Cycloamylose-nanogel drug delivery system-mediated intratumor silencing of the vascular endothelial growth factor regulates neovascularization in tumor microenvironment

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RNAi enables potent and specific gene silencing, potentially offering useful means for treatment of cancers. However, safe and efficient drug delivery systems (DDS) that are appropriate for intra-tumor delivery of siRNA or shRNA have rarely been established, hindering clinical application of RNAi technology to cancer therapy. We have devised hydrogel polymer nanoparticles, or nanogel, and shown its validity as a novel DDS for various molecules. Here we examined the potential of self-assembled nanogel of cholesterol-bearing cycloamylose with spermine group (CH-CA-Spe) to deliver vascular endothelial growth factor (VEGF)-specific short interfering RNA (siVEGF) into tumor cells. The siVEGF/nanogel complex was engulfed by renal cell carcinoma (RCC) cells through the endocytotic pathway, resulting in efficient knockdown of VEGF. Intra-tumor injections of the complex significantly suppressed neovascularization and growth of RCC in mice. The treatment also inhibited induction of myeloid-derived suppressor cells, while it decreased interleukin-17A production. Therefore, the CH-CA-Spe nanogel may be a feasible DDS for intra-tumor delivery of therapeutic siRNA. The results also suggest that local suppression of VEGF may have a positive impact on systemic immune responses against malignancies.

Among the various types of nanogel that we investigated, CH-CA-Spe nanogel was used as a new siRNA carrier. CH-CA-Spe formed polymer nanoparticles in 3-D networks, composed of physical cross-linking of polymer chains (Fig. 1a, b). Cycloamylose (CA), which is produced by an enzymatic reaction between linear amylose and disproportionating enzymes, is a unique cyclic α,1,4-glucose polymer consisting of more than 100 glucose units. CA can form inclusion complexes with a variety of hydrophobic drugs. Destabilization of the endosomal membrane is essential to increase the transfection efficiency of non-viral nucleic acid delivery systems. Cycloamylose with spermine group acts as an effective plasmid DNA delivery carrier because CA can interact with endosomal membrane components, such as phospholipids or cholesterol, by forming a supramolecular complex, causing membrane instability. Although CA has high potential as a new polysaccharide-based biomaterial, its biomedical application has thus far been limited.
Fig. 1. Structure of CH-CA-Spe nanogel. Schemes of chemical structure (a) and self-assembly (b) of CH-CA-Spe nanogel are shown.

In the present study, we propose the application of cycloamyllose-nanogel as a DDS for siRNA-based cancer therapy. We targeted the VEGF gene that could play a key role in tumor-induced neo-angiogenesis. Moreover, we also analyzed whether the local suppression of VEGF in tumors could affect systemic immunity in mice.

Materials and Methods

Synthesis of CH-CA-Spe nanogel. CH-CA-Spe nanogel (Fig. 1a) was synthesized as described previously. Briefly, cationic spermine groups were attached to CA (Mn = 1.9 × 10^6 g/mol, Mn/Mw = 1.08, DP ≈ 100, gifted from Ezaki Glico, Osaka, Japan) by conventional 1,1-carbonyldiimidazole (CDI) activation. Spermine derivatives showed superior activity for the transfection of siRNA. The spermine-bearing CA (CA-Spe) thus obtained was reacted with cholesteryl N-(6-isocyanato-hexyl) / carbamate. The degrees of substitution of cationic groups and cholesterol in the CA derivative (CH-CA-Spe) were 25 and 3.1, respectively, in 100 glucose units of CA (Fig. 1a).

siRNA and siRNA/CH-CA-Spe nanogel complex. The three siRNA duplexes targeting murine VEGF-A, that are, MSS212359 (siVEGF#59), MSS278683 (siVEGF#83) and MSS278684 (siVEGF#84), were purchased from Invitrogen (Carlsbad, CA, USA). Among them, the MSS278684 (siVEGF#84) showed the highest silencing efficiency (Fig. S1a,b), so this VEGF-specific short interfering RNA (siVEGF) was used as the representative siVEGF in the present study. Control non-silencing siRNA (SiCon) (MISSION siRNA Universal Negative Control), FITC-labeled non-silencing siRNA and human VEGF-A specific siRNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). 6-Carboxyfluorescein (FAM)-labeled negative control siRNA was purchased from Life Technologies (Waltham, MA, USA). To form siRNA/CH-CA-Spe nanogel complex, 10 µL each of siRNA (50 nM) and CH-CA-Spe nanogel (21.8 mg/mL) solutions were swiftly mixed, and incubated for 30 min at 25°C, so that the cation / phosphate (C/P) ratio of the resultant complex equaled 10 (C/P = 10). In some experiments different volumes of CH-CA-Spe nanogel were added to the 10 µL of siRNA solution in such a manner that the resultant complex contained 0.8 µg siRNA/10 µL and C/P ratios were 5, 10 or 15. The mixture was diluted to 1.5 with culture medium before transfection.

siRNA transfection in vitro. A murine RCC cell line, Renca, and human RCC cell lines, ACHN and 786-O, were resuspended in RPMI1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FBS and seeded into 24-well tissue culture plates at a density of 5 × 10^4 cells per well at 37°C in 5% CO2/95% humidified air (standard conditions). The next day, siRNA/nanogel complex was added into culture (5, 10 or 15 µL/well), followed by culturing under standard conditions until analysis. In some experiments, siRNA was transfected by cationic liposome (Lipofectamine RNAiMAX Reagent; Invitrogen) according to the manufacturer’s instructions.

Real-time RT-PCR. Total RNA was reverse-transcribed and subjected to quantitative real-time PCR using a 7300 Real-time PCR System (Applied Biosystem, Carlsbad, CA, USA) using primers and probes shown in Table S1. mRNA levels were quantified by RQ software (Applied Biosystems) and standardized relative to the level of the 18S ribosomal RNA or human GAPDH mRNA.

ELISA. Concentrations of VEGF in culture supernatants were measured by ELISA using a mouse VEGF Assay kit (Immuno Biological Laboratories, Tokyo, Japan).

In vivo transfection on Renca tumor. Animal experiments were carried out in accordance with the institutional guidelines of the Kyoto Prefectural University of Medicine. To establish tumor-bearing mice, 5 × 10^5 Renca cells were inoculated s.c. into the right flank of female 7–9 week-old BALB/c mice (Shimizu Laboratory Supplies, Kyoto, Japan). Fourteen days later, tumors developed to an average volume of 50 mm^3 (day 0), into which 50 µL of siRNA/nanogel complex composed of 442.5 µg of CH-CA-Spe and 20 µg of siRNA was injected via a 30-gauge needle on days 0, 4, 8, 12 and 16. The diameters of subcutaneous tumors were measured using a digital caliper, and tumor volume was calculated as (A^2 × B)/2, where A and B are the longest diameter and shortest width of the tumor, respectively. The mice were killed 20 days later.

Flow cytometry. To analyze myeloid-derived suppressor cells (MDSC) populations, spleen cells were stained with allophycocyanin (APC)-conjugated CD11b, FITC-conjugated anti-Gr-1 (MFiteny Biotec, Bergisch Gladbach, Germany) antibodies. To analyze Tregs, the spleen cells were stimulated with 2 µg/mL of Mouse T-Activator CD3/CD28 antibodies (Life technologies), 10 ng/mL recombinant transforming growth factor (rTGF)-β1 (Peprotech, Rock Hill, SC, USA) and 200 IU/mL rIL-2 (Peprotech) for 48 h, followed by staining with PE-conjugated CD4 (eBioscience, San Diego, CA, USA), APC-conjugated CD25 (BD Biosciences, San Jose, CA, USA) and FITC-conjugated anti-Foxp3 (Mouse Th17/TregPhenotyping Kit; BD Biosciences) antibodies. Cells were analyzed by FACS Canto II (BD Biosciences) using the FlowJo software (Tree Star, San Carlos, CA, USA).

Statistical analysis. Data were expressed as means ± SD. The statistical analyses were performed using Student’s t-test. P < 0.05 was considered statistically significant.

Results

Effective delivery of vascular endothelial growth factor-specific short interfering into tumor cells by means of CH-CA-Spe nanogel. CH-CA-Spe nanogel was mixed at various C/P ratios with a siVEGF duplex that had been selected as the most effective among the three different siVEGF duplexes tested (Fig. S1a,b). After incubation under various conditions, the mixtures were...
Intratumor gene silencing by nanogel

(a) Relative VEGF mRNA level

(b) Image of 200 nm and 50 nm

(c) Relative VEGF mRNA level

(d) VEGF concentration in the supernatant

(e) Relative VEGF mRNA level

(f) Relative VEGF mRNA level

(g) Relative VEGF mRNA level
added to the culture of Renca cells. Efficient silencing of VEGF mRNA was obtained when the mixture was prepared at the C/P ratios of 10 and 15, regardless of the temperatures and incubation periods tested (Fig. 2a). Colloidal siRNAs/nanogel particles were formed under these conditions (Fig. 2b). The siVEGF/nanogel complex suppressed VEGF expression in Renca cells in a dose-dependent manner (Fig. 2c,d), and the silencing effect persisted for 2 days (Fig. 2e). A similar effect was obtained using B16 and MBT-2 (murine melanoma and bladder cancer cell lines, respectively) as well as 786-O and ACHN (human RCC cell lines) (Fig. 2f,g), suggesting effective nanogel-mediated delivery of siRNA into various tumor cells. The siVEGF/nanogel complex did not show any significant toxicity toward the cells (Fig. S1c).

**siRNA/nanogel complex was engulfed by tumor cells via lysosomal pathway.** Intracellular transport of the siRNA/nanogel complex was analyzed by confocal laser scanning microscopy.

![Fig. 3.](image)

6-carboxyfluorescein (FAM)-labeled siRNA (FAM-siRNA)/CH-CA-Spe nanogel complex was endocytosed by tumor cells. Renca cells were transfected with 6-carboxyfluorescein (FAM)-labeled siRNA by means of CH-CA-Spe nanogel or cationic liposome. Three (a) and seven (b) hours later, lysosomes were stained with LysoTracker Blue DND-22 (Life Technologies) and visualized by confocal laser scanning microscopy. Scale bars represent 10 μm.

**Fig. 2.** Vascular endothelial growth factor (VEGF)-specific short interfering RNA (siVEGF)/CH-CA-Spe nanogel complex suppressed VEGF expression in vitro. (a) siVEGF/nanogel complex was prepared under the indicated conditions and added to Renca cell culture (0.8 μg of siRNA/10 μL/well). Twenty-four hours later, VEGF mRNA levels were evaluated by real-time RT-PCR analysis. (b) Transmission electron micrograph image of siVEGF/CH-CA-Spe nanogel complex formed at the cation/phosphate (C/P) ratio of 40 at 25°C for 30 min (concentration: 0.8 μg of siRNA/10 μL). The white small dots represent nanogel particles, and the white arrow indicates colloidal siRNA/nanogel complex with a diameter of 50–100 nm. (c and d) Renca cells were transfected with siVEGF/nanogel complex (0.8 μg of siRNA/10 μL) at different doses, while control groups were transfected with control non-silencing siRNA (siCont)/nanogel (0.8 μg of siRNA/10 μL) or left untransfected (UT). Twenty-four hours later, real-time RT-PCR (c) and ELISA (d) analyses were performed. (e) VEGF mRNA was measured by real-time RT-PCR at the indicated period after transfection with siVEGF/nanogel complex (0.8 μg of siRNA/10 μL/well). (f and g) The indicated cells were transfected with siVEGF/nanogel complex (0.8 μg of siRNA/10 μL) at doses of 15 (f) and 10 (g) μL/well, and real-time RT-PCR analysis was performed 24 h later. Data represent the means ± SD (n = 3). *P < 0.05 versus siCont; **P < 0.01 versus siCont; ***P < 0.001 versus siCont; ****P < 0.005 versus siCont.
FAM (green fluorescence)-labeled siRNA was incorporated into CH-CA-Spe nanogel, and added to Renca cells. Lysosome was stained with LysoTracker (fluorescent blue) and observed at different time points. As shown in Figure 3a, FAM-siRNA was present on the surface of the cells 3 h after addition of the complex. Seven hours after the transfection, the FAM-siRNA was still present on the surface of the cells. Figure 4 shows the results of the nanogel-enabled effective intratumor siRNA delivery and silencing of vascular endothelial growth factor (VEGF). Tumor-bearing mice were injected with FITC-siRNA alone, FITC-siRNA/cationic liposome complex and FITC-siRNA/nanogel complex into preestablished subcutaneous renal cell carcinoma (RCC) tumors (20 μg of siRNA/50 μL/tumor). Three (a) and twenty-four (b) hours later, the tumor tissue was observed under confocal laser scanning microscopy. Representative bright field (left), fluorescent (middle) and merged (right) images are shown. Scale bar, 100 μm. (c) Tumor-bearing mice were given an intratumoral injection with siRNA/nanogel complex (20 μg of siRNA/50 μL/tumor) or drug delivery system alone, as indicated. Twenty-four hours later, VEGF mRNA levels were measured by real-time RT-PCR. Data represent the means ± SD (n = 5-7). *P < 0.05 versus control non-silencing siRNA (siCont) or cationic liposome group.

Fig. 4. CH-CA-Spe nanogel enabled effective intratumor siRNA delivery and silencing of vascular endothelial growth factor (VEGF). Tumor-bearing mice were injected with FITC-siRNA alone, FITC-siRNA/cationic liposome complex and FITC-siRNA/nanogel complex into preestablished subcutaneous renal cell carcinoma (RCC) tumors (20 μg of siRNA/50 μL/tumor). Three (a) and twenty-four (b) hours later, the tumor tissue was observed under confocal laser scanning microscopy. Representative bright field (left), fluorescent (middle) and merged (right) images are shown. Scale bar, 100 μm. (c) Tumor-bearing mice were given an intratumoral injection with siRNA/nanogel complex (20 μg of siRNA/50 μL/tumor) or drug delivery system alone, as indicated. Twenty-four hours later, VEGF mRNA levels were measured by real-time RT-PCR. Data represent the means ± SD (n = 5-7). *P < 0.05 versus control non-silencing siRNA (siCont) or cationic liposome group.

Fig. 5. Vascular endothelial growth factor (VEGF)-specific short interfering RNA (siVEGF) CH-CA-Spe nanogel complex suppressed growth of Renca tumors in vivo. Tumor-bearing mice (n = 6) were given intratumor injections with 50 μL of nanogel alone or the indicated siRNA/nanogel complex on days 0, 4, 8, 12 and 16. A group of mice were left untransfected (UT). (a) Growth curves of the tumors are shown. (b-g) Mice were killed on day 20. Representative macroscopic images (b) and weight (n = 6) (c) of the tumors are shown. VEGF mRNA levels in the tumors (n = at least 4/group) (d) and VEGF levels in the sera (n = 5) (e) were measured by real-time RT-PCR and ELISA, respectively. Cryosections of tumors were immunostained with CD31 antibody (f) and microvessel density was calculated (n = 4) (g). Data represent the means ± SD. *P < 0.05 versus control non-silencing siRNA (siCont); **P < 0.01 versus siCont; **P < 0.001 versus siCont. Original magnification in (f) was x200.
was localized at the lysosomal compartment as demonstrated by a cyan fluorescence in the merged image (Fig. 3b).

**Effective intra-tumor delivery of siRNA by CH-CA-Spe nanogel.** Successively, to assess in vivo localization and stability of the siRNA that was delivered into tumors by CH-CA-Spe nanogel, we injected FITC-labeled siRNA alone or in combination with cationic liposome or nanogel into pre-established RCC tumors in mice. In the tumors that received FITC-siRNA alone, we could detect an extremely sparse distribution of signals, while a sparse signal was scattered in the tumor tissues of the FITC-siRNA/cationic liposome complex. In sharp contrast, we could confirm greater fluorescence signals widely distributed in the FITC-siRNA/nanogel-injected tumors (Fig. 4a). Twenty-four hours after the injection, the FITC signal was clearly observed only in this group (Fig. 4b). Therefore, it appears that siRNA delivered by means of the CH-CA-Spe nanogel is stably maintained in tumor tissue.

Next, to evaluate silencing activity in vivo, we injected siVEGF/nanogel complex into the RCC tumors in mice. Twenty-four hours later, real-time RT-PCR demonstrated that the VEGF mRNA in siVEGF/nanogel-injected tumors was suppressed by 42.2% compared with that in an untransfected control. In contrast, a control complex containing the same amount of siRNA but without nanogel did not silence the expression of VEGF to any appreciable degree (Fig. 4c).

**Inhibition of tumor growth and neovascularisation by vascular endothelial growth factor-specific short interfering CH-CA-Spe nanogel complex in vivo.** To assess whether siVEGF/nanogel suppressed tumor growth in vivo, mice that had been transplanted with Renca cells were given repetitive administrations of the complex, and tumor growth was monitored. As shown in Figure 5a, the siVEGF/nanogel complex significantly suppressed tumor growth, while tumors treated with control complex or nanogel alone showed comparable growth rates as non-treated tumors. These observations were confirmed by macroscopic (Fig 5b) and gravimetric quantification (Fig. 5c). Real-time RT-PCR revealed that VEGF mRNA was significantly reduced in the tumors treated with siVEGF/nanogel (Fig. 5d). Serum levels of VEGF were also measured by ELISA, demonstrating a significant decrease in VEGF concentration in the sera of the siVEGF/nanogel-treated animals (Fig. 5e).

We confirmed the anti-tumor effect of siVEGF/CH-CA Spe nanogel complex using two different siVEGF duplexes, while some mice were given intra-tumor administration of siVEGF without nanogel. Both siVEGF duplexes significantly suppressed tumor growth (Fig. S2a) and reduced VEGF mRNA expression in the tumor (Fig. S2b), if they had been coupled with CH-CA Spe nanogel. Such effects were not obtained by siVEGF alone, strongly suggesting the validity of CH-CA Spe nanogel as a DDS for siRNA transfer into tumor.

To estimate whether the siVEGF knockdown therapy had an effect on neovascularization in Renca tumor tissues, vascular density in the tumor sections were immunostained with CD31 and inhibition of tumor growth by 85%. C.-J. Chen et al. (17) administered VEGF-specific siRNA by means of hydrophobic poly-(amino acid)-modified polyethyleneimine (PEI) into CT26 colorectal cancer transplants in mice, resulting in a 30% decrease in intra-tumor VEGF levels and inhibition of tumor growth by 85%. C.-J. Chen et al. (18) incorporated siVEGF in poly-amidoamine with cholesterol moieties, and injected the complex into MCF-7 breast cancer xenografts in mice, resulting in approximately 70% inhibition of tumor growth. Our CH-CA-Spe nanogel enabled successful transfection of siRNA into some human and murine cancer cells in vitro (Fig. 2f,g) as well as Renca tumors in vivo (Figs 4c and 5d), and the growth of Renca tumors was suppressed by 70% (Fig. 5a-c). Compared with previously reported DDS, an important advantage of our physically cross-linked (self-assembled) nanogels is that they are composed of biodegradable components, including polyethyleneimine (PEI).
lose that is digested by α-amylase, and spermine and cholesterol moieties that are also metabolized in the body. This may result in biodegradation in vivo of the nanogels after siRNA delivery. Moreover, toxic cross-linkers, catalysts and byproducts are not required in the preparation of the nanogels. The biodegradability and low toxicity may be extremely important for clinical application of RNAi therapeutics in the future.

It is noteworthy that the self-assembled nanogels have high retentivity in tumor tissue (Fig. 4b). The high retentivity in vivo of the nanogels was also demonstrated in nasal mucosa in our previous study. Because the nanogels have 80–90% water content, the nanogel suspension has a low viscosity and can be easily injected into the tumor. In tumor tissue, however, the water in the solvent permeates the surrounding tissue, while the penetration of nanogel is lower due to its particle size. As nanogel condenses at the injection site, an increase in viscosity and formation of macrogel-like structures by self-assembly of nanogels lead to sustained release of the nanogels from the macrogel (Fig. S5).

Recent reports showed that the therapeutic outcome achieved by VEGF-targeting therapy is much more complicated than previously considered, including modification of immune responses upon tumor progression. For example, Rosenberg’s group demonstrated that inhibition of tumor vasculature with anti-VEGF antibodies facilitated extravasation of adoptively transferred T cells into the tumor tissues and enhanced the clinical efficacy of adoptive cell transfer-based immunotherapy in mice tumor models. We analyzed the influence of intratumor VEGF knockdown on MDSC in the spleen. MDSC are immature myeloid cell populations, characterized by the cell surface expression of Gr-1 and CD11b. They suppress NK and T cells by producing reactive oxygen species (ROS) and inducing Tregs. In tumor-bearing animals and patients, MDSC accumulate in the blood, lymphoid and tumor tissues and are associated with promotion of cancer cell proliferation, angiogenesis and metastasis. In the present study, the percentage of MDSC was significantly decreased in the spleens of the siVEGF/nanogel-treated mice (Fig. 6a,b). This is consistent with a previous report that the number of circulating MDSC correlated with serum VEGF levels. Therefore, it is conceivable that intra-tumor silencing of VEGF resulted in a decrease in the serum level of cytokine and subsequent inhibition of MDSC, which otherwise contributes to immune escape of the tumors through production of immunosuppressive cytokines, NO and ROS, as well as elevation of arginase-1 activity and induction of Tregs.

In the tumor microenvironment, IL-17A-producing T cells are also induced, and they may be involved in the growth and immune escape of the tumors. It has been suggested that...
the IL-17A-producing T cells may promote tumor vessel formation and proliferation of tumor cells, although more detailed mechanisms underlying the linkage between the local VEGF suppression and systemic immunity should be analyzed in future studies.

In conclusion, our siRNA/CH-CA Spe nanogel complex may offer a promising therapeutic procedure characterized by low toxicity to patients, efficient intra-tumor delivery and high stability of siRNA in vivo. The present study strongly suggested that local regulation of VEGF may modulate systemic immune responses. More detailed investigation of the mechanism underlying the relation between anti-VEGF treatment and immune modulation might suggest novel therapeutic approaches against malignancies, such as a combination of nano DDS-mediated RNAi therapy and immunotherapy, while CH-CA-Spe nanogel can be also quite useful in analyzing pathophysiology of various diseases, including neoplasms at the molecular level.

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Disclosure Statement
The authors have no conflict of interest to declare.

References
1. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 2000; 101: 25–33.

2. Martinou JC, Martinou A. p53: a tumor suppressor and cellular apoptosis regulator. Ann Rev Genet 2001; 35: 391–415.

3. Gooding WE, Figg WD. Vascular endothelial growth factor (VEGF) and cancer. J Natl Cancer Inst 2003; 95: 1458–77.

4. Barbieri F, Scharfmann L, Huch M. Cancer-associated angio genesis: the basis for enhanced tumor perfusion and potential therapeutic targets. Int J Mol Sci 2009; 10: 4220–50.

5. Piao X, Lin T. Angiogenesis-targeted cancer therapy: current challenges and future perspectives. Cancer Metastasis Rev 2012; 31: 9–22.

6. Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small intercalating cationic polymer nanoparticles and tumor suppression in mice. Biomaterials 2010; 31: 2912–22.

7. Sasaki Y, Akiyoshi K. Nanogel engineering for new nanobiomaterials: From chaperoning engineering to biomedical applications. Chem Rev 2010; 110: 366–76.

8. Kabanov AV, Vinogradov SV. Nanogels as pharmaceutical carriers: Finite networks of infinite capability. Angew Chem Int Ed Engl 2009; 48: 5418–29.

9. Totta S, Soma Y, Morimoto N, Akiyoshi K. Cytosine-base modified branched聚meric nanoparticles of cholesterol-bearing cationic cycloamylose for siRNA delivery. Chem Lett 2009; 38: 1114–5.

10. Totta S, Sawa N, Akiyoshi K. Polysaccharide complexed with hydrophobic poly(amino acid)-modified polyethylenimine: A new delivery system with endosome-escaping function. Chem Pharm Bull 2002; 50: 267–70.

11. Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small interfering RNA targeting vascular endothelial growth factor (VEGF) receptor 2 (KDR, Flk-1) activity by a monoclonal antibody: Role of immune modulation and revascularization in cancer. Cancer Res 2008; 68: 2561–3.

12. Rolf AB, Jay PO, Victor AS et al. Selective inhibition of vascular endothelial growth factor (VEGF) receptor (KDR/Flik-1) activity by a monoclonal anti-VEGF antibody blocks tumour growth in mice. Cancer Res 2000; 60: 5117–24.

13. Verheul HM, Hammers H, Van Erp K et al. Vascular endothelial growth factor trap blocks tumor growth, metastasis formation, and vascular leakage in an orthotopic murine renal cell cancer model. Clin Cancer Res 2007; 13: 4201–8.

14. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature 2005; 438: 967–74.

15. Eisen T, Sternberg CN, Robert C et al. Targeted therapies for renal cell carcinoma: Review of adverse event management strategies. J Natl Cancer Inst 2012; 104: 93–113.

16. Chen J, Tran H, Dong X et al. Effective tumor treatment by VEGF siRNA delivered by a combination of hydrophobic poly(amino acid)-modified polyethylenimine. Macromol Biosci 2013; 13: 1438–46.

17. Chen CJ, Wang JC, Zhao EY et al. Self-assembly cationic nanoparticles based on cholesterol-grafted bioreducible poly(amo)doamine for siRNA delivery. Biomaterials 2013; 34: 5303–16.

18. Nochi T, Yuki Y, Takahashi H et al. Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines. Nat Mater 2010; 9: 572–8.

19. Cristina LR, Kristi DL, Jason ET et al. Cytokine levels correlate with immune cell infiltration after anti-VEGF therapy in preclinical mouse models of breast cancer. PLoS ONE 2009; 4: e7669.

20. Shoen F, Wu X, Malik AK et al. Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. Nat Biotechnol 2007; 25: 911–20.

21. Shirimani RK, Yu Z, Theoret MR, Chinnasamy D, Restifo NP, Rosenberg SA. Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer. Cancer Res 2010; 70: 6171–80.

22. Tu S, Bhagat G, Cui G et al. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. Cancer Cell 2008; 14: 408–19.

23. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009; 9: 162–74.

24. Dolcetti L, Marigo I, Mantelli B, Peranzoni E, Zanovello P, Bronte V. Myeloid-derived suppressor cell role in tumor-related inflammation. Cancer Lett 2008; 267: 216–25.

25. Tu SP, Jin H, Shi JD et al. Curcumin induces the differentiation of myeloid-derived suppressor cells and inhibits their interaction with cancer cells and related tumor growth. Cancer Prev Res 2012; 5: 205–15.

26. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol Immunother 2006; 55: 237–45.

27. Nakamura I, Shibata M, Gonda K et al. Serum levels of vascular endothelial growth factor are increased and correlate with malnutrition, immunosuppression involving MDSCs and systemic inflammation in patients with cancer of the digestive system. Oncol Lett 2013; 5: 1682–6.

28. Satoh T, Tajima M, Wakita D, Kitamura H, Nishimura T. The development of IL-17/IFN-gamma-double producing CTLs from Th17 cells is driven by epigenetic suppression of soc3 gene promoter. Eur J Immunol 2012; 42: 2329–42.

29. Das RL, Pathangey LB, Tender TL, Schettini JL, Gruber HE, Mukherjee P. Breast-cancer-associated metastasis is significantly increased in a model of autoimmune arthritis. Breast Cancer Res 2009; 11: R56.
Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Nanogel successfully delivers vascular endothelial growth factor-specific short interfering RNA (siVEGF) into Renca cells without significantly reducing cell viability.

**Fig. S2.** Requirement of nanogel for efficient *in vivo* delivery of vascular endothelial growth factor-specific short interfering RNA (siVEGF).

**Fig. S3.** Cytokine production by mitogen-stimulated spleen cells from the tumor-bearing mice given siVEGF/nanogel treatment.

**Fig. S4.** Treg cells in spleen were not influenced by siVEGF administration into tumors.

**Fig. S5.** Schematic representation of release of nanogel from nanogel-integrated macrogel.

**Table S1.** RT-PCR primer sequences.

**Data S1.** Materials and Methods.