

MICROBIOLOGY

Fusobacterium nucleatum extracellular vesicles are enriched in colorectal cancer and facilitate bacterial adhesion

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Fusobacterium nucleatum in colorectal cancer (CRC) tissue is implicated at multiple stages of the disease, while the mechanisms underlying bacterial translocation and colonization remain incompletely understood. Herein, we investigated whether extracellular vesicles derived from *F. nucleatum* (FnEVs) have impacts on bacterial colonization. In mice with colitis-related CRC, a notable enrichment of FnEVs was observed, leading to a significant increase in intratumor colonization by *F. nucleatum* and accelerated progression of CRC. The enrichment of FnEVs in clinical CRC tissues was demonstrated. Subsequently, we revealed that FnEVs undergo membrane fusion with CRC cells, leading to the transfer and retention of FomA on recipient cell surfaces. Given its ability to facilitate *F. nucleatum* autoaggregation through interaction with FN1441, the presence of FomA on CRC cell surfaces presents a target for bacterial adhesion. Collectively, the findings unveil a mechanism used by EVs to prepare a niche conducive for bacterial colonization in distal organs.

INTRODUCTION

The tumor microbiome plays a crucial role in the pathogenesis and progression of various gastrointestinal malignancies, including gastric, colorectal, liver, and pancreatic cancer (1, 2). Emerging evidence suggests that *Fusobacterium nucleatum* enrichment specifically in colorectal cancer (CRC) is implicated in multiple stages of CRC progression (3–6). The oral cavity has been identified as the primary source of *F. nucleatum* in CRC, as evidenced by numerous studies (7–10). Therefore, regardless of the underlying mechanisms used by *F. nucleatum* to facilitate CRC initiation and progression, the colonization of *F. nucleatum* in CRC consistently remain a prerequisite. Our previous study reveals that the metabolic reprogramming of CRC cells represented by ANGPTL4-mediated glycolysis is essential for the intratumor colonization of *F. nucleatum* (11). However, the precise mechanism by which *F. nucleatum* in the oral cavity is translocated to CRC tissue and its preferential adhesion to tumor tissue has not been fully elucidated. Several studies have revealed that *F. nucleatum* outer membrane exposed adhesins Fap2 and FadA might be involved in the bacterial adhesion to CRC tissue (12–14). Yet, an in-depth exploration of additional virulence factors and

the specific mechanisms governing translocation and adhesion of *F. nucleatum* would greatly contribute to the establishment of effective preventive and control measures.

The shedding of microbial extracellular vesicles (EVs) represents a ubiquitous mechanism for inter- and intra-kingdom communication, which is conserved across prokaryotic and eukaryotic microorganisms (15, 16). The recent detection of bacterial EVs (BEVs) in the systemic circulation and distal organs underscores the intricate interplay between bacteria and the human body, potentially implicated in various diseases through mechanisms such as immunomodulation (17, 18). Several studies have found that following oral administration of BEVs derived from diverse origins, including EVs from *F. nucleatum* (FnEVs), a substantial proportion of these vesicles were retained within the gastrointestinal tract (19–21), indicating their potential role as mediators in oral-gut communication. A recent study has revealed that intravenous administration of BEVs from *Escherichia coli* Δ *msbB* strain (endotoxin-free) exhibits selective tropism for tumor tissue (22). This phenomenon can be attributed to the passive accumulation of nanoparticles in tumors due to the enhanced permeability and retention (EPR) effect (22, 23). Further investigation is warranted to determine whether FnEVs have similar tropism toward CRC tissue. On the other hand, in most studies, the presence of FnEVs in various organs or tissues has been shown to elicit proinflammatory responses from the host immune system, thereby contributing to the development of diseases such as periodontitis, colitis, and rheumatoid arthritis (19, 21, 24–26). It remains poorly understood whether FnEVs can mediate host responses other than unilateral immune system defense. Do FnEVs prepare niches favoring bacterial colonization in distant host tissues, such as CRC? Within these niches, do FnEVs affect the adhesion mechanisms of *F. nucleatum*?

The present study demonstrated the enrichment of FnEVs in CRC in both animal models and clinical samples. In animal CRC models and coculture assays, we found that FnEVs facilitated the

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adhesion of *F. nucleatum* to CRC cells. Mechanistically, this phenomenon relied on the membrane fusion between FnEVs and recipient CRC cells, during which the outer membrane protein FomA in FnEVs was transferred to the surface of CRC cells. Given our discovery that FomA mediates autoaggregation of *F. nucleatum* likely through its interaction with another surface protein FN1441, the retention of FomA on the surface of CRC cells serves as an adhesive target for *F. nucleatum*. Together, we revealed that the enrichment of FnEVs in CRC promotes the adhesion of *F. nucleatum*, thereby potentially contributing to the translocation of *F. nucleatum* from the oral cavity to CRC tissue.

RESULTS

FnEVs are enriched in CRC

The FnEVs were isolated and characterized (Fig. 1, A and B), and their tissue distribution in the azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced CRC-bearing mice was explored (Fig. 1C). The DiIR (1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanineiodide)-labeled FnEVs were applied with oral administration and found to distribute in the small intestine after 2 hours in both control and AOM/DSS-treated mice (Fig. 1D and fig. S1A). However, the enrichment of FnEVs in the colon was observed exclusively in CRC-bearing mice (Fig. 1D and fig. S1A). After 8 hours, while no fluorescence signal of FnEVs was detected in any examined organs from control mice, it could still be observed in the colon of CRC-bearing mice (Fig. 1E and fig. S1B).

The protein FomA (42.4 kDa, 25586_FusoPortal_Genome_655), identified as the most abundant protein in FnEVs (Fig. 1F and data S1) and known as an outer membrane porin, (27) was chosen as a representative marker for the FnEV. A specific antibody against FomA was generated (fig. S1, C and D). By performing immunofluorescence staining of FomA, we observed a significant enrichment of FnEVs in mice CRC samples compared to their paired noncancerous colon counterparts (Fig. 1G). We further isolated and purified EVs from human CRC and paired adjacent normal tissues obtained immediately after surgical removal (fig. S1, E to G). As an indicator for the presence of FnEVs, we successfully detected FomA by Western blotting, exclusively in the EVs isolated from CRC tissue for four patients (Fig. 1H). Moreover, in patient #4, a higher abundance of FomA protein was observed in EVs derived from CRC compared to that from adjacent normal tissue (Fig. 1H), suggesting the enrichment of FnEVs in clinical CRC tissue.

FnEVs promote the intratumor adhesion of *F. nucleatum* and aggravate CRC

To investigate the impact of FnEVs on CRC progression and *F. nucleatum* colonization, mice were orally administrated with FnEVs and/or *F. nucleatum* subsequent to AOM/DSS treatment completion (Fig. 2A). In accordance with previous studies (28–30), oral administration of *F. nucleatum* exacerbated colitis-related CRC apparently, while the introduction of FnEVs further increased tumor number and size (Fig. 2B). Treatment with FnEVs alone exhibited a modest yet significant promotion of CRC progression (Fig. 2B). By using fluorescence in situ hybridization (FISH), we observed a significant increase in the intratumor colonization of *F. nucleatum* within the “FnEV + *F. nucleatum*” group compared to the “*F. nucleatum*” group (Fig. 2C), which potentially elucidated the

additive procarcinogenic effect resulting from combined FnEV and *F. nucleatum* treatment. Consistently, in MC38 xenograft model (Fig. 2D), intratumor injection of FnEVs resulted in accelerated tumor growth (Fig. 2E) and enhanced colonization by *F. nucleatum* (Fig. 2F). However, treatment with FnEVs alone did not demonstrate a significant impact on the growth of MC38 xenograft (Fig. 2E). Notably, the specific probe targeting *F. nucleatum* 16S ribosomal RNA used in FISH was unable to label FnEVs in either CRC tissues or cultured cells (fig. S2A), thereby validating the robustness of this bacterial quantification method in samples treated concurrently with both *F. nucleatum* and FnEVs.

To explore whether the enhancement of *F. nucleatum* colonization was related with bacterial adhesion, the cell-attached *F. nucleatum* was quantified after 1 hour of coculture with CRC cells. The FnEV pretreatment remarkably increased the initial adhesion of *F. nucleatum* to CRC cells (Fig. 2G and fig. S2, B and C). As anticipated, the colonization of *F. nucleatum* after overnight coculture with CRC cells was found to be significantly enhanced by pretreatment with FnEVs (fig. S2, D and E). These results implicate that FnEVs facilitate the adherence of *F. nucleatum* to CRC cells, thus promoting bacterial colonization and accelerating CRC progression.

To investigate the comparable ability of purer FnEV in promoting the adherence of *F. nucleatum* to CRC cells, we further purified FnEVs using a density gradient ultracentrifugation method (fig. S3, A to D) and confirmed its equivalent effect on enhancing *F. nucleatum* adhesion compared to crude FnEVs (fig. S3, E to G).

In both animal and in vitro experiments, we observed that treatment with FnEVs led to an increase in the number of *F. nucleatum* colonizing in CRC tissues or cell cocultures. One possible explanation is that FnEVs may directly or indirectly promote the growth of *F. nucleatum*. However, our study found no direct effect of FnEV treatment on the growth of *F. nucleatum* (fig. S4A), nor did the culture supernatant of CRC cells after FnEV treatment affect its growth (fig. S4B). Another possibility is that FnEVs may inhibit bacterial clearance capacity of CRC cells, thereby promoting higher levels of colonization by *F. nucleatum* in cell cocultures. Transcriptome analysis revealed that most up-regulated genes (fig. S5A) in DLD-1 cells treated with FnEVs were associated with responses to lipopolysaccharide (LPS), granulocyte chemotaxis, etc. (fig. S5B). The most highly up-regulated gene was *SAA1* (fig. S5A), which encodes a member of the serum amyloid A (SAA) family of apolipoproteins and has been reported to be associated with CRC (31, 32). The effect of FnEVs on SAA was also demonstrated in AOM/DSS-induced CRC tissues (fig. S5C), which may partially explain the promotion of colitis-related CRC by FnEVs observed in our experiment (Fig. 2, A and B). However, FnEVs did not have a significant impact on autophagy (fig. S5D) and only up-regulate one of the genes (*LCN2*, encoding lipocalin-2) related to the expression of antimicrobial peptides (fig. S5E), which have been reported to be involved in bacterial clearance capacity of epithelial cell (33–36).

The membrane fusion and surface proteins of FnEVs are indispensable for promoting bacterial adhesion

Membrane fusion is the most frequently reported mode of BEVs entry into non-phagocytic host cells (17). A self-quenching lipophilic dye octadecyl rhodamine B (R18) (37) was introduced into FnEVs. Upon membrane fusion with unlabeled recipient CRC cells, the dye was diluted and resulted in concomitant fluorescence (Fig. 3,

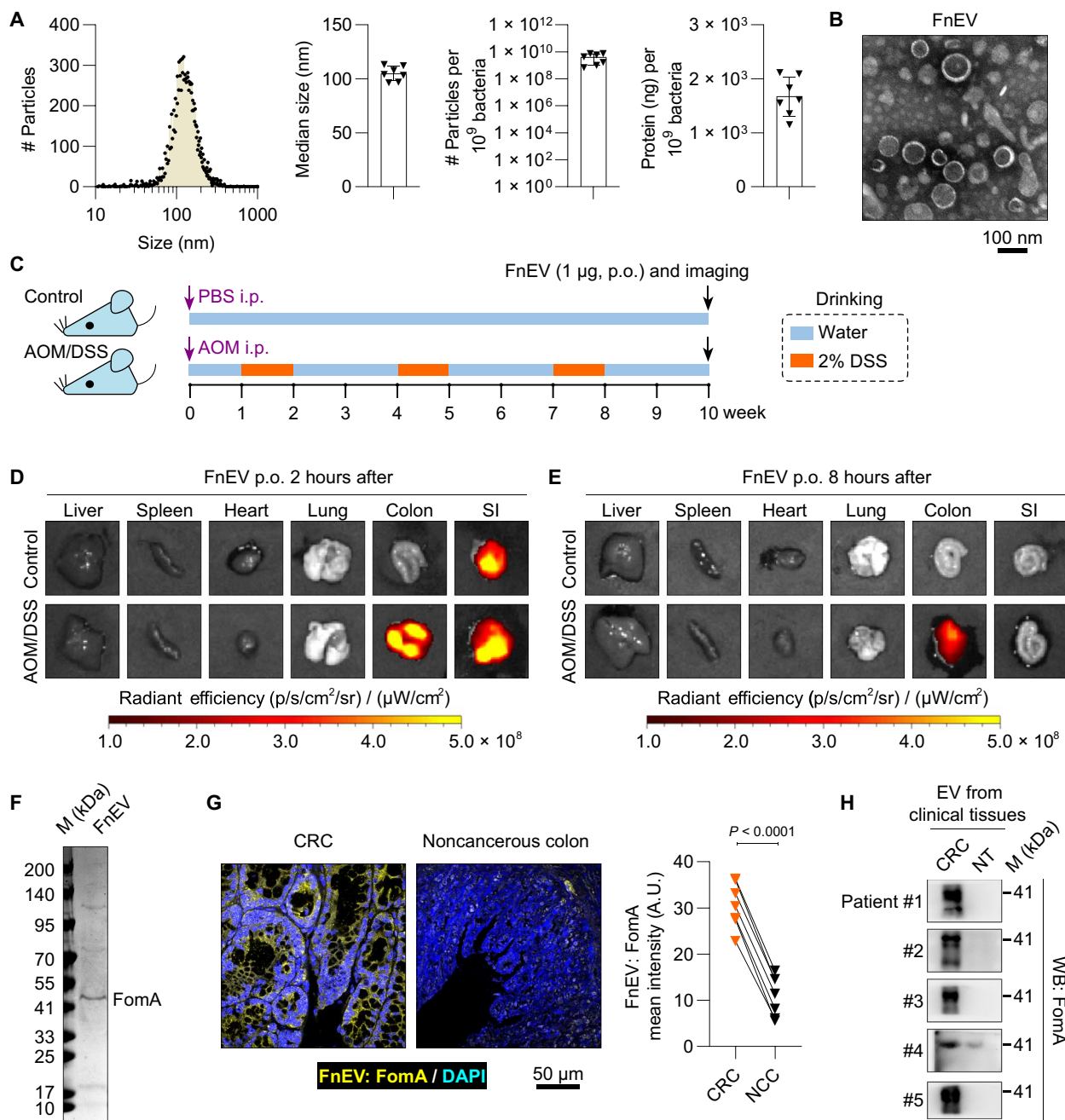


Fig. 1. The presence of FnEVs is significantly augmented in CRC tissue. (A and B) Characterization and representative transmission electron microscope (TEM) images of FnEVs. $n = 7$ biological replicates from three independent experiments. (C) Experimental design of investigating the tissue distribution of FnEVs in AOM/DSS-induced CRC model. The experiment was independently performed twice. i.p., intraperitoneal injection. p.o., oral administration. (D and E) Representative images of tissue distribution of DiR-labeled FnEVs, taken 2 hours [(D) $n = 4$ mice in control group and 6 in AOM/DSS group] and 8 hours [(E) $n = 4$ mice in control group and 5 in AOM/DSS group] after oral administration of FnEVs. (F) FnEV proteins were electrophoresed on an SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel and stained with Coomassie brilliant blue. FomA in an ~40-kDa band was identified using MS. M, the marker. (G) Representative images and quantification of FnEVs (2 hours after oral administration) uptake by CRC and paired noncancerous colon tissue in mice. $n = 7$ mice in each group from two independent experiments. A.U., absorbance unit. NCC, noncancerous colon. (H) Western blotting of FomA in EVs purified from clinical CRC tissue and paired adjacent normal tissue (NT). M, the marker. $n = 5$ patients. The experiment was independently performed twice. The data are presented as means $\pm$ SD in (A). Each triangle represents an individual sample in (A) and (G). The P value was determined by Mann-Whitney test in (G). DAPI, 6-diamidino-2-phenylindole.

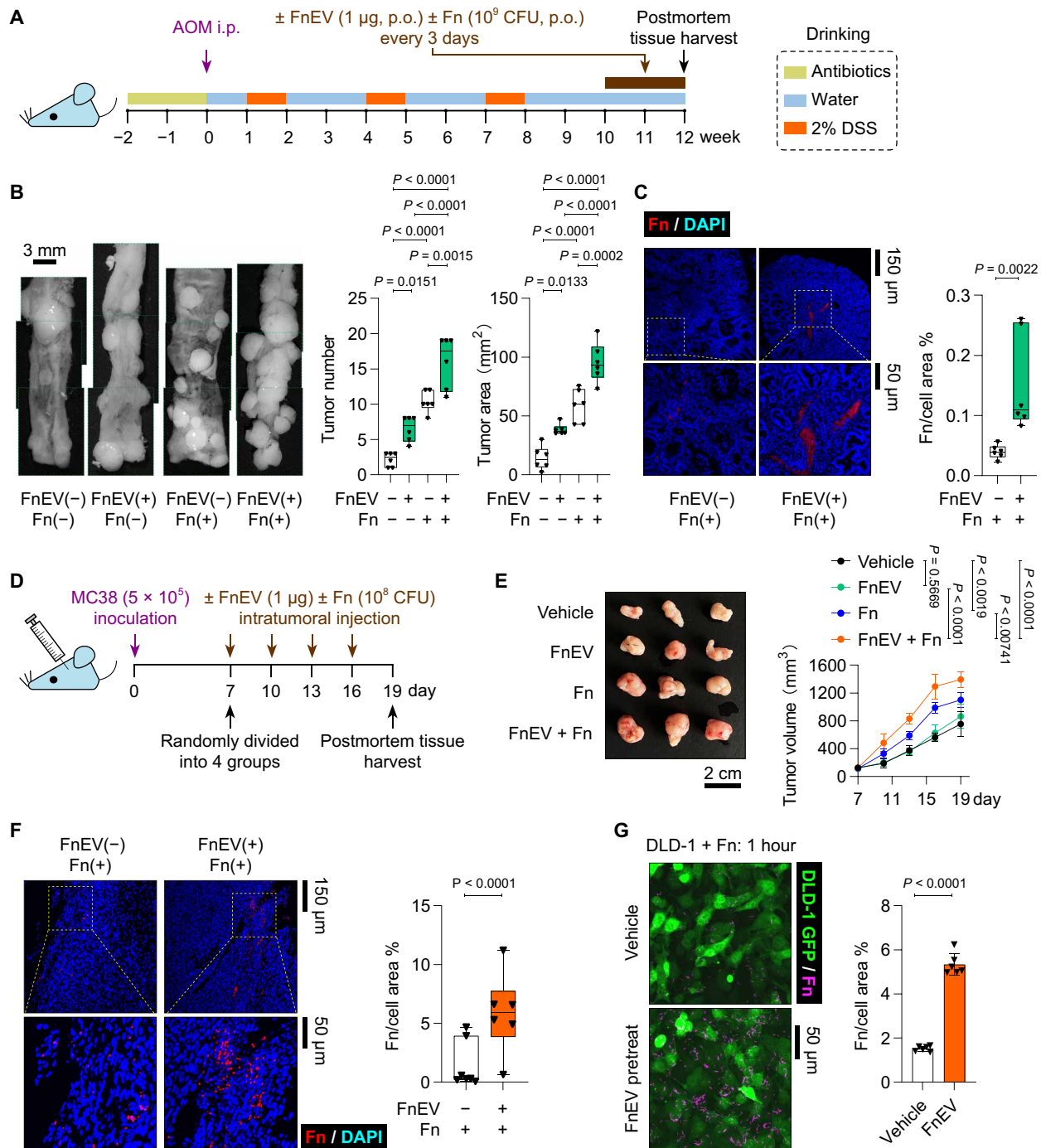


Fig. 2. FNEVs facilitate *F. nucleatum* adhesion and exacerbate CRC progression. (A) Experimental design of investigating the effects of FNEVs and *F. nucleatum* (Fn) on CRC progression in AOM/DSS model. The experiment was independently performed twice. i.p., intraperitoneal injection. p.o., oral administration. CFU, colony forming unit. (B) Representative stitched stereomicroscope images and quantitative evaluation of AOM/DSS-induced CRC in four groups. $n = 6$ mice in each group. (C) Representative images and quantification of *F. nucleatum* stained with FISH in AOM/DSS-induced CRC. $n = 6$ mice in each group. (D) Experimental design of evaluating the effects of FNEVs and *F. nucleatum* on CRC progression in xenograft model. The experiment was independently performed twice. (E) Representative images of xenografts and the monitoring of tumor volume of "Vehicle" ($n = 6$), "FnEV" ($n = 7$), "Fn" ($n = 7$), and "FnEV + Fn" ($n = 6$) groups. (F and G) Representative images and quantification of *F. nucleatum* stained with FISH in xenografts (F) or in coculture with DLD-1 [pretreated with PBS or FNEVs (5 μg/ml)] (G). (F) $n = 6$ mice in "FnEV (-) Fn (+)" group and seven mice in "FnEV (+) Fn (+)" group. (G) $n = 6$ biological samples in each group from three independent experiments. The data are presented as median with interquartile range in (B), (C), and (F) and as means $\pm$ SD in (E) and (G). Each triangle represents an individual sample in (B), (C), (F), and (G). The P values were determined by one-way analysis of variance (ANOVA) test [(B) and (E)], Mann-Whitney test [(C) and (F)], or Welch's t test (G).

A and B, and fig. S6A). Pretreating CRC cells with filipin, which binds to cholesterol and forms ultrastructural aggregates (37), effectively inhibited the fusion with FnEVs (Fig. 3, A and B, and fig. S6A), and abolished the promoting effect of FnEVs on *F. nucleatum* adhesion (Fig. 3C and fig. S6, B and C). BEVs can also enter host cells via dynamin-dependent endocytosis (17, 18). However, inhibition

of dynamin with dynasore (38) did not exert any impact on *F. nucleatum* adhesion in coculture with DLD-1 after FnEV pretreatment (fig. S6D).

The FnEVs, derived from a Gram-negative bacterium, contain outer membrane LPS, which is known to engage Toll-like receptor 4 (TLR4) (17). However, despite TAK-242 treatment inhibiting

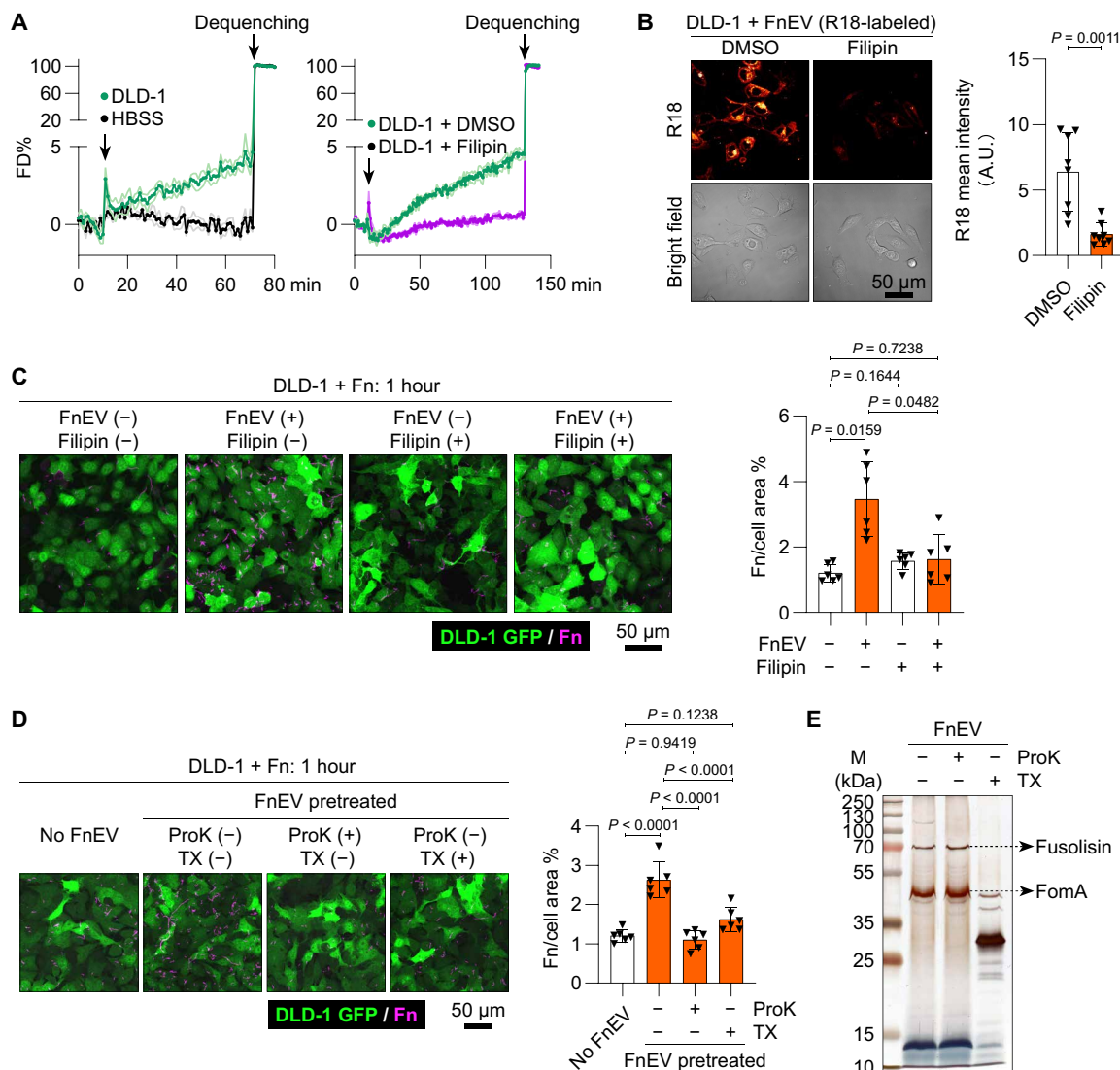


Fig. 3. The membrane fusion and surface proteins of FnEVs are indispensable for promoting *F. nucleatum* adhesion. (A) Spectrofluorimetric analysis after addition of DLD-1 to R18-labeled FnEVs. In Hanks' balanced salt solution (HBSS) control group, fluorescence increased only after disruption of FnEVs with TX (dequenching). DLD-1 cells were pretreated with 15 μ M filipin [dimethyl sulfoxide (DMSO) as vehicle control] to inhibit the membrane fusion of FnEVs. Two independent experiments were performed with similar results. The data of one representative assay with three replicates (means $\pm$ standard error of the mean) are shown. FD%, percentage of fluorescence dequenching level. (B) Representative fluorescent images depicting the membrane fusion of R18-labeled FnEVs with DLD-1. $n = 8$ biological replicates in each group from three independent experiments. A.U., absorbance unit. (C and D) Representative images and quantification of *F. nucleatum* stained with FISH in coculture with DLD-1. (C) Cells were pretreated with DMSO or 15 μ M filipin, followed by treatment FnEVs (5 μ g/ml), and then infected with *F. nucleatum* for 1 hour, $n = 6$ biological replicates in each group from three independent experiments. (D) FnEVs were pretreated with PBS, or ProK (2 μ g/ml) alone, or 1% TX alone, followed by inactivation of ProK with 200 μ M phenylmethylsulfonyl fluoride. $n = 6$ biological replicates in each group from three independent experiments. (E) Proteins of pretreated FnEVs were separated on an SDS-PAGE gel and visualized using silver staining. FomA in an ~40-kDa band and fusolisin in an ~70-kDa band were identified using MS. The experiment was independently performed twice. M, the marker. The data are presented as means $\pm$ SD in (B) to (D). Each triangle represents an individual sample in (B) to (D). The P values were determined by Mann-Whitney test (B), Welch's ANOVA test (C), or one-way ANOVA test (D). GFP, green fluorescent protein.

TLR-4, FnEVs still effectively facilitated *F. nucleatum* adhesion on DLD-1 cells to a similar extent (fig. S6E), indicating that the interaction between LPS and TLR-4 was not essential in this process.

To further elucidate the underlying mechanism of FnEVs in promoting bacterial adhesion, we observed that detergent-disrupted [Triton X-100 (TX)] FnEVs could no longer facilitate *F. nucleatum* adhesion (Fig. 3D and fig. S6F), potentially due to the absence of membrane fusion. Treatment of FnEVs with proteinase K (ProK), aimed at disrupting surface proteins, yielded a similar effect on *F. nucleatum* adhesion as TX treatment (Fig. 3D and fig. S6F), implying that both intact membrane structure and surface proteins were indispensable for promoting *F. nucleatum* adhesion.

FomA retained on CRC cell surface facilitates bacterial adhesion

Two major proteins in FnEVs, one is ~70 kDa and another is ~40 kDa, were digested by treatment with ProK (Fig. 3E and data S2). By mass spectrometry (MS), the ~70-kDa protein was identified as fusolisins, a serine protease bounded on the outer membrane (39). The ~40-kDa protein was FomA as aforementioned (Fig. 1F), which was again confirmed by mass spectrum (Fig. 3E and data S2) and Western blotting (fig. S6G). FomA is reported as an outer membrane protein that mediates adhesion (4, 40). A vaccine targeting *F. nucleatum* FomA could effectively inhibit bacterial co-aggregation and biofilm formation (41). FnEVs pretreated with the specific antibody against FomA (α FomA) could no longer promote *F. nucleatum* adhesion in

coculture with DLD-1 or MC38 (Fig. 4A and fig. S7A). Consistently, in MC38 xenograft model (Fig. 4B), there was no significant difference in tumor growth (Fig. 4C) and *F. nucleatum* colonization (Fig. 4D) between the “ α FomA FnEV + Fn” group and “phosphate-buffered saline (PBS) + Fn” group. The obtained results suggest that FomA plays a crucial role in FnEV-involved *F. nucleatum* adhesion.

A previous study demonstrated that the proteins of eukaryotic exosomes could be transferred to the surface of the recipient cells through membrane fusion (42). Therefore, we hypothesized that during the membrane fusion of FnEVs, FomA might be transferred to and retained on the surface of CRC cells, providing a target for *F. nucleatum* adhesion (fig. S7B). FomA protein was detected on the cell membrane of CRC cell by immunofluorescence staining (Fig. 5, A and B) and Western blotting (Fig. 5C and fig. S7C), with its abundance reduced by filipin-induced abrogation of membrane fusion (Fig. 5, A to C, and fig. S7C). To further validate the above hypothesis, we overexpressed FomA on the cytomembrane of CRC cells and evaluated its potential to enhance adhesion of *F. nucleatum*. FomA, conjugated with a membrane targeting signal peptide at the N terminus, was overexpressed in CRC cells. The membrane localization of FomA was confirmed by both Western blotting (Fig. 5D) and immunofluorescence staining (Fig. 5E). As anticipated, the heterologous expression of FomA in DLD-1 or MC38 significantly augmented the cell adhesion of *F. nucleatum* (Fig. 5F and fig. S7D).

To further confirm the essential role of FomA in mediating FnEV-induced *F. nucleatum* adhesion, we conducted genetic deletion

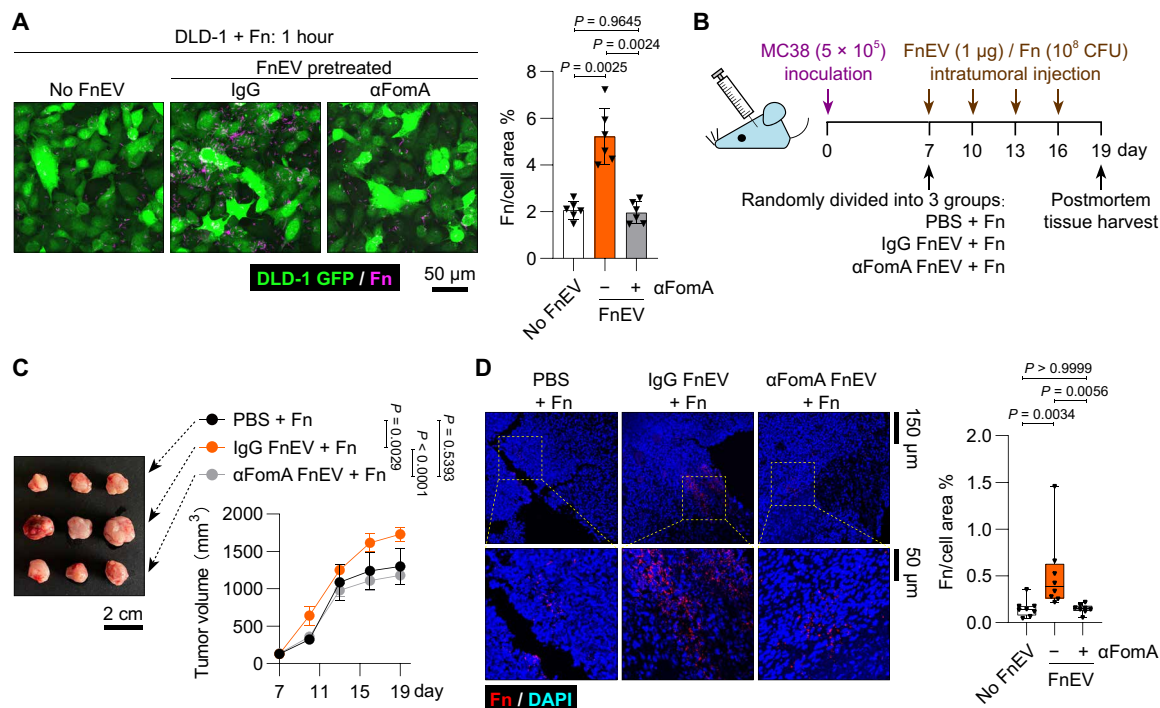


Fig. 4. FomA is required for FnEV to promote adhesion of *F. nucleatum*. (A) Representative images and quantification of *F. nucleatum* stained with FISH in coculture with DLD-1. FnEVs were pretreated with rabbit immunoglobulin G (IgG) or the homemade anti-FomA rabbit polyclonal antibody (α FomA). $n = 6$ biological replicates in each group from three independent experiments. (B) Experimental design of assessing the impacts of α FomA-pretreated FnEVs on tumor progression and *F. nucleatum* adhesion in xenograft model. The experiment was independently performed twice. (C) Representative images of xenografts and the monitoring of tumor volume of “PBS + Fn” ($n = 8$), “IgG FnEV + Fn” ($n = 8$), and “ α FomA FnEV + Fn” ($n = 8$) groups. (D) Representative images and quantification of *F. nucleatum* stained with FISH in xenografts. $n = 8$ mice in each group. The data are presented as median with interquartile range in (D) and as means $\pm$ SD in (A) and (C). Each triangle represents an individual sample in (A) and (D). The P values were determined by Welch’s ANOVA test [(A) and (C)] or Kruskal-Wallis’s test (D).

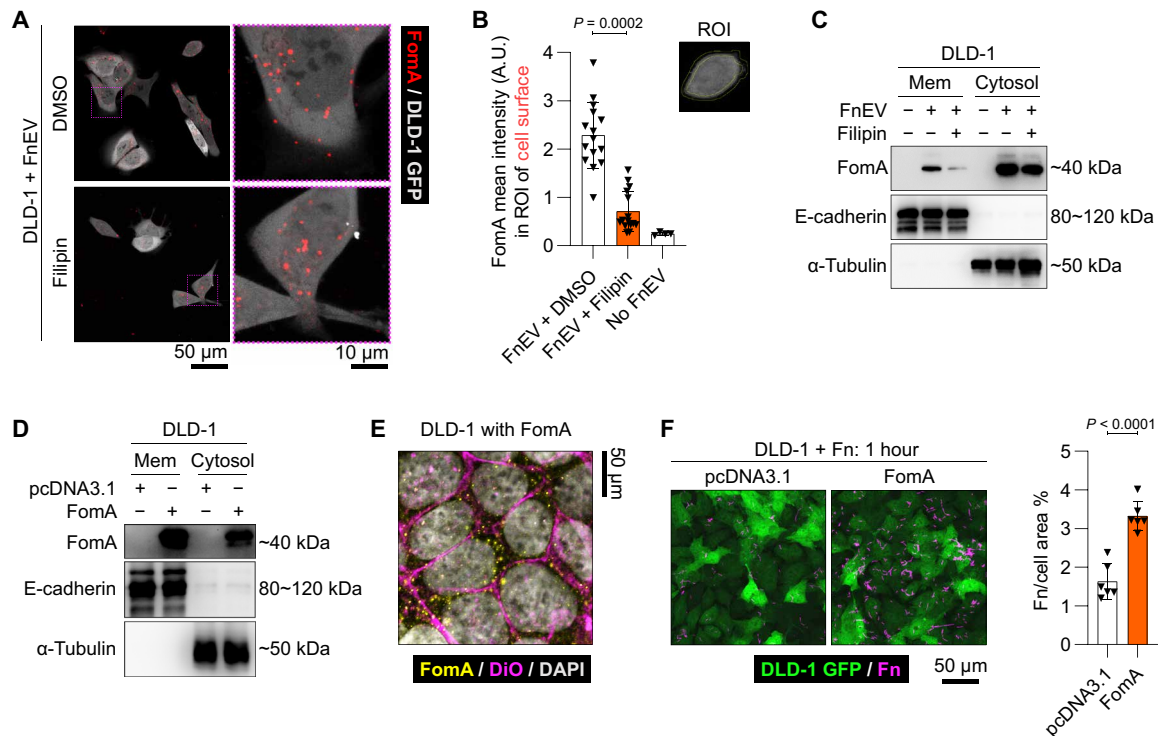


Fig. 5. FomA retains on CRC cell surface via FnEV membrane fusion and mediates adhesion of *F. nucleatum*. (A) Immunofluorescence staining of FomA. Cells were pretreated with DMSO or 15 μ M filipin, followed by treatment of FnEVs (5 μ g/ml). As a negative control, the cells were not treated with anything. (B) Mean intensity of FomA was calculated in the region of interest (ROI), which is defined as the periphery surrounding GFP-expressing cells. Each triangle represents a datum of a single cell. The data were collected from five biological replicates in "FnEV + DMSO" and "FnEV + filipin" groups, and from two biological replicates in "No FnEV" group. The experiment was independently performed twice. (C) Western blotting of FomA in DLD-1 treated with FnEVs and/or filipin. Mem, protein fraction of plasm membrane. Cytosol, protein fraction of cytosol. E-cadherin and α -tubulin were selected as the marker for cell membrane protein and cytosolic protein, respectively. The experiment was independently performed twice. (D to F) Heterologous expression of FomA in DLD-1 cells. The membrane localization of FomA was confirmed by Western blotting (D) and immunofluorescence staining (E). DAPI and DiO (3,3'-diiododecylcarbocyanine chlorates)-dye were used to visualize the nuclei and plasm membrane, respectively. The experiment was independently performed twice. (F) Representative images and quantification of *F. nucleatum* stained with FISH in coculture with DLD-1. $n = 6$ biological replicates in each group from three independent experiments. The data are presented as means $\pm$ SD in (B) and (F). Each triangle represents an individual sample in (F). The P values were determined by Kruskal-Wallis's test (B) or Student's t test (F).

of *fomA* in parent $\Delta galKT$ *F. nucleatum* ATCC23726 (Parent Fn) (fig. S8, A and B). The deletion of *fomA* had no impact on bacterial growth (fig. S8C). The median size and particle number of FnEVs derived from Parent Fn (Parent FnEVs) were comparable to that derived from $\Delta fomA$ Fn ($\Delta galKT\Delta fomA$ *F. nucleatum* ATCC23726) ($\Delta fomA$ FnEVs), while the protein concentration of the latter was reduced (Fig. 6A). The morphology of $\Delta fomA$ FnEVs did not show any obvious alterations (Fig. 6B). The absence of FomA protein in $\Delta fomA$ FnEVs was confirmed (Fig. 6C). $\Delta fomA$ FnEVs failed to facilitate the cell adhesion of Parent Fn in vitro (Fig. 6D and fig. S8D) and in MC38 xenograft model (Fig. 6, E to G).

FnEVs promote *F. nucleatum* autoaggregation via FomA

It has been reported that FomA can mediate the co-aggregation of *F. nucleatum* with other bacteria (40, 41), but whether it can mediate interspecies adhesion of *F. nucleatum*, namely autoaggregation, has not been reported. The addition of FnEVs significantly enhanced the autoaggregation of *F. nucleatum* (Fig. 7A). Scanning electron microscopy (SEM) observation also revealed that the presence of FnEV leads to increased adhesion between *F. nucleatum*

bacteria (Fig. 7B). After blocking with FomA antibodies, FnEVs could no longer promote autoaggregation of *F. nucleatum* (Fig. 7C). Similarly, the FnEVs derived from $\Delta fomA$ Fn also failed to promote autoaggregation of Parent Fn (Fig. 7D). Furthermore, a diminished adhesion (Fig. 7E) and impaired biofilm formation ability (Fig. 7F) was observed in $\Delta fomA$ Fn compared to Parent Fn strain, providing compelling evidence for the crucial role of FomA in interspecies adhesion and biofilm development.

To further investigate the interaction protein of FomA that mediates interspecies adhesion of *F. nucleatum*, we biotinylated the surface proteins of *F. nucleatum* and subsequently used streptavidin pull-down to isolate the surface proteins (Fig. 7G). The surface proteins potentially binding with purified glutathione S-transferase (GST)-FomA were further enriched by GST pull-down (Fig. 7, G and H). MS analysis revealed that the surface protein FN1441 (Fig. 7H and data S3) was a potential candidate for interacting with FomA, suggesting its involvement in mediating autoaggregation of *F. nucleatum*. FN1441, also known as FruA, belongs to a fructose phosphotransferase system (PTS). The fructose PTS has been reported to be associated with biofilm formation and bacterial

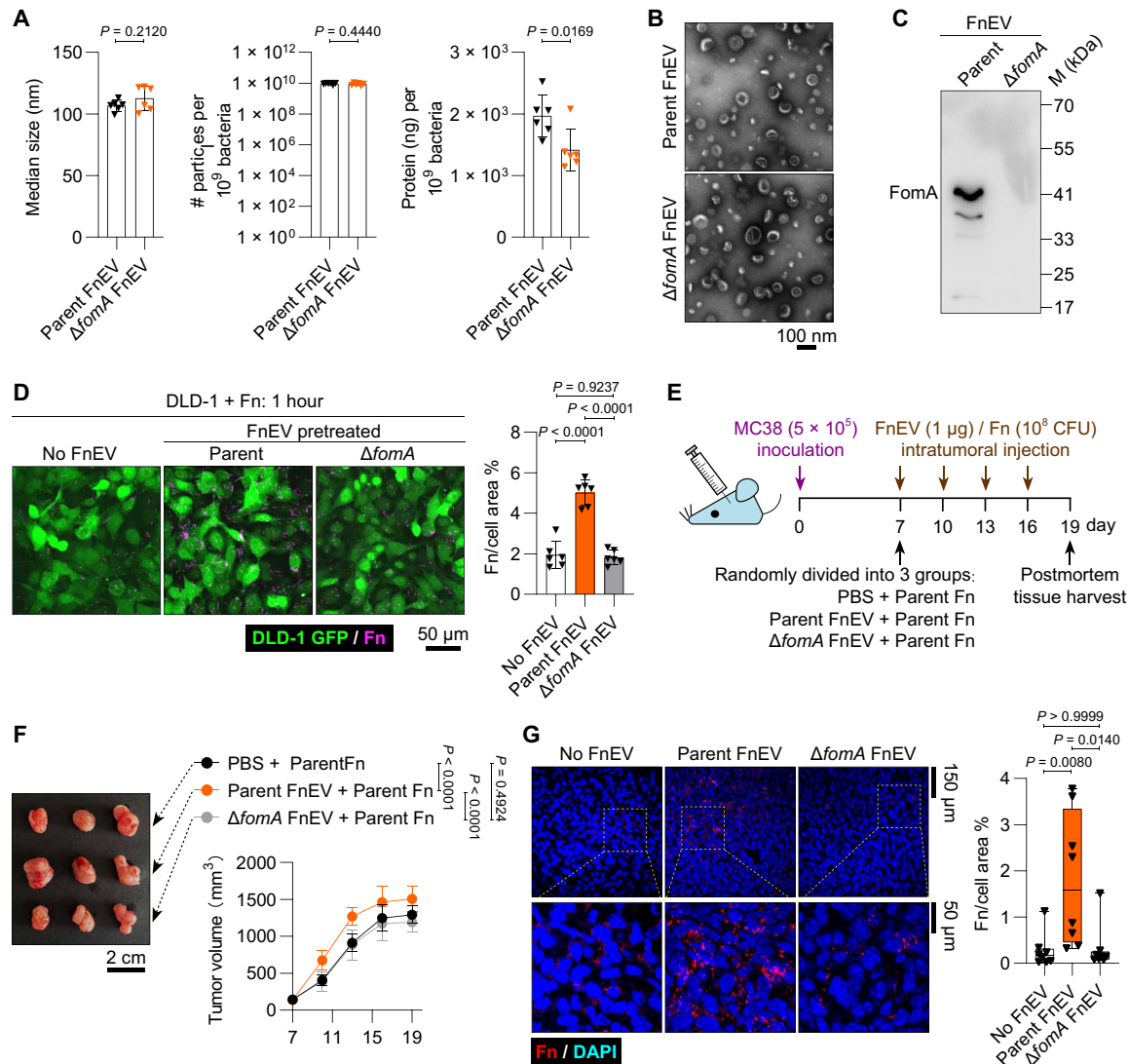


Fig. 6. FomA is indispensable for FnEVs facilitating *F. nucleatum* adhesion on CRC cells. (A and B) Characterization and representative TEM imaging of EVs generated by $\Delta galK$ ATCC23276 (Parent FnEVs) or $\Delta galK\Delta fomA$ ATCC23276 ($\Delta fomA$ FnEVs). $n = 6$ biological replicates from three independent experiments. (C) Western blotting of FomA in Parent FnEVs and $\Delta fomA$ FnEVs. The experiment was independently performed twice. M, the marker. (D) Representative images and quantification of $\Delta galK$ ATCC23276 (Parent Fn) stained with FISH in coculture with DLD-1. Cells were pretreated with Parent FnEVs ($5 \mu\text{g}/\text{ml}$) or $\Delta fomA$ FnEVs. $n = 6$ biological replicates in each group from three independent experiments. (E) Experimental design of assessing the impacts of Parent FnEVs or $\Delta fomA$ FnEVs on tumor progression and Parent Fn adhesion in xenograft model. The experiment was independently performed twice. (F) Representative images of xenografts and the monitoring of tumor volume of “PBS + Parent Fn” ($n = 8$), “Parent FnEV+Parent Fn” ($n = 8$), and “ $\Delta fomA$ FnEV+Parent Fn” ($n = 8$) groups. (G) Representative images and quantification of Parent Fn stained with FISH in xenografts. $n = 8$ mice in each group. The data are presented as median with interquartile range in (G) and as means $\pm$ SD in (A), (D), and (F). Each triangle represents an individual sample in (A), (D), and (G). The P values were determined by Student’s t test (A), one-way ANOVA test [(D) and (F)], or Kruskal-Wallis’s test (G).

adhesion (43, 44). We further purified FN1441 fused with a GST tag (GST-FN1441). As expected, the pull-down assay revealed that GST-FN1441 effectively enriched FomA in the protein lysis derived from *F. nucleatum*, confirming their interaction (Fig. 7I).

To demonstrate the involvement of FomA-FN1441 interaction in mediating interspecies adhesion of *F. nucleatum* and its essential role in FnEV-promoting *F. nucleatum* adhesion to CRC cells, we performed *fn1441* gene deletion in Parent Fn (fig. S9A). The growth of $\Delta fn1441$ Fn did not exhibit a significant difference compared to that of Parent Fn (fig. S9B). The autoaggregation ability of $\Delta fn1441$ Fn was significantly diminished (fig. S9C), and Parent FnEV failed

to enhance autoaggregation in $\Delta fn1441$ Fn (fig. S9D). Furthermore, Parent FnEV failed to enhance the adhesion level of $\Delta fn1441$ Fn to CRC cells (fig. S9, E to G).

DISCUSSION

Being an inherent form of nanoparticles, BEVs in the circulatory system have the potential to accumulate passively in tumor tissues due to the EPR effect (22, 23). Following oral administration, FnEVs were absorbed by the intestinal tract and might traverse the intestinal barrier to gain access into the circulatory system (20),

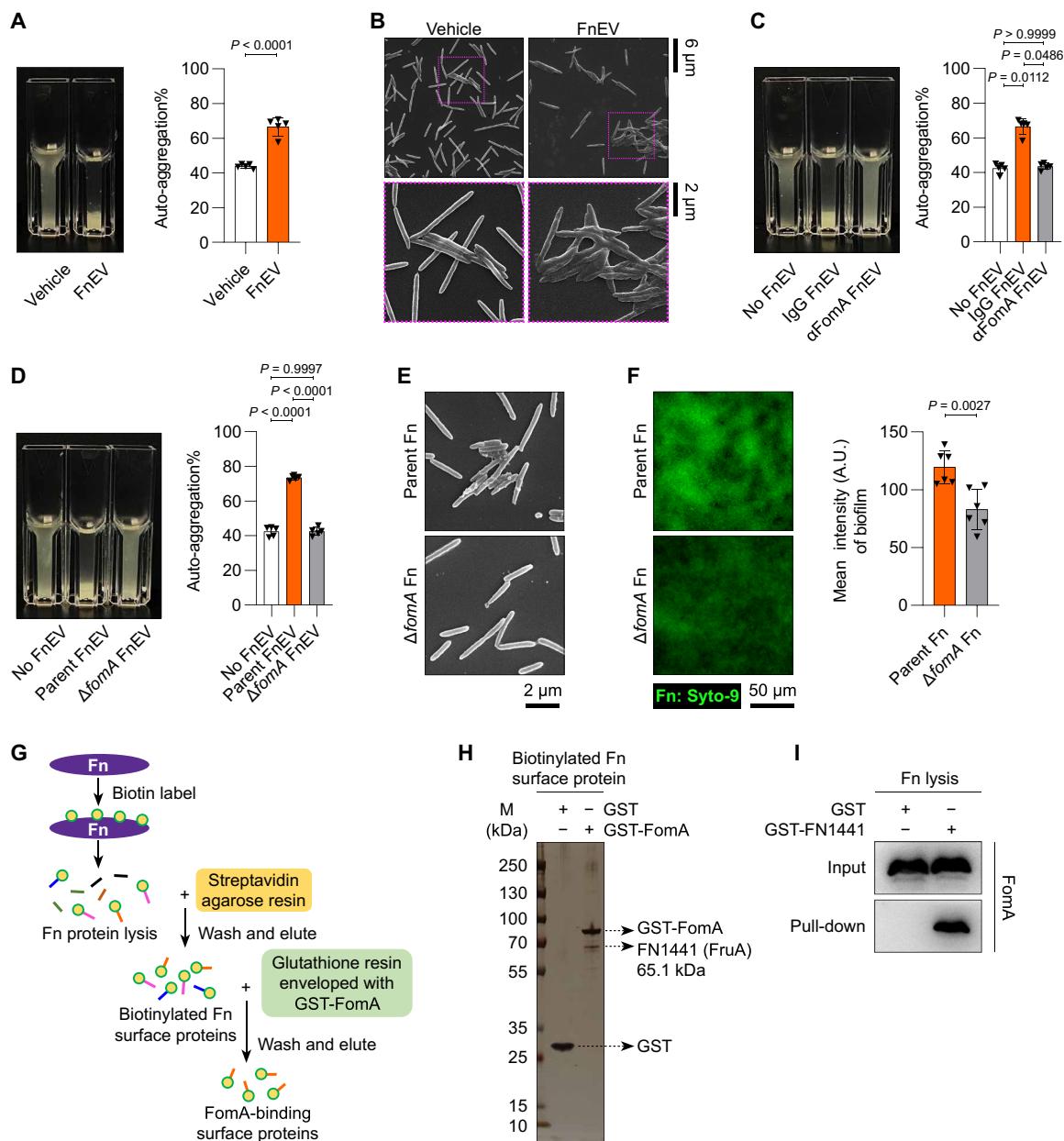


Fig. 7. FomA in FnEVs mediates autoaggregation of *F. nucleatum*. (A) Representative pictures and quantification of autoaggregation of *F. nucleatum* ATCC25586. One microgram FnEVs (in 5 μ l of PBS) was added when indicated. $n = 5$ biological replicates from three independent experiments. (B) Representative SEM images showing the autoaggregation of *F. nucleatum*, with or without FnEV treatment. The experiment was independently performed three times. (C and D) Representative pictures and quantification of autoaggregation of Parent Fn (Δ galKT ATCC23726) treated with indicated FnEV. $n = 5$ biological replicates from three independent experiments. (E) Representative SEM images showing the autoaggregation of Parent Fn and Δ fomA Fn (Δ galKT Δ fomA ATCC23726). The experiment was independently performed three times. (F) Representative images and quantification of Syto-9-labeled Parent Fn and Δ fomA Fn biofilm. $n = 6$ biological replicates from three independent experiments. (G) Schematic representation of the workflow for capturing biotinylated surface proteins of *F. nucleatum* ATCC25586 and FomA-binding surface proteins. (H) Biotinylated surface proteins of *F. nucleatum* ATCC25586 were pulled down with GST-FomA, separated on an SDS-PAGE gel, and visualized using silver staining. The experiment was independently performed twice. (I) GST pull-down assay with GST-FN1441 as the bait. The FomA protein in the protein lysis of *F. nucleatum* (input) and the eluted proteins (pull-down) was quantified by Western blotting. The experiment was independently performed twice. The data are presented as means $\pm$ SD in (A), (C), (D), and (F). Each triangle represents an individual sample in (A), (C), (D), and (F). The P values were determined by Student's t test [(A) and (F)], Kruskal-Wallis's test (C), or one-way ANOVA test (D).

potentially leading to their preferential accumulation in CRC tissues via the EPR effect. On the other hand, the acidic tumor microenvironment promotes the membrane fusion between eukaryotic exosomes and recipient cells, serving as a key factor for exosomes traffic in tumor cells (37). Similarly, since FnEVs were also uptake by CRC cells through membrane fusion in the present study, the acidic tumor microenvironment might also be one of the reasons why FnEVs absorbed in the intestine system would be enriched in CRC. We additionally identified the presence of FomA protein in EVs isolated from clinical samples, providing compelling evidence for the existence of FnEVs. Furthermore, a significantly higher abundance of FnEVs was observed in clinical CRC compared to adjacent normal tissues. The FnEV enrichment may be a consequence of the colonization of *F. nucleatum* in CRC tissues, which has been documented by numerous studies (3, 4, 6, 11, 12, 45). However, our findings also revealed that the presence of FnEVs facilitated the adherence of *F. nucleatum* to CRC. Therefore, it is highly plausible that they establish a causal relationship and form a positive feedback loop promoting the colonization of *F. nucleatum* in CRC. Whether FnEV serves as a driving force or a complicit actor for *F. nucleatum* adhesion, blocking its effect may potentially yield favorable outcomes by inhibiting the colonization of *F. nucleatum* in CRC.

BEVs encompass a diverse array of parent bacterial cargoes, including LPS, RNA, DNA, proteins, etc., and have the potential to elicit immune responses via host receptors such as pattern recognition receptors and signaling pathways like cyclic guanosine monophosphate–adenosine monophosphate synthase/stimulator of interferon genes (17). In diverse pathological contexts, BEVs derived from pathogens or commensal bacteria elicit distinct immune responses through disparate mechanisms, thereby exhibiting varying physiological significance. BEVs derived from pathogen play a pivotal role in the pathogenesis and progression of specific chronic inflammatory conditions, such as periodontitis (46) and rheumatoid arthritis (24). In some cases, BEVs can also promote immune defense, such as their mediated antitumor (22) and antiviral effects (47). In our study, FnEVs promoted the progression of inflammation-related CRC, potentially attributed to their ability to promote colitis (19, 21). Our in vitro experiments also found that FnEVs promote the expression of proinflammatory mediators such as SAA and CXCL8 in CRC cells. Previous studies have demonstrated that the secretion of CXCL8 induced by *F. nucleatum* is essential for its role in reprogramming the immune microenvironment of CRC (48) and facilitating CRC metastasis (49). Therefore, from this perspective, blocking the enrichment and proinflammatory effect of FnEVs would confer potential benefits in terms of managing colitis-associated CRC. Since the primary objective of this study is to investigate the impact of FnEVs on *F. nucleatum* adhesion and colonization, the influence of FnEVs on the initiation and progression of CRC through immune modulation remains unexplored in this context. Although we observed no correlation between the TLR4-mediated inflammatory response and FnEV-mediated *F. nucleatum* adhesion, it is worth exploring whether the influence of other cargoes within FnEVs on host immune response may affect the intratumor survival and colonization of *F. nucleatum*.

The global composition shift of microbiota is involved in CRC initiation, progression, and its response to treatment (1). Several studies have indicated that microbiota associated with CRC display higher species diversity, reduced levels of beneficial probiotics, and increases in bacteria potentially linked to tumor development (50,

51). The association between the presence of *F. nucleatum* and dysbiosis in CRC microbiota is highly plausible. In the dental plaque biofilm, *F. nucleatum* serves as a bridging organism due to its elongated shape and numerous surface adhesins, such as FomA, RadD, and Fap2, which facilitates bacterial adhesion and aggregation for biofilm formation (4). The process of bacterial adhesion is critical in determining the composition of bacteria within an ecological niche, thereby influencing the functionality of the entire community and host physiological responses. Our results have indicated that FnEVs may facilitate the translocation of bacterial adhesins to distant tissues, thereby providing targets for bacterial adherence. While our investigation primarily focused on the impact of FomA on *F. nucleatum* adhesion, it is important to acknowledge the presence of other adhesins within FnEVs (e.g., RadD: 25586_FusoPortal_Genome_30; see data S1), which theoretically have the potential to mediate the adherence of diverse bacteria. This phenomenon likely correlates with the composition and structure of microbial communities in CRC niches and warrants further exploration in future studies.

The limitation of this study is that it cannot be ruled out whether FnEVs promote the colonization of *F. nucleatum* in tumor tissues by affecting the proliferation and growth of *F. nucleatum* in tumors or the bacterial clearance capacity of host cells. In an in vitro coculture model, we demonstrated that FnEVs can facilitate *F. nucleatum* adhesion to CRC cells, thereby partially elucidating why FnEV treatment promote *F. nucleatum* colonization in AOM/DSS-induced CRC and xenograft. Although transcriptomic analysis indicated that FnEVs may not impair bacterial clearance capacity of CRC cells [such as autophagy (33) and antimicrobial peptide expression (34–36)] in this simple model, functional studies are lacking, and it cannot be ruled out whether FnEV can affect the bacterial clearance of other cell types in CRC. On the other hand, because of a lack of effective tools for monitoring *F. nucleatum* proliferation in tumor tissues or cell coculture in situ, we only confirmed that neither FnEV itself nor the supernatant from cells treated with FnEV had a significant effect on *F. nucleatum* growth. However, we could not completely exclude the potential for FnEVs to promote *F. nucleatum* proliferation and growth in vivo.

The human body maintains a symbiotic balance with resident microorganisms, while the translocation and colonization of these commensals can often trigger the occurrence and progression of diseases in distant organs or systems. This study has elucidated that BEVs generated by a commensal bacterium in the oral cavity, that is, FnEVs, accumulate within CRC tissue. FnEVs facilitate the transfer of an adhesin protein FomA to CRC cell surface through membrane fusion, thereby establishing a favorable niche for *F. nucleatum* adhesion and colonization. These findings not only provide a targeted explanation for the enrichment of *F. nucleatum* in CRC but also present a model for how resident microbiota disseminate to distant organs in humans.

MATERIALS AND METHODS

Bacterial culture

F. nucleatum ATCC25586, ATCC23726, and *Porphyromonas gingivalis* W83 was purchased from American Type Culture Collection (ATCC) and cultured in Columbia broth (Difco, USA) supplemented with hemin (5 µg/ml; Sigma-Aldrich, USA) and menadione (0.5 µg/ml; Sigma-Aldrich) (CBHK) at 37°C under anaerobic condition (90% N₂, 5% CO₂, and 5% H₂). Planktonic growth of *F. nucleatum* was

monitored by measuring the optical density at 600 nm (OD_{600nm}). Colony forming unit (CFU) counting was conducted by inoculating the bacteria onto solid agar plates made with CBHK supplemented with 5% defibrinated whole sheep blood. Before using in animal assays or in cell-coculture experiments, overnight bacterial cultures were back-diluted 1:1000 and grown to mid-exponential phase (~0.5 OD_{600nm}), centrifuged (2 min, 4°C, 12,000 rpm), and resuspended with PBS. ATCC23726 was used in *fomA* deletion-related assays (designated as Parent Fn and Δ *fomA* Fn), while ATCC25586 was used otherwise.

Purification, characterization, and treatment of FnEVs

The bacteria-free supernatant from 800 ml of overnight culture of *F. nucleatum* was collected by centrifugation at 10,000g for 15 min at 4°C. The supernatant was further filtered through a 0.45- μ m filter. FnEVs were pelleted by twice ultracentrifugation, at ~100,000g for 2 hours at 4°C, in an Avanti JXN-30 centrifuge (Beckman, USA). After removing the supernatant, FnEVs were resuspended in 2 ml of PBS. The median size and particle number were analyzed with the Zeta View (Particle Metrix, Germany). Protein concentration was quantified by Pierce bicinchoninic acid protein assay kit (catalog no. 23227, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Following a standard operation of negative staining (with 1% phosphotungstic acid for 1 min at room temperature), the morphology of FnEVs was evaluated with a JEM-1400FLASH transmission electron microscope (TEM) (JEOL, Japan).

For in vivo imaging assay, FnEVs were labeled with 2 μ M DiR-dye (catalog no. 60017, Biotium, USA), 30 min at 37°C, and subsequently cleaned-up by ultracentrifugation at ~100,000g for 1 hour at 4°C for removal of unincorporated dye. In membrane fusion assay, FnEVs were stained with 20 μ M octadecyl rhodamine B (R18) (catalog no. O0512, TCI, China), 30 min at room temperature, and ultracentrifuged (37).

To elucidate the underlying mechanism responsible for the pro-adhesion effect of FnEVs, we pretreated FnEVs with TX (catalog no. X100, Sigma-Aldrich), ProK (catalog no. CS203218, Millipore, USA), or the homemade α FomA. Specifically, (i) FnEVs were treated with 1% TX or ProK (2 μ g/ml) or PBS as a vehicle control for 5 hours at room temperature, followed by inactivation of ProK with 200 μ M phenylmethylsulfonyl fluoride (PMSF) and used in the indicated assays (52); (ii) FnEVs were incubated with α FomA or isotype immunoglobulin G as a vehicle control (1 μ g of antibody per 1 μ g of FnEVs) overnight at 4°C, followed by ultracentrifugation at ~100,000g for 1 hour at 4°C. The resulting pellet was resuspended in PBS and used in the indicated assays.

For a purer preparation of FnEV, we performed density gradient ultracentrifugation method using OptiPrep (catalog no. D1556, 60% w/v iodixanol, Sigma-Aldrich) according to previous studies with modifications (53, 54). Briefly, the crude FnEVs were resuspended in 40% w/v iodixanol and placed at the bottom of an Ultra-Clear tube (catalog no. 344058, Beckman). A discontinuous iodixanol gradient was created by layering 35, 30, 25, 20, 15, and 10% w/v iodixanol in Hepes buffer [50 mM Hepes (catalog no. 15630080, Thermo Fisher Scientific) and 150 mM NaCl (pH 6.8)]. Following centrifugation at 100,000g for 8 hours at 4°C, seven gradient fractions were collected from top to bottom. These fractions were then electrophoresed through 4 to 20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (catalog no. PG42015-S, Solarbio, China) and visualized with silver staining. Fractions showing protein bands were further

examined using TEM. Fractions 3 and 4 containing typical and pure vesicles were pooled as purified FnEV.

Cell culture and coculture assay

Human CRC cell lines DLD-1, SW620 and mice CRC cell line MC38 were obtained from ATCC and Kerafast (USA). Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere. For coculture of bacteria and cells, the cells were grown to >90% confluency. Before bacterial inoculation, FnEVs (5 μ g/ml) were added into the cultures and incubated for 2 hours. When indicated, 15 μ M filipin complex (catalog no. HY-N6716, MedChemExpress, China) (37), 80 μ M dynasore (catalog no. A1605, APEX BIO, China) (38), or 1 μ M TAK-242 (catalog no. A3850, APEX BIO) (55) was added into the cell cultures and incubated for 2 hours before FnEV treatment. The cells were washed three times with PBS, and the medium was refreshed. *F. nucleatum* at a multiplicity of infection of 10:1 was inoculated. After coculturing for 1 hour, the cells were washed three times with PBS to remove any unattached bacteria before undergoing FISH procedures to assess the bacterial adhesion.

Cell transfection

To construct green fluorescent protein (GFP)-reporting CRC cells, DLD-1, SW620, or MC38 were infected with lentivirus-delivered GFP vector (Hippo Biotechnology, China), alone with polybrene (8 μ g/ml; catalog no. TR-1003, Sigma-Aldrich). Infected cells were selected with puromycin (1 μ g/ml).

For heterologous expression of FomA in CRC cell lines, the coding region of *F. nucleatum* ATCC25586 *fomA* (FN1859) was amplified and cloned into pcDNA3.1/Zeo(+) vector (Thermo Fisher Scientific). To improve plasma membrane targeting, DNA encoding the first 45 amino acids of the rat somatostatin receptor subtype 3 was added to the 5' end of *fomA* (56). Cells at ~50% confluency were transfected with empty vector or FomA expression plasmid using Lipofectamine 2000 (catalog no. 11668019, Thermo Fisher Scientific) according to the instructions.

Animal experiments

All animal experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of animals in research and approved by the Institutional Animal Care and Use Committee at West China Hospital of Stomatology, Sichuan University (WCHSIRB-D-2021-071). The mice were housed under specific pathogen-free conditions.

In colitis-driven CRC model (57), 8-week male C57BL/6J mice (at least 20 g in weight) were used. The mice were injected intraperitoneally with AOM (10 mg per kg body weight) (catalog no. A5486, Sigma-Aldrich) on day 0. After 1 week, they were given water containing 2% DSS (catalog no. 9011-18-1, MP Biomedicals, USA) for another week, followed by 2 weeks of regular water. A total of three cycles of DSS treatment were conducted. For evaluation of FnEV distribution, the mice were given 1 μ g of DiR-labeled FnEVs either orally (p.o.) or through tail intravenous injection (i.v.) and then euthanized at specified times to collect tissues for imaging (Fig. 1C). To investigate the impact of FnEVs and *F. nucleatum* on CRC progression, the mice were given antibiotic cocktail [ampicillin (0.2 g/liter), vancomycin (0.1 g/liter), metronidazole (0.2 g/liter), and neomycin (0.2 g/liter)] in their drinking water for 2 weeks

before AOM injection. After three cycles of DSS treatment, they received oral administration of FnEVs (1 μ g in 100 μ l of PBS) and/or *F. nucleatum* (10^9 CFU in 100 μ l of PBS) every 3 days for approximately 2 weeks (a total of five treatments) (Fig. 2A). The mice were then euthanized to collect colon tissue for tumor evaluation. The colon was slit open and thoroughly washed in ice-cold sterile PBS. The numbers of tumors in each colon were counted. The tumors in each sample were captured using a stereomicroscope (Olympus, Japan), and the resulting images were stitched together and analyzed for tumor area using ImageJ. In addition, 1–3 tumors were excised in each colon, fixed, and embedded with paraffin. FISH was performed on tissue slices to quantify intratumor *F. nucleatum*.

In xenograft model, 8-week male C57BL/6J mice were used. 5×10^5 MC38 cells in 100 μ l of PBS were injected subcutaneously into the right flanks of each mouse. Seven days after injection, the mice were randomly divided into indicated groups, to which the investigators who collected the samples and performed analyses were blinded. As indicated in different experiments (Figs. 2D, 4B, and 6E), FnEVs (1 μ g in 10 μ l of PBS) and/or *F. nucleatum* (10^8 CFU in 10 μ l of PBS) were injected into the xenografts with a micro-syringe (RN 1801; Hamilton, USA) every 3 days (a total of four treatments). The length (L) and width (W) of the tumor were measured every 3 days using a digital caliper and converted into tumor volume with the formula $W^2 \times L/2$. At day 19, the mice were euthanized, and subcutaneous tumors were collected for follow-up analyses.

No statistical calculations were performed to predetermine sample size. Sample sizes were chosen on the basis of statistically significant results obtained in other publications that used similar methods (5, 11, 28, 29). The mice were randomly divided into indicated groups (simple randomization) and were assigned a specific number before data collection, and all data collection and analysis were blindly performed (Fig. 1, C, D, E and G, Fig. 2, A to F, Fig. 4, B to D, Fig. 6, E to G, fig. S1, A and B, and fig. S5C). No data were excluded.

Tissue distribution of FnEVs

Two or 8 hours after administering DiR-labeled FnEVs (described above), AOM/DSS-treated mice and control mice were euthanized to collect tissues, including the liver, spleen, heart, lung, colon (~2 cm, with visible tumors in mice treated with AOM/DSS), and small intestine (~2 cm jejunum). Immediately, the images of all tissues harvested from each mouse were captured and analyzed by IVIS-SPECTRUM imaging system (PerkinElmer, USA).

To further evaluate the distribution of FnEVs in CRC and non-cancerous colon tissues, the tumors and paired adjacent normal colon tissues were harvested 2 hours after oral administration of unlabeled FnEVs to mice with AOM/DSS-induced CRC, then fixed, and embedded with paraffin. Immunofluorescence staining of FomA was performed on tissue slices (5 μ m in thickness) to quantify the uptake of FnEVs.

MS and protein identification

The proteins of FnEVs were isolated by adding 4 volumes of lysis buffer (8 M urea and 1% protease inhibitor cocktail), followed by sonication on ice for 3 min using a high-intensity ultrasonic processor (Scientz, China). Subsequently, the mixture was centrifuged at 12,000g and 4°C for 10 min. The protein solution was mixed with 5 $\times$ SDS-PAGE protein loading buffer, boiled for 15 min, electrophoresed through 4 to 20% SDS-PAGE gels, and stained with Coomassie brilliant blue. The trypsin digestion of the protein solution or the

in-gel trypsin digestion of specific gel bands was performed by PTM Bio (China) using standard procedures.

MS was performed by PTM Bio (China). In brief, the tryptic peptides were dissolved in solvent A and directly loaded onto a home-made reversed-phase analytical column. The mobile phase consisted of solvent A (0.1% formic acid and 2% acetonitrile/in water) and solvent B (0.1% formic acid and 90% acetonitrile/in water). The peptides were separated on EASY-nLC 1200 UPLC system (Thermo Fisher Scientific). The separated peptides were analyzed in Orbitrap Exploris 480 with a nano-electrospray ion source. Precursors and fragments were analyzed at the Orbitrap detector.

The resulting MS/MS data were processed using Proteome Discoverer 1.3. Tandem mass spectra were searched against 25586_FusoPortal_Genes_AA.faa (downloaded from FusoPortal: <http://fusoportal.org/index.html>) (58) concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to one missing cleavage. Mass error was set to 10 parts per million for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys were specified as fixed modification and oxidation on Met was specified as variable modification. Peptide confidence was set at high, and peptide ion score was set >20.

Production of anti-FomA polyclonal antibody

Two FomA-derived polypeptides were designed, synthesized, and conjugated with keyhole limpet hemocyanin for immunization. The resulting immunogen was diluted with physiological saline and mixed 1:1 with corresponding adjuvant to form a stable emulsion, which was then injected into four New Zealand rabbits. Each rabbit received four immunizations on days 1, 21, 28, and 35. On days 45, 50, 65, and 70 after immunization, whole blood (20 ml) was collected from each rabbit and centrifuged to obtain serum for screening by enzyme-linked immunosorbent assay (ELISA). Positive serum samples were selected for antibody purification using Protein A affinity chromatography followed by specific enrichment of the target antibody using an equilibrated immunogen affinity chromatography column. The purified two antibodies were validated by ELISA and Western blotting assays. The one targeting the polypeptide KKFATYNKGDKKSQF exhibited superior specificity and was applied in indicated assays.

To test the specificity of the anti-FomA antibody, we performed Western blotting in protein lysis (methods described below) from *F. nucleatum* ATCC25586, Parent Fn, Δ fomA Fn, *P. gingivalis* W83, DLD-1, and MC38. In addition, we collected feces from mice treated with *F. nucleatum* ATCC25586 (oral administration of 10^9 CFU *F. nucleatum* in 100 μ l of PBS every 3 days for approximately 2 weeks) or untreated mice. DNA was isolated from feces using a fecal genome DNA extraction kit (catalog no. DP328, Tiangen, China). Protein was isolated as described below in the section Protein isolation and Western blotting. We conducted polymerase chain reaction (PCR) (see primers in table S2) to determine the presence or absence of *F. nucleatum* in mouse feces and performed Western blotting to evaluate the specificity of the anti-FomA antibody.

Immunofluorescent staining and analysis

Three types of samples were prepared for immunofluorescent staining: (i) Tissue slices of AOM/DSS-induced CRC, paired adjacent normal colon, and MC38 xenograft were prepared as aforementioned; (ii) GFP-expressing cells were seeded in eight-well μ Slide (Ibidi, Germany), grown to ~30% confluency, treated with FnEVs (5 μ g/ml)

for 2 hours, washed three times with PBS, and then fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature; and (iii) FomA-expressing cells were cultured in μ Slide until ~90% confluency and then washed and fixed.

Tissue slices or fixed cells were washed three times for 5 min with 1× PBS and blocked for 45 min with SuperBlock blocking buffer (catalog no. 37515, Thermo Fisher Scientific) supplemented with 0.3% TX and 2% donkey serum at room temperature. The samples were then incubated at 4°C overnight with the homemade anti-FomA antibody (1:1000 diluted in blocking buffer). After washing three times for 5 min with 1× PBS, the samples were incubated for 1 hour at room temperature with donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:500 diluted in blocking buffer) (catalog no. A-21206, Molecular Probes, USA). When indicated, 6-diamidino-2-phenylindole (DAPI) (catalog no. C0065, Solarbio, China) in deionized water was used to visualize the nuclei, and DiO-dye (catalog no. V22889, Thermo Fisher Scientific) was used to label plasm membrane.

The imaging was performed using a FLUOVIEW FV3000 confocal laser scanning microscope (Olympus, Japan). Z-series image stacks were captured with constant acquisition parameters (gain, offset, and pinhole settings) for every separated assay. Each sample was scanned at randomly selected positions. The mean intensity of FomA within the region of interest, defined as either the entire field or the periphery surrounding GFP-expressing cells (segmented using the MorphoLibJ plugin in ImageJ), was measured using ImageJ.

Purification of EVs from clinical tissue

The fresh colon adenocarcinoma and paired adjacent normal tissues ($n = 5$) were collected in West China Hospital, Sichuan University. All procedures were approved by the Institution Review Board of West China Hospital of Stomatology, Sichuan University (WCHSIRB-CT-2021-062). Written informed consent was obtained from all participants. Patients with a known synchronous cancer diagnosis or other cancer diagnosis within 5 years of the operation, or a history of radiotherapy, were excluded. No antibiotics were given preoperatively. Patient information was listed in table S1.

The EVs derived from human tissue were isolated following a previously described protocol with modifications (59). The fresh tissues were immersed in PBS on ice and promptly transported to the laboratory within 1 hour. The samples were weighed, gently sliced into small fragments (1 to 2 mm), and incubated for 30 min at 37°C in DMEM supplemented with collagenase D (2 mg/ml, catalog no. 11088858001, Roche) and deoxyribonuclease I (40 U/ml, catalog no. 11284932001, Roche). Following filtration using 70- μ m filters, cells and tissue debris were subsequently removed by centrifugation at 300g for 10 min and 2000g for 20 min at 4°C. The large vesicles were separated by centrifugation at 16,500g for 20 min at 4°C, followed by subjecting the resulting supernatants to centrifugation at 118,000g for 2.5 hours at 4°C to collect small vesicles. The small EV-enriched pellets were resuspended in PBS (with the volume normalized to 2.5 ml per 1 g of tissue) as crude preparation and stored at –80°C. We performed density gradient ultracentrifugation as mentioned before to further purified the crude EVs. The characterization and TEM imaging of EVs derived from clinical tissues followed the same protocol as FnEVs. Fraction 3 with the most abundant protein and typical vesicles observed in TEM examination were collected as purified EVs from clinical tissues. Equal volumes of purified EVs were loaded in Western blotting analysis to quantify the abundance of FomA protein.

Protein isolation and Western blotting

Four types of protein samples were prepared as follows: (i) Protein lysis of FnEVs or EVs derived from clinical samples were prepared as described above in the “MS and protein identification” section; (ii) the protein fraction from the plasma membrane and cytosol were isolated using a Mem-PER Plus membrane protein extraction kit (catalog no. 89842, Thermo Fisher Scientific) according to the manufacturer's instructions; (iii) *F. nucleatum* protein lysis was prepared as follows: An overnight culture of *F. nucleatum* was collected by centrifugation (20 min, 4°C, 4000g), resuspended in PBS containing lysozyme (1 mg/ml) and 1 mM PMSF, sonicated using a Q800R3 system (AMPL 50%, 15 s with a 30-s interval for 5 min) (Qsonica, USA), and followed by centrifuging (2 min, 4°C, 12,000g) to collect the supernatant; (iv) mouse feces were immersed in the same lysis buffer, sonicated (AMPL 50%, 15 s with a 30-s interval for 10 min), followed by centrifuging (2 min, 4°C, 12,000g) to collect the supernatant.

Proteins were electrophoresed through 4 to 20% SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad, USA). The membranes were blocked with 5% nonfat powdered milk (Sangon Biotech, China) for 1.5 hours at room temperature and subsequently incubated with indicated primary antibodies overnight at 4°C. The primary antibodies used included the homemade anti-FomA rabbit polyclonal antibody (1:1000), a rabbit monoclonal antibody to E-cadherin (1:1000) (catalog no. ab40772, Abcam, USA), and a rabbit polyclonal antibody to α -tubulin (1:500) (catalog no. 41517, SAB Biotech, China). After being washed with tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies for 1 hour at room temperature followed by TBST washing. The signals were detected using Immobilon enhanced chemiluminescence Ultra Western HRP substrate (catalog no. WBULS0500, Merck Millipore, German) and visualized with ChemiDoc XRS+ Imaging System (Bio-Rad).

FISH and quantification of *F. nucleatum*

FISH was performed as previously described (11). Cells and bacteria were permeabilized with 0.1% TX for 1 hour at room temperature, followed by lysozyme treatment (30 mg/ml) (catalog no. 302969, J&K Scientific, China) for 30 min at 37°C and subsequent washing with PBS. The samples were serially dehydrated in ethanol (50, 80, and 100%; each for 3 min), exposed to a hybridization buffer (0.9 M NaCl, 20 mM tris-HCl, 0.01% SDS, and 20% formamide) containing the FUS714 probe (5'GGCTTCCCCATCGGCATT3') (60) conjugated with Alexa Fluor 555 (200 nM), and incubated at 46°C for 90 min. After hybridization, the samples were immersed in a washing buffer (20 mM tris-HCl, 5 mM EDTA, 0.01% SDS, and 215 mM NaCl) for 15 min at 48°C and then rinsed with PBS. When indicated, DAPI was used to visualize the nuclei. The imaging procedures were the same as described in the “Immunofluorescent staining and analysis” section. The abundance of *F. nucleatum* was determined using ImageJ by calculating the ratio of *F. nucleatum*–positive area to GFP- or DAPI-positive area in percentage.

Immunohistochemistry

We performed immunohistochemistry (IHC) staining of SAA in AOM/DSS-induced CRC tissues with a standard method. Samples were permeabilized with PBS/0.1% TX for 1 hour and blocked with 1% bovine serum albumin for another 1 hour at room temperature, followed by incubation with the SAA primary antibody (catalog no.

DF6533, Affinity Biosciences, China) overnight at 4°C and subsequently with the HRP-conjugated second antibody (catalog no. ab6721, Abcam) for 1 hour at room temperature. The signals were detected with a diphenylene diamine system (catalog no. abs957, Absin, China). The nuclei were stained with hematoxylin. The percentage of IHC-positive cells with strong (S%), moderate (M%), or weak (W%) intensity, respectively, was analyzed by an IHC profiler plugin in ImageJ (61). The IHC score for SAA was calculated with the formula $3 \times S + 2 \times M + 1 \times W$.

Membrane fusion assay

The membrane fusion assay was conducted on the basis of previous studies with modifications (37). Ten micrograms of R18-labeled FnEVs were added to 2 ml of Hanks' balanced salt solution (HBSS) with Ca/Mg (pH 7) (Thermo Fisher Scientific) supplemented with glucose (2 g/liter), and fluorescence was measured continuously (Ex, 560 nm; Em, 590 nm; 37°C) with Flexstation III (Molecular Devices, USA). After a 10-min equilibration period, unlabeled DLD-1 cells (1×10^6 in 100 μ l of HBSS) were introduced to the FnEV solution. The fluorescence was monitored for an additional duration of either 60 or 120 min. To terminate the assay, 0.3% TX was added, resulting in maximal dilution of R18 dye. The increase in fluorescence was calculated as the difference relative to the initial fluorescence value obtained from labeled FnEVs and expressed as a percentage (%) compared to the maximum dequenching level (%FD), following this equation: $\%FD = [(F - F_i)/(F_{max} - F_i)] \times 100\%$, where F_i represents the initial fluorescence value observed for labeled FnEVs and F_{max} denotes the fluorescence intensity after detergent-induced disruption of membranes. For inhibition of membrane fusion, DLD-1 cells were pretreated with 15 μ M filipin complex for 2 hours at 37°C.

On the other hand, the cells were seeded in eight-well μ Slide, grown to ~30% confluency, pretreated with either 15 μ M filipin complex or dimethyl sulfoxide for 2 hours at 37°C, followed by treatment of R18-labeled FnEVs (5 μ g/ml) for 2 hours at 37°C, washed three times with PBS, and then fixed with 4% PFA for 15 min at room temperature. The imaging procedures were the same as described in the "Immunofluorescent staining and analysis" section. The mean intensity of R18 fluorescence was calculated using ImageJ.

RNA sequencing and analysis

The total RNA from DLD-1 treated with FnEVs (5 μ g/ml) or PBS was extracted and purified using a MiniBest Universal RNA extraction kit (catalog no. 9767, Takara, Japan) following the manufacturer's instructions. The quality of RNA was assessed using the 2100 Bioanalyzer (Agilent, USA), and quantification was performed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Subsequently, a cDNA library was prepared according to the TruSeq™ RNA sample preparation kit procedures (Illumina, USA) with 1 μ g of total RNA. Paired-end sequencing was conducted on an Illumina NovaSeq 6000 sequencer [2 $\times$ 150 base pair (bp) read length]. Raw paired-end reads were subjected to trimming and quality control using SeqPrep and Sickle tools with default parameters. Clean reads were then aligned separately to a reference genome in orientation mode using HISAT2 software (62). StringTie tool in a reference-based approach assembled mapped reads for each sample (63). The expression level of each transcript was determined on the basis of fragments per kilobase million method. Differential expression gene analysis using DESeq2 tool (64) with $P_{adj} \leq 0.05$ and $|\log_2FC| \geq 1$ criteria.

Genetic deletion of *fomA* and *fn1441*

A galactose-selectable gene deletion system was used to construct an in-frame *fomA* deletion ($\Delta fomA$) and *fn1441* deletion ($\Delta fn1441$) strains in *F. nucleatum* ATCC 23726, following a previously described method with slight modifications (figs. S8A and S9A) (49). Initially, a $\Delta galKT$ mutant was generated as the parent strain for gene knockout (Parent Fn) using the vector pDJSVT13 (synthesized by Sangon Biotech), which was electroporated into competent *F. nucleatum* ATCC 23726 and selected on solid agar plates containing thiamphenicol (5 μ g/ml) for single crossover, and on solid agar plates containing 0.25% 2-deoxy-D-galactose for second crossover. To generate $\Delta fomA$ and $\Delta fn1441$ Fn, ~750 bp of upstream and downstream homologous sequences of *fomA* (HMPREF0397_1003) or *fn1441* (HMPREF0397_0483) were assembled using overlap extension PCR. The resulting fragment was digested by XhoI and NotI enzymes and ligated into pDJSVT7 (synthesized by Sangon Biotech). The resulting vector (pKO*fomA* and pKO*fn1441*) was then transformed into competent *F. nucleatum* $\Delta galKT$ strains and selected on solid agar plates containing thiamphenicol (5 μ g/ml) for single crossover, and on solid agar plates containing 1% galactose for double crossover. The gene deletion of *galKT*, *fomA*, and *fn1441* was verified through PCR and sequencing. All primers used were listed in table S2.

Aggregation assay

The autoaggregation assay of *F. nucleatum* was conducted as previously described with modifications (65). Stationary-phase cultures of *F. nucleatum* were harvested by centrifugation (20 min, 4°C, 4000g), resuspended in coaggregation buffer [150 mM NaCl, 1 mM tris, 0.1 mM CaCl₂, and 0.1 mM MgCl₂ (pH 7)] and adjusted to an OD_{600nm} of ~2. Subsequently, aliquots of the *F. nucleatum* suspension (1 ml each) were added to cuvettes and vortex-mixed for 10 s before measuring the initial OD_{600nm}. The suspensions were then incubated anaerobically for 1 hour at 37°C, and the final OD_{600nm} was recorded. In cases where specified, 1 μ g of FnEVs (in 5 μ l of PBS) were added into each sample before incubation. Autoaggregation% was calculated by using the following formula: $(1 - \text{final OD}_{600nm} / \text{initial OD}_{600nm}) \times 100\%$.

The autoaggregation of *F. nucleatum* was also examined by an SEM (FEI, Hillsboro, USA). Aliquots of the *F. nucleatum* suspension (1 ml each) were inoculated onto cover glasses placed in a 24-well plate and incubated anaerobically for 1 hour at 37°C. The bacteria on the glasses were then fixed with glutaraldehyde for 12 hours, serially dehydrated in ethanol, and sputter-coated with gold. Specimens were examined at 10,000 $\times$ and 20,000 $\times$ magnifications.

Biofilm imaging and quantification

F. nucleatum in mid-exponential phase (~0.5 OD_{600nm}) was inoculated into μ Slide and anaerobically cultured at 37°C for 2 days, followed by washing the biofilm three times with PBS and fixing it using 4% PFA for 15 min at room temperature. Bacteria within the biofilm were labeled using SYTO9 green fluorescent nucleic acid stain (catalog no. S34854, Thermo Fisher Scientific). The imaging procedures were the same as described in the "Immunofluorescent staining and analysis" section. The fluorescent signal mean intensity of *F. nucleatum* biofilm was calculated using ImageJ.

Biotinylation and purification of surface proteins

The *F. nucleatum* ATCC25586 cells were collected by centrifugation (5000g, 15 min, 4°C) and washed once by cold PBS (pH 8.0)

containing 1 mM PMSF. After washing, the pellets were resuspended in 1 ml of PBS containing 1 mM PMSF and incubated with 100 μ l of EZ-Link Sulfo-NHS-LC-Biotin (10 mg/ml, catalog no. 21435, Thermo Fisher Scientific) for 1 hour on ice with gentle shaking. The biotinylation reaction was quenched by sedimenting the cells (4000g, 5 min, 4°C), removing the supernatant, and resuspending the cells in PBS containing 500 mM glycine buffer. Quenching was repeated three times to eliminate reactive Sulfo-NHS-LC-Biotin and soluble proteins that may have been released from lysed cells. Last, the cells were resuspended in 500 μ l of PBS with 1 mM PMSF and transferred to a 1.5-ml tube containing appropriate glass beads (0.1 mm). Cell disruption was performed mechanically using a Fast-Prep-24 5G Homogenizer (MP Biomedicals) with two cycles at 6 m/s² for 30 s, with an interval of cooling for 5 min. Cell debris was sedimented by centrifugation (20,000g, 30 min, 4°C). The Pierce streptavidin agarose resin slurry (catalog no. 20347, Thermo Fisher Scientific) was washed twice with PBS containing 1% NP-40. The supernatant of the cell lysate was incubated with the streptavidin agarose resins for 2 hours on ice with gentle shaking. Subsequently, the resins were washed twice with 1 ml of PBS containing 1% NP-40. Biotinylated proteins were eluted using 1 ml of elution buffer (0.1 M glycine, pH 2.8), and the pH was immediately adjusted to 7.5 to 9.0 by adding 100 μ l of Tris solution.

GST pull-down

The coding region of ATCC25586 *fomA* (FN1859), along with GST and 6His-tag coding sequences conjugated to its 3' end, was cloned into pET-41a(+) (primers used were listed in table S2). The resulting vector was transformed into *E. coli* BL21(DE3) (Takara). The GST-6His-FomA protein was purified using a standard protocol with Ni²⁺ affinity columns (Beyotime, China). The GST pull-down assay was conducted using the Pierce GST protein interaction pull-down kit (catalog no. 21516, Thermo Fisher Scientific). Briefly, glutathione agarose resin was equilibrated with a 1:1 wash solution of TBS and pull-down lysis buffer. The bait protein, GST-6His-FomA (86 kDa), or GST (26 kDa), was then incubated with the glutathione agarose resin at 4°C for 2 hours with gentle rocking motion. The immobilized bait proteins were incubated overnight at 4°C with biotinylated *F. nucleatum* surface proteins prepared as described above. Subsequently, the resins were washed with a wash solution and eluted using a 10 mM glutathione elution buffer. The eluted proteins were separated on 4 to 20% SDS-PAGE gels, followed by silver staining or MS analysis.

To further validate the interaction between FomA and FN1441, the ATCC25586 FN1441, fused with a GST tag and a 6His tag to its N-terminal, was purified using the same method described above (primers used were listed in table S2). The GST pull-down assay was performed as described above, with GST-6His-FN1441 as the bait and the protein lysis derived from *F. nucleatum* ATCC25586 as the input. The input proteins and eluted proteins were separated on 4 to 20% SDS-PAGE gels, followed by Western blotting to quantify FomA.

Statistical analysis

Statistical analyses were performed with the GraphPad Prism software. All data were first subjected to Kolmogorov-Smirnov test or Shapiro-Wilk test for normal distribution and to *F* test or Brown-Forsythe test for homogeneity of variance. Data that passed these two tests were analyzed by Students' *t* test or paired *t* test (two groups) or one-way analysis of variance (ANOVA) (multiple groups)

with the Tukey method as the post hoc test. Otherwise, data were analyzed by Welch's *t* test (two groups, data with normal distribution but do not have equal variance), Mann-Whitney test (two groups, data do not fit normal distribution), Welch's ANOVA (multiple groups, data with normal distribution but do not have equal variance) with Dunnett's T3 method as the post hoc test, or Kruskal-Wallis test (multiple groups, data do not fit normal distribution) with Dunn method as the post hoc test. Data were considered significantly different if the two-tailed *P* value <0.05. All experiments were repeated independently for two or three times. No data were excluded, and all data values (sample sizes <20) were present in table. S3.

Supplementary Materials

The PDF file includes:

Figs. S1 to S9

Tables S1 to S3

Legends for data S1 to S3

Other Supplementary Material for this manuscript includes the following:

Data S1 to S3

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