR-EGFP vector into the colon-26 cells. The vector solution (4 µg/250 µL RPMI-1640) and Lipofectamine 2000 (Thermo Fisher Scientific) solution (5 µL/250 µL RPMI-1640) were combined and incubated at room temperature for 20 min. The mixture was added to colon-26 culture medium and incubated at 37°C and 5% CO₂ for 3 h. The medium was then replaced with fresh

RPMI-1640 medium and the cells were cultured for 48 h. The *Nanog*⁺colon26 cells were then selected by culturing them in puromycin-containing RPMI-1640 medium for 2 weeks.

4.4. Quantification of colon metastasis to liver

To quantify the liver metastasis, colon-26 cells or *Nanog*⁺colon26 cells (1.0×10^6 cells/50 μ L PBS) were injected into the spleen of 8~9-week-old mice. Two weeks following the injection of cells, the mice were euthanised by cervical dislocation, and the livers were removed (Figure 1B). The livers were sectioned into lobes and photographed. The tumors in the images were filled in black using the Image J software, and the surface area of the tumors was calculated (Figure 1C).

4.5. Preparation of EVs

EV-depleted FBS was prepared from FBS by centrifugation at $100,000 \times g$ at 4°C for a total of 80 min. The cells were cultured in RPMI-1640 medium for 48 h, after which the medium was removed and the cells were washed with PBS. Following a 72-h incubation period in EV-depleted medium (RPMI-1640, 10% EV-depleted FBS, 1% penicillin-streptomycin), the culture supernatant was subjected to centrifugation at $2,000 \times g$ at 4°C for 20 min. Subsequently, the supernatant was centrifuged at $10,000 \times g$ at 4°C for 40 min, after which the resulting supernatant was centrifuged again at $120,000 \times g$ at 4°C for 80 min. The precipitate was suspended in 10 mL of phosphate-buffered saline (PBS) and filtered through a 0.22 μ m pore-size filter. The filtrate was divided into two fractions in a volume ratio of 1:9. The larger fraction was then centrifuged again at $120,000 \times g$ at 4°C for 80 min, after which the precipitate was suspended in PBS in order to use as an EVs solution. The remaining fraction was combined with 50 μ L of RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.6; Thermo Fisher Scientific) and employed as a protein sample for the BCA (bicinchoninic acid) assay (Pierce® BCA TM Protein Assay kit, Thermo Fisher Scientific). The mode diameter and mean diameter of EVs were approximately 90 nm and 130 nm, respectively. The presence of EVs was confirmed by the detection of CD81, a marker protein for EVs, by Western blot analysis.

4.6. Western analysis of CD81 and Gapdh

A protein sample of cells was prepared as follows: Following the rinsing of 70–80% confluent cells with PBS, a RIPA buffer was added to the culture dish. The culture dish was then placed on ice for a period of 15 min. Subsequently, the cells were detached from the culture dish with a cell scraper and collected in a 1.5 mL microtube. The cell suspension was subjected to sonication on ice and centrifugation at $20,000 \times g$ at 4 °C for 15 min. The supernatant was collected as a protein sample of the cells. A protein solution of EVs was prepared as follows: The pellet of EVs obtained as the precipitate of ultracentrifugation was suspended in a RIPA buffer and allowed to stand on ice for 15 min. The protein concentration was determined by BCA assay. A protein solution was prepared by mixing a 1/6 volume of a 0.375 M Tris-HCl (pH 6.8) buffer solution containing 93 μ g/mL DTT, 0.12 g/mL SDS, 0.6 mL/mL glycerol, and 0.6 mL/mL bromophenol blue. Subsequently, the solution was heated to 95°C for 5 min and applied to SDS-PAGE at 150 V. Blotting onto a PVDF membrane was conducted at 100 V for 3 h at 4°C. Subsequently, the PVDF membrane was immersed in a TBS-T solution (Tris-buffered saline (25 mM Tris, pH 7.4, 150 mM NaCl) containing 1 (v/v) % Tween 20) containing 5 (w/v) % skim milk at 25°C for 30 min. Subsequently, the PVDF membrane was incubated in a 5% skim milk TBS-T solution containing primary antibody at 25°C for 3 h. The primary antibodies used were against mouse Gapdh (1:1000, sc-32233; Santa Cruz Biotechnology, Dallas, TX, USA) and mouse CD81 (1:500, sc-166029; Santa Cruz Biotechnology, Dallas, TX, USA), respectively. Following three washes with TBS-T, the membrane was incubated in TBS-T containing a secondary antibody (anti-mouse immunoglobulin conjugated to alkaline phosphatase, Promega, Madison, WI, USA) at 25°C for 1 h. Membranes were washed three times with TBS-T and then incubated with Western Blue Stabilized Substrate (Promega) for alkaline phosphatase at 25°C for 5 min.

4.7. Analysis of the transfer of EVs to various organs in a mouse

EVs were stained with CellVue™ NIR815 (Invitrogen) and introduced into mice by tail vein injection at a dose of 5 µg per 100 µL of PBS. The mice were euthanized at 3 h and various organs were extracted for imaging analysis with Pearl Trilogy imaging system (LI-COR) at 800 nm. The integrated fluorescence intensity of the entire organ was divided by the organ area (signal/area) in order to estimate the density of EVs accumulated in each organ.

4.8. In vivo test of the effects of EVs on the metastasis

The introduction of EVs into mice was conducted via tail vein injection at a dose of 5 µg/100 µL PBS, administered three times per week for a period of three weeks (Figure 2Ca). Subsequently, *Nanog*⁺colon26 cells (1.0×10⁶ cells/50 µL PBS) were injected into the spleen of the mice. Two weeks later, the mice were euthanized by cervical dislocation, and the livers were removed. The quantification of colon metastasis was conducted according to the method described above (4.4).

4.9. Phagocytic activity test

The phagocytic activity of J774.1 cells was estimated by means of microbeads (MBs) uptake test. EVs were stained with a fluorescent dye, PKH26 (Ex: 551 nm, Em: 567 nm, DOJINDO). FITC-labelled microbeads (FITC-MBs) (Ex: 441 nm, Em: 486 nm, Sigma) were incubated at 37°C for 1 h in RPMI medium containing 10% heat-inactivated FBS for opsonization. J774.1 cells were incubated in RPMI medium with or without FITC-MBs and/or PKH26-EVs for a period of 2 h. Following this incubation period, the J774.1 cells were collected for flowcytometry analysis using the FACS Aria II (BD). Phagocytic activity was also analyzed at single-cell level by fluorescence microscopy. In this case, EVs were used without fluorescent labelling. The cell suspension was then placed in a hemocytometer, and bright-field and fluorescent images were taken with a fluorescent microscope. The bright-field and fluorescent images were merged using ImageJ to determine the percentage of cells that had taken up MBs and the number of MBs taken up by each individual cell. Three wells were prepared for the PBS, colon26EVs, and *Nanog*⁺colon26EVs groups, with 200 to 300 cells counted per well.

4.10. Analysis of miRNAs and their target genes

The differential expression analysis of miRNAs expressed in *Nanog*⁺colon26EVs and colon26EVs was outsourced to Macrogen Japan. The magnitude of variation (fold change, FC) and the statistical significance (p-value) of the respective miRNA components were obtained.

4.11. Statistical Analysis

Each test sample was subjected to two or three analyses, with the average of the two or three results recorded as the value for one test sample. The results are presented as the mean and standard deviation (SD) for the number of samples (n). The results of the metastatic colony analyses are presented in box plots. Outliers depicted in box plots were identified through the application of a Smirnov–Grubbs test, which determined that they exhibited a probability of greater than 0.05. The statistical significance between two specific data groups was analyzed by a two-tailed Student's t-test. The statistical significance of the results is indicated by a p-value or by the use of asterisks, ***: p < 0.001, **: p < 0.01, *: p < 0.05.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: M.S. designed studies, analyzed results, and wrote the manuscript. T.H. conducted the experiments and data analysis. H.M. conducted miRNA data analysis. All authors reviewed and approved the manuscript.

Funding: Please add: This study was supported by special fund donated to TUAT and JSPS KAKENHI Grant Number JP24K09432.

Institutional Review Board Statement: All animal experimental procedures were conducted according to the guidelines of the “Guide for the Care and Use of the Laboratory Animals” of Tokyo University of Agriculture and Technology, Japan, and approved by the Institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology (IACUC No. R04-74, R05-76).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: We thank Dr. Setsuko Hashimoto for stimulating discussion about cancer vaccine technologies.

Conflicts of Interest: The authors have no competing interests to declare that are relevant to the content of this article.

References

1. Kajiyama, H.; Shibata, K.; Terauchi, M.; Yamashita, M.; Ino, K.; Nawa, A.; Kikkawa, Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. *F. Int. J. Oncol.* 2007, 31, 277-283. <https://doi.org/10.3892/ijo.31.2.277>
2. Wattanawongdon, W.; Hahnvajjanawong, C.; Namwat, N.; Kanchanawat, S.; Boonmars, T.; Jearanaikoon, P.; Leelayuwat, C.; Techasen, A.; Seubwai, W. Establishment and characterization of gemcitabine-resistant human cholangiocarcinoma cell lines with multidrug resistance and enhanced invasiveness. *Int. J. Oncol.* 2015, 47, 398-410. <https://doi.org/10.3892/ijo.2015.3019>
3. Cao, W.; Wei, W.; Zhan, Z.; Xie, D.; Xie, Y.; Xiao, Q. Regulation of drug resistance and metastasis of gastric cancer cells via the microRNA647-ANK2 axis. *Int. J. Mol. Med.* 2018, 41, 1958-1966. <https://doi.org/10.3892/ijmm.2018.3381>
4. Speers, C.; Zhao, S.; Liu, M.; Bartelink, H.; Pierce, L.J.; Feng, F.Y. Development and validation of a novel radiosensitivity signature in human breast cancer. *Clin. Cancer Res.* 2015, 21, 3667-3677. <https://doi.org/10.1158/1078-0432.CCR-14-2898>
5. Carlos-Reyes, A.; Muñiz-Lino, M.A.; Romero-Garcia, S.; López-Camarillo, C.; Hernández-de la Cruz, O.N. Biological adaptations of tumor cells to radiation therapy. *Front. Oncol.* 2021, 11, 718636. <https://doi.org/10.3389/fonc.2021.718636>
6. Gurung, S.; Perocheau, D.; T ouramanidou, L.; Baruteau, J. The exosome journey: from biogenesis to uptake and intracellular signaling. *Cell Commun. Signal.* 2021, 19, 47. <https://doi.org/10.1186/s12964-021-00730-1>
7. Mitchell, M.I.; Ma, J.; Carter, C.L.; Loudig, O. Circulating exosome cargoes contain functionally diverse cancer biomarkers: from biogenesis and function to purification and potential translational utility. *Cancers* 2022, 14, 3350. <https://doi.org/10.3390/cancers14143350>
8. Kar, R.; Dhar, R.; Mukherjee, S.; Nag, S.; Gorai, S.; Mukerjee, N.; Mukherjee, R. Vatsa, R.; Jadhav, M.C.; Ghosh, A.; Devi, A.; Krishnan, A.; Thorat, N.D. Exosome-based smart drug delivery tool for cancer theranostics. *ACS Biomater. Sci. Eng.* 2023, 9, 577-594. <https://pubs.acs.org/action/showCitFormats?doi=10.1021/acsbiomaterials.2c01329&ref=pdf>
9. Santos, P.; Almeida, F. Exosome-based vaccines: history, current state, and clinical trials. *Front. Immunol.* 2021, 12, 711565. <https://doi.org/10.3389/fimmu.2021.711565>
10. Fernández-Delgado, I.; Calzada-Fraile, D.; Sánchez-Madrid, S. Immune regulation by dendritic cell extracellular vesicles in cancer immunotherapy and vaccines. *Cancers* 2020, 12, 3558. <https://doi.org/10.3390/cancers12123558>
11. Wang, C.; Huang, X.; Wu, Y.; Wang, J.; Li, F.; Guo, G. Tumor cell-associated exosomes robustly elicit anti-tumor immune responses through modulating dendritic cell vaccines in lung tumor. *Int. J. Biol. Sci.* 2020, 16(4), 633-643. <https://doi.org/10.7150/ijbs.38414>
12. Elashiry, M.; Elsayed, R.; Cutler, C.W. Exogenous and endogenous dendritic cell-derived exosomes: lessons learned for immunotherapy and disease pathogenesis. *Cells* 2022, 11, 115. <https://doi.org/10.3390/cells11010115>

13. van Neerven, S.M.; Tieken, M.; Vermeulen, L.; Bijlsma, M.F. Bidirectional interconversion of stem and nonstem cancer cell populations: A reassessment of theoretical models for tumor heterogeneity. *Mol. Cell. Oncol.* 2016, 3, 2, e1098791 <https://doi.org/10.1080/23723556.2015.1098791>
14. Chen, C.L.; Kumar, D.B.U.; Punj, V.; Xu, J.; Sher, L.; Tahara, S.M.; Hess, S.; Machida, K. NANOG metabolically reprograms tumor-initiating stem-like cells through tumorigenic changes in oxidative phosphorylation and fatty acid metabolism. *Cell Metab.* 2016, 23, 206–219. <https://doi.org/10.1016/j.cmet.2015.12.004>
15. Gawlik-Rzemieniewska, N.; Bednarek, I. The role of NANOG transcriptional factor in the development of malignant phenotype of cancer cells. *Cancer Biol. Ther.* 2016, 17, 1–10. <https://doi.org/10.1080/15384047.2015.1121348>
16. Saito, M. Novel roles of Nanog in cancer cells and their extracellular vesicles. *Cells* 2022, 11, 3881. <https://doi.org/10.1007/s11010-021-04110-8>
17. Saito, M.; Kishi, R.; Sasai, T.; Hatakenaka, T.; Matsuki, N.; Minagawa, S. Effect of Nanog overexpression on the metastatic potential of a mouse melanoma cell line B16-BL6. *Mol. Cell. Biochem.* 2021, 476, 2651–2661.
18. Hatakenaka, T.; Matsuki, N.; Minagawa, S.; May, C.K.S.; Saito, M., Anti-metastatic function of extracellular vesicles derived from Nanog-overexpressing melanoma. *Curr. Oncol.* 2022, 29, 1029–1046. <https://doi.org/10.3390/curroncol29020088>
19. Miyazaki, T.; Chung, S.; Sakai, H.; Ohata, H.; Obata, Y.; Shiokawa, D.; Mizoguchi, Y.; Kubo, T.; Ichikawa, H.; Taniguchi, H.; Aoki, K.; Soga, T.; Nakagama, H.; Okamoto, K. Stemness and immune evasion conferred by the TDO2-AHR pathway are associated with liver metastasis of colon cancer. *Cancer Science* 2022, 113, 170–181. <https://doi.org/10.1111/cas.15182>
20. Descarpentrie, J.; Araúz-Bravo, M.J.; He, Z.; François, A.; González, Á.; Garcia-Gallastegi, P.; Badiola, I.; Evrard, S.; Pernot, S.; Creemers, J.W.M.; Abdel-Majid, K. Role of furin in colon cancer stem cells malignant phenotype and expression of LGR5 and NANOG in KRAS and BRAF-mutated colon tumors. *Cancers* 2022, 14, 1195. <https://doi.org/10.3390/cancers14051195>
21. Tarique, A.A.; Logan, J.; Thomas, E.; Holt, P.; Sly, P.D.; Fantino, E. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages, *Am. J. Respir. Cell Mol. Biol.*, 2015, 53, 676–688. <https://doi.org/10.1165/rcmb.2015-0012OC>
22. Xiang, W.; Shi, R.; Zhang, D.; Kang, X.; Zhang, L.; Yuan, J.; Zhang, X.; Miao, H. Dietary fats suppress the peritoneal seeding of colorectal cancer cells through the TLR4/Cxcl10 axis in adipose tissue macrophages. *Signal Transduct. Targeted Ther.* 2020, 5, 239. <https://doi.org/10.1038/s41392-020-00327-z>
23. Khoo, C.S.M.; Hatakenaka, T.; Matsuki, N.; Minagawa, S.; Asami, K.; Henmi, T.; Morimoto, A.; Saito, M. Moderate hyperglycemia suppresses melanoma metastasis to the liver. *Exp. Anim.* 2023, 72(2), 183–192. <https://doi.org/10.1538/expanim.22-0078>
24. Deng, W.; Zhu, S.; Zeng, L.; Liu, J.; Kang, R.; Yang, M.; Cao, L.; Wang, H.; Billiar, T.R.; Jiang, J.; Xie, M.; Tang, D. The circadian clock controls immune checkpoint pathway in sepsis. *Cell Rep.* 2018, 24(2), 366–378. <https://doi.org/10.1016/j.celrep.2018.06.026>
25. Khoo, C.S.M.; Henmi, T.; Saito, M. Comparative study of metastasis suppression effects of extracellular vesicles derived from anaplastic cell lines, Nanog-overexpressing melanoma, and induced pluripotent stem cells. *Int. J. Mol. Sci.* 2023, 24, 17206.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.