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RESEARCH

Amelioration of Colorectal Cancer Using Negative Lipidoid Nanoparticles to Encapsulate siRNA Against APRIL by Enema Delivery Mode

Weifeng Ding · Guihua Wang · Keke Shao · Feng Wang · Hua Huang · Shaoqing Ju · Hui Cong · Huimin Wang

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Abstract A proliferation-inducing ligand (APRIL) is a key cell proliferation-regulatory molecule and have been investigated well enough in immunity regulation and a few of immune diseases. APRIL can stimulate tumor cell growth and is up-expressed in cancer tissues, especially in CRC (colorectal cancer). However, whether inhibition of APRIL can regulate tumor-relative genes expression in vivo and subsequently ameliorate the pathological progress of CRC remains obscure. To address this question, we developed a novel negative lipidoid nanoparticles (NLNs) encapsulating small interference RNA (siRNA) for selectively silencing APRIL in the parenchyma of CRC focus in vivo, which uptake proceeded through a lipid raft endocytotic pathway. Local enema delivery of APRIL-NLNs silenced APRIL in CRC cells and animal models, and then ameliorated experimentally the progress of CRC by suppressing CRC cell proliferation, metastasis, and apoptosis-related cytokine expression and did not affect the function of liver and kidneys and not trigger the immune response of CRC models. This study reveals APRIL to be a potential anti-CRC target by in vivo experiments, and suggests that the application of similar modes of siRNA delivery may be feasible in other therapeutic settings.

Keywords Colorectal cancer · A proliferation-inducing ligand · Negative lipidoid nanoparticles · siRNA · Enema · Small-molecule drugs

Abbreviations

NLNs	Negative lipidoid nanoparticles
PBS	Phosphate- buffered saline
APRIL	A proliferation-inducing ligand
DMH	Dimethylhydrazine
siRNA	Small interference RNA
H&E	Hematoxylin and eosin
CRC	Colorectal cancer
IMH	Immunohistochemistry
CDX 2	Caudal-related homeobox transcription factor 2
CK-18	Cytokeratin 18
TNF	Tumor necrosis factor
RNAi	RNA interference
qRT-PCR	Quantitative real-time PCR
2′OMe	2′-O-methylribonucleotide
ELISA	Enzyme-linked immunosorbent assay

Introduction

Colorectal cancer (CRC) is the third most common cancer and second leading cause of death from cancer in North America [1]. Its incidence is also rising at an alarming rate in the developing countries now. Although, thus far, significant progress has been made in the adjuvant systemic treatments

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of CRC, including chemotherapy and targeted therapy, clinical oncologists are still struggling to optimize adjuvant treatments in CRC patients due to their modest effects. Recently, APRIL (a proliferation-inducing ligand), a member of the tumor necrosis factor (TNF) family, has been reported as bio-markers for some tumors, such as CRC, pancreatic cancer and non-small cell lung cancer [2–4]. Notably, increased expression of APRIL has been observed in the parenchymas of human cancers of colon, rectum, thyroid and lymphoid tissues, as well as CRC cell line SW480 and lung cancer cell line A549 [5].

APRIL, also called TNFSF13, TRDL-1 or TALL-2, with homologous structure and function to several other cytokines in TNF family, can stimulate tumor cell growth in vitro and in vivo [5], has also been recognized as a carcinogenic factor [6–8]. It can act in a membrane-bound form or as proteolytically processed, soluble cytokines within the Golgi apparatus in an autocrine, paracrine, or endocrine manner [9]. Except for its higher expression level in the tissue of CRC, down-regulation of APRIL in SW480 cells can conduce to greatly decreased activity of phosphoinositide 3-kinase (PI3K)/Akt pathway [7]. Moreover, using the cell models, our previous study [10–12] have demonstrated that knockdown of APRIL gene can dramatically inhibit the invasion and proliferation of CRC cells and pancreatic cancer cells in vitro. However, it is still undetermined whether APRIL siRNA can improve the pathological progress and clinical manifestation of CRC in vivo.

Although many efforts to address the challenge of siRNA delivery have produced a variety of siRNA delivery platform [13, 14], among these, lipid-based delivery system is still widely accepted within the body for its high efficacy, ease of use in vivo, and reproducibility [15, 16]. Another important influential factor not be ignored is that the siRNA delivery method should be involved in a adopt delivery platform, because local or systemical delivery mode directly determines the efficacy of siRNA delivery.

Based on the aforesaid consideration, we hypothesized that reduced expression of APRIL through RNAi (RNA interference) could induce the apoptosis and necrosis, suppressed the metastasis of CRC cells in SW480 tumor implanted nude-mice and DMH-induced CRC mice, and prolonged the survival of CRC mice, which developed clysis as the delivery strategy of 2′OMe modified APRIL siRNA entrapped in negative lipidiod nanopaticles (NLNs), a novel lipid-like nanometer biomaterial synthesized by ourselves to CRC tissues [17]. Our study would verify the amelioration effection of APRIL siRNA enveloped by NLNs in vivo and may find considerable value in producing local target therapeutics that addresses CRC.

Methods and Materials

Cell Lines

Mouse CRC cell line (CT-26) and human CRC cell line (SW480) were maintained in DMEM (Biomics Biotech, China) supplemented with 10 % fetal bovine serum (Gibco, USA), 1 mM sodium pyruvate and 4 mM L-glutamine at 37 °C in 5 % CO_2.

Preparation of Synthetic siRNAs

Synthetic APRIL siRNAs, with 2′-O-methylribonucleotide (2′OMe)-sense strand modifications, were manufactured by Biomics Biotech, Inc.. Sequences of the oligoribonucleotides and their respective 2′OMe modifications were characterized by mass spectrometry and high-performance liquid chromatography.

Negative Lipidoid Nanoparticles Synthesis and NLNs-siRNA Formulation

The description of negative lipidoid nanoparticles (NLNs) synthesis and NLNs- siRNA formulation has been previously reported in detail [17]. Briefly, $98N_{12}$-5(1), mPEG2000-C12/C14 lipid, and cholesterol developed NLNs spontaneously at a molar ratio of 4:3:3 in 125 mM sodium acetate buffer (pH 5.2). NLNs-siRNA formulation was synthesized with NLNs and APRIL-siRNAs at the ratio of 8:1 (wt/wt), and then was stored at − 20 °C for preparing.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and reverse transcribed using Fermentas reverse transcriptase (Fermentas, Lithuania) according to the manufacturer's instructions. 2 μl of each 20 μl reverse transcription reaction was used as cDNA template for qRT-PCR, using SYBR Green 2x RT-PCR mix (Qiagen, USA) and 1 μM of each primer. Reactions were performed on a LightCycler PCR instrument (Roche, Germany), with the following cycle parameters: 94 °C, 3 min; 94 °C, 30 s; 50–65 °C, 20 s, 80–85 °C, 1 s×35–40 cycles. Melt curves were analyzed from 72 to 99 °C and products run on 2 % agarose gels to verify homogeneity and correct size of all products.

Antibodies and Immunoblotting

Protein extracts of cells or tissue specimens (10 mg/lane) were resolved by 5 %–12 % SDS-PAGE (Beyotime, China), transferred to a 0.22 μm PVDF membrane (Invitrogen), and analyzed by immunoblotting with affinity-purified polyclonal

anti-APRIL (Bioworld tech, Inc.) or other antibodies. Anti-actin mAb was from Abcam, anti-MMP-9 and anti-Villin mAbs were from Santa Cruz, anti-MMP-2 mAb was from Novus Biological, Llc., anti-TIMP-1 mAb was from Thermo Scientific, anti-PCNA mAb was from Maixin-Bio. Anti-Bcl-2, anti- Bcl-xL, and anti-Bax pAbs were from R&D systems.

Determination of sAPRIL Concentration

The sAPRIL content in cell culture supernatants or sera of mice was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA; Uscnk Life Science Inc., China) employing a non-competing pair of antibodies for each ligand as described in manufacturer's instructions. The concentration of sAPRIL was calibrated from a dose response curve based on reference standards. The experiment was repeated three times.

Cell Proliferation Potential Assay

Three different cell suspensions in a 24-well culture plate were prepared after transfection by NPCsiAP, random-siRNAs and non-transfected parental CT-26 cells. The protocol was as follows: 2×10^4 cells per well was as the initial cell number, counted once every 24 h by microscope after trypsinization for a total of seven times. The cell growth curve profile was drawn by using culture time as the x axis and cell number as the y axis.

Fluorescence Activating Cell Sorter (FACS) Analysis

For the apoptosis assay, 1×10^6 human SW480 cells were stained with PI and annexin V-FITC for 30 min after adding NPCsiAP or siRNA-random control by fluorescence activating cell sorter. The TUNEL assay was performed using the in

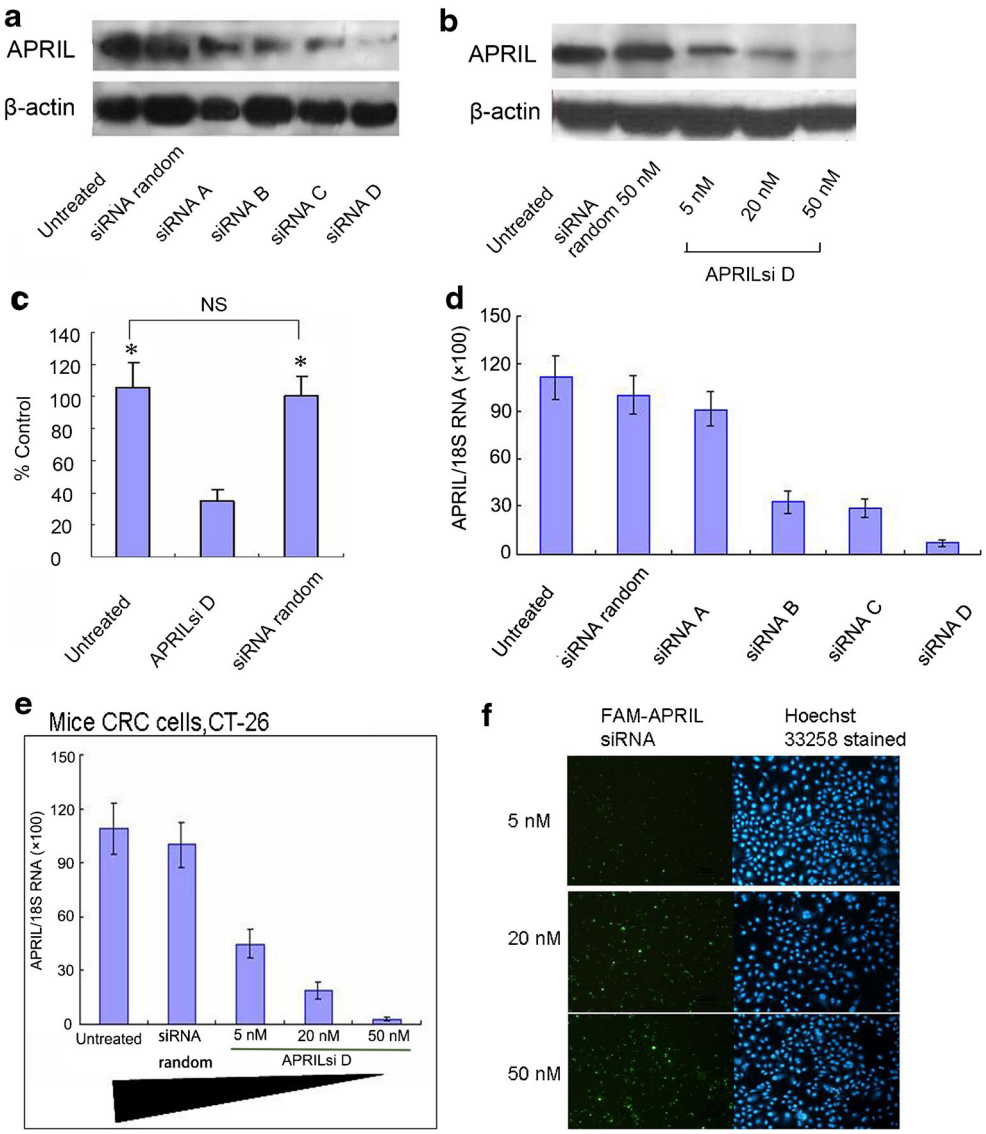

Fig. 1 Suppressive effect of APRIL-siRNAs on APRIL expression and sAPRIL secretion in CT-26 cells. **a** Western blot analysis was used to analyze the expression of APRIL or β-actin (normalization control) in CT-26 cells transfected with four types of APRIL-siRNAs (A, B, C, D) or random-siRNAs by using a commercial transfection reagent, Lipofectamine 2000. **b** Dose-dependent inhibition of APRIL expression by APRIL-siRNA D (APRILsi D). **c** sAPRIL secretion in CT-26 cells treated with APRILsi D or with random-siRNAs (control) was assayed by ELISA, 2 days after transfection. Data were expressed as mean ± s.d. calculated from five transfections and as a percentage of random-siRNAs control. *P<0.01 versus APRILsi D. NS, not significant. **d** Quantitative RT-PCR was performed to analyze the expression level of APRIL and 18S RNA (normalization control) in CT-26 cells transfected with 4 types of APRIL-siRNAs (A, B, C, D) by using Lipofectamine 2000. Substantial suppression of APRIL bands by siRNA (A), (B), (C), (D) compared with random-siRNAs. **e**, **f** Quantitative RT-PCR and FAM-labeled fluorescence (green) staining to optimize the transfection concentration of APRILsi D. Data were expressed as mean ± s.d. (n=3)

situ Apoptosis Detection Kit (Beyotime) to further evaluate tumor cell apoptosis. After incubation with proteinase K (20 μg/ml) at 25 °C for 30 min, the TUNEL reaction mixture containing BrdUTP, terminal deoxynucleotidyl transferase and reaction buffer was added to the slides, which were incubated in a humidified chamber at 37 °C for 60 s, followed by rinsing and incubation with a fluorescein isothiocyanate-labeled anti-BrdU monoclonal antibody at room temperature for 30 min. The reaction was visualized by fluorescence microscopy.

Treatment of Animals

Female BALB/c (nu/nu genotype) athymic mice and female ICR (Institute of Cancer Research) mice 4 weeks old were used. All animal procedures were approved by the Nantong University Institutional Animal Care and Use Committee. 2×10^6 SW480 cells in a final volume of 0.1 ml of PBS (phosphate-buffered saline) were injected subcutaneously (s.c.) on the right flank of BALB/c nude mice 4 times over 4 weeks. Mice were given 0.4 % DMH (dimethylhydrazine) dissolved in saline (20 mg/kg) subcutaneously on the back (b.w.) for once per week until week 20 to induce CRC to emerge.

Histological Observation

After week 20, the ICR mice were scarified by cervical dislocation and then saline perfusion, and the intestine, liver, and other organs (lung, spleen, kidneys, heart, stomach, and pancreas) were harvested. Colorectal tissues were carefully excised and fixed in 4 % buffered formalin for 24 h, followed by dehydration with ethanol and toluene series and embedded in paraffin. Approximately 4 μm-thick serial sections were obtained and stained with hematoxylin and eosin (H&E) for observation with a light microscope (Olympus BX51, Japan).

Immunohistochemistry

Specimens were rinsed with PBS, incubated with serial antibodies at 4 °C overnight, and then rinsed with PBS and incubated at room temperature for 30 min with secondary

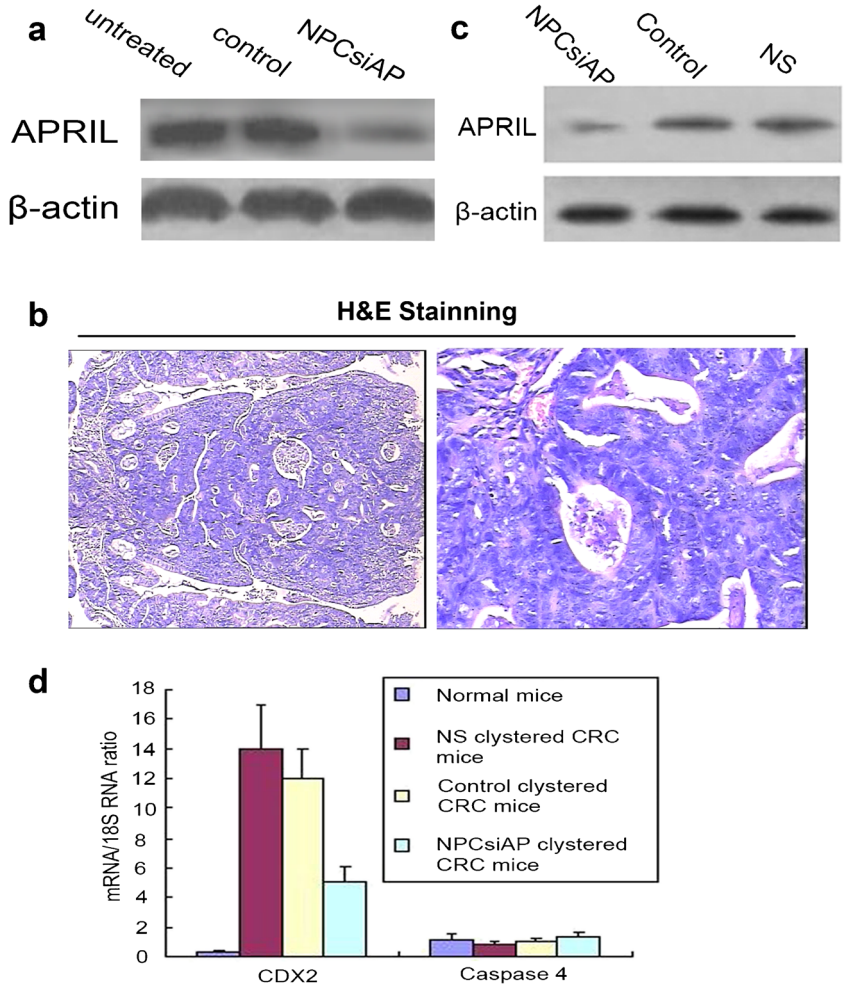

Fig. 2 Inhibition efficacy of APRIL with NPCsiAP nanoparticles **a** NPCsiAP transfected to CT-26 cells for knockdown effiency of APRIL gene expression was determined by western blotting (WB). β-actin was served as control. **b** The pathological photos of intestinal tissue in ICR mice by H&E staining, which were induced to form CRC with 0.4 % DMH (dimethylhydrazine). Magnification: Left, 40 folders; right, 200 folders. **c** WB was used to detect APRIL knockdown effiency of NPCsiAP transduced to CRC mouse models with enema delivery mode in CRC tissues. β-actin was served as control. **d** Quantitative real-time PCR to detect CDX 2 mRNA and Caspase-4 mRNA compared to normalization control, house-keeping gene 18S RNA. Three independent experiments were carried out

antibody, exposed to streptavidin-peroxidase for another 30 min. After rinsing with PBS, diaminobenzidine (DAB) solution was used and counterstained with haematoxylin. Evaluation of immunohistochemistry (IMH) was carried by Allred's score [18], as follows: negative grade (−), low grade (L, total score 1–6), high grade (H, total score 7–8).

Statistical Analysis

Statistical analyses were performed using the SPSS software package (version 13; SPSS Inc.). Results are presented as means (±s.d.) for each sample. Comparisons between groups were analyzed using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. Survival curves were constructed according to the Kaplan-Meier method and tested by Breslow-Gehan-Wilcoxon analysis.

Results

APRIL-siRNAs Suppress APRIL Expression and sAPRIL Secretion

Using Lipofectamine™ 2000 transfection reagents, we first elucidated the effects of four siRNAs (types A, B, C, D) on the abundance of APRIL transcripts (Fig. 1a and d) in a mouse CRC cell line, CT-26, previously be proved to overexpress APRIL [17]. Three siRNAs (types B, C, D) suppressed accumulation of APRIL protein (Fig. 1a) and APRIL mRNA (Fig. 1d), but the greatest efficacy, observed using type D siRNA (APRILsi D), led us to select it for subsequent experiments. Transduction to CT-26 cells with various dosages of APRILsi D revealed an apparent dose-dependent suppression of APRIL expression with almost complete suppression at 50 nM (Fig. 1b and e). Moreover, 50 nM APRILsi D had a highest transfection efficacy compared with 5 nM or 20 nM APRILsi D (Fig. 1f). We then used ELISA to test whether APRILsi D could inhibit sAPRIL (secret APRIL) secretion. The concentration of sAPRIL in the culture medium of CT-26 cells transduced with APRILsi D was significantly reduced compared with parental cells and cells transfected with randomly selected siRNAs (control) ($P<0.01$) (Fig. 1c).

NPCsiAP Suppresses APRIL Expression and sAPRIL Secretion

To validate the effect of NPCsiAP (APRIL siRNA enveloped by NLNs, we termed them as NPCsiAP) on APRIL protein expression and sAPRIL secretion from mouse CT-26 cells, we assessed the APRIL protein level of CT-26 cells by Western blot and the sAPRIL concentration in the culture supernatant by ELISA and found them to decrease significantly compared to that in untreated cells and control (random-siRNAs encapsulated by NLNs)-transduced cells ($P<0.01$) (Fig. 2a).

Furthermore, ICR mice were induced to form CRC animal model by DMH (dimethylhydrazine; 20 subcutaneous injections over 20 weeks), which was verified by H&E stain (Fig. 2b). Determined by PCR and immunohistological staining, almost no expression of APRIL gene was found in normal ICR mice intestine tissues, however, ICR mice induced with DMH highly expressed APRIL mRNA and protein. Then we

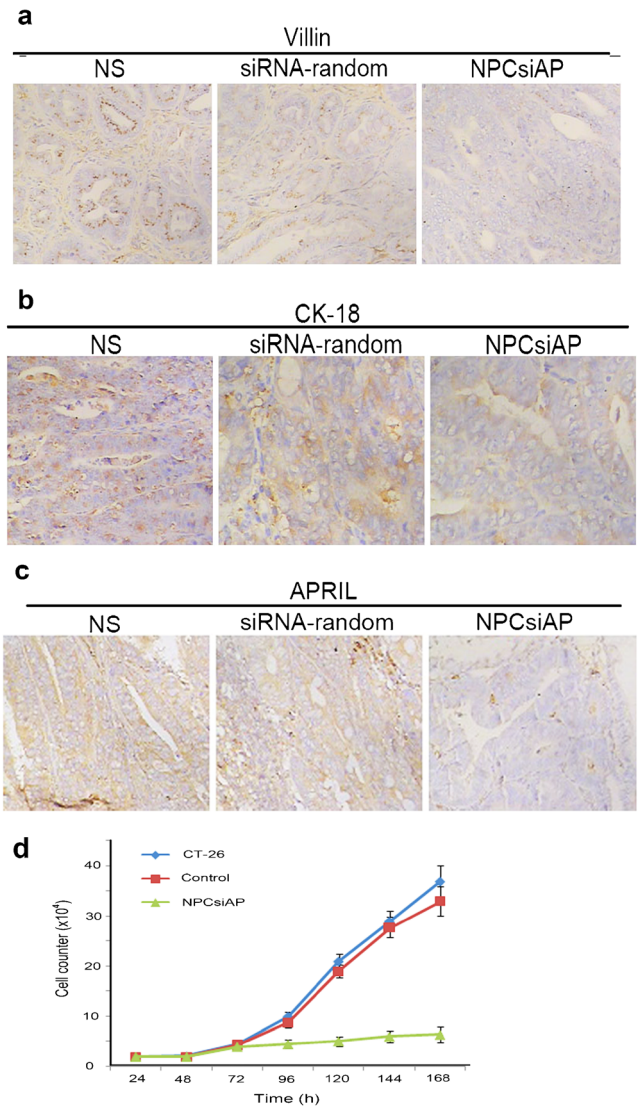

Fig. 3 Anti-proliferation effect of NPCsiAP in DMH-induced CRC mice and CT-26 cells. **a** Representative pictures by immunohistochemistry (IMH) staining of Villin protein after NPCsiAP enema delivery to CRC-induced ICR mice. **b** Representative IMH photos of CK-18 protein after NPCsiAP administration in CRC-induced ICR mice. **c** Representative IMH photos of APRIL protein after NPCsiAP treatment in CRC-induced ICR mice. Magnification: 200 folders. Three independent experiments were carried out. **d** Growth curve profile of CT-26 cells with NPCsiAP treatment, random-siRNAs transfection compared with parental cells. Data were expressed as mean ± s.d. ($n=3$)

delivered NPCsiAP to CRC mice with a unique method, colon-lavage (enema) [17] and also examined the knockdown effect of NPCsiAP towards APRIL in CRC tumor tissue compared to NS (normal saline) and control groups. As illustrated in Fig. 2c, NPCsiAP markedly inhibited the APRIL protein expression in vivo.

Antagonism of Proliferation of Mouse CRC Cells by NPCsiAP Treatment

The tissues in DMH-induced CRC ICR-mice (week 17) that received NPCsiAP (5 mg/kg) five times were examined with a CRC-specific protein, villin by IMH staining. The positive rate of tumorous enteric epithelium area detected by computerized image analysis scoring for villin staining was significantly lower in specimens from NPCsiAP-treated CRC mice than in control specimens ($P<0.001$) (Fig. 3a); these results were consistent with quantitative RT-PCR data for caudal-related homeobox transcription factor 2 (CDX-2) mRNA, a major regulator of intestine-specific genes, which showed substantial suppression of mRNA expression by NPCsiAP treatment (Fig. 2d). Similarly, cytokeratin 18 (CK-18) protein levels in NPCsiAP-treated CRC mice significantly decreased compared to that in control-mouse groups ($P<0.001$) (Fig. 3b). The effect of NPCsiAP on APRIL expression of CRC was also examined by IMH staining in intestine specimens of CRC mice douched with NPCsiAP and random-siRNAs five times (week 17). The positively stained area (brown) substantially reduced in the former (Fig. 3c), which also showed the same results with Fig. 2c.

In addition to antagonism of the growth of the mouse model, NPCsiAP also inhibited the proliferation of CT-26 cells compared to random-siRNAs and parental cells (Fig. 3d).

Inhibition of the Metastasis of CRC With NPCsiAP Treatment

The metastasis capacity of CT-26 cells that treated by NPCsiAP was examined in vitro (Fig. 4a). From Fig. 4b, it was found that the migration capacity of CT-26 cells was

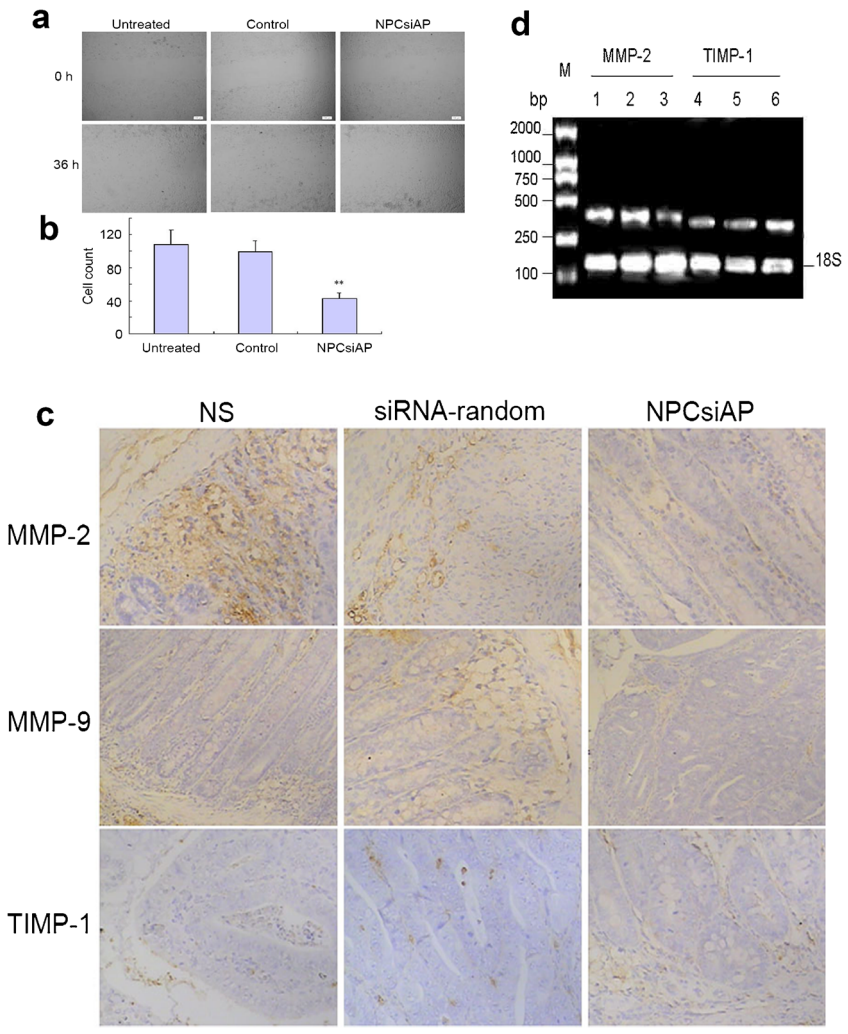

Fig. 4 Anti-metastasis capacity of NPCsiAP in CRC-induced ICR mice. **a** Observed pictures of the migration ability after NPCsiAP treatment by cell scratching experiment. **b** Statistics of the number of migrated cells that crossed the wound edge. Control, random-siRNAs; **, $P<0.03$ compared with control. **c** Representative IMH photos of extracellular matrix (ECM) protein- MMP-2, MMP-9, and TIMP-1, which are related to cellular metastasis. Magnification: 200 folders. **d** MMP-2 and TIMP-1 mRNA levels were determined by RT-PCR. 18S, 18S RNA, was served as the reference control. M, marker. Three independent experiments were carried out

suppressed after NPCsiAP transfection. When CRC mice douched with NPCsiAP five times, matrix metalloproteinase 2 (MMP-2), MMP-9 and tissue inhibitor of metalloproteinase 1 (TIMP-1) protein levels in specimens of CRC mice were observed by IMH to detect the metastasis of CRC cells in vivo. As elucidated in Fig. 4c, the protein levels of MMP-2 and MMP-9 reduced along with increase in expression of TIMP-1 after NPCsiAP treatment. By determining MMP-2 and TIMP-1 mRNA by RT-PCR, the result was similar with their protein expression (Fig. 4d). Together, these data indicated the metastasis capacity of CRC was obviously impaired through NPCsiAP administration.

Induction of Apoptosis in Mouse CRC Cells by NPCsiAP Administration

The aforesaid observation led us to speculate that NPCsiAP treatment might induce apoptotic death of CRC cells. Apoptosis was determined and TUNEL staining on colorectal specimens from DMH-induced CRC mice treated with NPCsiAP, NS, random-siRNAs. TUNEL-positive cells are apparently increased in CRC mice treated with NPCsiAP versus control or NS (Fig. 5d, $P<0.01$) and the results were also verified by the data of microarray analysis (data not shown).

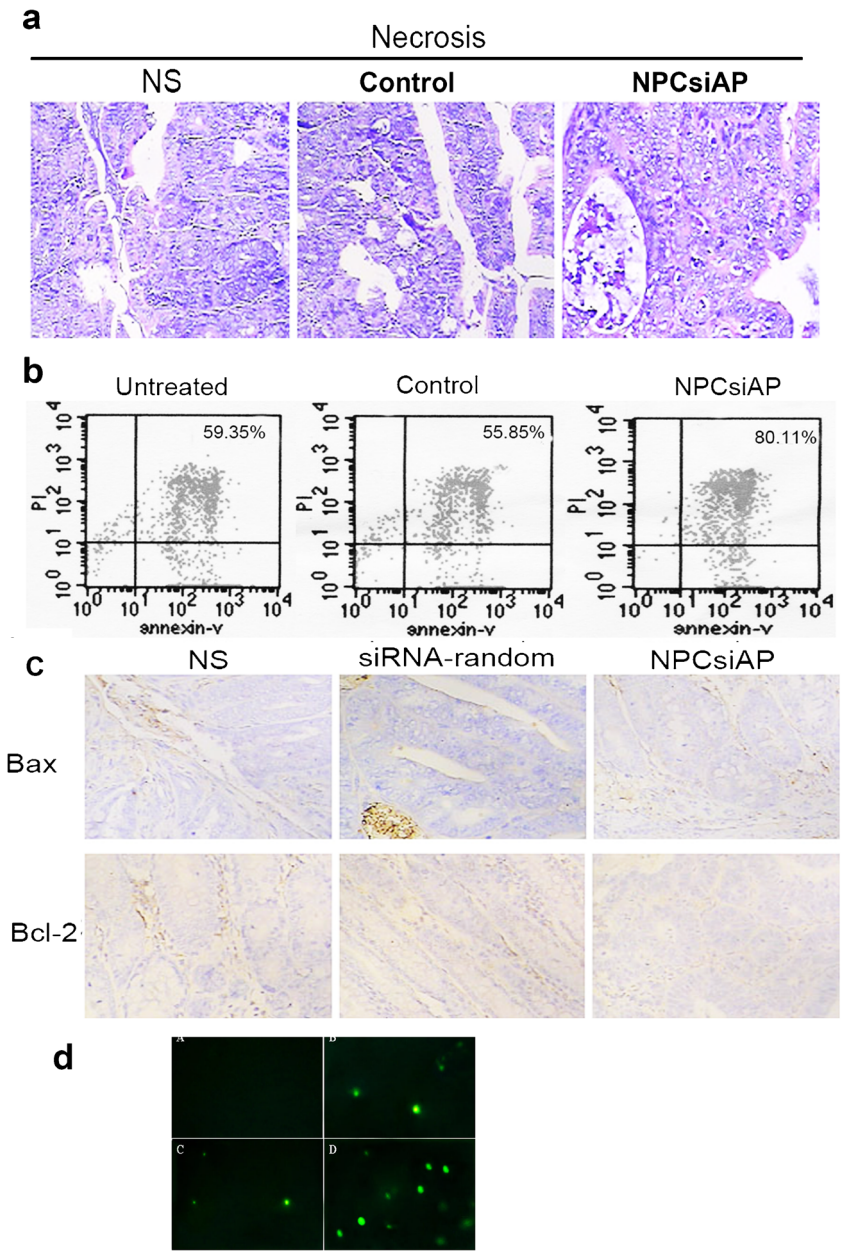

Fig. 5 Apoptosis and necrosis were induced by NPCsiAP enema delivery. **a** Necrosis on CRC tissues transduced with NPCsiAP through haematoxylin-eosin (H&E) staining. Magnification: 100 folders. **b** Apoptosis of CRC cells by FACS analysis with annexin V-FITC/PI double staining. **c** Representative IMH photos of Bax and Bcl-2 protein, which is related to cellular apoptosis. Magnification: 200 folders. **d** Apoptosis of CRC cells with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) experiment. Magnification: 100 folders. A, Background fluorescence. B, Control. C, NS. D, NPCsiAP. Three independent experiments were carried out

In CT-26 cell-cultivation experiments, apoptosis cells in groups which were transfected with NPCsiAP obviously elevated than those in control groups detecting by FCM through Annexin V-FITC/PI staining (Fig. 5b). Bcl-2 and Bax protein by immunohistological observation and mRNA level of Caspase-4 gene detected by PCR also confirmed the aforementioned results (Figs. 5c and 2d). Pathological state, that is, necrosis and architectural alteration, also clearly accumulated in NPCsiAP-treated CRC mouse tissues with haematoxylin-eosin (H&E) staining (Fig. 5a). These findings concluded that APRIL siRNA not only inhibited the metastasis of CRC cells but also abrogated CRC cells in colorectal tissue through apoptosis and necrosis.

Prolonged Survival of CRC Mice With NPCsiAP Treatment

To evaluate the prognosis of CRC mice with NPCsiAP treatment, we examined a cumulative survival rate of ICR-mice exposed to normally non-lethal DMH induction. In these series of experiments, NPCsiAP treatments begun (week 14 and 17) after induction to form CRC by constitutive administrations of DMH. Controls treated with NS or random-siRNAs

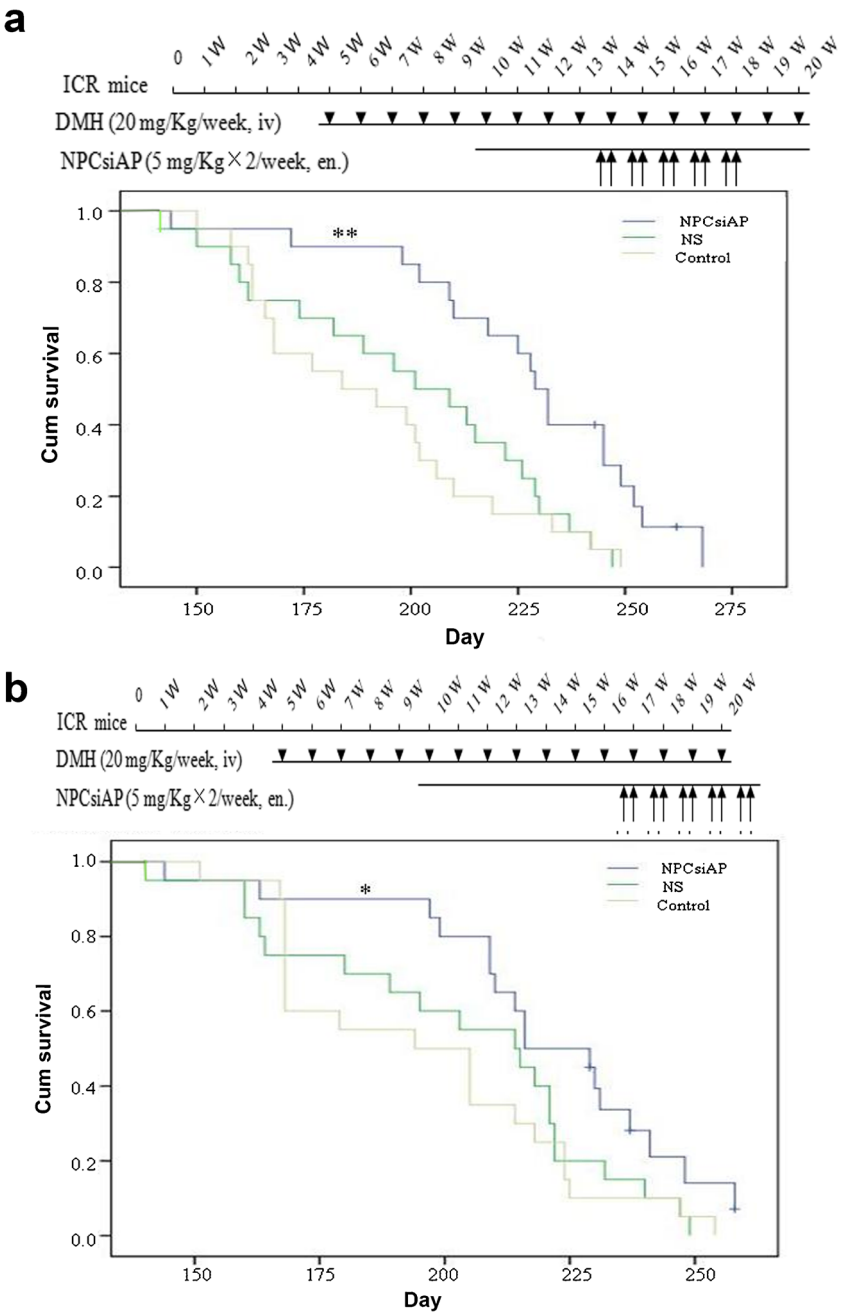

Fig. 6 Survival effect of NPCsiAP enema delivery on CRC mice. **a** Survival of CRC mice that were clystered with 5 mg/kg NPCsiAP twice a week at week 14 (n=20 per group). **b** Survival of CRC mice that were clystered with 5 mg/kg NPCsiAP twice a week at week 17 (n=20 per group). In the control groups, DMH-induced mice were douched with 5 mg/kg random-siRNA and NS (n=20 per group two times a week). Life-table analyses were presented as a Kaplan-Meyer plot. (*P<0.05; **P<0.001 compared with control mice)

twice weekly all died within 221 days after DMH induction (DMH induction began at 36th day).

DMH-indued mice appeared to die from hepatic failure and cachexia [19] when they developed ascites, gastrointestinal bleeding, tarry feces, muscle wasting, muscle atrophy, and weight decline (data not shown). In contrast, CRC mice treated with NPCsiAP twice weekly showed a prolongation of survival time and a much higher survival rate (63.2 %, 12/19 mice; 30.0 %, 6/20 mice or 26.3 %, 5/19 mice) at 5 mg/kg in 2 months (Fig. 6a and b).

Effects of APRILsiB and APRILsiC on CRC Mice

To eliminate the possibility that the in vitro and in vivo results obtained by treatment with APRILsi D (NPCsiAP) resulted from off-targeting effects, we tested the effect of two other siRNAs for APRIL, APRILsiB and APRILsiC, in CRC mice. Prolonged survival (Fig. 7a), reduced metastasis (Fig. 7b) and blockade of sAPRIL secretion (Fig. 7c) were clearly associated with exposure to either siRNA, indicating that the effects of APRILsi D were not related to off-targeting. And an altered pathway in the DNA microarray profiling of cells between high-expressed APRIL gene and low-expressed APRIL with APRIL knockdown were associated with apoptosis, matrix metalloproteinases and so on (data not shown), also confirming that off-target gene regulation was not trigered by APRILsi D.

Effect of H-siAPRIL on the Model of Human CRC Xenografts in Nude Mice

Serial subcutaneous injection of BALB/c nude mice with 2×10^6 SW480 cells (4 times over 4 weeks) leaded to human CRC xenografts. We used this additional implanted tumor

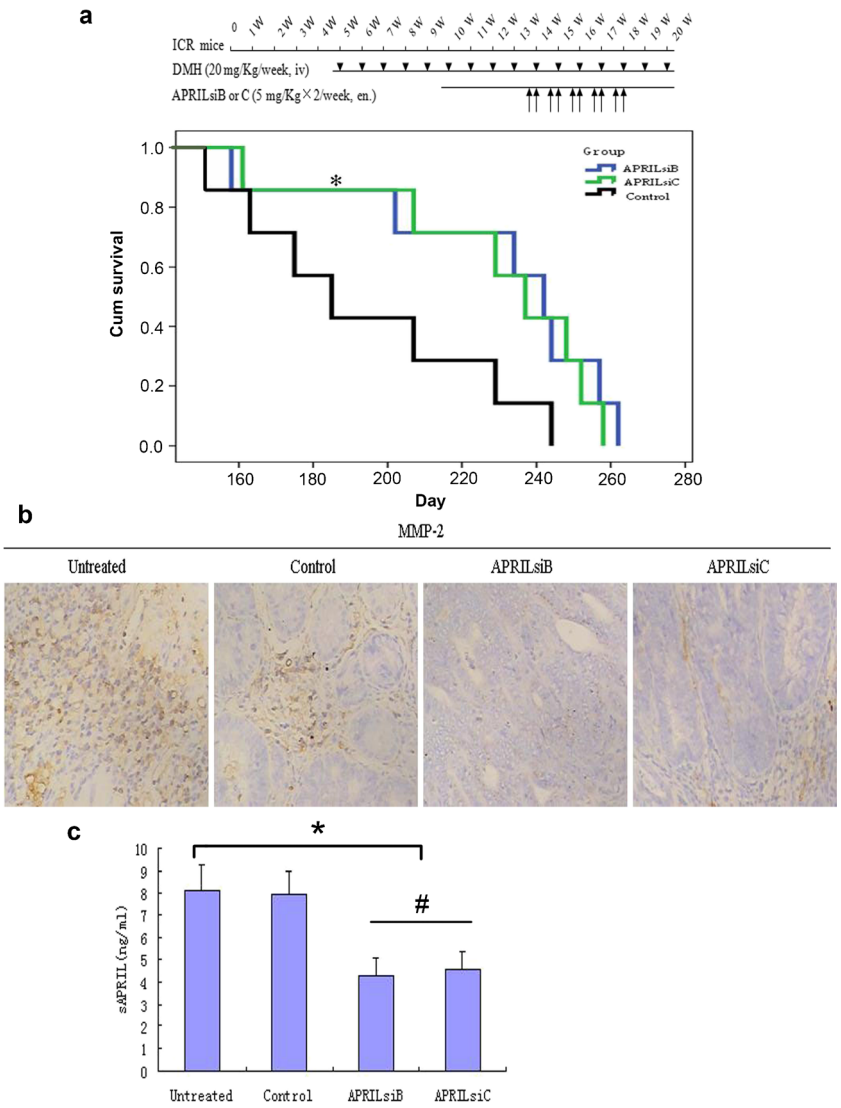

Fig. 7 Effects of APRILsiB and APRILsiC on DMH-induced CRC mice. **a** Schedule of APRILsiB and APRILsiC administration and results of survival rate in DMH-induced CRC mice. Samples were obtained from ICR mice treated with DMH and from PBS injected mice (n=6). Life-table analyses are presented as a Kaplan-Meyer plot (*P<0.05 compared with control mice). No significant prolongation of survival time by both treatments. **b** Representative IMH photomicrographs of APRILsiB and APRILsiC to inhibit MMP-2, which is related to metastasis (n=5, siRNA dose of 5 mg/kg, 3 times). **c** Examination of serum sAPRIL concentration in ICR mice induced with DMH. Data were expressed as mean ± s.d. (n=5). *P<0.05; #P>0.05

model to further assess the anti-proliferative potential of H-siAPRIL (7.5 mg/kg), which human APRIL siRNAs were entrapped in NLNs, by administering them once every 2 days for 2 weeks. The effect of H-siAPRIL was essentially the same as those of NPCsiAP in CRC mouse model induced by DMH with respect to shrinkage of the tumor size (Fig. 8a), obstruction of MMP-2, proliferating cell nuclear antigen (PCNA, Fig. 8b), and inhibition of APRIL protein expression (Fig. 8c) as well as APRIL mRNA level (Fig. 8e). Apoptosis (Fig. 8d), necrosis (Fig. 8f), and Bcl-2 or Bcl-xL (Fig. 8g) levels were also ameliorated in xenotransplantation CRC mice treated with H-siAPRIL.

Discussion

The specific reduction or silencing of gene expression through RNAi has considerable potential to create a new class of therapeutics that addresses previously untreatable diseases [20]. Selection of appropriate gene targets is an important parameter in the potential success of siRNA therapies to cancers. Candidate targets include genes associated with cell proliferation, metastasis, angiogenesis, and drug resistance. Importantly, silencing of such genes must not affect the functions of normal cells. Characters as a siRNA target are very suitable for APRIL gene, which can

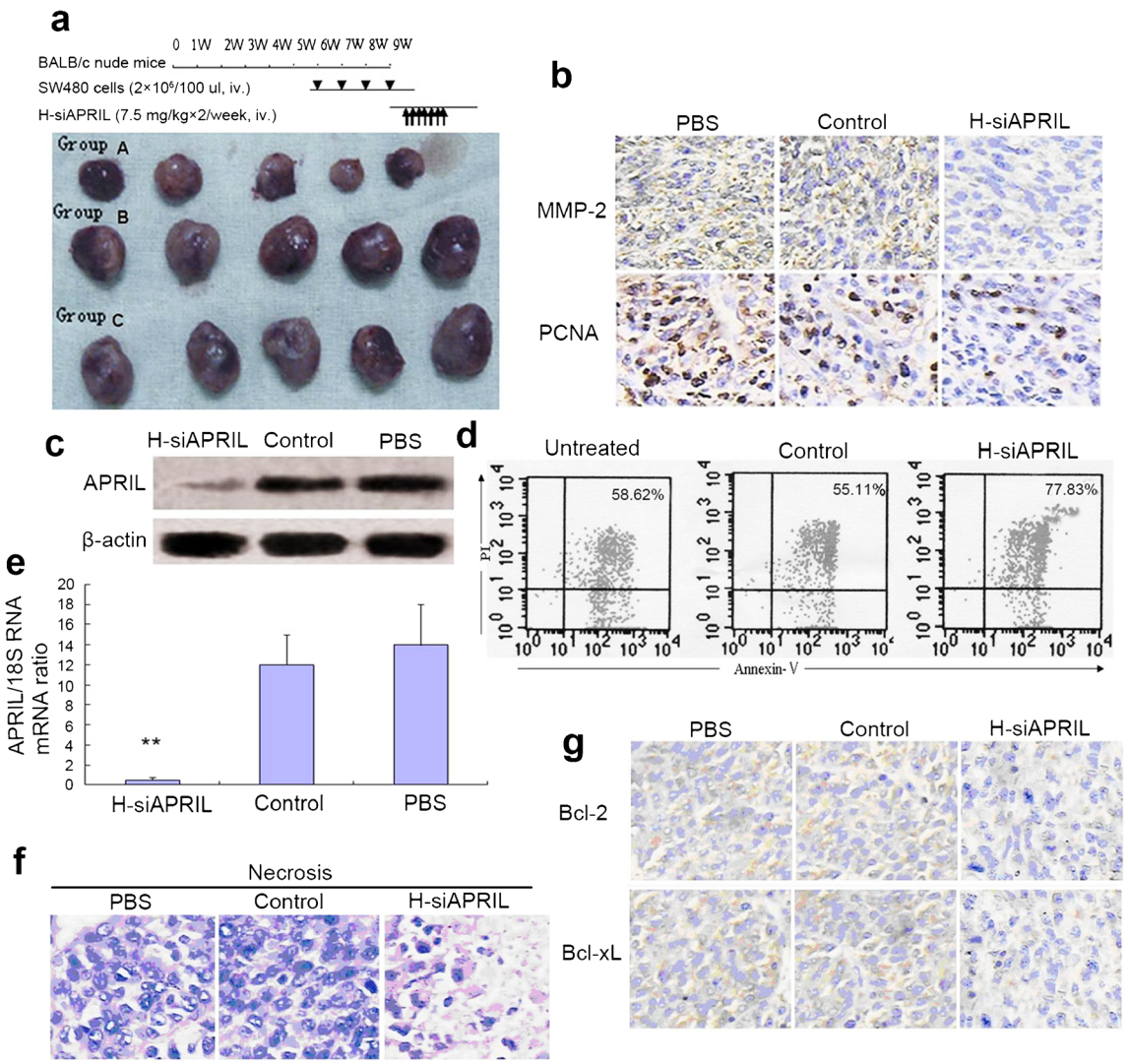

Fig. 8 Effect of i.v. injection of H-siAPRIL on nude mouse CRC xenograft. **a** Schedule of H-siAPRIL treatment in nude mice with SW480 cells implanted and tumor volume perfile after i.v. injection of H-siAPRIL. Group A, H-siAPRIL injection group. Group B, Random-siRNAs control group. Group C, PBS treatment group. (n=5 per group). **b** Representative photomicrographs of IMH staining with antibody protein of PCNA, MMP-2 to examine the effects of H-siAPRIL on anti-proliferation and anti-migration. **c** Western blot and quantitative PCR (**e**) to detect APRIL- knockdown effect of H-siAPRIL. Data were obtained from three randomly independent experiments and represent the mean ± s.d. β-actin was served as control. **d** Apoptosis effect of H-siAPRIL by FACS analysis. **f** Necrosis in CRC tissues transduced with H-siAPRIL through haematoxylin-eosin (H&E) staining. **g** Representative IMH images of Bcl-2 and Bcl-xL to examine the anti-proliferative potential of H-siAPRIL. Three independent experiments were carried out

stimulate tumor cell growth, especially promote colorectal tumorigenesis [5–8].

Development of suitable drug delivery systems (DDSs) is also an important issue. To date, the use of non-viral DDSs is preferred because it offers greater safety for clinical application than does the use of viral DDSs, and the non-viral DDSs includes direct conjugation of delivery agents to the RNA moiety [21], formulation using lipid-[22, 23], polymer-[24, 25] or peptide-based delivery systems [26] and, more recently, complexation with antibody fusion proteins [27, 28]. At these points, our research selected APRIL as a candidate target gene with a novel class DDS, negative lipidoid nanoparticles (NLNs) and a local delivery manner, enema, proved to be efficient to arrive in the parenchyma of colorectal diseases.

The study described here first utilized CRC mouse model obtained from DMH-induced ICR mice. After colon-calage (enema) with NPCsiAP, survival of DMH-induced CRC mice was prolonged in a dose- and duration- dependent manner, indicating a biologically specific effect of NPCsiAP treatment. Incidentally, CRC mice treated at 14 W had a more prolonged survival time than that treated at 17 W, which may be due to early intervention. Then we demonstrated this amelioration effect again with another mouse model, BALB/c nude mice xenografts, to mimic human CRC, which also were seen with the diminution of the tumor size, prolongation of survival time and suppression of tumor metastasis. The consistency of the findings between DMH-induced and xenograft nude mice models suggested APRIL siRNA could improve CRC manifestation by in vivo study and indicated the applicability of our approach for various types of CRC. More to the point, our research found APRIL knockdown can inhibit the CRC cells metastasis by immunohistological observation, PCR and migration experiments (Figs. 5 and 4d), also suggesting a hopeful targeted allopathy in the treatment of mCRC.

Off-target effect [29, 30] is an issue often associated with the use of siRNA that can lead to misinterpretation of siRNA experiments. We used three independent siRNAs against the same target (APRIL) mRNA and found comparable gene silencing efficacy and treatment effects in vivo (Fig. 1a), suggesting that the phenotype observed with downregulation of APRIL was indeed related to APRIL knockdown and not a bystander effect of the siRNA sequence. Immune response and toxic effect [31, 32] are another two issues often related to siRNA application, serum WBC (white blood count) and PLT (platelet) levels in CRC mice had little alteration [17] and DNA microarrays profile after NPCsiAP treatments, suggested no trigger of immune responses. Serum aminotransferase, bilirubin and BUN (blood urea nitrogen) concentrations, which reflect liver and kidney function, were not significantly affected by NPCsiAP treatment in CRC mice [17], also indicated the low toxicity of ongoing NPCsiAP treatment. These results underscore its promise for clinical translation to treat CRC.

Conclusions

Study described here consistently demonstrated that inhibition of APRIL with siRNA by a novel negative lipidoid nanoparticles (NLNs) significantly improved the symptom of CRC in vivo with CRC-induced mouse model and nude mouse xenograft model. This is the first attempt to examine the amelioration effect of APRIL siRNA to CRC through a special enema delivery mode in vivo, revealing APRIL to be a potential anti-CRC target and NLNs to be a promising drug delivery system.

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Competing Interests The authors declare that they have no conflict of interests.

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