ORIGINAL RESEARCH

Screening of potential molecular targets for colorectal cancer therapy

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Abstract: Colorectal cancer is a leading cause of cancer death worldwide. To identify molecular targets for colorectal cancer therapy, we tested small interfering RNAs (siRNAs) against 97 genes whose expression was elevated in human colorectal cancer tissues for the ability to promote apoptosis of human colorectal cancer cells (HT-29 cells). The results indicate that the downregulation of *PSMA7* (proteasome subunit, α-type, 7) and *RAN* (ras-related nuclear protein) most efficiently induced apoptosis of HT-29 cells. *PSMA7* and *RAN* were highly expressed in colorectal cancer cell lines compared with normal colon tissues. Furthermore, *PSMA7* and *RAN* were overexpressed in not only colon tumor tissues but also the other tumor tissues. Moreover, *in vivo* delivery of *PSMA7* siRNA and *RAN* siRNA markedly induced apoptosis in HT-29 xenograft tumors in mice. Thus, silencing of *PSMA7* and *RAN* induces cancer cells to undergo apoptosis, and *PSMA7* and *RAN* might be promising new molecular targets for drug and RNA interference-based therapeutics against colorectal cancer.

Keywords: colorectal cancer, molecular target, RNAi, *PSMA7*, *RAN*

Introduction

Colorectal cancer is one of the most common cancers in women and men worldwide. Nearly 1.2 million cases of colorectal cancer were expected to occur in 2007.[1] The highest incidence rates are found in Japan, North America, parts of Europe, New Zealand, and Australia.[1] Worldwide, some 630,000 people die from colorectal cancer per year, accounting for 8% of all cancer deaths.[1] The five-year survival for persons with colorectal cancer is about 65% in Japan; however, when this cancer is detected at advanced stages, the five-year survival rate decreases to 10%,[2] necessitating effective therapeutic targets.

A tumor is characterized by uncontrolled growth and spread of abnormal cells, which invade adjacent normal tissue and spread to other organs, a process that causes death.[3] Multiple molecular alterations are involved in a transformation from a normal cell into a cancerous cell and a progression from a pre-cancerous lesion to malignant tumors.

Angiogenesis is critical in tumor growth and survival. Its inhibition is a promising target for cancer therapy.[4–6] Vascular endothelial growth factor (VEGF) plays a key role in angiogenesis in cancer. In many human tumors including colorectal cancer, VEGF amd VEGF receptors (VEGFR) are overexpressed. Regulating the VEGF/VEGFR pathway is an effective approach to treat cancer.[6,7] A humanized anti-VEGF monoclonal antibody, Bevacizumab, is the first approved biological inhibitor against VEGF for the treatment of metastatic colorectal cancer.[4–6,8] Small-molecule tyrosine

kinase inhibitors against VEGFRs are also being developed for cancer therapy.[4,6,9,10]

Furthermore, the survival of abnormal cells is a characteristic feature of cancer. In colorectal tumors, some signal transduction pathways drive abnormal cell growth. The most important factor promoting cell survival is epidermal growth factor (EGF).[11,12] Its signaling is a potential target for cancer therapy.[4,5] In treating colorectal cancer, a monoclonal antibody against EGF receptor (EGFR) such as a cetuximab is active,[4,5,12–14] and small-molecular tyrosine kinase inhibitors of EGFRs have been shown to be effective.[4,12]

Additionally, one of the hallmarks of human carcinogenesis is the breakdown of cell apoptotic machinery.[15] Overexpression of anti-apoptotic Bcl-2 family members frequently relates to decreased sensitivity to anticancer drugs and radiotherapy in many types of cancer.[16] The antisense oligonucleotide drug targeting Bcl-2 mRNA expression such as an oblimersen is being investgated in some cancers.[17] The BH3-domain of anti-apoptotic Bcl-2 family proteins is required for the antiapoptotic function. BH-3 mimic peptides that interfere with Bcl-2 signaling are currently under development.[16] Moreover, therapies based on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which induces programmed cell death, have been studied.[18] Monoclonal antibodies against TRAIL receptors with an agonistic effect on the TRAIL pathway have been generated.[18,19] Thus, inducing apoptosis is a promising approach in the development of a molecular targeted therapy for cancer.

In this paper, we focused on apoptosis induction to identify molecular targets for colorectal cancer therapy. We tested siRNAs against 97 genes whose expression was elevated in human colorectal cancer tissues for the ability to promote apoptosis of human colon cancer cells (HT-29 cells). The results showed that the downregulation of proteasome subunit, α-type, 7 (PSMA7) and ras-related nuclear protein (RAN) strongly caused apoptosis of HT-29 cells. PSMA7 siRNA and RAN siRNA markedly induced apoptosis in HT-29 xenograft tumor tissues in mice. This silencing of PSMA7 and RAN that induces cancer cells to undergo apoptosis suggests that PSMA7 and RAN are potential key targets for future RNA interference (RNAi)-based therapeutics against colorectal cancer.

Materials and methods
Cell culture
Five colorectal cancer cell lines were obtained from the American Type Culture Collection (ATCC), and maintained at 37 °C under 5% CO_2 in a humidified incubator. Caco-2 (human colorectal adenocarcinoma) cells were cultured in

Eagle's minimum essential medium (EMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and nonessential amino acids (Invitrogen). Human colorectal carcinoma (HCT116) and human colorectal adenocarcinoma (HT-29) cells were cultured in McCoy's 5A medium (Invitrogen) containing 10% FBS. LoVo (human colorectal adenocarcinoma, derived from supraclavicular lymph node metastatic site) cells were cultured in Ham's F12 medium (Invitrogen) containing 10% FBS. T84 (human colorectal carcinoma, derived from lung metastatic site) cells were cultured in DMEM/Ham's F-12 medium (Invitrogen) containing 10% FBS. To obtain total RNA from these cell lines we plated cells at 1×10^5 cells per well (6-well plate) and culture for three days.

Design and synthesis of siRNAs
We designed siRNAs and synthesized them with four siRNA duplexes for each gene target (Dharmacon, Chicago, IL, USA). The siRNA sequences were described in Table 1.

Cell transfection array
For RNAi-based functional screening of genes, we used a reverse transfection based-cell transfection array.[20] HT-29 cells were plated into the cell transfection array in a 96-well format and transfected with siRNA. We evaluated the effects of the downregulation of genes on promotion of apoptosis, as mentioned below.

Measurement of cell proliferation
We plated HT-29 cells into a cell transfection array at a density of 5×10^3 cells per well and cultured. Three days after, we measured cell proliferation by resazurin reduction assay using CellTiter-Blue Reagent (Promega, Madison, WI, USA). Cells were incubated with CellTiter-Blue Reagent for one hour at 37 °C, and the fluorescence was then measured at 560Ex/590Em. After that, we subjected the same cell transfection array to a caspase-3/7 assay, Hoechst staining, and a cell-direct real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

Measurement of caspase activity *in vitro*
Cells were incubated with the Caspase-Glo 3/7 Reagent (Promega) for one hour at room temperature, and the luminescence was then measured.

Hoechst staining
Cells on a cell transfection array were washed with phosphate-buffered saline (PBS), and a fixative and staining solution was

Table 1 The sequences of siRNAs

	Sense strand	Antisense strand
PSMA7		
#1	5´-GAAGUAUGUUGCUGAAAUUUU-3´	5´-AAUUUCAGCAACAUACUUCUU-3´
#2	5´-GAAGAGACAUUGUUGUUCUUU-3´	5´-AGAACAACAAUGUCUCUUCUU-3´
#3	5´-GAAGAUCUGUGCUUUGGAUUU-3´	5´-AUCCAAAGCACAGAUCUUCUU-3´
#4	5´-CAUCGUGGGUUUCGACUUUUU-3´	5´-AAAGUCGAAACCCACGAUGUU-3´
RAN		
#1	5´-AGAAGAAUCUUCAGUACUAUU-3´	5´-UAGUACUGAAGAUUCUUCUUU-3´
#2	5´-GUGAAUUUGAGAAGAAGUAUU-3´	5´-UACUUCUUCUCAAAUUCACUU-3´
#3	5´-CCUAUUAAGUUCAAUGUAUUU-3´	5´-AUACAUUGAACUUAAUAGGUU-3´
#4	5´-ACAGGAAAGUGAAGGCGAAUU-3´	5´-UUCGCCUUCACUUUCCUGUUU-3´

Abbreviation: siRNA, small interfering RNAs.

added (4% paraformaldehyde, 0.1% Triton X-100, 1 µg/mL Hoechst 33342 in PBS). Twenty minutes after incubation, cells were washed with PBS. We determined the number of apoptotic cells was in three microscopic fields of each well by fluorescence microscopy.

Transfection of siRNA

We carried out transfection of HT-29 cells with siRNA using Liopfectamine 2000 (Invitrogen), according to the manufacturer's protocol. We plated HT-29 cells 24 hours before transfection, and we then transfected the cells, which were grown to 50% confluence, with 40 nM siRNAs.

Real-time RT-PCR

We purified total RNA from cells and tumor tissues with an RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN, Hilden, Germany), and produced cDNAs with an ExScript RT reagent Kit (Takara Bio, Shiga, Japan). We then subjected cDNA samples to real-time PCR using SYBR Premix Ex Taq (Takara) and specific primers as follows: for *PSMA7*, forward, 5´-CAAGTGGAGTACGCGCAGGA-3´; reverse, 5´-CTGCAGTTTGGCCACTGACTTC-3´; for *RAN*, forward, 5´-AAGTTGTCATGGACCCAGCTTTG-3´; reverse, 5´-GCTGGGCTCCAGCTTCATTC-3´. We carried out the reactions using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). We normalized gene-expression levels by 18S rRNA or *GAPDH*.

Cell-direct real-time RT-PCR

We used FastLane Cell cDNA Kit and QuantiTect SYBR Green PCR Kit (QIAGEN). We lysed cells in a well of cell transfection array and synthesized the first-strand cDNA.

The cDNA template was then directly subjected to real-time RT-PCR using specific primers.

siRNA treatment *in vivo*

We performed animal experiments in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute of Japan. We subcutaneously injected 5×10^6 HT-29 cells into athymic nude mice (six-week-old females; CLEA Japan, Tokyo, Japan). When the HT-29 tumor grew to approximately 5–6 mm in diameter, we injected mice with 1 nmol siRNA by intratumoral injection. We harvested tumor tissues for analysis of mRNA and apoptosis at 24 and 72 hours after treatment, respectively.

TUNEL technique

We harvested tumor tissues 72 hours after administration of siRNA and prepared frozen sections. We then performed TUNEL (TdT-mediated dUTP nick-end labeling) staining using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. DNA strand breaks in apoptotic cells can be labeled by the addition of fluorescein dUTP using terminal deoxynucleotidyl transferase (TdT). The nuclei were stained with DAPI. We determined the number of fluorescein-positive cells in three microscopic fields of each section by fluorescence microscopy.

Human samples

The study protocol for clinical samples was approved by the Institutional Review Board of Osaka University Medical School (Osaka, Japan), and written informed consent was

obtained from each patient. We obtained total RNA from tumor tissues and normal adjacent tissues (FirstChoice Human Tumor/Normal Adjacent Tissue RNA) from Ambion (Austin, TX, USA).

cDNA micro-array analysis

We performed cDNA micro-array analysis using AceGene (DNA Chip Research Inc., Yokohama, Japan) according to the manufacturer's instructions (http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf) to obtain an expression profile of human colorectal cancer.[21] As a standard normal control reference, a mixture of total RNA extracted from normal colorectal tissues was used. We synthesized cDNA from total RNA of normal colorectal tissues and colorectal tumor tissues, and labeled cDNA with Cy3 for normal colorectal tissue, and Cy5 for colorectal tumor tissues, respectively. After hybridization of cDNA and array, the array was scanned, and Cy5/Cy3 ratios were $\log_2$-transformed to compare levels of mRNA expression in tumor and normal tissues.

Statistical analysis

We conducted statistical analysis using the analysis of variance with Student's *t*-test. We considered a *P* value of 0.05 or less as a significant difference.

Results
RNAi-based screening for identification of molecular targets

To identify molecular targets for therapy, we conducted a study of RNAi-induced gene knockdown in HT-29 human colon cancer cells. The strategy for target identification is schematically shown in Figure 1. We performed gene expression profiling of 191 subjects with colorectal tumors and selected 97 genes whose expression was elevated in human colorectal cancer tissues by rank order of mRNA expression (Table 2). We used the siRNAs specific to these genes which are siRNA pools composed of four distinct siRNA species targeting different sequences of the same target transcript for RNAi-based screening. We transfected HT-29 cells with the
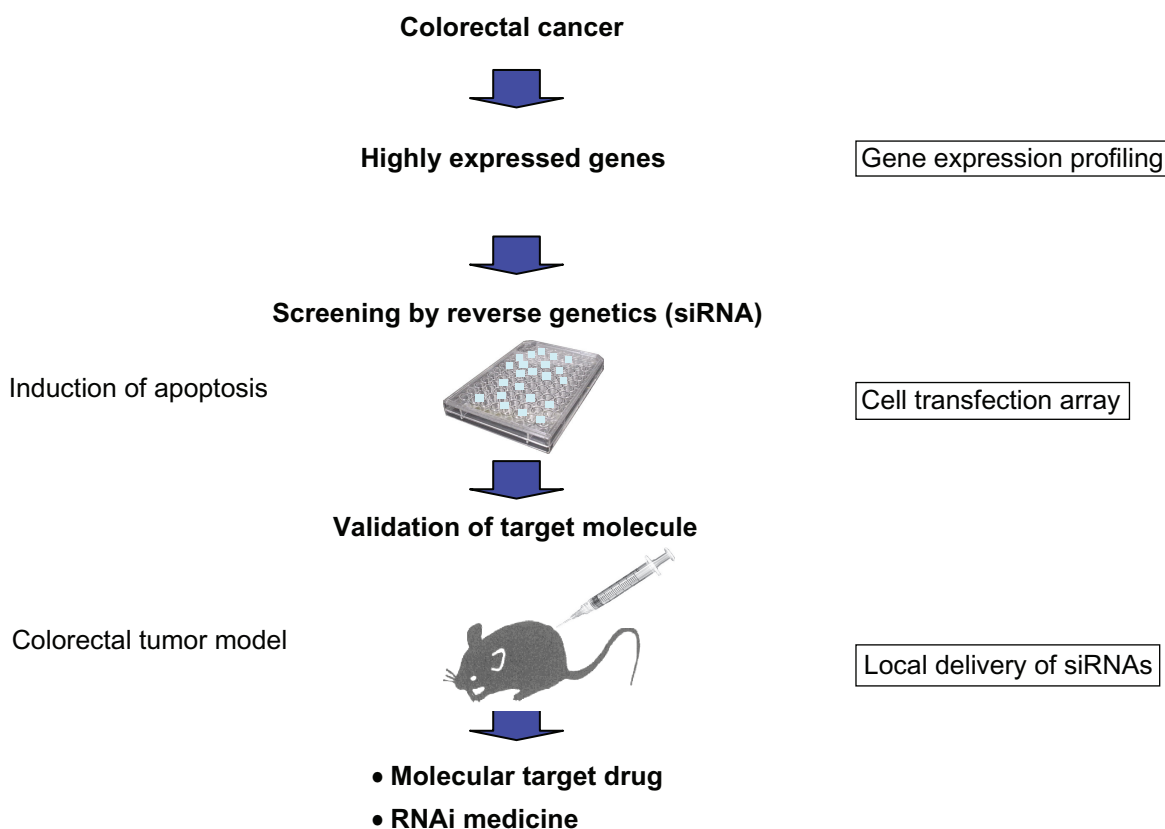
Colorectal cancer

Highly expressed genes | Gene expression profiling

Screening by reverse genetics (siRNA)

Induction of apoptosis | Cell transfection array

Validation of target molecule

Colorectal tumor model | Local delivery of siRNAs

- **Molecular target drug**
- **RNAi medicine**

Figure 1 Schematic representation of the strategy for targets identification by RNAi-based reverse genetics *in vitro* and *in vivo*. First, we carried out a gene expression profiling of human colorectal tumor tissues and selected genes whose expression was elevated. We performed a functional screening of genes by a cell transfection array to test the efficacy of a specific siRNA related to apoptosis induction in human colorectal cancer. Subsequently, the siRNAs against candidate genes were applied to an *in vivo* animal tumor model. Finally, we identified new molecular targets for drug and RNAi-based colorectal cancer therapy.
Abbreviation: siRNA, small interfering RNAs.

Table 2 The list of 97 genes elevated in human colorectal tumor tissues

No	Gene	Description	Accession no.	Log$_2$ ratio (tumor/normal)
1	COL1A1	Collagen, type I, α1	NM_000088	2.137
2	SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	NM_000582	1.531
3	CCL20	Chemokine (C-C motif) ligand 20	NM_004591	1.501
4	UBD	Ubiquitin D	NM_006398	1.392
5	TGFBI	Transforming growth factor, β-induced, 68 kDa	NM_000358	1.345
6	IFITM1	Interferon induced transmembrane protein 1 (9–27)	NM_003641	1.341
7	MMP12	Matrix metallopeptidase 12 (macrophage elastase)	NM_002426	1.248
8	CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	NM_002483	1.237
9	RPS21	Ribosomal protein S21	NM_001024	1.232
10	PFDN4	Prefoldin 4	NM_002623	1.209
11	TIMP1	TIMP metallopeptidase inhibitor 1	NM_003254	1.191
12	NEK6	NIMA (never in mitosis gene a)-related kinase 6	NM_014397	1.089
13	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	NM_002421	1.079
14	S100A11	S100 calcium binding protein A11 (calgizzarin)	NM_005620	1.055
15	IFNAR1	Interferon (α, β and ω) receptor 1	NM_000629	1.031
16	CPSF4	Cleavage and polyadenylation specific factor 4, 30 kDa	NM_006693	1.024
17	TMEPAI	Transmembrane, prostate androgen induced RNA	NM_020182	1.013
18	RPL31	Ribosomal protein L31	NM_000993	0.995
19	CPNE3	Copine III	NM_003909	0.988
20	UBE2C	Ubiquitin-conjugating enzyme E2C	NM_007019	0.979
21	NQO1	NAD(P)H dehydrogenase, quinone 1	NM_000903	0.968
22	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467	0.966
23	LCN2	Lipocalin 2 (oncogene 24p3)	NM_005564	0.959
24	PRKAA1	Protein kinase, AMP-activated, α1 catalytic subunit	NM_006251	0.958
25	GNGT2	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	NM_031498	0.932
26	PSMA7	Proteasome (prosome, macropain) subunit, α-type, 7	NM_002792	0.932
27	SLC3A2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	NM_002394	0.925
28	C10ORF137	Chromosome 10 open reading frame 137	NM_015608	0.917
29	CKS2	CDC28 protein kinase regulatory subunit 2	NM_001827	0.912
30	RPS6	Ribosomal protein S6	NM_001010	0.911
31	RPL39	Ribosomal protein L39	NM_001000	0.910
32	COL10A1	Collagen, type X, α1(Schmid metaphyseal chondrodysplasia)	NM_000493	0.910
33	MLLT1	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1	NM_005934	0.896
34	GABRD	γ-aminobutyric acid (GABA) A receptor, δ	NM_000815	0.895
35	COL1A2	Collagen, type I, α2	NM_000089	0.894
36	DKFZP564O0463	WD repeats and SOF1 domain containing	NM_015420	0.894
37	RIPK2	Receptor-interacting serine-threonine kinase 2	NM_003821	0.893

(*Continued*)

Table 2 (*Continued*)

No	Gene	Description	Accession no.	Log$_2$ ratio (tumor/normal)
38	FOXM1	Forkhead box M1	NM_021953	0.892
39	PIGL	Phosphatidylinositol glycan, class L	NM_004278	0.870
40	NIN	Ninein (GSK3B interacting protein)	NM_016350	0.870
41	ATP2B1	ATPase, Ca^{++} transporting, plasma membrane 1	NM_001682	0.870
42	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	NM_002415	0.861
43	PCYOX1	Prenylcysteine oxidase 1	NM_016297	0.850
44	ARF4	ADP-ribosylation factor 4	NM_001660	0.843
45	MEOX2	Mesenchyme homeo box 2 (growth arrest-specific homeo box)	NM_005924	0.840
46	HTR2B	5-hydroxytryptamine (serotonin) receptor 2B	NM_000867	0.836
47	HRASLS3	HRAS-like suppressor 3	NM_007069	0.827
48	GYPA	Glycophorin A (includes Ss blood group)	NM_002099	0.819
49	GDF15	Growth differentiation factor 15	NM_004864	0.817
50	NPHS2	Nephrosis 2, idiopathic, steroid-resistant (podocin)	NM_014625	0.816
51	FIBL-6	Hemicentin 1	NM_031935	0.804
52	AKAP8L	A kinase (PRKA) anchor protein 8-like	NM_014371	0.787
53	SLC12A2	Solute carrier family 12 (sodium/potassium/ chloride transporters), member 2	NM_001046	0.784
54	CDK10	Cyclin-dependent kinase (CDC2-like) 10	NM_003674	0.781
55	UFM1	Ubiquitin-fold modifier 1	NM_016617	0.780
56	TBX19	T-box 19	NM_005149	0.775
57	DPEP1	Dipeptidase 1 (renal)	NM_004413	0.775
58	NNMT	Nicotinamide N-methyltransferase	NM_006169	0.754
59	RPS20	Ribosomal protein S20	NM_001023	0.748
60	ZNF84	Zinc finger protein 84 (hpf2); znf84	NM_003428	0.740
61	HIG2	Hypoxia-inducible protein 2	NM_013332	0.639
62	SNAI2	Snail homolog 2 (Drosophila)	NM_003068	0.610
63	SLAMF7	SLAM family member 7	NM_021181	0.604
64	RAN	RAN, member RAS oncogene family	NM_006325	0.603
65	SNAI1	Snail homolog 1 (Drosophila)	NM_005985	0.580
66	MRAS	Muscle RAS oncogene homolog	NM_012219	0.543
67	ARHGEF4	Rho guanine nucleotide exchange factor (GEF) 4	NM_015320	0.536
68	MDK	midkine (neurite growth-promoting factor 2)	NM_002391	0.530
69	BRAF	Y-raf murine sarcoma viral oncogene homolog B1	NM_004333	0.528
70	FBXO11	F-box protein 11	NM_012167	0.519
71	AATF	Apoptosis antagonizing transcription factor	NM_012138	0.516
72	FIGN	Fidgetin	NM_018086	0.506
73	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	NM_004994	0.497
74	VEGFA	Vascular endothelial growth factor	NM_003376	0.496
75	FBXW5	F-box and WD-40 domain protein 5	NM_178225	0.481
76	LTA	Lymphotoxin α (TNF superfamily, member 1)	NM_000595	0.476
77	TRAP1	TNF receptor-associated protein 1	NM_016292	0.455
78	LGALS1	Lectin, galactoside-binding, soluble, 1 (galectin 1)	NM_002305	0.453
79	RRAS	Related RAS viral (r-ras) oncogene homolog	NM_006270	0.441
80	MMP10	Matrix metallopeptidase 10 (stromelysin 2)	NM_002425	0.440

(*Continued*)

Table 2 (*Continued*)

No	Gene	Description	Accession no.	Log$_2$ ratio (tumor/normal)
81	FBXW11	F-box and WD-40 domain protein 11	NM_012300	0.440
82	SAT	Spermidine/spermine N1-acetyltransferase	NM_002970	0.440
83	RPN2	Ribophorin II	NM_002951	0.400
84	RAB4B	RAB4B, member RAS oncogene family	NM_016154	0.398
85	FETUB	Fetuin B	NM_014375	0.395
86	ELF4	E74-like factor 4 (ets domain transcription factor)	NM_001421	0.388
87	SHAX3	Chromatin modifying protein 4C	NM_152284	0.387
88	ECT2	Epithelial cell transforming sequence 2 oncogene	NM_018098	0.384
89	HES6	Hairy and enhancer of split 6 (Drosophila)	NM_018645	0.383
90	DDEF1	Development and differentiation enhancing factor 1	NM_018482	0.378
91	RHEB	Ras homolog enriched in brain	NM_005614	0.375
92	CTNND1	Catenin (cadherin-associated protein), δ1	NM_001331	0.368
93	DNMT3B	DNA (cytosine-5-)-methyltransferase 3β	NM_006892	0.365
94	ASPM	ASP (abnormal spindle)-like, microcephaly associated (Drosophila)	NM_018136	0.360
95	PCDHA10	Protocadherin α10	NM_018901	0.349
96	VEGFC	Vascular endothelial growth factor C	NM_005429	0.347
97	RASGRF1	Ras protein-specific guanine nucleotide-releasing factor 1	NM_002891	0.346

siRNAs using reverse transfection-based cell transfection array. To evaluate the efficiency of the cell transfection array, we used GAPDH siRNA against the gene encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase). GAPDH siRNA transfer caused an approximate 75% reduction of the GAPDH mRNA expression in HT-29 cells relative to the control nontargeting siRNA (data not shown).

We assessed siRNAs for their ability to induce apoptosis in HT-29 compared with the control nontargeting siRNA. We measured cell viability by resazurin reduction and examined apoptosis by caspase-3/7 activity. Caspase-3/7 activity was normalized by viable cell number (measurement value of resazurin reduction) and Caspase-3/7 activation by siRNAs was compared with control nontargeting siRNA. The results indicated that the downregulation of 11 genes (*TIMP1*, *S100A11*, *TMEPAI*, *PSMA7*, *COL10A1*, *RAN*, *VEGFA*, *LTA*, *TRAP1*, *MMP10*, and *RHEB*) resulted in a marked induction of apoptosis in HT-29 cells (caspase-3/7 activity, percentage of nontargeting siRNA > 200, $P < 0.05$, Figure 2). In particular, the PSMA7 siRNA pool and the RAN siRNA pool strongly enhanced caspase-3/7 activity (caspase-3/7 activity, percentage of nontargeting siRNA > 300, $P < 0.001$, Figure 2). We validated these results by counting Hoechst-stained cells showing apoptotic nuclear condensation and fragmentation (Figure 3A) and found that there was a significantly higher apoptotic cell death rate in cells given PSMA7 siRNA pool and RAN siRNA pool relative to that in cells given control siRNA ($P < 0.01$, Figure 3B). At 72 hours after treatment with siRNA, there was substantial cell death induced by the PSMA7 siRNA pool and the RAN siRNA pool compared with the control nontargeting siRNA (Figure 3C).

We assessed the efficacy of PSMA7 siRNA pool and RAN siRNA pool for the knockdown of mRNA by cell-direct real-time RT-PCR analysis. This analysis revealed that PSMA7 siRNA pool and RAN siRNA pool inhibited the mRNA expression relative to the control nontargeting siRNA, 80% and 60% respectively (Figure 3D). To evaluate the knockdown efficiency of individual siRNAs of the siRNA pool, we performed a liposome-mediated siRNA transfection. PSMA7 siRNA #2 and RAN siRNA #4 most efficiently silenced PSMA expression (70% reduction of mRNA) and RAN expression (70% reduction of mRNA), respectively (Figure 3E). We also confirmed that PSMA7 siRNA #2 and RAN siRNA #4 strongly increased caspase-3/7 activity in HT-29 cells (data not shown). PSMA7 siRNA #2 and RAN siRNA #4 were used in further *in vivo* experiments.
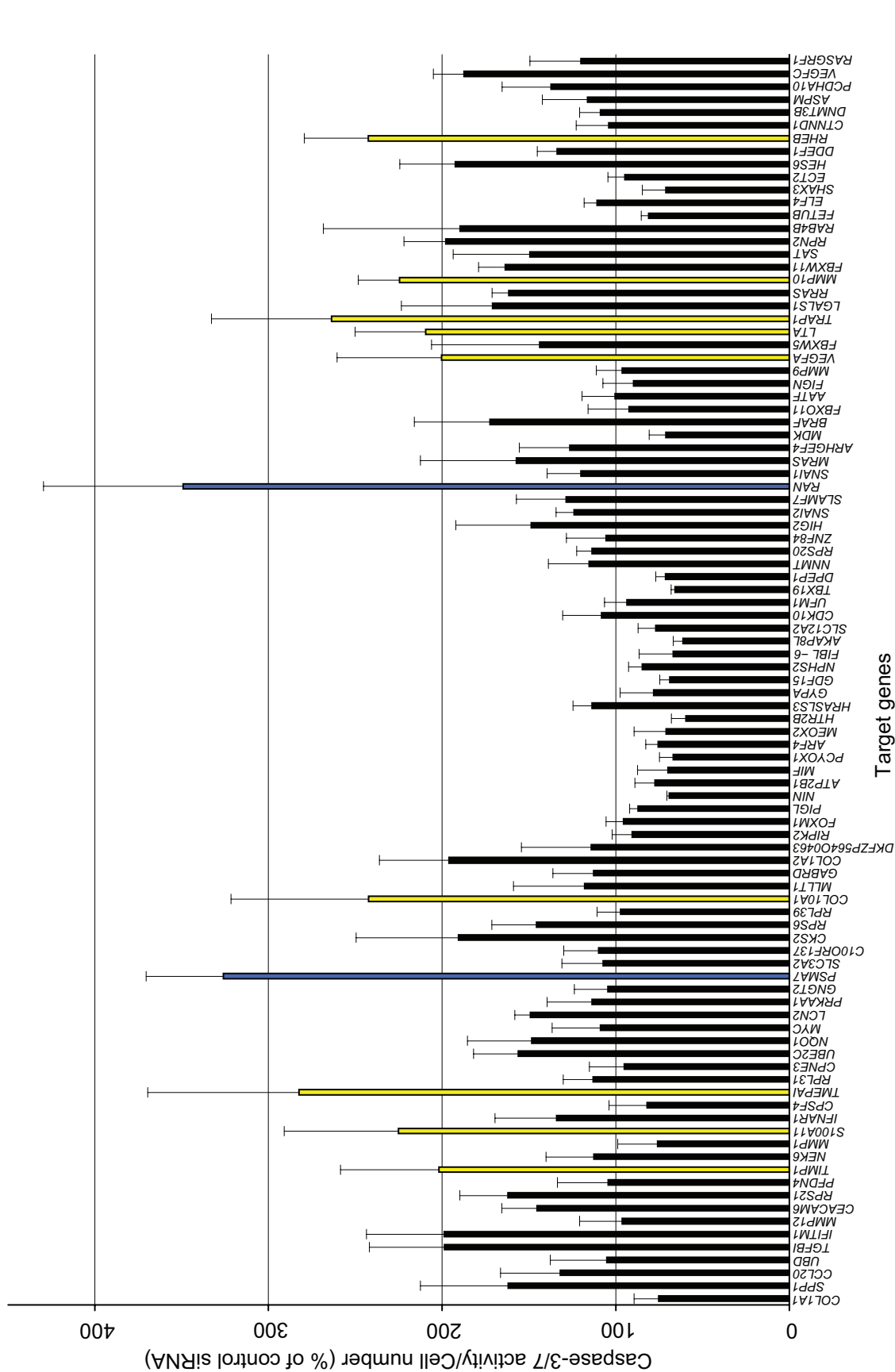
Figure 2 Functional screening of genes by RNAi-cell transfection array in cultured colorectal cancer cells. Cell number and caspase-3/7 activity were measured 72 hours after transfection of HT-29 cells. Caspase-3/7 activity was normalized by viable cell number to evaluate apoptosis induction. Control nontargeting siRNA is set to 100% and relative activity is shown. Yellow color bar: relative activity (%) >200, $P < 0.05$; blue color bar: relative activity (%) >300, $P < 0.001$. $n = 4$ per group. Values are mean ± SD.
Abbreviations: SD, standard deviation; siRNA, small interfering RNAs.
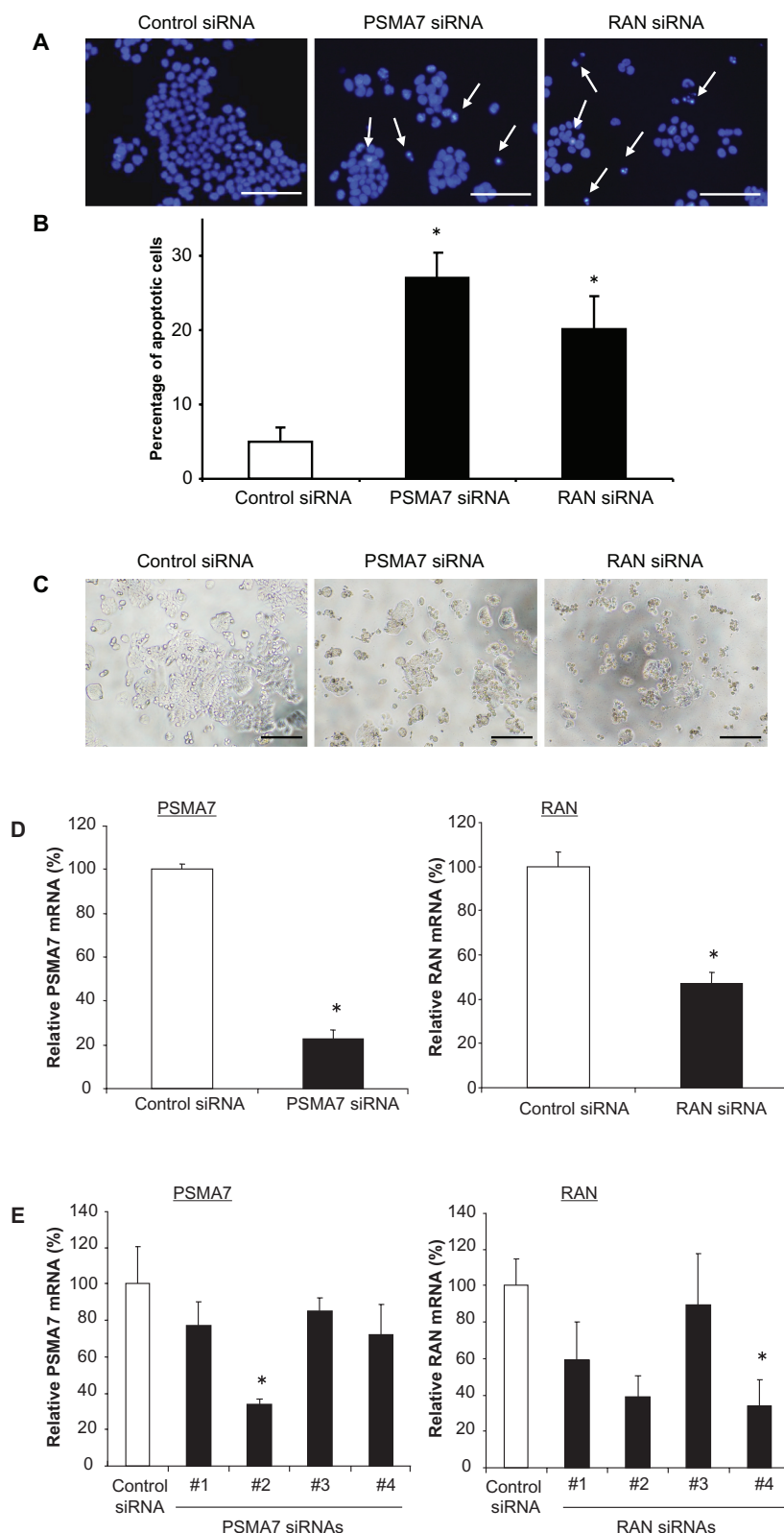
Figure 3 Apoptosis of HT-29 cells transduced with PSMA7 siRNA and RAN siRNA. **A**) Hoechst staining of cells 72 hours after the transfection of siRNA. Scale bar, 100 μm. The arrows indicate cells with nuclear condensation and fragmentation. **B**) Numbers of apoptotic cells from (A). The data show the percentage of apoptotic cells. As a control, nontargeting control siRNA was used (*n* = 4 per group, *P* < 0.01). **C**) Phase contrast micrograph of HT-29 cells 72 hours after the treatment of siRNA. Scale bar, 200 μm. **D**) Knockdown of mRNA by PSMA7 siRNA pool and RAN siRNA pool using cell transfection array was measured 72 hours after transfection. (*n* = 5 per group, *P* < 0.001). **E**) Knockdown of mRNA by PSMA7 siRNAs and RAN siRNAs. Expression of PSMA7 mRNA and RAN mRNA was measured 72 hours and 48 hours after transfection, respectively (*n* = 3 per group, *P* < 0.01). As a control, nontargeting siRNA was used. Values are mean ± SD.
Abbreviations: PSMA7, proteasome subunit, α-type, 7; RAN, ras-related nuclear protein; SD, standard deviation; siRNA, small interfering RNAs.
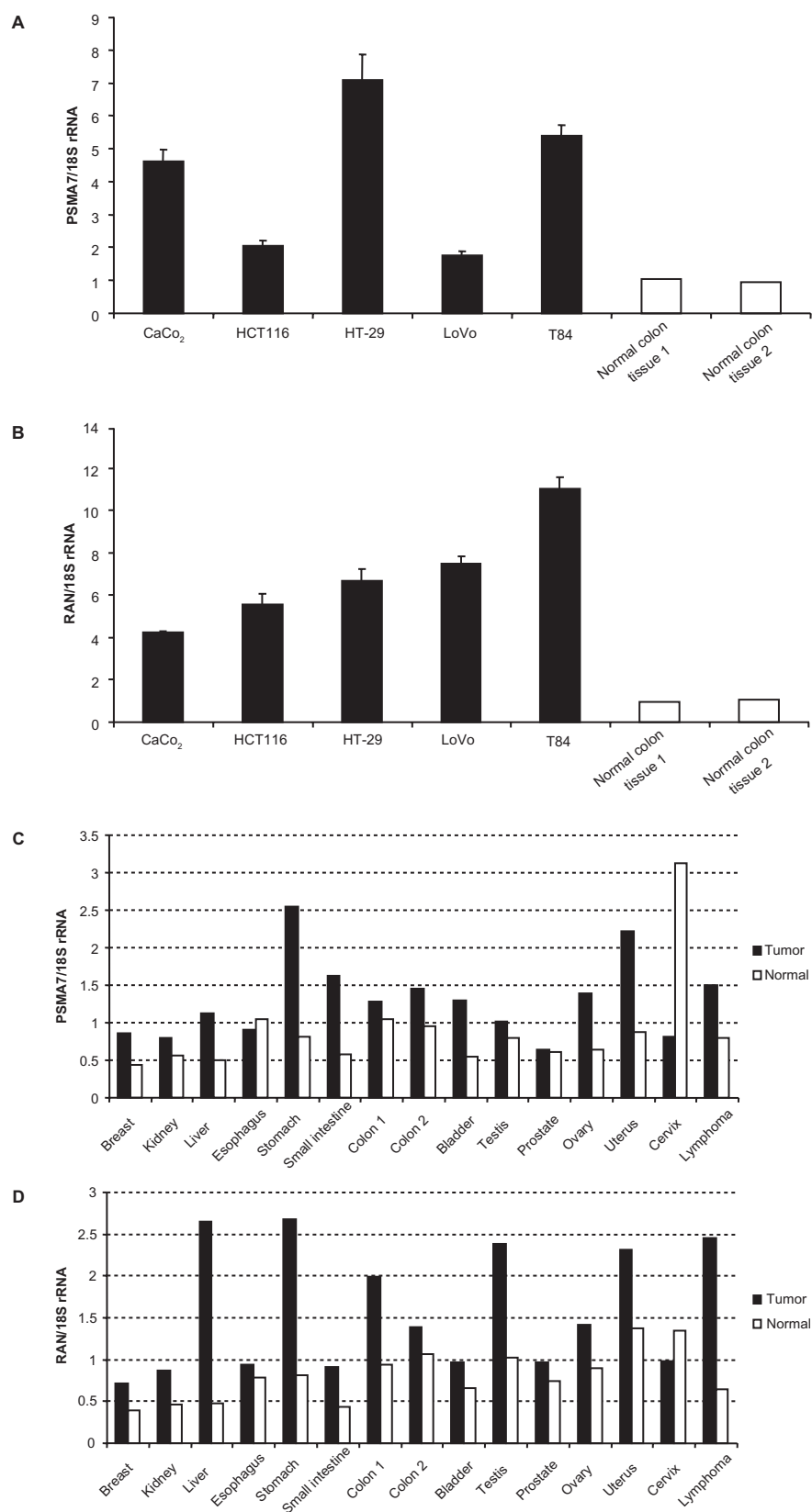
Figure 4 *PSMA7* and *RAN* expression in colorectal cancer cell lines and tumor tissues. PSMA7 mRNA and RAN mRNA expression were analyzed by real-time RT-PCR.
A) PSMA7 mRNA expression in colorectal cancer cell lines. $n = 3$ per group. Values are mean $\pm$ SD. **B**) RAN mRNA expression in colorectal cancer cell lines. $n = 3$ per group.
Values are mean $\pm$ SD. **C**) PSMA7 mRNA expression in various tumor tissues (■, $n = 1$) and their normal adjacent tissues (□, $n = 1$). **D**) RAN mRNA expression in various
tumor tissues (■, $n = 1$) and their normal adjacent tissues (□, $n = 1$). mRNA expression was normalized to 18S rRNA. Mean of normal colon tissues is set to 1.
Abbreviations: PSMA7, proteasome subunit, α-type, 7; RAN, ras-related nuclear protein; RT-PCR, reverse transcriptase-polymerase chain reaction; SD, standard deviation.

Thus, downregulation of PSMA7 and RAN expression by siRNA induces apoptosis in colon cancer cells, results that suggest that PSMA7 and RAN might act as a suppressor of apoptosis in colorectal cancer.

Overexpression of PSMA and RAN in colorectal cancer cell lines and tumor tissues

To investigate the role of PSMA and RAN in colorectal cancer, we analyzed the mRNA expression levels of PSMA7 and RAN by real-time RT-PCR. PSMA7 and RAN mRNA were significantly highly expressed in colorectal cancer cell lines Caco-2 (human colorectal adenocarcinoma), HCT116 (human colorectal carcinoma), HT-29 (human colorectal adenocarcinoma), LoVo (human colorectal adenocarcinoma, derived from supraclavicular lymph node metastatic site) and T84 (human colorectal carcinoma, derived from lung metastatic site), compared with normal colon tissues (Figures 4A, 4B). mRNA expression levels of PSMA7 and RAN in human tumoral colon tissues were higher than those of normal adjacent colon tissues (Figures 4C, 4D). These results indicate that PSMA7 and RAN strongly correlated with colorectal tumorigenesis and malignancy.

Furthermore, PSMA7 was overexpressed in other tumor tissues, especially uterus, stomach, and small intestine tumor tissues (Figure 4C). Similarly, RAN was overexpressed in other tumor tissues, in particular uterus, liver, testis, and stomach tumors and lymphoma tissues (Figure 4D). Thus, PSMA7 and RAN were overexpressed in not only colon tumor tissues but also in other tumor tissues, and PSMA7 and RAN might be involved in the development of multiple cancers.

Correlation of PSMA7 and RAN expression with liver and lung metastases in human colorectal cancer

We next investigated the levels of *PSMA7* and *RAN* expression in human colorectal cancers. The results are summarized in

Table 3. PSMA7 showed a borderline significance ($P = 0.076$) for correlation with liver metastasis in colorectal cancer patients. In contrast, no significant correlation between the PSMA7 expression and lung metastasis was found in patients ($P = 0.534$). Decreased expression of RAN showed a significance ($P = 0.023$) for correlation with lung metastasis; however, there is no correlation with liver metastasis ($P = 0.911$). However, to know whether or not the levels of *PSMA7* and *RAN* expression showed correlation with any other clinicopathological features such as depth of invasion, tumor size, lymphatic invasion, or the presence of lymph node metastasis, further analysis is needed.

PSMA7 siRNA and RAN siRNA induce apoptosis *in vivo*

To extend our *in vitro* findings and to determine whether PSMA7 and RAN could be effective therapeutic targets for colorectal cancer, we examined the effect of PSMA7 siRNA and RAN siRNA on an animal model of colon tumors by subcutaneously implanting HT-29 cells into mice. We injected the PSMA7 siRNA #2, RAN siRNA #4, or nontargeting control siRNA (1 nmol per tumor) into tumors that had reached 5–6 mm in diameter seven days after inoculation of HT-29 cells. The mRNA levels of PSMA7 and RAN in the tumors given siRNA were measured. mRNA expression was significantly reduced in mouse tumors 24 hours after treatment with PSMA7 siRNA and RAN siRNA, 40% and 30% reduction relative to nontargeting control siRNA, respectively ($P < 0.05$, Figure 5A).

HT-29 tumors treated with PSMA7 siRNA or RAN siRNA were investigated for apoptotic activity. Three days after siRNA treatment, we performed TUNEL staining which detects apoptotic DNA breaks *in situ*. TUNEL staining of tumor tissue treated with PSMA7 siRNA and RAN siRNA revealed a significant number of apoptotic cells relative to the number in the nontargeting control siRNA treated tumors

Table 3 Correlation of *PSMA7* and *RAN* expression with liver and lung metastases in human colorectal cancer

	No of subjects	*PSMA7*		*RAN*	
	(*n* = 191)	expression	*P*-value	expression	*P*-value
Liver metastasis					
Positive	41	1.108 ± 0.116	0.0762	0.597 ± 0.052	0.9112
Negative	150	0.884 ± 0.043		0.605 ± 0.031	
Lung metastasis					
Positive	29	0.870 ± 0.112	0.5336	0.460 ± 0.064	0.0233
Negative	162	0.943 ± 0.045		0.629 ± 0.029	

Note: Values are mean ± standard error.
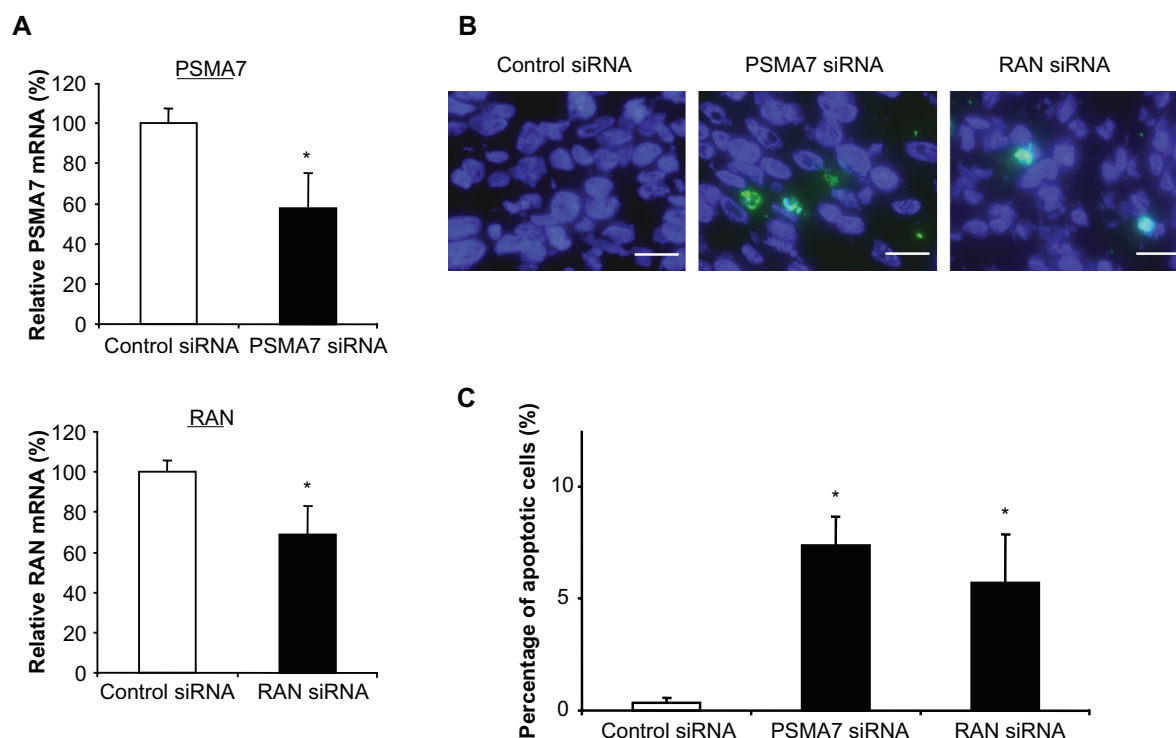
A

B

C

Figure 5 Apoptosis induction by PSMA7 siRNA and RAN siRNA treatment *in vivo*. **A**) Expression of PSMA7 mRNA and RAN mRNA in HT-29 tumors treated with siRNA. ($n = 4$ per group, $*P < 0.05$). **B**) TUNEL staining of HT-29 tumor tissues treated with siRNA. Scale bar, 20 μm. **C**) TUNEL-positive cells were counted and represented ($n = 3$ per group, $*P < 0.05$). As a control, nontargeting siRNA was used. Values are mean ± SD.
Abbreviations: PSMA7, proteasome subunit, α-type, 7; RAN, ras-related nuclear protein; SD, standard deviation; siRNA, small interfering RNAs.

($P < 0.05$, Figures 5B, 5C). These results indicate that PSMA7 siRNA and RAN siRNA induce apoptotic cell death *in vivo*.

Discussion

Molecular targeted therapy is one of the most promising approaches in cancer treatment. For cancer researchers it is now possible to understand the molecular mechanisms of the development and progression in many types of cancer. It is expected that many potential new molecular targets will be discovered for cancer therapy. To identify molecular targets for colorectal cancer therapy, we performed a sophisticated strategy using RNAi-based reverse genetics *in vitro* and *in vivo*. RNAi is a cellular mechanism for silencing of gene expression. Following the demonstration of RNAi mediated by siRNA in mammalian cells in 2001,[22] RNAi has provided new powerful tools for biological research and drug discovery. Additionally, therapeutics based on RNAi offers a new class of pharmaceutical drugs. The inhibition of gene expression through RNAi is applicable to all classes of molecular targets, including the "undruggable targets" of traditional pharmaceutical drugs such as small molecule and protein.

First, we carried out a gene expression profiling of subjects with colorectal tumors and selected 97 genes whose

expression was elevated in human colorectal cancer tissues as a screening source of molecular targets. This starting from human disease samples has a great advantage, because cultured cells and disease models cannot faithfully reproduce the pathology of cancer. We then analyzed the function of genes using RNAi *in vitro* and *in vivo*. We performed a functional screening of genes by a reverse transfection-based cell transfection array to obtain an unbiased evaluation on the efficacy of a specific siRNA related to apoptosis induction in human colorectal cancer. An unbiased evaluation is important in the discovery of a new and unique target that brings a novel concept of targeted therapy. Our results showed that the siRNA designed for PSMA7 and RAN significantly promoted apoptosis of HT-29 human colon cancer cells. In our next step, we demonstrated *in vivo* proof-of-concept by the administration of siRNA to a colon tumor model. The *in vivo* delivery of PSMA7 siRNA and RAN siRNA markedly induced apoptosis in HT-29 tumors in mice. Finally, we identified PSMA7 and RAN as new molecular targets for an RNAi-based colorectal cancer therapy.

PSMA7 (also known as XAPC7) is a proteasome subunit.[23] The 26S proteasome consist of a 20S proteasome core and two 19S regulatory subunits.[24] The 20S proteasome core

is a barrel-shaped structure arranged in four stacked rings. Of these four rings, two end rings are composed of seven α-subunits, and two central rings are composed of seven β-subunits. PSMA7 is one of the seven proteasome α-subunits of 20S proteasome. PSMA7 interacts specifically with hepatitis B virus X protein (HBX), which is important in the life cycle of the hepatitis B virus (HBV).[25] In addition, PSMA7 has a principal role in regulating activity of the hepatitis C virus (HCV) internal ribosome entry site (IRES), a function essential for HCV replication.[26]

The ubiquitin–proteasome pathway is the main extra-lysosomal system involved in intracellular proteolysis and is critical for the proliferation and survival of all cells.[24,27] In particular cancerous cells, the ubiquitin-proteasome pathway plays an integral role in the mechanisms underlying carcinogenesis and metastasis, including cell cycle regulation, apoptosis, and angiogenesis.[24,27] The cancer cells exploit the proteasome for their own proliferation through the cell cycle by degrading of cell cycle regulatory proteins. Furthermore, the proteasome regulates apoptotic activity via effects on the pro-oncogenic nuclear factor (NF)-κB pathway, which is a transcriptional activator and activated in many types of tumor.[28] Under normal conditions, NF-κB is bound to its inhibitor IκB (IκB) and in an inactive state. The proteasome degrades IκB in response to cellular stresses, and then NF-κB activates transcription of genes for growth factors and apoptosis inhibitors.[24,29,30] Therefore, proteasome is an attractive target for cancer therapy. A dipeptidyl boronic acid proteasome inhibitor, bortezomib, suppresses degradation of IκB by proteasome and blocks NF-kB signaling with resultant apoptosis.[24,27,29] The efficacy of bortezomib is investigated in various types of malignancies including advanced colorectal cancer.[24,31,32] However, the ubiquitin-proteasome pathway is essential for maintenance of cell function in all cells, and the proteasome inhibitor may attack normal cells and cause severe side effects in patients.[33,34] A proteasome inhibitor specific for cancer cells should provide a better treatment with minimal side effects. In this study, among proteasome subunits genes, *PSMA7* is the only gene listed for genes whose expression is elevated in human colorectal cancer tissues. RNAi-mediated specific inhibition of PSMA7 may be a smart colorectal cancer therapy. Although it is impossible at the moment to judge whether PSMA7 silencing inhibited degradation of IκB by proteasome and blocks NF-kB signaling, in fact, our results indicated that PSMA7 siRNA caused apoptosis in HT-29 colon cells and HT-29 tumor tissues. PSMA7 was overexpressed in not only colon tumor tissues but also in other tumor tissues, which suggests

that PSMA7 might be a potential target against various types of cancer.

Moreover, PSMA7 is interesting because of the correlation between its expression and the liver metastasis of colorectal cancer. Our study showed that expression of PSMA7 mRNA was elevated in colorectal tumor tissues from subjects with liver metastasis, although no statistical significance was found. Another research group also reported that overexpression of PSMA7 protein associates with liver metastasis in colorectal cancer.[35] PSMA7 may be a predictive marker and a molecular target for liver metastasis from colorectal cancer. Further investigations are needed to confirm the relationship between PSMA7 and colorectal cancer liver metastasis by gene expression profiling on a validation set of colorectal cancer subjects and analysis of PSMA7 expression on a colorectal cancer liver metastatic site at both the mRNA and protein levels.

RAN protein (Ran) is a small GTPase belonging to the Ras superfamily. Ran is essential for the translocation of RNA and proteins through the nuclear pore complex.[36,37] GTPase Ran regulates numerous cellular processes by switching between a GTP-bound and GDP-bound form.[38] Ran is also critical for the regulation of the cell cycle through mitotic spindle assembly and post-mitotic nuclear envelope assembly.[38,39]

It is reported that Ran is a suppressor of Bcl-2-associated X protein (Bax), a pro-apoptotic member of the Bcl-2 family of proteins, and that it inhibits apoptosis induced by the anticancer drug paclitaxel.[40] Furthermore, it is indicated that silencing of Ran in various tumor cell types causes aberrant mitotic spindle formation, mitochondrial dysfunction, and apoptosis.[41,42] Ran is abundantly expressed in most cancer cell lines and cancer tissues.[41] This suggests that Ran is associated with malignant transformation and/or the enhanced proliferation of cancer cells. There is a current finding that most tumor cells, but not normal tissues, become dependent on Ran signaling for cell mitosis.[41] Targeting the Ran signaling pathway may provide a selective anticancer strategy.

This study showed that RAN mRNA was elevated in colon cancer cell lines and tissues, and in some types of tumor tissue. *RAN* silencing using RAN siRNA induced apoptosis in HT-29 colon cancer cells and HT-29 tumor tissues. RAN siRNA can specifically inhibit Ran which is a main molecule of *RAN* signaling, and may be a selective inhibitor against colon cancer.

We identified PSMA7 and RAN as new molecular targets for colorectal cancer therapy using RNAi-based

screening *in vitro* and *in vivo*. In a further study, we are investigating the antitumor activity of PSMA7 siRNA and RAN siRNA in a colon cancer model and a colon cancer liver metastasis model. Induction of apoptosis is one of the main targets for cancer therapy. The inhibitors of PSMA7 and RAN may provide unique anticancer strategies based on novel mechanisms of action. Since the inhibition of gene expression through RNAi is highly specific and applicable to "undruggable targets", RNAi-based therapeutics using PSMA7 siRNA and RAN siRNA is a particularly promising approach for cancer treatment.

Acknowledgments

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