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Tumor-mediated 4-1BB induces tumor proliferation and metastasis in the colorectal cancer cells

Min-Kyoung Kim [a,1], Kyung-Ju Shin [a,1], Sijeong Bae [b], Jin-Myung Seo [c], Hosun Jung [c], Young-Ah Moon [b,*], Su-Geun Yang [d,**]

[a] Department of Biomedical Science, Inha University College of Medicine, Incheon 22332, South Korea
[b] Department of Molecular Medicine, Inha University College of Medicine, Incheon, South Korea
[c] Department of Biomedical Science, BK21 FOUR Program in Biomedical Science and Engineering, Inha University College of Medicine, Incheon 22212, South Korea
[d] Department of Biomedical Science and Inha Institute of Aerospace Medicine, Inha University, Incheon 22332, South Korea

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ABSTRACT

Aims: 4-1BB is a member of the tumor necrosis factor receptor superfamily that mainly expressed on activated T-cells and plays important roles in cell proliferation and survival of T-cells and natural killer cells. The roles of 4-1BB in immune cells have been intensively studied, whereas little is known about the expression and roles of 4-1BB in cancer cells.

Main methods: In the present study, we investigated 4-1BB expression in colorectal cancer tissues from human patients and established colorectal cancer cells, using mRNA expression, FACS, and immunostaining. Cancer cell proliferation and metastasis regulated by transfected 4-1BB was evaluated by cell growth rate, colony forming assay, cell migration, and Western blot with antibodies which are involved in epithelial-mesenchymal transition and anti-apoptosis. Expression of 4-1BB was knockdown by 4-1BB shRNA to prove that 4-1BB was involved in the cell proliferation. *In vivo*, 4-1BB transfected cancer cells were injected into mice, to induce tumor local region or lung.

Key findings: We found that colorectal cancer tissues from human patients and established colorectal cancer cells expressed 4-1BB at the high level. The higher expression of 4-1BB proliferated faster. In addition, we identified two forms of 4-1BB detected in colorectal cancer cells: full length form that was located on the plasma membrane and a short soluble form in the cytosol. The soluble form was also detected in the plasma from the mice with tumor xenografts expressed 4-1BB.

Significance: Tumor-mediated 4-1BB expression in the colorectal cancer cells showed effects on cancer cell proliferation, invasion, and metastasis.

1. Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer and ranks the second of cancer death worldwide [1]. South Korea also have increased in CRC incidence rapidly, and second highest incidence rate of CRC in 2018 [2]. The majority of CRC incidence, about 60–65 % of CRC, is sporadic without a family history and attributed by environmental risk factors, such as obesity, poor diet, smoking, and alcohol drinking. These risk factors can cause genetic and epigenetic mutations [1]. CRC caused by accumulated somatic mutations or damaged DNA repair deficient releases tumor-specific neoantigens [3], which enhance T cell reactivity and cytotoxic effect against tumors [4]. Recently immunotherapy, targeting not only tumor cells but also the immune cells, is expanding to treat cancers [5]. Immune checkpoint inhibitor therapy [6] is applicable and efficient for immunogenically active CRC. However, a significant feature of cancer development is immune modulation and evasion [7]. Cancer cells express molecules that block immune checkpoints, such as programed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), to avoid immune surveillance [8].

* Correspondence to: Y.-A. Moon, Department of Molecular Medicine, Inha University College of Medicine, 100 Inha-ro, Michuhol-Gu, Incheon 22212, South Korea.
** Correspondence to: S.-G. Yang, Department of Biomedical Science, Inha University College of Medicine, 366 Seohae-daero, Jung-gu, Incheon 22332, South Korea.
E-mail addresses: yamoon15@inha.ac.kr (Y.-A. Moon), Sugeun.Yang@inha.ac.kr (S.-G. Yang).
[1] These authors contributed equally to this work

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Nomenclature

APCs	antigen-presenting cells
Bcl-2	B-cell lymphoma 2
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CRC	colorectal cancer
H&E	hematoxylin and eosin
EMT	epithelial-mesenchymal transition
NF-kB	nuclear factor kappa B
PD-L1	programed death-ligand 1
PMA/Io	Phorbol 12-myristate 13-acetate and ionomycin
TNFR	tumor necrosis factor receptor
TNFRSF9	tumor necrosis factor receptor superfamily member 9
TRAF1	tumor necrosis factor receptor-associated factor 1

4-1BB is a costimulatory receptor [9] and known also as CD137 and tumor necrosis factor receptor superfamily member 9 (TNFRSF9). It is a member of the tumor necrosis factor receptor (TNFR) superfamily that expresses on T-cells, mainly $CD8^+$ and $CD4^+$, when activated [10]. The ligand of 4-1BB (4-1BBL, TNFSF9) present on activated antigen-presenting cells (APCs), such as B-cells, macrophages, and dendritic cells [11]. The interaction between 4-1BB and 4-1BBL maintains cell division, enables cell survival, and induces cytokine production [12]. The signaling pathways of the activated T-cell by binding of 4-1BB and 4-1BBL include recruitment of TNFR-associated factor adaptor molecules, pro-inflammatory signaling pathways involving phosphoinositide 3-kinases, protein kinase B, and NF-kB, and increased expression of anti-apoptotic B-cell lymphoma 2 (Bcl-2) family molecules [13].

In the present study, we found that some human colorectal cancer tissues and established human colorectal cancer cell lines expressed 4-1BB, and the expression levels were associated with cancer cell proliferation. We evaluated that the expression of 4-1BB and its regulation in colorectal cancer tissues and cell lines was independent from its roles in immune cells.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-1BB (3′-UTR-wt; vector pLightSwitch_3UTR) and the deletion sequence (3′-UTR-mut) were obtained from SwitchGear Genomics (Carlsbad, CA, USA). Plasmid containing the 4-1BB promoter −865 to +1 and −1742 to −866 were kindly provided by Dr. Kang from Seoul National University. Dulbecco's phosphate-buffered saline (DPBS), RPMI-1640, and 100× penicillin/streptomycin were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA), and 0.1 % trypsin-EDTA was obtained from Welgene Inc. (Daegu, Korea).

2.2. Studies using human tissues and animals

We used 5 samples of normal colon tissues and 10 samples of colon cancer tissues to evaluate 4-1BB gene expression in our research. Tissues were derived from 5 non-cancer patients and 15 colon cancer patients obtained from the Biobank of Inha University Hospital, and the study was approved by the Institutional Review Board of Inha University Hospital (INHAUH 2019-04-018). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Inha University (IACUC 170912-515-4).

2.3. Cell culture and cell viability

Human colorectal cancer cell lines HCT116, RKO, HT-29, and LOVO were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium with 10 % FBS and 1× penicillin/streptomycin and incubated in 5 % CO_2 at 37 °C. Cell viability was determined using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). To determine apoptosis, the cells were detached and stained with FITC-Annexin V and propidium iodide using an FITC/Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). The cells were separated using the BD Accuri C6 Flow Cytometer (BD Biosciences).

2.4. Transfection with plasmids p4-1BB or ps4-1BB, and shRNAs

The RKO cells were transfected with plasmids p4-1BB or ps4-1BB, or the empty vector (pcDNA) using the Lipofectamine 2000 Reagent (Invitrogen) according to the instructions of the manufacturer. Several clones and mass populations of transfectants were selected using G418 (Invitrogen) at concentration of 0.3 mg/ml for 2 weeks. The transfected RKO cells were expanded and evaluated for 4-1BB and s4-1BB. Plasmid p4-1BB contains full length of 4-1BB and ps4-1BB does the splice variant of 4-1BB.

An expression vector carrying 4-1BB shRNA (psi-H1-4-1BBshRNA) was obtained from GeneCopoeia (Rockville, MD, USA). A negative control vector was provided by GeneCopoeia, designated as shRNA-NC (psi-H1-scramble shRNA). Sh-4-1BB and sh-NC vectors contained enhanced green fluorescent protein (eGFP) cDNA. To stably establish the 4-1BB-knockdown HCT-116, cells were grown and transfected with lipofectamine 2000, and the transfectant efficiency was directly measured by the detection of eGFP expression in cells by fluorescence microscopy. The cells were grown in medium containing 1.5 μg/ml puromycin (Invitrogen) for 2 weeks. 4-1BB shRNA transfected cells were named as sh-4-1BB cells, whereas the control sh-NC-transfected cells were designated as mock cells. After transfection, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) or Western blot analysis was carried out to confirm 4-1BB expression (Fig. 4) or inhibition (Supplementary Fig. S1).

2.5. Immunofluorescence

Cells in 60 mm dishes were washed with 0.5 % bovine serum albumin (BSA) in PBS and added 4 % formaldehyde in PBS, followed by a 30 min incubation at room temperature. The cells were washed twice with 1 % BSA in PBS, and the cells were added 0.5 % saponin dissolved in 1 % BSA in PBS, followed by 15 min of incubation on ice. The mouse monoclonal anti-human 4-1BB antibody (ab197942; Abcam, Cambridge, UK) and ImmunoCruz rabbit ABC Staining System (SC-2018; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used for immunohistochemistry staining as instructed by each manufacturer's protocol. The specimens were mounted with Dako mounting solution (Glostrup, Denmark) and analyzed using confocal fluorescent microscopy (Zeiss LSM510 Meta Confocal Imaging System; Carl Zeiss, Thornwood, NY, USA).

2.6. Preparation of RNA and real time quantitative PCR (RT-qPCR)

Total RNA was isolated from tissues or cultured cells using Trizol (Invitrogen, Carlsbad, CA, USA), cDNA was synthesized using a reverse transcription kit (Takara), and real time qPCR was performed using SYBR Green Quantitative PCR Master Mixture (Applied Biosystems, Foster City, CA, USA).

2.7. Western blot

Whole cell lysates were prepared from tissues or cultured cells, and

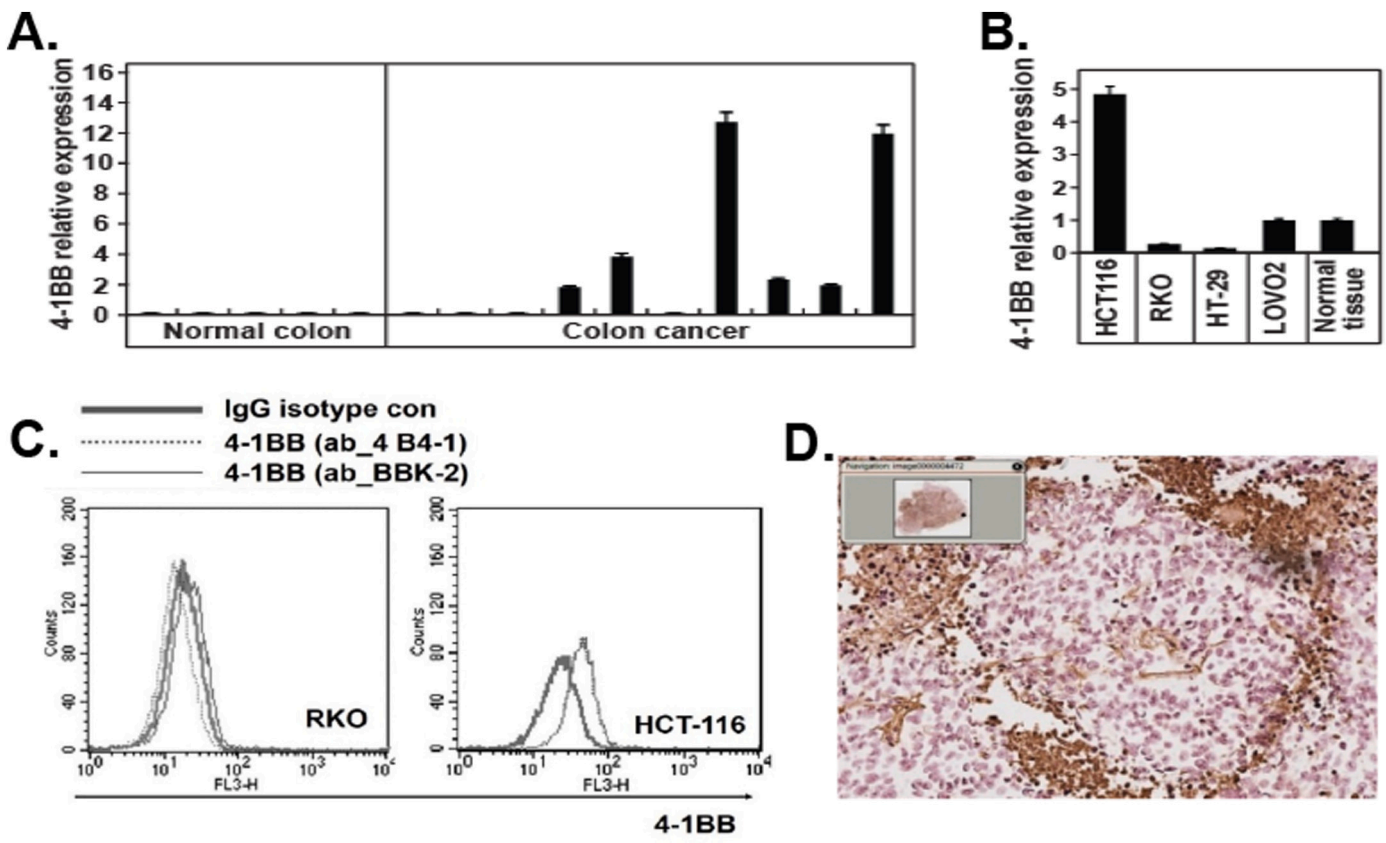

Fig. 1. 4-1BB expression in colorectal cancer cells.
(A) 4-1BB mRNA expression was determined in human normal colorectal tissues [5] and cancer tissues [10] derived from the patients. (B) 4-1BB mRNA expression was determined in the indicated human colorectal cell lines. (C) 4-1BB expression was determined using FACS analysis in HCT116 and RKO cells with two types of 4-1BB antibody: 4B4-1; ectodomain targeting Ab clone and BBK-2; cytoplasmic targeting Ab clone (D) 4-1BB protein was also detected by immunoblotting in the tumor tissue from xenograft cancer mouse.

blotting was performed as described previously [14]. E-cadherin and vimentin, human matrix metalloproteinase-2 (MMP-2), Bcl-2, and b-actin (Santa Cruz Biotechnology, Inc.) were used as primary antibodies. Western blot data were quantified based on the β-action using Image J (NIH, USA).

2.8. Cell migration and colony forming assays

HCT-116 cells were seeded at a density of 7×10^4 per well onto 24-well culture plates. When the cells reached 80 % confluency, a line (wound) with uniform width was created on the bottom of the plates with a sterilized tip. After cell growth, open area was determined relatively. For colony forming assays, HCT-116 cells were seeded at a density of 5×10^5 on day 0. On day 3, the cells were trypsinized and suspended in a mixture of 1 mL of 2× RPMI and 1 mL of 1 % Agar Select. Next, 1 mL of the cell suspension was poured over a 0.6 % agar/RPMI pre-layer (2 mL) in a 6-well plate. Feeder layers (2 mL) were overlaid on top of the hard cell layers. The cells were allowed to grow in a 37 °C incubator for 14 days.

2.9. Xenograft mouse model

HCT-116 cells (5×10^6 cells) were injected into the subcutaneous tissue on the flank region of 6-week-old male Balb/c nude mice (Orient Bio, Seongnam, Korea). The volume of the tumor was determined using the following equation: $(\text{width})^2 \times 0.5$ length. When the mean volume of the tumors reached approximately 100 mm^3, the mice were divided into 4 groups. Scrambled shRNA and 4-1BB shRNA (10 μg shRNA+lipofectamine/100 μL PBS and 100 μg vector+lipofectamine/100 μL PBS) were intratumorally injected into tumor-bearing mice once per day for 3 days. The changes in tumor volume were monitored for 30 days. At the end of the experiments, the mice were euthanized, and the tumor tissues were harvested. Plasma concentration of 4-1BB in control mice and the mice with HCT116 xenograft tumor was measured using 4-1BB-R (Human) OmniKine™ ELISA Kit (Assaybiotech, Fremont, CA).

2.10. Luciferase activity of the promoter and 3′-UTR on 4-1BB

To determine luciferase activity, a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was utilized following the manufacturer's protocols using a luminometer (Luminoskan Ascent; Thermo Fisher Scientific). 4-1BB promoter and 3′-UTR-constructs were transiently transfected into HCT-116 cells using Lipofectamine 2000 (Invitrogen) as described in the user manual. pCMV-b-galactosidase (pCMV-b-gal; BD Clontech, Palo Alto, CA, USA) was co-transfected to normalize the transfection efficiencies. For the NF-kB transactivation after transfection, the cells were incubated with 20 ng/mL PMA and 0.5 μM Io for 24 h. The cells were collected and lysed in 150 μl of reporter lysis buffer (Promega). Cell supernatant was detected in the luciferase and b-galactosidase (b-gal) assays.

2.11. Statistical assay

A one-way analysis of variance (ANOVA) or the Student's *t*-test was used to determine statistical significance, and $p < 0.05$ was regarded as significant.

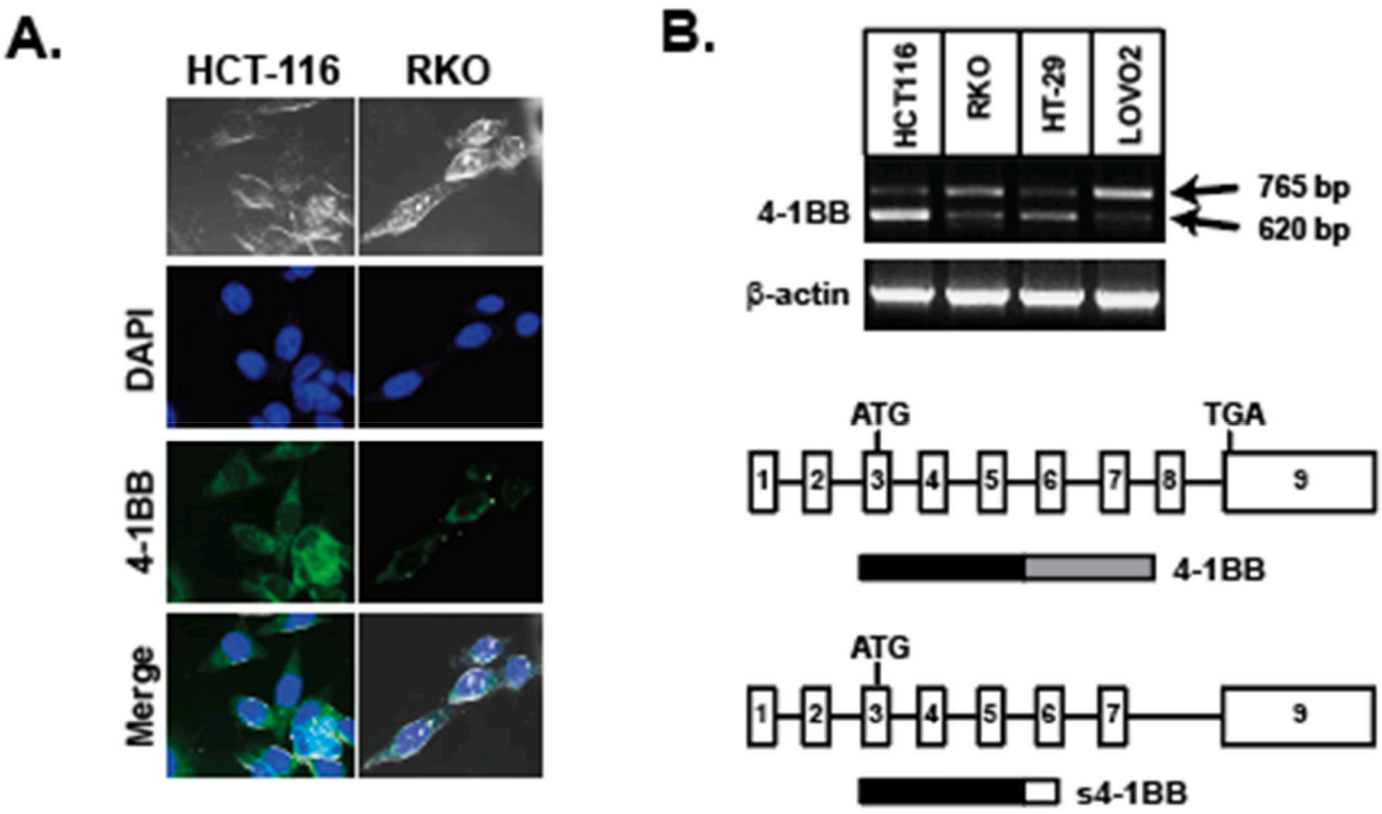

Fig. 2. Two forms of 4-1BB expressed in colorectal cancer cells
(A) Immunofluorescent image of 4-1BB (green) in HCT116 and RKO cells. The nuclei were stained with DAPI (blue) and merged with 4-1BB. (B) PCR products amplifying the 3′ end of the cDNA prepared from the indicated cells. The PCR product corresponding to the full length exhibited the 765 bp band, and that of the splicing variant missing exon 8 (short form) exhibited the 620 bp band. The splicing variant that was resulted in the early termination of translation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. 4-1BB expression in colorectal cancer cells

While the functions of 4-1BB and the interaction between 4-1BB and 4-1BBL on the plasma membrane of immune cells have been well characterized [12], limited information is available regarding 4-1BB expression and its functions in cancer cells. In this study we investigated that 4-1BB expression and its roles in colorectal cancer cells. We used 5 samples of normal colon tissues and 10 samples of colon cancer tissues to evaluate 4-1BB gene expression in this research. Tissues were derived from 5 non-cancer patients and 15 colon cancer patients, which were approved by the Institutional Review Board of Inha University Hospital (INHAUH 2019-04-018). First, we evaluated mRNA expressions of 4-1BB in human colorectal cancer tissues and human colorectal normal tissues. Some human colorectal cancer samples expressed 4-1BB and expressions of two samples were very high while others barely expressed 4-1BB as in normal colorectal tissues that expressed (Fig. 1A). Expressions of 4-1BB in established human colorectal cancer cell lines, including HCT116, RKO, HT-29, and LOVO2 cells were also determined. HCT116 cells expressed 4-1BB at the high level while RKO and HT-29 cells expressed relatively low levels of 4-1BB among the tested cells (Fig. 1B). Normal colorectal cell lines were generated from *intestinal* epithelial *cells. Unfortunately, we did not have normal colon cell lines.* Alternatively normal tissues derived as non-cancer patients were used to determine mRNA expression of normal cells cells as a basal level. Human aortic smooth muscle *cells* (HASMC) and human umbilical vein endothelial *cells* (HUVEC) were evaluated 4-1BB expression, but they were barely expressed 4-1BB (Supplementary Fig.S2).

4-1BB expressions at the protein level in HCT116 and RKO cells were compared using FACS analysis. The expression level of 4-1BB protein in cancer cells was confirmed by FACS through intracellular staining using an antibody that only targets (4 B4-1) the ectodomain of the cell and an antibody that targets (BBK-2) the cytoplasm. It confirmed that 4-1BB expression was higher in the HCT 116 than RKO cells compared to IgG levels (Fig. 1C). Expression of 4-1BB protein was also detected by immunoblotting in tumor tissue from xenograft cancer mouse (Fig. 1D).

3.2. Two forms of tumor-mediated 4-1BB expressed in colorectal cancer cells

4-1BB expression and localization in the cancer cells was determined by immunofluorescence in HCT116 and RKO cells, which displayed high and low expression levels of 4-1BB, respectively. The 4-1BB signal shown not only in cell membrane but the cytoplasm was higher in HCT-116 cells than that in RKO cells, which 4-1BB signal was only detected in the cell membrane (Fig. 2A). 4-1BB is a membrane protein with a transmembrane domain that usually exists at the plasma membrane where it interacts with 4-1BBL in immune cells. As in immune cells, 4-1BB was localized mainly on the plasma membrane in RKO cells. However, a significant amount of 4-1BB was detected in the cytoplasm as well as on the plasma membrane in HCT116 cells (Fig. 2A).

To determine how the cytoplasmic 4-1BB could be produced, the cDNA sequence of 4-1BB prepared from various colorectal cancer cells was analyzed. As the transmembrane domain of 4-1BB was in the exon 8 [15], the PCR primers were designed to amplify the exon 9 of the 4-1BB cDNA which covered the transmembrane domain. The PCR produced two forms: one was 765 bp which was considered the full length, while the other was 620 bp in length, considered short form of 4-1BB (Fig. 2B). Sequencing of two products revealed the 620 bp band as a splice variant

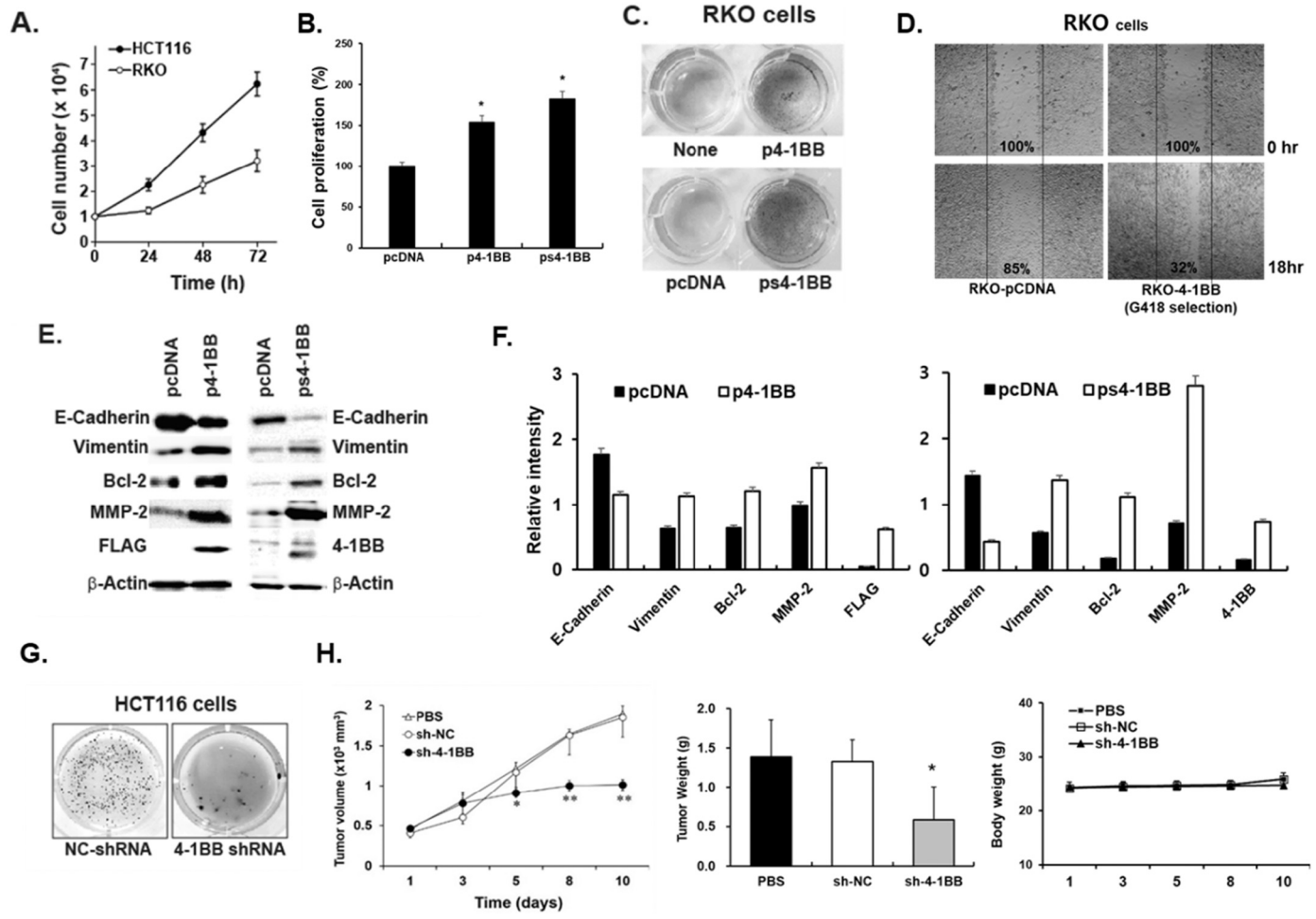

Fig. 3. 4-1BB effect on the proliferation and invasion of colorectal cancer cells
(A) Proliferation of HCT116 and RKO cells was investigated. The values are presented as the mean ± standard deviation (SD). (B) RKO cells were transfected with p4-1BB or ps4-1BB plasmids (C) Colony formation of the untransfected RKO cells or those transfected with an empty vector (pcDNA), p4-1BB, or ps4-1BB was determined using agar plates. (D) Cell migration was performed using RKO cells transfected with pcDNA or ps4-1BB, and open area were indicated in panel (E) Whole cell extracts prepared from the RKO cells transfected with pcDNA, p4-1B, or ps4-1BB were used for Western blotting of the indicated proteins. β-actin was used as a loading control. (F) Bar graphs showed normalized Western blot data. Each set of Western blot data was quantified based on the β-actin. (G) HCT116 cells were transfected with shRNA that inhibited 4-1BB or with a scrambled sequence (NC-shRNA). Colony formation of the cells was determined using agar plates. (H) HCT116 cells transfected with either NC-shRNA or 4-1BB-shRNA were injected into the flank region of mice, and tumor growth was determined after 30 days. Tumor weight and total body weight were also measured.

that was missed exon 8 of 4-1BB. The splice variant resulted in a frame shift and early termination of protein translation. Thus, the short form did not have the transmembrane domain so it localized in the cytoplasm of the cells consequently. The short form was highly detected in HCT116 cells compared to RKO cells.

3.3. 4-1BB effect on the proliferation and invasion of colorectal cancer cells in vitro and in vivo

The proliferation rate was compared with 4-1BB high expressed HCT116 cells and 4–1BB low expressed RKO cells. The result revealed that the proliferation of HCT116 cells was faster than it of RKO cells (Fig. 3A). To verify whether 4-1BB expression affected the proliferation of the cells, RKO cells were transfected with plasmids containing either the full length 4-1BB (p4-1BB) or short form (ps4-1BB) and confirm the expression of proteins by immunoblotting. The RKO cells were transfected with or the empty vector (pcDNA) using the Lipofectamine 2000 Reagent (Invitrogen) according to the instructions of the manufacturer. Two days later, G418 (Invitrogen) was added at concentration of 0.3 mg/ml transfected RKO. Several clones and mass populations of transfectants were selected for 2 weeks, expanded, and screened for 4-1BB and s4-1BB.

Transfected cells with either of the forms proliferated faster than the cells transfected with an empty vector (Fig. 3B). The RKO cells transfected with ps4-1BB formed most colonies and invaded more than the cells transfected with the empty vector (Fig. 3C and D). Proteins involved in epithelial-mesenchymal transition (EMT), invasion, and the anti-apoptosis were evaluated.

RKO cells transfected with either full 4-1BB (p4-1BB) or short soluble 4-1BB (ps4–1BB) exhibited higher levels of the proteins involved in tumor invasion and anti-apoptosis, such as vimentin, human matrix metalloproteinase-2 (MMP2), and B-cell lymphoma 2 (Bcl-2) expression than those transfected with the empty vector. We also detected that E-cadherin expression was downregulated. (Fig. 3E and F).

In contrast, HCT116 cells were transfected with 4-1BB shRNA to reduce 4-1BB expression. The cells with reduced expression of 4-1BB showed less colonies (Fig. 3G). The cells transfected with 4-1BB shRNA were injected into mice subcutaneously, and tumor growth was monitored. Tumor volume, tumor weight, and whole mouse body weight was measured (Fig. 3H). The size of the tumors that developed following injection with HCT116 cells transfected with shRNA was smaller than the ones that developed after injection with HCT116 cells

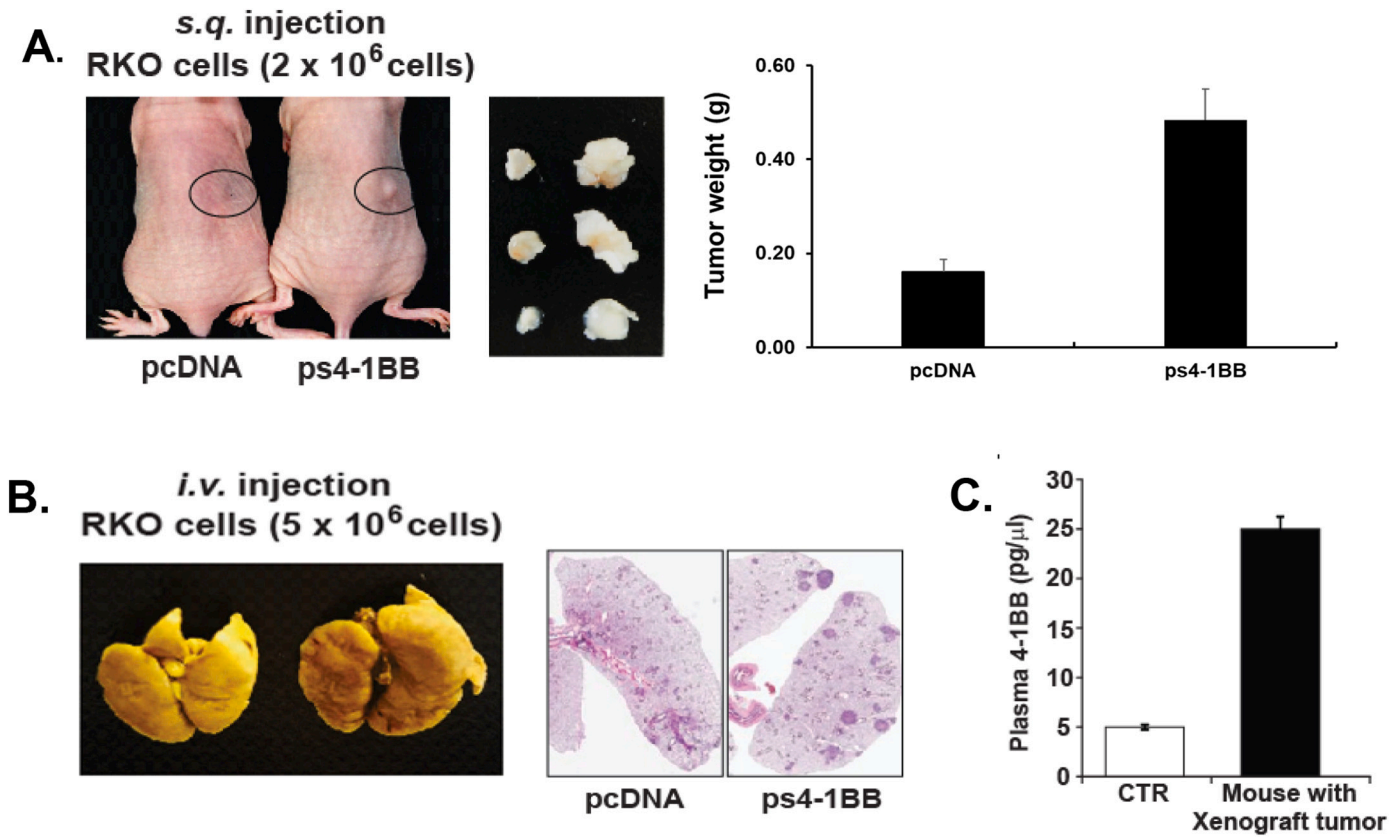

Fig. 4. Effects of 4-1BB on the tumorigenesis and metastasis of colorectal cancer cells *in vivo*
(A) RKO cells (2×10^6 cells) transfected with either pcDNA or ps4-1BB were injected into the flank region of mice, and tumor growth was determined. (B) RKO cells transfected with either pcDNA or ps4-1BB were injected intravenously into the tail vein of mice. Eight weeks after injection, the lungs were isolated. H&E staining of the lung tissue was performed (right). (C) 4-1BB concentration in the plasma of control mice (CTR) and mice with the HCT116 xenograft tumor was measured using enzyme-linked immunosorbent assay.

transfected with negative control shRNA or no transfected cells. These results suggested that 4-1BB expression was related to cancer development.

3.4. Effects of 4-1BB on the tumorigenesis of colorectal cancer cells in vivo

To identify whether 4-1BB expression in cancer cells could affect cancer development, RKO stably expression cells (empty vector or soluble 4-1BB, ps4-1BB) were injected into nude mice on a flank region and the growth of the tumor mass was monitored. The ps4-1BB expressed RKO cells displayed bigger tumor sizes than those empty vector (Fig. 4A). When ps4-1BB expressed RKO cells were injected intravenously into mice, tumor masses developed in the lungs (Fig. 4B). The hematoxylin and eosin (H&E) staining revealed cancer nodes in the lung injected ps4-1BB expressed RKO cells (Fig. 4B). Interestingly, we found the short form of 4-1BB in the blood of mice with the HC-T116 tumor xenografts, which suggested that short form 4-1BB could be secreted from the cancer cells that expressed it (Fig. 4C).

3.5. Role of NF-kB on 4-1BB expression in colorectal cancer cells

In the activated T-cells, NF-kB and activator protein 1 were the transcription factors activated through the phosphatidylinositol pathway that induced 4-1BB transcription. They regulated 4-1BB expression as *cis*-binding elements in the promoter of 4-1BB between 0.9 and 1.1 kb from the translation start site [16]. Furthermore, phenol myristate acetate (PMA) stimulation induced tumor necrosis factor receptor-associated factor 1 (TRAF1) and its expression was regulated by protein kinase C/NF-kB dependent pathway in human colorectal cancer [17]. To determine whether 4-1BB expression in colorectal cancer cells is regulated by NF-kB, PMA/Ionomycin (Io) was applied to stimulate HCT116 and RKO cells. The proliferation rates of both cell types were increased by PMA/Io treatment (Fig. 5A). Transcriptional expression of 4-1BB was induced by PMA/Io treatment in both HCT116 and RKO cells, although the expression level in HCT116 cells was markedly higher than in RKO cells (Fig. 5B). Next, we investigated whether the 4-1BB gene promoter activity was regulated by NF-kB. Plasmids with the full promoter region of the 4-1BB gene and with deletion of the NF-kB binding site in the promoter (Fig. 5C), which were previously characterized [16], were transfected to HCT-116 cells. Luciferase activity driven by the full promoter was induced by PMA/Io treatment, whereas the construct with deletion of the NF-kB binding site did not show induction of luciferase activity by PMA/Io (Fig. 5D). These results suggested that transcription factor NF-kB might regulate 4-1BB expression in colorectal cancer cells.

4. Discussion

The roles of 4-1BB in immune cells, which are immune cell proliferation, activation, survival, and anti-tumor function, have been intensively studied [5,18]. Interaction of 4-1BB and its ligand, 4-1BBL plays important roles in T-cells and natural killer cells with respect to cell division, induction of cytokine production, enhanced effector function, and increased cytotoxicity [12,13]. Although 4-1BB in immune cells ha intensively studied, expression of 4-1BB in the cancer cells was not much studied. Here, we identified 4-1BB expression in the colorectal cancer cells in both tissues from patients and established cell lines. There were

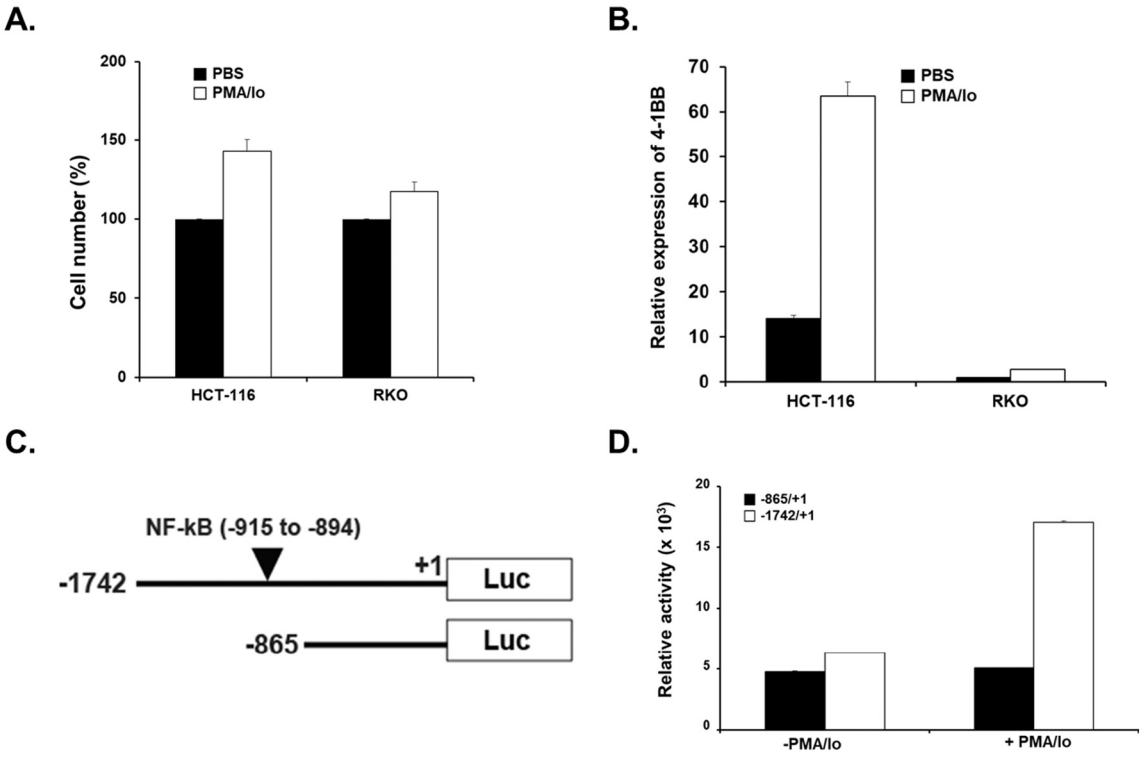

Fig. 5. Role of NF-kB on 4-1BB expression in colorectal cancer cells
(A) HCT116 and RKO cells were treated with PBS or PMA/Io, and changes in cell number were measured 3 days after treatment. (B) Relative mRNA expression of 4-1BB in HCT116 and RKO cells 2 days after PMA/Io treatment. (C) Reporter constructs containing the promoter region of 4-1BB. Nucleotide numbers were counted from the translation start site. (D) HCT116 cells were transfected with the reporter constructs. Two days after transfection, the cells were treated with PBS or PMA/Io, and luciferase activities were measured 24 h after the treatment. The values indicate the mean ± SD of three replicates.

two forms of 4-1BB, full size 4-1BB located in the plasma membrane and short form of 4-1BB, which both played roles in cancer cell proliferation, invasion, and metastasis. The expression level of 4-1BB was correlated to the cell proliferation rate in the colorectal cancer cells. We also detected that epithelial maker, E-cadherin expression was downregulated and mesenchymal maker, vimentin was increased (Fig. 3E). It explained EMT phenotypic feature that weakened cell-to cell adhesion and gained invasion capacity. EMT is an early step in the tumor metastasis [19,20]. However, 4-1BB effect on the colorectal cancer cell proliferation and metastasis was independent from the function of immune cells, and the mechanism and signaling pathways of 4-1BB involved in cancer cell proliferation and metastasis remain to be determined.

A lot of studies have reported that soluble form of 4-1BB that lacks the transmembrane domain was released from the activated lymphocytes like T cells. It has been detected in various circumstance in humans and mice, including in the blood of patients with rheumatoid arthritis and colorectal cancers [18,21–23]. Interestingly, in this study, we also found the higher level of soluble 4-1BB in the blood of mice with the HCT116 tumor xenografts than in the blood of control mice (Fig. 5C). It is possible that 4–1BB can be released from either immune cells or the cancer cells. Recently, CT26 (colon carcinoma) cells induced CD137 (4-1BB) expression and the higher concentration of soluble CD137 was detected in the culture supernatant of CT26 cells in hypoxia condition than in normoxia [24]. Probably, the 4-1BB released from hypoxic tumor microenvironment might affect the functions of other cells, such as endothelial and immune cells, resulting in increased tumor progression and metastasis. Since the cancer cells expressed soluble short 4-1BB proliferated faster (Fig. 3B) and induced proteins involved in the EMT (Fig. 3E) in this study, the secreted form of 4-1BB could be used as a cancer diagnostic and progressive marker that can be detected in the blood of patients.

The therapeutic potential of anti-4-1BB monoclonal antibodies has been assessed in both *in vitro* experiments and clinical studies, which have supported the notion of targeting the 4-1BB pathway for cancer immunotherapy [25]. It has reported that anti-4-1BB monoclonal antibody treatment stimulated the T cell immune system and brought antitumor effect through increasing cytotoxic T cell activity against the tumor [26]. However, the secreted 4-1BB from the cancer cells might be able to interact with administered anti-4-1BB monoclonal antibodies for therapeutic purposes and thereby affect its application potential in cancer immunotherapy, which should be considered upon testing of the therapeutic antibodies.

4-1BB expression was explored on leukemia [27], lymphoma [28], lung cancer [29], and pancreatic cancer [30]. Lung cancer and pancreatic cancer cells are frequently K-Ras mutated cancer cells. In this study 4-1BB expression confirmed in colorectal cancer was anticipated due to frequent K-Ras mutations of colorectal cancer. When K-Ras mutaion was induced, mitogen-activated protein kinase and NF-kB pathway could be activated [8]. NF-kB plays important roles in the cancer related processes. It enhances cell proliferation and angiogenesis, inhibits apoptosis, and promotes cell invasion and metastasis [31]. Studies on colitis-associated cancer have revealed that NF-kB directly promoted tumorigenesis in colitis-associated cancer [31]. Our results showed that 4-1BB gene expression might induce by NF-kB, which indicated that 4-1BB was a downstream player of NF-kB pathway in the development and progression of colorectal cancer.

One significant feature of cancer development is immune modulation and evasion [7]. Cancer cells express molecules that block immune checkpoints, such as PD-L1 and CTLA-4, to avoid immune surveillance [8]. Beyond the cancer cell proliferation, tumor-mediated 4-1BB expression in the cancer cells could modulate T cell immunity as competitive binding 4-1BBL on the T cells or other APCs, inhibiting T cell proliferating. The interaction between 4-1BB(or 4-1BBL) from cancer and 4-1BBL (or 4-1BB) from immune cells and their regulatory

mechanisms remain largely unknown. It needs to explore to understand complex 4-1BB signaling for cancer immunotherapy.

5. Conclusion

Function of 4-1BB expression in the colorectal cancer cells showed effects on cancer cell proliferation, invasion, and metastasis. In aspect of the transcriptional regulation, NF-kB might be one of transcription factors to regulate 4-1BB transcription as in activated T-cells. This study reported that colorectal cancer cells expressed 4-BB to promote cancer cell proliferation and metastasis. It implies that 4-1BB might be a new target for colorectal cancer therapy.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Inha University Hospital (INHAUH 2019-04-018). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Inha University (IACUC 170912-515-4).

Consent for publication

Not applicable.

Availability of data and materials

Available upon reasonable request.

CRediT authorship contribution statement

Min-Kyoung Kim: Conceptualization, Investigation, Writing – original draft. **Kyung-Ju Shin:** Conceptualization, Visualization, Writing – review & editing. **Sijeong Bae:** Investigation, Validation. **Jin-Myung Seo:** Investigation. **Hosun Jung:** Investigation. **Young-Ah Moon:** Conceptualization, Writing – review & editing, Supervision. **Su-Geun Yang:** Conceptualization, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare no competing of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.120899.

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