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ORIGINAL ARTICLE

Uptake and tumor-suppressive pathways of exosome-associated GKN1 protein in gastric epithelial cells

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Abstract

Background Gastrokine 1 (GKN1) is a stomach-specific tumor suppressor that is secreted into extracellular space as an exosomal cargo protein. The objective of this study was to investigate the uptake and tumor-suppressive pathways of exosome-associated GKN1 protein in gastric epithelial cells.

Methods Immunofluorescent and Western blot analysis were used to investigate gastric-specific uptake of HFE-145-derived exosomes. Binding affinity of HFE-145 derived exosomes with integrin proteins was examined using protein microarray chip. Tumor suppressor activities of exosome-carrying GKN1 protein were analyzed using transwell co-culture, MTT assay, BrdU incorporation, immunoprecipitation, and Western blot analysis.

Results HFE-145-derived exosomes were internalized only into HFE-145 gastric epithelial cells and gastric cancer cells. Gastric-specific uptake of stomach-derived exosomes required integrin α6 and αX proteins. Clathrin and macropinocytosis increased the uptake of exosomes into gastric epithelial cells, whereas caveolin inhibited the uptake of exosomes. Transwell co-culture of AGS cells with HFE-145 cells markedly inhibited viability and proliferation of AGS cells. Following uptake of HFE-145-derived exosomes in recipient cells, GKN1 protein bound to HRas and inhibited the binding of HRas to b-Raf and c-Raf which subsequently downregulated HRas/Raf/MEK/ERK signaling pathways in AGS, MKN1 cells, and MKN1-derived xenograft tumor tissues. In addition, exosomal GKN1 protein suppressed both migration and invasion of gastric cancer cells by inhibiting epithelial–mesenchymal transition.

Conclusions Gastric-specific uptake of exosomes derived from gastric epithelial cells requires integrin α6 and αX proteins in both gastric epithelial cells and exosomes. Exosomal GKN1 protein inhibits gastric carcinogenesis by downregulating HRas/Raf/MEK/ERK signaling pathways.

Keywords GKN1 · Exosome · Uptake · Ras signaling · Gastric cancer

Introduction

Exosomes are membrane-bound extracellular vesicles of 40–100 nm in diameter. They are secreted from all most cell types into the extracellular environment [1, 2]. In general, exosomes enclose a variety of contents, such as DNA, mRNA, miRNA, proteins, and bioactive lipids [3, 4], suggesting that exosomes play an important role in intercellular communication. Because exosomal cargo is a potential communicative agent, normal cell-derived exosomes are known to block key signaling pathways involved in cancer by tumor-suppressive contents, whereas oncogenic contents of tumor exosomes cause malignant transformation of recipient cells [5, 6]. Notably, proteins on the surface of exosomes have been reported to affect the uptake rate of exosomes into recipient cells [7]. Due to high organotropism of exosomes, exosome-based drug

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delivery systems could resolve insufficient targeting efficiency and translation difficulty. Previously, Hoshino et al. [8] have reported that integrins in exosomes can induce organ-specific colonization in a tissue-specific fashion. Integrins play a role in exosome adhesion to target cells by forming heterobimolecular complexes with tetraspanins such as CD9, CD63, and CD81 [9, 10]. Recent study has demonstrated that integrin α_v and β_4 are increased in exosomes from cancer patients with liver metastasis and lung metastasis, respectively, suggesting that integrin expression pattern is closely correlated with organotropism of exosome uptake [8]. In addition, the biological activity of exosomes that can interact with and be absorbed by specific cells is adjusted by various types of endocytosis such as phagocytosis [11], macropinocytosis [12], clathrin-mediated [13], caveolin-mediated [14], clathrin/caveolin-independent [15], and lipid raft-mediated [16] endocytosis. Although many studies have revealed the role of exosomes in cancer development and progression, the cell type-specific uptake pathways of exosomes are still unclear. Elucidating the mechanism underlying exosome internalization is important for understanding organ-specific targeting process of exosomes.

Human gastrokine 1 (GKN1), a stomach-specific protein, is produced by gastric mucosal epithelium, stored in cytoplasmic granules, and released into the extracellular environment as an exosomal cargo protein [17–19]. Notably, the role of GKN1 consists of maintaining mucosal integrity and regulating cell differentiation [20, 21]. We have previously demonstrated frequent absence of GKN1 expression in gastric cancer cells and tumor-suppressive activities of GKN1 by inhibiting cell proliferation, epithelial–mesenchymal transition (EMT), and cell migration [21–24]. In particular, GKN1-positive exosomes isolated from HFE-145 immortalized gastric epithelial cells can dramatically suppress cell growth and lead to cell cycle arrest and apoptotic cell death of AGS and MKN1 gastric cancer cells in vitro and in vivo studies [18]. Because exosomal secretion of proteins is expected to alter the phenotype and function of recipient cells, it is very important to understand roles of exosomal proteins in normal physiological processes and tumorigenesis.

Here, we focused on how exosomes derived from normal gastric epithelial cells and gastric cancer cells were specifically internalized into gastric cells and molecular pathways underlying tumor suppressor activity of exosomal GKN1 protein.

Materials and methods

Cell culture and transfection of integrins

AGS gastric cancer cells, MKN1 gastric cancer cells, HT29 colon cancer cells, H460 lung cancer cells, and SNU449 hepatocellular carcinoma cells were grown in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C with 5% CO_2. These cells did not express GKN1 protein. HFE-145 immortalized gastric epithelial cells with expression of GKN1 protein were also cultured in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated FBS at 37 °C with 5% CO_2. Complete integrin α6-, αX- and caveolin 1-cDNA were cloned into the expression vector pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA). AGS, HT29, H460 and SNU449 cells were transiently transfected in 60 mm-diameter dishes with expression plasmids (5 µg total DNA), using Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's recommendations. In addition, AGS and MKN1 cells were transfected with *siIntegrin* α6, *siIntegrin* αX, *siClathrin* and *siCaveolin 1*. To clarify the binding domain of GKN1 protein, we produced four deletion-formed plasmids, pGKN1$^{\Delta 68–199}$ containing the NH2-terminal hydrophophic region, pGKN1$^{\Delta 1–67,\ 165–199}$ with the BRICHOS domain, pGKN1$^{\Delta 1–164}$ with the COOH-terminus and pGKN1$^{\Delta 1–67}$ with the BRICHOS and COOH-terminus [25]. We also generated stably GKN1 knockdown HFE-145 cells (HFE-145^{shGKN1}) as well as non-targeting shRNA transfectant, HFE-145^{shCtrl} cells, as described previously [26]. Pitstop 2, genistein, and amiloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Co-culture of HFE-145 and AGS cells in transwell systems

To determine the effect of exosomes derived from HFE-145 gastric epithelial cells on viability and EMT of AGS gastric cancer cells, we developed a transwell cell-culture system as follows. First, we plated 3×10^3 AGS cells onto the bottom of a six-well transwell cell-culture system (Pore size 0.4 µm; Costar Corp., USA) with complete media and culture environment as described above. We seeded 3×10^3 HFE-145 cells onto the membrane of the transwell cell-culture inserts and allowed them to grow overnight under the above-mentioned condition. The next day, we washed these cells with serum-free media and cultured them for 24 h in serum-free medium without growth factors. For co-culture, we moved a membrane transwell insert containing HFE-145 cells into the six-well transwell cell-culture system containing 1×10^4 AGS cells and cultured them for 24 h in serum-free media. We performed cell viability, migration and invasion experiments after 10 days of culture using the complete media.

Human samples

A total of five patients with sporadic gastric cancer who underwent a gastrectomy were included. Gastric cancer

and corresponding non-cancerous gastric mucosae remote (≥ 5 cm) from the tumor were used in this study. In addition, normal liver ($n = 5$), lung ($n = 5$) and colon ($n = 5$) tissues were enrolled. The Institutional Review Board of The Catholic University of Korea, College of Medicine approved this study (MC16SISI0132).

Exosome isolation

We isolated exosomes from supernatants from HFE-145 immortalized gastric epithelial cells as described previously [18]. Briefly, cells at passages 3–8 were incubated in serum-free culture medium and supplemented them with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2 for 48 h before harvesting the medium. To remove cell debris, the medium was centrifuged at 2000*g* for 10 min at 4 °C and then passed through a 0.22 μm filter. The cleared supernatant was transferred to a new glass tube and placed on ice. The supernatant was mixed with A/B/C solution (101Bio company, CA, USA; 2 ml supernatant with 0.75 ml A/B/C solution) in a new tube, vortexed it for 30 s, and incubated it at 4 °C for 30 min. The mixture separated into two layers. After removing the top layer, the bottom layer was transferred to a microcentrifuge tube and spun it at 5000*g* for 3 min. The middle fluff layer was transferred to a new tube and we spun it at 5000*g* for 3 min. The cap was left open to air dry for 10 min at room temperature. We added 4 × volumes of 1 × PBS to the tube, vigorously pipetted it, placed the tube on a horizontal shaker at high speed for 15 min, and then spun it at 5000*g* for 5 min. We carefully transferred the supernatant to a PureExo® Column (101Bio company, CA, USA) and spun it at 1000*g* for 5 min. The flow-through fraction containing the isolated pure exosome suspended in PBS.

Exosome labeling

We labeled exosomes with PKH26 (Sigma) as described previously [18]. Briefly, we resuspended exosome pellets in 1 mL of Diluent C. 1 mL of Diluent C was mixed with 4 μL of PKH26 and we then mixed the exosome suspension with the stain solution followed by incubation for 20 min at 37 °C. We stopped the labeling reaction by adding an equal volume of 1% bovine serum albumin. We isolated the labeled exosomes using a Total Exosome Isolation kit (Invitrogen) according to the manufacturer's protocol. Briefly, we added 0.5 volumes of the isolation reagent to labeled exosomes and vortexed the mixture to mix well. We incubated these labeled exosomes at 4 °C overnight and subsequently centrifuged them at 10,000*g* for 1 h at 4 °C. We discarded the supernatant and resuspended each pellet in 100 μL of PBS.

Active Ras detection assay

We seeded gastric cancer cells (AGS and MKN1) into six-well plates at density of 3×10^5 per well in RPMI-1640 medium. After 24 h, cells were treated with exosomes carrying GKN1 for 48 h. We harvested cells under non-denaturing conditions and rinsed them with cold PBS. Cells were lysed in lysis buffer. An active Ras detection assay kit (Cell Signaling Technology) was used to perform affinity precipitation of active Ras, according to the manufacturer's protocol. We treated cell lysates (500 μg) with GTPγS for activation of Ras, acting as a positive or negative control, respectively. Subsequently, GST–Raf1–RBD fusion protein and cell lysate in glutathione resin were incubated. Electrophoresed and immunoblotting were performed with Ras mouse monoclonal antibody.

Statistical analysis

Student's *t* test was used to analyze effects of GKN1 on cell viability and proliferation. We carried out all experiments in duplicate to verify the reproducibility of findings. The results are expressed as means ± S.D. from two independent experiments. When *P* value was less than 0.05, the results were considered statistically significant.

Other methods are described in detail in the Supplementary materials and methods section.

Results

Cellular uptake of exosomes derived from gastric epithelial cells depends on the origin of the cells

To investigate whether exosomes derived from gastric epithelial cells were internalized into gastric and non-gastric cells, we isolated exosomes from cultured supernatants from HFE-145 immortalized gastric epithelial cells, AGS and MKN1 gastric cancer cells, H460 non-small-cell lung cancer cells, SNU449 hepatocellular carcinoma cells, and HT29 colorectal adenocarcinoma cells. Intact exosomes with the presence of vesicles ranging in size from 30 to 100 nm were seen in TEM analysis (Fig. 1a) and the expression of exosomal markers such as CD9, 63, and 81 was observed in exosomes (Fig. 1b). Interestingly, the expression of GKN1 was detected only in exosomes derived from HFE-145 immortalized gastric epithelial cells (Fig. 1b). When we treated HFE-145 immortalized gastric epithelial cells, AGS and MKN1 gastric cancer cells, H460 non-small-cell lung cancer cells, SNU449 hepatocellular carcinoma cells, and HT29 colorectal adenocarcinoma cells with exosomes derived from HFE-145 cells, exosomes were seen only in cytoplasms of HFE-145, AGS, and MKN1 cells (Fig. 1c). In

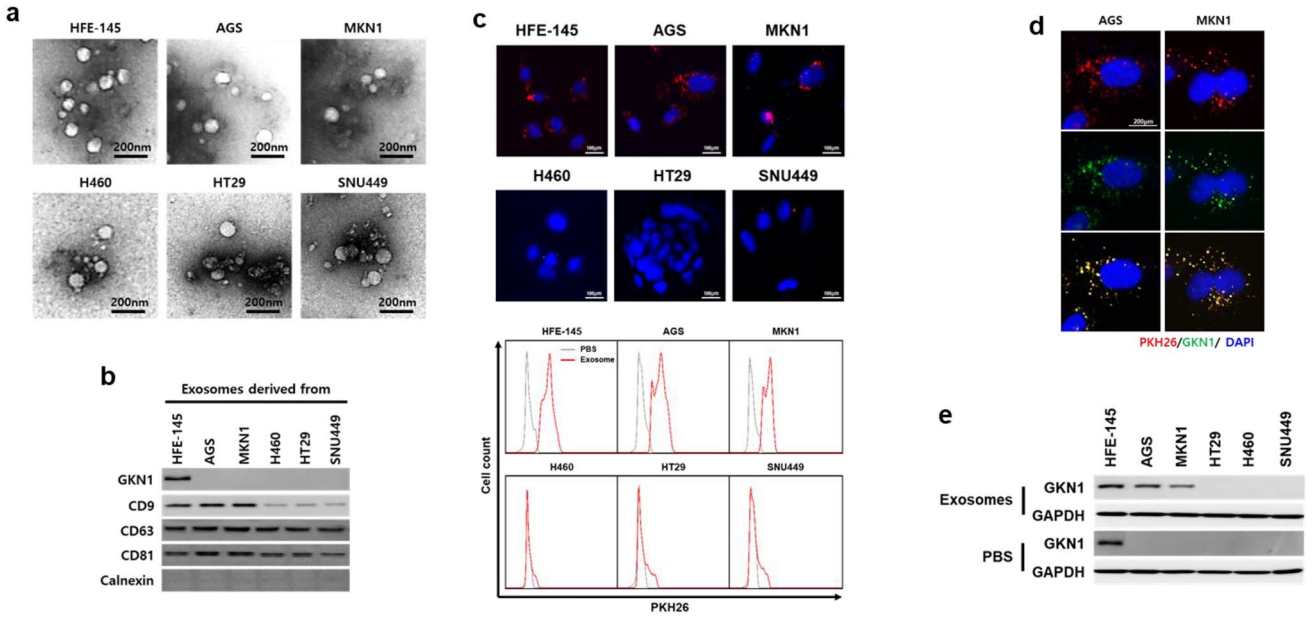

Fig. 1 Cellular uptake of exosomes derived from gastric epithelial cells depends on the origin of the cells. **a** TEM image of exosomes isolated from supernatants of HFE-145, AGS, MKN1, H460, HT29, and SNU449 cells. **b** In Western blot analysis, the expression of exosomal markers such as CD9, 63, and 81 was observed in exosomes derived from HFE-145, AGS, MKN1, H460, HT29, and SNU449 cells. However, the expression of GKN1 was only detected in HFE-145 cells. **c** In immunofluorescent and FACS analysis of HFE-145, AGS, MKN1, HT29, H460, and SNU449 cells treated with exosomes derived from HFE-145 cells, exosomes were only detected in cytoplasms of HFE-145, AGS, and MKN1 cells. **d** Co-localization of PKH26 labeled exosomes with GKN1 protein in cytoplasms of AGS and MKN1 gastric cancer cells. **e** In HFE-145, AGS, MKN1, HT29, H460, and SNU449 cells treated with exosomes derived from HFE-145 cells, expression of exosomal GKN1 proteins was observed in HFE-145, AGS, and MKN1, but not in HT29, H460, and SNU449 cells

immunofluorescence study, co-localization of GKN1 protein with PKH26 labeled exosomes derived from HFE-145 cells was found in cytoplasms of AGS and MKN1 cells treated with PKH26 labeled exosomes (Fig. 1d). Western blot analysis showed that GKN1 proteins were expressed only in HFE-145, AGS, and MKN1 cells treated with exosomes derived from HFE-145 cells (Fig. 1e), suggesting that HFE-145-derived exosomes were only internalized into HFE-145, AGS, and MKN1 cells (Fig. 1e).

Specific uptake of exosomes derived from gastric epithelial cells into the cytoplasm of gastric cells depends on integrin α6 and αX

To elucidate whether gastric-specific uptake of exosomes derived from gastric epithelial cells was associated with integrin proteins, we analyzed the binding affinity of exosomes derived from HFE-145 cells with integrin proteins including integrin β1, α6 and αX in a protein microarray chip. As shown in Fig. 2a, exosomes had high binding affinity with integrin α5, α11, αX, βL1, and β1 (Table 1). In addition, we examined integrin protein compositions in HFE-145 immortalized gastric epithelial cells, AGS and MKN1 gastric cancer cells, HT29 colorectal adenocarcinoma cells, H460 non-small-cell lung cancer cells, SNU449 hepatocellular carcinoma cells, and exosomes derived from these cells. In HFE-145, AGS, and MKN1 cells, expression levels of integrin α5, α6, α10, αε, and αX were higher than those in cancer cells of other tissue origins, although there was no significant difference in integrin protein composition among HFE-145, AGS, and MKN1 cells (Fig. 2b). In particular, integrin α6 and αX were expressed only in HFE-145, AGS, and MKN1 cells (Fig. 2b). To further confirm these results, we examined the expression levels of integrin α6 and αX in normal stomach, liver, lung and colon tissues. Consistently, the expression of integrin α6 and αX proteins was observed only in normal stomach tissues, but not in normal liver, lung and colon tissues (Fig. 2c). There was no significant difference in expression level of integrin α6 and αX proteins between non-cancerous gastric mucosae and gastric cancer tissues (Fig. 2d). Thus, these results indicate that integrin α1, α4, αε, β1 and β6 are required for membrane binding of exosomes derived from gastric epithelial cells. In addition, gastric-specific uptake of exosomes derived from gastric epithelial cells suggests that they are associated with different expression levels of integrin proteins such as integrin α6 and αX in both gastric cells and exosomes.

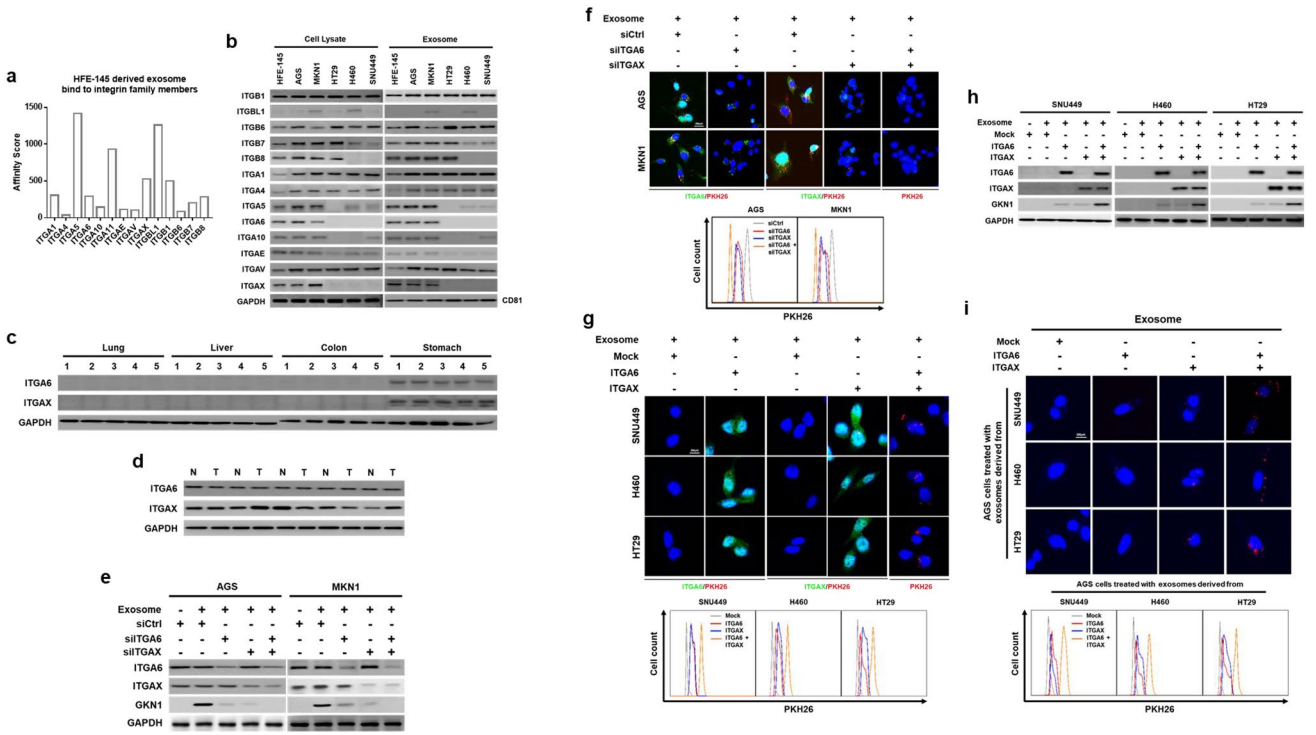

Fig. 2 Specific cellular uptake of exosomes derived from gastric epithelial cells through integrin α6 and αX. **a** Binding affinity of exosomes derived from HFE-145 cells to integrin proteins including integrin α1, β1, α6, and αX in a protein microarray chip. **b** Western blot analysis showing expression levels of integrin proteins, including integrin β1, α6, and αX, in cell lysates and exosomes from HFE-145, AGS, MKN1, HT29, H460, and SNU449 cells. **c** The expression of integrin α6 and αX proteins in normal liver, lung, colon and stomach tissues. **d** There was no significant difference in the expression of integrin α6 and αX proteins between corresponding non-cancerous gastric mucosae (N) and gastric cancer (T) tissues. **e** Knockdown of integrin α6 and αX in AGS and MKN1 cells treated with exosomes derived from HFE-145 cells reduced expression levels of integrin α6 and αX, and GKN1 proteins. **f** In immunofluorescent and FACS analysis, knockdown of integrin α6 and αX inhibited the internalization of exosomes into AGS and MKN1 cells' cytoplasms. **g** In immunofluorescent and FACS analysis, ectopic expression of integrin α6 and αX in SNU449, H460, and HT29 cells induced the internalization of exosomes derived from HFE-145 cells into these cells' cytoplasms. **h** Ectopic expression of integrin α6 and αX in SNU449, H460, and HT29 cells induced expression levels of integrin α6 and αX. In addition, GKN1 protein was detected in these cells treated with exosomes derived from HFE-145 cells. **i** In immunofluorescent and FACS analysis, exosomes derived from SNU449, H460 and HT29 cells expressing integrin α6 and αX were detected in the cytoplasm of AGS cells

Next, we examined the effect of integrin α6 and αX on uptake of exosomes derived from HFE-145 gastric epithelial cells in AGS and MKN1 cells. Knockdown of integrin α6 and αX with *siIntegrin α6* and *siIntegrin αX* in AGS and MKN1 cells markedly reduced expression levels of integrin α6 and αX, respectively (Fig. 2e). When AGS and MKN1 cells were treated with PKH26-labeled exosomes, these exosomes were clearly localized in the cytoplasm of these cells. However, knockdown of integrin α6 and αX markedly inhibited the internalization of PKH26-labeled exosomes into AGS and MKN1 cells' cytoplasms (Fig. 2f). In addition, level of GKN1 expression was dramatically reduced in AGS and MKN1 cells (Fig. 2e). We further confirmed the role of integrin α6 and αX in uptake of exosomes derived from gastric epithelial cells in non-gastric cells. In the transient transfection assay performed in HT29 colorectal adenocarcinoma cells, H460 non-small-cell lung cancer cells, and SNU449 hepatocellular carcinoma cells, ectopic expression of integrin α6 or αX slightly induced internalization of exosomes derived from HFE-145 gastric epithelial cells (Fig. 2g). However, expression of both integrin α6 and αX markedly induced internalization of exosomes derived from HFE-145 gastric epithelial cells (Fig. 2g). In Western blot analysis, ectopic expression of both integrin α6 and αX dramatically increased expression level of GKN1 in SNU449, H460, and HT29 cells (Fig. 2h). Additionally, ectopic expression of integrin α6 and αX in SNU449, H460, and HT29 cells markedly increased internalization of exosomes derived from these cells into the AGS cells' cytoplasm (Fig. 2i). Thus, these results suggest that integrin α6 and αX are required for organotropism of exosomes derived from gastric epithelial cells.

Table 1 Binding affinity of exosomes derived from HFE-145 cells with integrin proteins

Name	ID	F532 Median	F532 Mean	F532 SD	F532 CV	B532	B532 Median	B532 Mean	B532 SD	B532 CV	F532 Median—B532	F532 Mean—B532	F532 Total Intensity	A-score	SNR 532
ITGB1BP1	JHU00041.P001D10	2708	2752	586	21	2696	2696	2916	2683	92	12	56	220,148	5642	− 0.061
ITGB1BP1	JHU00041.P001D10	6684	13,920	15,122	108	2692	2692	2817	689	24	3992	11,228	167,034		16.115
ITGB3BP	JHU01593.P017B05	2634	2653	323	12	2646	2646	2679	410	15	− 12	7	212,222	1012	− 0.063
ITGB3BP	JHU01593.P017B05	3442	4560	4073	89	2543	2543	2599	314	12	899	2017	54,714		6.245
ITGB1	JHU08590.P090D05	3804	8104	11,010	135	2855	2855	2917	326	11	949	5249	97,250	2901	15.911
ITGB1	JHU08590.P090D05	2842	3346	3235	96	2793	2793	2856	634	22	49	553	267,699		0.773
ITGB7	JHU08686.P191B07	3475	3498	432	12	3314	3314	3369	604	17	161	184	279,833	219.5	0.214
ITGB7	JHU08686.P191B07	3641	3652	481	13	3397	3397	3422	485	14	244	255	43,829		0.474
ITGB1BP2	JHU10218.P107H11	2798	2796	372	13	2702	2702	2720	380	13	96	94	223,651	712	0.2
ITGB1BP2	JHU10218.P107H11	3352	4140	3268	78	2810	2810	2828	413	14	542	1330	49,676		3.177
ITGAV	JHU10517.P110H03	2760	2741	385	14	2717	2717	2758	428	15	43	24	219,289	81	-0.04
ITGAV	JHU10517.P110H03	2822	2797	315	11	2659	2659	2697	422	15	163	138	223,787		0.237
ITGB6	JHU11267.P118H03	2781	2801	423	15	2700	2700	2800	1186	42	81	101	224,061	113	0.001
ITGB6	JHU11267.P118H03	2778	2810	411	14	2685	2685	2715	358	13	93	125	224,817		0.265
ITGA1	JHU11749.P238B05	4388	4311	461	10	3925	3925	4236	2741	64	463	386	344,866	260	0.027
ITGA1	JHU11749.P238B05	4014	4003	530	13	3869	3869	3978	928	23	145	134	320,226		0.027
ITGA6	JHU11750.P123F09	3377	6494	9290	143	2941	2941	2952	375	12	436	3553	77,923	1825.5	9.445
ITGA6	JHU11750.P123F09	3034	2984	372	12	2886	2886	3134	2193	69	148	98	238,729		− 0.068
ITGA5	JHU13153.P138D04	2966	3036	796	26	2855	2855	2886	396	13	111	181	242,861	4264	0.379
ITGA5	JHU13153.P138D04	5630	11,255	12,153	107	2908	2908	2932	465	15	2722	8347	135,055		17.899

Table 1 (continued)

Name	ID	F532 Median	F532 Mean	F532 SD	F532 CV	B532	B532 Median	B532 Mean	B532 SD	B532 CV	F532 Median—B532	F532 Mean—B532	F532 Total Intensity	A-score	SNR 532
ITGB8	JHU13154.P138F04	3198	4093	3399	83	2750	2750	2800	350	12	448	1343	130,981	773.5	3.694
ITGB8	JHU13154.P138F04	2931	3020	673	22	2816	2816	2836	389	13	115	204	241,613		0.473
ITGB1	JHU13635.P143F05	2815	2856	393	13	2791	2791	2821	415	14	24	65	228,471	99	0.084
ITGB1	JHU13635.P143F05	3079	3115	505	16	2982	2982	3383	3479	102	97	133	249,200		− 0.077
ITGAE	JHU17061.P201A08	3459	3546	493	13	3449	3449	3461	450	13	10	97	283,646	147	0.189
ITGAE	JHU17061.P201A08	3747	3732	408	10	3535	3535	3549	503	14	212	197	298,589		0.364
ITGA4	JHU17446.P217F04	3980	3967	652	16	3974	3974	4015	1072	26	6	− 7	317,358	77	− 0.045
ITGA4	JHU17446.P217F04	4227	4317	550	12	4156	4156	4193	540	12	71	161	345,340		0.23
ITGA2	JHU17540.P218H08	3964	3957	371	9	4028	4028	4043	593	14	− 64	− 71	316,544	− 7	− 0.145
ITGA2	JHU17540.P218H08	4143	4172	565	13	4115	4115	4102	654	15	28	57	333,759		0.107
ITGA10	JHU17815.P221C11	4091	4068	444	10	3686	3686	3690	520	14	405	382	325,403	157	0.727
ITGA10	JHU17815.P221C11	3328	3376	387	11	3444	3444	3493	514	14	− 116	− 68	270,097		− 0.228
ITGA11	JHU17991.P223G05	4528	4614	617	13	4516	4516	4572	670	14	12	98	369,124	2682	0.063
ITGA11	JHU17991.P223G05	6266	9680	10,605	109	4414	4414	4420	531	12	1852	5266	116,158		9.906
ITGAD	JHU18095.P224B04	3764	3757	427	11	3923	3923	4047	1659	40	− 159	− 166	300,556	-62.5	− 0.175
ITGAD	JHU18095.P224B04	3949	4020	506	12	3979	3979	4006	459	11	− 30	41	321,593		0.031
ITGAX	JHU18282.P210B02	4346	4278	601	14	3478	3478	3513	431	12	868	800	51,331	541	1.775
ITGAX	JHU18282.P210B02	3769	3871	457	11	3589	3589	3619	497	13	180	282	309,646		0.507
ITGBL1	JHU18920.P226E10	6169	7154	2526	35	4137	4137	4170	541	12	2032	3017	85,847	1733.5	5.516

Table 1 (continued)

Name	ID	F532 Median	F532 Mean	F532 SD	F532 CV	B532	B532 Median	B532 Mean	B532 SD	B532 CV	F532 Median—B532	F532 Mean—B532	F532 Total Intensity	A-score	SNR 532
ITGBL1	JHU18920.P226E10	4643	4607	669	14	4157	4157	4208	835	19	486	450	368,565		0.478
ITGA4B7	JHU29142	4117	4204	447	10	4063	4063	4291	2893	67	54	141	134,541	− 0.5	− 0.03
ITGA4B7	JHU29142	4124	4140	373	9	4282	4282	4309	508	11	− 158	− 142	331,236		− 0.333

Effects of caveolin, clathrin and macropinocytosis on internalization of exosomes derived from gastric epithelial cells

We further examined whether vesicle trafficking-associated proteins including caveolin 1 and syntaxin 6 were associated with the internalization of exosomes derived from gastric epithelial cells. Vesicle trafficking-associated proteins in HFE-145, AGS, and MKN1 gastric epithelial origin cells showed expression levels similar to those in HT29 colorectal adenocarcinoma cells, H460 non-small-cell lung cancer cells, SNU449 hepatocellular carcinoma cells (Fig. 3a). However, caveolin 1 protein was not expressed in HFE-145 (Fig. 3a). AGS cells showed weak caveolin 1 expression, whereas MKN1 and non-gastric cancer cells had strong expression of caveolin 1 protein (Fig. 3a). Next, we examined the expression levels of clathrin and caveolin 1 in non-cancerous gastric mucosae and gastric cancer tissues. The expression of clathrin was slightly higher in gastric cancer tissues than non-cancerous gastric mucosae, whereas there was no significant difference in the expression of caveolin 1 proteins between non-cancerous gastric mucosae and gastric cancer tissues (Fig. 3b).

To further evaluate whether caveolin 1, clathrin, and macropinocytosis contributed to the uptake of exosomes in gastric epithelial cells, we treated AGS and MKN1 gastric cancer cells with genistein (an inhibitor of caveolin-dependent endocytosis) (27), Pitstop 2 (an inhibitor of clathrin-dependent endocytosis) [28], and 5-(*n*-ethyl-*n*-isopropyl)-amiloride (EIPA), a macropinocytosis inhibitor. Notably, inhibition of clathrin and macropinocytosis markedly reduced the uptake of exosomes into AGS and MKN1 cells (Fig. 3c, d), implying that the uptake of exosomes in gastric epithelial cells might require clathrin and macropinocytosis. When we treated MKN1 cells with strong caveolin 1 expression and AGS cells with weak caveolin 1 expression with exosomes carrying GKN1 protein, exosome uptake was higher in AGS cells than that in MKN1 cells. In contrast, caveolin 1 inhibition in MKN1 cells increased the uptake of exosomes into MKN1 gastric cancer cells (Fig. 3c, d). When we further confirmed the effects of clathrin and caveolin 1 expression on the uptake of exosomes in gastric epithelial cells, knockdown of clathrin inhibited the internalization of exosomes into AGS and MKN1 cells (Fig. 3e, f). Interestingly, ectopic expression of caveolin 1 in AGS cells inhibited the internalization of exosomes, whereas knockdown of caveolin 1 in MKN1 cells increased the internalization of exosomes (Fig. 3e, f). These results indicating that caveolin 1 could inhibit the uptake of exosomes into gastric epithelial cells.

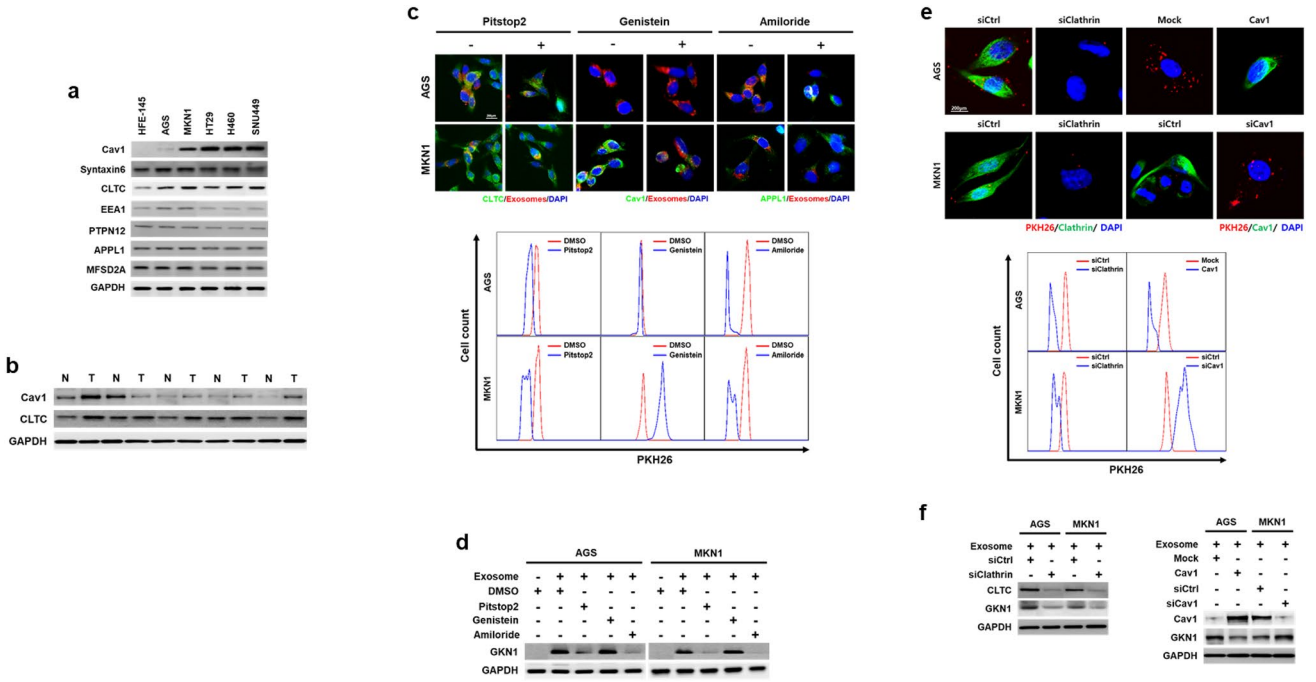

Fig. 3 Effects of caveolin, clathrin, and macropinocytosis on internalization of exosomes. **a** Expression levels of vesicle trafficking-associated proteins in HFE-145, AGS, MKN1, HT29, H460, and SNU449 cells. Cav1; caveolin 1, CLTC; clathrin, EEA1; early endosome antigen 1, PTPN12; protein tyrosine phosphatase non-receptor type 12, APPL1; adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1, MFSD2A; major facilitator superfamily domain containing 2A. **b** The expression of clathrin and caveolin 1 proteins in corresponding non-cancerous gastric mucosae (N) and gastric cancer (T) tissues. **c** Cellular uptake of exosomes following pre-treatment with 5 μM pitstop 2, 100 μM genistein, or 50 μM amiloride before addition of exosomes in AGS and MKN1 cells. **d** In AGS and MKN1 cells, pre-treatment with 5 μM pitstop 2 and 50 μM amiloride before treatment with exosome derived from HFE-145 cells reduced expression levels of GKN1, whereas treatment with 100 μM genistein increased expression levels of GKN1. **e** In immunofluorescent and FACS analysis, knockdown of Clathrin inhibited the internalization of exosomes into AGS and MKN1 cells. Ectopic expression of caveolin 1 in AGS cells inhibited the internalization of exosomes, whereas knockdown of caveolin 1 in MKN1 cells induced the internalization of exosomes. **f** Knockdown of clathrin in AGS and MKN1 cells treated with exosomes derived from HFE-145 cells reduced expression of GKN1 protein (left). Ectopic expression of caveolin 1 in AGS cells reduced expression level of GKN1 protein and knockdown of caveolin 1 in MKN1 cells increase expression level of GKN1 protein (right)

GKN1 inhibits cell growth by downregulating Ras/Raf/MEK/ERK pathway signaling in gastric cells

Transwell co-culture of AGS gastric cancer cells with HFE-145 gastric epithelial cells markedly inhibited viability and proliferation of AGS cancer cells (Fig. 4a), suggesting that exosomal components of HFE-145 gastric epithelial cells could inhibit cancer cells' survival and growth. Previously, we have reported that GKN1-positive exosomes derived from HFE-145 cells can significantly inhibit viability and proliferation of AGS and MKN1 gastric cancer cells [18]. To identify the molecular mechanism underlying tumor suppressor activity of GKN1, we here examined effects of GKN1 protein on the Ras/Raf/MEK/ERK kinase cascade. Expectedly, AGS cells co-cultured with HFE-145 cells showed markedly reduced expression levels of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk (Fig. 4b). When AGS and MKN1 cells were treated with HFE-145-derived exosomes, GKN1 decreased expression levels of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk in AGS and MKN1 cells (Fig. 4c). In addition, knockdown of GKN1 with *shGKN1* in HFE-145 cells increased expression of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk (Fig. 4d), suggesting that GKN1 could downregulate the Ras/Raf/MEK/ERK kinase signaling pathway.

Next, we examined the binding of HRas and GKN1 proteins to determine how GKN1 regulates the Ras/Raf/MEK/ERK kinase signaling pathway. We observed the binding of GKN1 protein to HRas in HFE-145 cells by immunoprecipitation assay (Fig. 4e). In addition, GKN1 protein inhibited the binding of HRas to b-Raf and c-Raf in AGS cells co-cultured with HFE-145 cells (Fig. 4f). Expectedly, Ras protein bound to both GTPγS and GST–Raf1–RBD in AGS cells, but not in AGS cells co-cultured with HFE-145 cells (Fig. 4g). When we treated AGS and MKN1 cells with HFE-145-derived exosomes, GKN1 protein bound directly to HRas and completely inhibited the binding of HRas to b-Raf and c-Raf in AGS and MKN1 cells (Fig. 4h). In

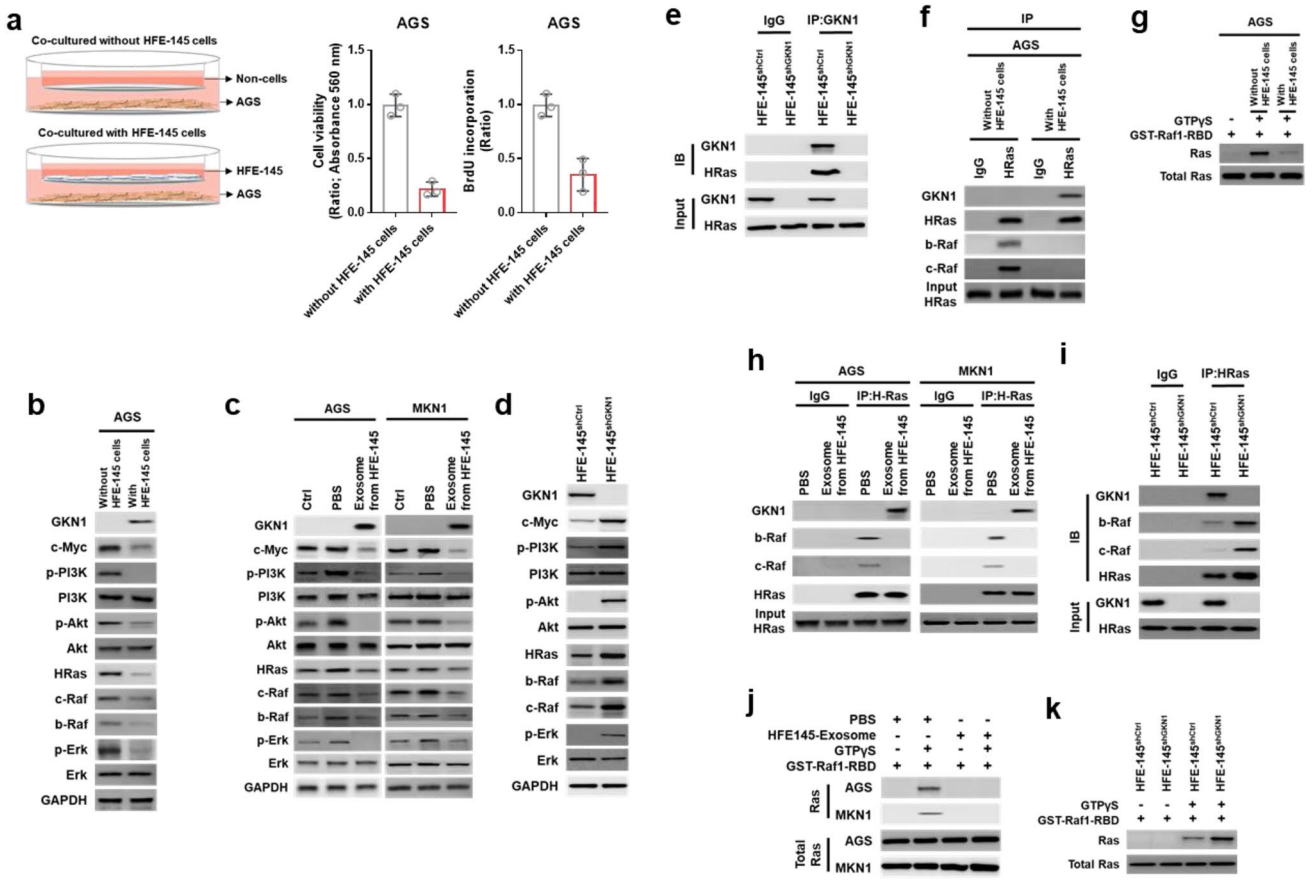

Fig. 4 GKN1 inhibits Ras/Raf/MEK/ERK signaling pathway. **a** Transwell co-culture system of AGS gastric cancer cells with HFE-145 immortalized normal gastric epithelial cells were used in this study. AGS and HFE-145 cells were separately cultured for 24 h and then co-cultured in serum-free medium for a further 96 h as described in Materials and methods (left panel). Transwell co-culture of AGS cells with HFE-145 cells inhibited cell viability and proliferation of AGS cells (right panel). **b** Expression levels of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk in AGS cells co-cultured with HFE-145 cells. **c** Treatment with exosomes derived from HFE-145 cells decreased expression levels of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk in AGS and MKN1 cells. **d** Knockdown of GKN1 in HFE-145 cells increased expression levels of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk. **e** Immunoprecipitation assay showed that GKN1 bound to HRas in HFE-145 cells. **f** In AGS cells co-cultured with HFE-145 cells, GKN1 bound to HRas and inhibited the binding of HRas to b-Raf and c-Raf. **g** Detection of active Ras (Ras-GTP) by pulldown assay in co-cultured AGS cells without or with HFE-145 cells. Ras activation was detected in co-cultured AGS cells without HFE-145 cells, but not detected in co-cultured AGS cells with HFE-145 cells. **h** Exosomes derived from HFE-145 cells inhibited the binding of HRas to b-Raf and c-Raf in AGS and MKN1 cells. **i** Binding of HRas to b-Raf and c-Raf was observed in GKN1 knockdown HFE-145 cells. **j** In AGS and MKN1 cells treated with PBS, Ras protein bound to GTPγS and GST–Raf1–RBD, but exosomes derived from HFE-145 cells inhibited the binding of Ras protein to GTPγS and GST–Raf1–RBD. **k** Binding of Ras protein to GTPγS and GST–Raf1–RBD was found in GKN1 knockdown HFE-145 cells

addition, binding of HRas to b-Raf and c-Raf was found in GKN1 knockdown HFE-145 cells (Fig. 4i). Consistently, Ras protein bound to both GTPγS and GST–Raf1–RBD in PBS-treated AGS cells and GKN1 knockdown HFE-145 cells, whereas Ras protein did not bound to both GTPγS and GST–Raf1–RBD in HFE-145 cells, and AGS and MKN1 cells treated with exosomes derived from HFE-145 cells (Fig. 4j, k). These findings demonstrate that normal exosomes containing GKN1 protein can inhibit the Ras activity.

When we examined the binding activity of GKN1 domains to HRas (Fig. 5a), amino terminal hydrophobic region ($GKN1^{\Delta 68-199}$) of GKN1 weakly bound to HRas, whereas the BRICHOS domain ($GKN1^{\Delta 1-67,165-199}$, $GKN1^{\Delta 1-67}$) strongly bound to HRas (Fig. 5b). In addition, Ras protein bound to both GTPγS and GST–Raf1–RBD in AGS cells transfected with amino terminal hydrophobic region ($GKN1^{\Delta 68-199}$) or carboxyl terminal region ($GKN1^{\Delta 1-164}$) of GKN1. However, Ras protein did not bind to both GTPγS and GST–Raf1–RBD in AGS cells transfected with the BRICHOS domain ($GKN1^{\Delta 1-67,165-199}$, $GKN1^{\Delta 1-67}$) of GKN1 (Fig. 5c). These findings demonstrate that the BRICHOS domain of GKN1 may be the

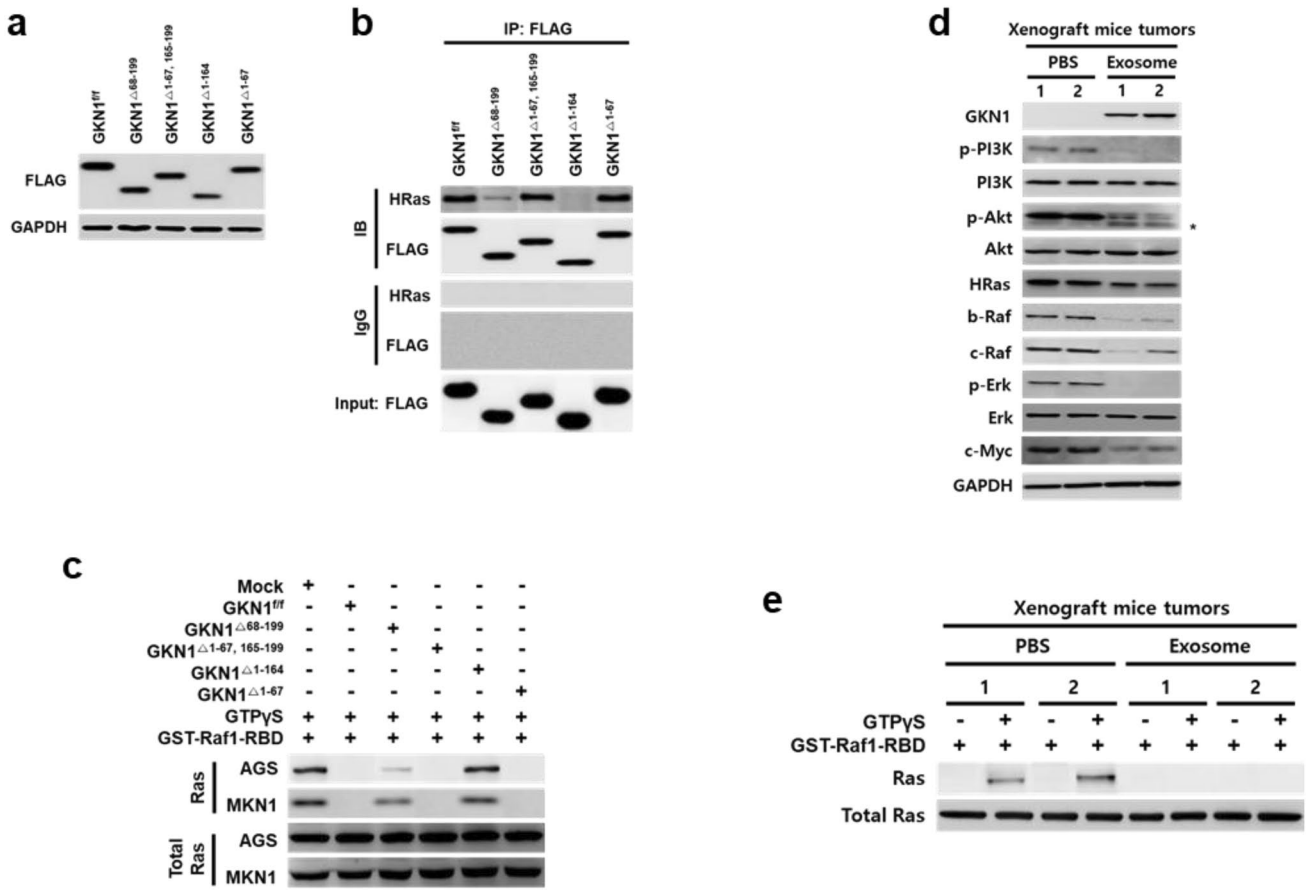

Fig. 5 Binding domain of GKN1 protein to Ras and inhibition of Ras/Raf/MEK/ERK signaling pathway by exosomes derived from HFE-145 cells. **a** Expression of $GKN1^{f/f}$ (full length), $GKN1^{\Delta 68–199}$, $GKN1^{\Delta 1–67,165–199}$, $GKN1^{\Delta 1–164}$, and $GKN1^{\Delta 1–67}$ and GAPDH were determined by Western blot analysis. **b** Amino terminal hydrophobic region ($GKN1^{\Delta 68–199}$) of GKN1 weakly bound to HRas, whereas the BRICHOS domain ($GKN1^{\Delta 1–67,165–199}$, $GKN1^{\Delta 1–67}$) strongly bound to HRas. **c** In AGS and MKN1 cells transfected with amino terminal hydrophobic region ($GKN1^{\Delta 68–199}$) and carboxy terminal region ($GKN1^{\Delta 1–164}$) of GKN1, Ras protein bound to GTPγS and GST–Raf1–RBD, but the BRICHOS domain ($GKN1^{\Delta 1–67,165–199}$, $GKN1^{\Delta 1–67}$) of GKN1 inhibited the binding of Ras protein to GTPγS and GST–Raf1–RBD. **d** Treatment with exosomes derived from HFE-145 cells decreased expression levels of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk in MKN1-derived xenograft tumor tissues. **e** MKN1-derived xenograft tumor tissues treated with PBS, Ras protein bound to GTPγS and GST–Raf1–RBD, but treatment with exosomes derived from HFE-145 cells inhibited the binding of Ras protein to GTPγS and GST–Raf1–RBD

physiological inhibitor of GTP binding to Ras protein in gastric epithelial cells. In addition, GKN1 decreased expression levels of c-Myc, HRas, c-Raf, b-Raf, and p-Erk in MKN1-derived xenograft tumors treated with exosomes derived from HFE-145 cells (Fig. 5d). Also, Ras protein bound to GTPγS and GST–Raf1–RBD in MKN1-derived xenograft tumors treated with PBS. However, exosomes derived from HFE-145 cells inhibited Ras activity (Fig. 5e), suggesting that GKN1 may induce Ras inactivation. Taken together, these results indicate that GKN1 protein in exosomes derived from gastric epithelial cells can inhibit cell growth by downregulating Ras/Raf/MEK/ERK kinase signaling pathway.

Exosomes derived from gastric epithelial cells inhibit epithelial–mesenchymal transition of gastric cancer cells

After co-culture of AGS gastric cancer cells with HFE-145 immortalized gastric epithelial cells, we analyzed effects of GKN1-positive exosomes on EMT of AGS cells. In transwell microchemotaxis and Matrigel assays, AGS cells co-cultured with HFE-145 cells showed decreased cell migration and invasion activity (Fig. 6a, b). In Western blot analysis, AGS cells co-cultured with HFE-145 cells showed increased E-cadherin expression but reduced expression of EMT-related proteins, including N-cadherin, ZEB1, Snail, Slug and Rho-GTP (Fig. 6c). Next, we treated AGS and

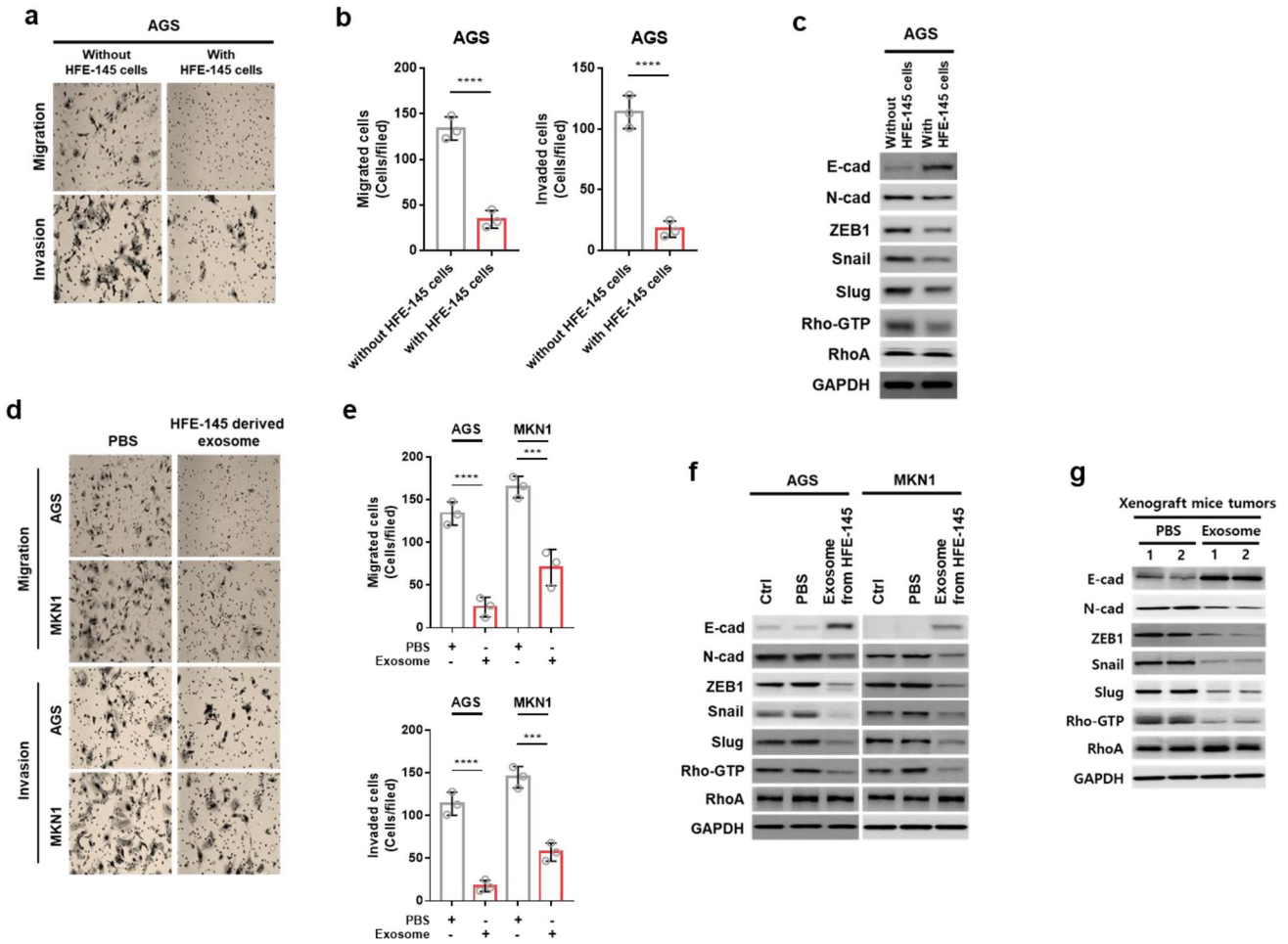

Fig. 6 Exosomes derived from gastric epithelial cells inhibit epithelial–mesenchymal transition. **a**, **b** In transwell microchemotaxis and Matrigel assays, AGS cells co-cultured with HFE-145 cells showed decreased cell migration and invasion activity, compared to AGS cells cultured without HFE-145 cells. **c** Expression levels of E-cadherin, N-cadherin, ZEB1, Snail, Slug, and Rho-GTP in AGS cells co-cultured without or with HFE-145 cells. **d**, **e** Migration and invasion activity of AGS and MKN1 cells were reduced in HFE-145-derived exosome-treated cells, compared to that of PBS-treated cells. **f**, **g** Expression levels of E-cadherin, N-cadherin, ZEB1, Snail, Slug, and Rho-GTP in AGS and MKN1 cells (**f**), and MKN1-derived xenograft tumor tissues (**g**) treated with PBS or exosomes derived from HFE-145 cells

MKN1 cells with exosomes derived from HFE-145 cells to investigate whether normal exosomes inhibited the progression of gastric cancer cells. As shown in Fig. 6d, e, migration and invasion of gastric cancer cells were dramatically reduced in AGS and MKN1 cells treated exosomes derived from HFE-145 cells, compared to those of control cells. Similar to co-culture of AGS with HFE-145 cells, treatment with exosomes derived from HFE-145 cells also showed increased E-cadherin expression but reduced expression of N-cadherin, ZEB1, Snail, Slug, and Rho-GTP in AGS, MKN1 cells, and xenograft tumors tissues (Fig. 6f, g). These findings suggest that GKN1 protein in exosomes derived from gastric epithelial cells can inhibit gastric cancer cell migration and invasion by regulating EMT-related protein expression.

Discussion

Here, we found that the uptake of exosomes derived from HFE-145 immortalized gastric epithelial cells occurred only in HFE-145, AGS, and MKN1 cells, but not in colon, liver, or lung cancer cells. In addition, expression levels of integrin α5, αX, α6, and αε were higher in gastric cells and exosomes derived from HFE-145, AGS, and MKN1 cells than those in exosomes derived from colon, liver, and lung cancer cells. In particular, integrin α6 and αX were expressed only in HFE-145, AGS, and MKN1 cells, and stomach tissues. Knockdown of integrin α6 and αX with *siIntegrin α6* and *siIntegrin αX* in AGS and MKN1 cells dramatically inhibited the internalization of PKH26-positive exosomes into AGS and MKN1 cells' cytoplasms, respectively. Furthermore,

ectopic expression of both integrin α6 and αX in HT29 colon cancer cells, H460 lung cancer cells, and SNU449 hepatocellular carcinoma cells markedly induced internalization of exosomes derived from HFE-145 gastric epithelial cells. Also, exosomes derived from HT29 cells, H460 cells, and SNU449 cells ectopically expressing integrin α6 and αX were internalized into AGS cells. These results suggest that exosome uptake depends on the expression pattern of integrin proteins of recipient cells and exosomes, and that gastric-specific uptake of exosomes derived from gastric epithelial cells requires higher expression of α6 and αX integrin proteins in both gastric epithelial cells and exosomes.

Next, we further investigated whether macropinocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis modulated the internalization of exosomes derived from gastric epithelial cells. Notably, no significant difference was found in the expression of vesicle trafficking-associated proteins, including syntaxin 6 or clathrin heavy chain 1 (CLTC), between HFE-145 gastric epithelial cells and cancer cells originating from stomach, lung, liver, and colon. Of these, caveolin 1 protein was not expressed in HFE-145 while AGS cells showed weak cavolin 1 expression. However, MKN1 and other cancer cells demonstrated strong expression of caveolin 1 protein. In human stomach tissues, expression of clathrin was slightly higher in gastric cancers than corresponding non-cancerous gastric mucosae, whereas there was no significant difference in the expression of caveolin 1 proteins between non-cancerous gastric mucosae and gastric cancer tissues. Interestingly, inhibition of clathrin and macropinocytosis markedly reduced the uptake of exosomes into AGS and MKN1 cells. Caveolin inhibition with genistein and *siCaveolin 1* increased the uptake of exosomes carrying GKN1 protein into MKN1 gastric cancer cells, whereas ectopic expression of caveolin 1 in AGS gastric cancer cells reduced the uptake of exosomes. These results are consistent with previous data showing that efficient exosome uptake is mediated by clathrin-dependent endocytosis [29] and that caveolin 1 negatively regulates endocytosis of exosomes [16]. Thus, we can conclude that the uptake of exosomes in gastric epithelial cells may require clathrin and macropinocytosis and that caveolin 1 can inhibit the uptake of exosome into gastric epithelial cells.

Cellular interactions with exosomes induce cell proliferation, apoptosis, invasion, and metastasis. Previously, we have reported that exosome-associated GKN1 protein can significantly inhibit viability and proliferation of AGS and MKN1 gastric cancer cells and reduce tumor volume and tumor weight of nude mice-bearing MKN1 xenograft tumors [18]. Here, co-localization of GKN1 protein with exosomes derived from HFE-145 cells was found in cytoplasms of AGS and MKN1 cells treated with PKH26 labeled exosomes. To further study the effect of exosomes-associated GKN1 protein on maintenance of homeostasis of gastric epithelium, we next sought to define the molecular pathway associated with cellular proliferation using a transwell co-culture system. Previously, we have reported that GKN1 can downregulate *H. pylori* CagA-induced overexpression of Ras and Raf family proteins in gastric cells and human gastric mucosae [30]. It is well known that the Ras/Raf/MEK/ERK kinase cascade is an essential effector cascade required for Ras GTPase signaling [31]. To trigger the Ras/Raf/MEK/ERK signaling pathway, growth factor needs to bind to its cognate receptor which in turn activates Ras-GTP by binding of GRB2 and SOS to the growth factor receptor [32]. Raf, a serine/threonine protein kinase and Ras effector, can promote protein phosphorylation via MEK/ERK activation [33]. Its upstream kinases MEK1/2 can activate ERK1/2 kinases known to mediate cell proliferation and apoptosis [34]. ERK1/2 can induce phosphorylation of various transcription factors such as Ets-1, c-Jun, c-Myc, and NF-kB [35]. p-ERK is a key downstream target of the Ras/Raf/MEK/ERK signaling pathway [33]. This cascade is involved in cell biology including cell proliferation, apoptosis, differentiation, and migration. Abnormal activation of this pathway is commonly detected in human cancers. It also influences chemotherapeutic drug resistance [32]. In the present study, we uncovered a new role of exosomes-associated GKN1 protein for inactivation of the Ras/Raf/MEK/ERK signaling pathway. In the transwell system, co-culture of AGS gastric cancer cells with HFE-145 immortalized gastric epithelial cells markedly inhibited viability and proliferation of AGS cancer cells and reduced expression levels of c-Myc, p-PI3K, p-Akt, HRas, b-Raf, c-Raf, and p-Erk. In addition, GKN1, especially the BRICHOS domain, bound to HRas, inhibited binding of HRas to b-Raf and c-Raf, and finally decreased the expression of p-ERK in AGS cells, MKN1 cells, and xenograft tumors treated with exosomes derived from HFE-145 cells, indicating that GKN1 protein could suppress cell proliferation of gastric epithelial cells by inhibiting the Ras/Raf/MEK/ERK signaling pathway. The BRICHOS superfamily consists of four distinct regions: hydrophobic, linker, BRICHOS, and C-terminal [36]. GKN proteins contain the BRICHOS domain, a COOH-terminal segment and hydrophobic NH_2-terminal signal peptide [37]. In the previous study, we found that BRICHOS domain is the main domain for the GKN1 tumor suppressor function [25]. Taken together, these findings strongly suggest that secreted exosomal GKN1 protein from gastric epithelial cells might be internalized into gastric cells, thus altering a set of signaling pathways involved in the regulation of cell growth.

Interestingly, GKN1 protein inhibited gastric cancer cell migration and invasion by downregulating c-Myc, RhoA, Snail, and Slug expression and inactivating NF-kB pathway [38]. Consistently, co-culture of AGS gastric cancer cells with HFE-145 immortalized gastric epithelial cells showed

dramatically decreased migration and invasion of AGS cells. In addition, increased E-cadherin expression and reduced expression of EMT-related proteins including N-cadherin, ZEB1, Snail, Slug, and Rho-GTP were detected in both AGS gastric cancer cells co-cultured with HFE-145 cells and xenograft tumors treated with exosomes derived from HFE-145 cells. These data indicate that exosome-associated GKN1 protein can suppress migration and invasion of gastric cancer cells by inhibiting EMT.

In summary, gastric-specific uptake of stomach-derived exosomes requires integrin α6 and αX proteins in gastric cells and exosomes derived from gastric cells. Clathrin and macropinocytosis can increase the uptake of exosomes into gastric epithelial cells, but caveolin can inhibit the uptake of exosomes. In the transwell co-culture, exosomes derived from HFE-145 cells markedly inhibited viability and proliferation of AGS and MKN1 cancer cells. GKN1 protein bound to HRas and downregulated PI3K/Akt and HRas/Raf/MEK/ERK signaling pathways. In addition, exosome-associated GKN1 protein suppressed migration and invasion of gastric cancer cells by inhibiting EMT. Thus, we can conclude that gastric-specific uptake of exosomes derived from gastric epithelial cells requires integrin α6 and αX proteins in both gastric epithelial cells and exosomes and that exosome-associated GKN1 protein can inhibit gastric carcinogenesis by downregulating the HRas/Raf/MEK/ERK signaling pathway.

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Author contributions JHY: concept and design; provision of study materials; data collection, analysis, and interpretation; article writing; and final approval of the article. WSP: concept and design; provision of study materials; article writing; and final approval of the article. HA and DTS: provided the HFE-145 cells before publication; and final approval of the article. SWN: manuscript drafting for important intellectual content; and final approval of the article. HH: performed in vivo experiments; and final approval of the article.

Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest. Dr. Park reports grants from The Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2018R1A2A2A14019713 to W.S.P).

Ethical standards

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent to be included in the study, or the equivalent, was obtained from all patients. For studies with animals, all institutional and national guidelines for the care and use of laboratory animals were followed.

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