Oncogenic role of *NALP7* in testicular seminomas

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To isolate novel molecular targets for treatment of testicular germ cell tumor (TGCT), we performed genome-wide expression profile analysis of testicular seminomas using a cDNA microarray. We here report identification of NACHT, leucine-rich repeat and PYD containing 7 (*NALP7*), that was significantly transactivated in testicular seminomas. Subsequent semi-quantitative RT-PCR and northern blot analyses confirmed an approximately 3.3-kb transcript that was expressed exclusively in testis, although the expression level of this gene in normal testis was much lower than in tumor cells, suggesting an important role of this gene in germ-cell proliferation. Immunohistochemical analysis using anti-NALP7 polyclonal antibody detected the endogenous NALP7 protein in the cytoplasm of embryonal carcinoma cells and testicular seminoma tissues. Transfection of small interfering RNA (siRNA) for *NALP7* significantly reduced the *NALP7* expression and resulted in growth suppression of testicular germ-cell tumors. These findings imply that *NALP7* may play a crucial role in cell proliferation, as well as testicular tumorigenesis, and it appears to be a promising candidate for development of targeted therapy for TGCTs. (Cancer Sci 2004; 95: 949–954)

Testicular germ-cell tumors (TGCTs) account for only 1–2% of all cancers in males. However, it is notable that, among males between the ages of 20 and 40, TGCTs are the most common type of cancer and their incidence has increased markedly over recent decades.[1–3] TGCTs are classified into two major histological types; seminomas and non-seminomatous tumors. Seminomas, accounting for approximately 60–65% of TGCTs, phenotypically resemble undifferentiated germ cells, while the non-seminomatous tumor cells resemble either embryonic or extra-embryonic tissues, and are able to differentiate to either type.[4,5]

About 15–20% of seminoma patients at clinical stage I relapse after orchidectomy if they are not given any additional treatment. However, most of the relapsed patients can be treated and cured with conventional doses of cytotoxic chemotherapy, although chemotherapy often causes severe adverse reactions. The protocol called BEP, a combination treatment of bleomycin, etoposide and cisplatin, that is usually the first choice for patients with metastatic germ-cell tumors, is quite effective, but approximately 30% of the patients show poor or no response to this therapy and consequently have a poor prognosis.[6] Therefore, novel treatment modalities must be developed to further improve clinical outcomes for TGCTs, especially seminomas.

Gene-expression profiles have been proven to provide detailed information about the nature of individual cancers, and can lead to improved clinical strategies for neoplastic diseases through development of novel drugs, as well as providing the basis of personalized treatment.[7] To search for molecular targets for such development, we have been performing expression profile analysis by using a cDNA microarray representing 23,040 human genes for cancers arising from various tissues, including TGCTs.[8–16] Through the expression profile analysis of synovial sarcomas (SSs) and subsequent functional analysis, for example, we showed that treatment of SS cells with small interfering RNA (siRNA) for the *PDZK4* gene effectively inhibited its expression and caused suppression of tumor growth.[17] Identification of such oncogenic elements should provide new opportunities for development of new drugs that target specific types of cancer.

We report here characterization of *NALP7*, which was commonly up-regulated in testicular seminoma cells and we show that gene silencing of *NALP7* with specific-siRNA suppressed its expression and resulted in inhibition of cell growth of testicular germ-cell tumors. Thus, *NALP7* appears to play an important role in promoting growth of testicular seminoma cells and may be a suitable target candidate for development of new agents to treat testicular germ-cell tumors.

Materials and Methods

Cell lines and tissue specimens. Human embryonal carcinoma cell lines, Tera-1 and Tera-2, human embryonic kidney cell line HEK293, and COS7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell lines were grown in monolayer in appropriate media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO), Dulbecco's modified Eagle's medium (Sigma) for COS-7, McCoy's 5A (Invitrogen, Carlsbad, CA) for Tera-1, Tera-2 and HEK293, and maintained at 37°C in humid air containing 5% CO_2. All 13 testicular seminomas were obtained with informed consent from patients who had undergone orchiectomy.

Semi-quantitative RT-PCR. Extraction of total RNA from Tera-1 cells and clinical samples of testicular seminoma were carried out using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Poly(A)$^+$ RNAs of normal human testis, heart, lung, kidney, liver, brain, and bone marrow were purchased from BD, Clontech (Palo Alto, CA). Reverse-transcription PCR was performed as described previously.[17] Primer sequences were as follows: for *TUBA3*, 5′-CTTGGGTCTGTAACAAAGCATTC-3′, and 5′-AAGGATTATGAGGAGGTTGGTGT-3′; for the common region of all three *NALP7* variants, F1 5′-GCTGACACACCTGTGCTTG-3′, and R1 5′-ACAGCTTCTTGATTTCCAAATTA-3′; for specific *NALP7*V3, F2 5′-GACCTTGGTTCTCTCAGAGGC-3′ and R1 5′-ACAGCTTCTTGATTTCCAAATTA-3′.

Northern blot analysis. Human multiple-tissue northern blots (BD, Clontech) were hybridized with a ^{32}P-labeled *NALP7* cDNA fragment as a probe. The probe of *NALP7* cDNA was prepared using F1 and R1 primers as described above. Pre-hybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at −80°C for 14 days.

Production of recombinant hNALP7 (rhNALP7) antigen and polyclonal antibody. A cDNA fragment corresponding to the N-ter-

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minal region (1–171 amino-acid residues) of NALP7 was subcloned into the pET21a vector (Merck, Novagen, Darmstadt, Germany) (pET21a-h*NALP7*). Recombinant human NALP7 (rhNALP7) protein was expressed in *Escherichia coli*, and purified from cell lysates prepared under native conditions with Ni-Superflow resins (Qiagen, San Diego, CA), according to the supplier's instructions. Further purification of rh*NALP7* was carried out on a MonoQ anion-exchange column using ÄKTA explorer 10S (Amersham Biosciences, Buckinghamshire, UK). Rabbits were then immunized with purified rh*NALP7* protein (Medical & Biological Laboratories, Nagoya, Japan). The antisera were subsequently purified by means of antigen-affinity columns using Affi-Gel 15 gel (Bio-Rad, Hercules, CA), according to the supplier's instructions.

Immunoblot analysis. We first constructed an expression vector, pCAGGS-*NALP7V1*, that was designed to express an entire coding region of *NALP7V1* using pCAGGS3.1-3nFlag/HA expression vector. We then transfected it into COS7 cells and performed immunoblot analysis. Cell lysates were separated on 5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane, followed by incubation with anti-NALP7 polyclonal antibody (anti-NALP7 pAb) as the primary antibody. After incubation with sheep anti-rabbit IgG-HRP as the secondary antibody (Amersham Biosciences), signals were visualized with an ECL kit (Amersham Biosciences).

Immunocytochemical staining. Tera-1 cells were washed three times with PBS(−) at room temperature, fixed with 4% paraformaldehyde solution at 4°C and made permeable with PBS(−) containing 0.1% Triton X-100 for 2.5 min at 4°C. Cells were then covered with blocking solution (3% BSA in PBS) for 60 min at room temperature. Endogenous NALP7 protein was detected with anti-NALP7 pAb as the primary antibody and goat IgG fraction to rabbit IgG labeled with green-fluorescent Alexa Fluor 488 (Molecular Probes, Eugene, OR) as the secondary antibody. Paraffin-embedded specimens of seminoma and of normal human tissues (heart, liver, lung, and kidney) (Biochain, Hayward, CA) were treated with xylene and ethanol to remove the paraffin. Antigen retrieval was carried out in Target Retrieval Solution High pH (DAKO, Carpinteria, CA) for 15 min at 108°C in an autoclave. We used ENVISION+ Kit/HRP (Dako Cytomation, Kyoto, Japan) to detect NALP7; after the endogenous peroxidase and protein-blocking reactions, affinity-purified rabbit anti-NALP7 pAb was added as the primary antibody, and the mixture was treated with HRP-labeled anti-rabbit IgG. Finally, substrate-chromogen was added and the tissue specimens were counterstained with hematoxylin. Experiments to inhibit immunostaining were also performed, as described previously.[18]

Treatment of testicular germ cell tumor cells with small interfering RNA (siRNA). We previously established a vector-based RNAi-expression system (psiU6BX vector).[18] The siRNA expression vector for *NALP7* (psiU6BX-*NALP7*) was prepared by cloning of double-stranded oligonucleotides shown in Table 1 into the B*bs*I site of the psiU6BX vector. A control plasmid, psiU6BX-EGFP, was prepared by cloning double-stranded oligonucleotides 5′-CACCGAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAAGTCGTGCTGCTTC-3′ and 5′-AAAAGAAGCAGCACGACTTCTTCTCTCTTGAAGAAGAAGTCGTGCTGCTTC-3′ into the B*bsI* site of the psiU6BX vector as well. Each siRNA expression vector was transfected with the aid of Fugene6 (Roche) into human embryonal carcinoma cell line, Tera-1, in which *NALP7* is endogenously highly expressed. After 2 weeks of selection with 0.1 mg/ml of Geneticin (Invitrogen), cell numbers were evaluated by means of colony formation assay using Giemsa staining and after 1 week by MTT assay using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).[18] The knockdown effect on *NALP7* mRNA was checked by semi-quantitative RT-PCR. Primer sequences were as follows: for β2-microglobulin (*β2-MG*), forward 5′-TTAGCTGTGCTCGCGCTACT-3′, and reverse 5′-TCACATGGTTCACACGGCAC-3′; for *NALP7*, same as for semi-quantitative RT-PCR.

Results

Up-regulation of NALP7 in testicular seminoma cells. To screen molecular targets for development of anticancer therapy for testicular germ-cell tumor, we previously performed cDNA microarray analysis (23,040 genes).[15] Among the genes overexpressed in testicular seminomas, we confirmed elevated expression of *NALP7* by semi-quantitative RT-PCR in most of the tumors examined. Subsequent analysis identified three different transcriptional variants, designated NALP7V1 (GenBank accession; AY154462), NALP7V2 (GenBank accession; NM_139176) and NALP7V3 (GenBank accession; NM_154462), respectively (Fig. 1A). Alternative splicing involved exons 5, 9 and 10, and the remaining 8 exons were commonly transcribed in all transcripts. Exon 5b, which is unique to the NALP7V2 variant, is 84-bp shorter at the 5′ portion than exon 5a of NALP7V1 and V3. Exon 9b of the NALP7V3 variant was 39-bp shorter at the 5′ portion than exon 9a of NALP7V1 and V2. Exon 10 consisting of 171 nucleotides was uniquely transcribed in NALPV2. The full-length cDNA sequences of NALP7V1, NALP7V2 and NALP7V3 transcripts contained 3244, 3331, and 3205 nucleotides, respectively. The translational starting site of all three transcripts is located within exon 2 and the translational termination codon is present in exon 11.

To confirm the expression pattern of each variant in testicular seminomas and also in normal human tissues, we performed semi-quantitative RT-PCR using F1 and R1 primers that were common to all the variants (Fig. 1B), and F2 and R1 primers that were unique to the V3 variant (Fig. 1C), respectively. We confirmed that the NALP7V1 variant was significantly up-regulated in five of the eleven testicular seminomas. However, no elevated expression of the NALP7V2 variant was observed in any seminoma. The transcript corresponding to the NALP7V3 variant showed lower expression in seminomas, compared with the V1 and V2 transcripts (Fig. 1C). Since, these results suggested that the elevated expression of the NALP7V1 variant

Table 1. Sequences of specific double-strand oligonucleotides inserted into siRNA expression vector

		Nucleotide
Si-1	5′-CACC**GAGGCTGATGGCAAGAAAC**TTCAAGAGA**GTTTCTTGCCATCAGCCTC**-3′	212–230
	5′-AAAA**GAGGCTGATGGCAAGAAAC**TCTCTTGAA**GTTTCTTGCCATCAGCCTC**-3′	
Si-2	5′-CACC**GAGATGAATCTCACGGAAT**TTCAAGAGA**ATTCCGTGAGATTCATCTC**-3′	299–317
	5′-AAAA**GAGATGAATCTCACGGAAT**TCTCTTGAA**ATTCCGTGAGATTCATCTC**-3′	
Si-3	5′-CACC**GTGATGCATTGTTCCTTCA**TTCAAGAGA**TGAAGGAACAATGCATCAC**-3′	1880–1898
	5′-AAAA**GTGATGCATTGTTCCTTCA**TCTCTTGAA**TGAAGGAACAATGCATCAC**-3′	

Bold letters indicate the specific sequences to *NALP7*, nucleotide indicates the number of nucleotide from 5′ end of full length V1 transcript.

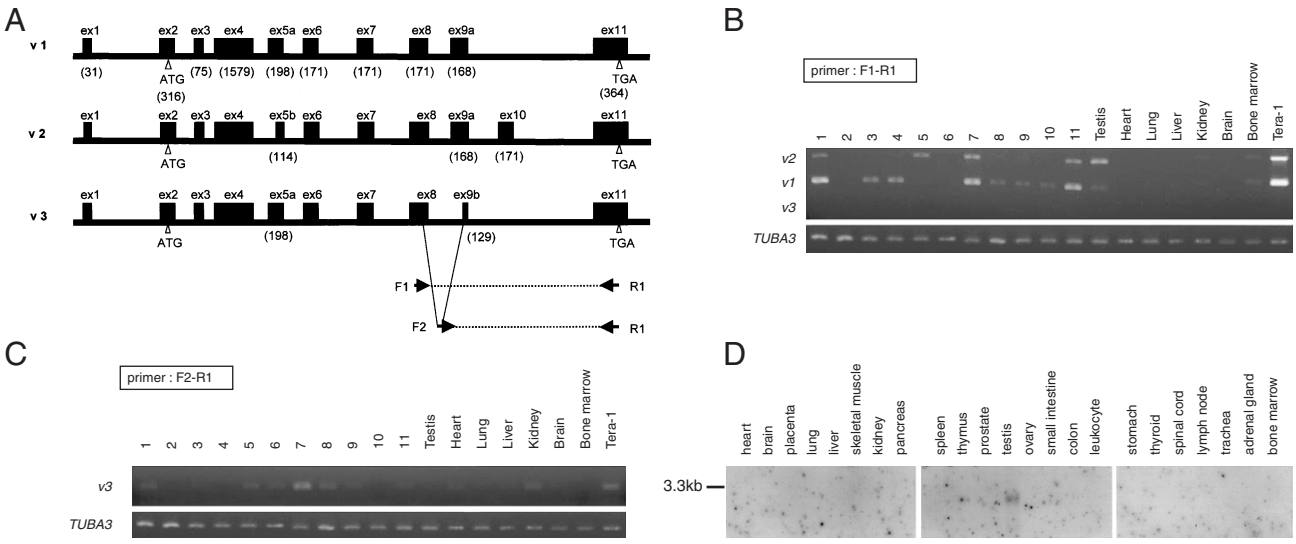

Fig. 1. Characterization of the *NALP7* gene. (A) Genomic structure of three transcriptional variants of *NALP7*, with variations in exons 5, 9 and 10 (exons are represented by black boxes). Open arrowheads indicate the first methionine codon and the stop codon, respectively. Numbers in parentheses indicate the length of each exon. Arrows indicated by F1 and R1, and F2 and R1 indicate the primer locations for semi-quantitative RT-PCR, respectively. (B) Expression of three *NALP7* transcriptional variants in 11 testicular seminomas, and normal human testis, heart, lung, liver, kidney, brain, and bone marrow as well as Tera-1 cells determined by semi-quantitative RT-PCR using F1 and R1 primers. Expression of *TUBA3* served as a quantitative control. (C) Expression of NALP7V3 variant in 11 testicular seminoma and normal human tissues determined by semi-quantitative RT-PCR using F2 and R1 primers. Expression of *TUBA3* served as a quantitative control. (D) Northern analysis using multiple-tissue blot with *NALP7* cDNA fragment as a probe (see "Materials and Methods").

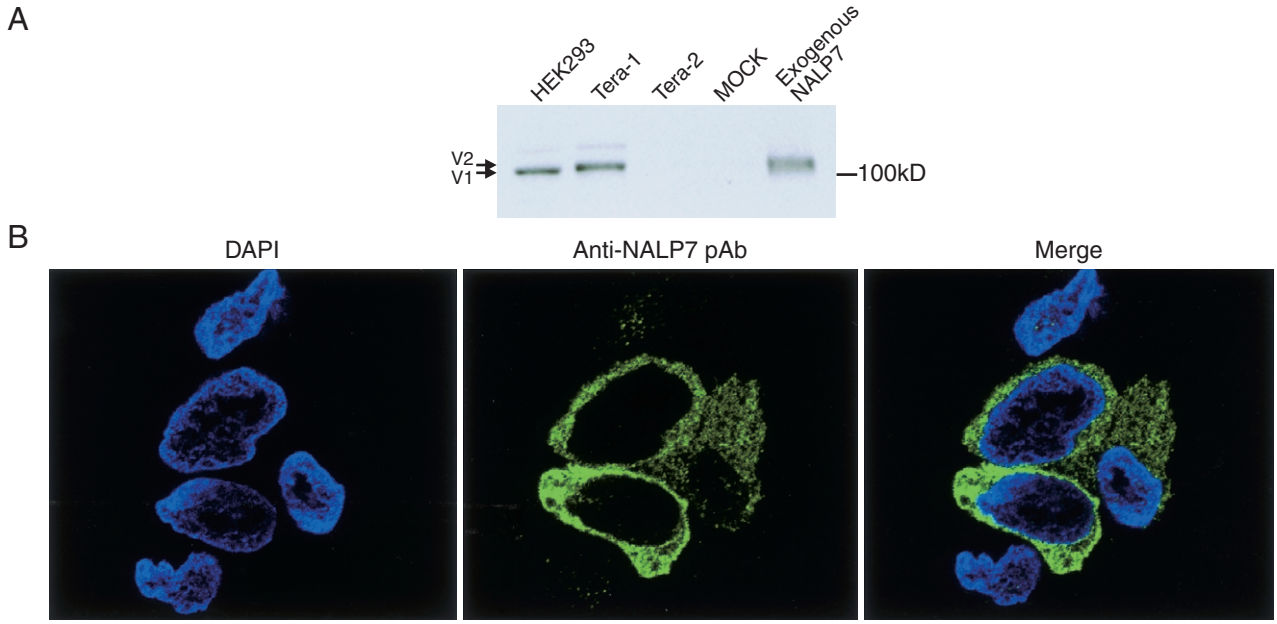

Fig. 2. Expression of NALP7 protein in mammalian cells. (A) Immunoblot analysis with anti-NALP7 pAb in HEK293, Tera-1, Tera-2 and pCAGGS3.1-3nFlag/HA vector-transfected COS7 (MOCK), and pCAGGS-*NALP7V1*-transfected COS7 cells (exogenous NALP7V1). (B) Subcellular localization of endogenous NALP7V1 in Tera-1 cells using anti-NALP7 pAb.

was specific in seminomas and might play some important role in the development of testicular seminomas, we focused on functional analysis of the NALP7V1 variant.

The *NALP7* gene belongs to the NALPs family, which currently consists of 14 members. NALP proteins were first identified as cytoplasmic proteins involved in inflammation.[19] The *NALP7* gene maps to chromosome 19q13.4, a locus where a gene cluster encoding NALP family members (NALPs 2, 4, 5, 7, 8, 9, 11, 12 and 13) is present. The SMART and PSORT II computer programs predicted that the NALP7V1 protein would contain PYD (PYRIN domain), NACHT, NAD (NACHT-associated domain) and LRRs (leucine-rich repeats) domains within its C-terminal portion. To further explore the function of *NALP7*, we performed multi-tissue northern analysis using a *NALP7* cDNA fragment that commonly hybridized to all variants as a probe (see "Materials and Methods") and detected a transcript of approximately 3.3 kb that was expressed exclusively in the testis (Fig. 1D).

Expression of NALP7 protein in embryonal carcinoma. We generated polyclonal antibody against rhNALP7 protein (see "Mate-

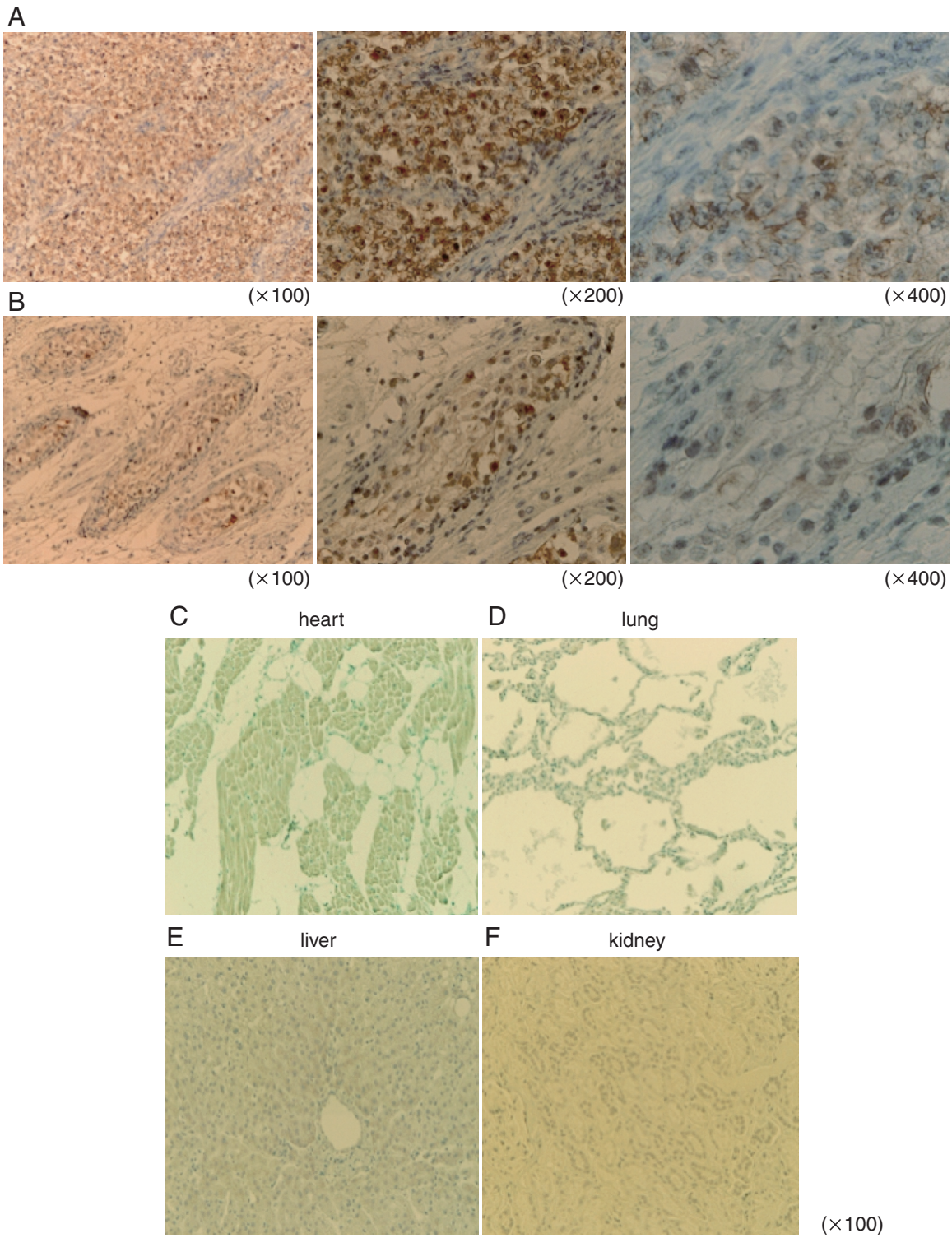

Fig. 3. Expression of NALP7 protein in testicular seminoma and normal tissues. (A) Immunohistochemical staining of NALP7 of testicular seminomas, and (B) the corresponding normal testis in the same tissue section with anti-NALP7 pAb. (C) Immunohistochemical staining of NALP7 in normal human heart, (D) lung, (E) liver and (F) kidney.

rials and Methods") and examined its expression in embryonal carcinoma cell lines and cancer tissues. Using the affinity-purified anti-NALP7 polyclonal antibody (anti-NALP7 pAb), NALP7 expression was hardly detectable in Tera-2 and HEK293 cells, or in mock-transfected COS7 cells (MOCK) by immunoblot analysis, but we identified a high level of endogenous NALP7V1 expression in human embryonal carcinoma Tera-1 cells. On the other hand, the expression of endogenous NALP7V2 was very low in Tera-1 cells (Fig. 2). We also carried out immunohistochemical analysis of NALP7 using testicular seminoma tissues and various normal human tissues (heart, liver, lung, and adult kidney). Strong staining for NALP7 pro-

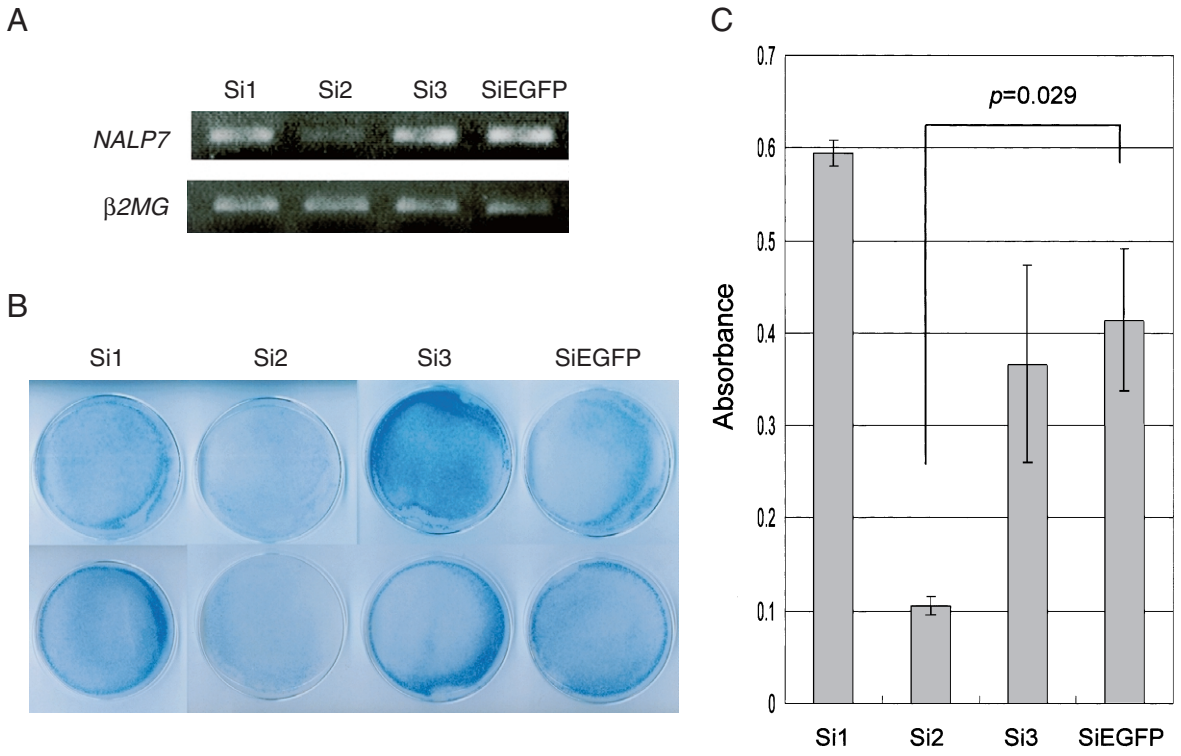

Fig. 4. Growth-inhibitory effects of small interfering RNAs (siRNAs) designed to reduce expression of *NALP7* in human embryonal carcinoma cell line, Tera-1. (A) Semi-quantitative RT-PCR showing suppression of endogenous expression of *NALP7* in Tera-1. β2-Microgloblin (*β2-MG*) was used as a quantitative control. (B) Colony-formation assay demonstrating a decrease in the number of colonies by transfection of psiU6BX-*NALP7* (Si-1, Si-2 and Si-3) into Tera-1 cells, compared with that of psiU6BX-EGFP (si-EGFP) as a control. (C) MTT assay of Tera-1 cells treated with Si-1, Si-2, Si-3 or Si-EGFP by using Cell Counting Kit-8. These experiments were carried out three times independently. Data are shown as mean±SD, and statistical analysis was performed by means of Student's *t* test.

tein was observed in testicular seminomas (Fig. 3A) as expected from the results of semi-quantitative RT-PCR analysis. However, positive staining was also observed in the corresponding normal testis, specifically in testicular germ cells, although its staining intensity was very weak compared with that of seminoma cells. NALP7 staining in the other normal tissues examined was not or was hardly detectable (Fig. 3, C–F).

Growth-inhibitory effects of NALP7-specific small interfering RNA (siRNA). To examine the role of *NALP7* in cell growth, we knocked down the expression of endogenous NALP7 in Tera-1 cells by means of the mammalian vector-based RNA interference (RNAi) technique (see "Materials and Methods"). As shown in Fig. 4A, introduction of the Si2 construct into Tera-1 cells clearly reduced the level of the NALP7 transcript, while no effect was observed in cells transfected with control plasmid (psiU6BX-*EGFP*). Colony-formation and MTT assays revealed that the Si2 construct suppressed the growth of Tera-1 cells (Student's *t* test) (Fig. 4, B and C). These results were verified in three independent experiments.

Discussion

Through the genome-wide expression profile analysis of testicular seminomas by means of the cDNA microarray approach, we identified up-regulation of *NALP7* in most of the testicular seminomas examined. Among the three transcripts of *NALP7*, the NALP7V1 transcript, but not the other two, showed significantly elevated expression in testicular seminomas compared with normal testis. NALP7 protein belongs to the NALPs family, consisting of 14 members that were recently identified as a subfamily of the CATERPILLER family. The CATERPILLER family is thought to function in apoptotic and inflammatory signaling pathways,[19] and is characterized by the presence of an amino-terminal PYD (pyrin domain), although NALP1 does not possess the PYD domain. Instead, NALP1 was shown to contain an additional domain, called FIIND (function to find) and CARD (caspase recruitment domain). The NALP7V1 protein on which we focused in this study also contains a single PYD motif at the N-terminal portion, as well as NACHT, NAD (NACHT-associated domain) and LRRs (leucine-rich repeats) domains. LRRs are generally 20–29 amino-acid motifs that are present in various proteins showing diverse functions. The LRRs potentially function to provide a versatile structural framework for the formation of protein-protein or protein-carbohydrate/lipid interactions with components of either bacterial or cellular origin; this domain was reported to interact with various bacterial substances, including lipopolysaccharide, bacterial lipoprotein, and peptidoglycan,[20, 21] suggesting LRRs might play a role in the detection of pathogen-derived molecules. The NACHT domain contains seven distinct motifs, including the ATP/GTPase-specific P-loop and the Mg^{2+}-binding site, and has sequence similarity to the nucleotide-binding motif of apoptotic protease-activating factor-1 (*APAF-1*). The PYD domain is predicted to form an α-helical bundle similar to the death domain, the death-effector domain and the caspase-recruitment domain (CARD).[19] As described above, the NALP family proteins are considered to play an important role in programmed cell-death; for example, the over-expressed NAPLP1 protein was shown to be involved in the apoptotic pathway through binding to the APAF-1 and caspase-9.[22] However, in view of the results of the immunohistochemical staining pattern, the *NALP7* gene may physiologically function in cell proliferation and development of the testis. In addition, among NALPs, TUCAN (tumor-upregulated CARD-containing antag-

onist of caspase nine) containing a CARD at the C-terminal portion was shown to be over-expressed in colon, ovarian and breast cancers, and to suppress activation of procaspase-9.[23] Hence, we suggest that *NALP7V1* overexpression in testicular seminomas may be involved in a critical step(s) of carcinogenesis through affecting interactions with apoptosis-mediating proteins. Furthermore, *NALP7*-specific siRNA significantly reduced *NALP7* expression and resulted in growth suppression of testicular germ-cell tumors (Fig. 4). Although we further examined the effect of *NALP7* on cell growth in COS7 cells by colony formation assay, over-expression of exogenous *NALP7* showed no significant enhancement of colony formation, compared with cells transfected with control plasmid (data not shown). These findings suggest that lack of the *NALP7* gene product has a critical effect on the survival of testicular tumor cells, although over-expression of this gene alone does not have growth-enhancing activity. These findings indicate that *NALP7* is a promising candidate for development of targeted therapy for testicular germ-cell tumors.

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